

A genetic and metabolomic analysis of the *Drosophila melanogaster* innate immune response

William Hugh Pearson

MRC Centre for Molecular Bacteriology and Infection
Imperial College London

Supervisor:

Dr Marc Dionne

Co-supervisor:

Dr Gerald Larrouy-Maumus

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Declaration of originality

I confirm that this thesis represents my own work and that any external contributions are acknowledged.

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Abstract

Immunity and metabolism are closely intertwined. Immune responses are resource-demanding and metabolic changes are therefore required to supply the immune system with the required energy and raw materials. In pathological infections where the immune response becomes dysregulated, these metabolic changes can contribute to pathology. Work using the model organism *Drosophila melanogaster* has been productive in uncovering how the immune system regulates metabolism, but details of actual metabolic changes that occur during infection, and their functions, are poorly known.

This project sought to uncover the metabolic changes that occur during the *Drosophila* innate immune response to infection and understand the consequences of these changes. By combining mass spectrometry-based metabolomic analysis with the genetic analysis of *Drosophila*, I found that the immune response to systemic bacterial infection caused an increase in nitrogenous waste metabolism. Further investigations suggested that this increase in nitrogenous waste is due to an increase in the upregulation of endogenous protein degradation via an ESCRT-III mediated process. Expression of CHMP2B, encoding a core ESCRT-III component, was indicated to be required in the fat body for optimal translation of antimicrobial peptide transcripts. Together these data suggest a model in which the fly upregulates endogenous protein degradation in order to rapidly provide in-demand amino acids for immune effector production, with catabolism of the resulting excess amino acids causing an increase in nitrogen waste metabolism. This project has thus revealed a profound metabolic change induced by a successful immune response in *Drosophila*. As the cost of this change is the degradation of the host's own protein, this is a probable mechanism of metabolic pathology in the context of a dysregulated immune response. Studying the etiology of such metabolic pathology will contribute to our understanding of conditions underpinned by metabolic dysregulation, such as sepsis and infection-induced cachexia.

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Abbreviations

4E-BP	eIF4E-binding protein
5-HIU	5-hydroxyisourate
Ala	Alanine
ALG-2	Alpha-1,3/1,6-mannosyltransferase 2
ALiX	ALG-2 Interacting protein X
ALS	Amyotrophic lateral sclerosis
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
Arg	Arginine
aru	Arouser (gene)
Asn	Asparagine
Asp	Aspartic acid
ATF4	Activating transcription factor 4
Atg	Autophagy-related (gene family)
ATP	Adenosine triphosphate
AU	Arbitrary units
BCAA	Branched chain amino acid
BCAT	Branched-chain amino acid aminotransferase
BDSC	Bloomington Drosophila Stock Centre
BHI	Brain heart infusion
BomS2	Bomanin Short 2 (gene)
BSA	Bovine serum albumin
cDNA	Complementary DNA
CEF	Agilent Compound Exchange File

CFU	Colony forming units
Chmp1	Charged multivesicular body protein 1
CHMP2B	Charged multivesicular body protein 2b
CMA	Chaperone mediated autophagy
crc	cryptocephal (gene)
Cys	Cysteine
DAG	Diacylglycerol
DAP	Diaminopimelic acid
DNA	Deoxyribonucleic acid
Dpt	Diptericin (gene)
Dro	Drosocin (gene)
Drs	Drosomycin (gene)
DRSC	<i>Drosophila</i> RNAi Screening Center
DSHB	Developmental Studies Hybridoma Bank
dsRNA	Double-stranded RNA
EDTA	Ethylenediamine tetra-acetic acid
EGFR	Epidermal growth factor receptor
EIC	Extracted ion chromatogram
eIF	Eukaryotic initiation factor
ELISA	Enzyme-linked immunosorbent assay
eMI	Endosomal microautophagy
ER	Endoplasmic reticulum
ESCRT	Endosomal sorting complexes required for transport
ESI	Electron-spray ionisation
ETC	Electron transport chain
EtOH	Ethanol

FC	Fold-change
FIP200	Focal adhesion kinase family interacting protein of 200kDa
GABA	γ -aminobutyric acid
GCN2	General control nonderepressible 2 (yeast gene)
GFP	Green fluorescent protein
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
GMP	Guanosine monophosphate
GSH	Glutathione (reduced)
GTP	Guanosine triphosphate
HA	Human influenza haemagglutinin
hep	hemipterous (gene)
His	Histidine
HOPS	Homotypic fusion and protein sorting (gene group)
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
HSC70	Heat shock cognate (70kDa) protein
Hsc70-4	Heat shock protein (70kDa) cognate 4
HSPA8	Heat shock (70kDa) protein 8
I-BAR	Inverse Bin/Amphiphysin/Rvs domain
IgG	Immunoglobulin G
IKK	I κ B Kinase (protein complex)
IL	Interleukin
Ile	Isoleucine
IM2	Immune induced molecule 2

imd	immune deficiency (gene)
IMP	Inosine monophosphate
IR	Inverted repeat
ISR	Integrated stress response
Ist1	Increased sodium tolerance 1 (gene)
IκB	Inhibitor of NF-κB
JAK	Janus kinase
KD	Knock-down
KEGG	Kyoto Encyclopedia of Genes and Genomes
key	Kenny (<i>Drosophila</i> IKKγ gene)
KFase	Kynurenine formamidase
KO	Knock-out
Kyat	Kynurenine aminotransferase
LAMP2	Lysosome-associated membrane protein 2
LAMP2A	Lysosome-associated membrane protein 2, splicing isoform A
LAP	LC3 associated phagocytosis
LB	Lysogeny broth
LC	Liquid chromatography
LCMS	Liquid chromatography-mass spectrometry
Leu	Leucine
Igd	Lethal (2) giant discs 1 (gene)
LPS	Lipopolysaccharide
Lys	Lysine
<i>m/z</i>	Mass-to-charge ratio
MA	Macroautophagy
MALDI-TOF	Matrix-assisted laser desorption/ionisation – time of flight mass spectrometry

MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MeOH	Methanol
MES	2-(N-morpholino)ethanesulfonic acid
Met	Methionine
MHC	Major histocompatibility complex
MI	Microautophagy
mim	missing-in-metastasis (gene)
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger RNA
MS/MS	Tandem mass spectrometry
Mtk	Metchnikowin (gene)
mTOR	Mechanistic target of Rapamycin
Mvb12	Multivesicular body subunit 12
Myd88	Myeloid differentiation primary response 88 (gene)
n.s.	Not statistically significant ($p>0.05$)
NEB	New England BioLabs Inc.
NF- κ B	Nuclear factor κ -light chain enhancer of activated B cells
NLR	NOD-like receptor
NOD	Nucleotide-binding oligomerization domain protein
OD ₆₀₀	Optical density at 600nm
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline

PBTw	PBS containing 0.2% Tween 20
PBTx	PBS containing 1% Triton X-100
pC	Protocol control
PC	Principal component
PCA	Principal component analysis
PCDL	Agilent Personal Compound Database and Library
PE	Phosphatidylethanolamine
PGRP	Peptidoglycan recognition protein
Phe	Phenylalanine
PI	Post infection
Pi3K59F	Phosphatidylinositol 3-kinase 59F
PI3KC3	Class 3 phosphoinositide 3-kinase
POSH	Plenty of SH3s (gene)
Pro	Proline
Pros β 2	Proteasome β 2 subunit
Pros β 6	Proteasome β 6 subunit
PRPP	5-phosphoribosyl diphosphate
PRR	Pattern recognition receptor
PtdIns	Phosphatidylinositol
PtdIns3P	Phosphatidylinositol 3-phosphate
PTPN23	Tyrosine-protein phosphatase non-receptor type 23
PVDF	Polyvinylidene fluoride
qPCR	Quantitative polymerase chain reaction
QTOF	Quadrupole time-of-flight mass spectrometer
RIPK2	Receptor-interacting serine-threonine kinase
RNA	Ribonucleic acid

RNAi	Interference RNA
ROI	Region of interest
RpL4	Ribosomal protein L4
rpm	Revolutions per minute
RSD	Relative standard deviation
RT	Retention time
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
RunC	Run control
ry	rosy (xanthine dehydrogenase gene)
S6k	Ribosomal protein S6 kinase
SD	(sample) Standard deviation
SDS	Sodium dodecyl sulphate
Ser	Serine
Sgt	Small glutamine-rich tetratricopeptide containing protein (gene)
shrb	shrub (gene)
SLC7A1	Solute carrier family 7 member 1
slif	Slimfast (gene)
SNAP	Soluble N-ethylmaleimide-sensitive factor attachment protein
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
STAT	Signal transducer and activator of transcription proteins
Stip1	Stress induced phosphoprotein 1
T2D	Type 2 diabetes
TAG	Triacylglyceride/triglyceride
Tak1	TGF- β activated kinase 1
TBS	Tris buffered saline
TBS-T	TBS with 0.1% Tween 20

TE	Tris buffered saline with 1mM EDTA
TGF- β	Transforming growth factor β
Thr	Threonine
TIC	Total ion chromatogram
Tor	Target of rapamycin (gene)
TORC1	Tor complex 1
TRIM21	Tripartite motif-containing protein 21
TRiP	Transgenic RNAi Project
tRNA	Transfer RNA
Trp	Tryptophan
TSB	Tryptic soy broth
Tyr	Tyrosine
UAS	Upstream activation sequence
ULK1	unc-51-like kinase 1
ULK2	unc-51-like kinase 2
unc-51	UNCoordinated -51 (<i>C. elegans</i> gene)
UPS	Ubiquitin proteasome system
Uro	Urate oxidase
UTR	Untranslated region
UV	Ultra-violet
Uvrag	UV-resistance associated gene
v	vermillion (Tryptophan 2,3-dioxygenase gene)
VDRC	Vienna Drosophila Resource Centre
Vps	Vacuolar protein sorting (gene family)
Vta1	Vesicle trafficking 1 (gene)
w/v	weight-to-volume ratio

α KG	α -ketoglutarate (synonym: 2-oxoglutarate)
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Chapter 1: Introduction

1.1 The complex interplay between metabolism and immunity

Metabolism is all the processes in an organism that either degrade biochemicals for energy and synthesise the chemicals needed for life. The number of these chemicals is vast and ranges from DNA, lipids, and proteins, to hormones, pigments, and neurotransmitters. The study of metabolism organises these processes into metabolic pathways: series of successive reactions, most often catalysed by enzymes, that lead from one compound to another, and can either consume or release energy. Many metabolic pathways are highly conserved across species and some, such as glycolysis and the pentose phosphate pathway, exist in all known living organisms (Grant and Grisham, 2012). Through providing energy, synthesising components, and manipulating biochemical conditions, metabolism underlies all aspects of physiology, including innate immune function.

The innate immune system in animals is the first line of defence against invading microorganisms. It consists of physical and chemical barriers to infection and colonisation, such as skin and stomach acid, as well as reactive elements. These reactive elements, such as activation of immune cells and synthesis of microbicidal compounds, are initiated upon detection of non-specific 'non-self' signals such as pathogen associated molecular patterns (PAMPs) as well as upon detection of damage to the host organism. To be effective, however, this reactive side of innate immunity needs both energy and raw materials. As these are in limited supply, the organism's metabolism needs to be engaged to divert energy and resources away from normal-state activities (such as storage and growth) and toward mounting a successful immune response.

Historically, immunology and metabolism have generally been studied separately. However, as with many aspects of physiology, the inter-connection between them becomes in apparent in a medical context when the healthy function of metabolism or immunity has been disturbed. The metabolic condition of undernutrition, for example, has a profound effect on immunity and can greatly increase susceptibility to infection. Around 1 million people are believed to have died in

the Great Famine in Ireland in the mid-19th century, yet the greatest mortality came not from starvation directly, but from a variety of infectious diseases striking in a undernourished population (Porter, 1997). Over 150 years later, undernutrition continues to affect millions of people globally, and remains the leading cause of immunodeficiency increasing the frequency and severity of infections, particularly in children (Ibrahim *et al.*, 2017). On the other end of the spectrum, obesity is associated with chronic inflammation in the absence of infection ("metainflammation") which is believed to be a key factor in the pathogenesis of type 2 diabetes (Hotamisligil, 2017; Lee, Wollam and Olefsky, 2018). Type 2 diabetes is a major, and growing, global health concern and involves defective insulin signalling, which can lead to a variety of damaging complications including reduced immune function (Zimmet, 2017; Berbudi *et al.*, 2019).

In the opposite direction, disturbed immune function can have dangerous metabolic consequences as seen in sepsis and infection-induced cachexia (wasting). Sepsis is a poorly understood condition in which an aberrant immune response to bacterial infection causes severe physiological and metabolic abnormalities that result in organ-dysfunction and in severe cases, organ failure (Singer *et al.*, 2016). In 2017, deaths from sepsis were estimated to account for a staggering 19.7% of global mortality (Rudd *et al.*, 2020). Infection-induced cachexia is classically associated with chronic tuberculosis but is also seen in other contexts, including in severe sepsis, and manifests as severe loss of fatty- and lean tissue (Schwenk and Macallan, 2000). Notably, infection-induced cachexia leads to loss of tissue mass, in particular muscle, at a rate far higher than in starvation, and this tissue loss is not ameliorated by increased feeding or even hypercaloric total parenteral nutrition (Kotler, 2000).

Importantly, progress in understanding these conditions requires dissecting the interplay between metabolism and the immune response and, indeed, immunometabolism is a highly active area of research. Nevertheless, translation into the clinic of the findings from immunometabolism research is in its youth. This at least in part due to the fact that much more is known about the interface between immune function and metabolism at a cellular level than at the level of the whole organism. For example, a substantial amount of work has uncovered the integral role of metabolic reprogramming in the activation of immune cells and revealed that considerable uptake of amino acid glutamine is important for the function of macrophages

and T-cells (Kelly and Pearce, 2020). These kinds of findings, in combination with the observation that lower plasma glutamine levels were associated with poorer outcome in intensive care patients, resulted in an expectation that administration of glutamine to all critically ill septic patients would be effective in improving outcomes. However, after numerous trials, this was found to be of no benefit, or possibly even harmful (Smedberg and Wernerman, 2016; De Waele, Malbrain and Spapen, 2020). Although *in vitro* research has been highly productive in understanding cellular immunometabolism, there is a need for research investigating the interplay between metabolism and the immune system at the organismal level (Lercher, Baazim and Bergthaler, 2020).

As the work in this thesis concerns metabolic changes during infections, this introduction will detail key pathways involved in of immunometabolism with a focus on amino acid and protein metabolism and outline where further insights could help advance the field.

1.2 Immunometabolism as resource allocation

Life has evolved in an environment of limited resources and so resource-consuming functions of an organism will trade off against each other; resources consumed by one process cannot also be used by another process. Life history theory considers how organisms have adapted to invest resources between different processes over the course of their lifespan to maximise the production of successful offspring (Stearns, 1976). While life history theory tends to focus on different strategies for managing trade-offs in growth and reproductive functions, other processes are critical for fitness including tissue maintenance, energy storage, and responses to stresses including infection by other organisms. As resources are in limited supply, multicellular organisms have evolved to mount immune responses from a low base-level as required rather than constitutively maintain a resource intensive, and potentially self-damaging, highly active anti-pathogen immune state. Consequently, organisms maintain constitutive systems with minimal cost requirements that function to prevent pathogen entry with cost requirements often kept low through the use of general components with other functions. Examples of these preventative systems include effective barriers that also prevent water loss and resist injury, such as skin and exoskeletons; stomach acid that aids in digestion but also ensures ingested microbes face an immediate hostile environment; or sensory systems that allow for avoidance of sources more likely to contain potentially dangerous microbes: innate (or learned) disgust responses induced by smell or taste. How much is invested in such constitutive preventative systems is highly dependent on ecology: vultures maintain their stomach acid at incredibly low pH (pH approaching 1) allowing them to feed on decaying carcasses with almost no risk of subsequent infections. Many herbivores, in contrast, are able to access and survive on food that carries a much lower risk of harbouring dangerous microbes and so do not need to invest in maintaining such a far-from-equilibrium gastric environment (pH around 6.4). These preventative systems have evolved to cost as little as possible whilst also substantially reducing the frequency at which the more costly immune processes are required.

To counter microbes or parasites that gain entry into its interior, an organism needs systems to detect potential pathogens, regulate the induction of an appropriate anti-microbial/anti-parasitic response, allocate the resources required for the response, and to downregulate the response once infection has been resolved. Multicellular organisms employ a whole host of

responsive mechanisms that function to kill potential pathogens or prevent their reproduction. Such mechanisms include the activation of highly active immune cells, mass synthesis of antimicrobial effector proteins such as complement and antibodies, and mechanisms that act to deprive pathogens of valuable resources required for their growth, for example by sequestering free iron (Nairz *et al.*, 2010). These responses are typically highly demanding in terms of energy and consumption of precursor metabolites. For example, sepsis in humans has been estimated to increase resting metabolic rate by 30% or more and protein synthesis by 38%; fever alone has been calculated to increase metabolic rate by 10-15% per 1°C rise in body temperature (Lochmiller and Deerenberg, 2000). The substantial resource requirements of the reactive immune response require both the mobilisation of metabolic stores and the diversion of resources from other processes such as growth, reproduction, and responses to other stresses (Okin and Medzhitov, 2012; Rauw, 2012).

Immune mechanisms that function to kill pathogens and/or preventing their growth are termed “resistance” mechanisms. A second, and less well understood, aspect of immunity is “tolerance”. Tolerance functions to maintain an organism’s health during infection by mitigating both the damage caused by the pathogen and any collateral damage resulting from the resistance arm of immune response (Schneider and Ayres, 2008). Thus, an individual with higher tolerance is able to maintain health at a pathogen load which would cause pathology and deterioration of health in a less tolerance individual. Tolerance mechanisms include repair processes; detoxification of harmful effectors, virulence factors, or by-products; mechanisms that negatively regulate the resistance response to reduce immune-mediated pathology. Given the resource demands of reactive immunity, prevention of excessive and/or extended resource prioritisation to the immune system at the expense of other processes is likely an important tolerance mechanism. Thus, an example of a successful immune response to infection would involve the rapid induction of robust resistance mechanisms with a corresponding appropriate allocation of resources. Tolerance mechanisms would be employed to repair any damage and to ensure that the immune response is promptly downregulated to limit immunopathology due to immune effector-mediated self-damage and ensure resources are not diverted from other important processes unnecessarily. A successful immune response may then involve a “metabolic-switch” which functions to induce an “immune-state metabolism”, in which the immune system has prioritised access to resources. Upon resolution of the infection, the metabolism is switched

back to homeostasis or “normal-state metabolism”. A failing immune response may take the form of an insufficient immune response in terms of resistance resulting in the host being overcome by the pathogen. Alternatively, a defect in tolerance mechanisms may result in a dysregulated, excessive and/or protracted immune response resulting in severe immunopathology, much of which will likely be mediated by the diversion of resources to the immune response becoming more harmful than beneficial. This form of immune failure, in very broad terms, likely underlies the pathological states of sepsis and infection-induced cachexia.

A successful tolerance adaption may involve having a high threshold of pathogen presence before a potentially costly immune response is mounted. Nevertheless, not mounting an immune response to a pathogen that has begun to undermine an organism’s homeostasis is near guaranteed to result in the organism’s death. Consequently, evolution in many organisms, including humans, has tended to favour strong, aggressive immune responses that will carry the risk of uncontrolled immunopathology. Such responses have presumably been maintained through evolution as the benefit of a robust immune response outweighs the risk of immunopathology (Okin and Medzhitov, 2012). In modern times, the medical treatments of antibiotics and vaccination plus very different living conditions and diets compared to those encountered during millions of years of evolutionary history, suggests that the benefit-to-risk balance of our immune response is no longer optimal to our environment. Considering our immune responses from an evolutionary perspective and with awareness of potential trade-offs is likely to be valuable in furthering our understanding of the etiology of aberrant immune responses.

Ultimately, this conceptual framework may be helpful in contextualising immunometabolism as it provides defined biological questions for experimental exploration: How does the immune system signal when it requires resources? What resources does the immune response need? How does an organism’s metabolism allocate resources as required? Where do the trade-offs fall when a costly immune response is mounted? What tolerance mechanisms may mitigate the cost of the trade-offs? What metabolic costs are there for tolerance mechanisms? And importantly, what factors influence whether an immune response is successful or maladaptive?

Considering immune response in terms of resource allocation may be critical for understanding immunometabolism at the level of the whole organism, but can it tell the whole story? The well-conserved phenomenon of infection-induced anorexia is perhaps difficult to square with the high resource use by the immune response; why not eat more to fuel immunity? The fact that this response is conserved between insects and mammals, and that it is mediated by host cytokine signalling, indicates that anorexia is an adaptive response that can be beneficial in infection (Sonti, Ilyin and Plata-Salamán, 1996). Research into the consequences of infection-induced anorexia have shown that whether it is beneficial or detrimental is highly pathogen dependent (Troha and Ayres, 2020). In *Drosophila*, for example, reducing feeding resulted in improved tolerance to systemic *Salmonella typhimurium* infection but reduced resistance to *Listeria monocytogenes* infection and had no observed consequence in *Enterococcus faecalis* infection (Ayres and Schneider, 2009). In mice, inhibiting glucose utilisation improved tolerance to *L. monocytogenes* infection; but in contrast, glucose utilisation was required for survival of viral inflammation (Wang *et al.*, 2016). There are numerous suggested roles for infection-induced anorexia, including preventing consumption of (more) potential pathogens, depriving the pathogen of nutrients, a mechanism to conserve energy that may otherwise be expended in foraging or hunting, or a way of promoting autophagy (Exton, 1997; van Niekerk *et al.*, 2016). The mixed consequences of anorexia or dietary restriction observed experimentally likely suggest multiple, complex functions of this response which may or may not be a result of resource allocation mechanisms.

Considering the roles of specific metabolic pathways or infection adds huge complexity to immunometabolism and many such roles will extend beyond the theme of resource allocation. Specific metabolites function as immune signals or are involved in mediating microbial killing. Nitric oxide, for example, has numerous functions within mammalian immunity that functions as an important intercellular and intracellular regulatory signal, has directly antimicrobial effects, and has recently been found to be important for the metabolic reprogramming in activated pro-inflammatory macrophages (Bogdan, 2015; Palmieri *et al.*, 2020). A growing number of endogenous metabolites are known to have a variety of specific immune functions or effects (Luan and Medzhitov, 2016). Further complexity in organismal immunometabolism is added by considering not just the host immune system, but the specific host-pathogen, and host-pathogen-microbiota, interactions at play (Troha and Ayres, 2020). Plenty of examples exist of

specific metabolites or metabolic pathways playing a key role in host-pathogen interactions; an interesting recent example is a study by Riquelme *et al.* that found, in the context of mouse lungs, *Pseudomonas aeruginosa* can adapt to resist the effects of macrophage derived itaconate, an antimicrobial metabolite. Beyond just adapting to resist the effects of itaconate, however, the bacterium, now favouring biofilm formation, was able to use itaconate as a carbon source and promote its continued production by host macrophages (Riquelme *et al.*, 2020).

In summary, the considerable resource demands of reactive immunity means that resource allocation is a key theme in organismal immunometabolism and is a process that can have profound effects on immune function. Yet consideration of the interplay between metabolism and immunity must also be aware of the great diversity in the possible roles played by a myriad of metabolites, and how such roles may vary in importance between particular infections. Certainly, *in vitro* studies of immune cell metabolism alone or observational studies of patients will be unable to untangle the complexity of organismal immune metabolism – calling for further investigations across areas of research within biology, biochemistry, biophysics, physiology, and biomedicine. The next section of this chapter will focus on the specific use of the *Drosophila* model to study this field.

1.3 Using *Drosophila melanogaster* to study immunometabolism

1.3.1 The fruit fly as a model organism

Drosophila melanogaster, the fruit fly or vinegar fly, has been studied in the laboratory for over 100 years and is one of the most established model organisms, as demonstrated by the fact that it was the second multicellular organism to have its whole genome sequenced (following shortly behind the nematode *C. elegans*) (Adams *et al.*, 2000). Research on *Drosophila* led by Thomas Hunt Morgan and Hermann Joseph Muller in the early 20th century was instrumental in the development of genetics from an observational to an experimental discipline in biology and famously revealed the chromosome to be the bearer of hereditary information.

Drosophila has numerous advantages as a model organism. Their small size, short life cycle, and large numbers of offspring mean they are easy and cheap to maintain in large numbers. Additionally, despite having just 4 chromosomes, *Drosophila* share a high degree of genetic similarity with humans, and it is estimated that around 75% of human genes associated with disease have an orthologue in fruit fly (Reiter *et al.*, 2001). Most significantly, the long history of *Drosophila* research has resulted in a vast array of available experimental resources including an extensive knowledge of the animal's genetics, plentiful well-established experimental protocols, and a multitude of mutants and genetically modified lines.

The metabolism, metabolic signalling, and the innate immune response of fruit flies are all highly conserved with humans. Consequently, *Drosophila* offers a promising model organism for use in exploring the relationship between metabolism and immunity.

1.3.2 Fly immunity

The innate immune system of *Drosophila* has many aspects that are well conserved with vertebrates, although they lack the vertebrate adaptive immune system. The value of *Drosophila* in immunology is exemplified by the discovery of the Toll receptor and its role in the activation

of the innate immune response to bacterial infection leading to the subsequent characterisation of the mammalian Toll-like receptors (TLRs, Imler, 2014).

1.3.2.1 The humoral immune response

Key to insect immunity to bacteria and fungi are secreted antimicrobial peptides. The *Drosophila* genome encodes multiple families of antimicrobial peptides which have differing expression and efficacy against different microbes (Hanson and Lemaitre, 2020). Many antimicrobial peptides have been found to demonstrate microbicidal activity *in vitro* as positively charged molecules which target and disrupt negatively charged bacterial cell-membranes. However, the mechanisms of action for many of these diverse effectors are yet to be characterised.

Some antimicrobial peptides are expressed constitutively by epithelial cells in tissues such as the gut, trachea, and reproductive tracts, which are constantly exposed to environmental microorganisms. These tissues can also mount localised responses to pathogens by upregulating expression of antimicrobial peptides, secreting lysozyme (anti-bacterial enzyme), and producing reactive oxygen species (Daffre *et al.*, 1994; Buchon, Silverman and Cherry, 2014).

Should infection progress beyond the epithelia, or microbes gain entry through the epidermis, a systemic humoral immune response is mounted. This is primarily mediated by the rapid synthesis and secretion of vast quantities of antimicrobial peptides into the hemolymph: some antimicrobial peptides reach 100 μ l-level concentrations in the hemolymph within hours (Ferrandon *et al.*, 2007; Hanson and Lemaitre, 2020). In the systemic response, antimicrobial peptides are synthesised and secreted by fat body cells. The insect fat body is a multifunctional organ analogous in function to both the mammalian liver and adipose tissues. In adult flies, the fat body is a diffuse organ, with most fat body tissue lining the inside of the abdomen cuticle but with cells also residing in the head and thorax. In addition to its role in immunity, fat body functions include nutrient sensing; the storage and mobilisation of lipids, and to a lesser extent, glycogen; and the production of circulating hemolymph proteins (Arrese and Soulages, 2010). The integration of metabolic and immune functions within a single tissue is a demonstration of the interconnectedness of metabolism and immunity.

1.3.2.2 The Imd and Toll signalling pathways

The systemic immune response is induced by bacterial or fungal infection via two signalling cascades, the Imd and Toll signalling pathways, which connect microbial recognition to the activation of Nuclear factor- κ B (NF- κ B)-like transcription factors and the subsequent expression of different, but overlapping, sets of immune responsive genes including the antimicrobial peptides (**Figure 1.1**). The Imd pathway responds to diaminopimelic acid (DAP)-type peptidoglycan, predominantly found in Gram-negative bacterial cell walls, and detected by peptidoglycan recognition protein (PGRP)-LC at the cell surface or PGRP-LE intracellularly (Buchon, Silverman and Cherry, 2014). The consequent signal transduction pathway is similar to the mammalian Tumor necrosis factor receptor (TNFR) signalling pathway (Myllymäki, Valanne and Rämetsä, 2014). Briefly, the activation of these PGRPs recruits and activates the Imd adaptor protein. This results both in the activation, via Fadd, of the caspase Dredd and the activation of the kinase Tak1. Downstream of Tak1, the signalling pathway splits with Tak1 able to induce the stress responsive JNK-signalling pathway as well activate the IKK complex formed of Kenny (homologous to mammalian IKK γ) and IKK β . Upon activation, the IKK complex acts together with Dredd to enact the cleavage of an ankyrin-repeat autoinhibitory domain from the NF- κ B transcription factor Relish. This cleavage allows Relish to enter the nucleus and induced the transcription of a variety of immune responsive genes including the genes encoding the Diptericin and Cecropin antimicrobial peptides (Buchon, Silverman and Cherry, 2014).

Toll pathway responds to Lys-type peptidoglycan which is primarily associated with Gram-positive bacteria and to β -1,3-glucan, a fungal cell wall component (Lindsay and Wasserman, 2014). Additionally, the Toll pathway can be activated in response to detection of microbial proteases. Activation of the Toll receptor is preceded by a complex set of extracellular protease cascades that converge on the activation of Spätzle processing enzyme (SPE) which subsequently cleaves Spätzle, allowing it to bind to the Toll receptor at the cell surface. The signal is transduced intracellularly via the adaptor proteins Myd88, Tube, and Pelle, which results in targeting of Cactus (homologous to mammalian I κ B) for degradation allowing the Dif transcription factor to translocate into the nucleus and induce a set of immune responsive genes including the antimicrobial peptide genes *Drosomycin* (*Drs*) and *Bomanin Short 2* (*BomS2*) (Lindsay and Wasserman, 2014; Hanson and Lemaitre, 2020).

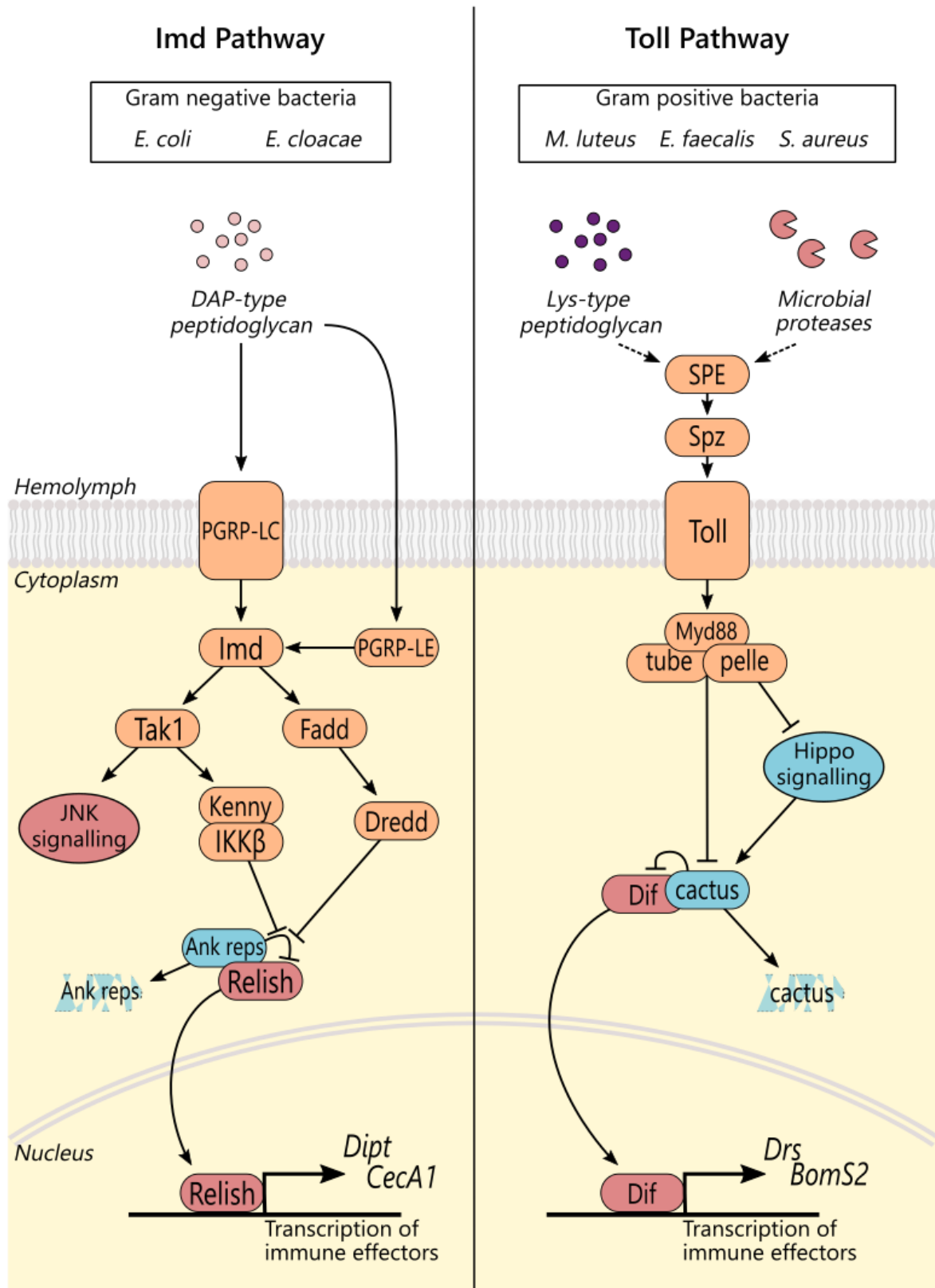


Figure 1.1: The Imd and Toll signalling pathways

See text for details.

1.3.2.3 *Drosophila* cellular immunity

Like mammals, insects possess professional immune cells called hemocytes. In adult *Drosophila*, the cellular component of the immune system is the plasmatocyte class of hemocytes. Plasmatocytes are phagocytic cells analogous to mammalian macrophages, which in addition phagocytosing invading microbes, coordinate the immune response by the secretion of cytokines and other signals (Williams, 2007). Additionally, plasmatocytes are important for the clearing of dead, apoptotic cells and extracellular matrix remodelling and, interestingly, have roles in maintaining metabolic homeostasis of tissues independent of infection. (Woodcock *et al.*, 2015; Kierdorf *et al.*, 2020).

The plasmatocytes of adult *Drosophila* are present from eclosion and do not proliferate, and so, remarkably, it seems that the adult fly retains this population, without replacement, for the remainder of their lifespan (Sanchez Bosch *et al.*, 2019). Larvae, in contrast, have a more complex cellular component to their immune system with lamellocytes and crystal cells in addition to plasmatocytes. Lamellocytes mediate the response to parasitic wasp eggs, in which the inserted egg is bound and encapsulated by the lamellocytes leading, if the response is successful, to its death (Lemaitre and Hoffmann, 2007). Crystal cells mediate the melanisation response in larvae, a clotting-like reaction in response to wounds, presumably functioning to block microbe entry, but that is also believed to be directly microbicidal. Adult flies also mount a melanisation response when wounded or infected, but, as they seemingly lack larval crystal cells, the cellular component mediating melanisation is unclear.

1.3.3 Immunometabolism and *Drosophila*

Drosophila provides an excellent model organism for the study of organismal immunometabolism due to a relatively well understood immune system, genetic tractability, and its small size which allows for straightforward metabolic measurements of the entire animal. The *Drosophila* systemic humoral immune response involves the rapid and considerable production of antimicrobial peptides following infection and so is likely to be highly resource consuming. This is suggestive of a good system in which to explore the mechanisms and consequences of resource allocation. Additionally, the integration of metabolism and immunity within a single

organ, the fat body, provides the opportunity to measure and genetically manipulate these processes within the same tissue.

The first key study in *Drosophila* of metabolism during infection, by Dionne *et al.*, found that infection of flies with *Mycobacterium marinum* resulted in a wasting phenotype with progressive loss of glycogen and lipid stores alongside hyperglycemia; a phenotype paralleling infection-induced cachexia in mammals (Dionne *et al.*, 2006). This phenotype was dependent on the Foxo transcription factor, which was canonically inhibited by insulin signalling, and *foxo* mutants showed increased tolerance to the infection with reduced wasting and longer survival despite similar bacterial loads compared with controls. Therefore, in this infection, defective insulin signalling resulted in an over-activation of Foxo leading to metabolic pathology. This study thus demonstrated that *Drosophila* can be successfully used to study the metabolic consequences of infection and the signals that regulate these changes.

Since this study, further work in *Drosophila* has confirmed the loss of metabolic stores following a variety of bacterial infections (Chambers, Song and Schneider, 2012; Bajgar and Dolezal, 2018; Vincent *et al.*, 2020). Additionally, numerous studies have indicated that immune signalling adjusts metabolism by inhibiting insulin signalling. In times of nutrient availability, the insulin signalling pathway is the crucial regulator of glucose storage in glycogen-synthesising cells, and together with Tor signalling (mTor in mammals), promotes lipid storage and growth. In adult *Drosophila*, a subset of Insulin like peptide (Ilps) hormones are homologous to mammalian insulin and are secreted by neurosecretory cells in the brain (Teleman, 2010). These Ilps subsequently bind the Insulin receptors on the surface of fat body cells resulting in the activation of a highly conserved intracellular signalling pathway that leads to the phosphorylation of Akt. Activated Akt then inhibits the Foxo transcription factor and activates Tor complex 1 (TORC1) to promote various anabolic processes (see **Section 1.4.1.1**).

The Toll signalling pathway, responding to infection or induced genetically, disrupts insulin signalling upstream of Akt as evidenced by a reduction in phosphorylated Akt and a decline in energy stores and reduced growth in larvae (DiAngelo *et al.*, 2009; Roth *et al.*, 2018). This presents an example of how the immune system may ensure diversion of resources for its use during infection; how immune signalling can induce a switch to immune-state metabolism.

There is also evidence that the Imd pathway interacts with insulin signalling; for example, adult *Imd* mutants have increased triglyceride stores and mildly impaired glucose regulation (Davoodi *et al.*, 2019). Notably these phenotypes were seen in the absence of infection, demonstrating an important theme in immunometabolism: the role of the immune system in supporting homeostasis independent of infection (Rankin and Artis, 2018).

A striking example of a mechanism that mediates a switch between normal-state and immune-state metabolism in *Drosophila*, is the transcription factor MEF2. MEF2 exists in two different forms which each promote the expression of different sets of genes (Clark *et al.*, 2013). In the absence of infection MEF2 is phosphorylated, likely by p70 S6 Kinase (S6K) which acts downstream of TORC1. Phosphorylated MEF2 promotes the expression of anabolic genes involved in glycogen and triglyceride synthesis and so supports metabolic homeostasis. However, MEF2 phosphorylation decreases upon infection. Instead of promoting the expression of anabolic genes, dephosphorylated MEF2 binds with the TATA-binding protein (TBP) forming the MEF2-TBP complex. This complex promotes the expression of antimicrobial peptide genes through binding TATA box sequences in their promoter regions. In this way MEF2 acts as a transcriptional switch, which dependent on upstream signalling, exclusively promotes either anabolic processes or the immune response.

Drosophila immunometabolism has also revealed the use of particular metabolites as immune signals. In a series of studies, Dolezal and colleagues investigated the signalling role of extracellular adenosine in *Drosophila* metabolism and immunity (Dolezal *et al.*, 2005; Zuberova *et al.*, 2010; Novakova and Dolezal, 2011; Bajgar *et al.*, 2015; Bajgar and Dolezal, 2018). These studies have suggested that extracellular adenosine secreted by hemocytes contributes to a switch from normal-state to infected-state metabolism. The role of extracellular adenosine was investigated in two different infections of *Drosophila*; parasitoid wasp infection of larvae and bacterial infection of adults (Bajgar *et al.*, 2015; Bajgar and Dolezal, 2018). In both cases, infection induced an increase in circulating glucose/trehalose with a corresponding decrease in glycogen. However, *Drosophila* mutant for *Adenosine receptor* (*AdoR*) demonstrated absent or delayed increases in circulating sugar and fared worse in both parasitoid wasp infection and infection with the bacterium *Streptococcus pneumoniae*. In the wasp infection, this reduced immunity was found to be due to impaired production of lamellocytes, the fly's anti-parasitoid immune cells.

In *S. pneumoniae* infection of adult flies, reduced host resistance was found to be due to impaired hemocyte phagocytic activity. As the resistance defect of these flies was rescued by high-sugar diet it appears this impairment of phagocytosis could be due to a lack energy available to these cells. Furthermore, genetically increasing adenosine signalling appeared to increase resistance to *S. pneumoniae* infection when compared with controls. Together these data suggest a model in which the cells of the immune system use increased levels of extracellular adenosine to promote the mobilisation of carbohydrates into circulation, thus directing energy stores to the immune response at the expense of other processes.

The findings above all demonstrate the value of *Drosophila* in studying organismal immunometabolism. The emerging theme is that activation of immune signalling induces a switch in metabolism that inhibits the allocation of resources to other processes, notably anabolic processes. Interestingly, several mechanisms for signalling this metabolic switch have been found so far including the inhibition of insulin signalling, the MEF2 transcriptional switch, and hemocyte extracellular adenosine signalling. The relationship between these different signals and the extent to which they may be redundant is unclear. It is conceivable that different signals are used in different contexts and also that the existence of multiple mechanisms for the immune system to regulate metabolism may allow for a level of fine-tuning in the resource allocation.

Despite the work in dissecting how the immune system and metabolism communicate, the consequences of this signalling switch on the animal's metabolism itself, and the functional consequences for the immune response, are less well understood. This is particularly the case for any changes in metabolism beyond the focus on circulating sugar, glycogen, and triglycerides.

1.4 Amino acid metabolism in *Drosophila*

Amino acid metabolism is an important and complex part of an organism's physiology. A subset of amino acids, the proteogenic amino acids, are well known as the building blocks of proteins. Controlled provision of these amino acids for protein synthesis is thus a key aspect of metabolism essential for life. However, amino acids are also required in non-proteogenic anabolism as precursors in the synthesis of a multitude of biological molecules from purines, pyrimidines, and neurotransmitters, to larger molecules like heme, melanin, and chitin. Additionally, amino acids can be catabolised, liberating their carbon skeletons to be used as an energy source. Finally, many amino acids themselves function as important signalling molecules, neurotransmitters, and osmolytes.

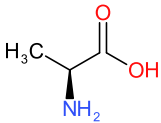
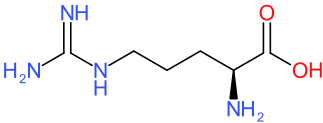
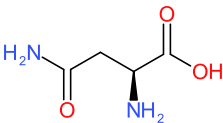
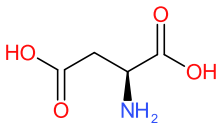
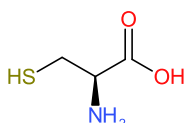
Animals are able to synthesise many amino acids in adequate amounts, but others must be obtained through their diet, primarily from ingested protein. Thus, amino acids can be described as non-essential or essential dependent on the animal's dietary requirement. Of the 21 proteinogenic acids, *Drosophila* are completely unable to synthesise 8. A further 2, methionine and arginine, can be enzymatically synthesised by the fly, but only from limited precursors, and in amounts far below what is required (Sang and King, 1961; Grandison, Piper and Partridge, 2009). Consequently, 10 amino acids are essential for *Drosophila* (see **Table 1.1**) and these are, with the exception of arginine, the same as those that are essential for humans. Additionally, normally non-essential amino acids may be required in an animal's diet under particular circumstances or stages of their lifecycle. Such amino acids are thus termed conditionally essential. Arginine, for example, is conditionally essential for humans as arginine synthesis is inadequate to meet metabolic demand under various stress conditions or to support growth in neonates (Morris, 2007). While adult *Drosophila* are able to survive for weeks on chemically defined diets entirely lacking in protein or amino acids, larvae and adult females require intake of essential amino acids for growth and viable egg-laying respectively. Additionally, optimal growth and egg laying require intake of non-essential amino acids (Sang and King, 1961; Lee and Micchelli, 2013; Lee, 2015; Consuegra *et al.*, 2020; Ma *et al.*, 2020).

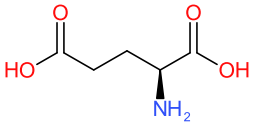
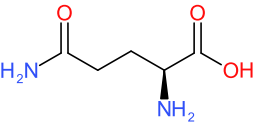
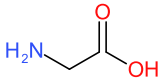
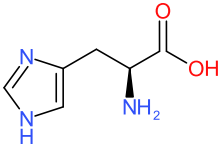
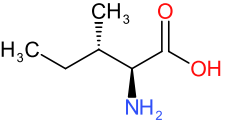
Amino acids have numerous roles in biology independent of ribosomal protein synthesis; however, such roles vary considerably between different amino acids. This is true for their roles

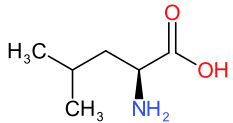
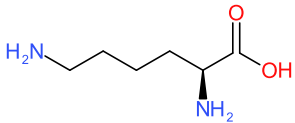
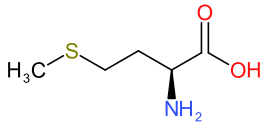
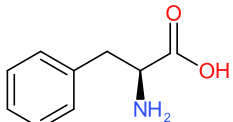
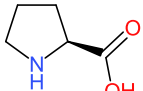
in metabolism, as different amino acids have different 'metabolic potentials', the collection of enzymatic reactions and metabolic pathways a metabolite is able to participate in. The metabolic potential of a metabolite depends on the repertoire of relevant enzymes at the animal's disposal: the enzymes encoded by their genome, supplemented by those encoded in the genomes of any symbiotic or commensal microorganisms. Some amino acids are the substrates of multiple different enzymes and utilised in numerous metabolic pathways, whereas some may only participate in one or two reactions outside of peptide synthesis. For example, in flies and humans, glutamine is required in the biosynthesis of numerous essential compounds including purines, pyrimidines, and amino sugars, and can be converted to glutamate. In contrast, histidine can undergo only a limited number of reactions: only one enzymatic reaction, decarboxylation to histamine, is annotated for histidine in *Drosophila melanogaster* in the BioCyc and KEGG databases (Caspi *et al.*, 2020; Kanehisa *et al.*, 2021). Importantly, many amino acids can be converted to at least one other amino acid, and indeed, synthesis of an animal's non-essential amino acids frequently involves another amino acid as a precursor.

Studies exploring the interaction between immunity and metabolism in *Drosophila* have tended to focus on carbohydrates and triglycerides. Nevertheless, an increasing number of studies in *Drosophila* have found that infection, or genetically altered immune signalling, induces transcriptional changes in genes related to amino acid metabolism (see for example (Clark *et al.*, 2013; Atilano *et al.*, 2017; Troha *et al.*, 2018; Davoodi *et al.*, 2019)). This is perhaps unsurprising as, *a priori*, it could be predicted likely that the mass-production of antimicrobial peptides by fat body cells in response to bacterial infection would require, or induce, changes in amino acid metabolism. Very few studies, however, have directly measured changes in the levels of the amino acids themselves or investigated the consequence of altered amino acid metabolism on immunity. Metabolomic analysis allows for simultaneous measurement of multiple metabolites and is a powerful technique that has been utilised in numerous studies in *Drosophila* (for a review see (Cox, Thummel and Tennessen, 2017)). To my knowledge, only two studies have performed metabolomics on bacteria-infected flies (Chambers, Song and Schneider, 2012; Bakalov *et al.*, 2016). While both studies found numerous changes in amino acid levels, these were not directly addressed in follow-up experiments. Thus, although multiple studies have directly, or indirectly, implicated changes in *Drosophila* amino acid metabolism, little is known about the nature, causes, or functions of these changes. There is, however, one notable exception, tyrosine, which

is important for the melanisation response. In the remainder of this section, I will first discuss the role of tyrosine in *Drosophila* immunity. Then, as carbohydrate and lipid levels and their regulation are known to be altered by infection, I discuss how amino acids contribute to carbohydrate/lipid metabolism, the resultant problem of nitrogenous waste, and how this is organised between different tissues. Finally, I will return to the most well-known role of amino acids by discussing how amino acid metabolism is coordinated with protein synthesis.

Amino Acid (Abbreviations)	Formula	Structure	Attributes	C- backbone fate	C- catabolism	N-catabolism	Precursor (if not essential)
Alanine (Ala, A)	$C_3H_7NO_2$		Hydrophobic	Pyruvate	Glucogenic	Aminotransferase	Pyruvate (+N)
Arginine (Arg, R)	$C_6H_{14}N_4O_2$		Basic, used for NO synthesis	α KG (via Glu)	Glucogenic	Urea (2N), aminotransferase (1N), leaves Glu (1N)	Essential (but recyclable from citrulline)
Asparagine (Asn, N)	$C_4H_8N_2O_3$		Neutral polar	Oxaloacetate	Glucogenic	Lysed to yield ammonia and Asp	Asp (+N)
Aspartic acid (Asp, D)	$C_4H_7NO_4$		Acidic	Oxaloacetate	Glucogenic	Directly into purine (or aminotransferase)	From Asn (-N) or oxaloacetate (+N)
Cysteine (Cys, C)	$C_3H_7NO_2S$		Sulphur atom easily oxidised, reactive component of GSH	Pyruvate	Glucogenic	S-oxidised then aminotransferase	From Met (via homocysteine)

Amino Acid (Abbreviations)	Formula	Structure	Attributes	C- backbone fate	C- catabolism	N-catabolism	Precursor (if not essential)
Glutamic acid (Glu, E)	C ₅ H ₉ NO ₄		Acidic, ammonia acceptor (forming Gln), neurotransmitter	αKG	Glucogenic	Synthesis of glutamine or cleavage yielding NH ₄ ⁺	αKG (+N)
Glutamine (Gln, Q)	C ₅ H ₁₀ N ₂ O ₃		Neutral polar, N-donor for amino sugars	αKG (via Glu)	Glucogenic	Side chain N into purine, leaving Glu	Glu (+N)
Glycine (Gly, G)	C ₂ H ₅ NO ₂		Achiral, involved in 1-C metabolism	Pyruvate (via Ser), or glycine cleavage system	Glucogenic	Directly into purine, cleavage into ammonia or AT	Ser (Hydroxymethyl transferase)
Histidine (His, H)	C ₆ H ₉ N ₃ O ₂		Basic	Not catabolised	NA	Not catabolised	Essential
Isoleucine (Ile, I)	C ₆ H ₁₃ NO ₂		Hydrophobic, branched chain	Acetyl-CoA and Succinyl-CoA	Both	Aminotransferase (BCAT)	Essential

Amino Acid (Abbreviations)	Formula	Structure	Attributes	C- backbone fate	C- catabolism	N-catabolism	Precursor (if not essential)
Leucine (Leu, L)	$C_6H_{13}NO_2$		Hydrophobic, branched chain, TORC1 agonist	Acetoacetate	Ketogenic	Aminotransferase (BCAT)	Essential
Lysine (Lys, K)	$C_6H_{14}N_2O_2$		Basic	Acetoacetate	Ketogenic	Multi-step, 2 x aminotransferase	Essential
Methionine (Met, M)	$C_5H_{11}NO_2S$		Hydrophobic, sulphur- containing, methionine cycle in 1-C metabolism	Succinyl-CoA	Glucogenic	Synthesis of cystathionine, then lysed to yield ammonia and Cys, (plus 2-oxobutanoate)	Essential (But recyclable in methionine cycle)
Phenylalanine (Phe, F)	$C_9H_{11}NO_2$		Hydrophobic	Acetoacetate and Fumarate	Both	To Tyrosine	Essential
Proline (Pro, P)	$C_5H_9NO_2$		Secondary amine, insect energy carrier?	α KG (via Glu)	Glucogenic	To Glu via Glutamate-5- semialdehyde	Glu (via pyrroline- 5-carboxylate)

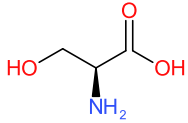
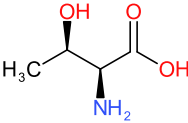
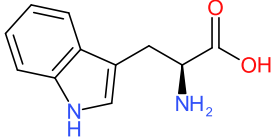
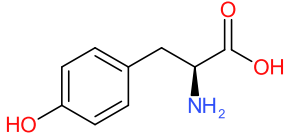
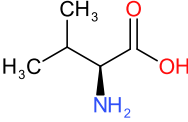
Amino Acid (Abbreviations)	Formula	Structure	Attributes	C- backbone fate	C- catabolism	N-catabolism	Precursor (if not essential)
Serine (Ser, S)	$C_3H_7NO_3$		Neutral polar, synthesised from glycolysis product, feeds 1-C metabolism	Pyruvate	Glucogenic	To Gly, aminotransferase, or lysed liberating ammonia (?)	3P-glycerate (via 3P- hydroxy- pyruvate +N)
Threonine (Thr, T)	$C_4H_9NO_3$		Neutral polar	Acetyl-CoA and pyruvate (via Gly)	Both	To Glycine or lysed to yielding ammonia	Essential
Tryptophan (Trp, W)	$C_{11}H_{12}N_2O_2$		Hydrophobic, alternative catabolic pathway	1x C to formate, rest to kynurenine	NA	2x N to kynurenine	Essential
Tyrosine (Tyr, Y)	$C_9H_{11}NO_3$		Hydrophobic, melanisation precursor	Acetoacetate and Fumarate	Both	Aminotransferase	From Phe (ring hydroxylation)
Valine (Val, V)	$C_5H_{11}NO_2$		Hydrophobic, branched chain	Succinyl-CoA	Glucogenic	Aminotransferase (BCAT)	Essential

Table 1.1: The structures and metabolism of the 20 encoded proteogenic amino acids. In “Precursor” column, +/- N signifies gain or loss of nitrogen in aminotransferase reactions.

Structures from the ChEBI Database (Hastings et al., 2016). BCAT, Branched chain aminotransferase; GSH, glutathione.

1.4.1 Tyrosine and melanisation

Tyrosine has an important role in the *Drosophila* innate immune response as it is the precursor for the melanisation reactions. Melanisation is part of insect innate immunity and contributes to wound healing, parasite encapsulation, and microbial killing. Adult *Drosophila* lacking a melanisation response show reduced survival to multiple Gram-positive bacterial and fungal infections (Binggeli *et al.*, 2014). How exactly the melanisation cascade results in microbial killing is unknown, though it is believed that reactive intermediates and by-products of the cascade are toxic for nearby microbes.

Melanisation is a complex cascade of reactions initiated by phenoloxidasases which are formed through the proteolytic cleavage of pro-phenoloxidasase zymogens. This activation is performed by extracellular serine proteases in a complex, and only partly known, cascade that overlaps with the serine proteases upstream of the Toll receptor (Dudzic *et al.*, 2019). In short, tyrosine and its derivative, L-dopa, are oxidised to quinones which undergo cyclization before reacting with each other in a double decomposition reaction generating dopachrome and regenerating L-dopa. Dopachrome is then converted to 5,6-dihydroxyindole which subsequently undergoes further oxidation and polymerisation to form eumelanin (Sugumaran and Barek, 2016; Nakhleh, El Moussawi and Osta, 2017). The conversion of dopachrome to 5,6-dihydroxyindole is likely enzymatically mediated, as has been found in other insect immune responses and in the synthesis of *Drosophila* cuticle melanin (Han *et al.*, 2002; Christensen *et al.*, 2005; Sugumaran and Barek, 2016). However, to my knowledge, this dopachrome decarboxylase/tautomerase enzyme, likely encoded by *yellow-f* and/or *yellow-f2*, has yet to be characterised in *Drosophila* immunity (Han *et al.*, 2002).

The cascade of reactions in melanisation has promiscuous involvement of metabolites and can react with other molecules and utilise other tyrosine-related substrates in the place of L-dopa. For example, if cysteine thiol groups are present during the cascade, the sulphur atoms are sequestered by the quinone rings resulting in the formation of the red-coloured pheomelanin. Pheomelanin, while far less common in insects than mammals, has been found in insect cuticular melanin. The tyrosine derivative, dopamine, is known to be used as a melanisation substrate

for cuticular melanin. Interestingly, the dopamine synthesising enzyme, dopa decarboxylase, is transcriptionally induced in infection, though the role of this induction is unknown (De Gregorio *et al.*, 2001; Davis, Primrose and Hodgetts, 2008). It is interesting to speculate that the use of different melanisation substrates, or the presence of sulphur, may play a functional role in immune-induced melanisation.

As tyrosine is required for melanisation it is unsurprising that a study by Chambers *et al.* found infection of adult *Drosophila* with *L. monocytogenes* resulted in depleted levels of tyrosine relative to uninfected controls (Chambers, Song and Schneider, 2012). The same study also found decreased phenylalanine levels following infection. The amino acid phenylalanine, essential for *Drosophila*, is able to be converted to tyrosine and so this result suggests phenylalanine could be being consumed to produce tyrosine to fuel the melanisation. Consequently, tyrosine and potentially phenylalanine are important in the fly response to bacterial infection beyond their inclusion into antimicrobial peptides.

1.4.2 Amino acids integrate with carbohydrate and lipid metabolism

Amino acids are important contributors to an animal's carbohydrate metabolism, lipid metabolism, and energy generating processes. How amino acid metabolism integrates with these demonstrates the different metabolic potentials of the amino acids. Each amino acid is converted to one, or two, of seven metabolites that are part of, or adjacent to, the tricarboxylic acid cycle (TCA): pyruvate, acetoacetate, acetyl-CoA, α -ketoglutarate (α KG), succinyl-CoA, fumarate, or oxaloacetate (see **Table 1.1**) Each of these metabolites can be used either to synthesise glucose or ketone bodies/fatty acids. Consequently, amino acids are termed glucogenic and/or ketogenic depending on the metabolic potential of their carbon skeleton (Grant and Grisham, 2012). This integration of amino acid metabolism with the TCA cycle and related metabolites, supports a number of amino acid functions. Firstly, the carbon from excess dietary amino acids can be stored for energy in glycogen or triglyceride. Secondly, when energy demand is not met by sugar and lipid supplies, such as in starvation, protein can be degraded to amino acids and their carbon skeletons oxidised for energy.

Finally, amino acids can be used for anaplerosis: the replacement of TCA cycle intermediates that have been used for various biosynthetic pathways such as fatty acid synthesis, gluconeogenesis, and indeed, synthesis of other, non-essential amino acids (Owen, Kalhan and Hanson, 2002). This role for amino acids in maintaining TCA cycle flux has been found to be especially important for highly anabolic and rapidly proliferating mammalian cells such as cancerous cells and various types of activated immune cells. These cells are associated with a shift towards aerobic glycolysis, also known as Warburg metabolism, in which a high proportion of pyruvate from glucose catabolism is reduced to lactate despite there being adequate oxygen for far more efficient ATP production through oxidising pyruvate in the TCA cycle to fuel oxidative phosphorylation. The role(s) of this shift is debated but it is generally considered as a way of prioritising anabolism over efficiency of energy production; shifting to aerobic glycolysis may aid in increasing the flux through the pentose phosphate pathway required for nucleotide synthesis, providing precursors for lipid synthesis, and may function to maintain NAD^+/NADH ratios, required due to increased demand for NAD^+ in anabolic reactions (Lunt and Vander Heiden, 2011; Luengo *et al.*, 2021). To maintain levels of TCA intermediates required for biosynthesis despite redirection of pyruvate to aerobic glycolysis, the carbon from amino acids can be used. For example, glutamine uptake is strongly increased in mammalian macrophages and lymphocytes upon their activation, and, amongst other roles, supports the metabolic reprogramming of these cells by providing a source of αKG . In activated macrophages, citrate, upstream of αKG in the TCA cycle, is redirected for the synthesis of Acetyl-CoA and itaconate. However, the pyruvate carbon that could be used for citrate production is instead being converted to lactate to regenerate NAD^+ . Consequently, citrate is no longer used to produce αKG leading to a break in the TCA cycle that is balanced by instead catabolising glutamine to αKG . In this way the cycle can continue to produce succinate, which is required by the macrophage for immune signalling (Kelly and Pearce, 2020; Ryan and O'Neill, 2020).

Adult *Drosophila* hemocytes do not appear to proliferate although there is some recent evidence of hemocytes shifting towards aerobic glycolysis in bacterial infection (Krejčová *et al.*, 2019; Sanchez Bosch *et al.*, 2019; Bajgar, Krejčová and Doležal, 2021). The extent to which uptake of different amino acids is required to support this hemocyte metabolic shift, or any other hemocyte activity for that matter, has not been investigated in *Drosophila*.

1.4.3 Nitrogenous waste

Amino acids are the main players in an animal's overall nitrogen metabolism, and dietary amino acids, primarily from the ingestion and digestion of protein, are its major source of nitrogen atoms. Beyond protein synthesis, much of metazoan amino acid anabolism (broadly speaking) involves using the nitrogen atoms from amino acids to synthesise the myriad of other nitrogen containing molecules needed for life. Conversely, a critical aspect of amino acid catabolism is the safe disposal of their constituent nitrogen atoms: build-up of free nitrogen, which exists as ammonia and ammonium ions, is toxic. Ammonia exists either as neutral NH_3 molecules or as NH_4^+ ammonium ions. Although the latter form is vastly more prevalent at physiological pH, both forms of ammonia are toxic to organisms, likely via multiple mechanisms (Cooper and Plum, 1987; Martinelle and Häggström, 1993; Davuluri *et al.*, 2016). Due to its toxicity, ammonia must be rapidly excreted or the excess nitrogen atoms sequestered in less toxic compounds which can then be excreted. Three main strategies for nitrogenous waste disposal exist in animals: ammoniotelism, ureotelism, and uricotelism. Ammoniotelism is the simplest method for disposal of excess nitrogen and simply involves excreting ammonia itself. This method, however, requires a large amount of water to dilute the highly soluble ammonia and so only aquatic organisms are able to use this method for the majority of their nitrogenous waste disposal. Terrestrial animals, therefore, have to prevent ammonia build up by incorporating nitrogen atoms into low toxicity compounds. Ureotelic animals, such as humans and other mammals, excrete most of their excess nitrogen as urea, a small, soluble, non-toxic molecule containing 2 nitrogen atoms that can harmlessly travel in the animal's circulation before being excreted in urine. Uricotelic animals, such as birds, reptiles, and many insects including *Drosophila*, use uric acid and/or closely related compounds as the primary end product of their nitrogen waste metabolism. Importantly, these strategies are not mutually exclusive, and while animals tend to utilise one primary method and end product for their nitrogenous waste disposal, other methods and end products may also be used to a lesser extent.

Both the ureotelic and uricotelic methods for nitrogenous waste disposal involve concentrating the nitrogen atoms from amino acid catabolism into a small number of nitrogen-carrying molecules. This is achieved through two general mechanisms. The first is through transamination reactions. These reactions transfer the amine groups from one

molecule to another, usually to yield glutamate. These reactions are mediated by a variety of enzymes, known as aminotransferases or transaminases, with different enzymes acting on different combinations of amino acid donors and acceptor molecules. There are multiple aminotransferases which each act on different amino acids, but all follow the same general reaction: the amino group of the donor amino acid is removed, leaving the amino acid's carbon-skeleton as an α -keto acid, and transferred to an acceptor α -keto acid to form a new amino acid. The most common acceptors are α -ketoglutarate (α KG), which aminates to form glutamate; oxaloacetate, which forms aspartate; and pyruvate, which forms alanine. These transamination reactions are also used to interchange amino acids to meet anabolic requirements, dependent upon the repertoire of aminotransferases the organism possesses.

The second mechanism for transferring nitrogen atoms between molecules is through the liberation of ammonium from one molecule (deamination) followed by its subsequent enzymatic reincorporation into a second molecule (ammonia-ligation). In animals, only a small number of reactions consume free ammonia including the formation of glutamate, by glutamate dehydrogenase; the synthesis of glutamine from glutamate; and, in some animals, the reaction of ammonia with ATP and bicarbonate to form carbamoyl phosphate. In ureotelic animals, this latter reaction is the penultimate step in delivering nitrogen to the urea cycle. The urea cycle is a series of reactions that synthesise arginine before cleaving it into urea, which is excreted, and ornithine, which continues around the cycle to regenerate arginine. *Drosophila* do not have a complete urea cycle as they lack both the enzyme that can produce carbamoyl phosphate from free ammonia, and the urea cycle enzyme that recycles ornithine.

1.4.3.1 Purine metabolism for nitrogen disposal

Drosophila are primarily uricotelic, excreting the majority of their nitrogenous waste in the form of uric acid and its degradation product, allantoin. Uric acid is a product of purine catabolism and so uricotelic animals synthesise purines *de novo*, packing the nitrogen atoms into purine rings, which are subsequently dehydrogenated to yield uric acid (**Figure 1.3**). To deliver nitrogen to purine synthesis, the two previously mentioned mechanisms, transamination and deamination/ammonia-ligation, are used to concentrate nitrogen atoms into glycine, glutamine, and aspartate. Each purine ring contains 4 nitrogen atoms. One nitrogen atom originates from the incorporation of glycine into the forming purine ring and

the other nitrogen atoms are delivered by donation from 2 glutamine molecules and 1 aspartate molecule (**Figure 1.3**). Purine synthesis begins with addition of 2 phosphate groups to ribose-5-phosphate, the end product of the pentose phosphate pathway, to form 5-phosphoribosyl diphosphate (PRPP). PRPP is used to for *de novo* synthesis of purines and pyrimidines, as well as donating ribose-5-phosphate to existing nucleotides. Formation of the purine ring involves a series of 10 enzymatic reactions starting with PRPP and resulting in inosine monophosphate (IMP). These reactions are mediated by a group of 6 cytosolic enzymes, 3 of which catabolise multiple reactions in the pathway. The synthesised IMP can then be used to produce AMP or GMP. Alternatively, the phosphate and ribose are removed to leave hypoxanthine. For nitrogen disposal, hypoxanthine is then converted to xanthine and then uric acid by xanthine dehydrogenase, encoded by *rosy (ry)* in *Drosophila* (Keith *et al.*, 1987). Uric acid is also the product of the degradation of excess guanosine and adenosine, the primary source of uric acid in ureotelic animals including humans. In uric acid is then either excreted or further catabolised by urate oxidase (Uro), an enzyme missing in humans and closely related primates. The product of urate oxidase is 5-hydroxyisourate (5-HIU) which is subsequently hydrolysed by 5-HIU hydrolase (CG30016) to form S-allantoin (Ramazzina *et al.*, 2006). *Uro* and *CG30016* are exclusively expressed in the Malpighian tubules, the insect excretory and osmoregulatory organ analogous to mammalian kidneys (Wallrath, Burnett and Friedman, 1990; Cohen *et al.*, 2020). Interestingly, while highly expressed in tubules, *ry* seems to have more broader tissue expression.

Compared with using the urea cycle, this form of nitrogen waste metabolism is highly demanding in terms of energy and resources (Klowden, 2007). In addition to the nitrogen-delivering amino acid contributions, the production of IMP from PRPP consumes 5 ATP molecules and the formyl groups donated from 2 formyltetrahydrofolate molecules, which each require a further ATP molecule to regenerate. What is the benefit of this expensive uricotelic strategy? Certainly, uric acid makes for an efficient packet for excess nitrogen as it has a high ratio of nitrogen to other elements, with a N:C ratio of 4:5 and a N:H ratio of 1:1 (**Figure 1.2**). In comparison, urea is more efficient in terms of carbon (N:C of 2:1) but less efficient in terms of hydrogen, with a N:H ratio of 1:2. The key benefit of uricotelism over ureotelism or ammonotelism is that uric acid has very low solubility at neutral or acid pH, and so can be excreted as a solid. Consequently, large volumes of water do not need to be

used for the disposal of nitrogenous waste. For flies, as small creatures with a high surface area to volume ratio, water conservation is critical. This is fact exacerbated by that fact that flying insects in particular have evolved to be very efficient in terms of mass in order to optimise the efficiency of flight as so do not have the capacity for storage of significant volumes of excess water. The further catabolism of uric acid, to the more soluble compound allantoin, is likely a method of regulating uric acid levels as the formation of uric acid from xanthine is irreversible. Uric acid levels require regulating in order to prevent an excessive and harmful build-up of solid urate crystals in the tubules faster than they can be excreted. Indeed, genetically causing the build-up of purine "stones" in *Drosophila* tubules has provided models to study human nephrolithiasis (Miller *et al.*, 2013). Under normal circumstances, the majority of uric acid is not further catabolised, and it is uric acid, not allantoin, that is the major excretory product of adult *Drosophila* (Yamauchi *et al.*, 2020).

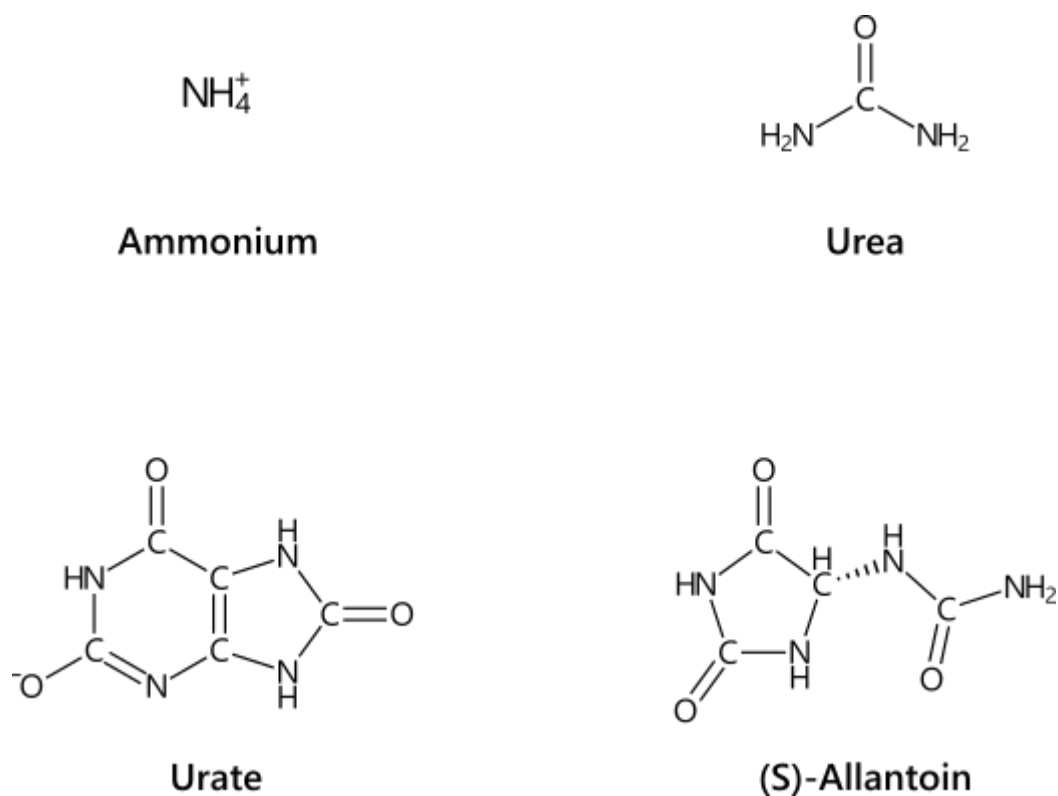


Figure 1.2: Nitrogenous waste metabolites

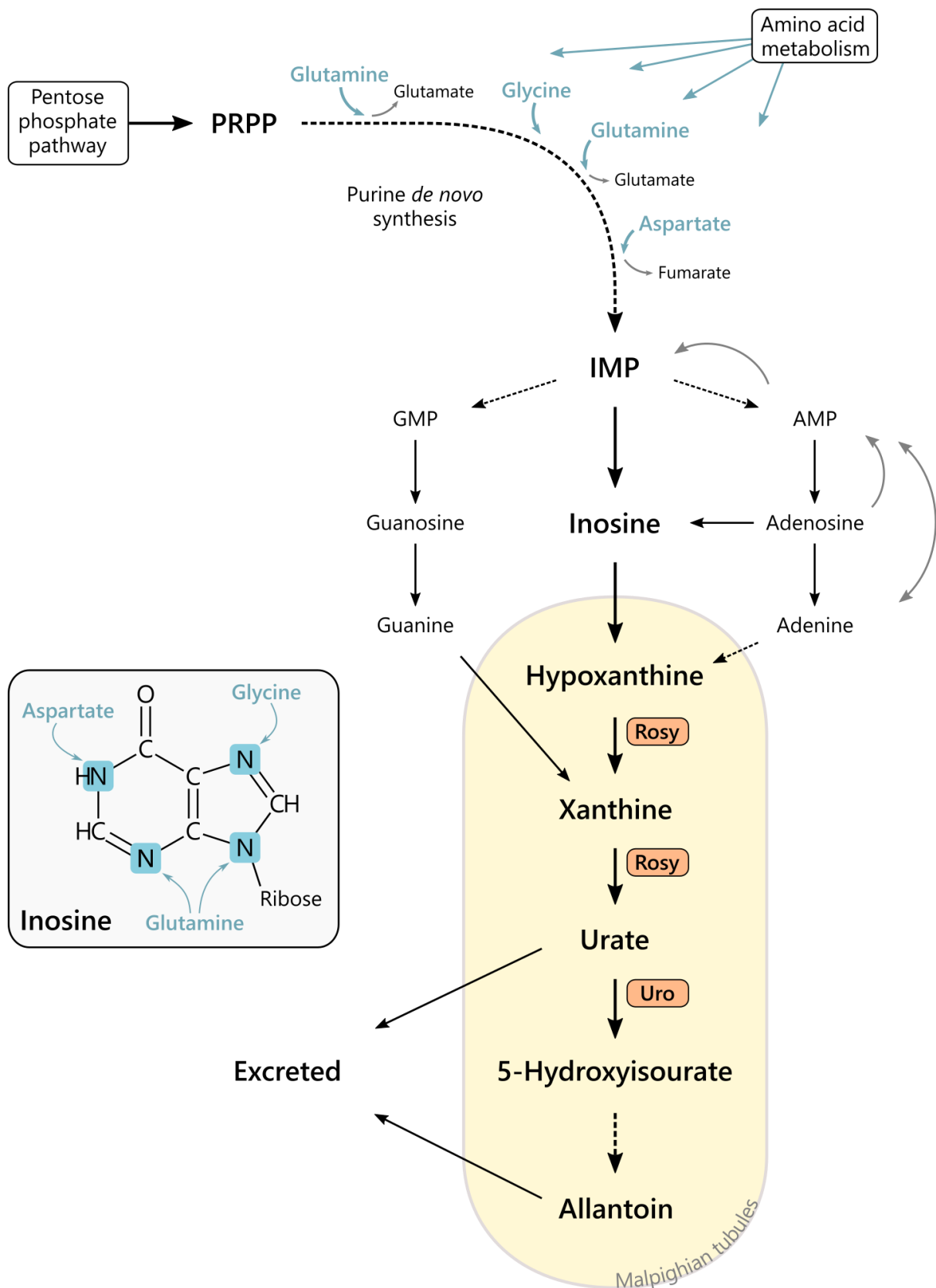


Figure 1.3: The nitrogenous waste pathway in *Drosophila melanogaster*

1.4.3.2 Non-uricotelic nitrogen disposal in *Drosophila*

While the majority of nitrogenous waste is disposed of via the uricotelic pathway, there are a number of exceptions. First, although lacking a complete urea cycle, the *Drosophila* genome encodes an arginase, the enzyme that hydrolyses arginine into ornithine and urea (Samson, 2000). This appears to be the only possible endogenous source of urea in flies. Ornithine is required as the precursor for polyamine synthesis.

Secondly, the enzymes that degrade histidine are not present in the *Drosophila* genome (Caspi *et al.*, 2020). Indeed, the only possible reaction for histidine in *Drosophila* appears to be its conversion to histamine which is important as a neurotransmitter in photoreceptor synapses and in cuticle pigmentation. Levels of histamine are controlled by its enzymatic condensation with β -alanine, forming carbinine, mediated by the enzyme encoded by *ebony* (Borycz *et al.*, 2002).

Thirdly, tryptophan has a separate catabolic pathway in which it is catabolised to kynurenine via the formyl-kynurenine intermediate (**Figure 1.4**). Kynurenine can then be converted to 3-hydroxy-kynurenine which is an important precursor in *Drosophila* for ommochrome synthesis, a class of pigments that function in the insect eye (Figon and Casas, 2019). The enzyme Kynurenine aminotransferase is able to convert kynurenine to kynurenic acid or 3-hydroxy-kynurenine to xanthurenic acid. Excess tryptophan is converted to kynurenine, but whether kynurenine is excreted or further catabolised is unclear. Interestingly, these tryptophan metabolites have been found to have various neurological and immune related activities in mammals (Cervenka, Agudelo and Ruas, 2017). A recent study by Kashio and Miura found that this pathway of tryptophan metabolism, and in particular kynurenic acid, was important in interorgan communication, with fat body-derived kynurenic acid promoting the regeneration of distal damaged tissues (Kashio and Miura, 2020).

Finally, there is some evidence that the Malpighian tubules are able to actively excrete ammonia itself (Browne and O'Donnell, 2013). The physiological importance of this finding is unclear.

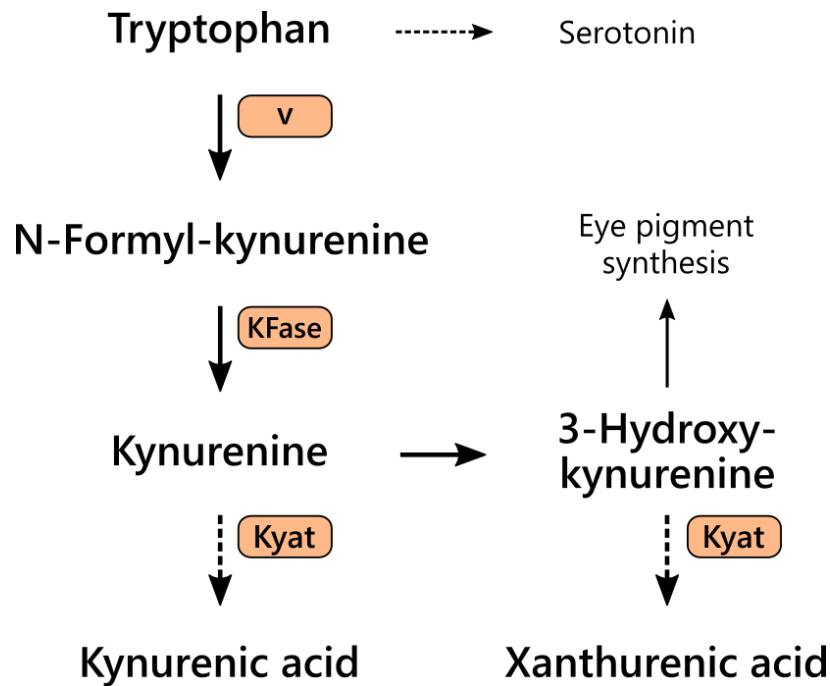


Figure 1.4: Tryptophan metabolism in *Drosophila melanogaster*.

1.4.4 Organisation of amino acid metabolism

At the level of the whole organism, the entirety of amino acid metabolism does not occur within one cell or cell type but involves multiple organs coordinating and compartmentalising synthesis, energy storage or generation, and waste disposal. For example, while the main source of protein that can be degraded is in muscle, gluconeogenesis occurs primarily in the liver in mammals, or in the fat body in insects. Furthermore, the disposal of the resulting nitrogenous waste is performed by the mammalian kidneys or the insect Malpighian tubules. Consequently, metabolic intermediates are transported in circulation and cycled between the organs allowing the required metabolic process to occur in the relevant tissue. In humans, for example, amino acid catabolism in the muscle is coordinated with gluconeogenesis and urea production in the liver. In the process, known as the Cahill cycle or the glucose-alanine cycle, aminotransferase reactions deliver nitrogen atoms from amino acid catabolism to pyruvate to form alanine which is then secreted into circulation. The alanine is subsequently converted back to pyruvate in the liver and used to fuel gluconeogenesis with the produced glucose entering circulation. In addition to providing energy to the rest of the body, the glycolysis of this glucose provides the skeletal muscle with more pyruvate to use as a nitrogen acceptor,

thus regenerating alanine. In the liver, the nitrogen atoms from alanine are delivered to the urea cycle, with the urea exported from the liver into circulation to be excreted by the kidneys.

1.4.4.1 The proline cycle

A fascinating amino acid metabolic cycle is found in many flying insects, which allows for continued catabolism of proline in the flight muscle to aid in meeting the extraordinary energy demand of these tissues during flight. Indeed, insect flight muscle has amongst the highest metabolic rate of any known animal tissue. Proline is catabolised to glutamate via the enzymes proline dehydrogenase, encoded by *sluggish A (SlgA)* in *Drosophila*, and pyrroline carboxylate dehydrogenase, encoded by *delta-1-pyrroline-5-carboxylate dehydrogenase (P5CDh1)*, and potentially also *CG6661*. This catabolism occurs in the mitochondria with the proline dehydrogenase reaction directly donating an electron to the electron transport chain (ETC), with ubiquinone the likely acceptor, and the pyrroline carboxylate dehydrogenase reaction generating NADH thus also contributing to the ETC. The proline-derived glutamate then donates the nitrogen atom to pyruvate forming α KG, which enters the TCA cycle, and alanine, which, similarly to the Cahill cycle, is exported to the hemolymph. Therefore, 2 carbons from the proline have been used for energy, with the remaining 3 carbons and the nitrogen atom remaining as alanine. The entry of the proline-derived α KG into the TCA cycle may also increase the efficiency of parallel carbohydrate fuel utilisation (Teulier *et al.*, 2016). The alanine is then taken up by fat body cells and used to resynthesise proline, using fatty acid derived acetyl-CoA to replace the lost carbon atoms, which is exported to the hemolymph for use by the flight muscle. In this way energy can be delivered, in the form of 2-carbon units, from the fat body lipid stores to the muscle. This proline cycle is believed to have a number of advantages. The high solubility of proline allows it to reach high concentrations in the hemolymph, providing a rapidly usable source of energy and an energy transport mechanism that does not rely on the use of bulky, poorly soluble lipid carriers; a potential advantage for small animals with limited water carrying capacity in their circulation. Additionally, in blood feeding insects, protein is the primary nutrient in their diet and storing large amounts of energy as circulating proline could be more efficient than directing all amino acids to gluconeogenesis and lipid synthesis. Different insects use this proline cycle to different extents: while tsetse flies predominantly use proline to fuel flight, blowflies and some beetles use

proline briefly before switching to carbohydrate, and honeybees may not use proline at all (Candy, Becker and Wegener, 1997; Teulier *et al.*, 2016). *Drosophila* are expected to rely predominantly on carbohydrate for flight but may possibly utilise proline as a supplemental fuel. Indeed, *Drosophila* with a deficiency in pyruvate import into the mitochondria show increased proline oxidation suggesting that they use proline as a mitochondrial fuel source under conditions of impaired carbohydrate metabolism (Simard *et al.*, 2020). Furthermore, proline is one of the most abundant metabolites in adult *Drosophila* hemolymph (data from flymet.org, University of Glasgow).

While the proline cycle in insects is associated with fuelling flight, could it perhaps be relevant to *Drosophila* immunity? This has not been investigated in *Drosophila*; however, proline metabolism has been implicated in insect host-pathogen interactions. For example, proline metabolism is essential for the parasite *Trypanosoma brucei brucei* in the tsetse fly stage of its lifecycle as it adapts to a low glucose environment and instead utilises host proline (Mantilla *et al.*, 2017). In another example, *in vitro* experiments suggest that the high levels of proline in insect hemolymph are used as a signal by the entomopathogenic bacteria *Photorhabdus luminescens* to trigger the production of virulence factors (Crawford, Kontnik and Clardy, 2010). Additionally, proline metabolism has been proposed as a clinically targetable node in a number of human host-pathogen interactions based on various, pre-dominantly *in vitro* studies, as reviewed in (Christgen and Becker, 2019).

Perhaps of relevance to fly immune function is the relationship between proline and reactive oxygen species (ROS). Indeed, proline derived ROS is been found to be involved in the *C. elegans* innate immune response (Tang and Pang, 2016). Proline catabolism by *C. elegans* proline dehydrogenase and pyrroline carboxylate dehydrogenase promoted survival in *Pseudomonas aeruginosa* infection via regulation of ROS, seemingly by the intermediate metabolite pyrroline-5-carboxylate (P5C). Production of P5C by proline dehydrogenase promoted a pro-survival response mediated by both ROS production by DUOX at the cell membrane and by activation of the SKN-1 transcription factor, which promotes expression of detoxification genes. As proline catabolism is linked to mitochondrial ROS generation, the authors posit that controlled mitochondrial ROS production via proline catabolism acts as an intracellular signal triggering the antibacterial and oxidative stress response in the cell.

Interestingly, in a model of high-sugar diet enhanced tumourigenesis in *Drosophila*, tumours upregulated the solute carrier transporter (SLC)-36, *pathetic (path)*, increasing their uptake of proline and promoting their growth (Newton *et al.*, 2020). This occurred in coordination with the induction of muscle tissue wasting, and thus replenishment of circulating proline, by tumour derived signals. As well as revealing that high-sugar diet enhanced tumours induce an organismal metabolic change to fuel their growth at the expense of the host, this work demonstrates the possibility that proline may function as a key energy carrier between organs in conditions of health or in responses to stresses such as infection.

This section on amino acids has emphasised their importance in metabolism beyond their function as the building blocks of proteins. Some amino acids perform specific metabolic roles; for example, tyrosine is required for the melanisation response. Additionally, amino acids can be catabolised for energy and/or used to support carbohydrate metabolism by replacing depleted TCA cycle intermediates. A critical aspect of amino acid catabolism is the disposal of excess nitrogenous waste, which, in *Drosophila* and other uricotelic animals, is a complex and costly process. Finally, amino acid metabolism is organised between tissues and some amino acids, potentially including proline in *Drosophila*, can play an important role in transporting resources between organ systems.

Nevertheless, the role of amino acids as the precursors for protein synthesis is clearly critical for immune function. The vast quantity of antimicrobial peptides produced by the *Drosophila* fat body in response to infection will in turn require a large quantity of amino acids for their synthesis. As amino acids used for protein synthesis cannot also be used for metabolic roles, demand for particular amino acids could potentially lead to trade-offs between different functions. Managing metabolism to optimise the total and individual levels of the proteogenic amino acids, as well as their location, during the immune response is likely to be of considerable importance.

1.4.1 Coordination of amino acid metabolism and protein synthesis

Amino acid metabolism must be carefully coordinated with protein synthesis and *vice versa*. A high level of coordination is required as there is no known dedicated, in-bulk storage form for amino acids in animal cells that is comparable to the storage of glucose in glycogen or lipids in lipid droplets. Although there are a number of possible examples of specialised storage of amino acids in animals. In humans, it is considered that one of the roles of the non-ribosomal tripeptide glutathione is to store the amino acid cysteine, as free cysteine is prone to auto-oxidation (Lu, 2009). Additionally, a mammalian epidermal protein, filaggrin, could be considered a protein used for amino acid storage as it is degraded by proteases at the skin surface to release its constituent amino acids, some of which are further metabolised, to form a collection of hygroscopic molecules. Together, these molecules, known as natural moisturising factor, aid the skin's moisture retention and barrier function (Rawlings *et al.*, 1994; Brown and McLean, 2012). Furthermore, there are two important examples of substances containing proteins that function to store amino acids: egg white and milk. However, these function to transfer amino acids to embryos or neonates rather than as amino acid storage for the producing organism.

This lack of bulk storage, combined with the fact that cells must avoid excessive build-up of free amino acids in their cytosol to prevent detrimental consequences due to changes in pH and osmolarity, means that a cell's amino acids are either in metabolic flux or incorporated in proteins. Consequently, a lack of free amino acids in a cell and its environment is remedied by upregulating endogenous protein degradation (discussed in **Section 1.5**), whilst also triggering what is known as the Integrated stress response (ISR) in which the cell inhibits the synthesis of all but the most high-priority proteins (Pakos-Zebrucka *et al.*, 2016). Connecting the detection of cellular amino acid depletion with these responses is primarily regulated by the mechanistic target of Rapamycin (mTOR) signalling pathway, inhibition of which triggers autophagy, and the kinase GCN2, which is able to initiate the ISR.

1.4.1.1 mTOR signalling

Free proteogenic amino acids must be available to charge tRNA for continued protein synthesis. As such, the level of free amino acids in the cell is an important input monitored by the mTOR signalling pathway, which coordinates cell growth and activity with resource availability and hormonal inputs, most notably from insulin signalling (Saxton and Sabatini, 2017; Texada, Koyama and Rewitz, 2020). Numerous signals converge to either promote or suppress activation of Tor Complex 1 (TORC1), the focal point of mTOR signalling which regulates a host of downstream effectors and signalling proteins. Under conditions of resource availability and permissive growth factor signalling, TORC1 promotes anabolic processes, including translation, synthesis of lipids and nucleotides, and cell growth/proliferation while also suppressing autophagy via inhibition of the ULK1 complex. Activated TORC1 promotes protein synthesis through a number of mechanisms, most notably by the phospho-activation of Ribosomal protein S6 kinase (S6k) and the phospho-inhibition of eukaryotic initiation factor 4E (eIF4E)-binding protein (4E-BP), also known as Thor in *Drosophila* (Saxton and Sabatini, 2017). Upon phosphorylation, S6k activates multiple targets to promote translation initiation, while inhibition of 4E-BP allows the assembly of the eukaryotic initiation factor 4F (eIF4F) complex. If a lack of resources is detected or hormonal signalling is inhibitory, TORC1 is inactivated leading to suppression of anabolic processes and the induction of autophagy. The resources that are monitored by mTOR signalling include glucose, oxygen, and ATP as well as amino acids. The activation of autophagic protein degradation when a deficit of amino acids is detected is essential to supply recycled amino acids to maintain continued protein synthesis.

How levels of amino acids are detected upstream of TORC1 in *Drosophila* is not yet fully known. Three sensors of cytosolic amino acid levels are so far known in mammalian cells: Sestrin2, CASTOR1, and SAMTOR specifically respond to leucine, arginine, and S-adenosylmethionine respectively, with the latter metabolite an important derivative of methionine (Saxton, Chantranupong, *et al.*, 2016; Saxton, Knockenhauer, *et al.*, 2016; Gu *et al.*, 2017). In all 3 cases, the sensors directly bind their cognate metabolite when it is present in sufficient concentration; however, when the concentration of their respective metabolite in cytosol falls, the sensor instead binds and inhibits the GATOR2 complex resulting in downstream inhibition of TORC1. While homologues for Sestrin2 and SAMTOR exist in *Drosophila*, they do not possess a

homologue of the arginine sensor CASTOR1. Lysosomal amino acid levels are also known to feed into mTOR signalling in mammals, but *Drosophila* have no known homologue of the implicated mammalian protein, SLC38A9 (Saxton and Sabatini, 2017). Given the role of the lysosome in protein breakdown and that activated TORC1 is located on the lysosomal surface, it is likely *Drosophila* also have a, so far unidentified, mechanism for linking amino acid levels in the lysosomal lumen to mTOR signalling.

Direct interaction between TORC1 and translation machinery further coordinates protein synthesis with amino acid availability. Work in *Drosophila* by Tsokanos *et al.* found that the eukaryotic eIF4F translation preinitiation complex physically associates with TORC1 and, furthermore, that the eIF4A subunit actively inhibits TORC1 in the absence of free amino acids (Tsokanos *et al.*, 2016). Thus, the regulation between TORC1 and the translation machinery is bi-directional: Activated TORC1 inhibits 4E-BP to allow the assembly of the eIF4F complex but, when amino acid supplies run low, the eIF4F complex itself deactivates TORC1. How the amino acids are sensed upstream of eIF4A is so far unknown, although NAT1, the fly homologue of mammalian eIF4G2, was found to be involved (Tsokanos *et al.*, 2016).

1.4.1.2 The integrated stress response

The integrated stress response (ISR) is a pathway that cells activate under various stresses including ER stress, oxidative stress, viral infection, and amino acid shortages. The main result of this response is an overall suppression of protein synthesis but with a contrary upregulation in the translation of stress responsive genes that promote recovery of cellular homeostasis (Pakos-Zebrucka *et al.*, 2016). The suppression of translation is mediated by the phosphorylation of eukaryotic initiation factor 2 subunit α (eIF2 α), preventing it from fulfilling its role in delivering initiator methionine to the translation preinitiation complex (Harding *et al.*, 2003). This phosphorylation can be performed by a number of kinases which each respond to different stresses. GCN2 (named after the yeast gene *General Control Non-depressible 2*) is the eIF2 α kinase that specifically responds to amino acid shortage. In yeast, GCN2 detects amino acid deprivation by binding deacylated (uncharged) tRNA (Lageix *et al.*, 2015). In metazoans, however, the picture appears more complex with evidence for additional, or alternative, mechanisms for GCN2 activation (Masson, 2019).

In addition to suppression of protein synthesis, the phosphorylation of eIF2 α results in the contradictory translation of Activating transcription factor 4 (ATF4). ATF4, also known as Cryptocephal (*crc*) in *Drosophila*, is able to regulate the synthesis of stress responsive proteins despite the suppression of translation during the ISR. In this way the cell can mount a response to the stress and, should this successfully restore cellular homeostasis, terminate the ISR (Pakos-Zebrucka *et al.*, 2016). How translation of *ATF4* mRNA is inhibited under normal conditions but promoted during the ISR, while not fully understood, is due to regulation by the presence of 2 small upstream open reading frames (ORFs) in the 5' untranslated region (UTR) of the *ATF4* mRNA (Lu, Harding and Ron, 2004; Hinnebusch, Ivanov and Sonenberg, 2016; Young and Wek, 2016). The first upstream ORF promotes the continued attachment of the 40S ribosomal subunit to the mRNA after its stop codon, allowing the subunit to continue moving along the transcript scanning for the next ORF. Under normal conditions, the 40S subunit is rapidly re-initiated by the eIF2 complex and translates the second upstream ORF. This blocks ATF4 peptide synthesis as this second upstream ORF contains the start codon of the main ORF but terminates well before a functional peptide is produced (Pakos-Zebrucka *et al.*, 2016). However, when eIF2 α is phosphorylated, eIF2 complex formation is inhibited, leaving fewer available to re-initiate the 40S subunit. Consequently, according to the proposed model, the 40S subunit travels further along the mRNA before re-initiation, increasing the chance that it misses the start codon of the second upstream ORF, but is reinitiated in time for the start codon of the actual ATF4 peptide encoding ORF. Recently however, work from the Ryoo and Teleman labs indicates the requirement of noncanonical initiation factors in ATF4 translation in *Drosophila* and in human cells (Bohlen *et al.*, 2020; Vasudevan *et al.*, 2020).

When translated as part of the ISR, ATF4 upregulates a variety of target genes that mount the cell's response to the stressor or promote cell death should this response fail. Importantly, ATF4 function is influenced by numerous interacting partners allowing the ATF4-induced transcriptional response to be tuned dependent on the ISR-inducing stress (Pakos-Zebrucka *et al.*, 2016). In this way, a single mechanism of induction can lead to the upregulation of different sets of relevant target genes. One key ATF4 target is Growth arrest and DNA damage-inducible protein 34 (GADD34), which promotes the restart of normal translation through upregulating eIF2 α dephosphorylation. Crucially, GADD34 translation is regulated by its 5' UTR in a similar manner to ATF4, resulting in inhibition of GADD34 synthesis under normal

conditions but its promotion under conditions of eIF2 α is phosphorylation (Lee, Cevallos and Jan, 2009). Consequently, the ISR results in a stall in translation that aids recovery of cellular homeostasis in a variety of conditions: the reduction of amino acid incorporation into proteins would help recover cytosolic amino acid concentrations, reduced delivery of new synthesised proteins to the ER would relieve ER stress, and translation of any viral genes would be suppressed. Meanwhile, the mRNA transcripts awaiting translational restart would be enriched with the stress-responsive, ATF4-induced targets.

A second central target of ATF4, 4E-BP, further influences ongoing protein synthesis (Kang *et al.*, 2017). As previously mentioned in **Section 1.4.1.2**, 4E-BP inhibits eIF4E to prevent recognition of the 5' cap of mRNA by the eIF4F complex, thus blocking translation. However, cap-independent mechanisms of translation exist and these are known to be used in cellular stress responses (Lacerda, Menezes and Romão, 2017). Cap-independent translation requires the mRNA transcript to possess a feature that allows for ribosomal recruitment without the requirement of the eIF4F complex. Importantly, the most well-known of these, an internal ribosomal entry site (IRES), is present in the transcript for 4E-BP itself, allowing it to bypass its own translational inhibition (Vasudevan *et al.*, 2017). In this way, a model presents for the effect on protein synthesis induced by the IRS. First, phosphorylation of eIF2 α results in a short period of severe translational inhibition in which only small number of mRNAs, such as ATF4 and GADD34, are able to be translated due to the special nature of their 5' UTRs. Then, ATF4 promotes dephosphorylation of eIF2 α via GADD34 to reactivate delivery of initiator Met-tRNA, while simultaneously inhibiting cap-dependent translation via 4E-BP. This results in a second period of protein synthesis that is heavily biased towards cap-independent mechanisms of translation, allowing the prioritisation of relevant, stress-responsive protein production (Ryoo and Vasudevan, 2017).

Thus, upon amino acid shortage, synthesis of new protein is inhibited by the combined action of mTOR pathway downregulation and the GCN2 activation of the integrated stress response. Additionally, cap-independent translation of stress-responsive genes is promoted by the transcriptional induction of 4E-BP and its release from TORC1 inhibition. Amino acid levels are restored through activation of macroautophagy due to TORC1 inhibition, supported by the transcriptional induction of key autophagy genes by the ISR (B'Chir *et al.*, 2013; Pakos-

Zebrucka *et al.*, 2016). To further support restoration of amino acid homeostasis, the ISR has been found, in various studies in mammalian cell culture, to induce the transcription of amino acid synthesis and transport genes (Lopez *et al.*, 2007; Ye *et al.*, 2010; Zhao *et al.*, 2016; Yang *et al.*, 2018).

1.4.1.3 Translational inhibition in bacterial infection

The suppression of host translation, while well known to be important in viral infection, has also been implicated in the innate immune response to bacterial infection across animal models (Lemaitre and Girardin, 2013). A study by Chakrabarti *et al.* demonstrated that translational inhibition occurs in *Drosophila* adults orally infected with *P. entomophila*, and that this was mediated both by activation of 4E-BP due to reduced inhibition by TORC1 and through the activation of the ISR by GCN2 (Chakrabarti *et al.*, 2012). This suppression of protein synthesis appeared to be a response to cellular damage, rather than to direct detection of the bacteria, as it was dependent on oxidative stress due to the host immune response and on Monolysin, a *P. entomophila* pore forming toxin. Interestingly, the inhibition of translation was found to be detrimental to the host in *P. entomophila* infection, as survival was improved by knock-down of *GCN2* or *AMPK α* , with the latter encoding the α -subunit of AMP-activated protein kinase (AMPK), an inhibitor of TORC1 signalling. Conversely, these same knockdowns showed reduced survival after oral infection with *Erwinia carotovora* 15; a bacterium which, while a strong activator of the immune response, is not usually lethal to flies under the conditions used (Buchon *et al.*, 2009). The authors suggested that these findings indicate that the translational blockage is a stress response to cellular stress that, while contributing to gut immunity in mild infection, is over-activated by damage to the epithelia in severe infections. This over-activation then contributes to pathogenesis by inhibiting the translation repair genes and immune effectors (Chakrabarti *et al.*, 2012; Lemaitre and Girardin, 2013).

Is the translational inhibition in bacterial infection simply a stress response triggered by infection induced damage, rather than part of the immune response itself? In the *P. entomophila* gut infection study above, the ISR was triggered by GCN2, which is canonically activated by amino acid deficiency (Chakrabarti *et al.*, 2012). Amino acid deficiency also results in deactivation of TORC1, as does activation of AMPK, which Chakrabarti *et al.* implicated as a

relevant signal in their study. AMPK is primarily known to be activated by an increase in the levels of AMP and ADP relative to ATP, and thus responds to loss of cellular energy homeostasis (Hardie, Schaffer and Brunet, 2016). Both amino acid deficiency and disturbance in energy homeostasis are likely consequences of membrane damage and/or oxidative stress. Consequently, a model presents in which translational inhibition is merely part of a general cellular stress response that happens to be activated by damage suffered during bacterial infections. However, it is likely the relationship between translational inhibition and immunity in *Drosophila*, and other animals, is more complex. Firstly, mTOR signalling is regulated by immune signalling and influences infection outcome. While studies investigating mTOR and immunity have focused on carbohydrate and lipid metabolism, given its close relationship with amino acid metabolism and protein synthesis, it would be more surprising if this side mTOR function was irrelevant to its involvement in immunity. Indeed, many studies use S6K as the read out of TORC1 activity, yet S6K is more directly relevant to amino acid metabolism and protein synthesis than to carbohydrate and lipid regulation (Saxton and Sabatini, 2017).

Secondly, cap-dependent translational inhibition by 4E-BP has been found to be important in *Drosophila* immunity beyond a non-specific response to cellular damage. Strikingly, a study by Vasudevan et al. investigating the role the ISR in infection found that 4E-BP-induced suppression of cap-dependent translation actually enhanced the production of antimicrobial peptides (Vasudevan *et al.*, 2017). Indeed, further experiments demonstrated that transcripts of 2 antimicrobial peptides, *Drosomycin* and *Attacin A*, as well as *4E-BP* itself, were able to be translated in a cap-independent manner dependent on their 5' UTRs. Consequently, it appears that the translational inhibition induced in bacterial infection by the ISR, likely in cooperation with the inhibition of mTOR signalling, is important for the immune response as it creates a cellular environment where immune effectors are preferentially translated. This finding is supported by their own observation that *4e-bp* mutant larvae show higher bacterial load after infection as well as by previous reports that found *4e-bp* mutants to have reduced survival in some bacterial infections and a fungal infection (Bernal and Kimbrell, 2000; Levitin *et al.*, 2007)

The upregulation in production of antimicrobial peptide transcripts in systemic immune activation in *Drosophila* is immense. Although so far unexplored directly, it can be speculated that suppression of cap-dependent translation may help to mitigate the increased strain on

the ER seen in infection, likely due, in part, to antimicrobial peptide production: reducing translation of low-priority transcripts would ensure only proteins of critical importance would be taking up trafficking, secretion, and quality control machinery (Troha *et al.*, 2018).

The production of large quantities of antimicrobial peptides requires the cell to have a ready, sustained supply of free amino acids. Consequently, we might venture that aspects of the cellular response will mirror responses seen in amino acid starvation. We have already seen that both situations can lead to inhibition of cap-dependent translation by the ISR and reduced TORC1 activity. It is also interesting to note that the same eIF2 α kinase, GCN2, initiates the ISR in both cases. How exactly GCN2 is activated by infection is not clear. It is feasible that damage to cell membrane would reduce intracellular amino acid levels and thus trigger GCN2 in the *P. entomophila* gut infection model used by Chakrabarti *et al.* (Chakrabarti *et al.*, 2012). This is unlikely, however, to explain the GCN2 activation in fat body seen in the Vasudevan *et al.* study; in this case, it could be that damage to the intestinal epithelium results in GCN2 activation due to systemic nutrient deprivation (Vasudevan *et al.*, 2017). Alternatively, it is interesting to speculate that the sudden induction of antimicrobial peptides, in itself, leads to an amino acid deficit triggering GCN2 and the ISR.

As well as inducing translational inhibition, reduction in mTOR signalling and the triggering of the ISR are associated with the induction of autophagy. Autophagy, along with regulation of amino acid transporters and enzymes, functions in nutrient starved cells to restore amino acid supply. Whether such responses in amino acid metabolism are important for proper humoral immunity in *Drosophila* has not, to my knowledge, been investigated.

1.5 Cellular protein degradation

How, and why, a cell breaks down its own protein is a large and complex field of study relevant to many aspects of physiology in both health and disease. Degradation of cellular protein in eukaryotes occurs either through the ubiquitin proteasome system (UPS) or through autophagy. Whereas the UPS targets proteins for degradation within protease complexes

called proteasomes, autophagy transports protein to the lysosome for degradation by hydrolytic enzymes. Two broad forms of autophagy mechanism exist in metazoans: macroautophagy (MA) and microautophagy (MI), both of which are highly conserved across eukaryotes. A third form, chaperone-mediated autophagy (CMA), is present in mammals and possibly birds (Mizushima *et al.*, 2008; Galluzzi *et al.*, 2017). When compared to the breakdown of ingested protein in the digestive system, these processes of cellular protein degradation are complex and elaborately regulated. Such careful regulation is necessary to prevent unchecked proteolytic activity which could lead to wanton destruction of critical cellular components.

1.5.1 The ubiquitin proteasome system

Proteasomes are highly abundant complexes found in the both the cytosol and nucleus that are chiefly responsible for the targeted degradation of both short-lived regulatory proteins and proteins that are misfolded or damaged. The structure of the complete proteasome (26S proteasome) is formed from two types of sub-complex; a core particle (also known as the 20S proteasome) resembling a hollow cylinder, plus a regulatory particle (19S proteasome) bound to either or both ends (Dikic, 2017). The core particle contains multiple, internally facing proteases of different types that rapidly and efficiently degrade protein into small peptides. Although the core particle can function on its own to degrade oxidised proteins, degradation of targeted proteins requires the regulatory particle. This complex recognises targets for degradation, unfolds the protein ready for proteolysis, and finally mediates its translocation into the core particle (Finley, Chen and Walters, 2016).

A protein is targeted for proteasomal degradation by being ubiquitinated; a reversible post-translational modification involving a cascade of enzymatic reactions mediated by ubiquitin-activating enzymes (E1), the ubiquitin-conjugating enzymes (E2), and the ubiquitin ligases (E3) (Yu and Matouschek, 2017). Together these enzymes sequentially add ubiquitin molecules to the target protein resulting in it being covalently labelled with one, or more, mono- or polyubiquitin tails. There are multiple types of ubiquitination with different forms resulting in different consequences for the target protein (Oh, Akopian and Rape, 2018). The most well-known of these is indeed the targeting to the proteasome via recognition of the polyubiquitin

chain by the regulatory particle. Other consequences include, the activation of the protein or its targeting to the secretory or endocytic pathways (Mukhopadhyay and Riezman, 2007; Komander and Rape, 2012). As such, ubiquitination can target proteins for degradation via autophagy; with the form of ubiquitination playing a role, along with other factors, in determining whether the protein is targeted to the UPS or to autophagy (Zientara-Rytter and Subramani, 2019).

In contrast to autophagy, the proteasome itself does not degrade proteins down to individual amino acids. Instead, the combination of proteases in the core particle cleaves the unfolded protein into numerous oligopeptides. In the case of *in vitro* rabbit muscle proteasomes, the majority of these oligopeptides were shorter than 8 amino acid residues in length (Kisselev *et al.*, 1999). Interestingly, this size distribution was found to be broadly comparable to that of the oligopeptides produced by prokaryotic proteasomes found in archaea suggesting the size of proteasomal products is conserved (Kisselev, Akopian and Goldberg, 1998). The fact that the proteasome produces oligopeptides means the complete degradation of the target protein to amino acids requires the activity of cytosolic proteases. Little is known about the identity of the proteases mediating this step, but the breakdown of these peptides is likely to be fairly rapid; an *in vitro* study using a human cell line (in the context of antigen presentation) found an oligopeptide of 9 amino acid residues in length had an intracellular half-life of around 7 seconds (Reits *et al.*, 2003).

Proteasomes are highly active in maintaining proteostasis: the proper functioning and turnover of the proteome. Damaged and terminally-misfolded proteins are targeted for degradation by the proteasome in order to prevent the build-up of harmful protein aggregates (Klaips, Jayaraj and Hartl, 2018). Additionally, the function of many regulatory proteins is in part controlled by their degradation by the UPS. Indeed, an example of this can be found in *Drosophila* immune signalling with the UPS involved in negatively regulating the Imd pathway via degradation of Tak1 or Imd (Khush *et al.*, 2002; Tsuda *et al.*, 2005; Thevenon *et al.*, 2009; Chen *et al.*, 2017; Kleino and Silverman, 2019). This UPS-mediated regulation of particular immune signalling components is the only published role for the proteasome in *Drosophila* immunity to date. In contrast, the mammalian proteasome plays an important additional role in immunity which is

absent in insects: the preparation of antigens for presentation to the adaptive immune system (Blum, Wearsch and Cresswell, 2013).

Traditionally the role of UPS has been considered mainly in light of its functions in protein control and regulation of cellular processes under normal conditions. In comparison, autophagy has usually been considered in its role as an adaptive response helping the cell survive and function under stress conditions, in particular nutrient starvation. However, the UPS is likely of importance in many stress responses whereupon factors are expressed that maintain proteasome assembly and abundance (Dikic, 2017). Under conditions of nutrient starvation, suppression of Tor signalling classically induces anabolic arrest and autophagy in order to provide resources for cell survival. However, inhibition of Tor signalling, or expression of the starvation-responsive FoxO3 transcription factor, in mammalian cell culture has been found to increase the activity of the UPS in degrading protein alongside the increased autophagy (Zhao *et al.*, 2007, 2015). Although this UPS upregulation could primarily be in order to degrade pro-growth proteins, it could be of critical importance for resource recycling. Indeed, mammalian cells and *Drosophila* with drug-inhibited proteasomes suffer lethality due to amino acid deprivation, rather than build-up of mis-folded protein, indicating the importance of the proteasome in providing amino acids for translation (Suraweera *et al.*, 2012)

In jawed vertebrates, the UPS is important for antigen presentation. In this process, proteins are degraded to small peptides, antigens, which are then presented on cell surface via major histocompatibility complexes (MHCs). Once on the cell surface the antigens are available for recognition by T-cells allowing for activation of the adaptive immune system. MHC class I (MHC-I) mediated antigen presentation occurs in all nucleated cells (Neefjes *et al.*, 2011). In this process a small proportion of the peptides produced by UPS-mediated protein degradation are rescued from complete degradation and are instead bound to MHC class I (MHC-I) molecules. The peptides are then transported to the cell surface where they are presented as antigens. An alternative form of the proteasome, the immunoproteasome, can be expressed in vertebrate cells and degrades proteins into peptides more optimised for antigen function (Kaur and Batra, 2016). *Drosophila* do not have vertebrate adaptive immunity and so do not process proteins for antigen presentation.

1.5.2 Macroautophagy

Macroautophagy is characterised by the formation of a large double-membrane vesicle called an autophagosome that forms around a part of the cytoplasm (Galluzzi *et al.*, 2017). The autophagosome subsequently fuses with a lysosome forming an autolysosome which results in the digestion of the internal phagosome membrane and, importantly, its contents (**Figure 1.5**). Unlike the UPS which exclusively acts on individual proteins, MA can process a broad range of substrates from proteins and lipid droplets to entire organelles and intracellular bacteria. MA can be non-selective, leading to the bulk degradation of large portions of cytoplasm, or selective, in which either particular substrates are targeted to an existing phagophore (the newly forming, incomplete autophagosome; also known as an isolation membrane), or a new phagophore is formed closely around the selected substrate (Zaffagnini and Martens, 2016). The mechanism of MA is well characterised and involves a host of highly conserved genes first identified in the yeast *Saccharomyces cerevisiae* together known as the autophagy-related gene (Atg) family (Galluzzi *et al.*, 2017; Mizushima, 2018).

1.5.2.1 Macroautophagy mechanism

As a full review of the regulation and mechanism of MA is beyond the scope of this chapter introduction, I will briefly outline the key stages and a selection of important effectors (**Figure 1.5**). Initiation of MA occurs via two complexes: the ULK1 (unc-51-like kinase 1) complex and the PI3KC3 (Class 3 phosphoinositide 3-kinase) complex 1 (Hurley and Young, 2017). The ULK1 complex in *Drosophila* is formed from Atg1, the yeast and *Drosophila* homologue of human ULK1 and ULK2 (unc-51-like kinase 2) isoforms; Atg13; Atg17, homologous to FIP200 (focal adhesion kinase family interacting protein of 200kDa); and Atg101 (Érdi *et al.*, 2012; Larkin *et al.*, 2020). Dependent upon its integration of multiple upstream signals, notably including TORC1 signalling which classically suppresses MA, the ULK1 complex triggers the creation of the new phagophore, a step known as nucleation, through phospho-activation of the PI3KC3 complex 1 (Russell *et al.*, 2013; Zachari and Ganley, 2017). This second complex is formed from Atg6, the yeast and *Drosophila* homologue of human Beclin1; Atg14 (also known as Barkor in humans); Pi3K59F (Phosphatidylinositol 3-kinase 59F), homologous to Vps34; and Vps15 (Érdi *et al.*, 2012; Larkin *et al.*, 2020). 2 other PI3KC3 complexes exist which lack Atg14 and are

involved in other processes including MI (see **Section 1.5.3**). In MA the PI3KC3 complex 1 performs the essential step of phosphorylating phosphatidylinositol (PtdIns) on the source membrane to PtdIns 3-phosphate (PtdIns3P) which allows elongation of the nascent phagophore around the autophagy substrate (Nascimbeni, Codogno and Morel, 2017).

The next key step in MA is the decorating of the growing autophagosome membrane with the small, ubiquitin-like Atg8 proteins. These proteins are also known as LC3 proteins after their mammalian orthologue, Microtubule-associated protein 1 light chain 3 (MAP1LC3 but commonly further abbreviated to LC3). *Drosophila* have 2 genes encoding Atg8 proteins, *Atg8a* and *Atg8b* (Scott, Schuldiner and Neufeld, 2004). *Atg8a* is ubiquitously and highly expressed whereas *Atg8b* expression is more restricted; however, any functional differences have not been characterised. Atg8 is incorporated into the phagophore membrane via its lipidation with phosphatidylethanolamine (PE). This is mediated by a cascade of reactions managed by the sequential involvement of Atg4, Atg7, Atg3, and the Atg16-Atg5-Atg12 conjugate and is essential for the formation of the autolysosome (Bento *et al.*, 2016; Nguyen *et al.*, 2016). Atg8 is also important in selective MA as it is able to link to target substrates in a process mediated by ubiquitination and autophagy receptor proteins such as p62, and its *Drosophila* orthologue refractory to sigma P (ref(2)P) (Nezis *et al.*, 2008; Gatica, Lahiri and Klionsky, 2018).

Prior to fusion with the lysosome, the autophagosome needs to be completed to form a double membrane vesicle by the sealing of the phagophore, a process recently identified as requiring the ESCRT-III (endosomal sorting complexes required for transport) complex (Takahashi *et al.*, 2018; Zhou *et al.*, 2019; Zhen *et al.*, 2020). Fusion of the outer autophagosome membrane with the lysosome is mediated by assorted tethering factors including Rab7 GTPase, the homotypic fusion and protein sorting (HOPS) complex, and the soluble N-ethylmaleimide-sensitive factor-attachment protein (SNAP) receptor (SNARE) proteins (Zhao and Zhang, 2019; Lőrincz and Juhász, 2020). The plethora of hydrolytic enzymes within the lysosome then breakdown the internal membrane and the contents within. Proteins are degraded down to individual amino acids which are exported from the lysosomal lumen by permeases.

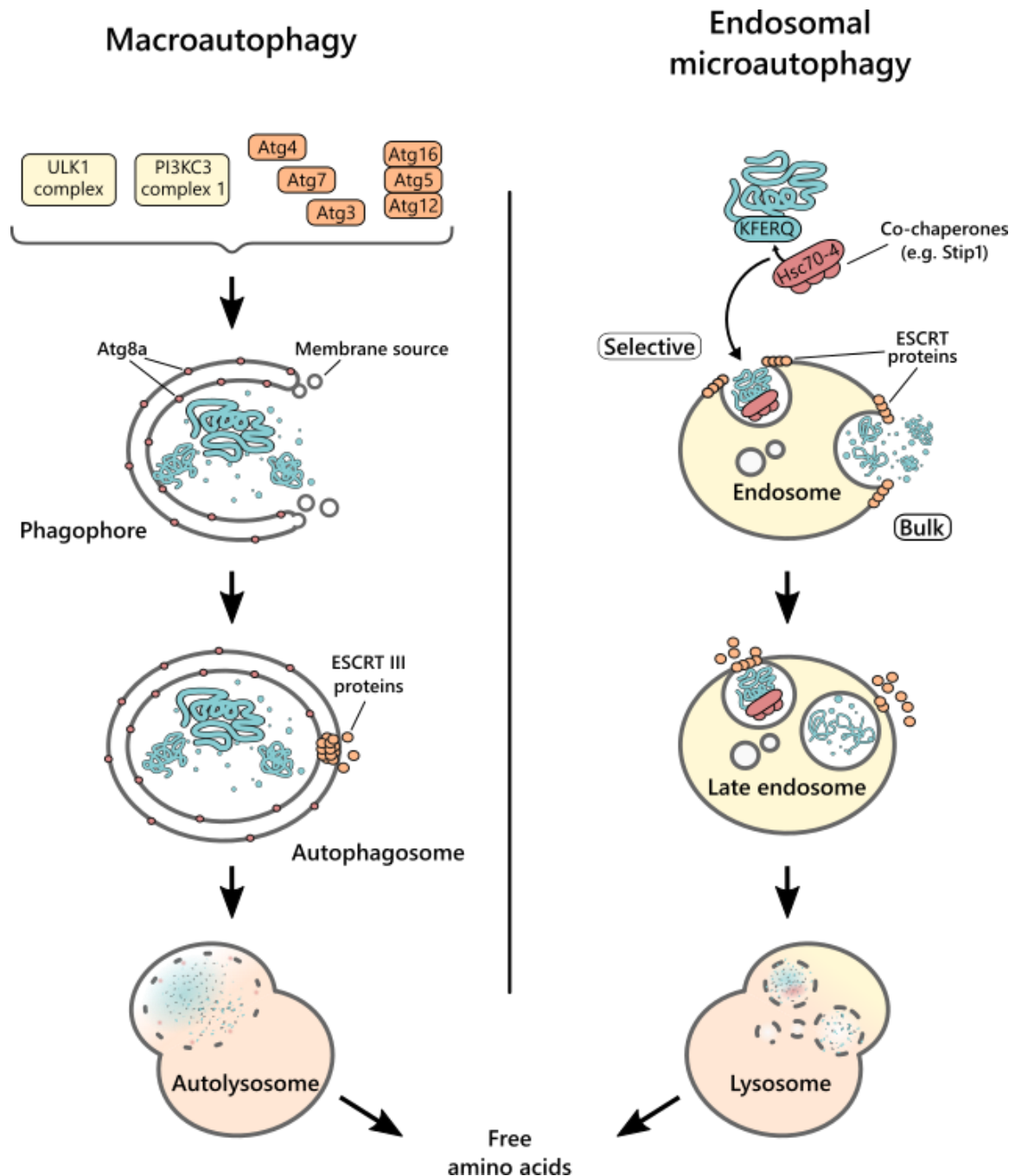


Figure 1.5: The two forms of autophagic protein degradation in *Drosophila melanogaster*.

Macroautophagy (left): ULK1 complex and PI3KC3 complex 1 act sequentially to initiate MA. Elongation of the phagophore requires the co-ordinated activity of Atg4, Atg7, Atg3 and the Atg16-Atg5-Atg12 conjugate to incorporate Atg8a into the phagophore membrane. ESCRT-III proteins seal the phagophore to form the autophagosome which then fuses with a lysosome forming the autolysosome. Endosomal microautophagy (right): Proteins are internalised into endosomes via membrane invagination either in bulk or selectively. In the latter, Hsc70-4 and its co-chaperones target proteins containing a KFERQ-like motif. ESCRT proteins are required for membrane invagination and formation of the intra-luminal vesicles. In both MA and eMI fusion with the lysosome leads to the degradation of internal membranes and their contents. Lysosomal hydrolases degrade proteins down to individual amino acids which are exported to the cytosol.

1.5.2.2 Functions of macroautophagy

Macroautophagy has numerous and varied roles and defective MA genes are implicated in a wide variety of human diseases (Levine and Kroemer, 2019). *Drosophila* have proved a valuable tool to investigate the functions of MA, as well as its mechanisms and regulation, *in vivo* (Nagy *et al.*, 2015; Bhattacharjee *et al.*, 2019). The multiplicity of MA's roles in an organism is nicely demonstrated by a single example: *Drosophila* lacking *Atg7*, while viable, show reduced oxidative-stress tolerance, signs of neuropathology, delayed development, reduced life-span, and an increased susceptibility to starvation presumably due to the observed inability of the *Atg7* deficient fat body cells to induce MA (Juhász *et al.*, 2007). Intriguingly, while *Atg7*-null mutants were viable, *Atg1*-null mutants die at the pupal stage indicating either that supposedly essential parts of the MA machinery are redundant for MA in some circumstances, metamorphosis in this case; or that *Atg1* has an essential function outside of its role in MA. Indeed, parts of the MA machinery are known to be used in non-autophagic processes such as in endosomal trafficking including LC3-associated phagocytosis (LAP) in which phagocytosed material is rapidly degraded by co-opting autophagy components (Heckmann *et al.*, 2017). To my knowledge, LAP has not yet been directly investigated in *Drosophila*. However, the key MA components involved, including LC3 (*Atg8a*) and the LC3 lipidation machinery, are present in fly phagocytic cells, suggesting they may perform LAP.

The functions of MA can be split into two broad categories: those that are based on MA providing raw materials, and those that are based on MA sequestering and disposing of unneeded or potentially damaging intracellular material (Morishita and Mizushima, 2019). The first category of roles of MA, provision of raw materials, is important for maintaining cellular homeostasis when resource demand outstrips supply. This situation occurs during cellular starvation and under perturbed metabolic conditions such as hypoxia. Continuing cell survival and function in these circumstances requires synthesis of prioritised proteins which in turn requires available amino acids. Should amino acids not be forthcoming from the cell's environment, MA can be upregulated to recycle amino acids from existing proteins which are likely to be of a lower priority. As well as providing the raw materials for translation, amino

acids liberated by MA can be catabolised for energy or used for gluconeogenesis to support the energy demands of other tissues (Ezaki *et al.*, 2011; Morishita and Mizushima, 2019). MA is also able to recycle and mobilise lipids, glucose, and iron by targeting lipid droplets, glycogen, and ferritin respectively (Kaur and Debnath, 2015).

The second category focuses on what MA is targeting, rather than what it is producing. An example that falls into this second category of sequester-and-eliminate functions is the proteostatic activity of MA performed in co-ordination with the UPS. Most cells are believed to be conducting MA at a low, basal level to promote protein turnover and, importantly, MA is able to target and eliminate protein aggregates which are too large to for proteasomal degradation. This is especially significant for long-lived cells such as neurons where old, damaged proteins have more time to accumulate and form toxic aggregates, a characteristic of neurodegenerative human diseases such as Alzheimer's disease, amyotrophic lateral sclerosis (ALS), and Parkinson's disease. Indeed, *Drosophila* has been used as a model to study the role of MA in these diseases (Bhattacharjee *et al.*, 2019). Other important cellular targets of MA include damaged or unneeded organelles; the best characterised of these processes is mitophagy: the MA-mediated clearance of mitochondria (Gatica, Lahiri and Klionsky, 2018; Pickles, Vigie and Youle, 2018).

1.5.2.3 Xenophagy

An important function of MA, included in the second category of sequester-and-eliminate roles, is its targeting of intracellular microbes and viruses; a process called xenophagy). Many microbes have evolved the ability to reside inside eukaryotic cells, either free in the cytosol or within vacuoles. Not only is the internal environment of the host cell rich in resources, it confers the invader protection from many aspects of the host's immune response such as professional phagocytes and circulating anti-microbial peptides or antibodies. Consequently, eukaryotic cells have innate, cell-autonomous immune processes that target intracellular invaders; processes which include using the MA machinery to selectively sequester the trespassers and deliver them to the lysosome (Randow, MacMicking and James, 2013). Of course, intracellular pathogens have evolved numerous ways to evade, and even exploit, host cell autophagy (reviewed in Siqueira, Ribeiro and Travassos, 2018; Jiao and Sun, 2019). Although, compared

with mammals, less is known about the cell autonomous immune response to intracellular invaders in *Drosophila*, the high conservation seen in MA across evolution suggests much of the xenophagy process is likely to be very similar (Moy and Cherry, 2013).

The key problem the host cell needs to overcome to perform xenophagy is the detection and targeting of the intracellular interloper. Once this is achieved, the MA machinery proceeds as usual, sequestering the target in an autophagosome before fusion with a late endosome/lysosome for degradation. There are 3 mechanisms for the targeting of intracellular bacteria for xenophagy in mammalian cells: ubiquitination, detection of membrane damage, and recognition of previously bound host proteins. Additionally, intracellular canonical pattern recognition receptors (PRRs) have been linked to xenophagy, though here the picture is less clear.

The first of these mechanisms is mediated by a number of E3 ligases including LRSAM1, Parkin, RNF166, and Smurf1. These are all able to coat the bacterium with ubiquitin leading to the recruitment of MA cargo receptors such as p62, NDP52, and optineurin to initiate xenophagy; however, the exact molecular substrates for this ubiquitination are poorly known (Huett *et al.*, 2012; Manzanillo *et al.*, 2013; Heath *et al.*, 2016; Franco *et al.*, 2017; Mitchell and Isberg, 2017). As well as triggering xenophagy, the ubiquitin coat can act as a signalling platform to induce NF- κ B signalling (Noad *et al.*, 2017; Van Wijk *et al.*, 2017). The study by Manzanillo *et al.* that found that the E3 ligase Parkin had a role in resistance to intracellular pathogens used *Drosophila* to validate their finding, strongly indicating this mechanism for pathogen targeting is conserved. Similar to their mouse counterparts, *Drosophila* mutants deficient for parkin were found to be sensitive to infection by a number of facultative intracellular bacteria, namely *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*), *M. marinum*, and *L. monocytogenes* (Manzanillo *et al.*, 2013). Interestingly, in both mammals and flies, Parkin is more well known for being a key mediator of mitophagy. These dual functions of Parkin nicely correlate with the endosymbiont-origin of mitochondria (Randow and Youle, 2014).

The second mechanism does not detect the bacterium itself but instead triggers xenophagy upon detection of damaged vesicular membranes (Randow and Youle, 2014). Such damage

can occur when pathogens, such as *S. Typhimurium* replicate within, or escape from, endosomal compartments. The internally facing membrane of endosomes possess host glycans that are not normally present in the cytosol. Consequently, these glycans can act as damage signals that are recognised by cytosolic proteins such as galectin-8 which accumulate on the damaged membrane (Thurston *et al.*, 2012). This accumulation of galectin-8 results in the recruitment of the NDP52 cargo receptor which acts as an “eat-me” signal both by recruiting the MA-initiating complexes ULK-1 and TBK1, and by binding to LC3 on the elongating phagophore membrane (Thurston *et al.*, 2016; Ravenhill *et al.*, 2019). This process culminates in the selective MA of the damaged vesicle and any residing bacteria. *S. Typhimurium*-containing vacuoles have also been found to be targeted for xenophagy by the presence of diacylglycerol (DAG) (Shahnazari *et al.*, 2010). What triggers the association of DAG with the *Salmonella* containing vacuole is unknown, but it seems that membrane damage is a likely candidate (Gomes and Dikic, 2014). The presence of host-enzyme-produced DAG, independently of ubiquitination, induced MA via PKC, JNK signalling, and NOX activity. The involvement of NOX activity indicates a similar induction mechanism to that seen in LC3-assisted phagocytosis (Heckmann *et al.*, 2017). Although much of the machinery is present, the occurrence of xenophagy triggered by the recognition of damaged membranes is not described in *Drosophila*.

Thirdly, the presence of host immunity proteins on intracellular microbes could potentially be an important inducer of xenophagy. Sorbara *et al.* recently found that *L. monocytogenes* and adherent-invasive *E. coli* that have been opsonised by the C3 complement protein prior to cytosolic invasion are targeted for xenophagy in epithelial cells (Sorbara *et al.*, 2018). As well as binding the bacterial surface, the C3 protein interacted directly with Atg16L resulting in increased xenophagy. Furthermore, bacteria that expressed proteases that detached their bound C3 were resistant to xenophagy in the epithelial cells. In addition to this apparent role in initiating xenophagy, there is increasing evidence that the complement system is able to positively regulate MA in a number of contexts, including in the absence of infection (King *et al.*, 2020; Viret *et al.*, 2020). In a broadly comparable detection mechanism, viruses that are bound by circulating antibodies prior to cytosolic invasion are recognised and targeted for UPS-mediated degradation by the E3 ligase Tripartite motif-containing protein 21 (TRIM21) (Mallery *et al.*, 2010). Recognition of intracellular antibodies by TRIM21 also induces NF- κ B

activity and other immune signalling pathways (McEwan *et al.*, 2013). As the binding is to the antibody rather than the pathogen itself, antibody coated bacteria are also recognised by TRIM21. There is some evidence that this may induce xenophagy as TRIM21 bound to antibody coated *Salmonella enterica* colocalises with LC3 (Rakebrandt *et al.*, 2014; Di Rienzo *et al.*, 2020). In this study, induction of xenophagy by TRIM21 was not fully confirmed; however, in the different context of immune-regulation, TRIM21 is able to induce selective MA of both IKK β and activated Interferon regulatory factor 3 (IRF3) (Niida, Tanaka and Kamitani, 2010; Kimura *et al.*, 2017). Consequently, it seems feasible that TRIM21 could mediate the xenophagy of antibody-labelled intracellular bacteria. *Drosophila* secrete a wide variety of antimicrobial peptides but the activity of many of them are poorly characterised. It is interesting to speculate that one or more of these antimicrobial peptides could act analogously to C3 in inducing xenophagy.

Pattern recognition receptors are also implicated in targeting bacteria for xenophagy. In mammals, the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), Nod1 and Nod2, are cytosolic PRRs. Both proteins bind specific fragments of bacterial peptidoglycan and subsequently initiate immune signalling by recruiting receptor-interacting serine-threonine kinase 2 (RIPK2) (Mukherjee *et al.*, 2019). Upon recruitment of RIPK2, both Nod1 and Nod2 have been found to associate with the MA protein Atg16L1. Travassos *et al.* found that, upon bacterial invasion of epithelial cells, the Nods were found to interact with Atg16L at the site of entry through the cell membrane (Travassos *et al.*, 2010). This was independent of their role in inducing downstream signalling and was found to induce autophagosome formation. Whether this mechanism acts to induce xenophagy of the invading bacterium or is triggering another function of MA is unclear (Sorbara and Girardin, 2015). Such an alternative function could be acting to control damage caused by the bacteria as has been observed in two cases (Keller, Torres and Cadwell, 2020). In first, Maurer *et al.* found that mice with a hypomorphic *Atg16L* allele had increased mortality in *S. aureus* infections due to a reduction in tolerance rather than resistance (Maurer *et al.*, 2015). Further investigation indicated that Atg16L was acting to counter damage caused by the pore-forming *S. aureus* α -Toxin. In the second study by Tan *et al.* the Atg16-Atg5-Atg12 conjugate was shown to be critical for repair of membrane damage caused by the *L. monocytogenes* toxin listeriolysin O in mouse embryonic fibroblasts

(Tan *et al.*, 2018). Intriguingly, this process was found to be independent of much of the rest of the canonical MA machinery.

Drosophila PGRP-LE is a PRR that has both a shortened extracellular form and a full-length intracellular form with functional parallels to mammalian Nod1 and Nod2 (Kaneko *et al.*, 2006; Moy and Cherry, 2013). As in mammals, *L. monocytogenes* is facultatively intracellular when infecting *Drosophila*. *L. monocytogenes* is phagocytosed by hemocytes but subsequently escapes the phagosome and invades the cytosol. Cytosolic bacteria are then recognised by PGRP-LE binding to their DAP-type peptidoglycan resulting in activation of JAK-STAT signalling and antimicrobial peptide expression (Goto *et al.*, 2010). In addition, Yano *et al.* demonstrated that PGRP-LE also induces xenophagy of the bacterium as green fluorescent protein- (GFP) labelled LC3 autophagosomes co-localised with cytosolic *L. monocytogenes* (Yano *et al.*, 2008). This co-localisation was dependent on PGRP-LE expression and also did not occur when the hemocytes were infected with a mutant *L. monocytogenes* that was unable to escape into the cytosol. The mechanism of how PGRP-LE recruits the autophagosome machinery has not yet been characterised. Although PGRP-LE is widely expressed in different tissues with high expression in the midgut, whether non-phagocytosing *Drosophila* cells can perform PGRP-LE induced xenophagy is unknown (Larkin *et al.*, 2020). Of note, the cytosolic presence of lysine-type peptidoglycan, which is not bound by PGRP-LE, also induced autophagy, indicating the presence of at least one unidentified intracellular PRR in *Drosophila* (Yano *et al.*, 2008; Péan and Dionne, 2014).

1.5.3 Microautophagy

Microautophagy (MI) is the second autophagic process found in eukaryotes and is less well characterised than MA. In MI, substrates are directly invaginated through the membrane of lysosomes to form a substrate-containing intraluminal vesicle which is subsequently degraded together with its contents. Alternatively, in a process termed endosomal microautophagy (eMI; see **Figure 1.5**), the substrate is delivered in the same way to the lumen of a late endosome, also known as a multi-vesicular body, which then fuses with the lysosome (Sahu *et al.*, 2011; Galluzzi *et al.*, 2017). In this way, eMI integrates with the endocytic pathway used to deliver

extracellular material and cell membrane proteins to the lysosome for degradation. Consequently these processes use much of the same machinery notably including the ESCRT proteins (Lefebvre, Legouis and Culetto, 2018).

1.5.3.1 Selective endosomal microautophagy

Like MA, eMI is able to degrade proteins non-selectively or proteins can be selectively targeted. In non-selective eMI, vesicles forming at the endosomal membrane simply trap any encircled cytosolic proteins. In contrast, selective eMI requires the binding of the HSC70 (heat shock cognate 70kDa protein), also known as HSPA8 (Heat shock 70kDa protein 8), chaperone to proteins containing a KFERQ-like motif; a process conserved between mammals and flies (Uytterhoeven *et al.*, 2015; Mukherjee *et al.*, 2016). *Hsc70-4* (Heat shock protein cognate 4), the *Drosophila* orthologue of HSC70, is highly, and ubiquitously, expressed in *Drosophila* tissues (Larkin *et al.*, 2020). A thorough study of Hsc70-4 activity in synapses by Uytterhoeven *et al.* revealed that Hsc70-4 function in eMI relies on its ability to deform membranes allowing for invagination of itself and its bound cargo into late endosomes (Uytterhoeven *et al.*, 2015). Hsc70-4 also has an alternative, and better characterised, function in binding and refolding misfolded proteins in a wide variety of contexts. Which function Hsc70-4 performs is regulated by co-chaperones and ATP levels, and both roles are important for maintaining optimal synaptic function (Uytterhoeven *et al.*, 2015). Co-chaperones include Small glutamine-rich tetratricopeptide containing protein (Sgt), which promotes the re-folding function of Hsc70-4 and inhibits its degradative function, and Stress induced phosphoprotein 1 (Stip1). Stip1 mediates the interaction of HSC70 with HSP90, another chaperone involved in protein refolding. However, Stip1 may promote Hsc70-4's degradative function as it has been found to co-localise with Hsc70-4 at the lysosome and is required for lysosomal translocation of targeted protein *in vitro* (Dice, 1990; Agarraberes and Dice, 2001). Whether Stip1 is involved in selective eMI in *Drosophila* is unknown; however, it has been implicated as having a role in protective protein degradation in a genetic screen of a *Drosophila* model of tau-induced neuropathy (Ambegaokar and Jackson, 2011)

The KFERQ-like motif, as the name suggests, is not necessarily an amino acid sequence of K-F-E-R-Q (lysine-phenylalanine-glutamate-arginine-glutamine), but a sequence that

biochemically resembles KFERQ (Dice, 1990; Kirchner *et al.*, 2019). Such a sequence must contain: 1 or 2 positively charged residues of either K or R; a hydrophobic portion of one or two of I, L, V, or F; and a negatively charged E or D. These amino acids can appear in any order, but the sequence must have a glutamine either at the end or the start. Increased flexibility of the motif is added by the fact that post-translational acetylation or phosphorylation can convert a “putative” KFERQ-like motif into a targetable KFERQ-like motif. For example, acetylation of lysine can allow it to take the place of the glutamine. KFERQ-motifs are common in the proteome; one or more KFERQ-like motifs are present in about 51% of reviewed *Drosophila* proteins covering a wide variety of protein classes and functions (Kirchner *et al.*, 2019; Jacomin *et al.*, 2020). Additionally, approximately a further 31% of the reviewed proteome contains sequences theoretically able to be post-translationally modified into a KFERQ-motif (Kirchner *et al.*, 2019). Whether a protein’s KFERQ-like motif is accessible to the Hsc70-4 chaperone is an important consideration; targeting by eMI could be prevented by the motif being buried within the protein structure or the protein’s location could prevent eMI degradation, as would be the case for extracellular and transmembrane proteins. Interestingly, however, in humans, KFERQ-like motifs are enriched in protein domains associated with protein-protein interactions and in cytosolic proteins indicating a higher-than-chance accessibility of the motif. Thus, the fraction of the proteome able to be targeted by eMI is likely to be considerable.

1.5.3.2 Functions of endosomal microautophagy

Very little is known about the physiological roles of eMI in animals, especially in comparison to the many roles of MA that have been described. Studies in *Drosophila* have begun to establish roles for eMI in both constitutive and stress response processes. Shortly after the finding by Uytterhoeven *et al.* that selective eMI plays a constitutive role in synapse function, Mukherjee *et al.* demonstrated the induction of selective eMI in *Drosophila* larval fat body during starvation (Mukherjee *et al.*, 2016). The process was identified as eMI due to the demonstrated dependence on ESCRT-I, -II, and -III as well as the recognition of the KFERQ-like motifs by Hsc70-4. Furthermore, the induction was found to be independent of essential MA genes *Atg5*, *Atg7*, and *Atg12*, demonstrating the separate machinery of these processes. Interestingly, this study found that the induction of selective eMI was, like MA, regulated by

Tor signalling and the ULK1 complex. However, the temporal separation between MA (from 1-hour post-starvation) and eMI (from 12 hours post-starvation) indicates such regulation is not identical.

Later work by the same group (the Jenny lab) found eMI to also be upregulated in the larval fat body in response to both oxidative stress and DNA damage (Mesquita, Glenn and Jenny, 2020). In these cases, the induced eMI was observed to sequester protein both non-selectively and selectively in a Hsc70-4/KFERQ-like motif manner. In oxidative stress, MA is induced by JNK signalling, and this was also found to be the case for eMI. Interestingly however, JNK signalling appears to work differently in inducing the two forms of autophagy with genetic activation of the JNK kinase *Hep* sufficient for inducing MA but insufficient for the induction of eMI (Wu, Wang and Bohmann, 2009; Mesquita, Glenn and Jenny, 2020). Curiously, Mesquita *et al.* found that eMI was not induced in response to ER stress, indicating that the triggering of eMI upregulation discriminates between cell stresses.

Selective eMI also appears to have a low, basal level of activity in fat body important for metabolic regulation. *Arouser* (*aru*) encodes a signalling protein that is important for regulation of lipid metabolism during starvation (Jacomin *et al.*, 2020). *Aru* contains a KFERQ-like motif and, interestingly, under fed conditions *Aru* is degraded by selective eMI. Upon starvation, *Aru* is somehow protected from being targeted for eMI which allows it to accumulate and promote organismal adaption to starvation (Jacomin *et al.*, 2020). Consequently, selective eMI appears to have both basal regulatory activity and starvation-responsive, high induction within the same tissue.

1.5.4 Chaperone-mediated autophagy

An additional form of autophagy related to MI, chaperone-mediated autophagy, is found in some animals. CMA consists of the targeted delivery of proteins into the lysosome through a translocation complex containing the splicing isoform LAMP peptide2A (Lysosome-associated membrane protein 2, isoform A) (Levine and Kroemer, 2008; Galluzzi *et al.*, 2017). Like selective eMI, the targeting of proteins requires the HSC70 chaperone binding to KFERQ-like motifs. In

contrast to MI however, proteins must be unfolded for translocation into lysosome (Salvador *et al.*, 2000). As the essential LAMP peptide2A isoform is restricted to mammals and possibly birds and fish, *Drosophila* are assumed to be unable to perform CMA (Galluzzi *et al.*, 2017; Lescat *et al.*, 2018). This assumption is supported by the fact that Lamp1, the closest *Drosophila* homologue to LAMP peptide2A, lacks the important amino acid residues for CMA and does not bind Hsc70-4 in co-immunoprecipitations (Uytterhoeven *et al.*, 2015).

In mammals, the physiological functions of CMA are much better known than those of MI. Like the UPS, CMA supports the maintenance of proteostasis by degrading individual oxidised proteins and defects in CMA are associated with neurodegeneration (Kaushik and Cuervo, 2018). In addition, CMA performs numerous cellular roles based on the targeted degradation of particular proteins, often in response to stresses. For example, in response to cellular starvation in mouse livers, CMA has been found to degrade both lipogenic enzymes and perilipins thereby downregulating TAG synthesis and enabling lipolysis and lipophagy by exposing lipid droplets (Schneider, Suh and Cuervo, 2014; Kaushik and Cuervo, 2015). Given the sharing of KFERQ-like motif containing substrates, *Drosophila* selective eMI could be performing many of the roles of CMA found in mammals.

1.5.5 Endocytosis

Endocytosis mediates transport of extracellular and cell surface material into the cell whereupon the endocytic pathway sorts and delivers endocytosed material to its appropriate destination. If the material endocytosed for degradation it is trafficked to lysosomes. In endocytosis a portion of the plasma membrane buds into the cell and detached from the cell surface to form an intracellular vesicle. This occurs by a number of mechanisms, but all lead to the internalisation of plasma membrane along with any bound proteins and enclosed extracellular material (Doherty and McMahon, 2009). The endocytic pathway is the subsequent sorting and trafficking of these newly formed intracellular vesicles culminating in their recycling, secretion, or degradation (Scott, Vacca and Gruenberg, 2014). Shortly after internalisation, intracellular vesicles fuse with early endosomes which tend to reside near the cell periphery. These act as sorting hubs. Much of the endocytosed membrane and bound

protein is normally recycled back to the cell surface, however endocytosed material can also be sorted for degradation in which case the material is retained by the endosome. Membrane bound proteins targeted for degradation are internalised into the endosome lumen by the budding of the membrane to form intraluminal vesicles. This membrane shaping is in the opposite direction to that of the earlier endocytosis as the membrane buds away from the cytosol. This requires the activity of ESCRT proteins (see **Section 1.5.6.2** below) and is a similar process to that seen in MI. As an early endosome matures into a late endosome, its pH decreases, it travels away from the cell surface, and it accumulates intraluminal vesicles (Huotari and Helenius, 2011). At this point the endocytic pathway integrates with the autophagic processes: eMI is conducted by late endosomes and autophagosomes can merge with late endosomes to form amphisomes. As in autophagy, the degradation of the endocytosed membrane bound proteins, and any internalised extracellular proteins, occurs upon fusion with the lysosome.

All metabolically active cells perform endocytosis continuously for numerous purposes. The main constitutive purposes of endocytosis are uptake of nutrients not amenable for transmembrane transport, notably including those bound to carrier proteins such as lipids, cholesterol, and iron, and the turnover of cell membrane and cell membrane proteins (Antonescu, McGraw and Klip, 2014). As well as these functions, endocytosis has several more selective, situational, or cell-dependent roles which frequently involve the use of endocytosis to regulate responses to extracellular signalling through removing receptors or transporters from the cell surface. The endocytosis of transmembrane proteins can be non-selective due to bulk membrane turnover or highly selective through ubiquitination of the cytoplasmic portion of the target. Sorting of cell surface receptors at the early endosome can also regulate the cellular response to future extracellular signalling: signal receptors can be recycled back to the surface after ligand removal to ensure continued sensitivity of the cell to the signal or directed to the lysosome for degradation to downregulate sensitivity of to the signal (Platta and Stenmark, 2011). As the cytosolic portion of endocytosed transmembrane proteins remains available on the surface of the early endosome, signalling can continue until protein internalisation via intraluminal vesicle formation. This presents further opportunity for modulation of signalling activity, indeed, TGF β signalling may even require endocytosis of the

receptor before continued signal transduction (Hayes, Chawla and Corvera, 2002; Sorkin and Von Zastrow, 2009).

Endocytosis has multiple activities in the context of immunity. Firstly, phagocytosis is in fact a form of endocytosis which, in multicellular organisms, is only performed by specialist cells with hemocytes being the sole phagocytosing cells in *Drosophila*. Secondly, endocytosis is, by its definition, the mode of entry for intracellular pathogens into the cell though they do this through a wide variety of different mechanisms. This can be a passive process for the pathogen, for example being phagocytosed, or the pathogen may actively induce its endocytosis (reviewed in (Cossart and Helenius, 2014)).

Endocytosis is important to consider in the context of endogenous protein degradation as it is able to access proteins inaccessible to the UPS and autophagy, namely membrane bound proteins and extracellular proteins. Additionally, machinery is shared between endocytosis and autophagy, particularly as the pathways converge.

1.5.6 Shared autophagic machinery

Mechanistically there are overlaps between endocytosis, eMI, and MA. In particular, late endosomes and autophagosomes share many similarities including how they are recognised and fused with lysosomes. Indeed, in animals, autophagosomes can fuse with late endosomes to form amphisomes prior to lysosome fusion. Completion of MA, eMI, and endocytosis requires tethering factors including Rab7, the HOPS complex, SNARE proteins, and the presence of PtdIns3P on the membrane. Furthermore, ESCRT proteins are, to greater or lesser extent, necessary for the re-modelling of vesicular membranes in all these processes. Of these shared machineries, I will discuss the PI3KC3 complex and the ESCRT proteins as these were found to be of particular importance in this project.

1.5.6.1 The PI3KC3 complexes

Whereas the phosphorylation of PtdIns on the phagophore membrane is performed by PI3KC3 complex 1, endosomal membrane phosphorylation is mediated by PI3KC3 complex 2 (Backer, 2016). PI3KC3 complex 2 regulates endosome maturation and endosome-lysosome fusion and is thus required for both eMI and endocytosis. This second PI3KC3 complex is made of the same components as complex 1, but with the protein Uvrag (UV-resistance associated gene) taking the place of Atg14 in a mutually exclusive manner (**Figure 1.6**) (Itakura *et al.*, 2008). Whether Atg6 is bound to Atg14 or Uvrag consequently determines the target of the complex.

In the context of autophagy, the function of PI3KC3 complex 2 is contentious with research conflicting on its requirement in MA for autophagosome-lysosome fusion (Liang *et al.*, 2008; Takáts *et al.*, 2014; Backer, 2016; Quach *et al.*, 2019). Research in *Drosophila* suggests that while PI3KC3 complex 2 is required for various endosome-related functions including endocytosis and indeed eMI, it is dispensable for MA (Lee *et al.*, 2011; Lőrincz *et al.*, 2014; Hegedűs *et al.*, 2016). Observations of PI3KC3 complex 2 requirement for completion of MA could potentially be explained by the importance of endosomal trafficking for biogenesis of lysosomes (Jacomín, Fauvarque and Taillebourg, 2016). This explanation holds that apparent defects in MA seen upon inhibition of PI3KC3 complex 2 are due, not to defective autophagosome-lysosome fusion, but to failure of defective lysosomes in degrading the autophagy substrates (Takáts *et al.*, 2014; Hegedűs *et al.*, 2016)

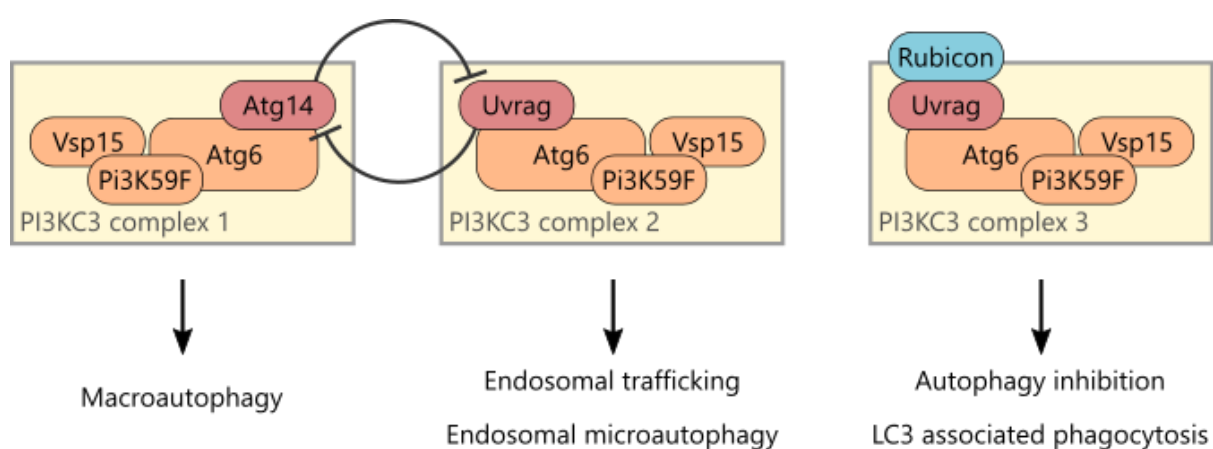


Figure 1.6: The three PI3KC3 complexes and their functions.

The PI3KC3 complexes all contain the 3 core proteins Vps15, the kinase Pi3k59F, and Atg6. Which function the complex performs is dictated by the binding of additional proteins, most importantly Atg14, Uvrag, and Rubicon. The functions associated with each complex are shown. Of note, LC3-associated phagocytosis is uncharacterised in *Drosophila* and some evidence in mammalian studies suggests PI3KC3 complex 2 also functions in MA.

The PI3KC3 complexes 1 and 2 are both heavily regulated by post-translational modifications and the attachment of additional proteins (reviewed in Backer, 2016). One such protein is Rubicon which binds to Uvrag in PI3KC3 complex 2 resulting in the inhibition both of this complex and of the PI3KC3 complex 1 as Atg14 is blocked from usurping Uvrag (Matsunaga *et al.*, 2009; Zhong *et al.*, 2009). In mammalian cells, this inhibitory complex, termed PI3KC3 complex 3 (**Figure 1.6**), is promoted by mTORC1 phosphorylation of Uvrag and suppresses both MA and, by blocking endosome maturation, eMI (Kim *et al.*, 2015). Interestingly, kinase activity of Vps34 is not entirely blocked as PI3KC3 complex has been found to function as the PtdIns-kinase mediating LAP in mouse macrophages (Martinez *et al.*, 2015; Wong, Sil and Martinez, 2018). The inhibition of autophagy by Rubicon is conserved between mammals, *C. elegans*, and *Drosophila* (Nakamura *et al.*, 2019). However, whether it aids phagocytosis in fly hemocytes is so far unknown.

1.5.6.2 The ESCRT proteins

The discussions of MA, eMI, and endocytosis above all mentioned the involvement of ESCRT and related proteins. These proteins mediate processes that involve the budding of a membrane out from the cytosol and its subsequent scission to form a vesicle on the non-cytosolic side of the membrane. This membrane manipulation can be viewed as the opposite of the type involved in endocytosis and the (conventional) secretory pathway in which the vesicle buds off into the cytosol. Consequently, the ESCRT proteins mediate what is described as “reverse-topology” membrane budding and scission (Schöneberg *et al.*, 2016). Multiple cellular processes require this form of membrane remodelling and so require the activity of some, or all, of the ESCRTs. As well as eMI and MA, such processes include cytokinetic abscission, neural pruning, plasma membrane repair, post-mitotic sealing of the nuclear envelope, and viral budding (Vietri, Radulovic and Stenmark, 2020).

The ESCRT machinery comprises of 5 complexes, ESCRT-0, -I, -II, -III, and the vacuolar protein sorting 4 (Vps4)-complex, plus a number of accessory proteins (see **Table 1.2**). While ESCRT-III and Vps4 are so far believed to be involved in all ESCRT-related activities, the other complexes are differentially involved dependent on the process being performed (Vietri, Radulovic and Stenmark, 2020). ESCRT-0, -I, -II are involved in recruiting and activating ESCRT-III proteins, in part through initiating membrane curvature. Upon activation, the ESCRT-III proteins polymerise forming membrane-interacting filaments. How exactly these filaments remodel the membrane and what roles each ESCRT-III component performs are not well characterised and a number of mechanistic models exist (Schöneberg *et al.*, 2016; McCullough, Frost and Sundquist, 2018; Caillat *et al.*, 2019). Broadly speaking, the ESCRT-III filaments are believed to form a circular or spiral-like structure on the curved membrane surface. Then, with the involvement of Vps4 hexamers, this structure constricts or buckles, possibly into 3-dimensional helix or dome, in order to pull the membrane “neck” together, eventually resulting in scission. Finally, the Vps4 complex, comprising Vps4 and Vesicle trafficking 1 (Vta1), mediates the disassembly of the ESCRT-III machinery in an ATP hydrolysis-dependent manner.

Complex	<i>Drosophila</i>	Human
ESCRT-0	Hrs	HRS
	Stam	STAM-1
ESCRT-I	TSG101	TSG101
	Vps28	VPS28
	Vps37A, B	VPS37A, B, C, D
	Mvb12	MVB12A, B
ESCRT-II	Lsn	EAP30
	Vps25	EAP20
	Vps36	EAP45
ESCRT-III	Vps2	CHMP2A
	CHMP2B	CHMP2B
	Vps20	CHMP6
	Vps24	CHMP3
	Shrb	CHMP4A, B, C
ESCRT-III auxiliary	Chmp1	CHMP1A, B
	Vps60	CHMP5
	IST1	IST1
	CG5498	CHMP7
Vps4 complex	Vps4	VPS4A, B
	Vta1	LIP5

Table 1.2: The ESCRT proteins.

The *Drosophila* ESCRT components are listed together with their corresponding human protein(s) (Li and Blissard, 2015; Larkin *et al.*, 2020; Vietri, Radulovic and Stenmark, 2020).

<i>Drosophila</i>	Human	Notes
ALiX	ALIX	Alternative ESCRT-III recruitment. Binds TSG101, Shrub
Mop	PTPN23 (AKA: HD-PTP)	Stabilisation of Hrs. Implicated in EGFR-and Toll signalling. Alternative ESCRT-III recruitment?
POSH	SH3RF1	Endosome surface scaffold? E3-ligase activity vs. HRS? Binds ALiX
Lgd	CC2D1A, B	lethal (2) giant discs 1, physically interacts with shrub and might regulate shrub activity

Table 1.3: ESCRT interacting proteins.

Some known ESCRT-interacting proteins in *Drosophila* are listed together with their human orthologues and notes on known or possible roles and interactions (Tsuda, Seong and Aigaki, 2006; Pradhan-Sundd and Verheyen, 2015; McMillan *et al.*, 2017; Bäumers *et al.*, 2020; Vietri, Radulovic and Stenmark, 2020)

The formation of intraluminal vesicles in the endocytic pathway requires the activity of all ESCRT complexes. Membrane proteins are targeted for internalisation into the endosomal lumen by ubiquitination which triggers the binding of the ESCRT-0 proteins Hrs and Stam. With the help of clathrin, which coats many endocytic vesicles, ESCRT-0 is believed to gather ubiquitinated targets together, thus supporting the sorting function of endosomes by segregating targets for internalisation apart from other membrane proteins (Sachse *et al.*, 2002; Vietri, Radulovic and Stenmark, 2020). ESCRT-I and -II are then recruited to the gathering target proteins and begin to push the local membrane inwards. ESCRT-II then recruits ESCRT-III and Vps4 which accumulate rapidly before mediating the constriction and scission of the membrane to form the intracellular vesicle and the simultaneous dissociation of the ESCRTs (Wenzel *et al.*, 2018). A number of ESCRT-III auxiliary proteins exist that have been found, in yeast, to be involved in directly regulating ESCRT-III and Vps4 activity; however, their roles and requirements in animals are, in general, poorly understood. A recent example of ESCRT-III auxiliary protein function in *Drosophila* is a study by Bäumers *et al.* which indicated that Vps60 and Ist1 are involved in regulating Shrub accumulation on endosomal membranes, with this regulation found to be particularly critical for development at lower temperatures (Bäumers *et al.*, 2019).

Dependent on the circumstances, the requirement for one or more of the ESCRT-0, -I, -II complexes is dispensable. For particular ESCRT mediated processes, the function of one, or more, of these complexes is instead performed by various ESCRT-interacting proteins (see **Table 1.3**). For example, ALG-2 interacting protein X (ALiX) or Myopic (Mop) are able to bind ESCRT-I and recruit ESCRT-III and Vps4, thus bypassing the requirement for ESCRT-II (Vietri, Radulovic and Stenmark, 2020). In *Drosophila*, studies suggest that ALiX is involved in the ESCRT activity mediating cytokinetic abscission whereas Mop is involved in neuronal pruning (Loncle *et al.*, 2015; Lie-Jensen *et al.*, 2019).

Given the high similarity between the formation of intraluminal vesicles in endocytic sorting and in eMI, it would be expected for the ESCRT requirements to be near identical. Indeed, studies so far indicate that *Drosophila* eMI requires the activity of all ESCRT complexes apart from ESCRT-0 which may be dispensable (Mukherjee *et al.*, 2016; Jacomin *et al.*, 2020). However, it is not known which particular ESCRT-III proteins are involved, aside from Shrb (the only ESCRT-III tested), or what requirements there are for ESCRT-interacting proteins. In contrast to eMI, MA only requires ESCRT-III and Vps4 complex for autophagosome closure, with CHMP2A being the demonstrated ESCRT-III participant in mammalian cells (Takahashi *et al.*, 2018; Vietri, Radulovic and Stenmark, 2020).

1.5.7 Cellular protein degradation in immunity

Aside from xenophagy, there are numerous known roles for cellular protein degradation in mammalian immunity. These include vertebrate specific functions in antigen processing and presentation as well as in immune cell development and inhibition of inflammasome signalling (Matsuzawa-Ishimoto, Hwang and Cadwell, 2018; Germic *et al.*, 2019).

In *Drosophila*, cellular protein degradation has been found to be important for the negative regulation of immune signalling; Imd and Tak1 for example are both targeted, post activation, to the UPS for degradation thus preventing excessive signalling activity (Tsuda *et al.*, 2005; Kleino and Silverman, 2014). Additionally, selective MA-mediated remodelling of the cytoskeleton has been found to be important for hemocyte motility (Kadandale *et al.*, 2010). A

potential role for autophagy in immunometabolism has not, to my knowledge been investigated in *Drosophila*.

1.6 This project

The *Drosophila melanogaster* model organism offers an excellent tool to investigate the complex interplay between metabolism and immunity. While the communication between immune and metabolic signalling is a highly active area of research, previous work investigating the metabolic changes themselves and their functions during the *Drosophila* immune response have tended to focus on simple carbohydrate and lipid measurements. The aim of the work outlined in this thesis is to combine the genetic tractability of *D. melanogaster* with metabolomic techniques to explore the following two questions:

1. What metabolic changes occur during bacterial infection?
2. Why do these metabolic changes take place?

The broad methodology I used to explore these questions is shown in **Figure 1.7**. An untargeted metabolomic approach is first used to identify metabolites showing changes in concentration following infection (the “What?”). Consideration of what processes, signalling pathways, or enzymes may be behind these changes aims to come up with hypotheses that can be explored using the wealth of genetic tools available in *Drosophila*. A variety of experiments can then be performed to determine the cause and function of the observed metabolic changes (the “Why?”).

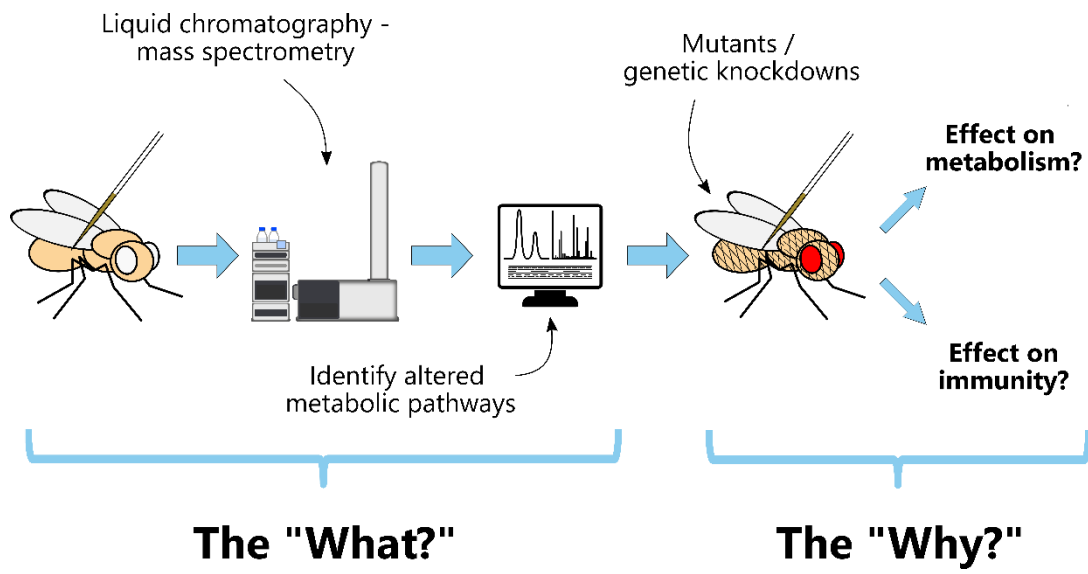


Figure 1.7: Project methodology

Chapter 2: Materials and methods

2.1 Fly husbandry

2.1.1 Fly culture

The *Drosophila melanogaster* lines were kept in vials containing a standard yeast- and fructose-based medium with the ingredients shown in **Table 2.1** below. Food was cooked in batches of 12L tap water plus ingredients: enough for around 1400-1500 vials, which were stored at room temperature, or 4°C for longer periods, before use. The vials were sealed with “flugs” and kept in incubators at 18°C, 25°C, or 29°C or on shelves at room temperature depending on desired speed of development (flies develop faster at higher temperatures). As circadian rhythms are important for fly physiology and immunity, the incubators ran a 12-hour light/dark cycle (Shirasu-Hiza *et al.*, 2007; Allada and Chung, 2010; Stone *et al.*, 2012).

Ingredient	Amount per batch	Percent (w/v)	Supplier
Tap water	12L	-	-
Brewer’s yeast	1200g	10	Acros Organics, 10088951
Fructose	960g	8	Rigest Trading Ltd
Polenta	240g	2	Wilton Wholefoods
Agar	96g	0.8	VWR, A0917
Propionic acid	90ml	0.75	Sigma-Aldrich, 402907
Nipagin (15% w/v in ethanol)	60ml	0.075	Acros Organics, 126960025

Table 2.1: Components of the fly medium and their proportions and suppliers

2.1.2 Fly stocks and crosses

Fly stocks were kept at 18°C with each line in duplicate. The fly lines used in this project are listed in **Table 2.2** below along with their full genotype and source. Sources include the Vienna Drosophila Resource Centre (VDRC), The Bloomington Drosophila Stock Centre (BDSC), gifts courtesy of particular labs, and lines generated in-house in the Dionne lab. The crosses performed to generate experimental genotypes are stated in the relevant results sections. Anaesthetisation of flies was performed with CO₂ on gas-porous pads (FlyStuff, Flypad) and paint brushes were used to move the flies around. For crosses, virgin females were collected and crossed with males with 3 or 4 flies of each sex to one vial. Flies were then kept in the same vials for 2 to 4 days to mate and lay eggs before being transferred to fresh vials.

Many experiments in this project utilised the UAS-Gal4 system to knock-down genes of interest in a target tissue (Brand and Perrimon, 1993). In this system the transgenic transcriptional activator Gal4 is placed under the control of an endogenous promoter with the desired pattern of expression. When expressed, Gal4 binds to a transgenic UAS enhancer to drive expression of the target gene. To knock-down a gene, fly lines containing an RNA inverted repeat (IR) under UAS control are crossed to lines expressing Gal4. Offspring of the cross will then express double-stranded RNA (dsRNA) knocking-down the target gene in the desired tissue. UAS-IR lines used in this project come from the KK and GD libraries stocked by the VDRC, and the Transgenic RNAi Project (TRiP) library from the Drosophila RNAi Screening Center (DRSC) at Harvard Medical School. The crosses performed to generate the Gal4-UAS experimental genotypes and one or both of the appropriate control genotypes all take the form of the example cross shown in **Figure 2.1**. In this example, the generated experimental genotype expresses Gal4 under control of the *c564* promoter (which predominantly drives expression in the adult fat body cells). Gal4 expression in the fat body results in the transcriptional activation of the inserted construct gene due to Gal4 binding the UAS. Expression of the inverted repeat targeting the desired gene results in knockdown of target gene expression. In the example case in **Figure 2.1**, the gene *CHMP2B* is thus targeted for knockdown in fat body cells in the experimental line only. The appropriate genotype controls are flies carrying the Gal4 driver without a target UAS construct ("Driver-only controls") and flies carrying only UAS construct without the Gal4 driver ("UAS-only controls"). Some UAS constructs show some expression even in the absence of the Gal4 transcriptional activator (see

Figure 6.2 for an example). Consequently, I used the driver-only control in this thesis, with the UAS-only control genotype occasionally used as an auxiliary control line, particularly in experiments with only one target gene.

Experimental genotype	Cross	$w^{1118}; c564Gal4$	X	$w^{1118}; UAS-CHMP2B-IR$
$c564 > CHMP2B-IR$	Collected offspring	$w^{1118}; \frac{c564Gal4}{UAS-CHMP2B-IR}$		
Driver-only control genotype	Cross	$w^{1118}; c564Gal4$	X	w^{1118}
$c564 > +$	Collected offspring	$w^{1118}; \frac{c564Gal4}{+}$		
UAS-only control genotype	Cross	w^{1118}	X	$w^{1118}; UAS-CHMP2B-IR$
$+ > CHMP2B-IR$	Collected offspring	$w^{1118}; \frac{UAS-CHMP2B-IR}{+}$		

Figure 2.1: The crosses performed, and the offspring collected for an example knockdown experiment using the Gal4-UAS system.

The terms on the left are used to describe the indicated offspring genotypes used in experiments. The abbreviated forms of the example experimental and control fly lines are shown beneath. The left shows the full genotypes of the example parental crosses and the offspring used for experiments. In the parental crosses, the virgin female genotype is shown on the left. Only male offspring were collected for experiments.

Line	Genotype	Identifiers and notes	Source
<i>ALiX</i>	<i>w¹¹¹⁸; ALiX³</i>	<i>ALiX³</i> , amorphic allele, 860bp deletion	(Eikenes <i>et al.</i> , 2015)
<i>Atg12</i> -IR	<i>w</i> ; UAS- <i>Atg12</i> -IR	GD29791	VDRC
<i>Atg13</i> -IR	<i>w</i> ; UAS- <i>Atg13</i> -IR	KK100340	VDRC
<i>Atg5</i> -IR	<i>w</i> ; UAS- <i>Atg5</i> -IR	KK104461	VDRC
<i>Atg7</i> -IR	<i>w</i> ; UAS- <i>Atg7</i> -IR	GD11671	VDRC
c564Gal4	<i>w¹¹¹⁸; c564Gal4</i>	Fat body Gal4 driver	BDSC, (Zaidman-Rémy <i>et al.</i> , 2006)
Gal80 ^{ts} c564Gal4	<i>w¹¹¹⁸; c564Gal4; TubGal80^{ts}</i>	Fat body Gal4 driver with temperature sensitive inhibition	Dionne lab
<i>CecA1^{HFA}</i>	<i>w¹¹¹⁸; CecA1^{HFA} / TM6cSb</i>	<i>CecA1^{HFA}</i> gene with HA and FLAG tags (third chromosome balancer)	Dionne lab
c564 <i>CecA1^{HFA}</i>	<i>w¹¹¹⁸; c564Gal4; CecA1^{HFA} / TM6cSb</i>	Fat body Gal4 driver plus <i>CecA1^{HFA}</i> gene with HA and FLAG tags (third chromosome balancer)	Dionne lab
<i>Chmp1</i> -IR	<i>w¹¹¹⁸; UAS-Chmp1</i> -IR	HM05117	BDSC
<i>CHMP2B</i> -IR ^{TRIP}	<i>w¹¹¹⁸; UAS-CHMP2B</i> -IR ^{TRIP} / (SM6a)	HMS01844	BDSC
<i>CHMP2B</i> -IR ^{KK}	<i>w¹¹¹⁸; UAS-CHMP2B</i> -IR ^{KK}	KK108600	VDRC
<i>Hsc70-4</i> -IR ^{KK}	<i>w¹¹¹⁸; UAS-Hsc70-4</i> -IR ^{KK}	KK101734	VDRC
<i>BomS2^{HFA}</i>	<i>w¹¹¹⁸; BomS2^{HFA}</i>	<i>BomS2</i> (also known as <i>IM2</i>) gene with HA and FLAG tags	Dionne Lab
c564 <i>BomS2^{HFA}</i>	<i>w¹¹¹⁸; c564Gal4 BomS2^{HFA}</i>	Fat body Gal4 driver plus <i>BomS2</i> gene with HA and FLAG tags	Dionne lab
<i>imd</i>	<i>w¹¹¹⁸; imd¹⁰¹⁹¹</i>	Amorphic allele	Dionne lab
<i>Ist1</i> -IR	<i>w</i> ; UAS- <i>Ist1</i> -IR	KK100771	VDRC

<i>key</i>	<i>w¹¹¹⁸; key^{c02831}</i> / (SM6a)	Transposable element insertion	BDSC
KFERQ-mCherry ^{AM93}	<i>w¹¹¹⁸; UAS-KFERQ-mCherry^{AM93}</i> / (SM6a)	Fluorescent mCherry tagged with KFERQ motif	Gift of Andreas Jenny (New York)
KFERQ-mCherry ^{AM94}	<i>w¹¹¹⁸; UAS-KFERQ-mCherry^{AM94}</i> / (SM6a)	Fluorescent mCherry tagged with KFERQ motif	Gift of Andreas Jenny (New York)
<i>mim^Δ</i>	<i>w¹¹¹⁸; mim^Δ</i> / (SM6a)	<i>mim^{null}</i> amorphic allele	Gift of Tom Millard (Manchester)
<i>Mvb12</i> -IR	<i>w¹¹¹⁸; UAS-Mvb12</i> -IR	HMS02004	BDSC
<i>Myd88</i> ^{2.8}	<i>w¹¹¹⁸; Myd88^{2.8}</i> / SM6a	Amorphic <i>Myd88</i> allele maintained with 2 nd chromosome balancer	Gift of Alicia Hidalgo (Birmingham)
<i>Myd88</i> ^{KRA}	<i>w¹¹¹⁸; Myd88^{KRA56}</i> / SM6a	Hypomorphic <i>Myd88</i> allele maintained with 2 nd chromosome balancer	Gift of Alicia Hidalgo (Birmingham)
<i>POSH</i>	<i>w¹¹¹⁸; POSH⁷⁴</i> / (SM6a)	Hypomorphic allele	Gift of Sean Sweeney (York)
Prosβx2-DN	<i>w¹¹¹⁸; UAS-Prosβ6¹; UAS-Prosβ2¹</i>	2x temperature sensitive dominant negative alleles	BDSC
r4Gal4	<i>w¹¹¹⁸; r4Gal4</i> / TM6cSb	Fat body Gal4 driver, 3 rd chromosome balancer	BDSC, (Lee and Park, 2004)
<i>shrb</i> -IR ^{KK}	<i>w¹¹¹⁸; UAS-shrb</i> -IR ^{KK}	KK108557 (lethal with c564)	VDRC
<i>shrb</i> -IR ^{TRIP}	<i>w¹¹¹⁸; UAS-shrb</i> -IR ^{TRIP}	HMS01767	BDSC
<i>Stip1</i> -IR ^{GD}	<i>w¹¹¹⁸; UAS-Stip1</i> -IR ^{GD}	GD9818	VDRC
<i>Stip1</i> -IR ^{KK}	<i>w¹¹¹⁸; UAS-Stip1</i> -IR ^{KK}	KK101758	VDRC
<i>Tak1</i>	<i>w¹¹¹⁸ Tak1²⁵²⁷</i>	Amorphic allele, 1 st chromosome	BDSC
<i>Uvrags</i> ^{B21}	<i>w¹¹¹⁸; Uvrags^{B21}</i> / SM6a	Amorphic allele maintained with 2 nd chromosome balancer	Gift of Jongkyeong Chung (Seoul)
<i>Uvrags</i> ^{KG}	<i>w¹¹¹⁸; Uvrags^{KG}</i> / SM6a	KG04163 hypomorphic allele maintained with 2 nd chromosome balancer	Gift of Jongkyeong Chung (Seoul)
<i>Vps13</i> -IR ^{GD}	<i>w¹¹¹⁸; UAS-Vps13</i> -IR / (SM6a)	GD29972	VDRC
<i>Vps13</i> -IR ^{TRIP}	<i>w¹¹¹⁸; UAS-Vps13</i> -IR / (SM6a)	HMS01715	BDSC

<i>Vps4</i> -IR	<i>w</i> ; UAS- <i>Vps4</i> -IR	KK101722	VDRC
<i>Vta1</i> -IR	<i>w</i> ; UAS- <i>Vta1</i> -IR	KK108142	VDRC
<i>w</i> ¹¹¹⁸	<i>w</i> ¹¹¹⁸	Isogenic wild-type control and background line	BDSC

Table 2.2: *Drosophila melanogaster* stocks used in this thesis

The fly lines used in experiments or crossed to generate experimental and control genotypes are listed. The "Line" column indicates the abbreviated line names used in this thesis with their full genotypes listed in the "Genotype" column. Brackets around balancer chromosomes (SM6a, TM6cSb) indicate that this balancer is not needed for viability and so flies may be homozygous for the construct/mutation carrying chromosome.

2.1.3 Preparing flies for experiments

Experimental fly stocks were kept in vials in an incubator set at 25°C. Experimental flies were separated within a few days of eclosion and transferred to fresh food with up to around 25 flies/vial. All experiments utilised adult male flies that were both aged-matched and 5-10 days post-eclosion (inclusive).

2.2 Bacterial infection experiments

2.2.1 Bacterial strains and growth conditions

The bacterial strains used in this study are listed in **Table 2.3**. For infection of flies, bacteria from stocks stored at -80°C were cultured in 50ml centrifuge tubes containing 5ml lysogeny broth (LB, Merck or Fisher Scientific), tryptic soy broth (TSB, VWR or Fisher Scientific), or brain heart infusion (BHI, Oxoid) and incubated overnight with agitation (200 rpm) at 37°C (see **Table 2.3**). Growth media were autoclaved before use except for BHI when used to grow *E. faecalis*; in this case the BHI was sterile filtered. For plating colonies, bacteria were plated on the appropriate media with 1-1.5% agar and grown overnight statically at 37°C with the exception of *E. cloacae* which was grown at 29°C or room temperature.

Species	Strain	Relevant characteristics	OD ₆₀₀ used in infections	Overnight growth conditions
<i>E. coli</i>	P2987	Gram-negative	1.0	LB, agitation
<i>M. luteus</i>	Clinical isolate, gift of W. Wade, KCL	Gram-positive	1.0	LB, agitation
<i>E. cloacae</i>	Type strain	Gram-negative	1.0	TSB, agitation
<i>S. aureus</i>	SH1000	Gram-positive	0.01 or 0.1	TSB, agitation
<i>E. faecalis</i>	NCTC00075	Gram-positive	0.3 (BHI) or 1.0 (TSB)	Sterile-filtered BHI or autoclaved TSB, agitation

Table 2.3: Bacterial strains, growth conditions, and OD₆₀₀ used for infections

2.2.2 Bacterial infection of *D. melanogaster*

Overnight bacterial cultures were centrifuged at 4000G for 5mins. The supernatant (spend growth media) was then tipped off and the bacteria resuspended in 5ml sterile phosphate-buffered saline (PBS). An aliquot of this bacterial suspension in PBS was then further diluted in sterile PBS to the required optical density (see **Table 2.3**) at a wavelength of 600nm (OD₆₀₀) measured using a cell density meter (Biochrom).

Injection of anaesthetised flies was performed using pulled glass needles injecting 50nl of the prepared bacterial suspension or sterile PBS for injury-only controls. The volume of injection was calibrated by injecting a droplet into oil (mixture of mineral oil with Halocarbon oil 700, Sigma-Aldrich H8898) and using the microscope eyepiece reticule to measure the width, and thus volume, of the droplet. Droplet size was changed by adjusting the length of time (in milliseconds) liquid is pumped through the needle.

The site of injection was the anterior of the abdomen around the dorsal-ventral midline with the needle heading in the posterior direction. Infected flies were transferred to fresh vials and kept at 29°C with their vials horizontal, to reduce deaths due to flies getting stuck to the food.

In some experiments, flies were transferred to “starvation media”, in order to provide the flies with moisture but no nutrition. This was made up of 2% PBS and 1% (w/v) agar in ultra-pure H₂O. For the metabolomic experiments in Chapter 3, experimental flies were transferred to vials containing this moisture-only starvation media one hour prior to sample collection. This aimed to reduce the influence of any recently ingested contents of the gut on the detected metabolome but without any metabolic change occurring due to an extended time without food.

2.2.3 Bacterial quantification

Bacterial quantification was performed by homogenising single flies in 100µl sterile PBS using a pestle, followed by serial 1 in 5 dilutions of the lysate in sterile PBS, performed in 96-well plates. 20µl of each dilution was then spotted onto agar plates. Plates were then cultured overnight (room temperature or 29°C for *E. cloacae*, 37°C for all other strains) and colonies counted the following morning.

The Mann-Whitney *U* statistical test was used for pairwise comparisons when analysing the data as a normal distribution was not assumed. Bacterial quantification data is presented in box plots (see **Section 2.6**). Origin Pro software was used to plot and analyse bacterial quantification data.

2.2.4 Survival experiments

Survival experiments were conducted by injecting bacterial suspension, or sterile PBS for injury control, into age-matched flies in cohorts of approximately 20-25 flies per vial per condition. Uninjected control flies were anaesthetised for a comparable length of time to the injected flies. Vials were checked regularly and the number of dead and living flies recorded. Surviving flies were transferred onto fresh food twice a week.

Survival data are presented as survival curves using the Kaplan–Meier method with the Log-Rank test used for pairwise comparisons. Origin Pro software was used to plot and analyse survival data.

2.3 Metabolomics

2.3.1 Sample collection

Three flies were placed in an Eppendorf containing 180µl cold LCMS solution; a solution of 2:2:1 methanol (Fisher): acetonitrile (Fisher): ultra-pure LCMS-grade H₂O (Fisher) with a 1µM caffeine 'spike' (Sigma-Aldrich) and a 4 mm glass bead. Microcentrifuge tubes containing flies were placed immediately into ice or dry ice before the flies were homogenised using a tissue homogeniser (FastPrep 24, MP Biomedicals). For each experiment 3 protocol control samples (pCs) were taken in which no flies were placed but were otherwise treated identically. Samples were stored at -80°C overnight or longer before preparation for LCMS.

2.3.2 Sample Preparation

Samples were thawed at -20°C before being centrifuged at 15,000G for 5 mins at 4°C. The supernatant was then transferred to Spin-X 0.22µl centrifuge filters (Corning) and these filters centrifuged at 15,000G for 75 mins at 4°C. 5µl of the filtrate from all samples (excluding pCs) was combined in a microcentrifuge tube to create a sample pool. This pool was then diluted 1 in 5 in clean LCMS solution (as above but without caffeine spike) and then further serially diluted (1 in 2) to create a dilution series of 1/5, 1/10, 1/20, 1/40, 1/80. In addition, another 1 in 10 dilution was made from the sample pool to use for "run controls" (RunCs). An aliquot of the filtrate of each sample was then diluted to 1 in 10 in clean LCMS solution for analysis, with the remainder stored at -80°C.

All samples for analysis, including pCs, RunCs, the serial dilutions of the sample pool, and blank samples (clean LCMS solution only) were diluted 1 in 2 in LCMS priming solution (0.2% acetic acid in acetonitrile) before centrifuging at 17,000G for 10 mins at 4°C. 100µl of the supernatant was then transferred to LCMS vials (Agilent). The serial dilutions of the sample pool were placed at the beginning of the run. RunCs and blank samples were placed at least as frequently as every 18 samples throughout the run. RunCs were used to check for any changes in

sensitivity of the mass spectrometer over the course of the run and to gauge the inherent level of variation in the quantification of different ions. The blank samples were used to check for changes in the baseline of ions detected and to help keep the high-performance liquid chromatography (HPLC) column clean, to prevent run-over of metabolites between samples.

2.3.3 HP-LCMS

Liquid chromatography was performed on the above samples using an Agilent 1290 Infinity II LC (liquid chromatography; Agilent Technologies, Santa Clara, CA, USA) system with a Cogent Diamond Hydride Type C silica HPLC column. Mass spectrometry was carried out in both positive and negative ion-modes using an Agilent Accurate Mass 6545 QTOF (quadrupole time-of-flight) mass spectrometer.

2.3.3.1 Chromatography details

The Agilent 1290 Infinity II liquid chromatography system had used an auto-sampler system kept at 4°C. The column compartment was maintained at 25°C. Chromatography was performed using a flow rate of 0.4ml/min using 2 solvents: 0.2% acetic acid in ultra-pure H₂O (solvent A) and 0.2% acetic acid in acetonitrile (solvent B). These 2 solvents were used to create a solvent gradient with the ratio of solvent A to solvent B increasing over time: from 0-2 min the solvent mix contained 85% solvent B; 3-5min, 80% solvent B; 6-7min, 75% solvent B; 8-9min, 70% solvent B; 10-11min, 50% solvent B; 11.1-25min, 20% solvent B. The run ended with a 5-minute re-equilibration period with 85% solvent B.

2.3.3.2 Mass spectrometry details

Ionisation was performed by electron-spray ionisation (ESI) using a nozzle voltage of 2,000V and a fragmentor voltage of 100V. The nebuliser pressure was 50psi and the nitrogen drying gas was maintained at 300°C with a flow rate of 5 L/min. Data was acquired at a rate of 1.5 spectra/min detecting a mass-to-charge ratio (m/z) range of 50-1,200.

2.3.4 Data analysis

2.3.4.1 Feature extraction

The data from the LCMS experiments were extracted and analysed using the Agilent Mass hunter software suite. Initial data quality control was performed by checking total ion chromatograms (TICs) using Agilent Masshunter Qualitative Navigator and removing samples where the chromatography had failed (for example loss of pressure during a sample run can be seen when TIC drops well below the apparent baseline). This software was also used for quickly and qualitatively checking the retention times (RTs) or presence of compounds in samples. For peak extraction and quantification, Agilent Profinder software was used.

For untargeted analysis the "batch recursive feature extraction (small molecules/peptides)" protocol was used. For targeted analysis the "targeted feature extraction" protocol was used with a database of compounds contained in a MassHunter "Personal Compound Database and Library" (PCDL) file. The PCDLs used were curated by myself using data from METLIN (Guijas *et al.*, 2018) or entered manually. Extracted peaks were manually checked within the program for quality control purposes using the quality control samples for guidance. In cases where the automatic integration of a peak varied across samples, this was manually corrected. The totality of the data was then exported to a comma separated values (.csv) file using the peak area for quantification (unless stated otherwise).

2.3.4.2 Feature identification

For identification of peaks in untargeted analyses, the peak data were saved in Agilent Compound Exchange File (CEF) format and the CEFs loaded into the Agilent Mass Profiler Pro compound identification tool. For speed and ease, this was only done with the CEFs from the RunCs and pooled sample dilutions, as these should contain all peaks detected in the experiment. Peak identification was performed using a PCDL containing data of compounds for which I had run standards (see **Section 2.3.5** below). Features were matched to metabolite identities using m/z and retention time, provided the feature also showed the expected distribution of isotopomer peaks (i.e., there were peaks at the same retention time with m/z matching the putative metabolite when containing natural levels of elemental isotopes). Peaks that remained unidentified were run through the compound identification tool a second time

but with identification performed using the METLIN database (Guijas *et al.*, 2018). This second method only allows for getting a list of possible identities for these peaks based on the measured m/z . If any of these possible identities were judged to be feasible, they were noted down for potential future investigation but were not applied to the data.

2.3.4.3 Data preparation and quantitative quality control

The extracted quantitative data was organised and prepared using Microsoft Excel. At this stage data from positive and negative modes could be combined. Quality control is an important part of untargeted quantitative metabolomic analysis and to ensure quality of the sample data, data from the pCs, RunCs, and serial dilution of pooled sample were used. Firstly, for each feature, the mean ($\bar{x}$) of the abundances from the pCs was calculated (for most features this is usually 0) and then subtracted from the abundances of the samples and the other quality controls. The abundance of a feature minus the mean of the abundance of that feature in the pCs was termed the "corrected abundance". Should any feature subsequently have a corrected abundance in the RunCs equal to or less than 0, this feature was deemed to not be from the biological samples and removed from analysis. For the "targeted analysis" workflow, when only 1 or 2 compounds were quantified as done in the screen for allantoin production in **Chapter 5**, the corrected abundance calculated above was used, expressed in arbitrary units (A.U.). For untargeted analysis or "Semi-targeted" analysis, in which the LCMS data was interrogated for a large number of pre-decided target compounds (typically 30+), three further data preparation and quality control steps were performed as follows.

Firstly, to assess the inherent variability in the detection of each feature, I calculated the relative standard deviation (RSD, also known as the coefficient of variation) of the signal for each feature in the RunCs. RSD is the sample standard deviation (s) over the sample mean ($\bar{x}$) expressed as a percentage:

$$RSD = \frac{s}{\bar{x}} \times 100$$

Features with a high RSD in the RunCs were removed (untargeted analysis) or interpreted with caution (a known compound in targeted analysis). A high RSD was arbitrarily decided to be any RSD greater than 30%.

Secondly, to check whether feature was measured within the linear range and correlated with the concentration of biological material, the coefficient of determination (r^2) of each feature in the serial dilution was calculated using the inbuilt Microsoft Excel function. Any feature that had a r^2 value less than 0.85 (decided arbitrarily) was removed from analysis as the measurement of the feature was considered to be out of linear range or the reliability of quantification poor. The caffeine spike present in the samples was found to not be reliable quantitatively but remained useful for troubleshooting and assessing the sensitivity of the mass spectrometer.

Finally, the data was trimmed to ensure each detected compound was only represented by one set of measured abundances. The most straight forward example of this is if the feature was detected in both positive and negative mode when these data sets were combined. In this case the only mode that had the highest abundance was retained (assuming all quality control checks had been passed). As features can in fact be made up of multiple peaks due to different ionisations, this can lead to a feature appearing multiple times in the data. Though the Agilent Profinder software does combine multiple peaks representing different ionisations of the same compound, some ionisations are missed and are instead outputted as additional, separate features in untargeted analyses. The most troublesome of these were ionised fragments of whole compounds which required careful identification based on RT, use of metabolite standards, and m/z analysis utilising online resources such as the METLIN Fragment Similarity Search tool (Guijas *et al.*, 2018).

Consequently, to ensure each feature was only represented once in the final data set, I manually checked the data to remove features known to be extracted from peaks corresponding to already represented features due to ionisations not picked up by the Agilent Profinder software. For example, in positive mode, leucine presents peaks with mass-to-charge ratios of 132.1019 ($[M+H]^+$) and 154.0838 ($[M+Na]^+$) and these are both combined into a single feature corresponding to Leucine by the software. However, leucine has a third peak with an m/z of 86.0969 (an ionised fragment). This third peak is not combined with the other two peaks by the software and instead is outputted as an additional feature. To ensure leucine was not overrepresented in the data set, this additional leucine-corresponding feature was removed.

2.3.4.4 Metabolomic data analysis

Analysis of data was performed using R Studio (version 1.4) with R (version 4.1) using Tidyverse packages (tidyverse.org).

The workflow for preparation of data is as follows. Firstly, values ≤ 0 were replaced with a minimum value equalling half the minimum corrected abundance >0 for that feature in the dataset. The samples were then normalised to the sum of the corrected abundance of all features in each sample. The resulting data was checked to ensure that normalisation reduced the RSD for more features than it increased the RSD. Other normalisation methods were also checked by this method, however sum normalisation performed best in every case. These sum-normalised abundances are the values used for box plots after multiplying them all by the same factor (normally 10,000,000) to make the data more readable.

Prior to principal component analysis (PCA), data was centred and scaled using median level scaling in which, for each feature:

$$\text{Scaled data} = \frac{\text{norm'd abundance} - \text{feature median norm'd abundance}}{\text{feature median norm'd abundance}}$$

2.3.5 Standards

For proper identification of the features measured by LCMS, the use of standards is required. These are solutions of a known compound (or compounds) at a known concentration. Multiple compounds can have the same mass, and so can the same detected m/z ; such compounds are termed *isobars*. Thus, to distinguish different isobars, a second dimension of measurement is taken: the retention time (RT). The RT is the length of time the compound spends travelling through the column during the liquid chromatography phase of LCMS. Consequently, it is a measurement of the solubility of a compound in the particular solvent gradient used in the particular column used. It is unreliable to predict retention times *a priori*, and so standards are required. Although there can be a shift in a compound's RT between the standards and the biological samples, the relative order of the isobars' RTs is stable (so long as the liquid chromatography set-up the same). For example, Valine and glycine-betaine are detected at the same m/z (118.0863 in positive mode) and so cannot be differentiated by mass alone. By

running standards of both compounds in the LCMS, it can be seen that (in our set up) valine has a lower RT than glycine-betaine. Even if valine's RT of 9.1min shifts in a biological sample, it will always be lower than the RT of glycine-betaine within the same run.

To make standards, the compound of interest was dissolved in ultra-pure H₂O to a defined concentration, usually either 1mM or 50mM depending on compound mass and availability, to create a stock solution. For compounds with low solubility in water, alternative solutions were made. For example, uric acid required dissolving in 1M NaOH. An aliquot of the stock solution was then diluted to 1mM (if not already at this concentration) in ultra-pure H₂O before diluting 1/64 in LCMS solution to create a solution of 1.0×2^{-6} mM. This solution was then serially diluted 1/2 in LCMS solution, normally to 1.0×2^{-13} mM (122nM). The dilutions to be run were then diluted 1/2 in LCMS priming solution before centrifuging at 17,000G for 10mins. 100µl of supernatant was then added to Agilent LCMS vials and LCMS performed on the standards in the same way as performed on the biological samples. The standards I ran, along with their observed RTs, are shown in **Table 2.4**. The identities of all compounds from metabolomic analysis in this thesis were confirmed using standards with the exception of taurine. The identity of taurine was assumed to be safe as taurine has no biological isobars in the METLIN database; all isobars are classed as non-biological "toxicants" (Guijas *et al.*, 2018). Additionally, the relatively high abundance of taurine in *Drosophila melanogaster* samples has previously been described and confirmed via a standard (Chintapalli *et al.*, 2013).

Compound	KEGG ID	Exact Mass	Isobars	Detected Retention Time (min)	Notes
Acetaminophen	C06804	151.0633		1.1	Drug
Adenosine	C00212	267.0968	Deoxyguanosine	7.5	
Alanine	C00041	89.0477	Sarcosine, β -alanine	9.9	
Allantoin	C02348	158.0440		1.6	Also KEGG ID: C02350
Arginine	C00062	174.1117		13.5	
Asparagine	C00152	132.0535		9.8	
Aspartic acid	C00049	133.0375		8.7	
Biopterin	C06313	237.0862	Sepiapterin, pyruvoyl-tetrahydropterin	3.1	
Caffeine	C07481	194.0804		1.5	Drug, used as spike, positive mode only

Compound	KEGG ID	Exact Mass	Isobars	Detected Retention Time (min)	Notes
Citric acid	C00158	192.0270	Isocitric acid	1.2	Negative mode only, indistinguishable from putative iso-citric acid peak on most runs
Cysteine	C00097	121.0197		7.9	
Dopamine	C03758	153.0790	Octopamine	12.4	Indistinguishable from octopamine
Fructose	C00095	180.0634	Glucose, mannose	1.6	Double EIC peak, multiple <i>m/z</i> s
Fumaric acid	C00122	116.0110		1.1	Negative mode only
GABA	C00334	103.0633	Other aminobutyric acids, dimethylglycine	12.4	
Glucose	C00031	180.0634	Fructose, mannose	1.6	Multiple <i>m/z</i> s
Glutamic acid	C00025	147.0532		9.1	

Compound	KEGG ID	Exact Mass	Isobars	Detected Retention Time (min)	Notes
Glutamine	C00064	146.0691		10.1	
Glutathione (Reduced)	C00051	307.0838		8.5	
Glycine	C00037	75.0320		10.3	
Glycine-betaine	C00719	117.0790	Valine	9.8	
Guanosine	C00387	283.0917		3.2	
Histidine	C00135	155.0695		13.3	
Homoserine	C00263	119.0582	Threonine	10.2	
Hypoxanthine	C00262	136.0385	Inosine fragment	2.3	Indistinguishable from inosine fragment on some runs
Inosine	C00294	268.0808		2.1	

Compound	KEGG ID	Exact Mass	Isobars	Detected Retention Time (min)	Notes
Isoleucine	C00407	131.0943	Leucine	9.7	Indistinguishable from leucine on some runs
Kynurenic acid	C01717	189.0426		1.6	
Kynurenine	C00328	208.0848		6.4	
Lactic acid	C00186	90.0317		1.2	Negative mode only, likely indistinguishable from peaks found in monosaccharide mass spectra
Leucine	C00123	131.0946	Isoleucine	9.6	Indistinguishable from isoleucine on some runs
Lysine	C00047	146.1055		13.5	
Malic acid	C00149	134.0215		2.0	Negative mode only
Maltose	C00208	342.1162	Trehalose, sucrose	2.0	Multiple m/z s

Compound	KEGG ID	Exact Mass	Isobars	Detected Retention Time (min)	Notes
Mannose	C00159	180.0634	Glucose, fructose	1.7	Double peak, multiple <i>m/z</i> s
Methionine	C00073	149.0510		8.9	
Methionine-S-oxide	C02989	165.0460		10.5	
Nicotinic acid	C00253	123.0320		2.1	
Octopamine	C04227	153.0790	Dopamine	12.4	Indistinguishable from dopamine
Ornithine	C00077	132.0899		14.4	
Phenylalanine	C00079	165.0790		8.4	
Proline	C00148	115.0633		9.4	
Pyruvic acid	C00022	88.0160		1.1	Negative mode only

Compound	KEGG ID	Exact Mass	Isobars	Detected Retention Time (min)	Notes
Sarcosine	C00213	89.0477	Alanine, β -alanine	9.4	
Serine	C00065	105.0426		9.5	
Succinic acid	C00042	118.0266		1.1	Negative mode only
Sucrose	C00089	342.1162	Trehalose, maltose	2.0	Multiple m/z s
Threonine	C00188	119.0582	Homoserine	9.6	
Trehalose	C01083	342.1162	Sucrose, maltose	2.3	Multiple m/z s
Tryptophan	C00078	204.0899		8.7	
Tyrosine	C00082	181.0739		8.7	
Uracil	C00106	112.0273		1.4	

Compound	KEGG ID	Exact Mass	Isobars	Detected Retention Time (min)	Notes
Urea	C00086	60.0324		1.8	
Uric acid	C00366	168.0283		1.5	
Valine	C00183	117.0790	Glycine-betaine	9.1	
Xanthine	C00385	152.0334		1.5	
α -ketoglutaric acid	C00026	146.0215		1.1	Negative mode only
β -Alanine	C00099	89.0477	Sarcosine, alanine	11.8	

Table 2.4: Compound standards for LCMS.

Compounds for which standards were run are shown along with their exact mass, potential isobars, and observed RTs. The isobars listed do not include all theoretically possible compounds, just compounds I judged to be most likely detected in biological samples. KEGG identifiers for the compounds are also shown (Kanehisa *et al.*, 2017; Kanehisa, 2019). Many of the standards for the proteinogenic amino acids were prepared by **Ashleigh Cheyne** of the Larrouy-Maumus lab.

2.3.6 Isotopologue analysis

To incorporate stable isotope-labelled glycine into the developing fly proteome, flies were reared on food supplemented with ^{15}N -glycine. To make the labelled food, 4g of ^{15}N -glycine (Cambridge Isotope Laboratories Inc, NLM-202-1) was added per 100g of brewer's yeast prior to food preparation. As brewer's yeast has previously been found to contain 4g of glycine (free and protein bound), this aimed to ensure that approximately half of the glycine present in the fly food was labelled (Bass *et al.*, 2007). Flies were also reared in parallel on food supplemented in the same way with non-isotope-labelled glycine.

Agilent Profinder was also used for isotopologue analysis. Isotope data extraction was checked via comparison of samples from flies reared on labelled supplemented food to those reared on non-labelled supplemented food. I found that the settings for Profinder feature extractions required adjusting, decreasing the mass defect tolerance parameters to 15ppm, to avoid mistaken detection of large M+4 allantoin peaks in non-labelled samples and pCs.

2.4 Gene expression analysis

Gene expression analysis was performed using reverse transcription followed by quantitative, also known as real-time, polymerase chain reaction (RT-qPCR).

2.4.1 RT-qPCR protocol

Three flies per sample were homogenised directly in 100 μl TRI reagent (Sigma T9424) in a 1.5ml microcentrifuge tube using either pestles or a 4mm glass beads and tissue-homogeniser. There was a minimum of 3 samples per condition per genotype per timepoint. After at least 5 minutes at room temperature, the samples were stored at -20°C before RNA extraction. RNA extraction was performed by adding 20 μl chloroform to each sample, samples shaken thoroughly and, after 3 minutes at room temperature, samples centrifuged at 12,000G for 5 minutes at 4°C . The upper aqueous layer of each sample was then pipetted into fresh microcentrifuge tubes containing 50 μl of isopropanol and vortexed. After 10 minutes at room temperature, samples were centrifuged at 12,000G for 10 minutes, supernatant discarded, and

200µl of 75% ethanol added to the remaining pellet. If required, these pellets were stored at -20°C before continuing.

For DNase treatment, the samples were centrifuged at max speed for 5 minutes and the ethanol removed twice before the following was added to each sample: 34µl ddH₂O, 4µl 10x DNase buffer (Thermo Scientific), 2µl RNase-free DNase I (1U/µl, Thermo Scientific). Samples were then incubated at 37°C for 30 minutes. The reaction was stopped by the addition of 4µl 25mM EDTA (Thermo Scientific) and incubation at 65°C for 10 minutes.

For reverse transcription, 1µl of random hexamers (200µg/ml, Thermo Scientific) was added to 10µl of each DNase treated sample and samples incubated at 70°C for 5 minutes. To each sample the following was then added: 4µl 5X reverse transcriptase buffer (Thermo Scientific), 1µl ddH₂O, 1µl RiboLock RNase inhibitor (Thermo Scientific), 1µl RevertAid M-MuLV reverse transcriptase (200U/µl, ThermoFisher EP0441), and 2µl 10mM dNTPs (Thermo Scientific). After incubation at room temperature for 10 minutes, samples were incubated at 37°C for 2 hours followed by a 70°C incubation for 10 minutes. 10µl of each sample was then combined in a single microcentrifuge tube to create a pooled sample to be used to create standard curves. An aliquot of this pooled sample was then serially diluted 1 in 5 to a concentration of pooled sample $\times 5^{-8}$. The remaining 10µl of each sample was diluted 1 in 40 by adding 390µl of TE (10mM Tris, 1mM EDTA). Diluted samples and pooled sample dilution series were stored at -20°C.

PCR reactions were set up on ice with 5µl of SensiMix SYBR Green (Bioline QT650) and 0.2µl 10µM primer mix (forward and reverse target gene primers in TE) added to 4.8µl of prepared complementary DNA (cDNA) samples or pooled sample dilutions. qPCR was performed using Corbett RG-6000 (red) with 1 amplification at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds, 57°C for 30 seconds, 72°C for 30 seconds. The sequences of the primers used (Invitrogen) are listed in **Table 2.5**.

A standard curve was generated for each gene in every run using the pooled sample dilution series. In this curve the fluorescence after each cycle is plotted against the “known” concentration of cDNA (in actuality, the dilution of pooled sample). Expression of genes in the samples was calculated using this standard curve and was also normalised to expression of *RpL4* housekeeping gene.

Gene	Left	Right
<i>RpL4</i>	TCCACCTTGAAGAAGGGCTA	TTGCGGATCTCCTCAGACTT
<i>Dipt</i>	ACCGCAGTACCCACTCAATC	CCCAAGTGCTGTCCATATCC
<i>Dro</i>	CCATCGAGGATCACCTGACT	CTTTAGGCGGGCAGAATG
<i>Drs</i>	GTACTTGTTGCCCCTCTTCG	CTTGCACACACGACGACAG
<i>BomS2 / IM2</i>	ACCGTCTTTGTGTTGCGTCT	GATTACCACATTTCTGGATCG
<i>CecA1</i>	TCTTCGTTTTCGTCGCTCTC	CTTGTTGAGCGATTCCCAGT
<i>CHMP2B</i>	GCTGGACGACATGCTGAAT	TTGTTGACTACGGCATTGGA

Table 2.5: Primers used for RT-qPCR

2.5 Protein expression analysis

The antibodies used in protein expression analysis are listed in **Table 2.6** along with details on their source, type, supplier, and use.

Target	Source and type	Supplier	Application	Concentration used
Rabbit IgG	Goat, HRP-linked	Cell Signalling 7074	Western blots (secondary)	1:5000
Mouse IgG	Horse, HRP-linked	Cell Signalling 7076	Western blots (secondary)	1:5000 (1:10000 vs α -tubulin)
α -tubulin	Mouse, monoclonal	DSHB 12G10	Western blots (normalisation)	1:5000
FLAG	Mouse, monoclonal (M2)	Sigma-Aldrich F1804	Sandwich ELISA capturing antibody	2.5 μ g/ml
HA	Rat, monoclonal, conjugated with peroxidase	Roche 3F10	Sandwich ELISA detecting antibody	5ng/ml

HA	Mouse IgG1, monoclonal.	Invitrogen 26183	Immunohistochemistry	1:500
mCherry	Rabbit, polyclonal IgG	Invitrogen PA5- 34974	Immunohistochemistry/ Western Blots	1:500 / 1:1000
Rabbit IgG	Goat, polyclonal, Cy3 fluorophore	Jackson ImmunoResearch 111-165-003	Immunohistochemistry	1:500
Mouse IgG	Goat, polyclonal, Cy5 fluorophore	Jackson ImmunoResearch 111-605-003	Immunohistochemistry	1:500

Table 2.6: Antibodies used for protein expression analysis

2.5.1 Western blots

The buffers, and their constituents, used for Western blotting are shown in **Table 2.7** below. Three flies per sample were homogenised using a pestle directly in 75µl 2x Laemmli sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE) buffer in a microcentrifuge tube and samples were stored at -80°C. Prior to electrophoresis, samples and protein ladder (NEB P7706) were heated at 85°C for 5mins. The samples and ladder were then vortexed and 5µl added to gel wells. Electrophoresis was performed using pre-cast 4-12% gradient gels (Invitrogen NuPAGE NP0329) in running buffer (see **Table 2.7**) contained in a gel chamber (Novex Life Technologies). If the target protein had a mass less than 75KDa, the running buffer used was 2-(N-morpholino)ethanesulfonic acid (MES). For proteins with a mass of 75KDa or higher, 3-(N-morpholino)propanesulfonic acid (MOPS) buffer was used (see **Table 2.7**).

Buffer	Components	Notes
2x Laemmli SDS-PAGE buffer	4% SDS 20% glycerol 10% 2-mercaptoethanol 0.004% bromophenol blue in 0.125M Tris HCl (pH 6.8)	Stored at -80°C

MES running buffer (20x stock solution)	97.6g Anhydrous MES 60.6g Tris-Base 10g SDS 3g EDTA in ultra-pure H ₂ O (brought to 500ml)	Diluted 1/20 in ultra-pure H ₂ O before use
MOPS running buffer (20x stock solution)	104.6g MOPS 60.6g Tris-Base 10g SDS 3g EDTA in ultra-pure H ₂ O (brought to 500ml)	Diluted 1/20 in ultra-pure H ₂ O before use
Transfer buffer	6.06g Tris Base 28.8g Glycine 400ml MeOH 1600ml ultra-pure H ₂ O	For 2L. MeOH added last. Cooled to 4°C before use
TBS 10x stock solution	24.2g Tris-base 80g NaCl in ultra-pure H ₂ O (brought to 1L)	Adjusted to pH 7.6 with HCl/NaOH as required
TBS-T	100ml 10X TBS stock solution 10ml 10% Tween20 (Sigma-Aldrich P1379) in 890ml ultra-pure H ₂ O	For 1L

Table 2.7: Western blot buffers

Protein was transferred via electroblotting onto polyvinylidene fluoride (PVDF) Immobilon Transfer membrane (Millipore) in transfer buffer (see **Table 2.7**) in a transfer chamber (Bio-rad). The transfer chamber contents were continually stirred using a magnetic spinner and kept at 4°C.

Following transfer, the membrane was blocked with 5% milk (Cell Signalling) in TBS containing 0.1% tween-20 (TBS-T) at room temperature for 1 hour. Primary antibody incubation was done overnight in 5% bovine serum albumin (BSA, Serva) in TBS-T before washing in TBS-T. Incubation with the secondary antibody, conjugated with Horseradish peroxidase (HRP), was performed in 5% milk in TBS-T for 2 hours at room temperature (see **Table 2.6** for antibody details and usage). After another wash in TBS-T, the antibody-stained membrane was incubated for 5 minutes in chemiluminescent HRP-substrate solution (SuperSignal West Pico

PLUS, Thermo Scientific 34580) and blots were imaged using a Fuji LAS-3000 luminescent image analyser.

In order to normalise the blot quantification, the membrane was incubated in 0.2M NaOH on a rocker at room temperature for 1 hour. This has the effect of stripping the bound antibodies off the sample protein, with minimal loss of the sample protein itself. The membrane was then washed in TBS-T and the steps above repeated using anti- α -tubulin antibody as the primary antibody.

Images were analysed in ImageJ (Schneider, Rasband and Eliceiri, 2012). Mass of sample bands was determined by comparison to the bands of known mass in the protein ladder. For each sample, signal from the bands of target protein was divided by the signal from the α -tubulin band for normalisation.

2.5.2 ELISAs

A sandwich ELISA assay was used for the detection of antimicrobial peptides tagged with both a FLAG-tag (DYKDDDDK) and a HA-tag (YPYDVPDYA). 100 μ l of 2.5 μ g/ml anti-FLAG antibody (Sigma-Aldrich) in 0.2M sodium carbonate-bicarbonate buffer (pH 9.4) was added to each well of a Nunc MaxiSorp 96-well ELISA plate (Thermo Scientific) and the plate incubated overnight at 4°C. The wells were then washed 3 times with 200 μ l PBS containing 0.2% Tween 20 (PBTw_{0.2}). After blotting with paper towel, the wells were blocked with 200 μ l PBS with 2% BSA for 2 hours at room temperature. Wells were then washed again 3 times with 200 μ l PBTw before use.

Single flies were homogenised in microcentrifuge tubes containing 200 μ l PBS with 1% Triton X-100 (PBTx, Sigma) using pestles or tissue homogeniser. There was a minimum of 7 samples per condition per genotype per timepoint. These samples were centrifuged at 17,000G for 5 minutes and the supernatants transferred into a 96-well plate and stored at -80°C.

Alongside the samples, a dilution series of FLAG(G5)HA peptide (DYKDDDDKGGGGSYPYDVPDYA, amidated, Sigma) of known concentration was prepared: 80ng/ml was serially diluted 1:2 in PBTx 13 times to create 14 standards. Two blank samples of just PBTx were also prepared. 50 μ l of anti-HA-peroxidase antibody (Roche) at 5ng/ml in PBTx was added to 50 μ l of each sample, serial dilutions, and blanks. After mixing, these were

transferred to the wells of the prepared ELISA plate which was sealed with sealing tape (Thermo Scientific 232698) and incubated overnight at 4°C. The following day, the samples were tipped out and the wells washed 6 times with 200µl PBTw with the plate blotted dry with paper towel between washes. 100µl of 1-Step Ultra TMB-ELISA Substrate (Thermo Scientific 34029) was added to each well used and the plate covered and incubated at room temperature for 30 minutes. The reaction was stopped by adding 100µl of 2M sulphuric acid to each well and the absorbance at 450nm measured using a microplate reader (Biochrom EZ Read).

2.5.3 Immunohistochemistry and confocal microscopy

Immunohistochemistry combined with confocal microscopy was used to analyse protein expression in fat body tissue. To dissect out fat bodies, anaesthetised flies were dipped in 70% EtOH before being placed in PBS. The abdomen was then cut from the rest of the fly and a small part of the posterior end snipped off. The cuticle was then cut through lengthways on one side to open up the abdomen and the unwanted organs were removed (gut, tubules, reproductive organs). Fat body tissue was carefully scraped off the inside of the cuticle using a tungsten wire loop and placed onto glass slides coated with poly-L-lysine (Sigma P1524). Dissected fat bodies were fixed immediately in 4% paraformaldehyde (Alfa Aesar 43368) to prevent post-mortem changes in cell morphology. After 40 minutes the fat bodies were washed in PBS and permeabilised by washing with 0.2% Triton X-100 (Sigma X100). All subsequent incubations were done in PBS with 0.2% Triton X-100 and 4% horse serum (Gibco 26050070). After a 1-hour blocking wash, fat bodies were incubated with primary antibody overnight. The fat bodies were then washed and incubated with the fluorophore-conjugated secondary antibody. The fixed, stained tissue was then mounted in glycerol containing the lipid stain LipidTOX (Thermofisher H34475) and, in some experiments, the nucleotide stain DRAQ7 (Abcam 109202). The stained tissues were imaged using a Zeiss LSM510 confocal microscope and images were processed and analysed using the Fiji distribution of ImageJ (Schindelin *et al.*, 2012; Schneider, Rasband and Eliceiri, 2012).

2.5.3.1 Quantification of tagged antimicrobial peptides by confocal imaging

For the immunohistochemical quantification of the tagged antimicrobial peptides (the same as described in **Section 2.5.2**), unconjugated anti-HA was used as the primary antibody. Using a Zeiss LSM510 confocal microscope, three random areas of each fat body were selected for analysis under bright field. Then the Fiji software was used to select a region of interest (ROI) in each image for analysis. These ROIs were selected using the LipidTOX (green) channel to ensure that the tissue analysed was indeed fat body (i.e., intracellular lipid droplets were present) and that no operator bias affected selection of the antibody (red) channel. The mean antibody fluorescence signal for each ROI was recorded. Additionally, an area of background was selected and subtracted from the mean antibody signal. For each fat body preparation, background-subtracted signal from three random ROIs was calculated and their mean was taken to represent the 'fluorescence value' of each fat body. As all confocal settings (laser gain and offset and pinhole size) were kept constant for all imaging experiments, the resulting fluorescent signal was proportional to the absolute amount of HA-tagged peptide present in each fat body. For infected flies, 6-8 individual fat bodies per genotype per timepoint were analysed in this way. Only 2 flies per timepoint were analysed for the uninjected controls.

2.6 Statistical analysis and data visualisation

Data handling, statistical analysis, and data visualisation were performed using Origin Pro, Microsoft Excel, and R (version 4.1) using R Studio (version 1.4) and Tidyverse packages (tidyverse.org). Metabolomic data analysis also used additional programmes (see **Section 2.3.4**).

The majority of results in this thesis are presented in the form of box plot graphs. In these graphs the box denotes the interquartile range (IQR), the horizontal line denotes the median, and the whiskers denote the range of all data points that were within $\pm 1.5 \times \text{IQR}$ of the median. Data points outside of this range were still included in calculations for statistical significance. Unless otherwise stated, the Mann-Whitney test (also known as Wilcoxon rank-sum test) was used when statistically analysing the data as a normal distribution was not assumed and occasionally minimum/maximum values were used to replace values beyond the limits of

quantification. For the majority of pairwise tests, no correction for multiple comparisons was used. This is for two reasons: firstly, the Mann-Whitney test is already a fairly conservative pairwise test, and secondly, in this project, the consequences of false positives would be minimal as results were subsequently experimentally verified.

RT-qPCR results, in which $n = 3-5$, are visualised as bar graphs representing the mean with the error bar denoting ± 1 standard deviation. All standard deviations are calculated as sample standard deviations (i.e., the Bessel correction is used).

For the data acquired via immunohistochemistry (**Figures 6.6** and **6.7**), statistical analysis was performed through pairwise comparisons using two-way, unpaired Student's t -test with Welch's correction as equal variance was not assumed. As a pairwise comparison was performed for each timepoint within an experiment, the calculated p -values were corrected for the multiple comparisons via the Bonferroni adjustment: corrected $p = p(\text{number of comparisons made})$.

Origin Pro software was used to plot graphs and to perform the statistical tests. Where relevant, the p -value calculated is represented in figures using the standard asterisk method:

(*) denotes $p \leq 0.05$

(**) denotes $p \leq 0.01$

(***) denotes $p \leq 0.001$

(n.s.) denotes $p > 0.05$

Diagrams were drawn using Inkscape (inkscape.org).

2.6.1 Statistical analysis of metabolomic data

For the untargeted and semi-targeted metabolomics in **Chapter 3**, hypothesis testing was not performed as these experiments were exploratory and merely sought to indicate which metabolites and timepoints would be best to examine metabolic changes following infection in our experimental set-up. Instead, a principal component analysis (PCA) was conducted to

identify potential metabolites of interest. Quantification and hypothesis testing of the resulting metabolites of interest was then conducted when performing targeted metabolomics in **Chapters 3-5**. The Mann-Whitney test was used for pairwise comparisons of these data as above.

2.6.2 RNA-seq data analysis

This thesis contains box plots of gene expression using data from an RNA-seq dataset generated from an experiment previously performed and analysed by **Dr Marc Dionne**. This data set was analysed using kallisto to assign reads to transcripts and sleuth to identify differentially-expressed genes (Pimentel et al., 2017). The q values for the pairwise comparisons of gene expression between conditions presented in these graphs were generated in sleuth as described previously (Yi *et al.*, 2018). In brief, for each gene, the transcript p -values generated using the likelihood ratio test are then aggregated via the Lancaster method, to obtain a p -value for the comparisons at the gene level. To account for multiple comparisons, the Benjamini–Hochberg method was used to generate the q -values presented.

Chapter 3: What metabolic changes occur during the immune response?

3.1 Introduction

3.1.1 Metabolomics as a tool to study immunometabolism in *Drosophila*

Metabolomics is a powerful analytical technique that can measure the abundance of a large number of metabolites within the same sample. The metabolome refers to the entirety of all the small molecules present in a biological sample which could be a biofluid, a cell culture, or an entire multi-cellular organism. There is a vast variety of small molecules present in living things and some, including the proteogenic amino acids and ribonucleotides, are essential for all known forms of life. An organism's metabolome is highly dynamic and is regulated genetically via enzyme activity, small molecule transporters, and compartmentalisation whilst also being heavily influenced by extrinsic factors such as temperature, diet, and the metabolism of its microbiome. Consequently, metabolomic analysis can provide valuable insight into an organism's metabolism and physiology and how these may be affected in a variety of conditions.

Numerous studies have combined the analytical capability of metabolomics with the genetic tractability and high-throughput potential of the *Drosophila melanogaster* model organism (Cox, Thummel and Tennessen, 2017). However, only a handful of published studies have used this combination to study immunometabolism. The study in closest alignment to my aim of investigating the metabolic changes associated with an immune response is by Chambers *et al.* who performed metabolomic analysis on flies infected with the bacterium *Listeria monocytogenes* (Chambers, Song and Schneider, 2012). As expected, given the close linking of metabolism and immunity, numerous metabolites were found to have altered concentrations following infection. In particular they found depletion of long-chain fatty acids, modulation of beta-oxidation, and complex effects on sugar metabolism. Interestingly, they also observed changes in the levels of amino acids and purine catabolites. Many of the findings in the study

are hard to interpret due to the fact that *L. monocytogenes* causes a lethal infection in *Drosophila*. Consequently, it is unclear whether a particular metabolic change is occurring due to the host immune response or due to infection-induced pathology, particularly as the infection progresses. Notably, large changes were seen in metabolism by 6 hours following infection, the earliest time point examined. Given that the *Drosophila* humoral immune response is rapid, with strong expression of a number of antimicrobial peptides by 1.5 hours after infection (Lemaitre, Reichhart and Hoffmann, 1997; Clark *et al.*, 2013), this suggests that earlier changes in metabolism that may function to support the immune response could have been missed in this study.

3.1.2 Aims

The first aim of this project was to identify metabolites and metabolic pathways that are altered during a successful immune response to a systemic bacterial infection in *Drosophila*. To do this I, in collaboration with my co-supervisor **Dr Gerald Larrouy-Maumus** (Department of Life Sciences, Imperial College London), created and optimised high-performance liquid chromatography-mass spectrometry (LCMS)-based protocols for metabolomic analysis of whole-fly samples. Extensive data analysis could then be performed to find, and identify, metabolites that increase or decrease following infection. Consideration of any changing metabolites within the context of their metabolic pathways and known functions would then be used to generate testable hypotheses concerning the function of altered metabolic pathways in the immune response. Any such hypotheses could then be investigated and tested using the genetic tools available in *Drosophila*.

3.2 Untargeted metabolomic analysis of infected flies

3.2.1 The experimental protocol

The first step was to decide on the details for the infection and the samples to be taken. As the aim was to investigate the metabolic alterations occurring due to the immune response, bacterial infections that were reliably non-lethal, whilst also being strong activators of the fly immune system, were chosen. The kinetics of such infections would ideally result in an infection course in which the host, upon detecting the presence of bacteria, mounts an effective humoral immune response which successfully controls the infection and so results in host survival. In this way, metabolic changes due to host pathology or bacterial activity were expected to be minimised.

The fly humoral immune response is activated by detection of invading microbes by the Imd and Toll pathways, which primarily respond to Gram-negative and Gram-positive bacteria respectively. As these pathways transduce signals separately and result in the expression of different, although overlapping, sets of immune genes, I infected flies with Gram-negative and Gram-positive bacteria in parallel. For the initial metabolomic experiments I used the Gram-negative bacterium *E. coli* and the Gram-positive bacterium *M. luteus*. While resulting in a robust immune response, injection of either of these bacteria does not result in lethality in *w¹¹¹⁸* flies (used as “wild-type”) under the infection conditions used in our lab. These particular infections had the added advantage that they mirrored the conditions of a transcriptomic dataset previously generated in the Dionne Lab using RNA extracted from whole flies 3 hours after infection with either *E. coli* or *M. luteus* alongside injury-only, and anaesthesia-only controls. As a result, the transcriptomic data could provide context in which to interpret the metabolomic results. One issue in interpreting metabolomic data is that if the concentration of a particular metabolite is found to increase, one cannot determine whether this is due to increased production or decreased consumption of this metabolite without further information on the context. Use of transcriptomic data can add context and perhaps suggest the underlying cause for a metabolic change; for example, strong transcriptional up-regulation of an enzyme producing a metabolite found to increase in concentration would indicate

upregulation of its production. Consequently, aligning the metabolomic experiment with this transcriptomic dataset may provide valuable context in which to interpret the results.

The systemic immune response in *Drosophila* is fast and so the timing of sample collection is critical. Antimicrobial peptides have different temporal patterns of expression following infection; however, in response to systemic infection with *E. coli* or *M. luteus*, very strong expression of a variety of antimicrobial peptides is evident by 3 hours post-infection (Lemaitre, Reichhart and Hoffmann, 1997; Boutros, Agaisse and Perrimon, 2002). Studies investigating metabolic changes have tended to look at later time points, however changes in the transcription of numerous metabolic genes are seen as early as 1.5 hours following these infections (Clark *et al.*, 2013). For these reasons, and in order to mirror the aforementioned transcriptomic dataset, I used a 3-hour timepoint for sample collection following injection.

The method to induce systemic infection involved injecting 50 nl of a bacterial suspension diluted in PBS to a defined OD₆₀₀ to ensure consistent bacterial numbers between experiments utilising the same bacterial strains. Injection of fluid into the fly abdomen results in a mild immune response independent of bacterial infection, as indicated by a brief induction of antimicrobial peptide transcript expression (Lemaitre, Reichhart and Hoffmann, 1997). To differentiate between the responses to injury and infection, flies injected with sterile PBS were used as sterile-injury controls.

For LCMS analysis, initial preliminary tests, conducted with **Dr Gerald Larrouy-Maumus**, indicated that samples generated from homogenates of 3 whole flies were sufficient for good detection of a variety of metabolites. Furthermore, early qualitative analysis of the generated data appeared to confirm that changes in metabolite levels between conditions could be measured quantitatively at the 3-hour timepoint.

3.2.2 Untargeted metabolomics can be used to investigate metabolic changes during a successful *Drosophila* immune response

To investigate metabolic changes during the immune response, I first utilised an “untargeted” approach in which all detected and quantifiable features within the LCMS data are analysed.

Identification of metabolites that show changes in concentration between experimental conditions can then be used to generate hypotheses concerning the underlying biology.

In the experiment outlined here, flies were infected with *E. coli* or *M. luteus* and, alongside sterile PBS-injected and uninjected controls, harvested 3 hours later for LCMS. After quality control and removal of known duplicate features, 199 features were analysed using Agilent Mass Profiler software and an in-house workflow using R. Feature abundance was normalised to the sum of all features in each sample. While the identity of the majority of the features was undetermined, numerous features could be matched with metabolite identities based on the use of standards. A further set of features, while their identity could not be fully established due to being isobaric (same determined mass) with close retention times (RTs), were highly likely to be one of a small number of isomers. For example, our experimental set-up has little ability to differentiate between sugars of the same mass, and so some features are annotated as monosaccharides, disaccharides, or alcohol sugars. The median log₂ fold changes of identified features, and some putatively identified features, relative to the median of the uninjected samples are shown in **Tables 3.1–4**: the proteogenic amino acids (except for cysteine which was not quantified) are shown in **Table 3.1**; non-proteogenic amino acids and amino acid-related metabolites are shown in **Table 3.2**; purines and related metabolites are shown in **Table 3.3**; and identified TCA cycle and related metabolites are shown in **Table 3.4**. The cells of the tables are coloured with a colour gradient with blue highlighting metabolites found to decrease following sterile injury or infection, red highlighting increased metabolites, and the intensity of the colour reflecting the extent of change. The largest changes observed in the amino acids and related metabolites was a strong decrease in the median concentration of the branched-chain amino acids, leucine, isoleucine, and, albeit to a lesser extent, valine, following infection (**Table 3.1**). Leucine, for example, showed a log₂ fold change, relative to the uninjected samples, of -3.96 following *M. luteus* infection; this was the largest fold-decrease seen amongst the identified metabolites. Levels of glycine, phenylalanine, and tyrosine also showed strong decreases, with concentrations following one, or both, of the infections less than half that of the controls. In contrast, few amino acids or related metabolites showed comparable increases in concentration, with the exception of nicotinic acid which demonstrated a doubling in median concentration in either bacterial infection. Lysine appeared to show a modest elevation in concentration following *E. coli* or *M. luteus* expression

(median log2FCs of 0.58 and 0.38 respectively, **Table 5.1**). Interestingly, tryptophan showed a modest increase in level after *M. luteus* infection (log2FC of 0.73) which was not seen in response to *E. coli* infection. This pattern was mirrored to some extent by kynurenine, a product of tryptophan catabolism, which appeared increased in response to *M. luteus*, but, in contrast, decreased in response to *E. coli* (**Table 5.2**).

Metabolite	PBS	<i>E. coli</i>	<i>M. luteus</i>
Alanine	-0.07	-0.12	-0.24
Arginine	0.08	-0.04	0.07
Asparagine	0.22	-0.03	-0.17
Aspartic Acid	-0.48	-0.33	-0.41
Glutamate	-0.21	0.00	-0.04
Glutamine	0.10	0.12	0.11
Glycine	-0.47	-0.74	-1.72
Histidine	0.05	0.04	0.15
Isoleucine	-0.97	-2.65	-3.96
Leucine	-0.86	-2.76	-3.33
Lysine	0.24	0.58	0.38
Methionine	0.35	0.07	0.27
Phenylalanine	-0.29	-1.31	-1.19
Proline	-0.08	0.08	0.08
Serine	0.69	0.30	-0.07
Threonine	-0.10	-0.26	-0.15

Tryptophan	0.32	0.15	0.73
Tyrosine	-0.28	-1.09	-1.40
Valine	-0.48	-1.26	-1.57

Table 3.1: Log2 fold changes in the abundance of identified proteogenic amino acids

Median log2 fold changes relative to median uninjected for each of the proteogenic amino acids 3 hours following injection with sterile PBS, *E. coli*, or *M. luteus*. Cysteine was not successfully quantified in this experiment and thus absent from this list.

Metabolite	PBS	<i>E. coli</i>	<i>M. luteus</i>
Beta-Alanine	-0.03	-0.24	-0.13
Betaine	-0.32	0.28	-0.13
GABA Isobar 1	-0.05	0.02	0.04
GABA Isobar 2	-0.27	-0.27	-0.76
Glutathione	-0.14	-0.23	-0.16
Kynurenine	0.28	-0.48	0.45
Methionine S-oxide	0.03	-0.17	0.00
Sarcosine	-0.03	0.31	0.28
Taurine	0.06	0.13	0.27
Nicotinic acid	0.41	0.97	1.06

Table 3.2: Log2 fold changes in the abundance of identified non-proteogenic amino acids and amino acid-related metabolites.

Median log2 fold changes relative to median uninjected for various non-proteogenic amino acids and related metabolites 3 hours following injection with sterile PBS, *E. coli*, or *M. luteus*.

Changes in the abundance of identified purines and related metabolites showed a decrease in median adenosine concentrations for both infections (log2FCs of -0.78 and -0.83 following *E. coli* and *M. luteus*, **Table 3.3**) markedly contrasting with increased levels of the purine catabolites uric acid and, in particular, allantoin which showed striking increases in observed levels following infection with either *E. coli* (log2FC=2.30) or *M. luteus* (log2FC=1.95). These increases in allantoin levels were the largest amongst the identified metabolites. A feature that likely represented another purine catabolite, 5-hydroxyisourate (5-HIU), increased in a similar manner following infection (log2FCs of 1.51 and 1.85). I assigned this feature (mass=184.0226, RT=1.17) the putative identification 5-HIU based on matching mass (exact mass of 5-HIU=184.0233, therefore a difference of 3ppm); the fact that no other biological compound is annotated with this mass in the Metlin database (Guijas *et al.*, 2018); and a retention time similar to, but not overlapping with, uric acid, a closely related compound. This latter observation suggests a similarity in chemistry to uric acid but also shows that this feature is not duplicated with uric acid (i.e., this peak is not also a uric acid peak). This likely identification, in addition to a pattern of regulation mirroring allantoin, led me to include this feature in the tabulated log2 fold change data.

Metabolite	PBS	<i>E. coli</i>	<i>M. luteus</i>
Inosine	0.54	0.42	0.55
Adenosine	0.41	-0.78	-0.83
Guanosine	0.30	0.07	0.17
Hypoxanthine	0.33	0.48	0.52
Uric acid	0.67	0.91	0.91
5-HIU (Putative ID)	1.16	1.51	1.85
Allantoin	0.95	2.30	1.95

Table 3.3: Log2 fold changes in the abundance of identified purines and related metabolites.

Median log2 fold changes relative to median uninjected for identified purines and related metabolites 3 hours following injection with sterile PBS, *E. coli*, or *M. luteus*. The identification of 5-hydroxyisourate (5-HIU) is putative as a standard for this compound was not run.

The levels of identified tricarboxylic acid (TCA) cycle metabolites showed no such striking changes in median abundances (**Table 3.4**). The largest fold change of the fully identified metabolites was seen in the increased median level of α -ketoglutaric acid following *M. luteus* infection ($\log_2\text{FC}=0.75$). Other modest increases were seen in two of the partially identified metabolites. Two features matched aconitic acid and had close RTs, with the less abundant of these, aconitic acid isobar 2, showing increased levels following infection with *M. luteus* (0.72), and to a lesser extent *E. coli* (0.58). One of the features representing either citric acid or iso-citric acid, (iso)citric acid 1, was increased following *E. coli* infection (0.77), and to a lesser extent, following *M. luteus* infection (0.56).

Metabolite	PBS	<i>E. coli</i>	<i>M. luteus</i>
Aconitic acid isobar 1	0.09	0.12	0.35
Aconitic acid isobar 2	0.22	0.58	0.72
α -Ketoglutaric acid	0.04	0.19	0.75
(iso)Citric acid 1	0.22	0.77	0.56
(iso)Citric acid 2	0.21	0.44	0.38
Fumaric acid	-0.10	0.12	0.16
Malic acid	0.15	0.24	0.25
Succinic acid	0.24	0.32	0.32

Table 3.4: Log2 fold changes in the abundance of identified TCA cycle metabolites

Median $\log_2$ fold changes relative to median uninjected for identified TCA cycle metabolites 3 hours following injection with sterile PBS, *E. coli*, or *M. luteus*. Citric acid and iso-citric acid were not differentially identified with standards due to very close RTs. Two features matched aconitic acid but close RTs prevented determination of the 'true' aconitic acid peak. Consequently, the data for both features are shown as aconitic acid isobars, with isobar 1 having the larger peak area.

3.2.3 Principal component analysis

Next, principal component analysis (PCA) was performed on the full data from the above experiment to observe whether the samples cluster by experimental condition, thus indicating differing metabolic responses. Should the samples cluster, the PCA also shows which features (metabolites) contribute the most to the clustering. Consequently, this analysis can be used to highlight potential metabolites of interest for hypothesis generation and further investigation. The data were first centred and scaled by median level scaling in which the abundance of each feature in each is expressed as a change relative to the median abundance of that feature in all samples. This method ensures that multivariate statistical techniques, including PCA, are not biased in favour of higher abundance metabolites (van den Berg *et al.*, 2006). The results from principal component analysis of this data are shown in **Figure 3.1** and **Table 3.5**. On the first two principal components (PCs), the samples showed clear, although overlapping, clustering by experimental condition for the uninjected, PBS-injected, and bacterially infected samples but with the *E. coli*-infected and *M. luteus*-infected samples overlapping considerably (**Figure 3.1B**). This perhaps indicates similar metabolic responses to these infections at this timepoint. Consistent with sterile injury producing a mild immune response, the PBS-injected fly samples cluster between the uninjected and infected samples. Variation along the third PC did not correlate with experimental conditions (**Figure 3.1B, C**). The shape of the scree plot suggests the use of the first 3 PCs, as afterwards the curve begins to flatten, with these 3 PCs explaining 58.05% of the variation in the data (**Figure 3.1D**).

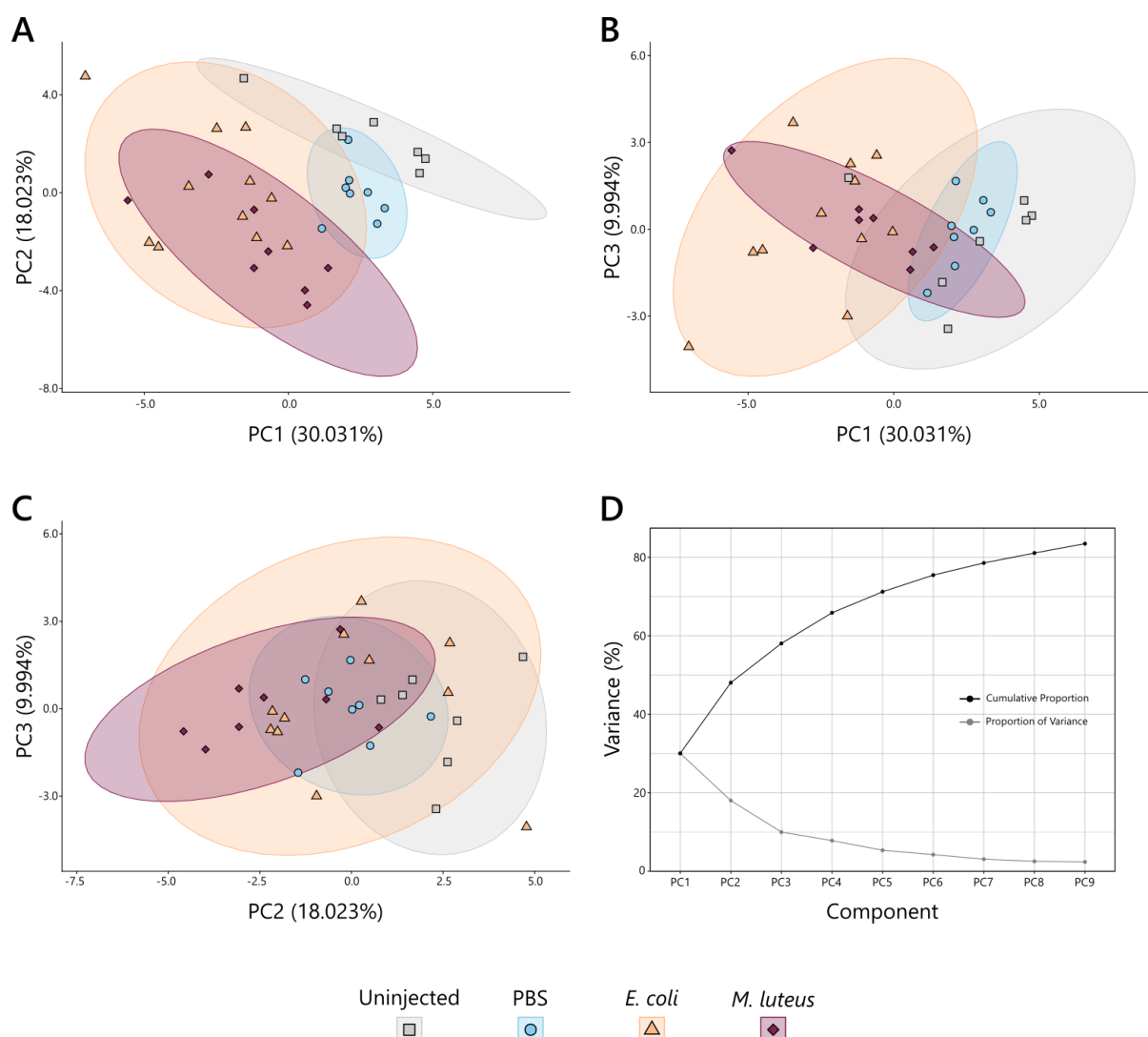


Figure 3.1: Principal component analysis indicates metabolic shifts 3 hours after infection.

PCA was performed on LCMS data from fly samples after infection with *E. coli* or *M. luteus* alongside PBS-injected and uninjected controls. 2-dimensional plots of the first 3 principal components are shown in **A-C**. The scree plot from the analysis, indicating the variance explained by each PC is shown in **D**.

The top 10 features contributing to the loadings of each of the first three PCs, are shown in **Table 3.5**. Of these features, only 5 were able to be identified with confidence (in bold): 4 proteogenic amino acids plus the nitrogen waste metabolite allantoin. The feature most contributing to PC1 was also amongst the top 10 features contributing to PCs 2 and 3 but was of unknown identity. Notably this unknown feature had almost the same mass (as determined by the Agilent MassHunter Profiler software) as the feature third-most contributing to PC1. The weight of these features in the PCA indicates their importance in explaining the variation between the samples, and as the samples cluster by experimental condition when plotted on

PCA1 and 2, these features are suggested to be regulated by infection. Indeed, graphing the normalised (but not centred or scaled) data for these features showed increased median abundance following *E. coli* or *M. luteus* infection. However, abundances were highly variable in the infected samples, which accounts for the inclusion of the first unknown feature (**Figure 3.2A**) in all three tabulated PCs (**Table 3.5**).

Metabolite	Exact mass	RT (min)	PC1	PC2	PC3
Unknown	162.0533	5.15	-0.483	0.302	0.331
Disaccharide 2	342.1158	3.85	-0.430	0.172	0.295
Unknown	162.0529	5.53	-0.318	-	0.251
Monosaccharide 2	180.0630	2.11	-0.293	0.372	-0.667
Possible sugar alcohol 1	182.0788	2.18	-0.205	-	-0.116
Possible sugar alcohol 2	182.0789	2.29	-0.198	-	-0.110
Isoleucine	131.0947	9.20	0.195	0.315	-
Allantoin	158.0437	1.62	-0.185	-	-
Leucine	131.0947	8.90	0.173	0.295	-
Unknown	132.0422	1.00	-0.161	-	-0.174
Glycine	75.0320	9.84	-	0.243	-
Phenylalanine	165.0791	7.79	-	0.223	-
Unknown	198.0386	1.13	-	-0.221	-
Unknown	166.0131	1.76	-	-0.189	-
Possible 5-HIU	184.0226	1.17	-	-0.185	-
Unknown	196.0580	1.99	-	-	-0.218
Unknown	127.0390	11.33	-	-	-0.151
Monosaccharide 1	180.0632	2.52	-	-	-0.119

Table 3.5: The features contributing the most to the loadings of the first three principal components.

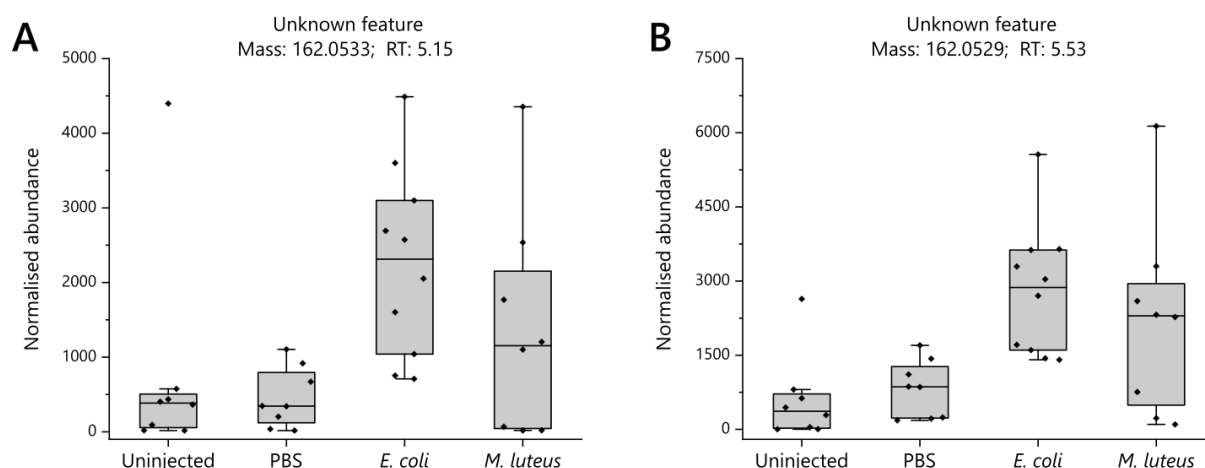


Figure 3.2: Two unknown features of the same mass show increased concentration following infection.

Data extracted from the untargeted metabolomic analysis. 3 flies per sample, $n = 8-10$ per condition. In the graphs, the line indicates median, the box shows the IQR, and the whiskers show the range of data within $\pm 1.5 \times \text{IQR}$ of the median.

A further 5 features highlighted by the PCA were identified as corresponding to sugars or sugar alcohols (**Table 3.5**). The second ranked feature in terms of PC1 contribution (342.1158 at 3.85min) was the second most abundant peak likely corresponding, in terms of mass and RT, to a disaccharide (therefore termed "disaccharide 2"). This feature appeared to be strongly upregulated by *E. coli* infection, and to a lesser extent, by *M. luteus* infection (**Figure 3.3A**). The feature contributing the most to both PC2 and PC3 (180.0630 at 2.11min) was the second most abundant feature likely corresponding to a monosaccharide ("monosaccharide 2", **Figure 3.3B**). More precise identification of the sugar peaks was not possible with standards due to close and highly variable retention times. These features, especially monosaccharide 2, showed high variation regardless of condition. While such variation may be reflective of the biology, it could be due to poor quantification of sugars in whole fly samples using our instrumental set-up. The features that likely correspond to 6-carbon sugar alcohols (sorbitol/mannitol), and highlighted by the PCA, were elevated following infection (**Figure 3.3C, D**).

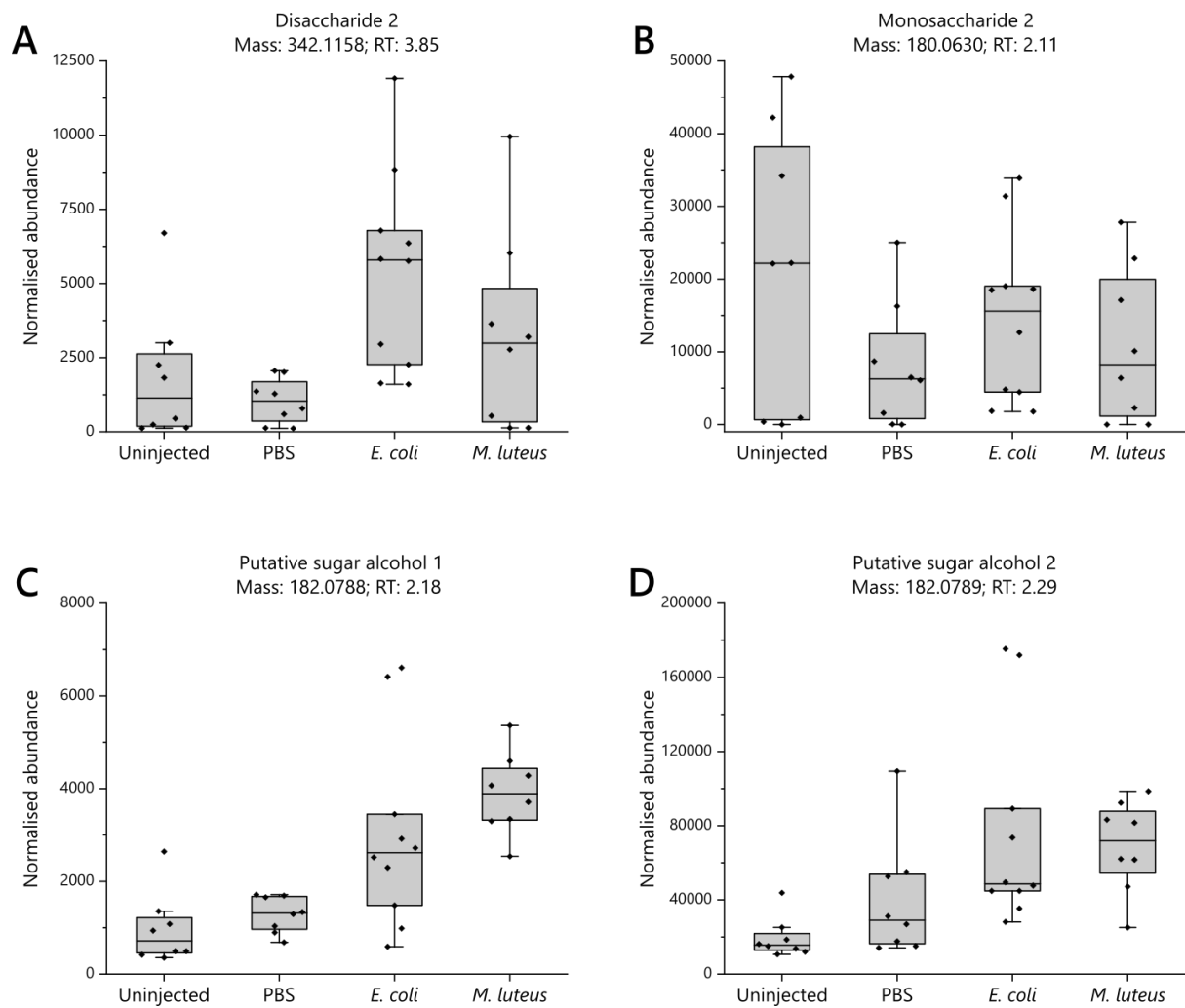


Figure 3.3: The concentrations of some, but not all, sugar and sugar alcohol metabolites are raised following infection.

Data extracted from the untargeted metabolomic analysis. 3 flies per sample, $n = 8-10$ per condition. In the graphs, the line indicates median, the box shows the IQR, and the whiskers show the range of data within $\pm 1.5 \times \text{IQR}$ of the median.

3.2.3.1 Levels of the branched chain amino acids decrease following infection

The branched chain amino acids (BCAAs), isoleucine and leucine, were indicated to be of interest in the PCA (**Table 3.5**) and, as can be seen in box plots of this data (**Figure 3.4A, B**), concentrations of both metabolites markedly decreased following infection, and to a lesser extent, following sterile injury. While valine, the other BCAA was not highlighted by the PCA, it also showed a similar pattern of substantially decreased levels following infection and an intermediate phenotype after sterile injury (**Figure 3.4C**). The similar pattern of changes in the BCAAs may reflect the fact that they are all deaminated by the same enzyme, BCAA aminotransferase (BCAT). The transcriptomic data set previously generated in the Dionne lab indicates transcriptional upregulation of the putative *Drosophila* BCAT gene, *CG1673*, 3 hours following injury or *M. luteus* infection, but not following *E. coli* infection (**Figure 3.4D**). While the increased expression of *CG1673* correlates with the decrease in the BCAAs in *M. luteus* infection, the results do not correlate in *E. coli* infection. The BCAAs are essential amino acids, so cannot be synthesised by *Drosophila*, and BCAT is the only enzyme known to act on BCAAs in animals. Consequently, this the reduction seen in levels of free BCAAs is due either to BCAT activity or incorporation into protein. As multiple other essential amino acids, such as histidine, lysine, methionine and tryptophan, do not appear to show any reduction in levels, it seems unlikely the reduction is caused by incorporation of the BCAAs into protein unless such protein is hugely enriched with BCAAs. Therefore, it seems more likely that upregulation of BCAT activity is responsible for this metabolic phenotype and the discrepancy in the *E. coli*-infection between the metabolomic and the transcriptomic results may be due to variation between the independently performed experiments.

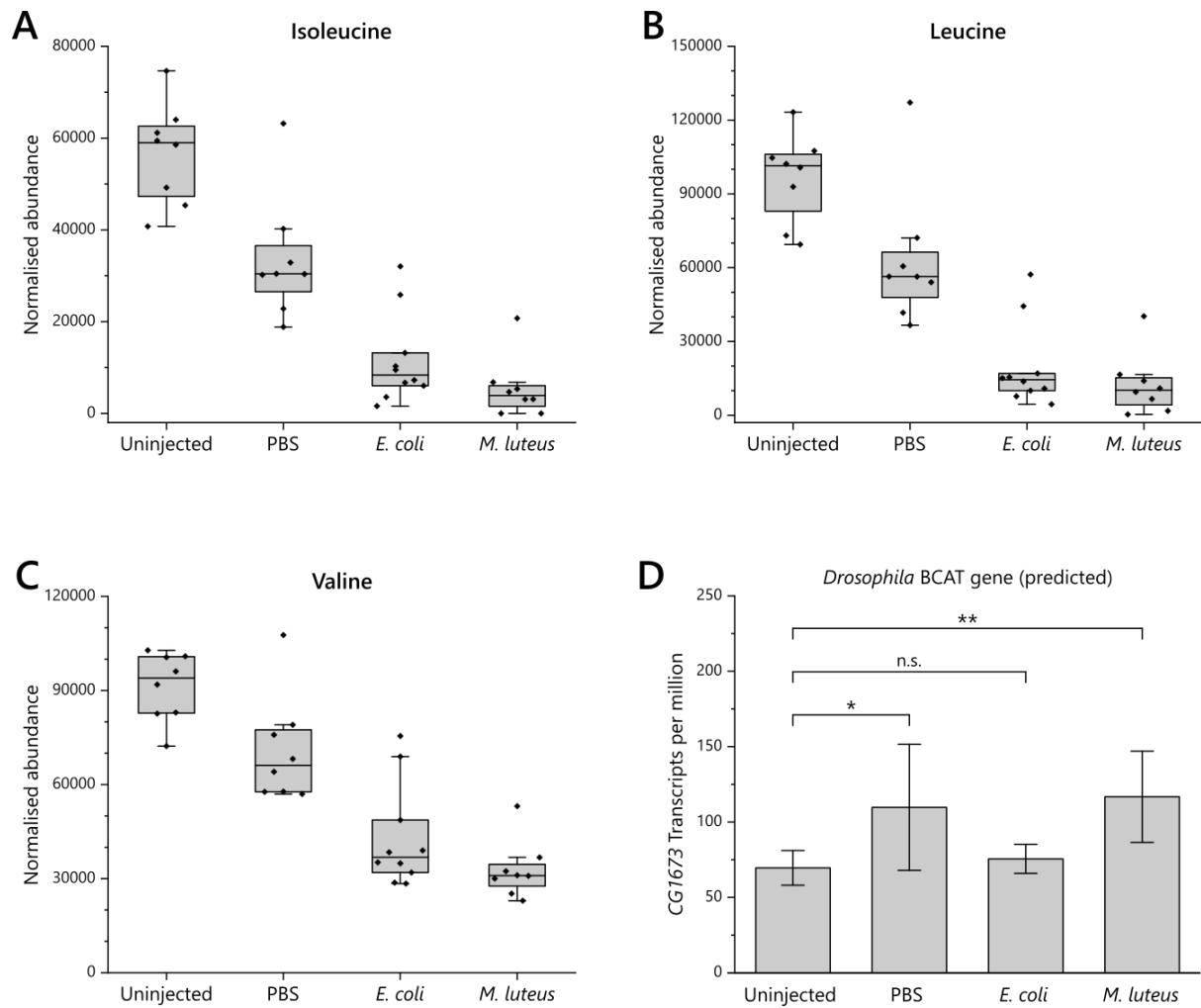


Figure 3.4: The branched chain amino acids are depleted in *Drosophila* following bacterial infection.

The data in **A–C** is extracted from the untargeted metabolomic analysis. 3 flies per sample, $n = 8–10$ per condition. In the graphs, the line indicates median, the box shows the IQR, and the whiskers show the range of data within $\pm 1.5 \times \text{IQR}$ of the median. **D**: Data from a transcriptomic data set previously generated in our lab using the same experimental conditions found transcriptional expression of *CG1673*, the predicted *Drosophila* BCAA aminotransferase, increased following sterile injury ($q = 0.0326$) or *M. luteus* infection ($q = 0.0061$), but not following *E. coli* infection ($q = 0.9477$). $n = 5$ per condition. Graph bars show mean transcripts per million, whiskers show ± 1 standard deviation from mean. Data analysis performed by **Dr Marc Dionne**.

3.2.3.2 Purine catabolites show increase in concentration following infection or injury in *Drosophila*

Allantoin, which showed the largest median fold change of the identified metabolites (**Table 3.3**), was also the 8th ranked feature contributing to PC1 (**Table 3.5**). Allantoin is the end product of purine catabolism in *Drosophila* (see **Section 1.4.3**) and the purines and related metabolites showed changes in median abundance, with most increasing following infection and sterile injury (**Table 3.3**). Consequently, I was particularly interested in further exploring the data from this experiment regarding this particular metabolic change. The normalised abundances of allantoin and its upstream metabolites, the putatively identified 5-HIU and uric acid, all show clear elevation following infection and sterile injury (**Figure 3.5A-C**). While the difference between the infected samples and the uninjected samples is clear, the difference between the infected samples and the sterile injury samples is not so apparent, perhaps indicating a general response to injury. Indeed, the transcriptomic data show expression of *Urate oxidase (Uro)*, which encodes the enzyme that oxidises uric acid to 5-HIU, is upregulated to a similar extent following sterile injury and infection (**Figure 3.5D**). The metabolic pathway containing these metabolites and the enzyme Uro is shown in **Figure 3.5E** (see **Figure 1.3** for more detail). These data mirror an earlier observation by Chambers *et al.* of strongly elevated allantoin levels 6-, 24-, and 48 hours following infection with *Listeria monocytogenes*, (Chambers, Song and Schneider, 2012). However, as this bacterium causes lethal infection in flies and sterile injury controls were not performed in their study, this result could suggest this metabolic change is not a product of the humoral immune response to the bacteria, but a response to tissue damage caused either by the mechanism of infection or infection induced pathology. As previously mentioned, *Drosophila* do show an immune response to sterile injury, as indicated by upregulation of antimicrobial peptide transcript expression, though this response is not as prominent or as sustained as the response infection (Lemaitre, Reichhart and Hoffmann, 1997). Consequently, given the early 3-hour timepoint, the result seen in my experiment could also be interpreted as due to the host response to tissue damage rather than to the detection of bacteria. Untangling whether this upregulation is a response to tissue damage independent of bacterial detection, or whether it is related to the humoral immune response against infection requires further work.

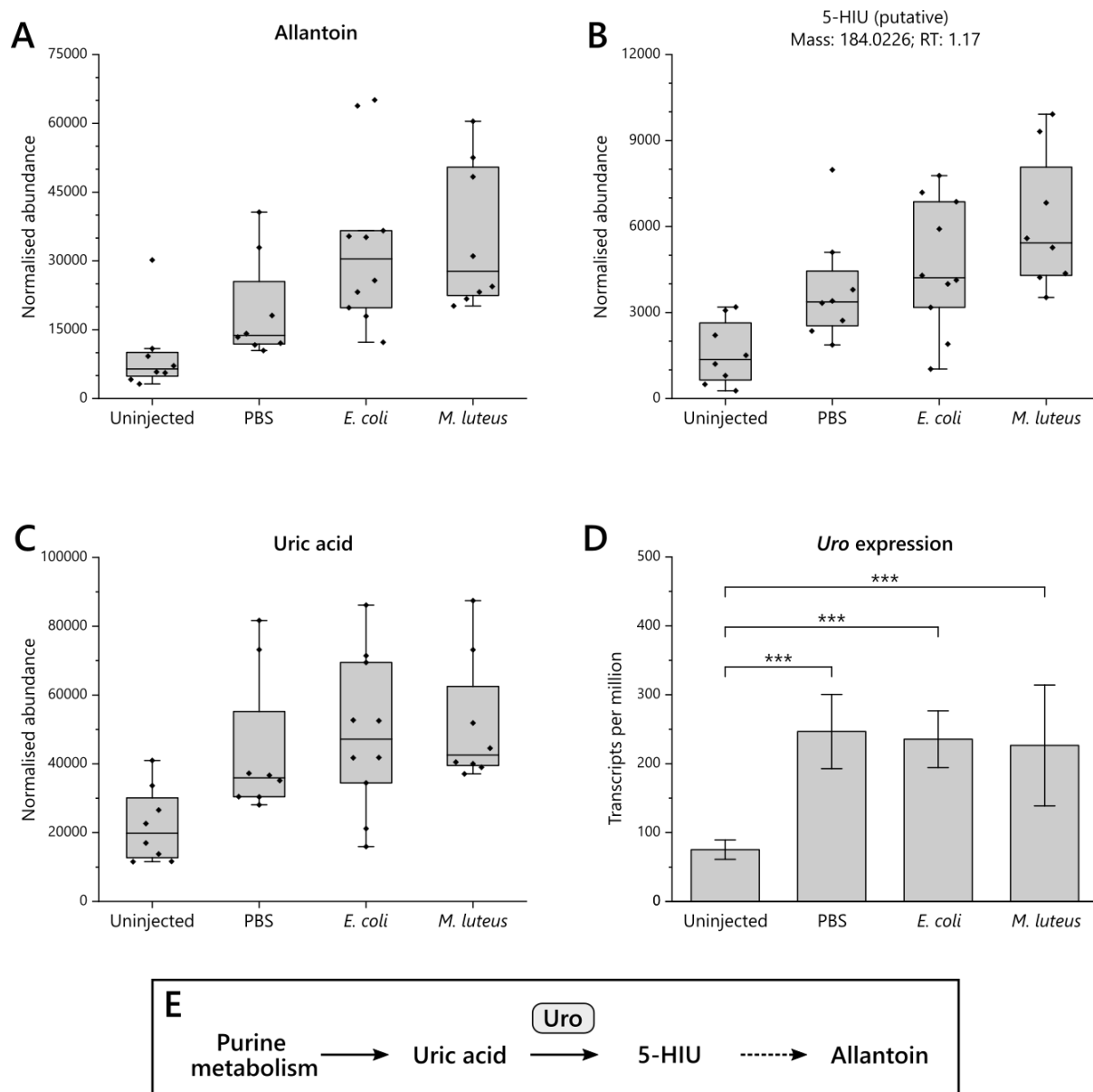


Figure 3.5: Purine catabolites show increase concentration following infection or injury.

Data extracted from the untargeted metabolomic analysis. 3 flies per sample, $n = 8-10$ per condition. In the graphs, the line indicates median, the box shows the IQR, and the whiskers show the range of data within $\pm 1.5 \times \text{IQR}$ of the median. **D:** Data from a transcriptomic data set previously generated in our lab using the same experimental conditions found transcriptional expression of *Urate oxidase* (*Uro*) increased following PBS injection ($q \leq 0.0001$), *E. coli* infection ($q \leq 0.0001$), and *M. luteus* infection ($q \leq 0.0001$). $n = 5$ per condition. Graph bars show mean transcripts per million, whiskers show ± 1 standard deviation from mean. Data analysis performed by **Dr Marc Dionne**. The relationship between the purine catabolites and the *Uro* enzyme is shown in **E**.

3.2.3.3 The levels of free glycine and phenylalanine appear to be depleted following infection

The amino acids glycine and phenylalanine were also picked out of data by PCA, and both show decreased concentration in infected samples as shown in by the extracted normalised abundance of these metabolites (**Figure 3.6**). The reduced glycine concentration in *M. luteus* infected samples is perhaps particularly striking in light of the very high measured abundance of glycine in whole fly samples (**Figure 3.6A**). As with the BCAAs, reduction in the free levels of these amino acids could be due to protein synthesis or enzyme activity. The decrease in phenylalanine could potentially be due to its conversion into tyrosine to fuel the melanisation response following injury and infection (see **Section 1.4.1**). The metabolomic study performed by Chambers *et al.* also showed reduced phenylalanine levels, but at 6-, 24-, and 48 hours following *Listeria* infection (Chambers, Song and Schneider, 2012). No reduction in glycine was observed in their data indicating that the reduction seen here is perhaps specific to this earlier 3-hour time-point.

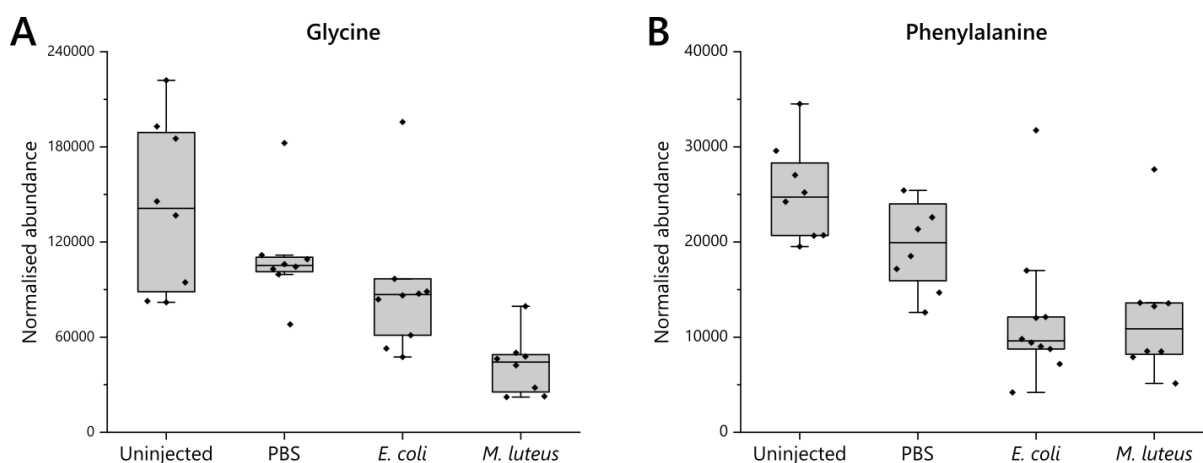


Figure 3.6: Glycine and phenylalanine show reduced levels following infection.

Data extracted from the untargeted metabolomic analysis. 3 flies per sample, $n = 8-10$ per condition. In the graphs, the line indicates median, the box shows the IQR, and the whiskers show the range of data within $\pm 1.5 \times \text{IQR}$ of the median.

3.2.4 The results from metabolites of interest were replicated in *E. faecalis* infection

3.2.4.1 Which metabolic changes to focus on?

Untargeted metabolomic analysis of whole-fly samples showed changes in metabolism in response to infection and, to a lesser extent, sterile injury. Metabolites indicated by PCA to have altered concentrations between the experimental conditions included unidentified features, sugar metabolites, the BCAAs and purine catabolites, as well as the amino acids glycine and phenylalanine. The unknown features will require further work for identification before their changes can be usefully interpreted.

While the levels of various sugars were elevated following infection, our experimental set up was unable to distinguish between different sugars of the same mass and the abundance many of these features was very variable. This may reflect biology, but I also believe that our specific LCMS set-up was not well suited to the quantification of these metabolites and so may be adding substantial noise into the data. Running metabolite standards for various sugars (see **Section 2.3.5**) showed that while their measured abundance correlated well with concentration, each sugar presented a large number of peaks with different m/z ratios but despite having the same RT, many were not identified by the Agilent software and appear in the untargeted data as multiple unknown features. The chromatography of the sugars also appeared to indicate that each sugar gave a signal comprising of two overlapping peaks with the same m/z , this could make quantification less accurate. These problems seen in the sugar standards are likely exacerbated in the chemically complex whole-fly samples. Due to these issues with measuring sugar compounds, in addition to the fact that regulation of sugar metabolism by immune signalling is already a highly active area of research in *Drosophila* (Roth *et al.*, 2018; Davoodi *et al.*, 2019; Dolezal *et al.*, 2019), I decided to concentrate on the changes seen in the amino acids and purine catabolites.

3.2.4.2 The observed changes in leucine and allantoin concentrations were replicated in *E. faecalis* infection

The BCAAs showed a marked decrease in abundance following infection in this experiment, whereas the purine catabolite allantoin showed substantially increased levels. To validate these two results seen in this untargeted analysis and to determine if they are general responses to bacterial infection, rather than specific to *E. coli* and *M. luteus*, I performed a targeted metabolomic analysis on flies following infection with the Gram-positive bacterium *E. faecalis*. While injection of *M. luteus* strongly induces the Toll pathway, it rarely kills the fly host, even when the Toll pathway is inhibited. In contrast, *E. faecalis* reliably kills flies that have reduced Toll pathway activity (this is confirmed in my hands in the next chapter) demonstrating that a successful humoral immune response is required for the fly host to survive this infection. Consequently, I considered that infection with *E. faecalis* would likely be more useful in follow up experiments investigating metabolic responses in systemic immunity as this infection would presumably be more likely than *M. luteus* infection to show an immune phenotype in flies with a genetically disturbed immune-metabolic response.

To add temporal detail to the results, I tested an additional time-point of 4.5 hours following infection. Metabolism can change rapidly but the untargeted data above only provides information regarding metabolite levels at a single moment in time. Consequently, adding even one time-point can provide value information regarding the kinetics of the changes: how transient is increase in allantoin? Is the concentration of allantoin in the process of further increasing or is it recovering from an earlier peak? I reasoned that data from this additional time-point could potentially aid in differentiating the response to injury from the response to bacterial infection as the injury response is likely more transient.

For this experiment, I analysed the results using a simple targeted method, quantifying abundance of leucine and allantoin only. I choose these as, of the identified metabolites in the above metabolomic experiment, leucine showed the largest median decrease in concentration following infection, whereas allantoin showed the largest median increase. Testing just two metabolites is far less time-demanding but also allows for simple statistical hypothesis testing that is not possible when testing numerous metabolites. Notably, although this analysis takes

a very restricted approach, the sample collection and LCMS data acquisition is the same as in the untargeted experiment. Consequently, additional targeted or untargeted analyses can be performed on the collected data as desired.

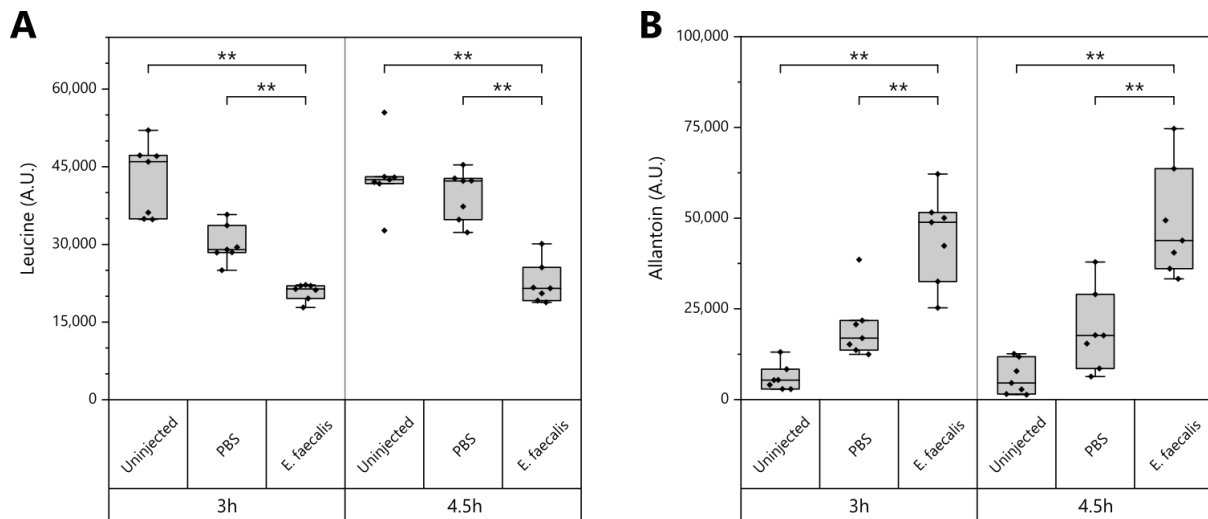


Figure 3.7: The changes in leucine and allantoin concentrations also occur in response to *E. faecalis* infection.

A: Leucine abundance was lower in the *E. faecalis* infected samples compared with both the uninjected and the PBS-injected controls at 3 hours following infection (median log₂FCs = -1.10 vs. uninjected, -0.44 vs. PBS; $p = 0.0021$ vs uninjected and 0.0021 vs PBS) and at 4.5 hours following infection (median log₂FCs = -0.98 and -0.97; $p = 0.0022$ and 0.0022). **B:** Allantoin levels were significantly elevated in the *E. faecalis* infected samples compared with the control conditions both at the 3-hour timepoint (median log₂FCs = 3.19 vs. uninjected and 1.53 vs. PBS; $p = 0.0022$ and 0.0049) and the 4.5-hour timepoint (median log₂FCs = 3.26 and 1.31; $p = 0.0022$ and 0.0049). Pairwise comparisons were conducted using the Mann-Whitney test. $n = 7$ per condition per time-point, 5 flies per sample. The horizontal line indicates median, box shows IQR, and whiskers show range of data within $\pm 1.5 \times \text{IQR}$ of the median.

The results from this experiment clearly showed that leucine concentration was decreased in flies infected with *E. faecalis*, compared with both uninjected and PBS-injected controls (**Figure 3.7A**). The results at the 3-hour and 4.5-hour timepoints were very similar. In contrast to the effect on leucine levels, infection with *E. faecalis* resulted in strongly elevated allantoin abundance at both the 3-hour and 4.5-hour time-points compared with both the uninjected controls and the PBS-injected controls (**Figure 3.7B**). As with the leucine result, little difference was seen in the data between these two time-points. Consequently, two metabolites of interest from the untargeted metabolomic data following *E. coli* or *M. luteus* infection, showed the same pattern of changes following *E. faecalis* infection. These results validate the use of the untargeted data analysis to identify metabolites of interest, and show that these metabolic changes, decreased leucine and increased allantoin, occur during the innate immune response to three different bacterial infections. These results also indicate that both of these metabolic changes are not just due to injury caused by the injections, as the levels of both metabolites

were significantly different between the infected and sterile-injured samples. Instead, injection of PBS appeared to show a milder, intermediate effect, consistent with the induction of a minor immune response to injury alone.

3.3 Infection with *E. cloacae* results in metabolic changes that are synchronous with the immune response

The results from the *E. faecalis* infection showed little difference in the altered metabolite levels between the 3h and 4.5h timepoints, indicating a metabolic response that is actively sustained for at least 1.5 hours or that normal-state metabolism is not rapidly recovered. To further explore the temporal nature of the metabolic changes, I conducted a time-course experiment in which samples were taken for metabolomic analysis at 1-, 2-, 3-, 4.5-, 6-, 7.5-, and 24 hours following infection. Such data would potentially allow me to resolve the timing of the metabolic changes following infection revealing the onset of the metabolic changes and whether the fly host is able to rapid restore normal-state metabolism.

By this point in the project, I had switched to using *E. cloacae* for Gram-negative bacterial infection experiments. This is because, while *E. cloacae* is closely related to *E. coli*, flies lacking a functional Imd pathway are rapidly and reliably killed by *E. cloacae* infection, whereas *w¹¹¹⁸* ("wild-type") flies invariably survive this infection. I also found *E. cloacae* to be more experimentally robust for bacterial quantifications, in my hands, compared with our *E. coli* strain.

For this data, I used a "semi-targeted" metabolomic analysis approach in which only features matching a predetermined set of known metabolites are quantified. It is thus similar to the targeted approach used above but quantifies many more metabolites, in my case the data is searched for features matching a set of around 100 metabolites, although extracted features are only given a positive identification if I had run a standard for the target metabolite. I found this method of analysis to perform better than the untargeted method on LCMS data that was particularly noisy due to instrumental variation in chromatography or detection within the same LCMS data acquisition run. Such variation was observed in the data from this time course experiment.

3.3.1 Allantoin increase after *E. cloacae* infection is synchronous with the immune response.

The data from this experiment showed a number of interesting results. Allantoin, found in the previous experiments to have markedly raised elevation 3 hours after *E. coli*, *M. luteus*, and *E. faecalis* infection also showed strong elevation 3 hours after *E. cloacae* infection (**Figure 3.8A**). In addition, the data indicate that the increase in allantoin occurs primarily between 2 and 3 hours post infection and that allantoin levels remain stably elevated until at least 7.5 hours following infection before beginning to recover to uninfected levels by 24-hour timepoint. The allantoin levels in the PBS-injected flies appeared to follow a similar pattern but with a weaker increase and more variability than the infected samples. Intriguingly, the uninjected flies appeared to show a marked decrease in allantoin levels specifically at the 6-hour timepoint. I speculate that this may be due to circadian behaviour regulating the excretion of allantoin either directly or indirectly as a result of feeding behaviour (these samples would have been harvested in the early evening, between around 5-6pm). Whatever the cause, this change was not reflected in the infected or sterile-injured flies, resulting in a very large difference in allantoin concentration between the conditions at this time point.

Notably, the increase in allantoin concentration between 2 and 3 hours is synchronous with the onset of bacterial clearance in *E. cloacae* infection, as shown in an independent bacterial quantification experiment (**Figure 3.8B**). This indicates that the timing of increased allantoin production occurs at a similar time to when the antimicrobial peptides reach a concentration that kills the bacteria faster than they can replicate. This synchronicity hints that this metabolic change is related to the production of antimicrobial peptides. Unfortunately, quantification of the metabolites immediately upstream of allantoin, such as uric acid, failed due to high variation between the run controls (RunCs; aliquots of a pooled sample).

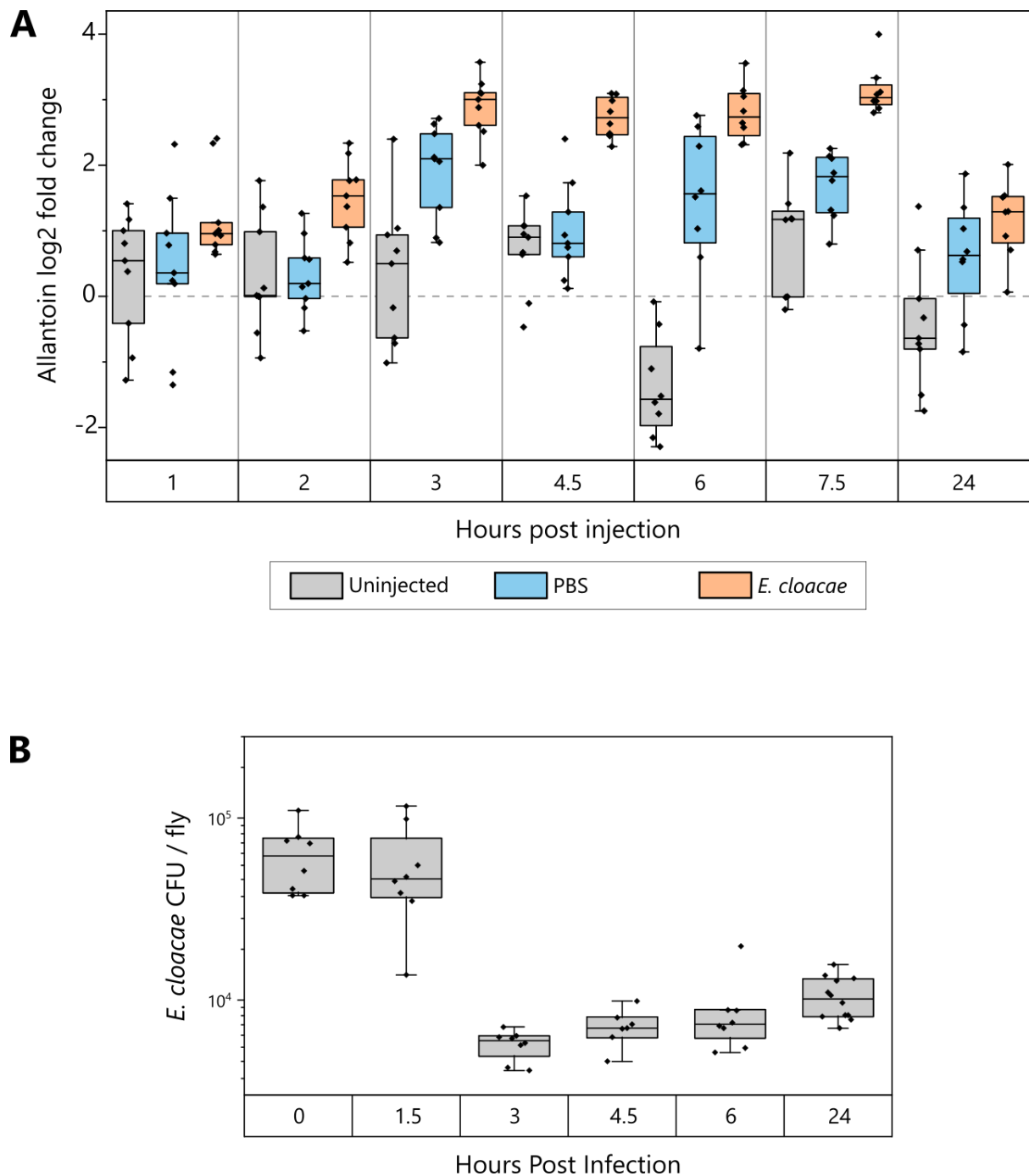


Figure 3.8: Allantoin increase after *E. cloacae* infection is synchronous with the immune response.

Flies (*w¹¹¹⁸*) were infected with *E. cloacae* or injected with PBS and collected for metabolomic analysis at the timepoints indicated along with uninjected controls. The level of allantoin in these samples (**A**) is shown as the log₂ fold change relative to median of all uninjected samples. Compared with uninjected and PBS-injected samples, the infected flies showed substantially elevated levels of allantoin from 3 to 7.5 hours following injection with levels appearing to recover, though incompletely, by 24 hours. The sharp increase in allantoin levels between 2 and 3 hours in the infected flies parallels the onset of bacterial

clearance as shown by bacterial quantification results (**B**) from an independent experiment using the same bacteria, *E. cloacae*, at the same dose ($OD_{600} = 1.0$). Compound abundances (**A**) were quantified by the semi-targeted metabolomic analysis method (33 compounds quantified, negative mode only). Allantoin abundance was normalised to the sum of all compound abundances in each sample. $n = 7-9$ per condition per time point, 3 flies per sample. For bacterial quantification, 1 fly was used per sample, $n = 7-12$ samples per time point. In both graphs, horizontal line indicates median, box shows IQR, and whiskers show range of data within $\pm 1.5 \times IQR$ of the median.

3.3.2 A selection of essential amino acids accumulates following infection

In addition to the changes over time in allantoin concentration after *E. cloacae* infection, the data also suggest changes in the levels of a number of amino acids and related metabolites. Unfortunately, quantification of the BCAAs, which I found to be depleted following infection in the experiments above, failed due to high variation between the RunCs. Tryptophan, which was earlier found to be elevated following *M. luteus* infection, but not *E. coli* infection (**Table 3.1**), showed increased levels from 3 hours post-infection onwards (**Figure 3.9A**). Though this increase in concentration is modest in terms of fold change, with median $\log_2FC$ s of around 0.5 from the 3-hour time-point, it is especially notable as tryptophan is an essential amino acid for *Drosophila*: it cannot be synthesised. Furthermore, although levels of kynurenine, a tryptophan metabolite, varied considerably over time in the uninjected-fly samples they appeared to be generally increased following *E. cloacae* infection, with apparent higher levels than uninjected at the 2-, 3-, and 6-hour timepoints (**Figure 3.9B**). As kynurenine is produced by tryptophan catabolism (see **Section 1.4.3.2**), this suggests “production” of tryptophan is greater than implied by the detected tryptophan levels, as some of the tryptophan is converted to kynurenine. Indeed, the modest but stably elevated levels of tryptophan measured from 3 hours following infection may be indicative of an upper limit in the concentration free tryptophan is able to, or permitted to, reach within the fly, with excess tryptophan requiring catabolism to kynurenine, and possibly other tryptophan products not measured in this experiment.

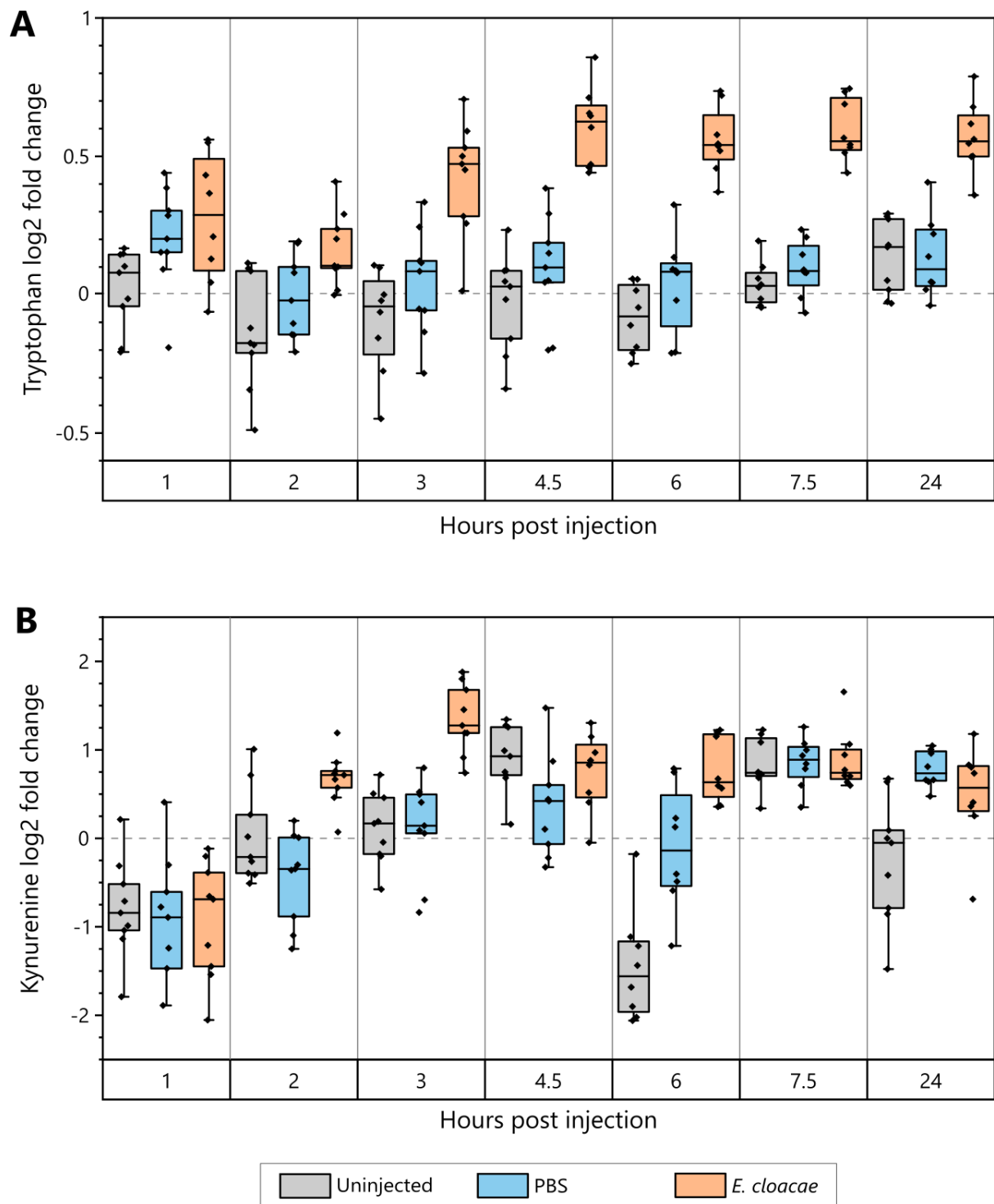


Figure 3.9: The concentrations of tryptophan and kynurenine increase following *E. cloacae* infection

Compound abundances were quantified by the semi-targeted metabolomic analysis method (34 compounds quantified, positive mode only). Abundance was normalised to the sum of all compound abundances in each sample. $n = 7-9$ per condition per time point, 3 flies per sample. The horizontal line indicates median, box shows IQR, and whiskers show range of data within $\pm 1.5 \times \text{IQR}$ of the median.

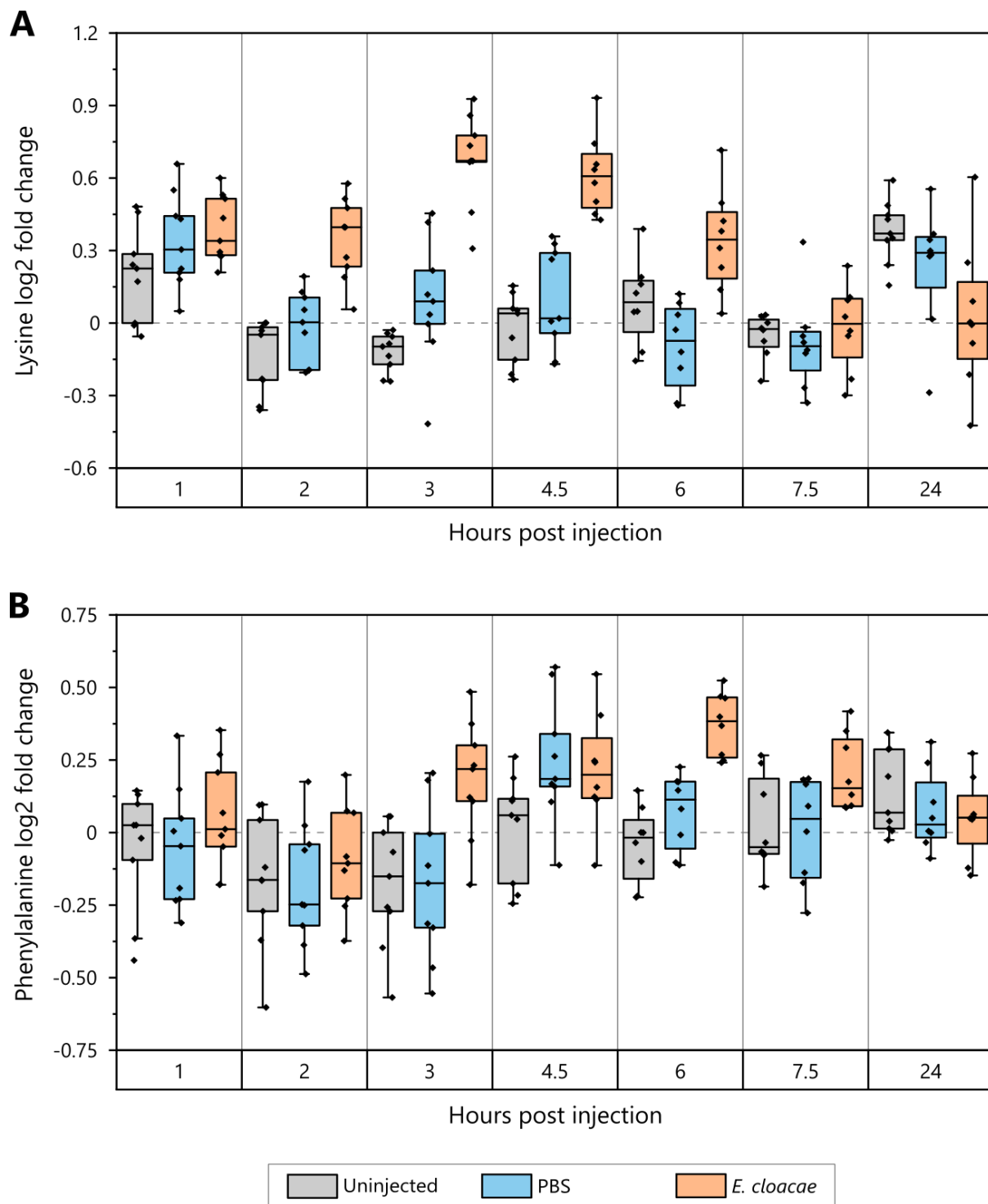


Figure 3.10: The concentrations of the essential amino acids lysine and phenylalanine increase during *E. cloacae* infection.

Compound abundances were quantified by the semi-targeted metabolomic analysis method (34 compounds quantified, positive mode only). Abundance was normalised to the sum of all compound abundances in each sample. $n = 7-9$ per condition per time point, 3 flies per sample. The horizontal line indicates median, box shows IQR, and whiskers show range of data within $\pm 1.5 \times \text{IQR}$ of the median.

It is perhaps significant that the concentration of kynurenine in the uninjected flies, while variable over time, shows a nadir at the 6-hour time point similar to that seen in the allantoin levels at this time-point (**Figure 3.8A**). This may suggest that these metabolites are regulated by circadian rhythms in a similar manner and leads me to speculate that kynurenine may potentially be excreted alongside allantoin, should excretion be the cause of the reduced allantoin concentration at the 6-hour time-point.

In light of the observed increase in the levels of the essential amino acid tryptophan, I examined the data from other essential amino acids that were successfully quantified. Interestingly, the essential amino acid lysine (**Figure 3.10A**) also appeared to show clearly, although perhaps modestly, elevated levels following *E. cloacae* infection. These raised levels were present from 2-6 hours following *E. cloacae* infection, with a peak median log₂ fold change of around 0.7 relative to the uninjected median. Phenylalanine, another essential amino acid, again showed evidence of raised concentration following infection, with demonstrating a clear, though, small increase in concentration at the 3 and 6-hour timepoints following infection (**Figure 3.10B**). Notably, for unknown reasons, this 3-hour result conflicted with the marked decrease in phenylalanine seen following *E. coli* and *M. luteus* infection (**Figure 3.6**).

Together, the results of this time-course experiment indicate that the peak of bacterial killing by the immune response is synchronous with a sharp increase in the concentration of the purine catabolite allantoin and is also accompanied by increases in the levels of (at least) 2 essential amino acids: tryptophan, lysine, and possibly phenylalanine. Additionally, the tryptophan catabolite kynurenine is upregulated, suggesting that the increase in tryptophan is mitigated, or controlled, via its catabolism.

3.3.3 Upregulation of endogenous protein degradation could explain these results

The marked increase in allantoin concentration and the increased levels of a subset of essential amino acids both hint a shared underlying metabolic response: an upregulation of protein degradation. Allantoin is the end product of purine catabolism in *Drosophila*, but this additionally means that it is the end product of nitrogenous waste metabolism. *Drosophila* are

uricotelic animals meaning that the majority of their excess nitrogen is detoxified and excreted by purine *de novo* synthesis, followed by the catabolism of purines to uric acid, and subsequently allantoin via 5-HIU (see **Section 1.4.3** for more detail). Consequently, the observed increases in these three metabolites suggests an increase in nitrogenous waste. Importantly, the primary source of nitrogenous waste is the catabolism of amino acids. Amino acid catabolism usually occurs to convert the carbon skeletons of excess amino acids from the digestion of dietary protein into sugars or lipids for energy or energy storage. Consequently, the increase in nitrogenous waste following infection, as well as the rise in tryptophan and lysine which *Drosophila* cannot synthesise, could be a result of increased feeding in response to infection. I considered this possibility unlikely for a number of reasons. Firstly, in these experiments, flies were transferred to “starvation-media” 1-hour prior to sample collection. This was done with the aim of reducing the contribution of recently ingested food in the fly gut to the detected metabolome. This would limit the amount of food the infected flies could consume in the short time between the detection of bacteria and the transfer to the starvation media, particularly at the early 3 hour-time point by which time allantoin levels are already strongly elevated. Secondly, infection in animals, including insects, is normally associated with a decrease, rather than an increase, in feeding behaviour (Ayres and Schneider, 2009; McMillan, Miller and Adamo, 2018). Consequently, we considered that the increase in amino acid catabolism, implied by the accumulation of nitrogenous waste metabolites, was most likely a result of an upregulation of protein degradation. The free amino acids from degraded protein cannot accumulate too much as this would disturb cellular osmolarity and biochemical state, and so they are catabolised. An upregulation in protein degradation would also explain the increased concentration of the essential amino acids, tryptophan and lysine, following infection. Additionally, the fact the these increases in amino acids are modest in comparison to the increase seen in allantoin could support the idea that the concentrations of free amino acids are prevented from rising above a certain upper limit. Finally, in *Drosophila*, tryptophan cannot be catabolised like most of the other amino acids and is instead converted into kynurenine (see **Section 1.4.3.2** for more detail). Thus, kynurenine could perhaps be considered as a specialised nitrogenous waste metabolite responsible for detoxification and disposal of the nitrogen atoms from tryptophan, and so the increase in kynurenine following infection parallels the increase in allantoin, the primary nitrogenous waste metabolite.

3.4 Is allantoin production downstream of endogenous protein degradation?

To explore whether the increased allantoin levels following infection could be downstream of increased protein degradation, I performed an experiment utilising the ability of LCMS to detect stable isotope labelling of molecules. In this experiment, I raised flies on normal food supplemented with ^{15}N -glycine such that approximately 50% of the total glycine present in the food (free or incorporated in protein) was labelled and would be incorporated into the protein of the developing fly (**Figure 3.11A**). Shortly after eclosion, flies were transferred to vials containing normal, unlabelled food. Flies were left in these vials for 3-5 days so that any residual free ^{15}N -glycine would either have been eliminated or incorporated into protein. These animals were then infected with *E. cloacae* and samples harvested for isotopologue analysis after 3 hours along with PBS-injected and uninfected controls. Matching the previous experiments, there was a large increase in the total allantoin present in the infected samples compared to the uninjected and PBS-only controls (**Figure 3.11B**). Additionally, there was a clear increase in the proportion of allantoin molecules containing at least 1 ^{15}N atom in the infected samples relative to the uninjected samples (**Figure 3.11C**). This indicates that ^{15}N -glycine was liberated by degradation of endogenous host protein and subsequently used for purine *de novo* synthesis upstream of allantoin production. Importantly however, glycine is required for the synthesis of the purine rings and so it could be argued that, rather than being indicative of increased degradation of endogenous protein, free glycine in flux may be redirected to meet demand for purine synthesis. Indeed, a decrease in free glycine was seen earlier in response to *M. luteus* infection. Thus, further investigation will be required to confirm that fly is degrading its own protein in response to infection.

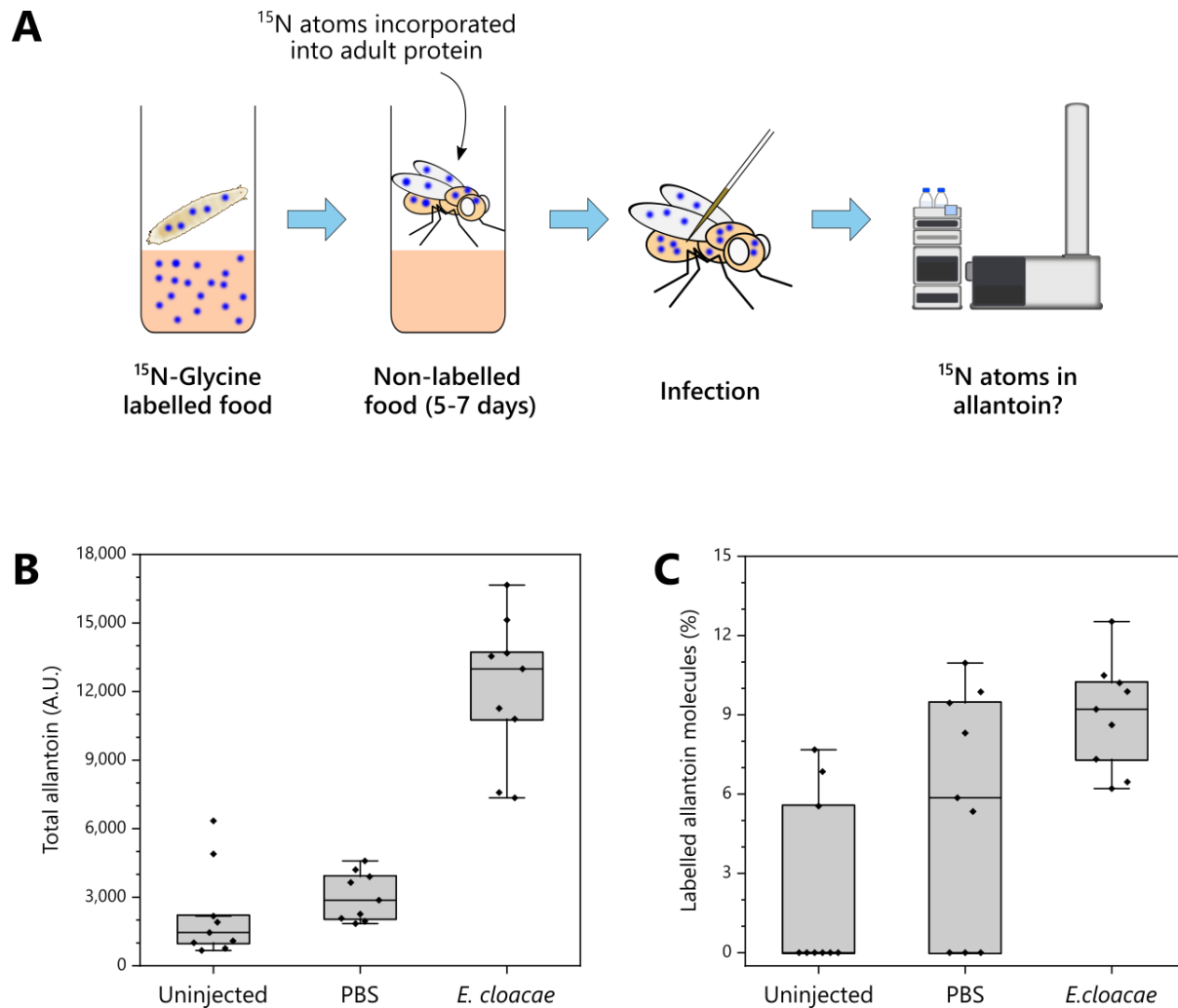


Figure 3.11: ¹⁵N atoms ingested during development are enriched in the allantoin produced following *E. cloacae* infection.

Flies (*w¹¹¹⁸*) were raised on standard food supplemented with ¹⁵N-labelled glycine (**A**). The amount of ¹⁵N-glycine added aimed to result in approximately 50% of the total glycine in the food being labelled. In parallel, flies were also raised on identical food supplemented with the equivalent amount of unlabelled glycine. Following eclosion, flies were transferred to normal food for at least 5 days before the injection. Flies were injected with *E. cloacae* or PBS and harvested 1-, 2-, and 3 hours later for isotopologue-metabolomic analysis along with uninjected controls. At 3 hours following infection total allantoin levels (labelled and unlabelled) were substantially increased in the *E. cloacae* infected flies (**B**) compared with uninjected or PBS injected flies. In each sample, the percent of allantoin molecules labelled with at least 1 ¹⁵N atom was calculated (**C**), correcting for natural isotope abundance and validated by checking for the absence of corresponding signals in flies raised on unlabelled food. At 3 hours, labelled allantoin accounted for a greater percentage of the total allantoin in the infected flies relative to uninjected controls, with the PBS injected flies presenting an intermediate phenotype. Notably, all infected samples contained measurable levels of labelled allantoin compared with only 3/9 and 6/9 uninjected and PBS-injected samples respectively. *n* = 7–9 per condition, 3 flies per sample. In the graphs, horizontal line indicates median, box shows IQR, and whiskers show range of data within $\pm 1.5 \times \text{IQR}$ of the median.

3.5 Discussion

3.5.1 A hypothesis: The fly upregulates the degradation of endogenous protein in response to bacterial infection

Untargeted metabolic analysis of flies infected with *E. coli* or *M. luteus* revealed infection-induced changes in organismal metabolism. These changes were found to include alterations in the concentrations of unidentified metabolites and various sugar metabolites. Additionally, changes were observed in the levels of various free amino acids, particularly the BCAAs, such as leucine, which showed strongly decreased levels following infection. In contrast, the purine catabolites, uric acid and allantoin, plus a feature putatively identified as 5-HIU, were found to show strongly increased levels following injury and infection. The reduction in leucine concentration and the increase in allantoin concentration were also found to occur following *E. faecalis* infection, with this result also showing that these changes were greater in response to infection than in response to sterile injury. A time course experiment showed that the increase in allantoin following *E. cloacae* infection was synchronous with the onset of bacterial clearance. This experiment also indicated that the levels of the essential amino acids, tryptophan, lysine, and possibly phenylalanine, may be elevated following infection. Together, these results led to the hypothesis that the fly is upregulating the degradation of endogenous protein following infection. This hypothesis was supported by an experiment demonstrating that ¹⁵N-labelled glycine ingested during development was enriched in the allantoin produced after infection.

While this hypothesis of upregulated protein degradation appears to have good explanatory power, and was supported by the stable isotope labelling experiment, other explanations for the observed metabolic changes could be put forward. For example, could the metabolic changes seen in the amino acids and purine catabolites be due to the metabolism of the bacteria rather than the host? I consider this possibility unlikely as PBS injection appeared to cause an intermediate phenotype in multiple cases. Additionally, any bacterial metabolites are unlikely to be detected in the whole-fly samples due to the vastly larger number of fly cells. This is supported by an early, preliminary LCMS test I performed in which samples containing the bacterial doses injected directly into LCMS solution without the flies, but otherwise treated

identically in terms of dilutions, lacked detectable signal above background noise. I accept that particular metabolites may be synthesised by the infecting bacteria to such an extent that they are detectable in whole fly samples, and more work can be done to investigate this possibility. However, I think it is unlikely that, in the context of a successful immune response, bacterial metabolism is drastically influencing the detected metabolome in the whole fly samples.

Could the increase in allantoin perhaps serve a purpose other than nitrogenous waste disposal? In their earlier metabolomic analysis, Chambers *et al.* also found increased allantoin levels following infection with *Listeria monocytogenes* (Chambers, Song and Schneider, 2012). In this study, the authors hypothesised a role for *Uro* in redox regulation since uric acid is a potent antioxidant and, additionally, its enzymatic oxidation by *Uro* produces hydrogen peroxide. Their follow-up experiments showed that, dependent on the infecting bacteria, *Uro* mutants either showed no apparent immune-defect or mildly increased bacterial clearance and survival. The *Uro* mutants also showed increased survival after wounding, but lifespan when unmanipulated was no different to controls. Consequently, the immunity-related role of *Uro*, and the increased allantoin production, remained mysterious. Our interpretation of the infection-induced increase in allantoin sees it as a consequence of an upstream metabolic change rather than a specific reaction used to regulate redox state. This is not to say that the influence of these reactions on cellular redox state is not important, and it is certainly conceivable that extended allantoin production in a lengthier immune response, as seen in the Chambers *et al.* study, may be damaging for the host due to hydrogen peroxide production. Indeed, the costs of this metabolic response would be an interesting line of inquiry as it indicates that the immune response may place substantial metabolic pressure upon the cells of the Malpighian tubules, the site of uric acid catabolism, and homologous to the mammalian kidneys. It could be that this stress may contribute to host pathology, particularly if allantoin production is sustained for an extended period. It is also a strong possibility that the accumulation of nitrogenous waste during infection has important implications for water balance and potentially may increase the requirement for hydration following infection. The negative effect of increased purine catabolism on water balance has been observed in *Drosophila* in the context of high sugar diets, which, for reasons that are not entirely clear, are associated with high levels of uric acid in flies and in humans (Lang *et al.*, 2019; van Dam *et al.*,

2020). Consequently, study of these metabolites can have implications in fields beyond immunity.

An alternative explanation for the implied increase in amino acid catabolism could be that is due to a reduction in overall protein synthesis rather than an increase in protein degradation. While gut infections have been found to lead to suppression of protein synthesis by the integrated stress response, this is a cell autonomous response to stress and so is perhaps likely to be localised to epithelial cells at the site of infection (Chakrabarti *et al.*, 2012). The systemic immune response in *Drosophila* is mediated by the secretion of vast numbers of antimicrobial peptides by the fat body and so a more global repression of protein synthesis may be considered unlikely. Interestingly, work by Vasudevan *et al.* found that at least two antimicrobial peptides are in fact preferentially translated by a cap-independent mechanism during the ISR-mediated suppression of protein synthesis (Vasudevan *et al.*, 2017). This indicates that during translational suppression, free amino acids are required for the expected production of antimicrobial peptides. Indeed, it is our view that role of the hypothesised increase in protein degradation following infection is to supply amino acids for the massive induction of antimicrobial peptide synthesis.

The depletion in branched chain amino acids following infection was striking and certainly warrants further investigation. Indeed, work investigating this change has continued separately in our lab, with a particularly interesting future line of enquiry being the relationship of this metabolic response with Tor signalling. Leucine is well-known activator of TORC1, and one can speculate that leucine is being degraded in order to prevent inappropriate activation of Tor signalling during infection. In this case, the reduction in the other BCAAs, isoleucine and valine, would simply be due to the lack of specificity of the first degradation enzyme. This method for preventing TORC1 activation may be particularly relevant in an environment of high protein degradation, and thus high amino acid flux, to ensure that amino acids are prioritised for the synthesis of immune effectors. Furthermore, if confirmed that the BCAAs are being enzymatically degraded this would certainly influence nitrogen waste catabolism. Indeed, it could be speculated that BCAT activity is upregulated specifically to synthesise glutamate, that could subsequently be used to react with any accumulated ammonium ions, or for other metabolic purposes such as donating nitrogen for *de novo* serine production.

3.5.2 How to improve the untargeted metabolomic analysis

The untargeted metabolomic analysis presented a number of metabolites of interest that were indicated to be altered in infection. That these changes were replicated in separate experiments utilising different bacteria suggests that these tested changes, for example the increase in allantoin, are a response to bacterial infection in general, rather than due to a more specific host-pathogen interaction. Additionally, replication of these changes indicates the success of the untargeted metabolomic workflow. However, I believe that the analysis could likely be improved substantially. Indeed, the untargeted metabolomic analysis described at the beginning of this chapter was performed relatively early on in my PhD, and since then many aspects of the metabolomic workflow have been optimised, for example increased use of the run controls (RunCs) to assess the variability in detection of each feature. These have revealed that some features seem to inherently show variation in their detection independent of the biology. Better use of RunCs for quality control may well have reduced the presence of highly variable features that may have undermined the untargeted analysis. Additionally, as mentioned earlier, our LCMS set up is not well suited to the quantification and identification of sugars, particularly in these chemically complex whole-organism samples. The analysis would likely be improved simply by removing the features that most likely represent sugars during data quality control rather than taking these features through the entire analysis only to be sceptical of their quantification at the end.

Many more tools exist for the analysis of metabolomic data including the commonly used partial least squares discriminant analysis (PLS-DA) and workflows incorporating machine learning. The use of such techniques in analysis is far from simple and they are commonly used inappropriately or insufficiently validated (Szymańska *et al.*, 2012; Ruiz-Perez *et al.*, 2020). Even the scaling or transformation during the pre-treatment of metabolomic data can have profound effects on the results (van den Berg *et al.*, 2006). Consequently, while there are many options for the analysis of the untargeted metabolic data sets, improving the analysis is not straight forward. To design a robust and useful statistical analysis workflow, artificial model data sets could be produced mimicking the biological ones (Ruiz-Perez *et al.*, 2020). Such a dataset would contain known “important” features correlating with conditions present amongst non-correlating features, with both correlating and non-correlating features assigned

a spectrum of different mean abundances and variation. This kind of dataset, with alterable parameters, would allow for informative testing of the strengths and weaknesses of different multivariate analysis workflows by seeing to what extent they can successfully, and exclusively, extract the correlating features. This testing would likely enable the construction of a robust, accurate, and useful workflow for the untargeted metabolomic analysis of *Drosophila* samples. In the meantime, however, the use of *Drosophila* allows for rapid, low-cost generation of experimental samples and so any findings in metabolomic analyses can simply be tested in independent experiments, reducing the need for elaborate and potentially misleading statistical analysis workflows.

Chapter 4: Nitrogenous waste metabolism and immune signalling

4.1 Introduction

In the last chapter, metabolomic analyses of *Drosophila* following bacterial infection showed numerous changes in metabolism, with increased purine catabolism one of the most robust and reproducible changes. Additionally, the amino acid tryptophan, which *Drosophila* cannot synthesise, showed elevated levels following infection. These observations led to the following hypotheses: firstly, infected flies upregulate purine catabolism to dispose of an increase in nitrogenous waste; and secondly, this increase in excess nitrogen is due to amino acid catabolism downstream of protein degradation.

These hypotheses, however, assume that the metabolic changes observed are regulated by the host and connected to the immune response. Though this is suggested by the results in the last chapter, the well-characterised *Drosophila* immune signalling pathways and the availability of viable null- or hypomorphic mutants allowed me to test this assumption directly.

Both the Imd and Toll pathways have previously been demonstrated to regulate metabolism in the presence and absence of infection. For example, genetic activation of either pathway in the fat body of larvae inhibits growth and triglyceride accumulation, a phenotype that suggests inhibited insulin signalling (Roth *et al.*, 2018; Davoodi *et al.*, 2019). While the signalling mechanisms behind this are not fully characterised, Toll pathway activation has been found to inhibit insulin signalling by impeding the phosphorylation of Akt (DiAngelo *et al.*, 2009; Roth *et al.*, 2018). The Imd pathway does not appear to inhibit insulin signalling in a similar, direct manner, so how Imd signalling antagonises insulin signalling is less clear. One possible mechanism is the activation of the Foxo transcription factor by Imd signalling via the JNK signalling pathway. As the inhibition of Foxo is a crucial activity of insulin signalling, this activation downstream of Imd could override many of the transcriptional effects of insulin signalling. Research on the metabolic effects of Imd and Toll signalling have predominantly measured larval growth, triglyceride content, and the levels of glycogen and circulating sugars:

all canonically promoted by insulin signalling. To my knowledge, how Imd or Toll signalling may affect purine metabolism or amino acid metabolism following infection has not been directly investigated.

4.1.1 Aims

The experiments described in this chapter aimed to investigate further the upregulation of purine catabolism and tryptophan metabolism following infection. I sought to explore the relationship between these metabolic changes and the immune response by using flies with genetic impairments in the Imd or Toll signalling pathways: the pathways that connect the detection of bacteria to the induction of the immune response.

4.2 The Imd pathway is involved in the alteration of metabolism following infection

4.2.1 The increase in allantoin following *E. cloacae* infection is dependent on Imd

The Imd signalling pathway connects the detection of DAP-type peptidoglycan, primarily associated with Gram negative bacteria, to the induction of antimicrobial peptide expression by Relish, a NF- κ B transcription factor (**Figure 4.1**). Additionally, a component of the signalling cascade, Tak1, also activates JNK signalling, one of the MAPK signalling pathways, which promotes expression of genes involved in stress responses, wound healing and additional immune responsive genes (Silverman *et al.*, 2003). To determine whether the Imd pathway is upstream of the increases observed in the levels of purine catabolites, tryptophan, and tryptophan catabolites, I utilised *Imd*¹⁰¹⁹¹ and *Tak1*²⁵²⁷ mutant flies. The *Imd*¹⁰¹⁹¹ allele (hereafter *Imd*) contains a 26-nucleotide deletion resulting in a frame shift and is presumed to be an amorphic allele as homozygous flies are viable but severely immunocompromised in Gram negative bacterial infection (Pham *et al.*, 2007; Chambers *et al.*, 2014). The *Tak1*²⁵²⁷ allele (hereafter *Tak1*) contains a premature stop codon disrupting its kinase domain and is thus considered amorphic (Polaski *et al.*, 2006). Infection of either *Imd* or *Tak1* flies with the Gram-negative bacterium, *E. cloacae*, at OD₆₀₀ = 1.0 (approximately 60,000 CFUs) show 100%

mortality within 24 hours, whereas the vast majority (>95%) of w^{1118} flies survive (data not shown).

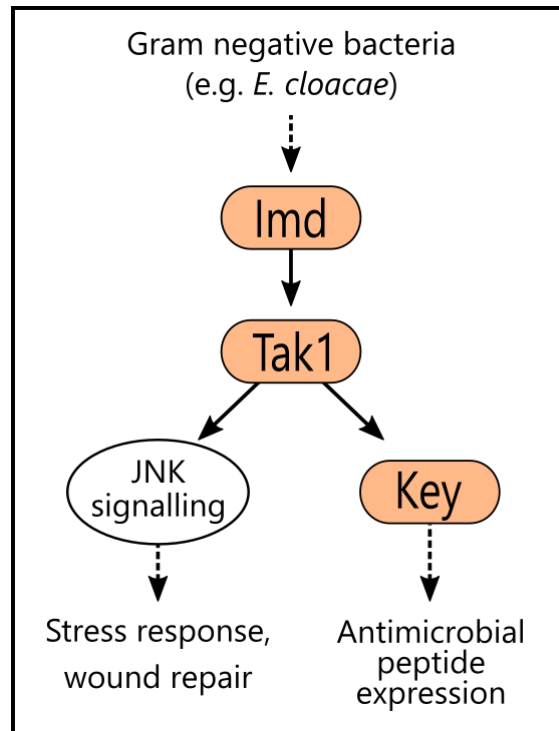


Figure 4.1: The positions of Imd, Tak1, and Key in the Imd signalling pathway.
For more detail see **Figure 1.1**.

To test whether the Imd signalling pathway is upstream of increased nitrogenous waste metabolism, *imd* and *Tak1* mutant flies and w^{1118} control flies were infected with *E. cloacae* and harvested after 3-hours for metabolomic analysis (**Figure 4.2**). As seen in the previous chapter, allantoin levels were markedly increased in the infected w^{1118} flies when compared with uninjected controls (**Figure 4.2A**). In contrast, *imd* mutants showed no change in allantoin levels following infection, whereas *Tak1* mutants showed an increase, albeit to a lesser extent than that seen in the w^{1118} line. In this experiment, uric acid, the other key purine catabolite, showed high variation within sample groups with no clear difference between any genotypes or conditions (data not shown). While the failure of *imd* flies to increase allantoin levels following infection places this metabolic change downstream of Imd signalling, this is perhaps undermined by the intermediate phenotype seen in the *Tak1* mutants. Taken alone, this intermediate phenotype may suggest the involvement of other signals in the induction of

allantoin production. However, as Tak1 is considered to function downstream of Imd and upstream of both Relish and JNK signalling, it is perhaps difficult to align these two results if indeed both alleles are completely amorphic and this experimental result is confirmed.

Kynurenine levels were increased following *E. cloacae* infection in *Tak1* mutants in a manner that was near-identical to that in the *w¹¹¹⁸* controls (**Figure 4.2B**). The *imd* mutants, however, showed large within-condition variation masking any clear, possible alteration in kynurenine levels, although the fold change between the medians of the infected *imd* samples and the uninjected *imd* samples ($\times 1.75$) approached the fold change seen in the *w¹¹¹⁸* flies ($\times 2.29$). Tryptophan showed a similar result with increased levels in *w¹¹¹⁸* and *Tak1* flies following *E. cloacae*, but a possible corresponding increase in the *imd* flies did not reach statistical significance by Mann-Whitney test ($p = 0.520$, data not shown). These results suggest that the upregulation of tryptophan and related metabolites following infection is independent of Imd signalling.

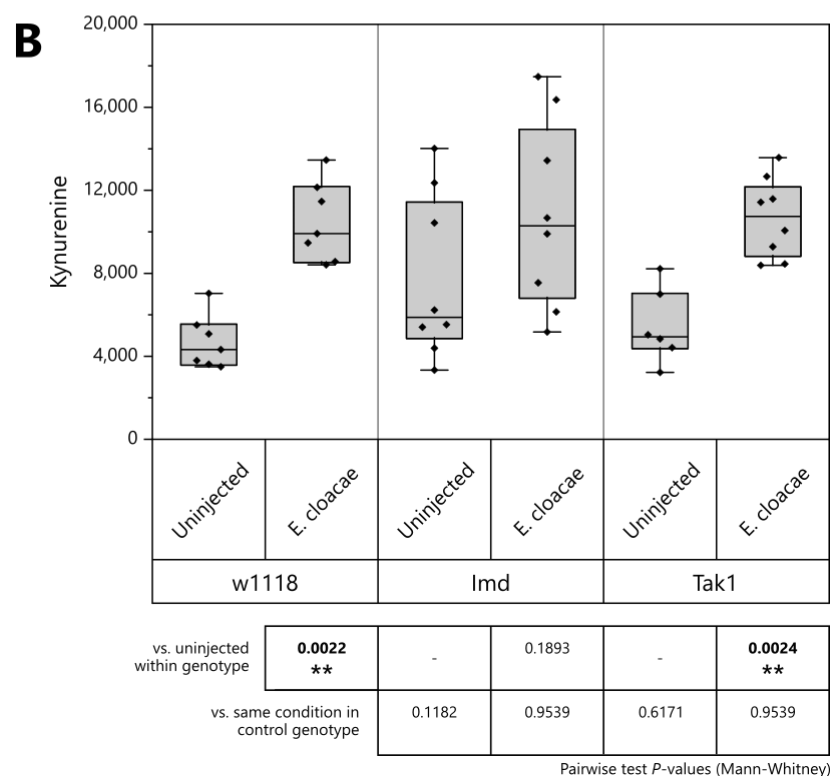
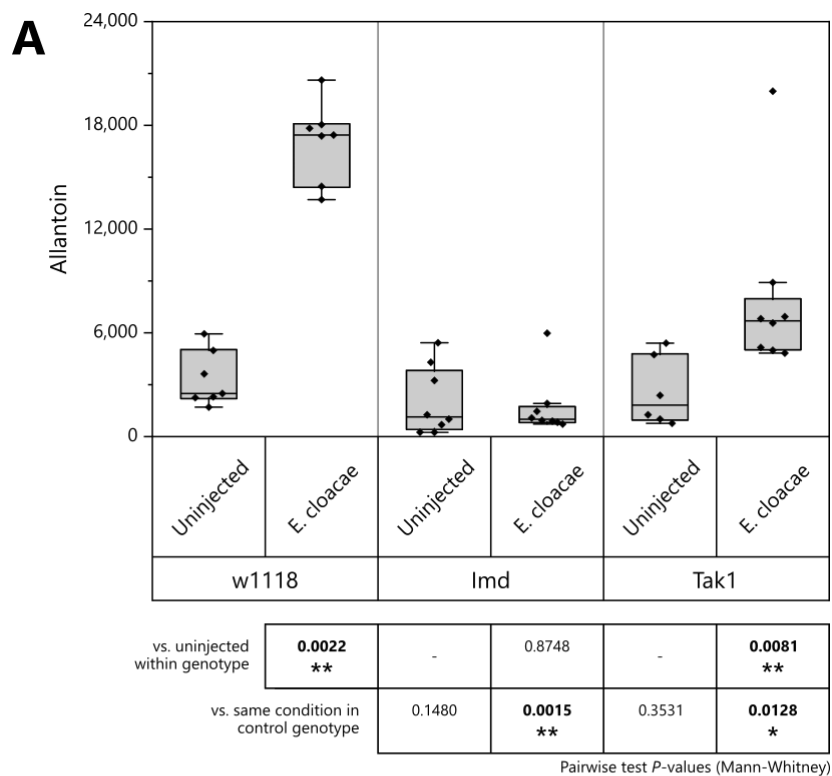


Figure 4.2: A functional Imd pathway is required for upregulation of allantoin levels following infection with *E. cloacae*.

Imd and *Tak1* mutant flies and genotype control *w*¹¹¹⁸ flies were infected with *E. cloacae* and harvested 3-hours later for metabolomic analysis along with uninjected controls. The increase in

allantoin levels (**A**) after infection, as seen in *w¹¹¹⁸* control flies, was completely ablated in *Imd* mutants. *Tak1* flies showed an intermediate phenotype as they appeared able to induce allantoin production following infection though not to the same extent as the *w¹¹¹⁸* control flies. Levels of kynurenine (**B**) were elevated in both *w¹¹¹⁸* and *Tak1* flies following infection however *Imd* flies showed comparatively variable kynurenine levels regardless of infection, which may be masking any infection induced change. Tabulated beneath each graph are the *p*-values from pairwise comparisons: the top row shows results of the comparisons between infected samples and uninjected controls for each genotype; the bottom row shows the results for comparisons for each condition between experimental genotypes and the *w¹¹¹⁸* control genotype (Mann-Whitney tests). Compound abundances were quantified by the semi-targeted metabolomic analysis method (70 compounds quantified, positive and negative mode) and normalised to the sum of all compound abundances in each sample. *n* = 6–9 per genotype per condition, 3 flies per sample. In the graphs, the horizontal line indicates median, box shows IQR, and whiskers show range of data within $\pm 1.5 \times \text{IQR}$ of the median.

4.2.2 Key is required for allantoin production in *E. cloacae* infection

To further test the involvement of Imd signalling in the induction of purine catabolism and the increase in tryptophan and its catabolites, I used *key* deficient flies. Key, is a component of the IKK complex which functions downstream of Tak1 to phospho-activate Relish (**Figure 4.1**). The *key* mutants I used, *key*^{c02831}, have a large PiggyBac transposable element inserted in the coding sequence. As with the *Imd* and *Tak1* mutants, homozygous *key* mutants are viable but highly susceptible to *E. cloacae* infection, with infected flies succumbing to infection within 24 hours (Bou Aoun *et al.*, 2011). Importantly, the IKK complex functions downstream of Tak1 and so while relish activation is blocked in these *key* mutants, Tak1-induced JNK signalling should be intact. Utilising *key* mutants should thus allow for differentiation between the roles of Relish NF-κB transcription factor activity and JNK signalling.

Metabolomic analysis demonstrated that, as with the *imd* flies, *key* flies did not show any upregulation of allantoin levels by 3-hours after *E. cloacae* infection (**Figure 4.3A**). In this experiment, I also infected the flies with *M. luteus*, a Gram-positive bacterium and potent Toll pathway agonist. While their allantoin production did not respond to *E. cloacae* infection, *key* mutants showed increased allantoin levels following *M. luteus* infection, with levels at 3-hours not statistically different from *w*¹¹¹⁸ flies by Mann-Whitney test ($P=0.2246$). In this experiment, uric acid was increased following both *E. cloacae* and *M. luteus* infection in the *w*¹¹¹⁸ line (**Figure 4.3B**). Notably, *key* flies had much higher levels of uric acid than *w*¹¹¹⁸ regardless of condition with approximately double the concentration of uric acid detected in uninjected *key* flies compared with their genotype controls. Nevertheless, as seen with allantoin, *key* mutants showed no change in uric acid abundance following *E. cloacae* infection, but an increase in response to *M. luteus*. These results clearly implicate the Imd pathway in regulating purine catabolism, directly or indirectly, in response to Gram-negative bacterial infection. Data from the *key* mutants suggests, however, that Relish activation is not required for modulation of purine catabolism in response to Gram-positive bacteria.

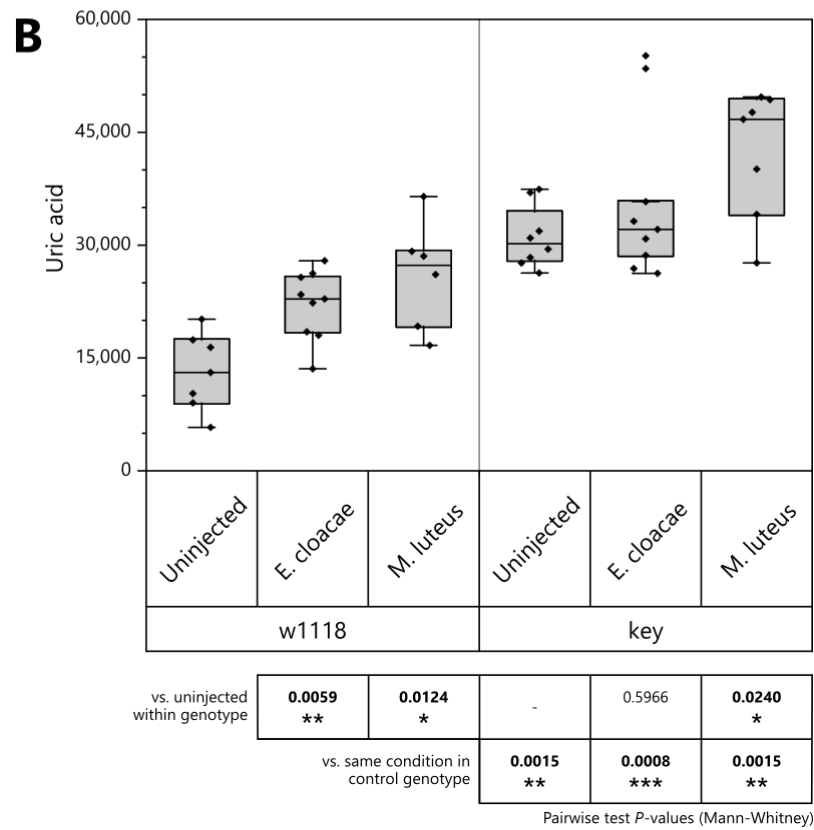
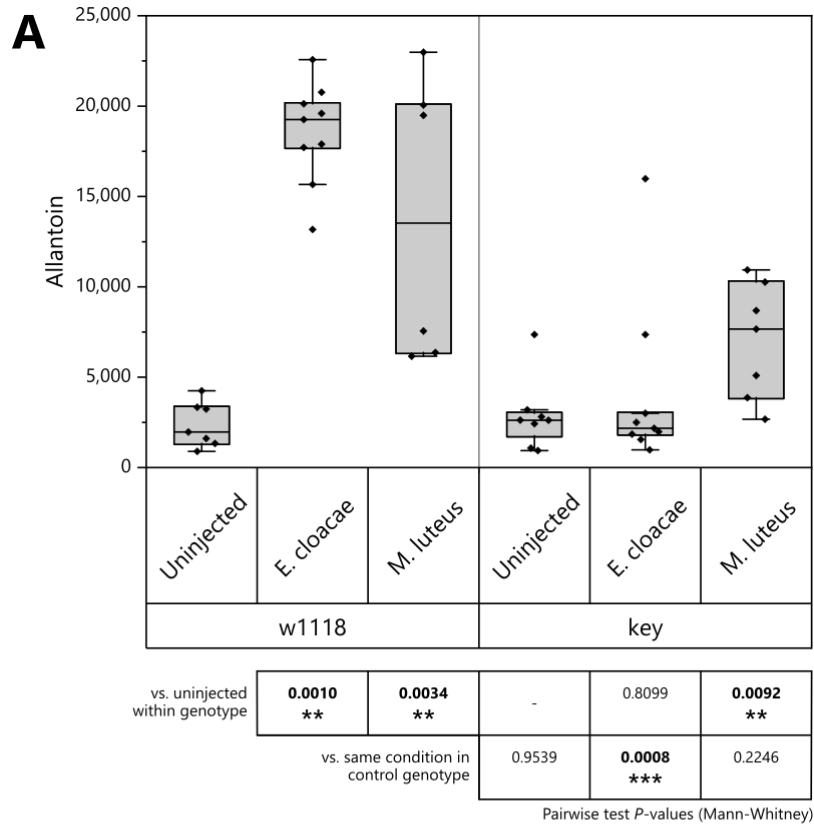


Figure 4.3 (previous page): *Kenny* mutants show defective purine catabolism after *E. cloacae* infection.

w¹¹¹⁸ and *key* flies were infected with *E. cloacae* or *M. luteus* and harvested 3-hours later for metabolomic analysis along with uninjected controls. The levels of allantoin (**A**) were elevated following both infections in *w¹¹¹⁸* flies as previously seen. In contrast, while *key* flies showed upregulation of allantoin following *M. luteus* infection, they failed to show any increase in allantoin levels following *E. cloacae* infection. Uric acid (**B**) was also elevated following infection in *w¹¹¹⁸* flies, though to a lesser extent than allantoin. Although *key* flies had higher levels of uric acid than the *w¹¹¹⁸* flies in all conditions, uric acid concentration rose in response to *M. luteus*, but not *E. cloacae*, infection. Tabulated beneath each graph are the *p*-values from pairwise comparisons between infected samples and uninjected controls for each genotype, and between genotypes for each condition (Mann-Whitney tests). Compound abundances were quantified by the semi-targeted metabolomic analysis method (60 compounds quantified, positive mode) and normalised to the sum of all compound abundances in each sample. *n* = 6–9 per genotype per condition, 3 flies per sample. In the graphs, horizontal line indicates median, box shows IQR, and whiskers show range of data within $\pm 1.5 \times \text{IQR}$ of the median.

4.2.3 *key* appears to be required for the alteration of Tryptophan metabolism following *E. cloacae* infection

While the tryptophan metabolism of *imd* and *Tak1* mutants did not show any clear difference from that of *w¹¹¹⁸* flies following infection, *key* mutation did suppress the *E. cloacae* infection-induced increase in levels of kynurenine (**Figure 4.4A**) and tryptophan (**Figure 4.4B**). These results suggest the tryptophan metabolic alterations in Gram-negative infection are in fact dependent on *Imd* pathway activation, contrasting with the results seen in the *imd* and *Tak1* mutants (**Figure 4.2B**). Interestingly, while *key* mutants showed an increase in free tryptophan very similar to *w¹¹¹⁸* flies following *M. luteus* infection, they only showed a comparably marginal increase in kynurenine. This may suggest that while the upregulation of free tryptophan in response to *M. luteus* is independent of *Key*, *Key* may still regulate the corresponding accumulation of its catabolite, kynurenine.

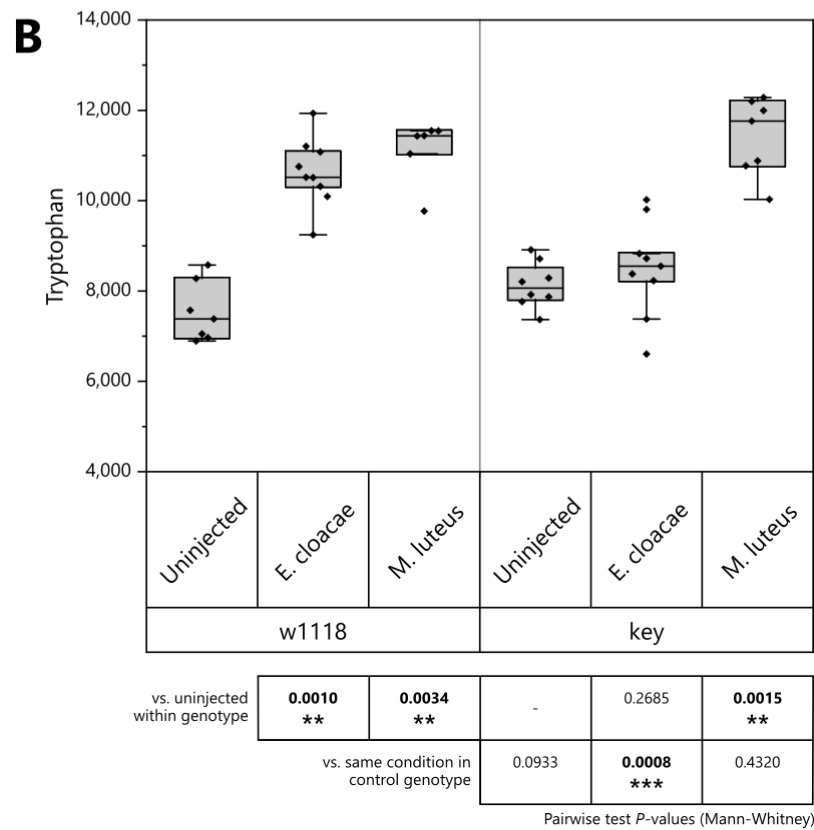
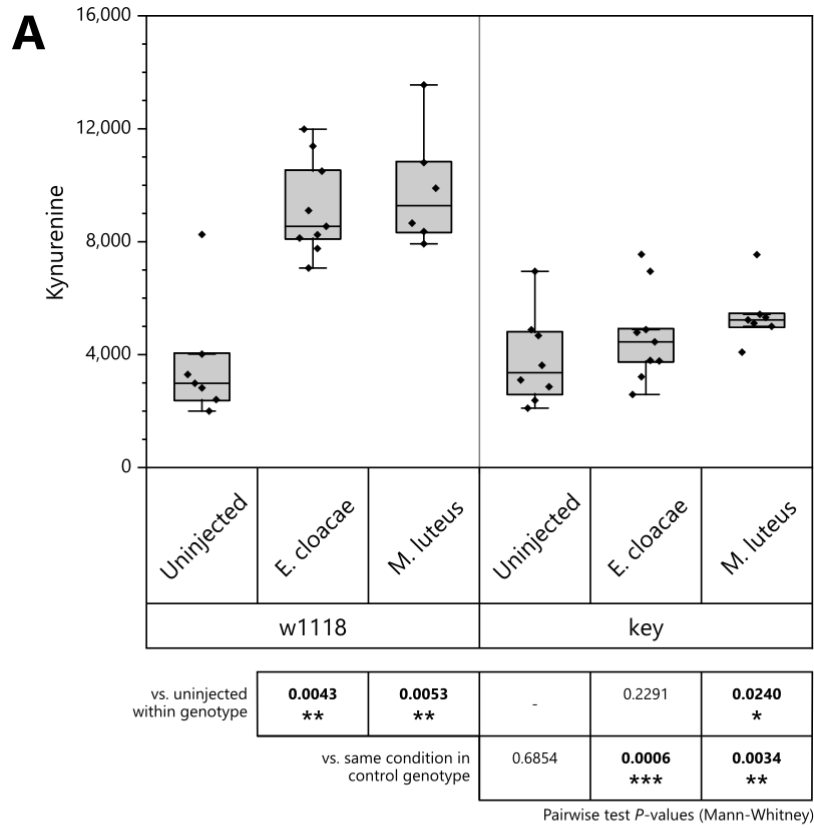


Figure 4.4 (previous page): *Kenny* mutants show defective tryptophan catabolism after *E. cloacae* infection.

w¹¹¹⁸ and *key* flies were infected with *E. cloacae* or *M. luteus* and harvested 3-hours later for metabolomic analysis along with uninjected controls (Data from the same experiment as **Figure 4.3**). While the levels of kynurenine (**A**) were elevated following both infections in *w¹¹¹⁸* flies, *key* flies showed no increased kynurenine in response to *E. cloacae* and the increase seen after *M. luteus* infection was impaired relative to the increase seen in control genotype flies. *key* flies also showed no rise in tryptophan (**B**) in response to *E. cloacae*, though their increase in tryptophan in response to *M. luteus* matched that seen in the *w¹¹¹⁸* control genotype samples. Tabulated beneath each graph are the *p*-values from pairwise comparisons between infected samples and uninjected controls for each genotype, and between genotypes for each condition (Mann-Whitney tests). Compound abundances were quantified by the semi-targeted metabolomic analysis method (60 compounds quantified, positive mode) and normalised to the sum of all compound abundances in each sample. *n* = 6–9 per genotype per condition, 3 flies per sample. In the graphs, horizontal line indicates median, box shows IQR, and whiskers show range of data within $\pm 1.5 \times \text{IQR}$ of the median.

4.3 Signalling through the Toll pathway appears dispensable for changes in purine catabolism and tryptophan metabolism in infection

The Toll signalling pathway induces the humoral immune response in response to Lysine-type peptidoglycan, which is primarily associated with Gram-positive bacteria including *M. luteus* and *E. faecalis*. The Toll pathway is also triggered by the detection of fungi and extracellular microbial virulence factors. The Toll protein itself is a cell surface receptor that is activated by Spz downstream of an extracellular protease cascade. Intracellular signal transduction from the Toll receptor begins with the adaptor protein Myd88 that recruits Tube and Pelle (**Figure 4.5A**). This then results in the translocation of the Dif (and/or Dorsal) transcription factor into the nucleus where it promotes antimicrobial peptide expression.

To test whether the Toll pathway is involved in the changes in purine and tryptophan metabolism following bacterial infection I utilised an *Myd88* trans-heterozygote (*Myd88^{2.8}/Myd88^{KRA56}*) that displays strongly reduced Myd88 function but is viable. This genotype was used as entirely amorphic alleles of core Toll pathway components are non-viable due to the role of the Toll pathway in development. I confirmed the survival defect of the *Myd88* trans-heterozygote line in Gram-positive bacterial infection by monitoring survival after *E. faecalis* infection (**Figure 4.5B**). Injection of *E. faecalis*, at a dose that is generally survived by control lines, killed 100% of *Myd88* trans-heterozygotes within 24 hours.

Metabolomic analysis of *Myd88* trans-heterozygotes following infection with *E. cloacae* or *E. faecalis* as well as PBS injection and uninjected controls showed no apparent defect in purine catabolites (**Figure 4.6A**) or tryptophan metabolism (**Figure 4.6B**) compared with the *w¹¹¹⁸* genotype controls. This result suggests normal Toll pathway function is not required for these metabolic changes following either Gram-positive or Gram-negative bacterial infection, nor following sterile injury.

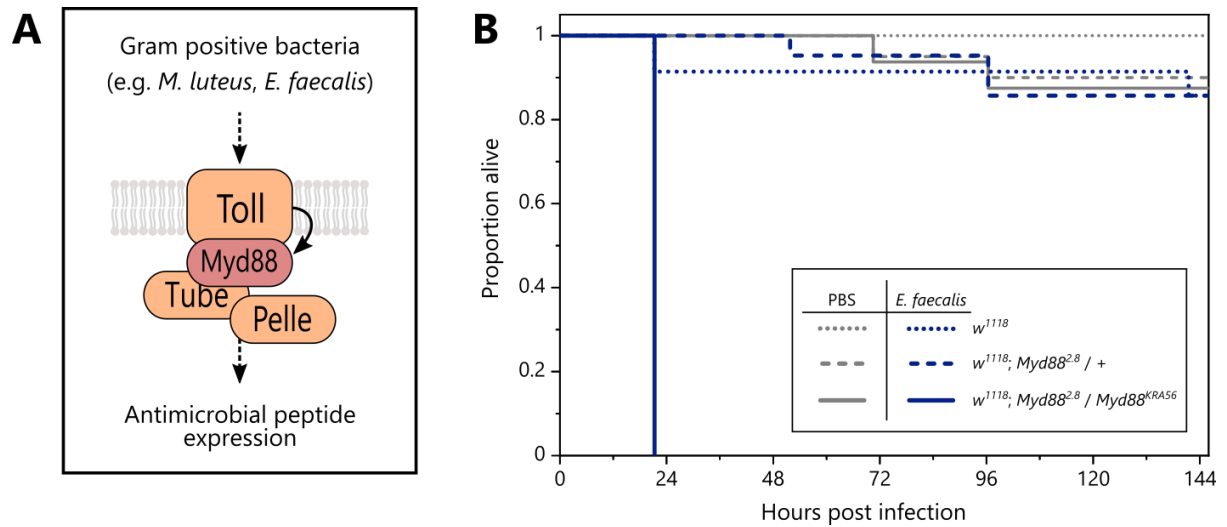


Figure 4.5: The role of Myd88 in the *Drosophila* immune response

Myd88 functions downstream of the Toll receptor in the Toll signalling pathway (**A**). $Myd88^{2.8}/Myd88^{KRA56}$ trans-heterozygotes are highly vulnerable to *E. faecalis* infection ($OD_{600} = 0.3$, **B**), showing 100% lethality within 24 hours, whereas at least 80% of control genotype flies survive. $n = 16-24$ per genotype per condition. Experimental result in **B** was representative of two other independent experiments with the same genotypes and infection conditions.

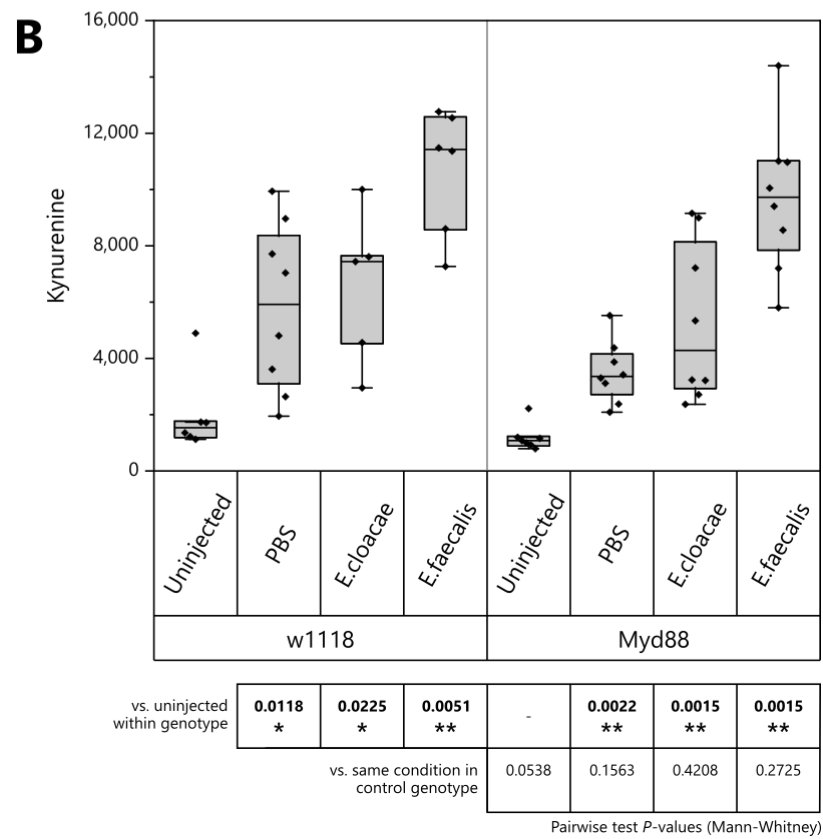
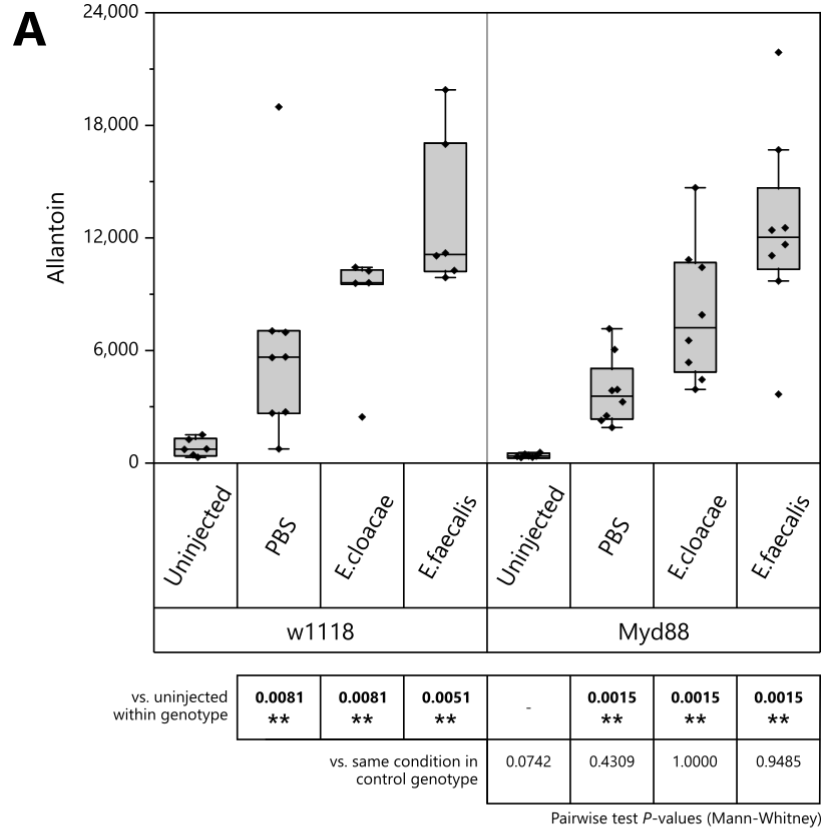


Figure 4.6 (previous page): *Myd88* knockdown has no effect on the increase in allantoin and kynurenine following infection with *E. cloacae* or *E. faecalis*.

Myd88 hypomorphic mutant flies (*Myd88^{KRA}/Myd88^{2.8}*) and *w¹¹¹⁸* controls were infected with *E. cloacae* or *E. faecalis* and harvested 3-hours later for metabolomic analysis along with PBS-injected and uninjected controls. The levels of allantoin (**A**) and kynurenine (**B**) were elevated following injury and both infections in both the experimental *Myd88* and the control *w¹¹¹⁸* genotypes, indicating that normal function of the Toll pathway is not required for the rise in the levels of these metabolites. Tabulated beneath each graph are the *p*-values from pairwise comparisons between infected/PBS-injected samples and uninjected controls for each genotype, and between genotypes for each condition (Mann-Whitney tests). Compound abundances were quantified by the semi-targeted metabolomic analysis method (50 compounds quantified, positive mode) and normalised to the sum of all compound abundances in each sample. *n* = 5–8 per genotype per condition, 3 flies per sample. In the graphs, horizontal line indicates median, box shows IQR, and whiskers show range of data within $\pm 1.5 \times \text{IQR}$ of the median.

4.4 Discussion

I conducted experiments on flies with genetically impaired immune signalling to investigate whether the metabolic changes observed in the last chapter, namely an increase in purine catabolism and an increase in free tryptophan and kynurenine, are dependent on the Imd or Toll signalling pathways. The results in this chapter indicate that the Imd pathway component Key is required for the increases in purine catabolites and tryptophan metabolites following infection with the Gram-negative bacterium *E. cloacae*. Interestingly, Toll pathway function appeared to be dispensable for these metabolic changes, including following Gram-positive infection, leaving how these metabolic changes are induced after Gram-positive infection mysterious.

Mutation of Key, immediately genetically upstream of the activation of the relish NF- κ B transcription factor, had a strong effect on the metabolic response to Gram-negative bacterial infection. Notably, this contrasted with the effect of the Tak1 mutation and, to a lesser extent, with the Imd mutation. Flies with the *Tak1*²⁵²⁷ mutation were severely immunocompromised yet were able to show a metabolic response similar to their genotype controls, albeit with a less pronounced elevation of allantoin (**Figure 4.2**). Tak1 is considered immediately upstream of Key and so that their metabolic phenotype did not match with that of the *key* mutants used (**Figures 4.3, 4.4**) suggests that the *Tak1*²⁵²⁷ allele may not be entirely null, perhaps being null with respect to the activation of JNK signalling but able to activate key. However, this seems unlikely as the allele contains a premature stop codon fairly early in the transcript that disrupts the kinase domain (Polaski *et al.*, 2006; Larkin *et al.*, 2020). Alternatively, activation of Key may be independent of Tak1, at least in adult flies following systemic bacterial infection. This matches a previous report that key is not downstream of Tak1 *in vivo*, but that Tak1-mediated activation of JNK signalling is required, in parallel with Relish activation, for antimicrobial peptide induction (Delaney *et al.*, 2006). Should this alternative model of the Imd pathway be more accurate, the experiments in this chapter would suggest that while both relish and JNK activation are required for survival in *E. cloacae* infection, only relish activation is required for the induction of purine catabolism following infection. Further investigation is required to delineate the role of Tak1 within the Imd pathway. Experiments utilising flies with genetically disrupted JNK signalling could be beneficial in confirming the suggestion that Tak1 activation

of JNK signalling is required for antimicrobial peptide production but not the changes in purine and tryptophan metabolism following Gram-negative bacterial infection.

While the *Imd* mutation showed a clear effect on allantoin following *E. cloacae* infection, interpreting whether *Imd* is required for the change in tryptophan metabolism following infection was impossible as kynurenine and tryptophan levels were considerably more variable in the *Imd* mutants than in the other genotypes (**Figure 4.2B**). This is perhaps related to my observation that the detected metabolome of the *Imd* mutants was noticeably different to those of the *w¹¹¹⁸* and *Tak1* flies, regardless of infection (data not shown). This is perhaps unsurprising as *Imd* mutants have previously been shown to be metabolically different in the absence of infection, at least in part due to dysregulation of insulin signalling (Davoodi *et al.*, 2019). However, it is interesting that the *Tak1* mutants were metabolically much closer to that of *w¹¹¹⁸* than the *Imd* flies. This supports the alternative model of the *Imd* pathway, in which *Tak1* is not required for relish activation, and also suggests that Relish is more important than JNK signalling in the regulation of normal-state metabolism by the *Imd* pathway. The *key* mutants used also had differences in their metabolomes compared with their genotype controls, however, at least some of these differences appeared due to altered pigment metabolism as a result of a *white* rescue marker within the inserted element disrupting the *key* locus. Direct comparison of *Tak1* and *Relish* mutants could be particularly useful in determining the comparative roles of the different branches downstream of *Imd* both in the presence and absence of infection.

Disruption of the Toll pathway by knocking down *Myd88*, while severely impairing the immune response (**Figure 4.5**), had no observable effect on purine catabolism or tryptophan metabolism following *E. faecalis* infection (**Figure 4.6**). Intriguingly, some of the results seen in the *key* mutants hint that the *Imd* pathway may regulate some of the metabolic changes after Gram-positive infection, although in this case the infection was performed with *M. luteus* rather than *E. faecalis*. For example, kynurenine levels did not increase in the *key* flies following *M. luteus* infection. Additionally, the increase in allantoin following *M. luteus* was perhaps not as strong in the *key* line as in the control line; although this difference was not statistically significant, the small number of *M. luteus*-infected *w¹¹¹⁸* samples (6) is potentially problematic for this comparison. Further work is clearly required to identify the signalling pathway(s) that

induces increased purine catabolism and tryptophan metabolism following Gram-positive infection.

It could be argued that metabolic changes seen after infection could perhaps be due to damage to host cells caused by the bacteria or the immune response, or the production or consumption of metabolites by the bacteria themselves. Alternatively, the induction of antimicrobial peptide production by the fat body may cause alterations in metabolism due to amino acid demand, rather than these metabolic changes being mediated directly by immune signalling pathways. The results in this chapter indicate that the changes in purine catabolism and tryptophan metabolism following infection are actively induced by the host. That an intact Imd pathway is required for these metabolic changes in *E. cloacae* infection shows that these changes are not due to bacterial metabolism or bacterially induced damage. Additionally, the uncoupling of the humoral immune response and the metabolic changes, as seen in the *Myd88* mutants in response to *E. faecalis*, indicates that these metabolic changes are not downstream of antimicrobial peptide production or antimicrobial peptide mediated immunopathology.

The key hypotheses of this project are that the fly is upregulating purine catabolism in order to cope with increased nitrogenous waste and that increase in nitrogenous waste is due to increase amino acid catabolism which, in turn, is due to increased protein degradation. While the results in this chapter do not directly support these hypotheses, they do support the underlying assumption that the relevant metabolic changes seen are actively upregulated by the host following activation of immune signalling.

Chapter 5: Exploring the mechanism behind infection-induced protein degradation

5.1 Introduction

In the previous chapter, the results from experiments investigating the infection-induced increase in nitrogenous waste indicated that the source of this waste is due to catabolism of amino acids liberated through host-mediated degradation of endogenous protein. Consequently, the working hypothesis at this point in my project was that the fly upregulates the degradation of its own protein in response to bacterial infection, and that this is the source of the observed infection-induced increase in nitrogenous waste. If this is the case, it raises the question of what degradative process the fly is upregulating. As discussed in **Section 1.5** the fly has a number of mechanisms for degrading endogenous protein. The ubiquitin proteasome system (UPS) targets ubiquitinated proteins to the proteasome for degradation whereas the autophagic processes, macroautophagy (MA) and endosomal microautophagy (eMI), deliver proteins, selectively or in bulk, to lysosomes for degradation. Plasma membrane proteins or extracellular proteins can also be transported to lysosomes after internalisation by endocytosis. These processes are known to have roles in *Drosophila* immunity; however, work in this area has focused on how immune signalling is modulated by the targeted degradation of proteins in the Imd or Toll signalling pathways. Any potential roles for these degradative processes in the metabolic response to infection are unknown.

5.1.1 Aims

The aim of the experiments in this chapter was to further test the hypothesis that the fly is upregulating endogenous protein degradation and to elucidate the underlying mechanism. To do this I investigated the requirement for a variety of protein degradation genes in infection. By this approach, should the hypothesis be supported, I would also gain insight to

the mechanism governing the hypothesised protein degradation. Utilising flies with various genetic defects in protein degradation processes, I sought to identify candidate genes that were required for the infection-induced increase in nitrogenous waste. If flies are indeed upregulating protein degradation in response to infection, the mechanism is clearly of interest, and identified genetic manipulations that block this process can then be used to investigate the function of this metabolic response.

5.2 Autophagic protein degradation and infection

5.2.1 Which protein degradation genes, if any, play a role in the immune response to bacterial infection?

To begin the search for genes involved in the hypothesised upregulation of protein degradation in infected flies we chose to test the ability of various knockdown lines to kill infecting bacteria. If the fly is degrading protein in response to infection, it could be assumed that this process is important for the immune response's ability to clear bacteria. Though this approach has the weakness that it relies on this assumption, the advantage of this approach is that quantifying bacterial numbers in infected flies is relatively time- and resource efficient when compared with screening numerous lines for nitrogenous waste production via LCMS.

The fly fat body is both the key metabolic organ and the site of antimicrobial peptide production during systemic infection. In light of this, I used the UAS-GAL4 system to knockdown genes of interest specifically in the fat body (Brand and Perrimon, 1993). This approach allows for tissue-specific knockdown of genes that, if knocked-down or knocked-out in the whole organism would be lethal. Initially I used the r4Gal4 driver; later I switched to the c564Gal4 driver (Lee and Park, 2004; Zaidman-Rémy *et al.*, 2006). This switch was made because, although both these drivers express in fat body cells, work by Dr Jake Jacobson in our lab demonstrated that the c564Gal4 driver was both more specific to fat body tissue and more strongly and uniformly expressed within fat body when compared with r4Gal4.

For the first experiments in this chapter, I aimed to inhibit either selective eMI or MA in fat body and test for any consequent defect in bacterial clearance (**Figure 5.1**). To inhibit selective eMI, I knocked down the chaperone *Hsc70-4*. Hsc70 co-chaperone *Stip1* may also be required for selective eMI and so knockdown of *Stip1* was also tested. To inhibit MA, I knocked down either *Atg7* or *Atg13*, both of which are essential for MA with *Atg13* also required for eMI induction in the context of starvation (Mukherjee *et al.*, 2016). These flies were subsequently infected, and the number of bacteria present determined at 3- and 6 hours post-injection. Flies expressing RNAi for *Hsc70-4* or *Stip1* demonstrated a similar defect in bacterial clearance in both Gram-negative (*E. cloacae*; **Figure 5.1A-D**) or Gram-positive (*E. faecalis*; **Figure 5.1E, F**) bacterial infections as compared to the Gal4 driver-only controls. This defect was not seen in flies expressing *Atg7* RNAi in fat body (**Figure 5.1A, B**). *Atg13* RNAi expression had little or no effect on bacterial clearance (**Figure 5.1A-D**). The effect on the killing of *E. cloacae* in the *r4>Hsc70-4-IR* and *r4>Stip1-IR* lines was replicated in a further independent experiment and was also replicated in two independent experiments utilising *c564Gal4* as the fat body driver. Interestingly, despite their defect in bacterial killing, knockdown of *Hsc70-4* or *Stip1* had no effect on survival to these infections (data not shown). This suggests that knockdown of selective eMI in fat body results in a delay, rather than a complete failure, in bacterial clearance.

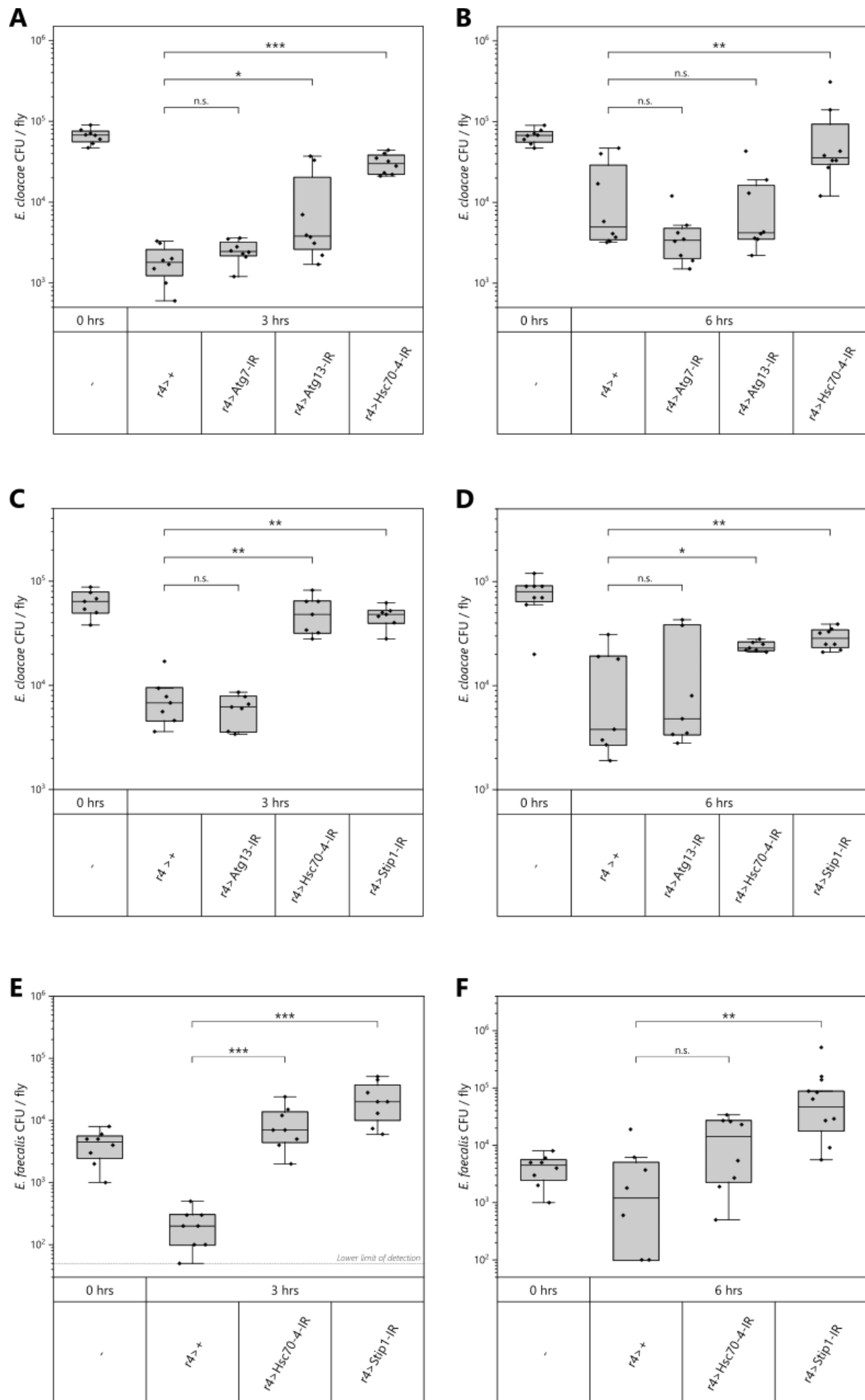


Figure 5.1: Flies with the selective endosomal microautophagy related genes, *Hsc70-4* and *Stip1*, knocked down in fat body show reduced ability to clear bacteria

Bacterial quantifications of *E. cloacae*-infected flies 3 hours (**A** and **C**) and 6 hours (**B** and **D**) after infection indicate that knockdown of the selective eMI genes *Hsc70-4* (**A–D**) and *Stip1* (**C** and **D**) impair the ability of the immune response to clear the bacteria. This was replicated in *E. faecalis* infection (**E** and **F**). In contrast, knockdown of MA via expression of RNAi for *Atg7* (**A** and **B**) or *Atg13* (**A–D**) had little or no effect on bacterial clearance. Box shows interquartile range, line indicates median, and whiskers indicate the range of data points not determined as outliers. Statistical significance was determined using the Mann-Whitney U test, *n* = 8 per genotype.

In systemic infection of *Drosophila* with *E. cloacae* or *E. faecalis*, killing of the bacteria is mediated by antimicrobial peptides expressed downstream of the Imd or Toll signalling pathways. The defect in bacterial clearance seen in the selective eMI knockdown flies could conceivably be due to some impairment of antimicrobial peptide expression rather than the hypothesised defect in protein catabolism. To test for this, antimicrobial peptide expression in *Hsc70-4* knockdown flies was measured via RT-qPCR following infection with *E. coli* and *M. luteus*, well characterised inducers of the Imd and Toll pathways respectively. Induction of the three antimicrobial peptides tested (*Dipt*, *Dro*, and *Drs*) was not reduced in *Hsc70-4* knockdown line when compared to the driver only control; indeed, antimicrobial peptide induction was frequently stronger in the knockdown (**Figure 5.2**). Interestingly, this does not seem to be due to the increased bacterial load, as induction was also higher in the sterile injury controls. The lack of reduction in antimicrobial peptide transcript expression indicates that the apparent role for eMI in the immune response is not part of bacterial recognition, Imd or Toll immune signalling, nor expression of antimicrobial peptide mRNA.

Taken together these data suggest that selective eMI, but not MA, in fat body is important for the prompt killing of bacteria by the activated systemic immune response. Interestingly this role for selective eMI appears independent of *Atg13*, hinting that selective eMI may be regulated by a different mechanism in infection than in starvation.

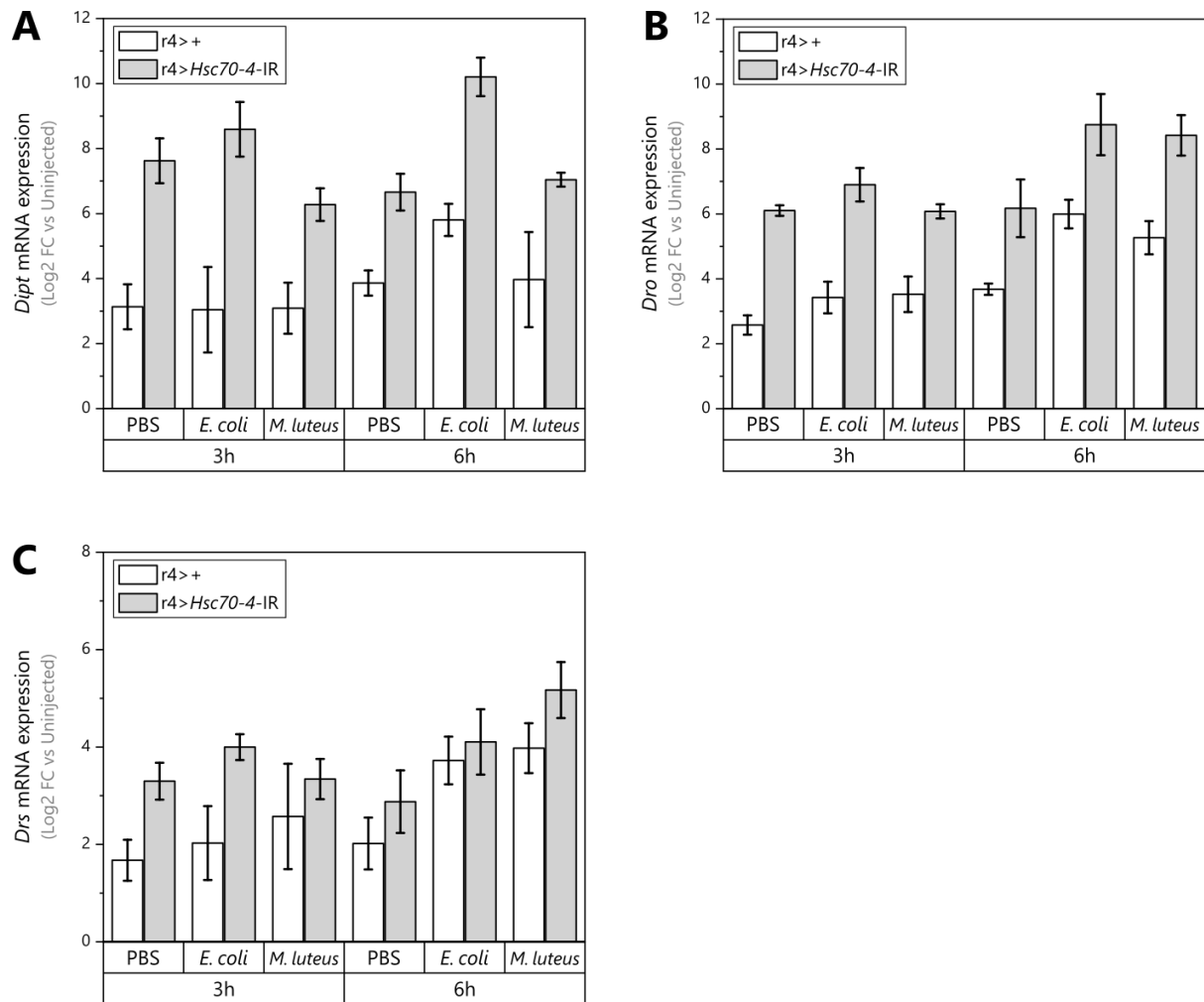


Figure 5.2: The bacterial clearance defect in flies with Hsc70-4 knocked down is not due to reduced antimicrobial peptide mRNA expression

Gene expression analysis via RT-qPCR shows that expression of the antimicrobial peptide genes *Dipt* (A), *Dro* (B), and *Drs* (C) is not reduced in *Hsc70-4* knockdown flies 3- or 6-hours following infection with either Gram-negative (*E. coli*) or Gram-positive (*M. luteus*) bacteria; indeed, in most cases antimicrobial peptide expression was higher in *Hsc70-4* knockdown flies. Expression was normalised to that of the *RpL4* house-keeping gene in each sample. Three flies were used per sample, $n = 3$ per condition per time point. In the graphs, the bars indicate mean, and the whiskers indicate ± 1 standard deviation from the mean. FC = fold change.

5.2.2 Is selective endosomal microautophagy is upregulated in infection?

To confirm selective eMI as the mechanism behind the hypothesised endogenous protein degradation in infected flies, we next looked to detecting its activation following infection. Importantly, whilst *Hsc70-4* and *Stip1* were found to be important for a normal immune response, it does not necessarily follow that the process of selective eMI is important. This is because *Hsc70-4*, and to a lesser extent *Stip1*, is known to have a variety of roles within the cell. For example, *Hsc70-4* is also involved in refolding misfolded proteins (Uytterhoeven *et al.*, 2015).

To investigate eMI following infection, we utilised flies expressing mCherry tagged with the KFERQ-motif in the fat body using the UAS-Gal4 system (Mukherjee *et al.*, 2016). Upon activation of eMI, the KFERQ-motif of the mCherry should be bound by *Hsc70-4* resulting in the targeting of the mCherry to the late endosomes for degradation. To see if I could detect this degradation, I breed flies bearing the UAS-KFERQ-mCherry^{AM93} construct under control of the r4Gal4 fat body driver. Then, after infection with *E. cloacae*, I performed western blots to quantify the KFERQ-mCherry (**Figure 5.3**). As the bacterial clearance defect in eMI gene knockdowns was evident by 3 hours, I took samples at 1.5 and 3 hours after infection. Samples from uninfected driver only controls (r4>+) were also taken which successfully confirmed specificity of the anti-mCherry antibody. No change in the levels of KFERQ-mCherry was detected compared with the uninjected controls after infection (**Figure 5.3**). This experiment was repeated independently using a second allele, UAS-KFERQ-mCherry^{AM94}, with the same result.

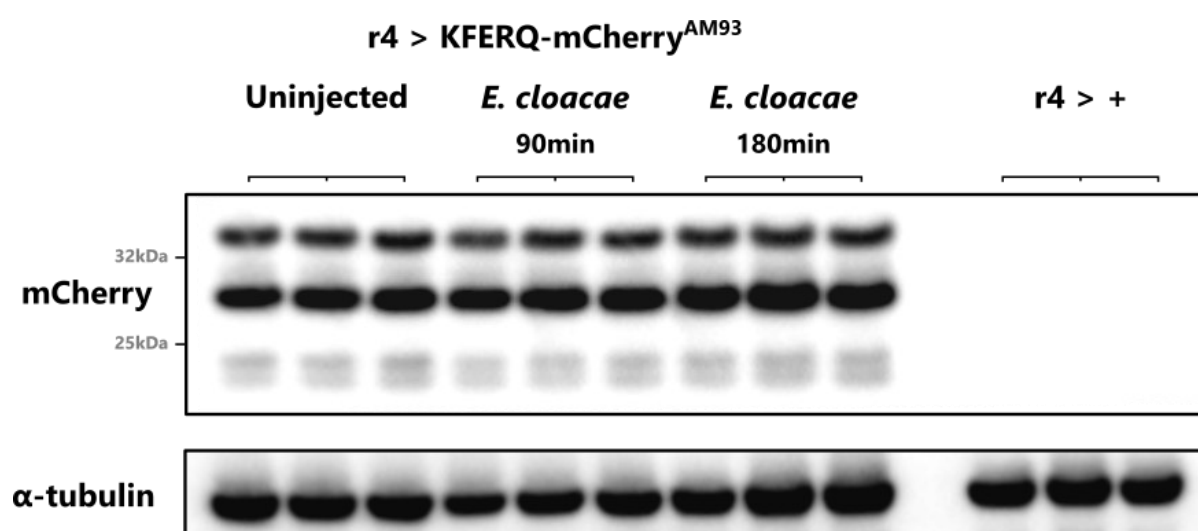


Figure 5.3: No change in detected levels of KFERQ-mCherry following infection with *E. cloacae*.

KFERQ-mCherry protein levels in whole-fly lysate were measured via western blot. Anti- α -tubulin antibody was used as a loading control. Flies collected 90 minutes or 180 minutes following infection with *E. cloacae* showed similar levels of KFERQ-tagged mCherry to the un-injected controls. Specificity of the anti-mCherry antibody was confirmed by the absence of signal in the r4>+ genotype control flies. Three whole flies per sample.

The observed lack of change in the levels of KFERQ-mCherry could be due to a lack of selective eMI activation. Alternatively, the mCherry may be being targeted to the late endosomes correctly but subsequently not being successfully internalised or degraded. In addition, it is possible that the mCherry signal is so high that a potential reduction is not readily measurable. To test the possibility that the KFERQ-mCherry is being successfully targeted for selective eMI but is then failing to be degraded, I (in collaboration with Dr Jake Jacobson) used immunohistochemistry combined with confocal microscopy. Following Mukherjee et al. (Mukherjee *et al.*, 2016), we examined the distribution of mCherry within the fat body cells. As illustrated in **Figure 5.4**, if the eMI is activated but the KFERQ-mCherry is not being immediately degraded, then the mCherry signal should present as puncta due to the protein being localised and sequestered in (or on the surface of) late endosomes/lysosomes. In the absence of eMI or successful targeting of the mCherry, the signal should be diffuse in the cytoplasm. We infected flies with either *E. cloacae* and *E. faecalis* alongside uninjected or sterile-injury controls and then, at various time points following infection, dissected out fat body tissue for immunohistochemistry. In these experiments we could clearly see that fat body cells had either a diffuse or punctate distribution of mCherry. Indeed, in the initial experiments

the frequency of cells showing punctate signal, and thus had activated selective eMI, appeared far greater in *E. cloacae* or *E. faecalis* infected samples relative to the controls (**Figure 5.5**). These results, however, could not be replicated in several independent experiments with the frequency of punctate cells not immediately obvious to be different across conditions. At best, the frequency of cells in a sample performing eMI between conditions was one of degree rather than type. We observed that, in all conditions, the fat bodies frequently contained regions of cells with punctate mCherry signal and regions with diffuse mCherry signal raising the question of how to reliably, and reproducibly, compare different samples. As the fly fat body is an anatomically diffuse organ that cannot be dissected from the rest of the fly as a complete structure, we decided a quantitative approach to comparing cellular mCherry distribution in different fat bodies would not be feasible.

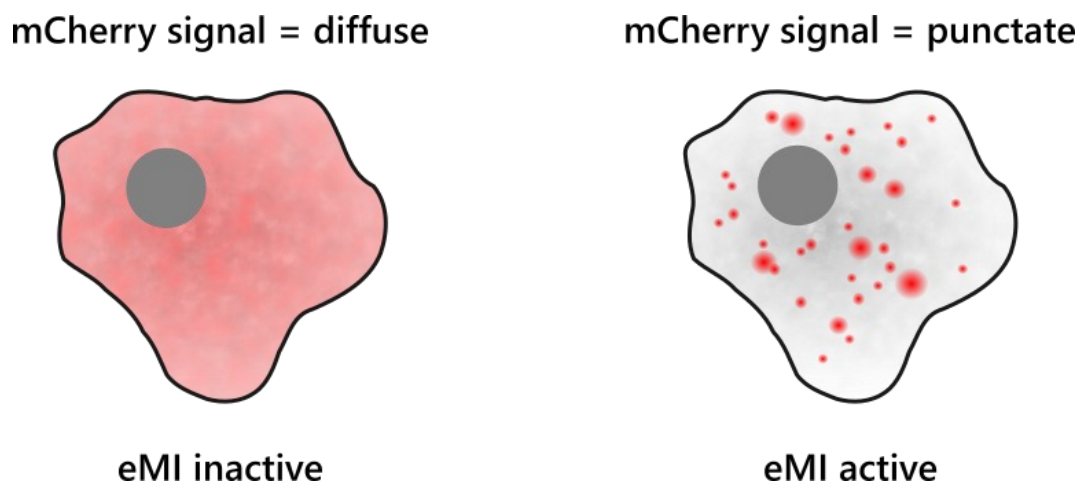


Figure 5.4: How KFERQ-tagged mCherry was used to attempt to detect selective endosomal microautophagy activation via confocal microscopy.

A diffuse signal of KFERQ-mCherry (left) in the cytoplasm indicates the protein is not being targeted to any organelle such as endosomes or lysosomes. In contrast, a punctate signal (right) indicates the protein is being targeted to organelles. Should this localisation be dependent on the KFERQ-like motif, this suggests eMI is active.

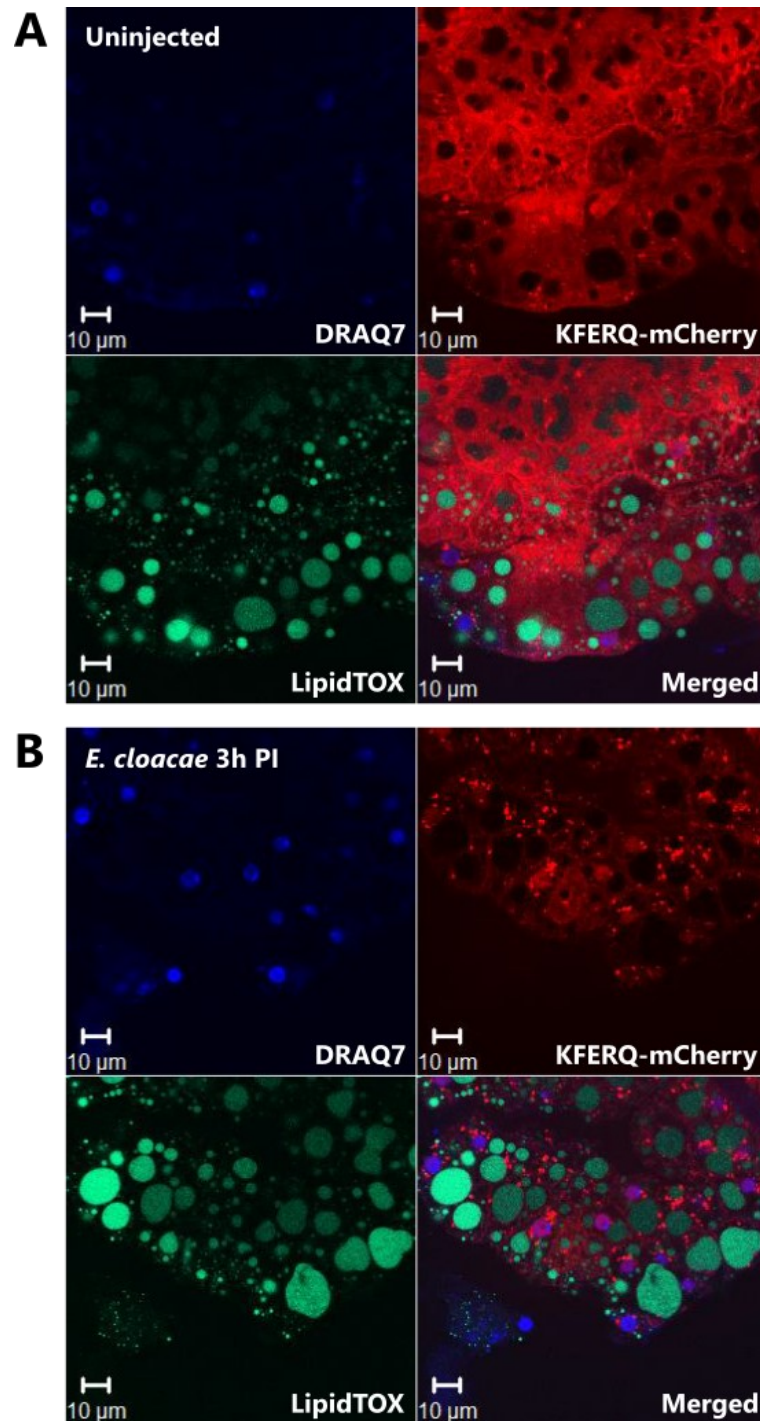


Figure 5.5: Selective eMI can appear upregulated in fat body following *E. cloacae* infection.

Confocal microscopy images of (A) Fat body of uninjected fly 3 hours after CO₂ and (B) Fat body of fly 3 hours post infection with *E. cloacae*. KFERQ-tagged mCherry was stained with anti-mCherry antibody (red, top right of each panel), nuclei were stained with DRAQ7 (blue, top left), and lipid droplets stained with LipidTOX (green, bottom left). The bottom right box in each panel shows a merged image of the three stains. Images were representative of samples within the same experiment but were not replicated in a second independent experiment. Dissections and imaging were performed by, or with the help of, **Dr Jake Jacobson**.

Up to this point, we have been unable to demonstrate that selective eMI is induced by infection in *Drosophila* despite knockdown of selective eMI genes resulting in a defective immune phenotype. To test whether the role of *Hsc70-4* in bacterial killing is connected to the increase in nitrogenous waste following infection, I performed a metabolomic analysis using flies with *Hsc70-4* knocked down in fat body (**Figure 5.6**). The level of the nitrogenous waste product, allantoin, in the *c564>Hsc70-4-IR* flies was found to be significantly increased 3 hours after infection with *E. cloacae*, following the driver-only controls (**Figure 5.6A**). The fold-change of the median amount of allantoin after infection was comparable between the genotypes; a median fold increase of 3.73 and 3.69 after infection in the controls and the knockdowns respectively. The level of the tryptophan catabolic product, kynurenine, was also found to increase after infection in the *Hsc70-4* knockdown flies (**Figure 5.6B**), although this increase was smaller than that seen in the driver-only control line. This could perhaps be a result of a higher level of kynurenine in the uninfected knockdown line samples relative to the driver-only controls. In summary, knockdown of the selective eMI gene, *Hsc70-4*, had no clear suppression of the normal nitrogenous waste production after infection. This result, combined with a lack of evidence of an induction of selective eMI during the immune response, left our hypothesis of an upregulation of protein degradation unsupported. The cause in the delay in effective bacterial killing seen in the *Hsc70-4* or *Stip1* fat body knockdowns remains mysterious but could potentially be due to functions of these genes independent of eMI.

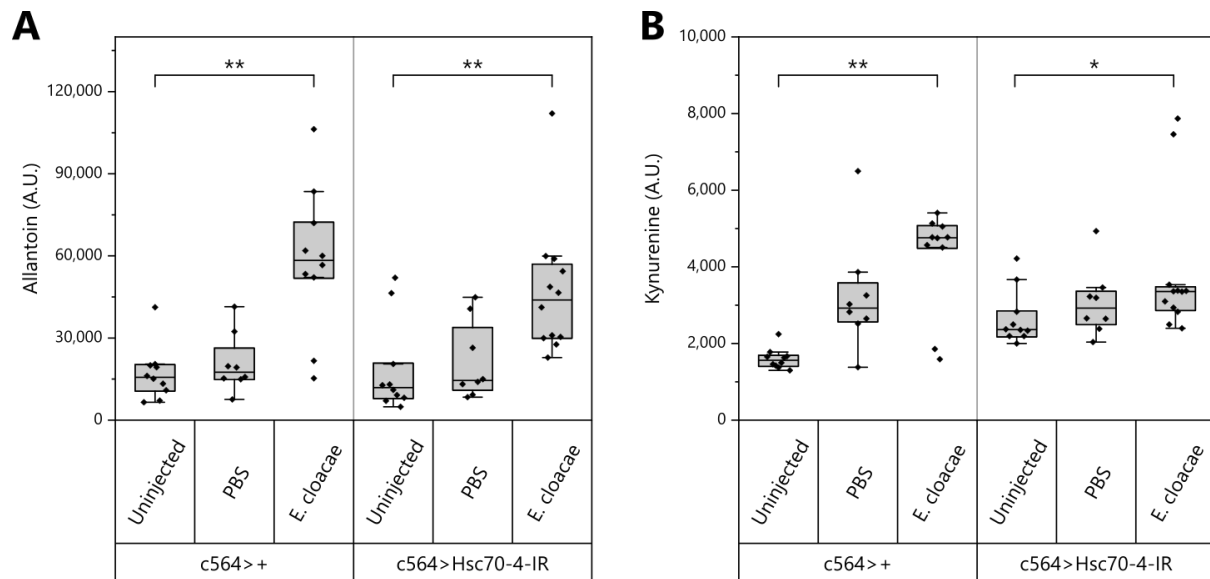


Figure 5.6: No clear defect in nitrogenous waste production following infection in *Hsc70-4* knockdown flies.

The levels of allantoin (**A**) and kynurenine (**B**) in flies 3 hours post-infection with *E. cloacae* were measured. The usual increase in the nitrogenous waste product allantoin (**A**) was still seen in flies with *Hsc70-4* knocked down in fat body. In contrast, the infection-induced production of the tryptophan degradation product, kynurenine, appeared impaired upon knockdown of *Hsc70-4* (**B**). Three flies were used per sample, $n = 8-12$ per genotype per condition. In the graphs, the horizontal line indicates median, box shows IQR, and whiskers show range of data within $\pm 1.5 \times \text{IQR}$ of the median. The Mann-Whitney test was used for the pairwise comparisons. In **A**: c564>+ $p = 0.001$; c564>*Hsc70-4-IR*, $p = 0.0027$. For **B**: c564>+, $p = 0.001$; c564>*Hsc70-4-IR*, $p = 0.0229$.

5.3 A genetic screen to identify genes required for the infection-induced increase in allantoin

5.3.1 Screen rationale and tested genes

As the previous experiments had failed to support the hypothesis that flies are upregulating eMI in response to infection, I decided to screen directly for genes required for allantoin production post-infection. The genes tested encode proteins that are known, or at least suspected, to be involved in autophagic processes at various stages. I also tested flies with impaired proteasomes to test necessity of the UPS. Should knockdown/knockout of any of these genes interfere with the infection-induced increase in allantoin, this would both strongly support allantoin being downstream of protein degradation and hint at the degradation process involved.

5.3.1.1 Proteasome and macroautophagy genes

As part of this screen, I tested the requirement for the proteasome and MA in triggering the increase in allantoin. These two processes are well characterised and more frequently implicated in protein degradation when compared to MI. The involvement of the proteasome was tested by using two temperature-sensitive dominant-negative alleles, *Prosβ6¹* and *Prosβ2¹* (together denoted Prosβx2-DN), of two essential proteasome subunits under UAS-Gal4 control. At 25°C these mutant alleles, when heterozygous, appear to have limited effect in the cells expressing them; however, at 29°C proteasome function is seriously compromised (Schweisguth, 1999; Belote and Fortier, 2002). Flies were bred expressing both of these constructs under control of the c564Gal4 driver and were kept at 29°C for a few days prior to the infection experiment in order to impair proteasome function in fat body cells. Requirement for MA was tested using flies expressing RNAi against *Atg5* or *Atg12*; both of which encode proteins required for Atg8a lipidation and are thus essential for MA.

5.3.1.2 ESCRT genes

The ESCRT proteins form 4 distinct complexes, plus disassembly machinery, that act in a sequential manner to mediate membrane budding and scission. The vacuolar protein sorting gene (Vps) family is highly conserved in eukaryotes and includes genes encoding the ESCRTs as well as the HOPS complex (Li and Blissard, 2015). The ESCRT function is required by multiple processes, including eMI and, in the case of ESCRT-III, MA (Vietri, Radulovic and Stenmark, 2020). To determine if ESCRT proteins are required for allantoin production following infection, I included in the screen RNAi knockdowns of *Mvb12* (ESCRT-I); *shrub*, *Chmp1*, *CHMP2B*, and *Ist1* (ESCRT-III); as well as *Vps4* and *Vta1* (Vps4 complex). Interestingly, transcriptomic data previously generated in the lab indicated most of the ESCRT-III genes are upregulated early after injury and infection (**Table 5.1**)

Gene	<i>p</i> -value	<i>q</i> -value	Log2 fold change vs uninjected		
			PBS	<i>E. coli</i>	<i>M. luteus</i>
<i>Chmp1</i>	<0.0001	0.0006	0.81	0.87	0.68
<i>CHMP2B</i>	0.0001	0.0015	1.37	1.74	1.85
<i>shrb</i>	0.0004	0.0036	0.46	0.58	0.37
<i>Ist1</i>	0.0019	0.0129	0.92	1.15	0.85
<i>Vps2</i>	0.0003	0.0027	0.69	0.76	0.44
<i>Vps24</i>	<0.0001	0.0002	0.63	0.69	0.52
<i>Vps20</i>	0.1274	0.2753	0.22	0.35	0.30
<i>Vps60</i>	0.0003	0.0028	0.45	0.57	0.36
<i>CG5498</i>	0.0008	0.0062	0.14	0.17	0.36

Table 5.1: ESCRT-III genes are transcriptionally upregulated 3 hours after injury and infection.

Data from a transcriptomic experiment previously conducted in our lab shows 7 out of 9 ESCRT-III genes are significantly upregulated 3-hours after injury/infection (*p*- and *q*-values <0.01 are in bold type, darkness of red colour corresponds with increase Log2 fold change vs uninjected flies). *n* = 5. Data analysis performed by **Dr Marc Dionne**.

5.3.1.3 ESCRT-interacting genes

The product of the ESCRT associated gene *ALG-2 interacting protein X (ALiX)* is able to recruit ESCRT-III proteins to the endosomes independently of ESCRT-II and is involved in a number of ESCRT mediated processes. In flies these include the membrane scission during cytokinesis and preparation of intraluminal vesicles for exocytosis (Corrigan *et al.*, 2014; Eikenes *et al.*, 2015). In mammals, Alix has also been found to be involved in membrane repair and the targeted endocytosis and sorting of subset of G protein-coupled receptors (Dores and Trejo,

2019). Additionally, ALiX has been found to interact with Atg12 and Atg3 and promote basal MA in mouse embryonic fibroblasts (Murrow, Malhotra and Debnath, 2015). This, along with the ability of ALiX to induce ESCRT-III activity, means ALiX could conceivably be involved in endocytic or autophagic protein degradation, and so an *ALiX* mutant was included in the screen. This mutant is believed to be amorphic due to an 860bp deletion removing the start codon, the first two exons, and much of the third exon. These *ALiX*³ are viable, but with defective cell cytokinetic abscission in the germ line (Eikenes *et al.*, 2015).

ALiX also has a number of ESCRT-independent roles including, of potential importance here, a possible role in JNK-signalling. The nature of this role is unclear but involvement of ALiX is inferred from the finding that it physically interacts with the JNK signalling scaffold protein POSH, in *Drosophila*- and mammalian cell culture, and that *ALiX* overexpression induces JNK signalling in *Drosophila in vivo* (Tsuda, Seong and Aigaki, 2006; Votteler *et al.*, 2009). Interestingly, vertebrate POSH has been found to localise with Hrs, a component of the ESCRT-0 complex, on endosomes and is able to target Hrs for degradation via ubiquitination (Kim *et al.*, 2006). This activity of POSH appears to be mutually exclusive with its role as a JNK signalling pathway scaffold, as the presence of JNK inhibited POSH-Hrs interaction. Whether POSH interacts with ESCRT-0 function in *Drosophila* has not, to my knowledge, been investigated. However, both POSH and the ESCRTs, as well as JNK signalling, are highly conserved between flies and vertebrates so such an interaction is likely to be present in flies. In light of POSH's interactions with ESCRT function via ALiX and likely Hrs, along with its involvement in Imd and JNK signalling, I included *POSH* mutants in the genetic screen. The *POSH*⁷⁴ flies used have a large deletion in *POSH* including the start codon and 1282bp of coding region (Tsuda *et al.*, 2005). However, expression of a C-terminal end transcript has been reported and some observations suggest *POSH*⁷⁴ is not phenotypically completely null (Zhang, Zhang and Xu, 2010; West *et al.*, 2015).

5.3.1.4 Other genes tested

PI3KC3 complexes 2 and 3 contain Uvrag and so, in the absence of Uvrag, only the MA-mediating PI3KC3 complex (containing Atg14) can form. Consequently, the hypomorphic trans-heterozygote *Uvrag^{B21}/Uvrag^{KG}* was tested for allantoin production, with the idea that these flies would have defective eMI and endocytic activities but intact MA machinery.

As knockdown of the Hsc70-4 co-chaperone *Stip1* was earlier seen to have a defect in bacterial clearance, I also decided to include *Stip1* in our screen. Activities of *Stip1* in protein degradation are poorly known, but it was found to be required for lysosomal translocation of HSC70 substrates *in vitro* in the context of CMA prior to the discovery of selective eMI (Agarraberes and Dice, 2001). Given the role of HSC70 targeting in both CMA and selective eMI, *Stip1*-Hsc70-4 interaction could perhaps be required for selective eMI in *Drosophila*.

Expression of a poorly characterised gene, *Vps13*, was upregulated by bacterial infection in the previously generated transcriptomic data. *Vps13* proteins are highly conserved in yeast and metazoans, with *Drosophila Vps13* being orthologous to human *Vps13A*, and possibly also *Vps13C* (Rzepnikowska *et al.*, 2017). Mutations in *Vps13A* cause chorea-acanthocytosis, a fatal neurodegenerative disease with patients presenting characteristically deformed “thorny” red blood cells. Studies in the amoeba *Dictyostelium discoideum*, humans, and *Drosophila* have linked *Vps13(A)* to autophagy, by showing that a lack of *Vps13A* function leads to impaired autophagic flux and/or build-up of protein aggregates (Muñoz-Bracerás, Calvo and Escalante, 2015; Lupo *et al.*, 2016; Vonk *et al.*, 2017; Muñoz-Bracerás *et al.*, 2019). Although the details of how *Vps13(A)* is involved in autophagic function are unclear, this association, combined with the upregulation seen in infected flies, led us to include *Vps13* knockdowns in the screen.

Missing in metastasis (mim) encodes an inverse Bin/Amphiphysin/Rvs (I-BAR) domain containing protein. This domain remodels membranes by inducing negative curvature, i.e., pushing membrane out from the cytoplasm. Studies in mammalian cell culture indicate the involvement of MIM in cell polarisation, motility, and the endocytosis of ligand-bound receptors including transferrin receptor and C-X-C chemokine receptor type 5 (CXCR4) (Yu *et*

al., 2011; Li *et al.*, 2019). *Drosophila mim* is poorly characterised, but it has been found to be involved in directed cell migration through inhibiting endocytosis of guidance receptors (Quinones, Jin and Oro, 2010). However, the yeast homologue of *mim*, *Ivy1*, appears to have a role in regulating vacuolar (yeast lysosome) membrane invaginations and also interacts with the yeast homologue of Rab7, a key regulator of the fusion of late endosomes with lysosomes/vacuoles (Numrich *et al.*, 2015; Ishii *et al.*, 2019). Consequently, *Ivy1* may be involved in regulation of yeast microautophagy and so we included its *Drosophila* homologue, *mim*, in the screen.

5.3.2 Screen methodology

For some of the genes I tested, namely *ALiX*, *mim*, *POSH*, and *Uvrag*, viable null- or hypomorphic mutants were available. However, most of the genes screened were knocked down in fat body using UAS RNAi constructs under control of the c564Gal4 driver (Hrdlicka *et al.*, 2002). In the case of the *Vps4* knockdown line and one of the two *shrb* knockdown lines (UAS-*shrb*-IR^{KK}), the UAS-Gal4 crosses resulted in no, or very few, viable offspring. To test these genes, the temperature sensitive Gal80 (Gal80^{ts}) system was used allowing for knockdown of the gene only in the adult stage thus removing any lethal effect during development (McGuire *et al.* 2003). The fly lines used as the genotype control for each form of knockdown/knockout are shown in **Table 5.2**.

Type of experimental genotype	Control genotype
RNAi knockdown	<i>w</i> ¹¹¹⁸ ; c564Gal4 / +
Temperature-sensitive RNAi knockdown	<i>w</i> ¹¹¹⁸ ; c564Gal4 / +; TubGal80 ^{ts} / +
Mutant	<i>w</i> ¹¹¹⁸

Table 5.2: Control genotypes used in genetic screen

The 3 types of genetic knockdown that were utilised and their 3 corresponding genotype control lines.

The screen was performed as a number of independent experiments with the appropriate genotype controls and uninjected controls used in each case. Flies were infected with either *E. cloacae* or *M. luteus* to stimulate the Imd- and Toll pathways respectively. Three hours after infection, flies were collected (3 flies per sample) along with uninjected controls for LCMS. The data was analysed by a targeted approach to quantify allantoin levels only. However, the full raw data from each experiment was retained to allow for more thorough metabolomic analysis should the experimental genotypes prove interesting.

5.3.3 Screen interpretation

A screen result was considered a “Hit” if the median log₂ fold change in allantoin of the experimental genotype was less than, or equal to, the median log₂ fold change of the control genotype minus 0.75. A result was termed “N/A” if the particular infection was not performed (often due to low numbers of viable experimental flies), or the log₂ fold change in allantoin in the genotype control infection was less than 1 (i.e., the level of allantoin did not at least double in the controls following infection, highlighted in red in the table). Additionally, it should be noted that the levels of allantoin occasionally differed between the uninjected genotypes. Such cases, where the allantoin level in uninjected flies was either less than half or more than double that of the controls, are highlighted yellow in the table. This can be seen, for example, in the *mim* mutants.

5.3.4 Screen results

Over the course of the genetic screen experiments, I used a total of 20 different fly lines (plus controls) to test a total of 18 genes for involvement in the infection-induced increase in allantoin. The results of the screen are summarised in **Table 5.3**. Infection with the Gram-negative bacterium *E. cloacae* resulted in hits for 9 genes: *Atg12*, *Chmp1*, *CHMP2B*, *Mvb12*, *Vps13*, *shrb*, *mim*, *POSH*, and *Uvrag*. The *E. cloacae* results for 3 lines, *c564>Ist1-IR*, *c564>Vps13^{GD}*, and *c564>Vta1-IR*, were discarded as the control line failed to present the usual

allantoin increase. Four further lines were not tested with *E. cloacae* infection: c564>Pros β x2-DN, c564>*Stip1*-IR^{GD}, Gal80^{ts} c564>*Vps4*-IR^{KK}, and the *mim* mutants.

In contrast to the *E. cloacae* infections, the Gram-positive *M. luteus* infections resulted in only 3 hits, *mim*, *shrb*, and *Vps13*. The results from *M. luteus* infection of c564>*Chmp1*-IR and c564>*CHMP2B*-IR^{TRIP} were discarded due to the control line not showing a normal induction of allantoin synthesis. *M. luteus* infections were not performed on the *POSH* or *Uvrag* mutants due to small numbers of viable adult flies. Finally, 2 knockdown lines, c564>Pros β x2-DN and c564>*Ist1*-IR, showed a markedly greater increase in allantoin levels following *M. luteus* injection in comparison to their control lines. As the log₂ fold change in allantoin of these experimental genotypes was greater than the log₂ fold change in the control genotype plus 0.75, (i.e., symmetrically opposite to the “Hit” cut-off), I have labelled these results as “Higher” in final column of **Table 5.3**.

Many of the negative results in the screen should be treated with caution as the efficacy of many of the knockdowns was not measured; a lack of effect on allantoin production seen in a particular knockdown line may be due to little, or no, suppression of the target mRNA rather than due to dispensability of the target gene. Consequently, the involvement of MA machinery in the allantoin synthesis following infection cannot be ruled out despite the *Atg5* RNAi line showing no effect in both infections. Testing the efficacy of the RNAi in the c564>*Atg5*-IR line in particular would greatly aid in interpretation of this result. Interestingly, knockdown of *Atg12* resulted in a hit in response to *E. cloacae* infection, though it had no effect on allantoin production following *M. luteus* infection. These ambiguous results mean that more work will be needed to determine whether MA plays a role in upregulating nitrogenous waste. It is also conceivable that individual MA genes, such as *Atg12*, may have a role in the immune response independent of their function in MA.

Knockdown of the proteasome did not hinder allantoin production in response to *M. luteus* infection; indeed, the increase in allantoin levels was particularly strong in these flies. Impairment of the UPS could possibly be causing a compensatory upregulation of autophagic protein degradation. However, the allantoin levels were not elevated in the uninfected animals

contrary to what may be expected if autophagy was constitutively induced. As the UPS has been found to be involved in negative regulation of immune signalling, it is interesting to speculate that the increased upregulation of allantoin levels was perhaps due to excessive induction of the immune response when proteasome activity is impaired.

Two different knockdowns of the putative eMI gene *Stip1* resulted in no clear defect in the induction of allantoin production following infection. This matched the result seen earlier in flies with knockdown of the eMI gene *Hsc70-4* (**Figure 5.6**). Together these results indicate that, although resulting in bacterial clearance defect (**Figure 5.1**), knockdown of selective endosomal microautophagy genes in fat body does not appear to result in impairment of the infection induced increase in purine catabolism.

Experimental genotype	Gene function	Type of genetic manipulation	Control genotype log2 FC vs uninjected		Experimental genotype log2 FC vs uninjected		Identified as hit?	
			<i>E. cloacae</i>	<i>M. luteus</i>	<i>E. cloacae</i>	<i>M. luteus</i>	<i>E. cloacae</i>	<i>M. luteus</i>
c564>Prosβx2-DN	Proteasome	RNAi knockdown	Not perf.	1.77	Not perf.	3.41	N/A	Higher
c564>Atg5-IR	Macroautophagy	RNAi knockdown	2.09	2.88	2.78	3.41	No	No
c564>Atg12-IR	Macroautophagy	RNAi knockdown	Not perf.	1.77	Not perf.	2.25	N/A	No
c564>Atg12-IR (repeat)	Macroautophagy	RNAi knockdown	2.09	2.88	1.08	3.18	Hit	No
c564>Stip1-IR^{GD}	Endosomal microautophagy (?)	RNAi knockdown	Not perf.	1.77	Not perf.	1.36	N/A	No
c564>Stip1-IR^{KK}	Endosomal microautophagy (?)	RNAi knockdown	2.09	2.88	2.55	2.37	No	No
mim	Microautophagy (?)	Amorphic mutant	Not perf.	2.28	Not perf.	0.71	N/A	Hit*
Uvrq	Endosomal microautophagy (?) and endocytosis	Heterozygous hypomorphic mutant	3.90	Not perf.	0.50	Not perf.	Hit*	N/A
POSH	JNK scaffold and ESCRT-0 associating	Hypomorphic mutant	4.37	Not perf.	1.37	Not perf.	Hit	N/A

Experimental genotype	Gene function	Type of genetic manipulation	Control genotype log2 FC vs uninjected		Experimental genotype log2 FC vs uninjected		Identified as hit?	
			<i>E. cloacae</i>	<i>M. luteus</i>	<i>E. cloacae</i>	<i>M. luteus</i>	<i>E. cloacae</i>	<i>M. luteus</i>
ALiX	ESCRT associated	Amorphic mutant	4.17	4.20	4.09	4.12	No	No
c564>Mvb12-IR	ESCRT-I	RNAi knockdown	3.04	1.12	1.08	1.35	Hit	No
c564>CHMP2B-IR^{TRiP}	ESCRT-III core	RNAi knockdown	1.82	0.54	1.03	1.22	Hit	N/A
c564>shrb-IR^{TRiP}	ESCRT-III core	RNAi knockdown	3.04	1.12	0.61	0.91	Hit*	No
Gal80^{ts} c564>shrb^{KK}-IR	ESCRT-III core	Temperature-sensitive RNAi knockdown	3.14	2.92	1.26	1.65	Hit	Hit
c564>Chmp1-IR	ESCRT-III accessory	RNAi knockdown	1.82	0.54	0.74	1.75	Hit	N/A
c564>Ist1-IR	ESCRT-III accessory	RNAi knockdown	0.78	1.25	1.51	2.63	N/A	Higher
c564>Vta1-IR	VPS4 complex	RNAi knockdown	0.78	1.25	1.16	1.44	N/A	No
Gal80^{ts} c564>Vps4-IR^{GD}	VPS4 complex	Temperature-sensitive RNAi knockdown	3.14	2.92	2.10	3.36	Hit	No

Experimental genotype	Gene function	Type of genetic manipulation	Control genotype log2 FC vs uninjected		Experimental genotype log2 FC vs uninjected		Identified as hit?	
			<i>E. cloacae</i>	<i>M. luteus</i>	<i>E. cloacae</i>	<i>M. luteus</i>	<i>E. cloacae</i>	<i>M. luteus</i>
Gal80^{ts} c564>Vps4-IR^{KK}	VPS4 complex	Temperature-sensitive RNAi knockdown	3.14	2.92	Not perf.	3.51	N/A	No
c564>Vps13-IR^{GD}	Lysosome function?	RNAi knockdown	0.78	1.25	0.41	0.27	N/A	Hit
c564>Vps13-IR^{TRIP}	Lysosome function?	RNAi knockdown	3.04	1.12	1.56	1.60	Hit	No

Table 5.2: A genetic screen highlighted a number of genes indicated to be required for a normal infection-induced increase in allantoin

The genotypes tested in the screen, and their function and the type of genetic manipulation used, are shown together with summary data on their effect on allantoin production 3 hours after infection with either *E. cloacae* or *M. luteus*. The columns entitled “Control genotype log2 FC vs uninjected” and “Experimental genotype log2 FC vs uninjected” give the median fold change, in log2, of allantoin levels measured after the indicated infection compared with the median allantoin level in the uninjected samples of the same genotype. For the control genotype data, the median log2 fold changes below 1 are highlighted in red and were removed from further analysis. The last column pair indicates whether the result in the previous column was considered a “Hit”: Experimental genotype log2 FC $\leq$ (Control genotype log2 FC) – 0.75. “Higher”: Experimental genotype log2 FC $\geq$ (Control genotype log2 FC) + 0.75. * denotes a Hit in which there was a log2 FC $\geq$ 1 in the uninjected experimental genotype vs. the uninjected control genotype. Not perf., experiment not performed; FC, fold change.

5.3.5 ESCRT activity appears to be required for the increase in allantoin following *E. cloacae* infection

The screen results strongly indicate ESCRT-III components are important for the increased production of allantoin following *E. cloacae* infection. Flies expressing RNAi for *Chmp1* or *CHMP2B* in fat body were unable to upregulate allantoin to the same extent as the driver-only control flies (**Figure 5.7A**). Knockdown of the ESCRT-III core component *Shrb* also appeared to potentially impair allantoin production, though the measured allantoin levels were very variable in these flies (**Figure 5.7B, C**). Two different *shrb* RNAi lines were used: UAS-*shrb*-IR^{KK} and UAS-*shrb*-IR^{TRiP}. As mentioned earlier, crossing of UAS-*shrb*-IR^{KK} with the c564Gal4 fat body driver resulted in no viable adult offspring and so it was crossed with the c564Gal4; TubGal80^{ts} temperature sensitive driver line at 18°C. This cross did produce viable adults, yet yield was low. This potentially indicates that expression of the KK RNAi results in a potent suppression of *shrb* expression whereas the TRiP RNAi line may result in comparatively weaker suppression. This may explain why the temperature sensitive driving of the *shrb*-IR^{KK} gave a hit in the *M. luteus* infection whereas the TRiP knockdown did not. Two of the Gal80^{ts} c564>*shrb*-IR^{KK} samples also showed very high levels of allantoin in the absence of infection. In any case, knockdown of *CHMP2B*, and possibly *shrb*, both core ESCRT-III genes, appeared to result in impairment in allantoin production following infection with *E. cloacae*. A similar impairment was seen in flies with knockdown of the *Chmp1* ESCRT-III accessory gene. Together these results point to a requirement of ESCRT-III activity in fat body for infection-induced changes in nitrogen metabolism.

Interestingly, knockdown of *Ist1*, an accessory ESCRT-III gene, did not hinder allantoin production in *M. luteus* infection. In fact, these flies showed increased nitrogenous waste induction relative to the infected controls. While the *E. cloacae* infection of the relevant control line failed, the *Ist1* knockdown flies also showed a strong increase in allantoin levels in response to *E. cloacae*, indicating a similar result may have been seen for this infection. Although the individual roles of each of the ESCRT-III accessory proteins is poorly characterised, these results appear to indicate a differing requirement for the *Chmp1* and *Ist1* accessory components in the implicated ESCRT-III activity occurring in response to *E. cloacae* infection.

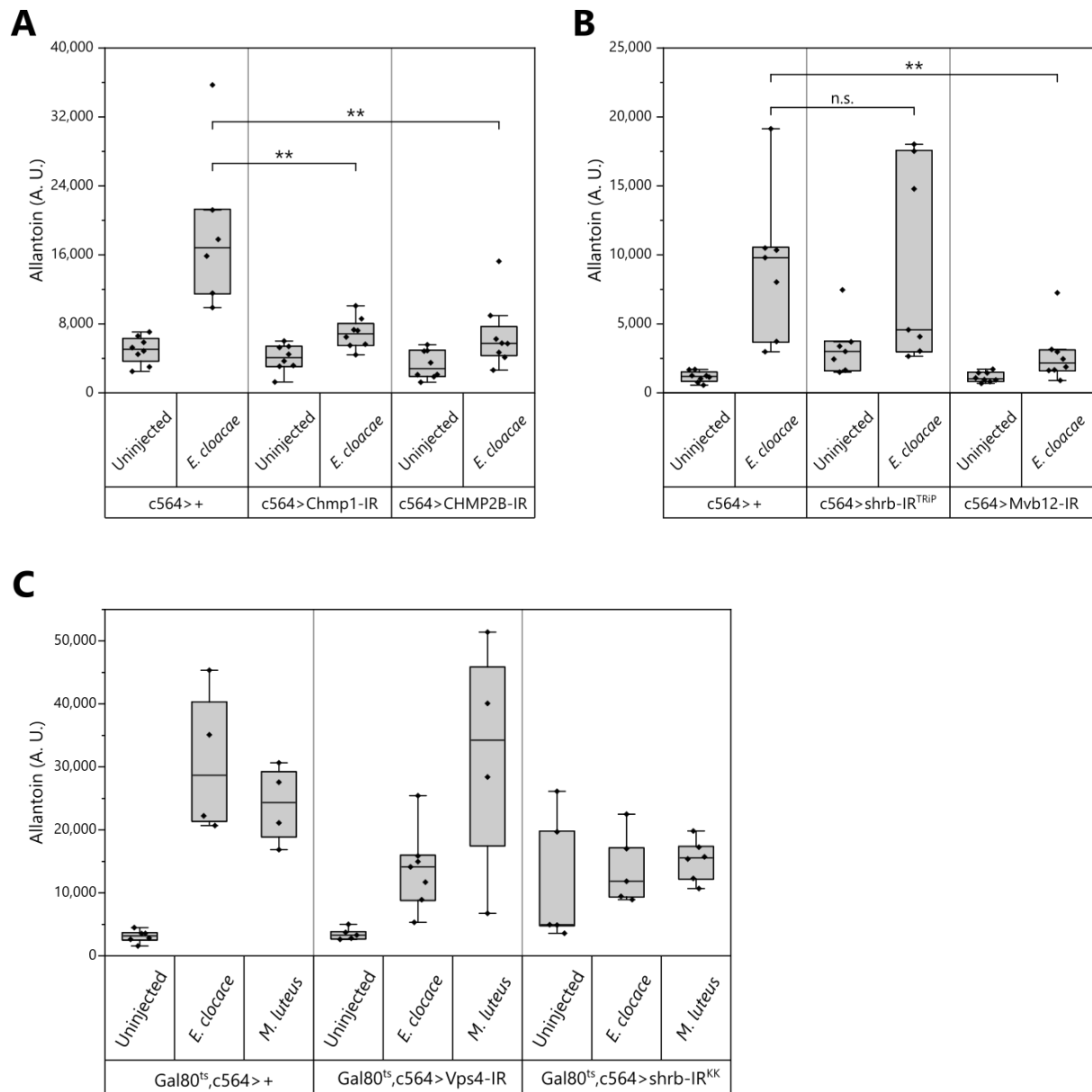


Figure 5.7: The infection-induced allantoic acid increase is dampened in multiple ESCRT RNAi knockdown lines.

The levels of allantoic acid 3 hours after infection from selected experiments performed as part of the screen are shown. c564Gal4-driven fat body knockdown of the ESCRT-III component genes, *Chmp1* and *CHMP2B* (A), resulted in reduced allantoic acid production in comparison to the control lines (c564>*Chmp1*-IR, $p = 0.0037$; c564>*CHMP2B*-IR, $p = 0.0055$). Additionally, knockdown of the ESCRT-I gene, *Mvb12* (B), also reduced allantoic acid production following infection as compared to the control line ($p = 0.0046$). Knockdown of *shrb*, another ESCRT-III gene, using the TRiP line (B) did not show a clear effect on allantoic acid levels following *E. cloacae* infection when compared to controls ($p = 0.8983$). However, infection of the KK *shrb* knockdown line under Gal80^{ts} control (C) indicated a possible requirement for *shrb* in the induction of allantoic acid production (statistical test not performed due to low sample number). Knockdown of the ESCRT-III disassembly gene *Vps4* showed a mild potential effect following *E. cloacae* infection only (C). Data is from experiments conducted as part of the genetic screen measuring allantoic acid levels by LCMS. Three flies were used per sample, $n = 6-8$ (A and B) or 4-8 (C); although enough samples were generated for an $n \geq 7$, the chromatography

of many samples in **C** failed QC. Consequently, statistical tests were not performed on the data in (**C**). In the graphs, horizontal line indicates median, box shows IQR, and whiskers show range of data within $\pm 1.5 \times \text{IQR}$ of the median. The Mann-Whitney test was used for the pairwise comparisons.

Proper functioning of ESCRT-III requires the Vps4 complex. Knockdown of *Vps4* in fat body, by both RNAi lines used, required Gal80^{ts} temperature sensitive control of the c564Gal4 driver for viable offspring. However, both *Vps4* knockdown lines were still able to solidly induce allantoin production following *E. cloacae* infection (**Figure 5.7C**). Though, in the case of *Vps4*-IR^{GD} line, the induction of allantoin production was noticeably lower than that of the control without reaching the cut-off for a partial hit.

In addition to ESCRT-III components, the ESCRT-I component Mvb12 appears to be required for full upregulation of allantoin levels following *E. cloacae* infection (**Figure 5.7B**). The knockdown of *Mvb12* did not, however, show much of an effect in the response to *M. luteus* infection relative to the controls. The ESCRT-I complex is required for recruitment of ESCRT-II proteins which subsequently recruit the ESCRT-III components. Alternatively, ESCRT-III can be recruited by the ESCRT associated protein ALiX. The *ALiX* amorphic mutants tested in this screen did not show any impairment of allantoin production following infection with *E. cloacae*. Together these results hint that the sequential activity of ESCRT-I and -II, the "classical" route of ESCRT-III recruitment, may mediate the implicated ESCRT activity following infection. However, it must be noted that no ESCRT-0 or ESCRT-II components were tested in this screen.

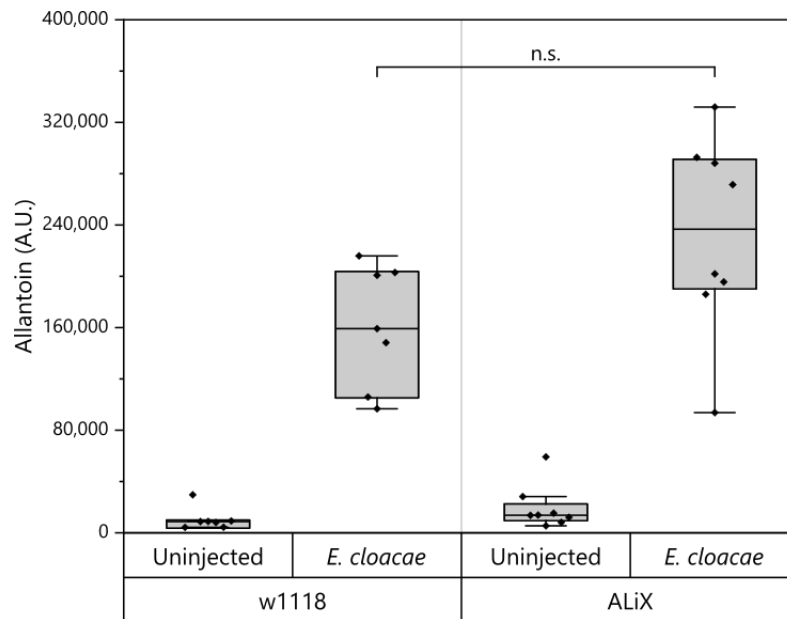


Figure 5.8: ALiX is not required for allantoin production following infection.

Flies mutant for *ALiX* were tested for allantoin production after infection with *E. cloacae*. The increase in allantoin was not hindered in *ALiX* flies ($p = 0.1480$). Data is from an experiment conducted as part of the genetic screen measuring allantoin levels by LCMS. Three flies were used per sample, $n = 7-8$. In the graph the horizontal line indicates median, box shows IQR, and whiskers show range of data within $\pm 1.5 \times \text{IQR}$ of the median. The Mann-Whitney test was used for the pairwise comparison.

5.3.6 *Uvr*ag is required for infection-induced increase in allantoin

The protein *Uvr*ag is a component of PI3KC3 complex 2 and so is important in endosomal trafficking after endocytosis (Backer, 2016). This role means it is likely also important for eMI, though this has not been shown directly. *Uvr*ag is not required for MA in *Drosophila*, and may even act as an inhibitor of MA induction as it competes with Atg14 to bind the PI3KC3 complex (Lőrincz *et al.*, 2014). The *Uvr*ag-deficient flies tested in the genetic screen did not upregulate allantoin production in response to *E. cloacae* infection (**Figure 5.9**). This result indicates that proper endosomal trafficking, but not MA, is required for the infection-induced increase in nitrogenous waste.

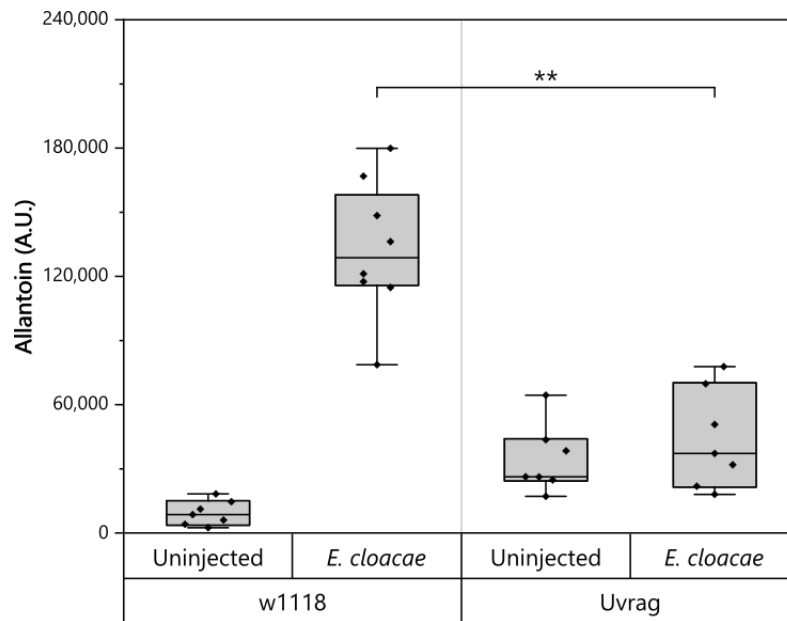


Figure 5.9: *Uvrage* is required for the induction of allantoin production following *E. cloacae* infection.

Uvrage^{B21}/*Uvrage*^{KG} hypomorphic trans-heterozygote flies do not show the increase in allantoin levels seen in the control flies 3 hours after infection with *E. cloacae* ($p = 0.0015$). Data is from an experiment conducted as part of the genetic screen measuring allantoin levels by LCMS. Three flies were used per sample, $n = 7-8$. In the graph the horizontal line indicates median, box shows IQR, and whiskers show range of data within $\pm 1.5 \times \text{IQR}$ of the median. The Mann-Whitney test was used for the pairwise comparison.

Together with the observed requirement of ESCRT-III components and the ESCRT-I component *Mvb12*, this requirement for *Uvrage* supports the hypothesis that the increase in nitrogenous waste following infection is downstream of protein degradation as all these genes are involved in autophagic and endocytic protein degradative processes. Furthermore, while ESCRT-III components, such as *CHMP2B* and *shrb*, are necessary for autophagosome closure, *Mvb12* and *Uvrage* are not required for MA in *Drosophila*. Consequently, the totality of the screen data indicates that proteins are being degraded either by a form of eMI that is independent of *Hsc70-4* and *Stip1*, or by endocytosis as both of these processes require the activity of ESCRT-I, ESCRT-III, and the *Uvrage*-containing PI3KC3 complex 2.

5.3.7 What is the role of POSH in infection?

Flies homozygous for a hypomorphic allele of *POSH* show a lack of allantoin increase following infection (**Figure 5.10**); however, the reason for this is unclear owing to multiple functions of POSH. *POSH* was included in the screen as it interacts with the ESCRT-0 component Hrs and ALiX. However, POSH also functions as a scaffold protein for JNK signalling and a regulator of both the JNK- and Imd signalling pathways. Particularly pertinent in the context of this experiment is the finding by Tsuda *et al.* that *POSH*⁷⁴ flies show a delay in the induction of the Imd pathway-responding antimicrobial peptide *Diptericin* after Gram-negative bacterial infection (Tsuda *et al.*, 2005). Consequently, it is possible that the reduced allantoin response seen here in the *POSH* flies, may represent dysregulation of signalling upstream of metabolic changes, rather than reduced ability to degrade protein. However, it is interesting to note that flies lacking *Tak1*, which connects the Imd- and JNK signalling pathways and interacts with POSH, still demonstrated an upregulation of allantoin levels 3-hours after *E. cloacae* infection, albeit to levels lower than the genotype controls (**Figure 4.2A**). Interpreting the metabolic phenotype of POSH mutants will require careful separation of its function in regulating immune signalling and any function it may have in ESCRT-mediated activities.

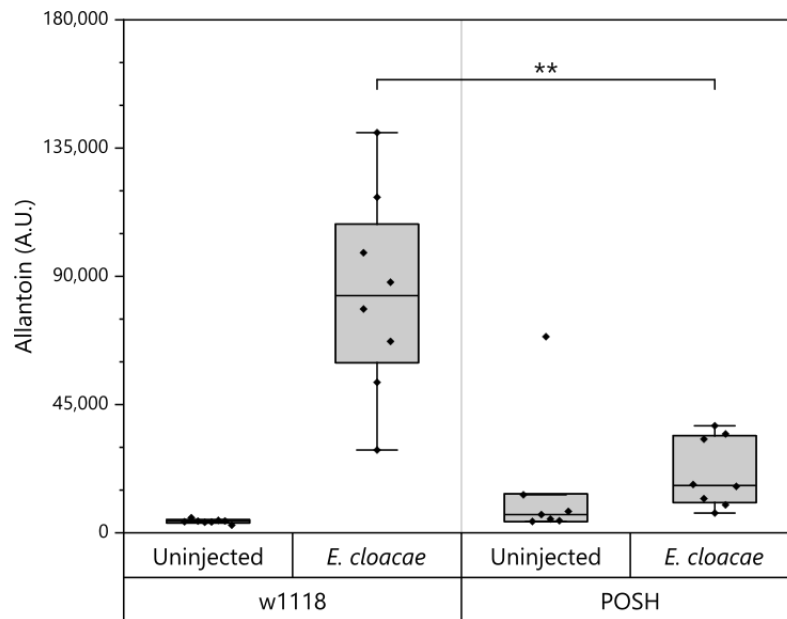


Figure 5.10: *POSH* mutants do not show elevated allantoin following infection with *E. cloacae*.

*POSH*⁷⁴ flies infected with *E. cloacae* do not show the increased allantoin levels 3 hours post infection that is seen in *w*¹¹¹⁸ flies ($p = 0.0028$). Data is from an experiment conducted as part of the genetic screen measuring allantoin levels by LCMS. Three flies were used per sample, $n = 7-8$. In the graph the horizontal line indicates median, box shows IQR, and whiskers show range of data within $\pm 1.5 \times \text{IQR}$ of the median. The Mann-Whitney test was used for the pairwise comparison.

5.3.8 Vps13

Flies with knockdown of *Vps13* in the fat body also may be unable to fully upregulate allantoin levels following infection. Two UAS constructs were used. The first, from the GD RNAi library, was one of only 3 lines that was a hit in the screen following *M. luteus* infection. Though this line was not tested in *E. cloacae* infection, a second line, from the TRiP library, showed reduced allantoin increase after *E. cloacae* infection but not after *M. luteus* infection. One possible reason for the different results between the lines in response to *M. luteus* infection is that the GD line resulted in more pronounced knockdown of *Vps13* expression. This possibility could be checked by comparison of *Vps13* transcript expression in these lines. Nevertheless, these results together indicate that *Vps13* is involved in the increase in allantoin following infection. Interpretation of this result is difficult owing to the poor understanding of *Vps13*. Studies in *Drosophila* indicate that *Vps13* is important for proteostasis in the nervous system, with lack of *Vps13* function leading to accumulation of protein aggregates (Vonk *et al.*, 2017). Studies

using cells from a variety of organisms indicate that Vps13 binds membranes and is located on lipid droplets and at sites where the membranes of different organelles meet, leading to the suggestion that it may function in exchanging lipids between membranes (Kumar *et al.*, 2018; Yeshaw *et al.*, 2019). Interestingly, a recent study by Faber *et al.* found that *Drosophila* Vps13 mutants had defective clearance of the remains of ovary nurse cells that had undergone programmed cell death (Faber *et al.*, 2020). Intriguingly the authors found that in these cells, Vps13 was localised to an unknown membrane structure near the plasma membrane, and they speculate that this Vps13-enriched structure may function in regulating the lipid content of intracellular membranes during programmed cell death. Consequently, it may be the case that the protein degradation I have been investigating relies on a mechanism in which organelle membrane contact sites and/or the lipid content of intracellular membranes is important. As these factors could be important for macroautophagy, endosomal microautophagy, or endocytosis, the apparent requirement of Vps13 for full allantoin production following infection aligns with the hypothesis that the increased allantoin is downstream of protein degradation, but it does not yet provide an indication of which degradative mechanism is being employed.

5.4 Discussion

Results from the previous chapters led us to hypothesise that infected flies upregulate the degradation of their own protein. In this chapter, I sought to test this hypothesis by interfering with cellular processes involved in protein degradation. Initially I focused on selective endosomal microautophagy as fat body specific knockdown of Hsc70-4, the chaperone required to target proteins to the late endosome, or Stip1, an interacting partner, resulted in an impaired ability to kill bacteria in the early stages of infection. Further work, utilising flies expressing mCherry tagged with the KFERQ motif that is targeted by Hsc70-4, did not consistently show the induction of selective eMI following infection. Crucially, knockdown of *Hsc70-4* or *Stip1* did not reduce the production of the nitrogenous waste metabolite allantoin following infection. While these results indicate selective eMI is not the mechanism behind the putative increase in endogenous protein degradation, the question remains why knockdown of *Hsc70-4* or *Stip1* impairs the immune response. The observation that *Hsc70-4* is involved in

the *Drosophila* innate immune response was confirmed in a recent study by Kumada *et al.* in which they found that knockdown of *Hsc70-4* in the fat body (using the c564Gal4 driver) resulted in reduced survival in *Erwinia carotovora* infection (Kumada *et al.*, 2019). Work in the same study, using human embryonic cells, indicated that the human homologue of *Hsc70-4*, HSPA8, has a function in supporting the phosphorylation of NF- κ B in immune signalling. This would suggest the bacterial clearance defect I found in the *Hsc70-4* knockdowns may be due to impaired immune signalling, however I found no impairment in the induction of antimicrobial peptides in these flies. Additionally, in Chapter 4 I found that *key* mutant flies did not have raised allantoin levels following infection, placing the activation of the NF- κ B transcription factor upstream of nitrogenous waste production. If *Hsc70-4* knockdown interfered with the proper functioning of the Imd signalling pathway, then both antimicrobial peptide induction and allantoin production would have been impaired following Gram-negative bacterial infection; a phenotype I did not see. *Hsc70-4*/HSPA8 has been implicated in multiple processes based on its chaperone and refolding activities such as in supporting proper synapse function, stress responses, and RNA silencing (Stricher *et al.*, 2013; Uytterhoeven *et al.*, 2015; Tsuboyama, Tadakuma and Tomari, 2018). Pertinent for our protein degradation hypothesis is that, as well as endosomal microautophagy, *Hsc70-4* is indicated to be required for clathrin-dependent endocytosis (Chang *et al.*, 2002). That knockdown of *Hsc70-4* did not appear to reduce nitrogenous waste metabolism in response to infection may suggest that clathrin-dependent endocytosis is also not the mechanism of protein degradation used following infection. It should be noted, however, that the efficacy of the *Hsc70-4* and *Stip1* knockdowns used was not measured directly. It is conceivable that the reduction in transcript expression may have interfered with some, but not all, *Hsc70-4* activities depending on the required abundance of this protein in the fat body. Thus, testing the efficacy of this knockdown is required prior to further studies into the role of *Hsc70-4* in the immune response.

The imaging experiments utilising KFERQ-tagged mCherry were seemingly able to successfully detect selective eMI in fat body cells dissected from adult flies. While it did not appear that selective eMI is consistently upregulated following infection, this assay may be useful for studying the role of eMI in other physiological processes. A factor that may have confounded the results of these experiments is the possibility that many fat body cells may have induced

eMI due to the stresses caused by the dissection. A negative control in which fat body cells could not induce selective eMI, during dissection or otherwise, would validate the accuracy of this assay in detecting its upregulation. Combining the expression of KFERQ-mCherry with knockdown of *Hsc70-4* in the same fly could confirm the reliability of this assay.

The screen for allantoin production after infection identified the requirement of a variety of genes associated with protein degradation, strongly supporting the hypothesis that the production of allantoin is a result of increased amino acid catabolism downstream of the upregulation of endogenous protein degradation. However, the mechanism of this protein degradation remains unclear. The ESCRT-III complex was strongly implicated as required for the increase in allantoin, yet this complex is involved in MA, eMI, and endocytosis and so this result does not discriminate between these mechanisms. However, the requirement of *Uvrag* suggests endocytosis and/or eMI as the mechanism(s) of protein degradation induced during the immune response. If the finding that *Hsc70-4* is dispensable is confirmed, then the requirement for ESCRT-III and *Uvrag* seems to imply that protein is being degraded by a form of bulk, non-selective eMI, or by endocytosis of extracellular/membrane protein through a clathrin-independent mechanism. Non-selective eMI in animals is poorly characterised, and it has even been speculated that the untargeted uptake of protein into the intraluminal vesicles of endosomes is no more than the happenstance capturing of nearby protein into vesicles being formed for other functions. In contrast, endocytosis is far better characterised, although the clathrin-independent endocytic mechanisms are not as well studied as the clathrin-dependent forms (Ferreira and Boucrot, 2018). Consequently, exploring whether endocytosis is upregulated in fat body cells following infection is perhaps the preferable next avenue of investigation.

Interpretation of the screen results and further characterisation of the degradative mechanism being upregulated following infection will be complicated by the likelihood that defects in one mechanism of protein degradation will lead to the compensatory upregulation of an alternate mechanism. Indeed, the induction of MA upon proteasome impairment is seen in many mammalian cell types (Dikic, 2017). The high levels of allantoin relative to the control genotype seen after infection in the flies with defective proteosomes in the fat body could perhaps be

due to the induction of compensatory MA. However, the screen results indicate that the protein degradation upstream of allantoin production is mediated by ESCRT-III and Uvrag, rather than the proteosome. As the proteosome has been implicated in the negative regulation of the Imd signalling pathway (Tsuda *et al.*, 2005; Chen *et al.*, 2017), it is possible that the high levels of allantoin in infected proteosome-deficient flies could be due to hyper activation of immune signalling. Although, in this case, infection was with the Gram-positive bacterium *M. luteus* and the signalling pathway connecting the induction of purine catabolism in response to Gram-positive infection has yet to be identified, it may be this signalling pathway is negatively regulated by the proteosome and the lack of this negative regulation results in an excessive induction of protein degradation.

The screen results lead to two broad observations across the many experiments performed. Firstly, the *M. luteus* infections resulted in less hits compared with the *E. cloacae* infections (3 and 9 gene hits respectively). Notably, both the lines that had their *M. luteus* results discarded, and the two lines on which *M. luteus* infection was not performed, were hits in the *E. cloacae* infections. This likely goes some way to explaining the reduced number of hits resulting from the *M. luteus* infections. However, 5 lines that were successfully tested with both bacteria were hits in *E. cloacae* infection but not in *M. luteus* infection. These were knockdowns of *Atg12*, *Mvb12*, *shrb*, *Vps4*, and *Vps13*. This difference could be due to differing requirements of protein degradation machinery dependent on which immune signalling pathway is predominantly activated. Alternatively, these differences may be a result of different timings of the induction of allantoin production. Testing different timepoints may show a defect in allantoin production that is temporally different between the two infections due to differences in the timing of signalling pathway induction. Indeed, the Imd, Toll, JNK, and JAK-STAT signalling pathways have previously been observed to have temporally distinct profiles of induction following infection (Lemaitre, Reichhart and Hoffmann, 1997; Boutros, Agaisse and Perrimon, 2002). Sensitivity to timing across different experiments may explain some of the variation in induction of allantoin between the control lines; flies could have been collected slightly early, or late, within the course of their infections to see the sharpest increase in allantoin.

Secondly, while direct quantitative comparison of the different control genotypes is not possible as they were not infected within the same experiment, qualitatively the results suggest that the flies carrying the c564Gal4 construct show a reduced induction of allantoin production following infection when compared with the *w¹¹¹⁸* flies. Aside from the c564Gal4 construct, these lines should be isogenic, however these results may indicate some genetic divergence influencing their metabolic response to infection. Alternatively, the c564Gal4 construct itself may influence metabolism independent of Gal4-UAS activity. One possible mechanism for this is the fact that the construct carries a *white* rescue to enable easy genotyping based on eye colour. Despite its long history and extensive use as a marker, the exact function which the White transporter performs in pigment synthesis and transport is unknown. Interestingly, *white* is highly expressed in the Malpighian tubules and so it is certainly conceivable that the *w¹¹¹⁸* mutation has an effect on nitrogenous waste metabolism, transport, or excretion that leads to an increased production of allantoin relative to flies carrying a *white* rescue. Indeed, other genes with eye colour phenotypes are known to have functions beyond eye pigmentation and, in the case of *rosy* and *maroon-like*, can have “unexpected” effects on metabolism (Kamleh *et al.*, 2008, 2009). Metabolomic characterisation of the effects of *white* mutation may reveal metabolic effects that may potentially confound the results of experiments performed using flies with genetic backgrounds containing a *white* mutant allele.

Results from this chapter show that the protein degradation occurs in fat body, although other tissues may well contribute. Muscle in particular would presumably be able to supply large quantities of amino acids through endogenous protein degradation. If other tissues are also upregulating protein degradation in order to provide amino acids, changes in the metabolome of the hemolymph would presumably be detectable as the amino acids are “delivered” to fat body for antimicrobial peptide production. The role of other tissues in mobilising amino acids for the immune response could be investigated by testing for an additive effect when protein degradation genes are knocked down in other tissues as well as fat body, by measuring metabolite levels in hemolymph following infection, and through exploring whether amino acid transporters in fat body are required for infection-induced allantoin production.

We hypothesised that the fly is upregulating the degradation of endogenous protein in response to infection, leading to an increase in nitrogenous waste. The results described in this chapter support this hypothesis as genetic knock-down of genes involved in protein degradative processes suppressed the infection-induced increase in the nitrogenous waste metabolite allantoin. Additionally, the nature of the genes that were indicated to be involved in this process, namely ESCRT-III components and *Uvr**ag*, provides insight into the protein degradation mechanism used, indicating that lysosomal protein degradation is the primary source of the amino acids upstream of the increase in nitrogenous waste. Furthermore, the requirement of *Uvr**ag* seemingly rules out macroautophagy, leaving some form of endosomal microautophagy or endocytosis as the most likely candidates for the mechanism of delivery of protein to the lysosome.

Chapter 6: The role of CHMP2B in the *Drosophila* innate immune response

6.1 Introduction

The results in the preceding chapters lead us to a model in which, following the detection of infecting bacteria, the fly upregulates the lysosomal degradation of endogenous protein in the fat body. In the experiments described in this final results chapter, I sought to answer whether this observed metabolic response in infection is important for the function of the innate immune response, and if so, how.

The experiments performed in the previous chapter implicated ESCRT-III genes to be required for the infection-induced increase in allantoin. Among the wide variety of cellular activities that involve ESCRT-III activity is lysosomal protein degradation: ESCRT-III mediates the sealing of the autophagosome in macroautophagy and the formation of intraluminal vesicles in endosomes, a process required in both endosomal microautophagy and endocytosis (see **Section 1.5** for more detail). Consequently, knockdown of ESCRT-III activity should interfere with all (known) forms of lysosomal protein degradation present in *Drosophila*, allowing me to test the role of this process in the innate immune response.

I decided to focus initially on *CHMP2B* because it is one of the core ESCRT-III genes that showed the metabolic phenotype, and additionally, the transcriptomic data previously generated in the Dionne Lab showed that *CHMP2B* was transcriptionally induced 3 hours after *M. luteus* infection or sterile injury, although *CHMP2B* expression following *E. coli* infection was highly variable with strong induction in some, but not all, samples (**Figure 6.1**). Overall, the induction of *CHMP2B* expression was the strongest amongst the ESCRT-III components (**Table 5.1** in the previous chapter). This data found This suggests that CHMP2B may be the component that is most limiting for the ESCRT activity involved in the degradation of host protein upstream of allantoin production.

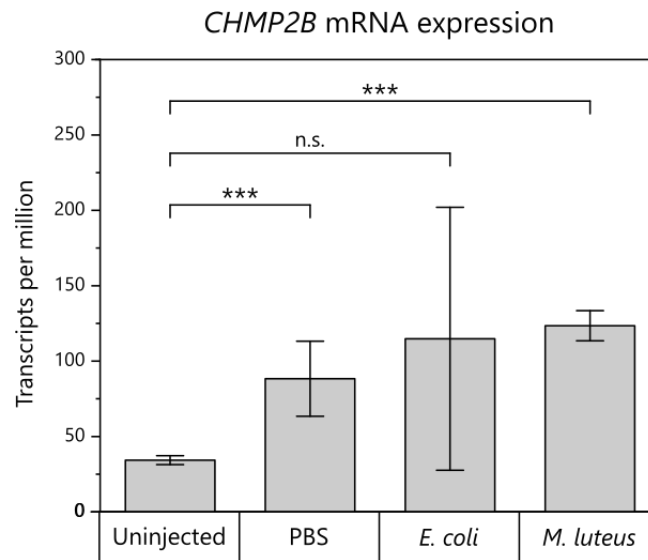


Figure 6.1: CHMP2B is transcriptionally induced 3 hours after injury and infection.

Data from a transcriptomic data set previously generated in our lab found statistically significant increases in the transcriptional expression of *CHMP2B* following sterile injury ($q \leq 0.0001$) or *M. luteus* infection ($q \leq 0.0001$), but not following *E. coli* infection ($q = 0.0525$). $n = 5 w^{1118}$ flies per condition. Graph bars show mean transcripts per million, whiskers show ± 1 standard deviation from mean. Data analysis performed by **Dr Marc Dionne**.

The most well-characterised ESCRT-III component in *Drosophila* is Shrub; however, as seen in the previous chapter, adult flies with *shrb* knockdown (when not lethal) appeared to have a defect in allantoin production following infection, but the samples produced highly variable results. This is likely due to the previously mentioned problems with the LCMS but could potentially also reflect the general unhealth of these flies. It is perhaps surprising then, that knockdown of *CHMP2B* using the same c564Gal4 driver produced far more viable flies, yet these flies did not show the same increase in allantoin levels as their genotype controls following infection. While this may simply be due to a higher efficacy of *shrb* knockdown compared with *CHMP2B* knockdown, it is tempting to speculate that the requirements of these two core ESCRT-III components differ depending on the ESCRT role being performed, and that *CHMP2B* is more important for the infection-induced protein degradation compared with whichever ESCRT-III functions mediate lethality in *shrb* knockdowns.

Little is known about the precise role of CHMP2B in ESCRT-III function. However, *Drosophila* have been used to model frontotemporal dementia by expressing a mutant form of human

CHMP2B. This mutant allele, *CHMP2B*^{Intron5}, lacks 35 amino acid residues at its C-terminus and was found to be the cause of familial, autosomal dominant frontotemporal dementia in a Danish family (Skibinski *et al.*, 2005; Lee *et al.*, 2007). Studies utilising mammalian cultured neurons and flies expressing the transgenic *CHMP2B*^{Intron5} indicate that it perturbs ESCRT-III function by failing to dissociate from Shrb after binding. This perturbation results in accumulation of autophagosomes in the cultured neurons, neurodegeneration, defects in synapse development, and disrupted endocytosis which leads to dysregulation of various signalling pathways (Lee *et al.*, 2007; Ahmad *et al.*, 2009; Cheruiyot *et al.*, 2014; West *et al.*, 2015). While informative for investigating the roles of *shrb*, and so the roles of ESCRT-III function in general, this model does not provide much insight into how wildtype CHMP2B itself functions within the ESCRT-III complex, other than that its binding to Shrb needs to be reversible.

6.1.1 Aims

The work in this project suggests that, upon bacterial infection, the fly host upregulates lysosomal degradation of its own protein which causes an increase in nitrogenous waste. While the mechanism of this protein degradation remains unclear, it appears to be dependent on the activity of the ESCRT-III complex. The aim of the experiments performed in this chapter was to explore the role of this metabolic response in the immunity. Why does the fly degrade its own protein shortly after detecting bacterial infection? Is this protein degradation important for the immune response? To explore these questions, I investigated the effect of knocking down the expression of the ESCRT-III gene *CHMP2B* on fly immunity. If, as suggested by our hypothesis, the fly is degrading protein in order to provide amino acids for antimicrobial peptide production, then interfering with this protein degradation should compromise translation, but not transcription, of antimicrobial peptides, potentially resulting in reduced immune function.

Please note that, although I was the primary planner for all the experiments described in this chapter, due to restricted lab access during the COVID-19 pandemic many of the experiments were performed by my colleagues in the Dionne lab. I have credited those

who performed the actual experiments in the figure legends. In all cases, they have agreed that this work can be presented here.

6.2 Is CHMP2B required for a normal immune response?

To investigate if CHMP2B, and thus ESCRT-III activity, has a role in the fly innate immune response, two different UAS-constructs were used to knockdown *CHMP2B* under control of the *c564Gal4* fat body driver: UAS-*CHMP2B*-IR^{KK} (KK101734) and UAS-*CHMP2B*-IR^{TRiP} (HMS01844). The latter, UAS-*CHMP2B*-IR^{TRiP}, was the construct used in last chapter that demonstrated reduced upregulation of allantoin levels following bacterial infection. Before characterising the effects of *CHMP2B* knockdown on immunity, the knockdowns were validated in an infection setting by measuring levels of *CHMP2B* mRNA by RT-qPCR in the experimental and control genotypes following infection (**Figure 6.2**).

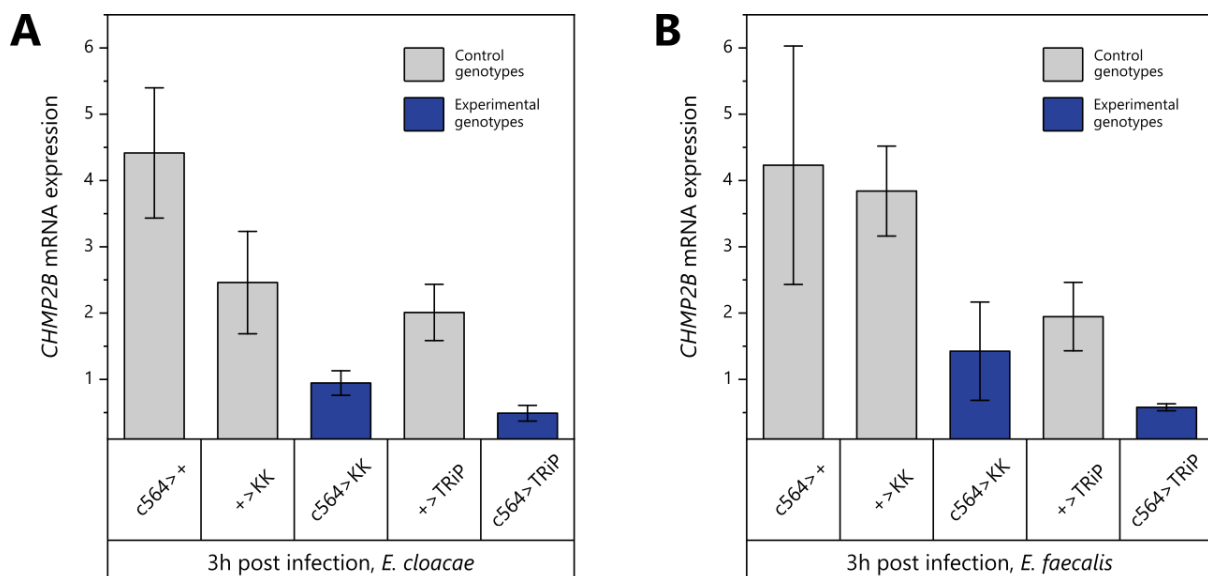


Figure 6.2: Both of the UAS-IR constructs strongly knockdown *CHMP2B* expression following infection, but also show an effect in the absence of the Gal4 driver.

Flies were crossed to generate offspring with the experimental genotypes *c564>CHMP2B*-IR^{KK} (*c564>KK*) and *c564>CHMP2B*-IR^{TRiP} (*c564>TRiP*) along with their driver-only control genotype (*c564>+*) and their respective UAS-only control genotypes (*+>KK* and *+>TRiP*). These lines were infected with *E. cloacae* or *E. faecalis* in parallel and mRNA was extracted from whole flies 3 hours later. Following *E. cloacae* infection (**A**) and *E. faecalis* infection (**B**) the experimental genotypes (in blue) showed substantially reduced expression of *CHMP2B* mRNA compared with the driver-only *c564>+* line. The UAS-only control genotype lines demonstrated intermediate levels of *CHMP2B* expression, with the exception of the *+>KK*

line in *E. faecalis* infection that behaved similarly to the c564>+ controls (**B**). *CHMP2B* expression is normalised to *RpL4* expression in each sample and so, in the graph, the bars indicate mean *CHMP2B/RpL4* expression, and the whiskers show ± 1 standard deviation from the mean. $n=4$ per genotype per condition, with 3 flies per sample. The experiment was performed by **Krista Grimes** and **Ashima Wadhawan**.

Under control of the c564Gal driver, both constructs showed greatly reduced *CHMP2B* mRNA 3 hours after infection with *E. cloacae* or *E. faecalis*. However, the 2 UAS-only control lines also showed knockdown of *CHMP2B* expression relative to the driver-only control. This showed *CHMP2B* mRNA levels that were intermediate between the experimental and driver-only controls, with the apparent exception of the w^{1118} ; UAS-*CHMP2B*-IR^{KK} line after *E. faecalis* infection that showed no knockdown compared to the w^{1118} ; c564Gal4 driver-only control. UAS constructs that show an effect in the absence of Gal4, such as seen here, are described as having “leaky” expression. In the experiment above, mRNA was extracted from whole fly and so the tissue in which this leaky expression is occurring is unclear. Although many of the following experiments use both control genotypes, this leaky expression means the driver-only w^{1118} ; c564Gal4 line is the appropriate genotype control. In comparison to this driver-only line, both experimental lines show substantial knockdown of *CHMP2B* mRNA. This validates the efficacy of the knockdown used in the last chapter and for the remaining experiments in this chapter.

To test whether *CHMP2B* expression is important for immunity, bacterial quantifications and survival experiments were performed. Although flies with knockdown of *CHMP2B* and their control genotypes all survived *E. cloacae* infection (>95% survival in all lines after 10 days), in comparison to the driver-only c564>+ controls, lines carrying either UAS-*CHMP2B*-IR construct showed reduced ability to kill *E. cloacae* at 3 hours post-infection regardless of whether the c564Gal4 driver was present (**Figure 6.3A, B**). Following *E. faecalis* infection, flies carrying the UAS-*CHMP2B*-IR^{TRIP} construct showed higher numbers of bacteria at 6 hours post-infection relative to the driver-only controls (**Figure 6.3C**). Again, no effect was found on survival (>90% survival in all lines after 10 days). The UAS-*CHMP2B*-IR construct from the KK library has not yet been tested in *E. faecalis* infection. As the UAS-*CHMP2B*-IR constructs were found to reduce *CHMP2B* mRNA whether or not Gal4 was present, these data indicate the requirement for *CHMP2B* expression for the optimal killing of bacteria. However, there was no

survival phenotype in these infections implying that the immune response in the knockdown flies is able to control bacterial numbers. As antimicrobial peptide expression is required for survival in these infections, this indicates that antimicrobial peptide levels are produced. Together these data suggest that, in the absence of proper *CHMP2B* expression, antimicrobial peptides do not reach the optimal concentration and/or accumulate at a slower rate following infection, but this hindered production is still sufficient to control bacterial numbers in the infections tested here. Consequently, these results support the idea that, although CHMP2B is not necessary for a humoral innate immune response, it may be necessary for an optimal humoral innate immune response by aiding in antimicrobial peptide production with respect to the amount and/or the timing of their synthesis.

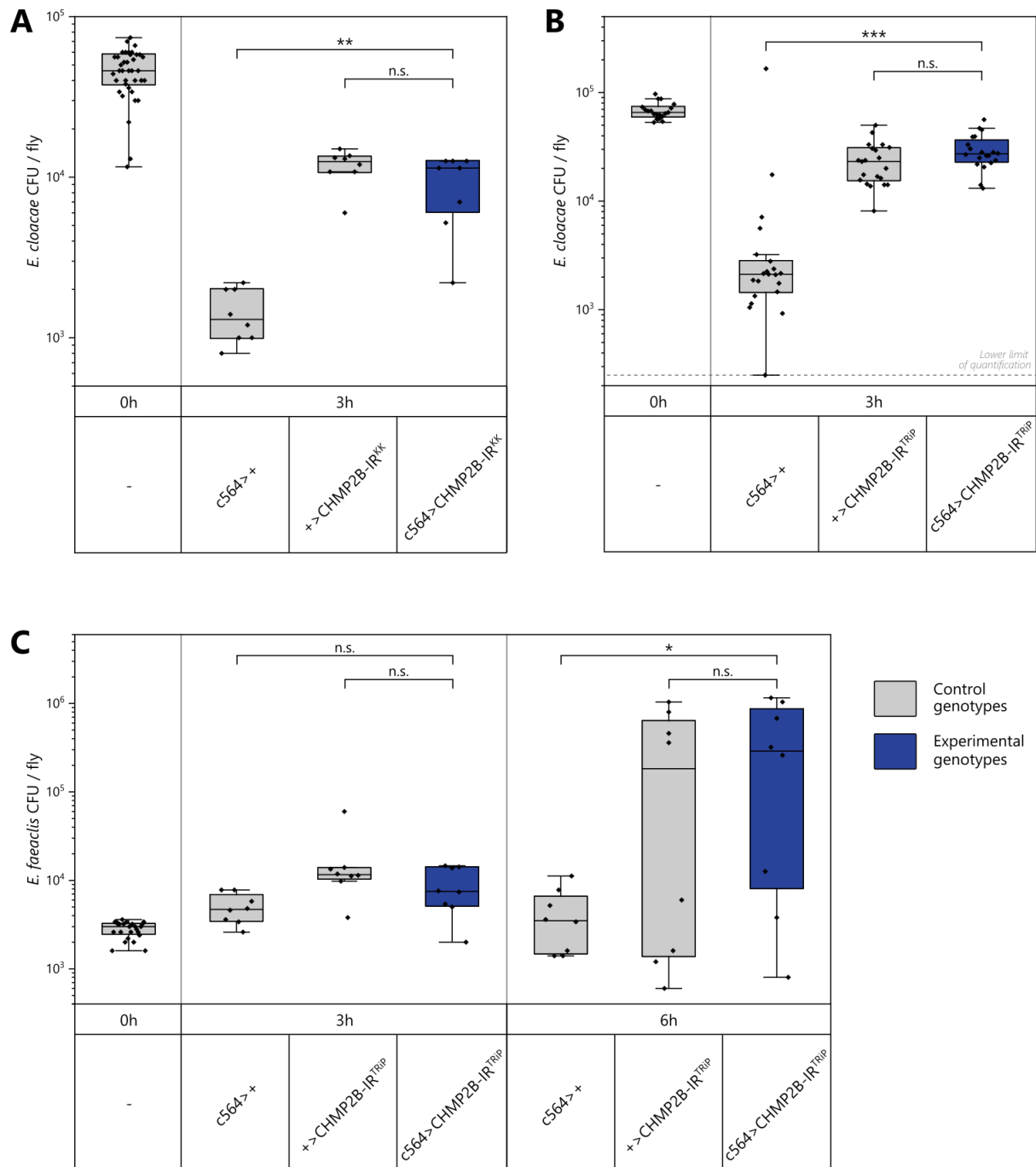


Figure 6.3: Flies with reduced *CHMP2B* expression show defective bacterial clearance in the early stages of infection.

Flies with *CHMP2B* knocked down in fat body under control of the c564Gal4 driver (c564>*CHMP2B*-IR, in blue) were infected, alongside their corresponding genotype control lines (c564>+ and +>*CHMP2B*-IR, in grey), with *E. cloacae* (**A**, **B**) or *E. faecalis* (**C**). 3 hours post-infection, flies carrying either the UAS-*CHMP2B*-IR^{KK} construct (**A**) or the UAS-*CHMP2B*-IR^{TRIP} construct (**B**) with the c564Gal4 driver demonstrated higher levels of *E. cloacae* in comparison to the driver-only c564>+ control line (**A**: $p = 0.0011$. **B**: $p < 0.0001$). The presence of the c564Gal4 driver had no apparent effect on the number of viable bacteria between the lines carrying either UAS-*CHMP2B*-IR construct (**A**: $p = 0.1873$. **B**: $p = 0.0949$). Although there was no clear difference in the number of viable *E. faecalis* 3 hours post-infection (**C**, $p = 0.1560$ vs. c564>+, $p = 0.4309$ vs. +>*CHMP2B*-IR^{TRIP}), by 6 hours the tested

c564>*CHMP2B*-IR^{TRIP} line showed greater numbers of viable bacteria in comparison to the c564Gal4 driver-only control line ($p = 0.0312$) there was no statistical difference in comparison to the +>*CHMP2B*-IR^{TRIP} UAS-only control line ($p = 0.5992$). In **A** and **C**, $n = 8$ per genotype per timepoint with the 0-hour timepoint data pooled together. For **B**, $n = 20$ – 21 per genotype, with data pooled from two independent replicate experiments. One fly per sample in all experiments. In the graphs, the line is the median, the box is the IQR, and the whiskers indicate the data points that fall within $\pm 1.5 \times \text{IQR}$ from the median. The Mann-Whitney test was used for the pairwise comparisons. The experiments in **A** and **C** were performed by **Ashima Wadhawan**.

6.3 Is the *CHMP2B* immune phenotype due to impaired production of antimicrobial peptides?

6.3.1 Tagged Antimicrobial peptides

Antimicrobial peptide production is typically monitored by RT-qPCR; however, this would not detect if there was reduced translation of the gene transcripts. To see if knockdown of *CHMP2B* is, as we hypothesise, leading to suboptimal antimicrobial peptide synthesis, I needed to be able to detect antimicrobial peptides directly. To develop assays for the quantification of antimicrobial peptides, as peptides rather than as transcripts, Dr Marc Dionne and Dr Jake Jacobson used the CRISPR-CAS9 system to produce flies encoding an antimicrobial peptide tagged with the FLAG- and HA (hemagglutinin) epitopes. This allows for the use of the widely available anti-FLAG and anti-HA antibodies to detect and quantify the translated, tagged peptide. Two methods were used to quantify the tagged antimicrobial peptides: immunohistochemistry with confocal microscopy, and sandwich enzyme-linked immunosorbent assay (sandwich ELISA, **Figure 6.4**).

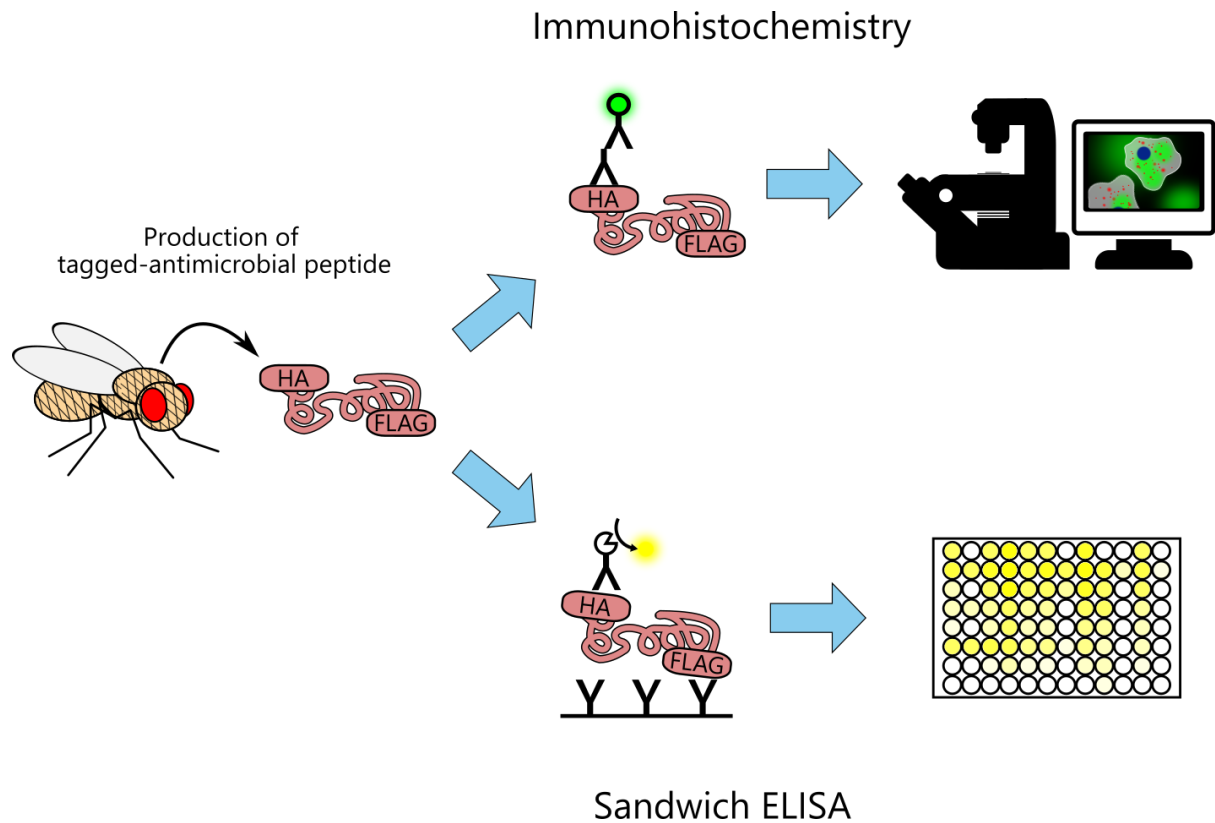


Figure 6.4: The two methods used quantify antimicrobial peptide production.

Fly lines were generated using the CRISPR-CAS9 system to express a mutant form of either the *CecA1* or *BomS2* antimicrobial peptide genes. Each mutant gene encodes the wildtype antimicrobial peptide tagged with both the HA and FLAG epitopes, with each epitope present towards different ends of the peptide. These epitopes allow for the use of commonly produced antibodies to detect and quantify the tagged *CecA1* and tagged *BomS2* by either immunohistochemistry or sandwich ELISA. These mutant alleles, *CecA1^{HFA}* and *BomS2^{HFA}*, were then further crossed to generate fly lines carrying one of these alleles plus the *c564Gal4* driver to allow for the combination of antimicrobial peptide quantification with the fat body-specific knockdown of a target gene by the UAS-Gal4 system. Consequently, these fly lines, with the antibody-based quantitative methods shown, can be used to investigate the role of target genes in the synthesis of these antimicrobial peptides when quantification of translated peptide is more relevant than quantification of transcripts.

The tagged antimicrobial peptide genes used were *CecA1^{HFA}* and *BomS2^{HFA}*. After confirming that these genes produced peptides detectable via immunohistochemistry and ELISA, they were crossed into flies carrying the *c564Gal4* driver to combine production of quantifiable antimicrobial peptides with fat body-specific knockdown of target genes. To test the hypothesis that *CHMP2B* is required for the optimal synthesis of antimicrobial peptides through involvement in providing amino acids for translation, these flies carrying UAS-*CHMP2B*-IR were crossed with these flies carrying both the *c564Gal4* construct and a tagged antimicrobial peptide to allow for peptide quantification. To confirm whether any effect on

antimicrobial peptide levels seen in these experiments is due to reduced translation rather than reduced transcription, the data was interpreted alongside the antimicrobial peptide mRNA expression as measured separately by RT-qPCR.

6.3.2 CecA1 synthesis in *E. cloacae* infection

Flies were generated with UAS-*CHMP2B*-IR mediated knockdown under control of the c564Gal4 driver in a background containing 1 copy of the tagged, endogenous *CecA1* allele (w^{1118} ; c564Gal4 / UAS-*CHMP2B*-IR^{KK}; *CecA1*^{HFA} / +). *CecA1* is primarily under the control of the Imd pathway and so these flies were infected with *E. cloacae*, a strong Imd pathway agonist, along with their corresponding, driver-only control-genotype flies (w^{1118} ; c564Gal4 / +; *CecA1*^{HFA} / +). Flies were harvested every hour for the first 6 hours post-infection and also at 24 hours post-infection. The fat body was dissected from samples and fixed for immunohistochemistry (**Figure 6.5**). The levels of tagged *CecA1*, as quantified by confocal microscopy of immuno-stained fat body cells, is shown in **Figure 6.6A**. *CecA1* peptide levels increase from 1 hour after *E. cloacae* injection, peaking at 4 hours in the *CHMP2B* knockdown flies and at 3 hours in the control flies. In the control line, the levels of *CecA1* remain elevated before recovering between 6- and 24 hours post-infection. In the knockdown line, this recovery potentially begins immediately after 4 hours, although the high variation in signal (demonstrated by the length of the error bars), particularly at the 4- and 5-hour time points, makes the kinetics of *CecA1* levels over time hard to decipher in this experiment. Most notably however, is the difference at 3 hours post-infection between the genotypes, when the *CHMP2B* knockdown flies show markedly reduced *CecA1* peptide levels. As expected, flies that were anaesthetised, but not injected, did not show any change levels of *CecA1*. Quantification of *CecA1* mRNA extracted from flies with the same *CHMP2B* knockdown at 3- and 6 hours following *E. cloacae* infection in a separate experiment (**Figure 6.6B**) suggests that any reduction in *CecA1* peptide levels is not due to a reduction of *CecA1* transcript expression. Indeed, at the 3-hour timepoint, expression of *CecA1* transcripts was higher in the c564>*CHMP2B*-IR^{KK} compared with the genotype controls, possibly reflecting the increased bacterial load of these flies as previously observed (**Figure 6.3A**).

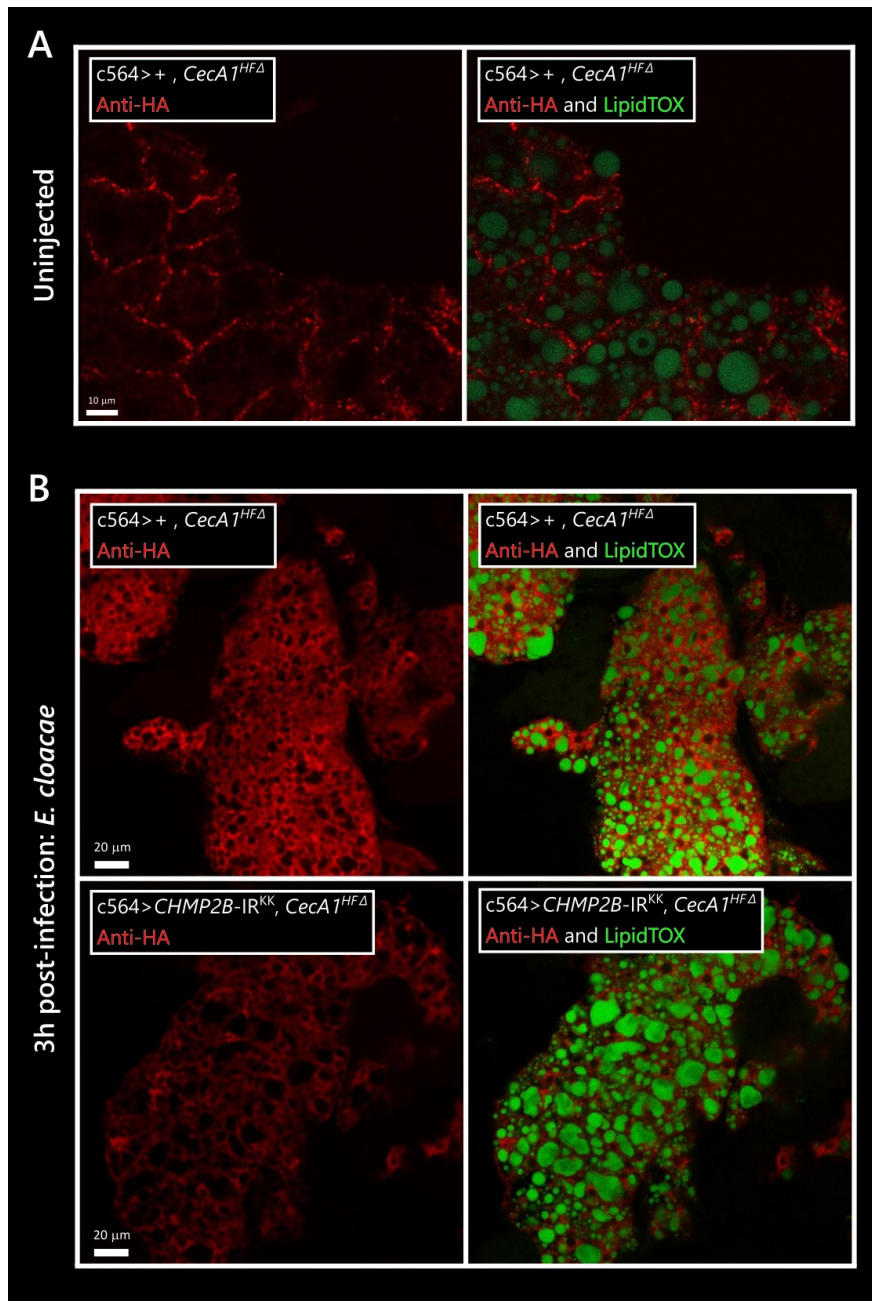


Figure 6.5: Immunohistochemistry and confocal imaging can successfully detect the induction of the tagged CecA1 peptide in fat body cells following infection and indicates that full CecA1 production may require *CHMP2B* expression in the fat body. Antimicrobial peptides can be detected by immunohistochemistry on flies expressing the *CecA1^{HFA}* tagged antimicrobial peptide. The fat body of uninjected flies (**A**) shows some punctate signal in the anti-HA channel (red), localised to the cell membrane. LipidTOX staining of lipid droplets (green channel), as shown in the combined images on the right-hand side, confirms that stained cells are fat body cells. 3 hours after infection with *E. cloacae*, the fat body cells show much higher levels of anti-HA staining indicating that the staining is effectively detecting the tagged CecA1 peptide. Knockdown of *CHMP2B*, lower panels in **B**, appears to reduce the antimicrobial peptide signal induction compared with controls. Quantitative analysis of images such as these allows for the measurement of tagged antimicrobial peptide levels. Experiment performed by **Dr Jake Jacobson**.

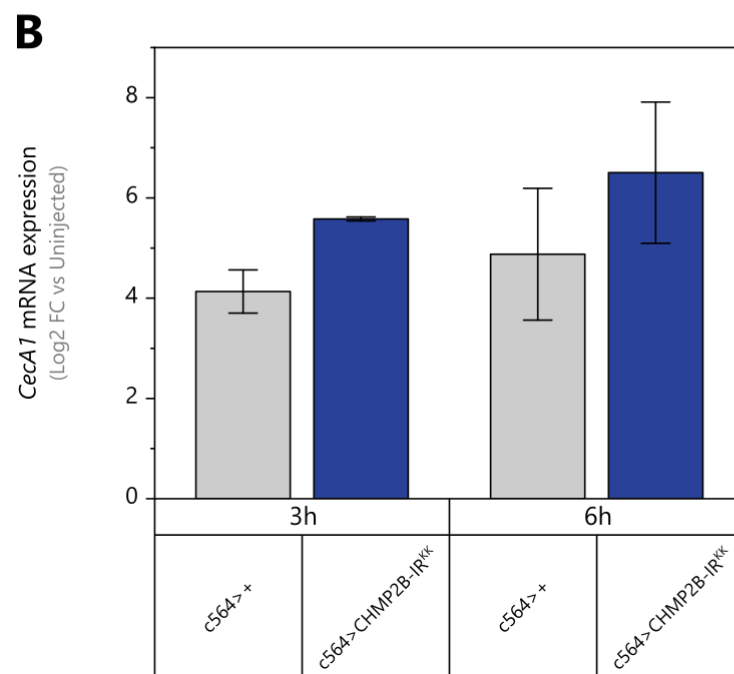
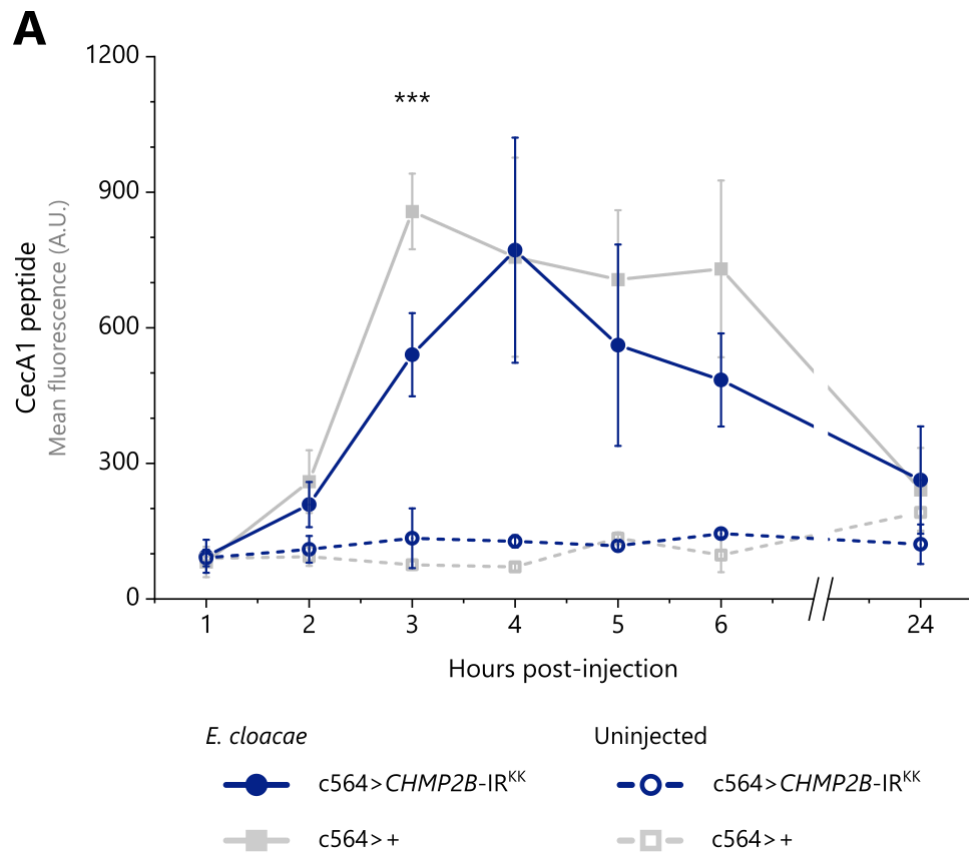


Figure 6.6 (previous page): Immunohistochemistry suggests knockdown of *CHMP2B* may impair translation of the CecA1 antimicrobial peptide in *E. cloacae* infection.

A: Flies with knockdown of *CHMP2B* expression in the fat body (*c564>CHMP2B-IR^{KK}*, in blue) and their corresponding driver-only genotype flies (*c564>+*), both carrying the tagged *CecA1^{HFA}* allele, were injected with *E. cloacae*. At the indicated timepoints following injection, flies were dissected, and fat body tissue fixed for immunohistochemistry. Anti-HA antibody staining was used to quantify tagged CecA1 peptide levels (details of quantification in **Section 2.5.3**). The results suggest that knockdown of *CHMP2B* may hinder or delay production of CecA1 in response to infection. However, when comparing between the infected genotypes via Student's *t*-test, only the 3-hour timepoint reaches statistical significance ($p \leq 0.0001$). $n = 7-8$ per time point for infected flies, and $n=2$ per time point for uninjected flies. **B:** Any impairment of CecA1 antimicrobial peptide production is not a result of reduced *CecA1* transcript expression. 3- and 6 hours following *E. cloacae* infection, the induction of *CecA1* mRNA expression relative to uninjected controls was comparable or even raised in the *CHMP2B* knockdown flies compared with their genotype control (*c564>+*). Statistical tests were not performed as this experiment was performed to test only if there was a reduction in *CecA1* transcript expression in the *CHMP2B* knockdown line; any such reduction was clearly absent. $n = 4$, 3 flies per sample. Graph shows mean log₂ fold change of *CecA1* mRNA relative to its mean expression in the uninjected controls for each genotype. *CecA1* mRNA expression in each sample was normalised to *RpL4* expression. Error bar shows ± 1 SD from the mean. The experiment in **A** was performed by **Dr Jake Jacobson** and the experiment in **B** was performed by **Krista Grimes** and **Ashima Wadhawan**.

6.3.3 BomS2 synthesis in *E. faecalis* infection

To examine the role of *CHMP2B* in antimicrobial peptide production induced by Toll pathway activation. Experiments were performed using flies with the same *CHMP2B* knockdown but carrying the tagged *BomS2^{HFA}* allele, an antimicrobial peptide that is strongly induced by the Toll pathway in response to *E. faecalis* infection (**Figure 6.7**). In these experiments, both genotypes showed rising levels of BomS2 peptide after infection with levels peaking at 4 hours post-infection (**Figure 6.7A**) or 5 hours post-infection (**Figure 6.7B**). Again, uninjected flies showed no such change in BomS2 peptide levels. While both experiments potentially suggest reduced BomS2 production when *CHMP2B* expression is reduced, the difference at 2- and 3 hours post-infection is certainly not clear, and the experiments clearly conflict over levels of BomS2 peptide 4 hours following infection. While **Figure 6.7A** shows levels of BomS2 are similar between genotypes, albeit very variable within genotypes; **Figure 6.7B** shows clearly reduced levels of BomS2 peptide at 4 hours and (albeit to a lesser extent) 5 hours following infection. The large difference in **Figure 6.7A** between the signal measured in the *c564>CHMP2B-IR^{KK}* flies at the 4-hour time-point compared with the adjacent 3- and 5-hour time-points may suggest that this measurement at 4 hours is erroneously elevated; further independent repeats will be required to confirm the accuracy of this particular measurement. As with the Imd pathway-activating *E. cloacae* infection above, any impairment of BomS2 peptide production does seem to be due to a reduction in the transcriptional induction of *BomS2* (**Figure 6.7C**). Although these data are not yet convincing due to high variation between samples of the same genotype and between independent experiments, they do suggest that knockdown of *CHMP2B* expression may interfere with optimal antimicrobial peptide production.

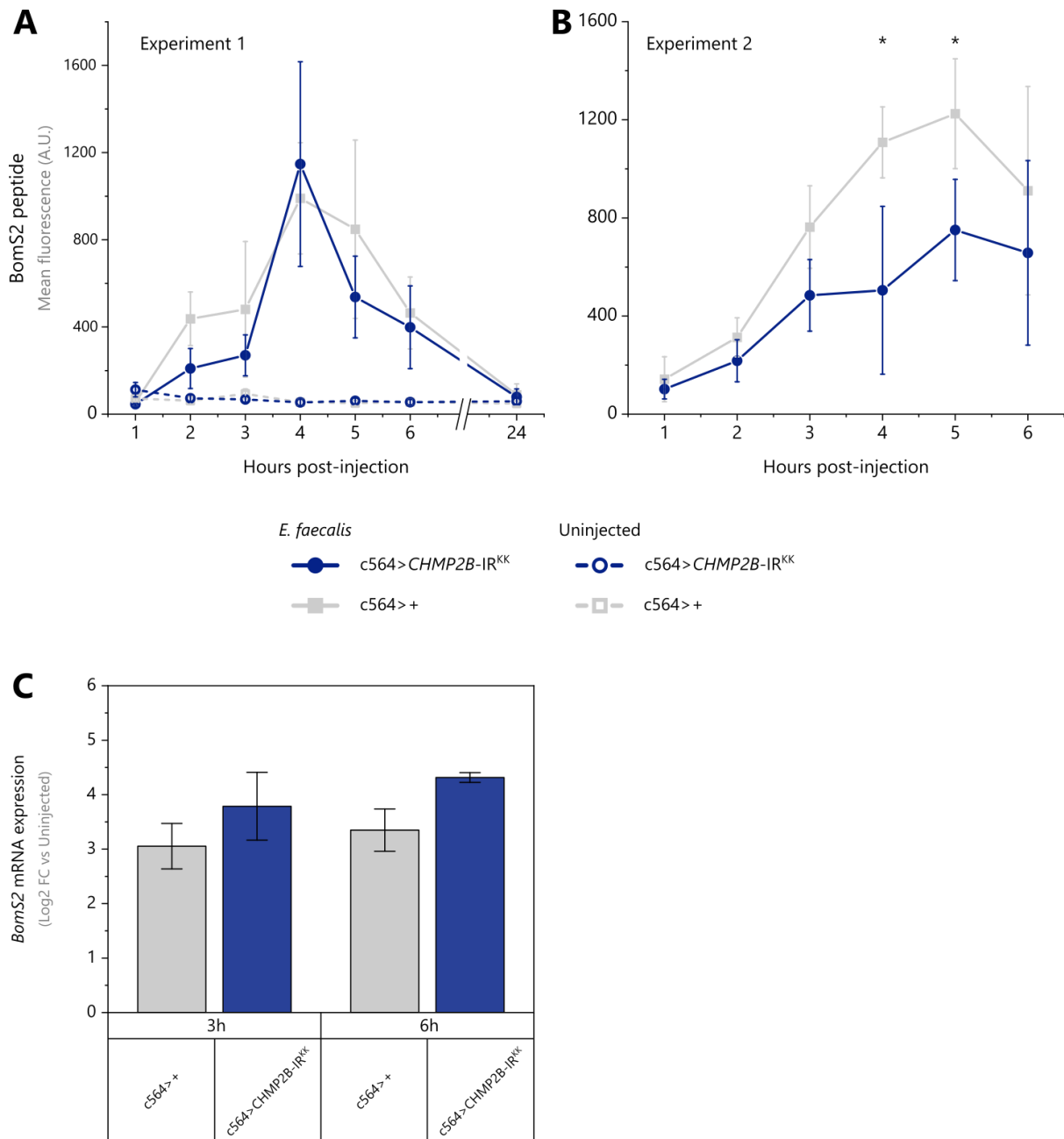


Figure 6.7: Immunohistochemistry suggests knockdown of *CHMP2B* may impair translation of the BomS2 antimicrobial peptide in *E. faecalis* infection.

Flies with knockdown of *CHMP2B* expression in the fat body (c564>*CHMP2B*-IR^{KK}, in blue) and their corresponding driver-only genotype flies (c564>+), both carrying the tagged *BomS2^{HFA}* allele, were infected with *E. faecalis* in two independent experiments (**A**, **B**). At the indicated timepoints following infection, flies were dissected, and fat body tissue fixed for immunohistochemistry. Anti-HA antibody staining was used to quantify tagged BomS2 peptide levels (details of quantification in **Section 2.5.3**). BomS2 levels peaked at 4 hours (**A**) or 5 hours (**B**) following infection in both the experimental and control genotypes. The results suggest that knockdown of *CHMP2B* may hinder total production of BomS2 in response to infection. Pairwise comparisons between the infected genotypes via Student's *t*-test resulted in statistical significance only in Experiment 2 (**B**) at the 4- and 5-hour timepoints ($p = 0.0347$ and $p = 0.0208$ respectively). $n = 6-8$ per genotype per timepoint for infected flies, and $n = 2$ per genotype per timepoint for uninjected flies. **B**: The possible

reduction in BomS2 peptide production is not a result of reduced *BomS2* transcript expression. In an independent experiment, 3- and 6 hours following *E. faecalis* infection, the induction of *BomS2* mRNA expression relative to uninjected controls was comparable or even raised in the *CHMP2B* knockdown flies compared with their genotype control (*c564>+*). Statistical tests were not performed as this experiment was performed to test only if there was a reduction in *BomS2* transcript expression in the *CHMP2B* knockdown line; any such reduction was clearly absent. Graph shows mean log₂ fold change of *BomS2* mRNA relative to its mean expression in the uninjected controls for each genotype. *BomS2* mRNA expression in each sample was normalised to *RpL4* expression. Error bar shows ± 1 SD from the mean. The experiment in **A** was performed by **Dr Jake Jacobson** and the experiment in **B** was performed by **Krista Grimes** and **Ashima Wadhawan**.

The immunohistochemical method for tagged antimicrobial peptide quantification can only measure the peptide levels in fixed cells. Consequently, tagged peptides that had already been secreted by the fat body cells into the hemolymph were not detected. This may be problematic as the hemolymph is both where antimicrobial peptides perform their function and likely where they accumulate. The alternative method for quantifying tagged antimicrobial peptide concentration is the sandwich ELISA. Instead of quantifying tagged antimicrobial peptide solely in the fat body cells where they are synthesised, this ELISA assay allows for quantification of the tagged peptide in whole fly samples. As these samples will include the hemolymph, this assay is likely more reflective of humoral immune function. In light of this, I used the sandwich ELISA assay to test the effect of *CHMP2B* knockdown in fat body on the levels of tagged antimicrobial peptide in the whole fly (**Figure 6.8A**). As in the previous experiment, the generated experimental fly line contained the *BomS2^{HFA}* allele along with *c564Gal4* driven knockdown of *CHMP2B*, on this occasion using the UAS-IR construct from the TRiP library. The ELISA assay allows for much higher sample numbers per timepoint than the immunohistochemical assay as no dissection is required. Consequently, I was able to quantify tagged-BomS2 levels in both the control genotypes (driver-only and UAS-only), as well as in PBS-injected flies and in a larger number of uninjected flies than tested via immunohistochemistry. The results in **Figure 6.8A** show that the *c564>CHMP2B-IR^{TRiP}* were able to upregulate the production of BomS2 by 3 hours following *E. faecalis* infection but the concentration of BomS2 reached in these flies was statistically lower than in both the infected control genotypes by Mann-Whitney test. Indeed, all the *c564>CHMP2B-IR^{TRiP}* samples had a lower concentration than all but one of the driver-only control lines. In part reflecting the earlier finding that the UAS-only control genotype demonstrates knockdown of *CHMP2B* expression in the absence of a driver, this control line appeared to have an intermediate

phenotype in its tagged-BomS2 peptide concentration following infection. Again, an independent experiment using the same UAS construct, infection conditions, and timepoint, indicated that the reduced concentration of BomS2 peptide seen in the *CHMP2B*-knockdown flies was not due to a reduced induction of *BomS2* transcript expression as measured by RT-qPCR (**Figure 6.8B**). Together, these data indicate that *CHMP2B* expression is required in the fat body for maximal and/or timely production of Toll pathway-responsive antimicrobial peptides following infection with *E. faecalis*.

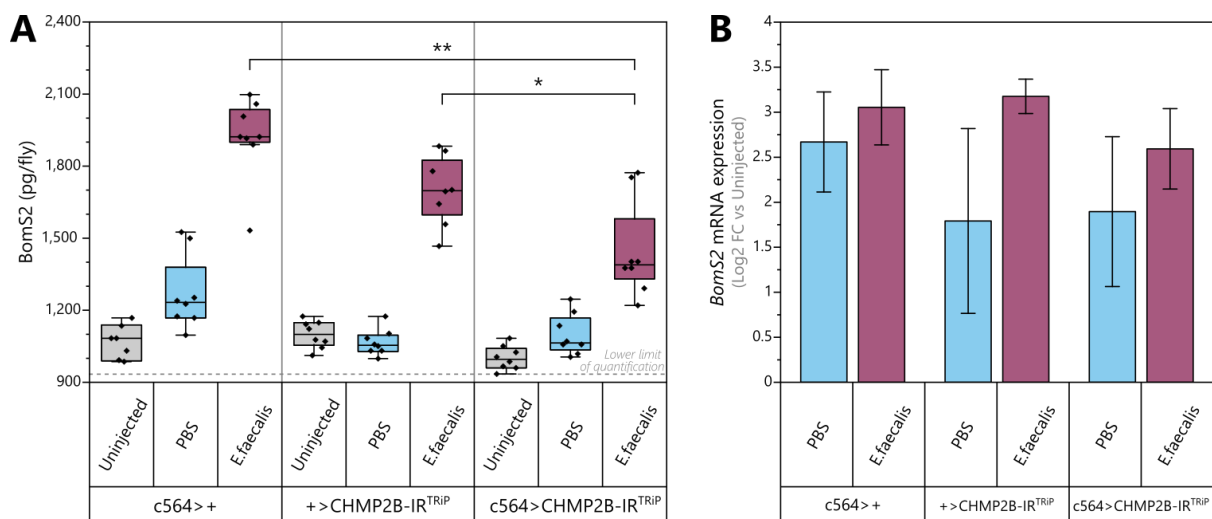


Figure 6.8: CHMP2B is required in the fat body for optimal translation, but not transcription, of the antimicrobial peptide BomS2 3 hours after *E. faecalis* infection.

A: Flies expressing tagged-BomS2 with *CHMP2B* knocked down in the fat body, plus control genotypes, were infected with *E. faecalis*. Infected fly samples (in red), along with PBS-injected (blue) and uninjected controls (grey) were harvested 3 hours following injection for quantification of tagged-BomS2 peptide levels by ELISA. While BomS2 peptide levels were upregulated following infection in all genotypes, flies with *CHMP2B* expression knocked down in the fat body showed lower levels of tagged-BomS2 following infection compared with both driver-only (c564>+) and undriven-RNAi (+>*CHMP2B*-IR) control lines: $p = 0.0019$ and $p = 0.0237$ respectively by Mann-Whitney test. This result was replicated in a second independent experiment ($p \leq 0.0001$ and $p = 0.0136$ for the same comparisons). $n = 7-8$ per genotype per condition, 1 fly per sample. In the graph, the horizontal line indicates median, the box shows IQR, and the whiskers show range of data within $\pm 1.5 \times \text{IQR}$ of the median. **B:** An independent experiment measuring mRNA expression of BomS2 3 hours after infection with *E. faecalis* by RT-qPCR indicates the defect in BomS2 production seen in the *CHMP2B* knockdown flies (**A**) was not due to suppressed transcriptional induction of the antimicrobial peptide following infection. Statistical tests were not performed as this experiment was performed to test only if there was a reduction in *BomS2* transcript expression in the *CHMP2B* knockdown line; any such reduction was clearly absent. $n = 4$ per genotype per condition, 3 flies per sample. Graph shows mean log2 fold change of *BomS2* mRNA relative to its mean expression in the uninjected controls for each genotype. *BomS2* mRNA expression in each sample was normalised to *RpL4* expression. Error bar shows ± 1 SD from

the mean. The experiments for **A** were performed with **Dr Esteban Beckwith**. **Ashima Wadhawan** and **Krista Grimes** performed the experiments for **B**.

6.4 Discussion

The results in this chapter suggest that *CHMP2B* expression is required in the fat body for optimal speed or maximal output in the production of antimicrobial peptides in response to both a Gram-negative and a Gram-positive infection. As *CHMP2B* is a core component of the ESCRT-III complex, the results presented here suggest that the ESCRT-III activity that I found to be important for the infection-induced change in nitrogen metabolism (see **Chapter 5**) is also important for the translation, though not the transcription, of antimicrobial peptides. Consequently, these results support our hypothesis that the fly is upregulating endogenous protein degradation in order to support the production of antimicrobial peptides, presumably through the prompt supply of free amino acids.

However, the results in this chapter do only *suggest* the importance of *CHMP2B* in the immune response: upon *CHMP2B* knockdown, no survival effect was seen in the two infections tested; the bacterial quantification results in *E. faecalis* infection (**Figure 6.3C**) are not yet convincing and more experiments are required, likely at a slightly later timepoint; and the tagged antimicrobial peptide quantification by immunohistochemistry demonstrated highly variable results (**Figure 6.6A** and **Figure 6.7A** and **B**). The quantification of tagged BomS2 in whole-fly samples by sandwich ELISA was consistent between the two independent replicates and produced a clearer result (**Figure 6.8A**). I would suggest that this is likely due to the fact that this assay, as it is performed on whole-fly samples, includes antimicrobial peptides present in hemolymph in the quantification. Indeed, it may be worth testing the sandwich ELISA on extracted hemolymph samples to see if this improves the consistency of quantification, although this will require greater effort in sample collection and processing which may add variation. Performing further experiments utilising the ELISA at different time-points following *E. faecalis* infection may add clarity to the effect of *CHMP2B* knockdown suggested by the confocal imaging. The ELISA has not yet been used to quantify production of tagged CecA1 following *E. cloacae* infection and such experiments would likely be useful in clarifying the effect *CHMP2B* knockdown has on CecA1 production. The higher throughput of the ELISA, and

the fact it measures antimicrobial peptide in the whole fly, means it may be more successful in teasing apart how knockdown of ESCRT-III activity is affecting the kinetics of antimicrobial production: is the peak in total antimicrobial peptide concentration reached lower in the ESCRT-III knockdowns? Is the speed of production reduced? Answering such questions may be informative in uncovering the mechanism of protein degradation used, and in exploring the metabolic cost of humoral immunity since more understanding of the speed and extent of peptide production will provide insight into the amount, and type, of energy and resources required for an effective immune response.

To confirm the requirement for ESCRT-III activity, and to investigate the mechanism of protein degradation, experiments similar to the experiments in this chapter can be performed using flies with knockdowns of other genes that were implicated by the screen for allantoin production in the last chapter. Flies with knockdown of other ESCRT-III or Vps4 complex genes (if viable) could be used to confirm the requirement for ESCRT-III activity, whereas knockdown of genes such as *Mvb12*, an ESCRT-I gene, or *Uvrag* may help in narrowing down the form of lysosomal degradation required: both ESCRT-I and *Uvrag* are believed to be dispensable for macroautophagy but are required for endosomal microautophagy and endocytosis.

The generation of the tagged antimicrobial peptide mutant alleles, *CecA1^{HAD}* and *BomS2^{HAD}*, allowed for the direct measurement of peptide levels via immunohistochemistry with confocal imaging or sandwich ELISA. Due to the small size of most of the antimicrobial peptides, measurement of their levels has previously been largely indirect, for example through the use of reporter constructs under control of an antimicrobial peptide promoter, or poorly quantitative, such as when they are detected via MALDI-TOF. Consequently, it may be tempting to interpret quantification of transcript levels as a proxy for measurement of actual peptide production. However, the results presented in this chapter highlight that it can be problematic to assume antimicrobial peptide transcript levels correspond proportionally to the levels of translated peptide. This problem with corresponding transcript levels to translated peptide is also highlighted by results in the study by Vasudevan *et al.* demonstrating that the translation of antimicrobial peptides is positively regulated during the integrated stress response, independently of transcriptional induction (Vasudevan *et al.*, 2017).

Nevertheless, while the results in this chapter highlight the disconnect between the transcription and translation of antimicrobial peptides, the tagged antimicrobial peptide genes present a novel and valuable experimental tool that can be used to quantify translated antimicrobial peptides with relative ease. The experiments utilising the tagged antimicrobial peptides provided insight into the requirement for *CHMP2B* in the humoral immune response, but also produced some interesting data that was independent of the genotype comparisons being made. Firstly, in the confocal images of fat body cells from the uninfected flies, the punctate signal near the cell membranes may be due to an artifact of the assay, for example antibody persisting in these locations during washes, but may be reflective of the biology. In the absence of infection, could fat body cells be storing antimicrobial peptides in vesicles at the cell membrane, perhaps to allow for rapid secretion upon detection of microorganisms? While this is a speculative question, it demonstrates the kind of question that could be investigated via the immunohistochemical assay used in this chapter. The wide variety of genetic tools available to the *Drosophila* researcher means that the tagged antimicrobial peptide gene can be combined, in the same animal, with a vast variety of organelle markers, transgenic fluorescent proteins, and reporters of cell processes or biochemical states. In the context of this project, the combination of tagged antimicrobial peptides with protein degradation reporters such as the KFERQ-mCherry construct used in **Section 5.2.2** could potentially be useful in investigating the mechanism by correlating induction of protein degradative processes with antimicrobial peptide production.

A second interesting observation was seen in the data from the experiments quantifying tagged antimicrobial peptides by ELISA. Interestingly, the concentration of tagged BomS2 appeared to be only marginally increased, if at all, following sterile PBS injection (**Figure 6.8A**). This contrasts with the strong induction of *BomS2* transcript levels seen at the same 3-hour timepoint following PBS injection as detected by RT-qPCR (**Figure 6.8B**), with the mean log₂ fold changes ranging from 1.7 to 2.7. This may be down to variation between the experiments, or it could be that transcriptional induction of *BomS2* is delayed after sterile injury compared to its induction after infection, which would result in lower levels of accumulated peptide by the 3-hour timepoint. An interesting, alternative possible explanation is that this discrepancy is due to strict post-transcriptional control of antimicrobial peptide production.

Finally, a fascinating question that could be tackled by combining the ELISA with bacterial quantifications in time-course experiments is what *in vivo* concentration of a particular antimicrobial peptide is required for sufficient bacterial killing. Hanson *et al.* previously found that some infections can be controlled by the activity of just one of the known antimicrobial peptides: Drosocin, for example, appears to be necessary for resistance to *E. cloacae* infection (Hanson *et al.*, 2019). In cases such as this, accurate quantification, at multiple time-points, of a required antimicrobial peptide's concentration, in parallel with quantification of its target bacteria, may provide fascinating insights into the kinetics of the host-pathogen interaction.

In summary, these assays for quantifying antimicrobial peptide concentration will be extremely useful in investigating a wide range of biological questions in *Drosophila*: the immunohistochemistry and confocal imaging assay can explore questions regarding the anatomical and cellular locations of antimicrobial peptides, while the use of ELISA allows for quantification of antimicrobial peptides in a large number of samples. Additionally, the experimental potential of these assays can be vastly enhanced by their combination with the wealth of other genetic tools available in *Drosophila*.

Chapter 7: Discussion

7.1 Summary of work presented in this thesis

7.1.1 Background and aim of the project

Metabolism and immunity are closely intertwined and this relationship is critical for health, as dysregulation in their interaction is implicated in a wide ranges of conditions from sepsis and infection-induced cachexia, to obesity and type-2 diabetes (Hotamisligil, 2017). Despite the importance of immunometabolism, it is poorly understood, particularly at the level of a whole organism (Ayres, 2020). Studies using the model organism *Drosophila melanogaster* have begun to reveal much about how immune and metabolic signalling interact in these animals, yet the resultant effects on metabolism, especially with regard to the metabolite concentrations themselves, are less well studied (Lee and Lee, 2018; Galenza and Foley, 2019).

In this project I sought to explore what metabolic changes occur during the systemic innate immune response of *Drosophila* to bacterial infection and to investigate the functions of these changes. To do this, I planned to use an untargeted metabolomic approach to identify metabolites with altered concentrations during the immune response. I would then use this information to construct hypotheses about the cause and possible functions of these changes that could then be tested using the wide variety of genetic tools available for *Drosophila* research.

7.1.2 Results

In **Chapter 3**, untargeted metabolomic analysis of a successful systemic immune response of *Drosophila* showed profound changes in metabolism just 3 hours after infection with either *E. coli* or *M. luteus*, which are known to activate the *Drosophila* Imd and Toll immune-signalling pathways, respectively. The identified metabolite with the largest increase in concentration was allantoin, the end product of purine catabolism. There were also numerous changes in the

free proteogenic amino acids with a substantial depletion of the branched chain amino acids contrasting with a mild increase in the levels of other essential amino acids including tryptophan. An initial follow up experiment replicated the observed changes in allantoin and leucine following infection with *E. faecalis*. A second follow up experiment showed that, following infection with *E. cloacae*, the increase in allantoin was synchronous with bacterial clearance. Additionally, tryptophan and lysine, both essential amino acids, showed elevated concentrations with the rise in tryptophan corresponding to an increase in its catabolite kynurenine. Based on these results, we hypothesised that the fly is upregulating the degradation of endogenous protein in response to bacterial infection. First, as the end product of purine catabolism, allantoin is also the end product of nitrogenous waste metabolism in *Drosophila*, and the primary source of excess nitrogen is the catabolism of amino acids. Secondly, an increase in the free concentration of any essential amino acid is suggestive of protein degradation as *Drosophila* are unable to synthesise these molecules.

Before testing this hypothesis, I tested whether the infection-induced increases in purine catabolism and in tryptophan and kynurenine levels were downstream of immune signalling (**Chapter 4**). Using flies with genetically disrupted immune signalling, I found that the Imd pathway, specifically the branch upstream of Relish activation, was required for upregulation of purine catabolism and tryptophan/kynurenine levels following a Gram-negative bacterial infection. Interestingly, I found that functioning Toll signalling was not required for these changes following Gram-positive infection. While the signalling mediating the changes in purine and tryptophan metabolism during Gram-positive bacterial infection remained unidentified, the fact that Imd signalling was required for these changes shows that they are downstream of the immune response.

The hypothesis that protein degradation is upregulated in *Drosophila* during the systemic immune response was tested in **Chapter 5**. Different mechanisms of protein degradation were investigated by using knockdowns or mutants of various relevant genes. While knockdown of genes involved in selective endosomal microautophagy (eMI), including *Hsc70-4*, resulted in a defect in bacterial clearance, I did not find evidence of an upregulation in selective eMI following infection, nor did this process appear to be upstream of the infection-induced increases in allantoin and kynurenine concentrations. A genetic screen for allantoin production

following infection indicated the requirement for ESCRT-III genes and for the PI3KC3 component Uvr_g. Taken together, the results of this chapter support the hypothesis that protein degradation is upstream of the increased allantoin concentration following infection and that the mechanism of this protein degradation relies on ESCRT-III activity and Uvr_g involvement. The data presented suggest that the protein degradation is mediated by a bulk form of eMI or by endocytosis, but more work will be required to properly identify the mechanism(s) involved.

Finally, in **Chapter 6**, the role of *CHMP2B*, encoding a core ESCRT-III component, in the systemic immune response was investigated. Knockdown of *CHMP2B* expression in the fat body resulted in reduced ability to kill infecting bacteria despite normal upregulation of antimicrobial peptide transcript expression. Immunohistochemistry and ELISA experiments using flies carrying genes encoding epitope-tagged antimicrobial peptides suggested that knockdown of *CHMP2B* results in a defect in the translation of these peptides. As *CHMP2B* is also implicated in protein degradation following infection, these findings support our hypothesis that the upregulation of protein degradation functions to provide a ready supply of amino acids for antimicrobial peptide synthesis.

7.2 Proposed model

Together, the results in this thesis suggest a model in which, following the detection of bacterial infection, the fly upregulates the degradation of endogenous protein through an ESCRT-III dependent mechanism, in order to provide amino acids for antimicrobial peptide translation. In Gram-negative bacterial infection, this is downstream of Imd pathway activation, but the picture in Gram-positive infection is less clear. As certain amino acids are more required for antimicrobial peptide production than others, excess amino acids are catabolised, resulting in increased nitrogenous waste metabolism as demonstrated by a large increase in allantoin concentration (schematic of the proposed model is shown in **Figure 7.1**).

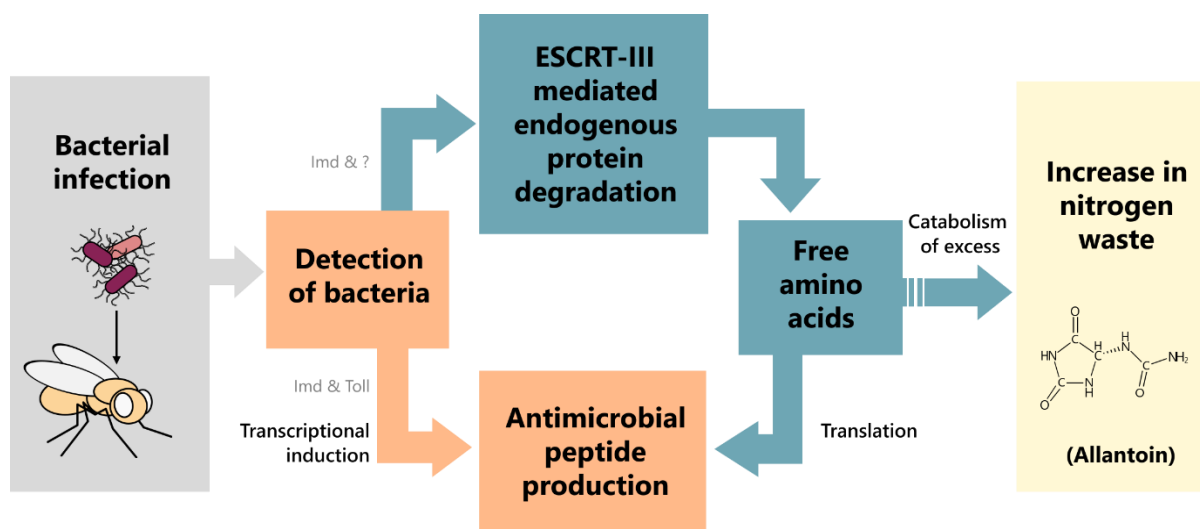


Figure 7.1: The proposed model for metabolic mobilisation of amino acids to support antimicrobial peptide translation

Bacterial infection of *Drosophila* results in a well characterised response in which the detection of bacteria leads to a large transcriptional induction of antimicrobial peptides under control of the Imd and Toll signalling pathways (in orange). The results in this thesis (blue) suggest that this response is accompanied by an increase in the degradation of endogenous protein, via an ESCRT-III dependent mechanism, that provides the free amino acids required for the translation of antimicrobial peptide transcripts. An imbalance between the amino acids required for antimicrobial peptide synthesis and the amino acids liberated by protein degradation results in an excess of amino acids that are catabolised. This results in an increase in nitrogenous waste presenting as a substantial increase in allantoin, the end product of nitrogenous waste metabolism in *Drosophila*.

7.2.1 Amino acid provision versus amino acid requirement.

The proposed model for the role of infection-induced protein degradation contains the assumption that the proteins upregulated during infection, primarily the antimicrobial peptides, have amino acid contents that are different relative to the proteome in general, or more specifically, to the proteins that are degraded in response to infection. In other words, the assumption is that certain amino acids are enriched in antimicrobial peptides. If this assumption is incorrect, then the model falls down as the protein degradation would not result in an excess of amino acids, as they would all be recycled equally into immune effector production; the model would fail to explain the increase in allantoin. However, this assumption was not made blind. Antimicrobial peptides are typically far smaller than most proteins with

most containing just a few tens of amino acid residues. The Cecropins, for example, contain 34-39 amino acid residues following cleavage of the secretory signal; the Drosomycins, 44-50; Metchnikowin, 26; and Drosocin, just 19 (Hanson and Lemaitre, 2020; Larkin *et al.*, 2020). These small sizes suggest an amino acid composition that is different from larger proteins, at least in part due to the resultant differences in the surface area to volume ratio which impacts the size and content of the hydrophobic core, if present (Carugo, 2008). More convincingly, a bioinformatic analysis by my supervisor, Dr Marc Dionne, which combined transcriptomic data with the known translated amino acid sequences from Flybase (Larkin *et al.*, 2020), demonstrated that the relative proportions of the amino acids present in the *Drosophila* proteome during infection with either *M. luteus* or *E. coli* are different to their proportions in the proteome of an uninfected fly (**Figure 7.2**). Consequently, when protein degradation is upregulated following infection, the degraded proteins are from the uninfected proteome and thus the proportions of the liberated amino acids do not match the proportions required for translation of the proteome during infection. The analysis indicates that, following infection, demand exceeds supply for amino acids such as glycine (G) and proline (P), and so more protein must be degraded to match this demand. As a result, the amino acids for which supply exceeds demand, such as glutamate (E) and lysine (K), will accumulate substantially. These amino acids will thus require elimination via catabolism causing an increase in nitrogenous waste metabolism.

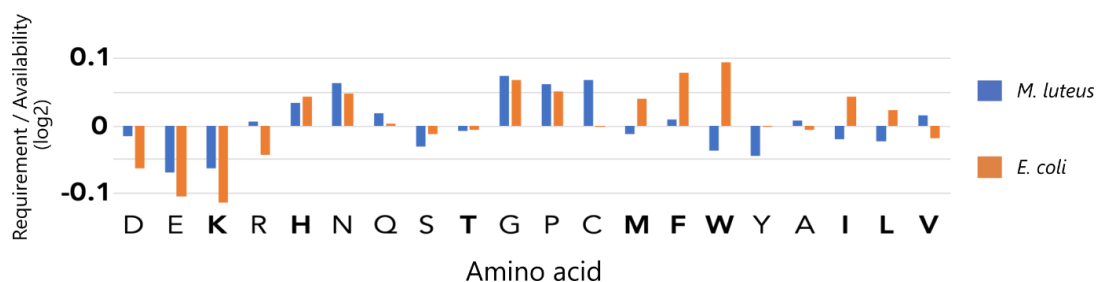


Figure 7.2: The amino acid content required by the infected fly proteome is dissimilar to the amino acid content of the protein available for degradation (the uninfected proteome).

The graph shows the amino acid content of the proteome, weighted by transcript expression, following *M. luteus* infection (blue) or *E. coli* infection (Orange) divided by that of the uninfected fly (in log2). Essential amino acids are in bold. Analysis by **Dr Marc Dionne**.

Interestingly, this analysis further shows that *M. luteus* and *E. coli* infection result in different demand for different amino acids. Tryptophan (W), for example, is indicated to be required in response to *E. coli*, but not *M. luteus* infection. This aligns with my results in the untargeted analysis (**Section 3.2.2**) in which an indicated increase in free tryptophan after *M. luteus* infection ($\log_2FC=0.73$) was not matched after *E. coli* infection ($\log_2FC=0.15$). Notably however, later experiments showed that *E. cloacae* infection resulted in increased levels in tryptophan suggesting that the metabolic requirements in *E. cloacae* infection is dissimilar those required in *E. coli* infection, despite the fact they both activate the Imd signalling pathway. This supports a previous observation that transcriptional induction following infection varies considerably dependent on the bacterial species involved and so is not simply dependent on which of the Imd or Toll pathways is most activated (Troha *et al.*, 2018).

Amino acids are not just required for incorporation into protein, and many individual amino acids will be required for different functions in the immune response. Consequently, the above analysis does not fully show the demand for tyrosine as this amino acid is consumed by the melanisation response. Along these lines, the indicated demand for glycine to use in protein synthesis following infection is particularly notable as glycine is also necessarily consumed by the synthesis of the purine rings required for the disposal of nitrogenous waste; in fact, an entire molecule of glycine is consumed for every two nitrogens excreted from other amino acids. As a result, this model predicts that glycine is in particularly high demand in the infected fly. Consideration of the relative importance of different amino acids for the immune response could be explored experimentally by reducing their supply by genetically interfering with synthesis enzymes or through dietary manipulation.

The analysis presented here assumes that the whole proteome is available for degradation. This is likely not to be the case, and the subset of the proteome available for degradation will depend heavily on the specific mechanism involved and would influence the spectrum of amino acids returned. Evolution would presumably drive selection for an infection-induced mechanism of protein degradation that targets proteins that are of low importance for immune tolerance (the ability to maintain health in infection), not transcriptionally induced by infection, easily targeted for degradation, and with amino acid compositions most closely matching the amino acids required by the immune response, likely with a bias towards the in-

demand essential amino acids as deficit in a non-essential amino acid could be mitigated by its synthesis. Finding proteins that match these criteria may indicate suitable target proteins that are *a priori* more likely to be targeted for degradation. For example, the apparent demand for glycine in infection, both for translation and purine synthesis, may suggest that collagens, which are enriched with glycine, would be an efficient source of this amino acid. As collagens exist in the extracellular matrix (ECM), this would implicate a form of endocytosis in the induced degradative mechanism. Indeed, the finding in this thesis that ESCRT-III activity and Uvrug involvement are required for increased nitrogen waste metabolism fits with the use of an endocytic mechanism. It is additionally tempting to speculate that using endocytosis for degradation of ECM or membrane proteins has the added potential advantage of recycling membrane from the cell surface to the interior which could support the function of the secretory pathway in delivering antimicrobial peptides in the opposite direction.

7.2.2 Organ-isation of amino acid supply and nitrogenous waste.

This thesis has focused on experimental measurement and manipulation of either the whole fly or of the fat body. However, the proposed model also requires the specific involvement of the hemolymph and the Malpighian tubules. Allantoin is the end waste-product of most amino acid catabolism. While the excess amino acids are being produced in fat body cells (and to a lesser extent in other tissues), the key enzymes in the allantoin synthetic pathway, *Urate oxidase* and *CG30016* (the gene predicted to encode 5-HIU hydrolase), are only known to be expressed in the Malpighian tubules. To follow up on this project, further metabolomic analysis of extracted hemolymph may illuminate whether the excess amino acids are exported from fat body cells as they are, or whether they are first partially catabolised before transport to the tubules. *A priori* I would predict that some level of catabolism would occur in fat body cells as the excess amino acids can, to an extent, be interconverted to more in-demand amino acids. Importantly, exporting the excess nitrogen once it has been further packed into nucleosides, such as inosine, would exert less osmotic pressure in the hemolymph; one molecule of inosine transports nitrogen atoms delivered from three separate amino acid molecules, aspartate, glycine, and glutamine (**Figure 1.3**). This consideration of osmotic and biochemical environment in the hemolymph is likely to be particularly pertinent during infection due to the

large amounts of secreted anti-microbial peptides as well as other metabolic changes, most notably the increase in circulating sugars and any biochemical consequences of the melanisation response (Bajgar and Dolezal, 2018; Valanne *et al.*, 2019).

Degrading protein in the fat body has the advantage that it provides free amino acids where they are most required. However, protein degradation in other tissues may be required for continued amino acid supply, particularly if infection is not rapidly resolved and immune effector synthesis continues for an extended period. Muscle tissue, being rich in protein, seems to be a likely source of amino acids that could be liberated by protein degradation and exported into the hemolymph for uptake by the fat body. The insect proline cycle (described in **Section 1.4.4**) demonstrates an example of inter-organ amino acid transport, the logic and components of which could conceivably be co-opted or mirrored in infection to deliver amino acids from muscle to fat body, and nitrogen waste from fat body to the tubules. Unravelling the possible contributions of different tissues to infection-induced metabolic change, perhaps through use of tissue specific knockdown of amino acid transporters, may highlight which tissues pay the highest cost during an immune response, revealing the trade-offs the host is having to make to support immunity.

7.3 Immunometabolism and resource allocation

Many metabolic changes infection have been found to, or are assumed to, mediate the reallocation of resources to the immune response (Rauw, 2012). Studies in *Drosophila* have repeatedly shown the depletion of glycogen and triglyceride energy stores during various infections or genetically induced immune activation (Dionne *et al.*, 2006; Roth *et al.*, 2018; Davoodi *et al.*, 2019). The breakdown of glycogen fuels an increase in circulating sugar which has been found to be important for resistance to systemic bacterial infection in adults and to parasitic wasp infection in larvae, in both cases via supporting immune cell function (Yang and Hultmark, 2017; Bajgar and Dolezal, 2018). Lipolysis has been shown to be required for anti-bacterial ROS production during enteric infection through providing NADPH for oxidation (Lee *et al.*, 2018). I contend that work in this thesis shows that amino acid metabolism is also of critical importance to immunity and that infection-induced change in metabolism is not restricted to mobilising energy storage for use by the immune response but involves

reallocation of the raw materials for protein synthesis. Additionally, particular metabolic changes observed during the immune response function to mitigate the *consequences* of shifted resource allocation: in this project, the observed increase in purine catabolites functioned in the disposal of the increased excess nitrogen which was seemingly formed as a by-product of a resource allocation process, namely the mobilisation of protein-incorporated amino acids for immune effector synthesis.

Consideration of immunometabolism in terms of resource allocation seemingly contradicts with infection-induced anorexia, a sickness-behaviour conserved between insects and mammals which can be beneficial or detrimental dependent on the infecting pathogen (Troha and Ayres, 2020). The mechanisms behind how reducing feeding can be beneficial in some infections despite a resource hungry immune response are unclear. One possible explanation for infection-induced anorexia is presented by my results: the increase in nitrogenous waste may result in a metabolic burden that would only be exacerbated by ingestion of more protein and thus more nitrogen atoms. This idea is similar to recent work suggesting that, in *Manduca sexta* larvae, infection induced anorexia is a result of a trade-off between immunity and food detoxification (McMillan, Miller and Adamo, 2018). The immune response in these animals caused a depletion in glutathione, but this metabolite is also important for the detoxification of compounds within the plant material on which the larvae feeds. Thus, in infection, glutathione resources are allocated to the immune response, meaning they are no longer available for food detoxification, and so the animal reduces feeding. In a similar manner, the fly may reduce feeding as there is substantially reduced metabolic capacity for handling any diet-derived excess nitrogen. Exploring feeding behaviour during infection where there is a choice of dietary sources that do or do not contain protein may be an interesting method to assess this idea.

A further explanation for reduced feeding following infection is that, if, as I speculated earlier, amino acids are being mobilised in circulation for delivery to the fat body, then this may inhibit the secretion of absorbed nutrients into the hemolymph from the gut. This would thus reduce the value of feeding. Alternatively, such secretion may actively interfere with a finely tuned inter-organ metabolic mobilisation that is delivering resources to the immune response and waste to the tubules.

7.4 Outlook

Resource allocation to the immune system is of clear importance in mounting an effective immune response. However, it can be maladaptive, particularly in cases of chronic and/or dysregulated immune activation. Infection-induced cachexia accounts for much of the morbidity and mortality seen in patients with chronic tuberculosis or severe sepsis (Schwenk and Macallan, 2000; Kaneki, 2017), and both of these conditions are major causes of mortality worldwide. Treatment options are few and are limited in effectiveness. Various strategies of nutritional support, including tailored supplementation of amino acids, have been shown to have no benefit and some may even be harmful (Gunst *et al.*, 2018). Research has tended to focus on immunometabolism in the context of immune dysregulation or highly pathogenic infections. There has been comparably less focus on the functions and mechanisms of immune-induced metabolic changes in a successful response to infection with a subsequent return to homeostasis. Understanding these will aid significantly in understanding how and why immunometabolism can become maladaptive and contribute to research into novel treatment strategies for conditions such as chronic tuberculosis and sepsis.

In this project, I used the *Drosophila* model organism to explore what metabolic changes occur during a successful immune response to systemic bacterial infection and why. My work suggests that the immune response results in an increase in nitrogenous waste metabolism due to the degradation of endogenous protein by an ESCRT-III-mediated process and that this protein degradation is important for the provision of in-demand amino acids to support the rapid synthesis of immune effectors. These results reinforce the usefulness of *Drosophila* in understanding immunometabolism at the level of the whole-organism and will support further research into alteration of metabolism in both adaptive and maladaptive immune responses. In addition, this project demonstrated that combining the genetic tractability and high throughput potential of the *Drosophila melanogaster* model organism with the analytical power of metabolomics provides an effective methodology for generating and testing hypotheses around metabolic responses; a methodology that could be applied to a wide variety of biological questions.

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