Galleria mellonella: A Model for Studying Pathogen Virulence and Insect Immunity

by

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Abstract

The greater wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae), has been used as a model organism to study numerous human pathogens due to low rearing costs, short generation time, and the ability to be reared at human body temperature. The use of *G. mellonella* for the study of enteropathogenic *Escherichia coli* (EPEC), an enterobacteria causing diarrhea in humans, has been proposed and demonstrated previously. However, very little is known regarding the virulence of EPEC in *G. mellonella* and *G. mellonella* immune responses against EPEC, both of which are essential in understanding the *Galleria*-EPEC model system.

Chapter 1 of this thesis provides relevant background information on *G. mellonella* immunity, EPEC pathogenesis, and the *Galleria*-EPEC system.

Chapter 2 examines: (1) the nature of EPEC virulence in the hemocoel of *G. mellonella* by monitoring insect mortality, survival time, time to pupation, pupal mass, pupal duration, fecundity, and egg hatch rate after injection; (2) the source of EPEC virulence in *G. mellonella* by comparing the intrahemocoelic median lethal dose (LD_{50}) of EPEC to the LD_{50} of an EPEC mutant (Δ*escN*) with disabled type III secretion system (T3SS) and to the LD_{50} of a benign *E. coli* strain (DH5α); (3) the degree of EPEC virulence in *G. mellonella* by comparing the intrahemocoelic LD_{50} of EPEC to that of a known entomopathogen (*Providencia rettgeri*) and a benign soil bacterium (*Bacillus clausii*); (4) the oral pathogenicity of EPEC in *G. mellonella* by comparing the intrahemocoelic LD_{50} to the *per os* LD_{50} of EPEC; and (5) the virulence of EPEC in *B. mori* and its suitability an alternative insect model for the study of EPEC. I found that EPEC-induced disease in *G. mellonella* was dose-dependent and manifested as increased mortality, decreased survival time, delayed pupation, decreased pupal mass, and increased pupal duration. The T3SS contributes to EPEC virulence in *G. mellonella* but unknown factors are

responsible for most of the virulence. EPEC displayed moderate virulence in *G. mellonella* relative to *P. rettgeri* and *B. clausii* but had low oral pathogenicity. *Bombyx mori* is inferior to *G. mellonella* for the study of EPEC virulence due to low EPEC susceptibility and low thermotolerance.

Chapter 3 examines: (1) the immune responses of *G. mellonella* against EPEC *in vivo* by hemolymph examination and larval dissection following intrahemocoelic EPEC injection; and (2) the temporal dynamics of circulating hemocytes, melanized particles, nodules, and EPEC replication/clearance during EPEC infection in *G. mellonella* by quantification using hemocytometer and the plate-count method. In addition to the typical insect immune responses (*i.e.* melanization, hemolymph coagulation, phagocytosis, and nodulation), a novel insect immune response in the form of extracellular DNA release similar to neutrophil extracellular traps (NETs) was present. The extracellular DNA immobilized EPEC and appeared to be hemocytic in origin. Hemocytopenia was temporarily induced in *G. mellonella* by EPEC between 3h - 6h post-injection but the circulating hemocyte count recovered by 48h post-injection. The immune responses of *G. mellonella* were unable to control EPEC replication in the early stage of infection (*i.e.* within 3h post-injection) but was eventually able to clear EPEC from the hemolymph by 48h post-injection. The clearance of circulating EPEC corresponded to the appearance of melanized particles and nodules, implicating these insect immune responses in EPEC clearance.

Chapter 4 examines: (1) the origin of the extracellular DNA by *ex vivo* stimulation of *G. mellonella* hemocytes using known inducers of NET release; and (2) the role of extracellular DNA *in vivo* in *G. mellonella* by monitoring disease severity after intrahemocoelic injection of EPEC in the presence and absence of DNase I, heat-inactivated DNase I, and *G. mellonella*

hemocyte DNA. The results confirm the involvement of *G. mellonella* hemocytes (likely granulocytes and oenocytoids) in DNA release *ex vivo* and demonstrate that extracellular DNA confers protection (increased EPEC clearance rate and prolonged insect survival) to *G. mellonella* against EPEC infection of the hemocoel *in vivo*, providing support to the hypothesis that insect hemocytes release extracellular DNA that protects the insect against microbial infection in the hemocoel.

Chapter 5 concludes this thesis and discusses future research utilizing the *Galleria*-EPEC model system.

Dedication

This thesis is dedicated to my parents Zhiwei Chen and Hong Bao, who sparked my curiosity towards science at a young age and continued to support me in my quest for knowledge.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

AMP	Antimicrobial peptide
βGRP	β-glucan recognition protein
BLAST	Basic local alignment search tool
CFU	Colony-forming unit
DAMP	Damage-associated molecular pattern
DIC	Differential interference contrast
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
Dscam	Down syndrome cell adhesion molecule
EPEC	Enteropathogenic *Escherichia coli*
GBP	Growth-blocking peptide
GFP	Green fluorescent protein
GLM	Generalized linear model
GNBP	Gram-negative bacteria-binding protein
HCl	Hydrochloric acid
IHET	Insect hemocyte extracellular trap
IMD	Immune deficiency
JH	Juvenile hormone
LB	Luria-Bertani
LD_{50}	Median lethal dose
LEE	Locus of enterocyte effacement
LPS	Lipopolysaccharide
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor κB
Nle	non-locus of enterocyte effacement encoded protein
PAMP	Pathogen-associated molecular pattern

PCR	Polymerase chain reaction
PGRP	Peptidoglycan recognition protein
PKC	Protein kinase C
PM	Peritrophic membrane
PMA	Phorbol 12-myristate 13-acetate
PO	Phenoloxidase
PP	Paralytic peptide
PPO	Prophenoloxidase
PRR	Pattern recognition receptor
PSP	Plasmatocyte-spreading peptide
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
T3SS	Type III secretion system
Tir	Translocated intimin receptor

Chapter 1 Introduction to *Galleria mellonella* and enteropathogenic *Escherichia coli*

1.1 Introduction to *G. mellonella* immunity

The greater wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae), is an economically important pest of honey bees worldwide (Kwadha *et al.*, 2017). The larvae feed primarily on honey, pollen, and wax in honey bee hives causing extensive damage to the honey combs and entrap emerging bees with silk in a condition known as galleriasis (Williams, 1997). In the laboratory, the larvae are used as a model organism to study numerous human pathogens including bacteria, fungi, and protozoans (Aperis *et al*., 2007; Fedhila *et al*., 2006; Fuchs *et al*., 2010; Jackson *et al*., 2009; Leuko & Raivio, 2012; Miyata *et al*., 2003; Mukherjee *et al*., 2010; Mylonakis *et al*., 2005; Peleg *et al*., 2009; Seed & Dennis, 2008; Tomiotto-Pellissier *et al*., 2016). Pathogen virulence in *G. mellonella* correlate with murine models, combined with low rearing costs, short generation time, the ability to be reared at 37°C required by many pathogens to synthesize virulence factors, and the lack of ethical constraints make these insects ideal alternative model hosts for infection (Brennan *et al.*, 2002; Jander & Rahme, 2000).

The first line of defense against pathogens in insects are physical barriers that consist of the cuticle and the peritrophic membrane (PM) (F. Liu *et al.*, 2017). The cuticle is the chitinous exoskeleton that covers the external surfaces of insects, including some internal surfaces that are connected to the environment such as the foregut, hindgut, and trachea (Andersen, 1979). The PM is a chitinous matrix that surrounds the food bolus in the midgut of most insects (Lehane, 1997). The cuticle and PM protect the epidermis and midgut epithelium, respectively, from damage and pathogen invasion (Lehane, 1997; F. Liu *et al.*, 2017). After the breach of physical barriers and the invasion of the hemocoel, the pathogen must contend with the insect immune system. The innate immune system of insects is divided into the humoral and the cellular

branches (Michael R. Strand, 2008a). Humoral immune responses include hemolymph coagulation, melanization, antimicrobial peptide (AMP) production, and the production of other hemolymph components such as pattern recognition receptors (PRRs) (Sheehan *et al.*, 2018). Cellular immune responses are mediated by hemocytes and include phagocytosis, nodulation, and encapsulation (Michael R. Strand, 2008b). The hemocytes of lepidopteran insects are classified into five distinct cell types: plasmatocytes, granulocytes, oenocytoids, spherulocytes, and prohemocytes (Price & Ratcliffe, 1974). The morphology and functions of *G. mellonella* hemocytes are summarized in Table 1.1. Insect hemocyte populations are maintained by hematopoiesis and mitotic division of differentiated hemocytes (Michael R. Strand, 2008a). Mitosis occurs in prohemocytes, plasmatocytes, and granulocytes of *G. mellonella* (Shapiro, 1968). However, the process of hematopoiesis in *G. mellonella* is currently unknown (İzzetoğlu, 2012). In the silkworm *Bombyx mori*, the hematopoietic organs are located in the thoracic segments of the larvae near the imaginal discs and contain prohemocytes, oenocytoids, and granulocytes (Akai & Sato, 1971; Ling *et al.*, 2005). Prohemocytes are released from the hematopoietic organs and can subsequently differentiate into all other types of hemocytes (plasmatocytes, granulocytes, and spherulocytes) except oenocytoids *in vitro* (Yamashita & Iwabuchi, 2001). Hematopoiesis in *G. mellonella* may be similar to that of *B. mori* since both are lepidopteran insects.

Table 1.1 Morphology and function of *G. mellonella* hemocytes.

Hemocyte Type	**Morphology** (G. Wu *et al.*, 2016)	**Abundance** (G. Wu *et al.*, 2016)	**Function**
Plasmatocyte	Round or spindle-shaped, 8-10 µm in diameter, eccentric nucleus, high nucleus:cytoplasm (N:C) ratio, adhesive.	~64% of total hemocytes	Phagocytosis (Tojo *et al.*, 2000) Nodulation (Ratcliffe & Gagen, 1977) Encapsulation (Schmit & Ratcliffe, 1977) Wound healing (Rowley & Ratcliffe, 1978)
Granulocyte	Round or oval shaped, 8-12 µm in diameter, eccentric nucleus, low N:C ratio, adhesive, numerous granules in cytoplasm, adhesive	~20% of total hemocytes	Phagocytosis (Tojo *et al.*, 2000) Nodulation (Ratcliffe & Gagen, 1977) Encapsulation (Schmit & Ratcliffe, 1977) Hemolymph coagulation Melanization (Schmit *et al.*, 1977) Wound healing (Rowley & Ratcliffe, 1978)
Oenocytoid	Round shaped, 12-20 µm in diameter, eccentric nucleus, low N:C ratio, opaque and homogeneous cytoplasm	~9% of total hemocytes	Melanization (Schmit *et al.*, 1977) Phagocytosis (G. Wu *et al.*, 2016)
Spherulocyte	Variable shapes, 10-15 µm in diameter, eccentric nucleus, low N:C ratio, cytoplasm filled with large granules	~7% of total hemocytes	Transportation and secretion of cuticular components (Sass *et al.*, 1994)
Prohemocyte	Round or oval shaped, 6-13 µm in diameter, high N:C ratio (Price & Ratcliffe, 1974)	Not observed by Wu *et al.*, (2016), but was observed by Ratcliffe & Rowley, (1974, 1975); Price & Ratcliffe (1974)	Hematopoiesis (Shapiro, 1968)

1.1.1 Pathogen recognition

Insects use cell membrane-bound and secreted pattern recognition receptors (PRRs) to detect pathogen presence in the hemolymph by binding to pathogen-associated molecular patterns (PAMPs) that are unique to microbes, resulting in the activation of downstream immune responses against the invading pathogens. The PRRs identified in lepidopteran insects include peptidoglycan recognition proteins (PGRPs), β-glucan recognition proteins (βGRPs), gram-negative bacteria-binding proteins (GNBPs), hemolins, immulectins, and apolipophorins (Wang *et al.*, 2019; Yu *et al.*, 2002). The properties of these PRRs are summarized in Table 1.2. The biochemical mechanisms of downstream immune response activation remain poorly understood (Wang *et al.*, 2019). The activation of Toll and immune deficiency (IMD) pathways by PGRPs and GNBPs are responsible for AMP production (Gottar *et al.*, 2006; Michel *et al.*, 2001; Neyen *et al.*, 2012). The opsonization of bacteria by hemolins and immulectins and the agglutination of bacteria by immulectins are responsible for the enhancement of phagocytosis (Jung *et al.*, 2019; Ling & Yu, 2006; Yu *et al.*, 1999).

Table 1.2 Properties of PRRs in lepidopteran insects.

PRR	Location	Ligand (PAMPs)	Pathogens recognized	Downstream immune responses
PGRPs	Plasma (Yoshida *et al.*, 1996) Membrane-bound (Werner *et al.*, 2000)	Peptidoglycan (Yoshida *et al.*, 1996)	Bacteria (Yoshida *et al.*, 1996)	AMP production (Iketani & Morishima, 1993) Melanization (Yoshida & Ashida, 1986)
βGRPs	Plasma (Ochiai & Ashida, 1988)	β-1,3-glucans (Ochiai & Ashida, 1988)	Fungi (Ochiai & Ashida, 1988)	Melanization (Ochiai & Ashida, 1988)
GNBPs	Plasma (W. J. Lee *et al*., 1996)	β-1,3-glucans and LPS (Kim *et al.*, 2000)	Fungi and Gram-negative bacteria (Kim *et al.*, 2000)	AMP production (Kim *et al.*, 2000)
Hemolins	Serum (Ladendorff & Kanost, 1990)	LPS (Daffre & Faye, 1997)	Gram-negative bacteria (Daffre & Faye, 1997)	Melanization (Terenius *et al.*, 2007) Phagocytosis and nodulation (Ioannis Eleftherianos *et al*., 2007) Encapsulation (Jung *et al.*, 2019)
Immulectins	Plasma (Koizumi *et al.*, 1997)	LPS (Koizumi *et al.*, 1997)	Gram-negative bacteria (Koizumi *et al.*, 1997)	Melanization (Yu & Kanost, 2000) Phagocytosis (Ling & Yu, 2006) Nodulation (Koizumi *et al.*, 1999) Encapsulation (Yu & Kanost, 2004)
Apolipophorins	Plasma (Kawooyas *et al.*, 1984)	β-1,3-glucans (Zdybicka-Barabas *et al.*, 2012) LPS (Leon *et al.*, 2006) LTA (Halwani *et al.*, 2000)	Fungi (Zdybicka-Barabas *et al.*, 2012) and bacteria (Halwani *et al.*, 2000; Leon *et al.*, 2006)	AMP production (Wen *et al.*, 2016) Melanization (Contreras *et al.*, 2013) Nodulation (Son & Kim, 2011) Encapsulation (Whitten *et al.*, 2004)

1.1.2 Melanization

Melanization is the biosynthesis of melanin by insects as a response to injury and pathogen presence (Nakhleh *et al.*, 2017). Melanization is initiated upon the detection of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) and lead to the release of the zymogen prophenoloxidase (PPO) from hemocytes followed by the activation of a serine protease cascade that converts PPO into the active enzyme phenoloxidase (PO) (Bidla *et al.*, 2009; Ma & Kanost, 2000; Takehana *et al.*, 2002; Yoshida & Ashida, 1986; Yu *et al.*, 1999). Active PO catalyzes the oxidation of hemolymph tyrosine to quinones, which are precursors to melanin (Nakhleh *et al.*, 2017). The oxidative damage by the reactive oxygen species (ROS) and cytotoxic quinones, in addition to the physical isolation of the pathogens by melanin deposition contribute to pathogen killing during melanization (Nappi *et al.*, 1995; Zhao *et al.*, 2007). Melanization is tightly regulated by serine protease inhibitors (serpins) and is only localized around pathogens and wounds due to toxicity to the host insect (Nakhleh *et al.*, 2017; Scherfer *et al.*, 2008). In *G. mellonella*, PPO is found in oenocytoids and granulocytes (Schmit *et al.*, 1977). Melanization synergizes with hemolymph coagulation, nodulation, and encapsulation to enhance pathogen killing (Ratcliffe & Gagen, 1977; Rowley & Ratcliffe, 1978; Schmit & Ratcliffe, 1977). In addition to immunity, melanization contributes to the hardening of the clot after injury and the sclerotization of the cuticle during development (Hiruma & Riddiford, 1988; Rowley & Ratcliffe, 1978).

1.1.3 Hemolymph coagulation

Coagulation of insect hemolymph involves the formation of insoluble clots after injury to limit water loss and prevent pathogen entry, and functions as an immune response to immobilize pathogens (Dushay, 2009). Hemolymph coagulation is initiated by the degranulation of activated

hemocytes that release clotting factors (*e.g.* hemolectin and Eig71Ee in *Drosophila melanogaster*, hemocytin in *B. mori*) into the hemolymph to form a clot through a protease cascade involving transglutaminase and hemolymph clotting factors (*e.g.* fondue in *D. melanogaster*, hemofibrin in *Manduca sexta*, and lipophorin in *Locusta migratoria*) (Dushay, 2009; Gellissen, 1983; Geng & Dunn, 1988; Kotani *et al.*, 1995; Li *et al.*, 2002; Lindgren *et al.*, 2008; Rowley & Ratcliffe, 1976). Subsequently, hemocytes release PPO and cause melanization and hardening of the clot (Rowley & Ratcliffe, 1978). Hemolymph coagulation is a Ca^{2+}-dependent process but the detailed biochemical and activation mechanisms of clot formation remain unclear (Dushay, 2009; Li *et al.*, 2002). The degranulation of granulocytes is responsible for the initiation of hemolymph coagulation in *G. mellonella* (Rowley & Ratcliffe, 1976). After wounding, the underlying fat body physically blocks the wound and forms a melanized clot with coagulated hemolymph to seal the wound (Rowley & Ratcliffe, 1978). Hemolymph coagulation traps bacteria, and extracellular nucleic acids (DNA and RNA) induce hemolymph coagulation (Altincicek *et al.*, 2008; Gagen & Ratcliffe, 1976).

1.1.4 Antimicrobial peptide production

Antimicrobial peptides (AMPs) in insects are short (< 100 amino acid residues) peptides and proteins that disrupt the membrane integrity of microbes (*e.g.* attacins, defensins, and cecropins) or inhibit microbial protein synthesis (*e.g.* attacins and apidaecins) (Carlsson *et al.*, 1998; Cociancich *et al.*, 1993; Engström *et al.*, 1984; Krizsan *et al.*, 2014; Moore *et al.*, 1996; Q. Wu *et al.*, 2018). Different AMPs target different microbes, including bacteria, fungi, protozoans, nematodes, and even viruses (Q. Wu *et al.*, 2018; Yi *et al.*, 2014). The production of AMPs occurs mainly in the fat body and is initiated upon the detection of PAMPs or DAMPs by PRRs and the subsequent activation of the well-characterized Toll and IMD pathways. The

signaling cascades result in the expression of AMPs that target gram-positive bacteria and fungi (Toll), and AMPs that target gram-negative bacteria (IMD) (Hoffmann & Reichhart, 2002). In *G. mellonella*, there may be as many as 18 different AMPs, including galiomycin, gallerimycin, cecropins, gloverin, moricin-like peptides, a heliocin-like peptide and proline-rich peptides (Brown *et al.*, 2009). The expression of AMPs in *G. mellonella* differ depending on the type of pathogen involved, showing some degree of specificity in the immune response (Mak *et al.*, 2010).

1.1.5 Phagocytosis

Phagocytosis is the process of particle engulfment by cells and is responsible for the removal of pathogens and apoptotic cells in metazoans (Stuart & Ezekowitz, 2005). In insects, phagocytosis is initiated when hemocyte surface receptors bind to invading pathogens directly, or indirectly through hemolymph opsonins bound to the pathogen surface (Nazario-Toole & Wu, 2017). Phagocytic hemocytes extend filopodia that surround and engulf pathogens to form phagosomes in the cytoplasm (Ratcliffe & Rowley, 1974). The phagosomes then fuse with lysosomes containing hydrolytic enzymes for pathogen killing (Stuart & Ezekowitz, 2005). Hemocytes undergoing phagocytosis also undergo oxidative burst and produce ROS that kill pathogens (Bergin *et al.*, 2005). Plasmatocytes and granulocytes are the phagocytic hemocytes in *G. mellonella* (Ratcliffe & Rowley, 1974; Tojo *et al.*, 2000). The phagocytic activity of *G. mellonella* hemocytes are similar to that of human neutrophils (Bergin *et al.*, 2005). The hemocytes are able to phagocytize a broad range of pathogens including bacteria, fungi, and protozoans (Banville *et al.*, 2011; Ratcliffe & Rowley, 1974; Tomiotto-Pellissier *et al.*, 2016).

1.1.6 Nodulation and encapsulation

Nodulation is an insect cellular immune response against aggregations of small pathogens such as bacteria and fungi, and functions to isolate and kill these pathogens (Dubovskiy *et al*., 2016; Michael R. Strand, 2008b). A nodule refers to a structure that consists of multiple layers of hemocytes surrounding a melanized mass of bacteria (Ratcliffe & Gagen, 1976). In *G. mellonella*, nodule formation is initiated by the degranulation of granulocytes in the presence of microbes, resulting in localized hemolymph coagulation and melanization that trap and kill the microbes. The resulting clump of granulocytes and coagulum is subsequently surrounded by multiple layers of flattened plasmatocytes to form a mature nodule (Ratcliffe & Gagen, 1976, 1977). The attachment and flattening of plasmatocytes is mediated by plasmatocyte-spreading peptide (PSP), an insect cytokine (Clark *et al.*, 1997).

Encapsulation is an insect cellular immune response against pathogens and parasites such as nematodes and parasitoid wasp eggs that are too large to be phagocytized (Dubovskiy *et al*., 2016; Michael R. Strand, 2008b). A capsule is a cellular sheath composed of multiple layers of hemocytes surrounding a foreign body. The process of encapsulation in *G. mellonella* is very similar to that of nodulation described above. Capsule formation is initiated by the degranulation of granulocytes around the parasite and is followed by the adhesion and flattening of multiple layers of plasmatocytes to the parasite surface (Schmit & Ratcliffe, 1977). The capsule is completed by the formation of a basement membrane-like layer surrounding the capsule, resulting in the termination of plasmatocyte recruitment (C. T. Liu *et al.*, 1998) . Pathogens are killed within nodules and capsules by cytotoxic products of melanization and physical isolation preventing nutrient and gas exchange (Dubovskiy *et al.*, 2016).

1.1.7 Adaptive immunity

Innate immunity is considered non-specific and elicit similar responses against a broad range of pathogens regardless of prior exposure. Insect innate immune system share many common features with vertebrate innate immune system (*e.g.* pathogen recognition by PRRs, pathogen killing by phagocytosis and AMP production, and the use of cytokines to coordinate immune cells) (Müller *et al.*, 2008). Unlike vertebrates, insects are generally believed to lack adaptive immunity, which is defined by the ability to mount specific and enhanced immune responses following prior exposure to a pathogen (Cooper & Eleftherianos, 2017). However, studies have shown that, after initial priming using dead or sub-lethal doses of bacteria, insects were able to survive an otherwise lethal subsequent dose (Ioannis Eleftherianos *et al*., 2006; Pham *et al*., 2007). The immune priming effect can be pathogen-specific: priming with one pathogen confers better resistance to subsequent infection by the same pathogen but not others (Pham *et al.*, 2007; Sadd & Schmid-Hempel, 2006). The priming effect can persist throughout the life of the insect and can even be passed onto the next generation (Dubuffet *et al.*, 2015; López *et al.*, 2014; Pham *et al.*, 2007). The enhanced insect resistance to pathogens after priming is attributed to the increased production of PRRs, AMPs, ROS, and the activation and proliferation of hemocytes (Ioannis Eleftherianos *et al*., 2006; López *et al*., 2014; Mikonranta *et al*., 2014; G. Wu *et al*., 2014). The immune system of *G. mellonella* can be primed with heat-killed pathogens, PAMPs, thermal stress, and physical stress (Browne *et al.*, 2014; G. Wu *et al.*, 2015, 2014). The magnitude and duration of the priming effect increases with priming dose (G. Wu *et al.*, 2015, 2014). In insects, the Down syndrome cell adhesion molecule homolog Dscam is a possible PRR and opsonin with tremendous isoform diversity (*e.g.* 1.8×10^4 potential isoforms can be somatically generated by alternative splicing in *Drosophila melanogaster*)

(Armitage *et al.*, 2015). Different Dscam isoforms expressed by individual hemocytes may differ in binding affinity to different pathogens and can potentially allow insects to recognize and differentiate between pathogens on a detailed level, similar to vertebrate antibodies. Immune memory may even be possible if the expression of specific Dscam isoforms can be maintained (Armitage *et al.*, 2015). The role(s) of Dscam in *G. mellonella* immunity is currently unknown and could be a topic of future research in insect adaptive immunity.

1.2 Introduction to enteropathogenic *Escherichia coli*

Escherichia coli is a Gram-negative, rod-shaped bacterium in the family Enterobacteriaceae. The six major pathotypes of diarrheagenic *E. coli* are enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), Shiga toxin-producing *E. coli* (STEC), and diffusely adherent *E. coli* (DAEC). The Kauffmann-White classification scheme using oligosaccharide (O) and flagellar (H) surface antigens are utilized in the serotyping of *E. coli* (*e.g.* O157:H7 is a serotype of STEC that causes hemorrhagic diarrhea in humans). EPEC differs from other pathotypes by displaying attaching and effacing (A/E) properties and lacking Shiga toxins. EPEC causes diarrhea in humans, especially in children of developing countries. The only known reservoir host of EPEC are humans and it is transmitted through the ingestion of contaminated material (fecal-oral). EPEC causes disease in the small intestine of the host and the colonization process is initiated by the attachment of EPEC to enterocytes by bundle-forming pili. The injection of effectors by EPEC into the enterocyte through a type III secretion system (T3SS) results in the destruction (effacement) of the microvilli and the formation of the characteristic actin pedestals on the apical surface of the enterocyte by cytoskeletal rearrangement. Diarrhea is likely caused by a

combination of EPEC effectors that inhibit intestinal fluid uptake, alter water and ion transport, and increase intestinal permeability (Croxen *et al.*, 2013).

1.3 *G. mellonella* as a model host for EPEC

Currently, the mouse (*Mus musculus*) is the model host used for *in vivo* studies of EPEC (Dupont *et al.*, 2016; Rhee *et al.*, 2011; Shifflett *et al.*, 2005). Mice are anatomically, physiologically, and genetically similar to humans, making them ideal animal models to study human pathogens and diseases (Vandamme, 2015). However, studies using mice are constrained by the high costs of maintenance, low sample sizes, and ethical concerns compared to invertebrate models (Pereira *et al.*, 2018). These constraints can be alleviated by the use of insect models such as *G. mellonella*, in which pathogens show similar virulence as murine models (Brennan *et al.*, 2002; Jander & Rahme, 2000). The use of *G. mellonella* as an alternative model host to study EPEC was first suggested and demonstrated by Leuko and Raivio (2012). In this study, *G. mellonella* larvae were shown to be susceptible to E2348/69 (serotype O127:H6), the prototypical EPEC strain, in a dose-dependent manner after intrahemocoelic injection. EPEC induces melanization, nodulation, and the expression of the AMPs cecropin and gloverin in *G. mellonella*. A mutant strain of EPEC without functional T3SS was approximately 100 times less virulent than the wild type, indicating that the secreted effectors are important in EPEC virulence in *G. mellonella*. The Cpx envelope stress response, that protect EPEC from envelope protein misfolding, is also implicated in EPEC virulence in *G. mellonella*. The activation or inhibition of the Cpx envelope stress response reduce the production of virulence factors in EPEC, resulting in reduced virulence in *G. mellonella*. The mechanisms of EPEC virulence in *G. mellonella* remain unknown. EPEC was not seen attached to *G. mellonella* tissues and the EPEC mutant lacking bundle-forming pili showed similar virulence as the wild-type, indicating that EPEC attachment

to host cells may not be required for virulence in *G. mellonella* (Leuko & Raivio, 2012). In a recent study, *G. mellonella* primed by the intrahemocoelic injection of probiotic bacteria *Clostridium butyricum* showed protective effects against subsequent intrahemocoelic injections of gastrointestinal pathogens *Salmonella enterica* and *Listeria monocytogenes*, but not EPEC, suggesting the possibility of EPEC resistance to *G. mellonella* immune responses (Scalfaro *et al.*, 2017).

1.4 Objectives of this thesis

G. mellonlella shows great promise as an alternative host model for the study of EPEC. However, very little is known regarding the virulence of EPEC on the insect host and the host immune responses against EPEC, which are essential for understanding this system. This thesis aims to provide important background knowledge on the *Galleria*-EPEC model system for future studies on EPEC virulence and *G. mellonella* immunity using this system. Chapter 2 of this thesis examines: (1) the nature of EPEC virulence in the hemocoel of *G. mellonella* by monitoring insect mortality, survival time, time to pupation, pupal mass, pupal duration, fecundity, and egg hatch rate after injection; (2) the source of EPEC virulence in *G. mellonella* by comparing the intrahemocoelic median lethal dose (LD_{50}) of EPEC to the LD_{50} of an EPEC mutant (Δ*escN*) with disabled T3SS and to the LD_{50} of a benign *E. coli* strain (DH5α); (3) the degree of EPEC virulence in *G. mellonella* by comparing the intrahemocoelic LD_{50} of EPEC to that of a known entomopathogen (*Providencia rettgeri*) and a benign soil bacterium (*Bacillus clausii*); (4) the oral pathogenicity of EPEC in *G. mellonella* by comparing the intrahemocoelic LD_{50} to the *per os* LD_{50} of EPEC; and (5) the virulence of EPEC in *B. mori* and its suitability an alternative insect model for the study of EPEC. Results from this chapter identify useful metrics for measuring EPEC virulence in *G. mellonella* and provide the basis for future research into

EPEC virulence in *G. mellonella* that may result in the identification of novel virulence factors that are important for EPEC pathogenesis in humans. Chapter 3 examines: (1) the immune responses of *G. mellonella* against EPEC *in vivo* by hemolymph examination and larval dissection following intrahemocoelic EPEC injection; and (2) The temporal dynamics of circulating hemocytes, melanized particles, nodules, and EPEC replication/clearance during EPEC infection in *G. mellonella* by quantification using hemocytometer and the plate-count method. Results from this chapter reveal how *G. mellonella* responds to EPEC and the fate of EPEC in *G. mellonella*, both of which are important information for future research using this system. Chapter 4 focuses on the release of extracellular DNA in *G. mellonella*, a novel insect immune response first discovered *in vivo* and described in Chapter 3. Chapter 4 examines: (1) the origin of the extracellular DNA by *ex vivo* stimulation of *G. mellonella* hemocytes using known inducers of NET release; and (2) The role of extracellular DNA *in vivo* in *G. mellonella* by monitoring disease severity after intrahemocoelic injection of EPEC in the presence and absence of DNase I, heat-inactivated DNase I, and *G. mellonella* hemocyte DNA. Results from this chapter support the hypothesis that insect hemocytes release extracellular DNA that protects the insect against microbial infection in the hemocoel, making the *Galleria*-EPEC system a novel model for the study of extracellular DNA release, a novel insect immune response against microbes.

Chapter 2 The virulence and pathogenicity of EPEC in *Galleria mellonella*

2.1 Introduction

The virulence of enteropathogenic *Escherichia coli* (EPEC) in *Galleria mellonella*, reviewed in Chapter 1, is not well understood. To date, EPEC injection into the hemocoel of *G. mellonella* is known to cause insect mortality, which depends on multiple factors including EPEC dose, the type III secretion system (T3SS) function, and the Cpx envelope stress response (Leuko & Raivio, 2012). Nothing is known about the sublethal effects of EPEC to *G. mellonella*, which could be used as metrics for measuring EPEC virulence in addition to mortality. The use of multiple metrics of virulence may help future studies identify virulence factors of EPEC in *G. mellonella* where the use of mortality alone is insufficient. Virulence factors identified using the *Galleria*-EPEC model system could subsequently be examined in the murine model to determine whether such factors are important in EPEC virulence in humans.

The present study aims to increase our understanding of the *Galleria*-EPEC model system by the characterization of EPEC virulence and pathogenicity in *G. mellonella*. The definitions of virulence and pathogenicity proposed by Thomas & Elkinton (2004) are used in this thesis, in which virulence refers to the ability of a pathogen to cause disease in infected hosts whereas pathogenicity includes virulence while also accounting for the infectivity of the pathogen. The nature of EPEC virulence in the hemocoel of *G. mellonella* was determined by monitoring insect mortality, survival time, time to pupation, pupal mass, pupal duration, fecundity, and egg hatch rate after intrahemocoelic injection at various doses of EPEC. EPEC virulence may manifest in the insect as increased mortality, decreased survival time, increased time to pupation, decreased pupal mass, increased pupal duration, decreased fecundity, and decreased hatch rate in a dose-dependent manner. The source of EPEC virulence in *G. mellonella*

was examined by comparing the intrahemocoelic LD_{50} of EPEC to the LD_{50} of an EPEC mutant (Δ*escN*) with disabled T3SS and to the LD_{50} of a benign *E. coli* strain (DH5α). If T3SS function contributes to EPEC virulence in *G. mellonella*, then the LD_{50} of Δ*escN* would be greater than the LD_{50} of the wild type EPEC. If the T3SS is the main or only source of EPEC virulence in *G. mellonella*, then the LD_{50} of Δ*escN* would be similar to the LD_{50} of DH5α. The degree of EPEC virulence in *G. mellonella* was examined by comparing the intrahemocoelic LD_{50} of EPEC to that of a known entomopathogen (*Providencia rettgeri*) and a benign soil bacterium (*Bacillus clausii*). If EPEC is virulent in *G. mellonella*, then the LD_{50} of EPEC would be closer to the LD_{50} of *P. rettgeri*. Conversely, if EPEC is not very virulent in *G. mellonella*, then the LD_{50} of EPEC would instead be closer to the LD_{50} of *B. clausii*. The pathogenicity of EPEC in *G. mellonella* was examined by comparing the intrahemocoelic LD_{50} to the *per os* LD_{50} of EPEC. The *per os* injection was conducted since it represents one of the most common portals of entry for bacterial pathogens in insects in nature as well as the portal of entry for EPEC in humans (Croxen *et al.*, 2013; Tanada & Kaya, 1993b). If EPEC is highly infective *per os* (*i.e.* can efficiently invade the hemocoel of *G. mellonella* through the gut and/or cause severe disease in the gut), then the LD_{50} of EPEC *per os* would be similar to or lower than the intrahemocoelic LD_{50}. Lastly, the virulence of EPEC in *G. mellonella* was compared to its virulence in *Bombyx mori* to determine the suitability of *B. mori*, a well-studied model organism, as an additional insect model to study EPEC pathogenesis. If *B. mori* is a suitable insect model compared to *G. mellonella*, then the LD_{50} of EPEC in *B. mori* should be similar to or lower than that of *G. mellonella*, since an insect model must be susceptible to the pathogen of interest. I found that EPEC-induced disease in *G. mellonella* was dose-dependent and manifested as increased mortality, decreased survival time, delayed pupation, decreased pupal mass, and increased pupal duration. EPEC had moderate

virulence in *G. mellonella* relative to an entomopathogenic bacterium and a soil bacterium but had low oral pathogenicity. The T3SS contributed to EPEC virulence in *G. mellonella* but unknown factors were responsible for most of the virulence, opening additional avenues for future research. A potential insect host, *B. mori*, was evaluated and determined to be inferior to *G. mellonella* for the study of EPEC virulence due to low EPEC susceptibility and low thermotolerance. Overall, this study provided insights into EPEC pathogenesis in *G. mellonella* needed for future research on the subject and identified life history metrics for the evaluation of EPEC virulence in *G. mellonella*.

2.2 Materials and methods

2.2.1 Insect rearing

Galleria mellonella larvae were purchased from Recorp Inc. (Georgetown, Ontario, Canada) and used to establish a laboratory colony. Insects were reared in 20 oz Atlas mason jars kept in a Percival I-41VL incubator at 30°C and 30% RH in total darkness (0L:24D). The larvae were fed *ad libitum* on artificial diet (Appendix 1.1). Last instar larvae approximately 300 mg in mass (Mettler College150 digital precision balance) were used for all experiments described in this thesis.

Bombyx mori eggs were purchased from Recorp Inc. and used to establish a laboratory colony. Once hatched, larvae were reared in 12 oz Solo paper cups in a Percival I-41VL incubator at 27°C and 75% RH on a 16L:8D photoperiod. Once the colony was established, new eggs were stored at 4°C within 48h after laying and egg diapause was later terminated by HCl treatment (Saheb *et al.*, 1990). Larvae were fed *ad libitum* on artificial diet (Appendix 1.2). Fourth (penultimate) instar larvae approximately 300 mg in mass were used for all experiments described in this thesis.

G. mellonella were reared at 30°C to be consistent with Leuko & Raivio (2012) and is within the optimum growth temperature range of 29°C to 33°C (Kwadha *et al.*, 2017). *B. mori* were reared at 27°C, which is within the optimum growth temperatures of 20°C to 28°C (Rahmathulla, 2012). Selection of the optimum temperatures ensures the most robust immune reponses in each of these species. The life cycles and development times of *G. mellonella* and *B. mori* are summarized in Appendix 3.

2.2.2 Bacteria strains, culturing, and quantification

Wild type enteropathogenic *Escherichia coli* (E2348/69 serotype O127:H6, henceforth referred to as simply EPEC) and an EPEC mutant Δ*escN* were obtained from T. L. Raivio (University of Alberta). The Δ*escN* mutant of EPEC lacks the ability to secrete effectors using the T3SS due to the loss of the EscN protein that functions as an ATPase for the T3SS (Andrade *et al.*, 2007). EPEC was transformed with the plasmid pXG-1, enabling the constitutive expression of green fluorescent protein (GFP) for *in situ* visualization by fluorescent microscopy and chloramphenicol resistance for isolation by selective media (J. H. Urban & Vogel, 2007). *E. coli* strain DH5α was obtained from P. D. Batista (University of Alberta). *P. rettgeri* was isolated from *G. mellonella* killed by *Steinernema carpocapsae* which were obtained from G. J. Hilchie (University of Alberta). *B. clausii* was isolated from a *Glossina* sp. homogenate sample from R. H. Gooding (University of Alberta). This bacterium does not kill *Malacosoma disstria* cells *in vitro* and was used to represent a non-pathogenic bacterium (personal observation). Both *P. rettgeri* and *B. clausii* were identified by 16S rRNA gene sequence using the Basic Local Alignment Search Tool (BLAST). DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen). Polymerase chain reaction (PCR) was performed with bacterial universal primers (27F: 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R: 5′-GGTTACCTTGTTACGACTT-3′ from

Integrated DNA Technologies, Inc.) (Lane, 1991). Sanger sequencing was performed by the Molecular Biology Service Unit (MBSU) at the University of Alberta. All bacteria were cultured in Luria-Bertani (LB) medium (Appendix 2.3) at 30°C to match *G. mellonella* rearing temperature. EPEC and *P. rettgeri* were cultured in glass culture tubes (KIMAX, 16 mm x 100 mm) on a shaker (Mistral Multi-Mixer Model 4600, Lab-Line). *B. clausii* was cultured in 50 mL Erlenmeyer flasks (PYREX) agitated by a magnetic stir bar at 200 rpm since *B. clausii* form long chains and grow unevenly in shaken media. Bacteria were quantified by optical density (OD_{600}, Appendix 4.2) using a Spectronic 20+ spectrophotometer pre-injection and by the plate-count method using LB agar post-injection. Log phase bacteria (Appendix 4.1) washed and suspended in insect Ringer's solution (Appendix 2.1, henceforth referred to as simply Ringer's) were used as inoculum for experiments.

2.2.3 Insect injections

A 1 mL glass tuberculin syringe (BD Yale) with a 33-gauge beveled needle mounted on a motorized microapplicator (Model M, ISCO Inc.; Lincoln, Nebraska, USA) was used to inject 5 µL of inoculum into the hemocoel (intrahemocoelic) through the plantar (*G. mellonella*) or the base (*B. mori*) of the left anteriormost proleg of the larva. These sites were disinfected by swabbing (using Kimwipes or cotton swabs soaked with 70% ethanol) immediately prior to injection. Injection sites were selected to minimize bleeding and underlying tissue damage. Alternatively, the inoculum was delivered directly into the midgut of the insect by the careful insertion of a 33-gauge blunt-end needle through the mouth (*per os*) as far as the anterior midgut. All injections were conducted under a stereo microscope at 12x magnification.

G. mellonella larvae were incubated at 30°C post-injection for the remainder of the experiment while *B. mori* larvae were incubated at 30°C for 72h post-injection to be consistent

with *G. mellonella* before returning to 27°C to avoid heat stress in this species. All insects were allowed to feed *ad libitum* on artificial diet post-injection.

2.2.4 EPEC and *G. mellonella* mortality

Various doses of EPEC (suspended in Ringer's) were injected intrahemocoelically (as previously described) into 349 *G. mellonella* larvae (Appendix 5.1). Insect mortality, melanization, survival time, and time to pupation were recorded. Insect mortality was recorded daily until day 20 post-injection at which point all insects had either died or emerged as adults. Larval melanization was evaluated visually at 24h post-injection and classified into two categories based on the severity of melanization (score 0 = no to slight melanization, score 1 = moderate to severe melanization). Survival time was recorded as the number of days an insect survived post-injection. Survival scores were calculated for each insect: $Survival\ Score = \frac{Survival\ Time}{20}$, which were used as proxies for survival time in the analysis to avoid heteroscedasticity. Survival time of insects that died as pupae could not be determined and were not included in the survival time analysis. Time to pupation was recorded as the number of days post-injection until pupation. Larvae were considered dead when no movement was observed after tactile stimulation.

2.2.5 Sublethal effects of EPEC on *G. mellonella*

Known sublethal doses of EPEC were injected intrahemocoelically into 45 *G. mellonella* larvae (Appendix 5.2). Time to pupation, pupal mass, adult eclosion, fecundity, and egg hatch rate were recorded. Pupae were carefully extracted from cocoons using micro scissors and fine tip forceps after the sclerotization (colour change from light yellow to dark brown) of the pupal cuticle. Pupae damaged during extraction were removed from the experiment. Each pupa was sexed and placed into a 1 oz Solo plastic cup. Pupal mass was measured by a digital precision

balance (Mettler College150) and recorded within 24h post-pupation to minimize the effects of water loss. Pupal duration was calculated as the time difference between pupation and eclosion (adult emergence) post-injection. Each adult female, within 24h after eclosion, was transferred into a 1 oz Solo plastic cup with an untreated adult male and incubated at 30°C. A thin film of artificial diet was smeared onto the inner surface of each cup to facilitate oviposition. The number of eggs in each cup was recorded after the death of the female. 20 eggs were randomly collected from each cup and placed into 2 mL microfuge tubes at 30°C to monitor hatching.

2.2.6 Route of infection and EPEC pathogenicity

Various doses of EPEC were injected *per os* into the midgut of 70 *G. mellonella* larvae (Appendix 5.3). Insect mortality, survival time, time to pupation, pupal mass, fecundity, and egg hatch rate were recorded as previously described.

2.2.7 EPEC virulence compared to other bacteria

Various doses of *E. coli* (Δ*escN*, n = 110; DH5α, n = 85), *P. rettgeri* (n = 50), and *B. clausii* (n = 55) were injected intrahemocoelically into *G. mellonella* larvae (Appendix 5.4). Insect mortality was recorded.

2.2.8 Virulence of EPEC in a different insect species

Various doses of EPEC were injected intrahemocoelically into 169 *B. mori* larvae (Appendix 5.5). Insect mortality was recorded.

2.2.9 Statistical analyses

Statistical analyses in this chapter were conducted using R (R Core Team, 2019). Generalized linear models (GLMs) were constructed to determine the relationships between bacteria dose and insect survival time, time to pupation, pupal mass, pupal duration, fecundity,

and egg hatch rate. The GLM family used in each model was determined by the type of data: Gaussian family for continuous data such as pupal mass, Poisson family for count data such as time to pupation, and binomial family for proportion data such as egg hatch rate. Overdispersion (dispersion parameter > 2.0) and underdispersion (dispersion parameter < 0.5) were accounted for by using quasi-families. Model assumptions (*i.e.* absence of heteroscedasticity and non-normality of errors) were checked graphically. The minimum adequate models were obtained by stepwise deletion of non-significant factors and interactions when applicable. The median lethal dose (LD_{50}) was defined in the context of this thesis as the number of bacteria injected that would kill 50% of the insects by the end of the experiment. The LD_{50} values of each bacteria species and strain were determined by binomial or quasi-binomial GLMs using the probit link function. Model comparisons were conducted using F tests when dispersion parameters were estimated or χ^2 tests (deviance change) when dispersion parameters were fixed. Model coefficients (β) were used to infer relationships (*i.e.* positive or negative) between variables.

2.3 Results

2.3.1 EPEC and *G. mellonella* mortality

The intrahemocoelic LD_{50} of EPEC in *G. mellonella* larvae is $1.58 \times 10^4 \pm 1.26 \times 10^3$ CFU (± 95% CI) (Figure 2.1). No mortality was observed in insects injected with $\leq 5.0 \times 10^3$ CFU. EPEC dose was a significant predictor of insect mortality (Binomial GLM, deviance = 301, df = 1 and 347, $p < 0.0001$), melanization (Quasi-binomial GLM, $F = 821$, df = 1 and 347, $p < 0.0001$), survival score (Quasi-binomial GLM, $F = 670$, df = 1 and 342, $p < 0.0001$), and time to pupation (Quasi-Poisson GLM, $F = 188$, df = 1 and 219, $p < 0.0001$). Increase in EPEC dose was associated with increase in mortality ($\beta = 1.79 \times 10^{-4}$, $p < 0.0001$), increase in melanization ($\beta = 4.16 \times 10^{-4}$, $p < 0.0001$), decrease in survival score ($\beta = -2.51 \times 10^{-4}$, $p < 0.0001$), and

increase in time to pupation ($\beta = 3.13 \times 10^{-5}$, $p < 0.0001$) (Figures 2.1 and 2.2). Melanization was a significant predictor of insect mortality (Binomial GLM, deviance = 339, df = 1 and 347, $p < 0.0001$), survival score (Quasi-binomial GLM, F = 551, df = 1 and 342, $p < 0.0001$), and time to pupation (Poisson GLM, deviance = 12.8, df = 1 and 219, p = 0.0004). No signs of melanization were observed in control larvae injected with Ringer's. Larvae that displayed moderate to severe melanization (Figure 2.3c and 2.3d) showed significantly higher mortality ($z = 13.6$, $p < 0.0001$), lower survival score ($t = - 9.44$, $p < 0.0001$), and longer time to pupation ($z = 3.77$, $p = 0.0002$) compared to insects that showed slight to no melanization (Figure 2.3a and 2.3b) (Figure 2.5). Insect mortality occurred at either the larval stage (96.1%) or the pupal stage (3.9%). EPEC dose was not a significant predictor of whether insect mortality occurs during the larval stage or the pupal stage (Binomial GLM, deviance = 2.23, df = 1 and 127, p = 0.14). Insects that died within 24h post-injection showed massive EPEC presence in the hemolymph (personal observation). Moribund larvae cease to feed (anorexia), show minimal movement (lethargy), gradually shrink over time (brachytosis), and can remain alive for up to 20 days post-injection before death eventually occurs. Black feces (frass) and diarrhea were observed from moderately to severely melanized larvae by 24h post-injection (Figure 2.4a). Larvae injected with Ringer's produced normal brown frass without any diarrhea (Figure 2.4b). Careful dissection of dead pupae (usually shriveled and slightly deformed) revealed fully developed adults under the pupal cuticle (pharate). Adults that successfully eclosed from pupae appear normal in all treatments.

2.3.2 Sublethal effects of EPEC on *G. mellonella*

EPEC dose was a significant predictor of time to pupation (Quasi-Poisson GLM, F = 17.8, df = 1 and 37, p = 0.0002). Increase in EPEC dose was associated with increase in time to pupation in *G. mellonella* regardless of sex ($\beta = 5.20 \times 10^{-5}$, p = 0.0001) (Figure 2.6a). EPEC

dose and insect sex were significant predictors of pupal mass (Gaussian GLM; $F = 5.99$, $df = 1$ and 36, $p = 0.02$; $F = 27.8$, $df = 1$ and 36, $p < 0.0001$; respectively). EPEC dose did not affect the sexes differently ($F = 0.119$, $df = 1$ and 35, $p = 0.73$). Pupal mass decreased as EPEC dose increased ($\beta = -8.24 \times 10^{-3}$, $p = 0.02$), with female pupae being 55.1 mg more massive on average than male pupae ($t = 5.27$, $p < 0.0001$) (Figure 2.6b). EPEC dose and insect sex were significant predictor of pupal duration (Quasi-Poisson GLM; $F = 32.0$, $df = 1$ and 36, $p < 0.0001$; $F = 18.4$, $df = 1$ and 36, $p = 0.0001$; respectively). EPEC dose did not affect the sexes differently ($F = 0.234$, $df = 1$ and 35, $p = 0.63$). Pupal duration increased as EPEC dose increased ($\beta = 6.34 \times 10^{-5}$, $p < 0.0001$), with female pupae taking 1.15 days longer on average than male pupae to complete metamorphosis ($t = 4.23$, $p = 0.0002$) (Figure 2.6c). EPEC dose was not a significant predictor of fecundity (Gaussian GLM, $F = 0.459$, $df = 1$ and 28, $p = 0.50$) or egg hatch rate (Quasi-binomial GLM, $F = 1.30 \times 10^{-3}$, $df = 1$ and 23, $p = 0.97$). Pupal mass was not a significant predictor of fecundity (Gaussian GLM, $F = 0.433$, $df = 1$ and 28, $p = 0.52$) or egg hatch rate (Quasi-binomial GLM, $F = 1.97$, $df = 1$ and 23, $p = 0.17$).

2.3.3 Route of infection and EPEC pathogenicity

The *per os* LD_{50} of EPEC in *G. mellonella* larvae was greater than 2.50×10^7 CFU (5% mortality at this dose, $n = 20$), which was more than 3 orders of magnitude higher than the intrahemocoelic LD_{50} ($1.58 \times 10^4 \pm 1.26 \times 10^3$ CFU) (Table 2.1). EPEC dose was not a significant predictor of insect survival score (Quasibinomial GLM, $F = 1.38$, $df = 1$ and 68, $p = 0.24$), time to pupation (Poisson GLM, deviance = 0.546, $df = 1$ and 66, $p = 0.46$), pupal mass (Gaussian GLM, $F = 0.226$, $df = 1$ and 63, $p = 0.64$), pupal duration (Quasi-Poisson GLM, $F = 9.88 \times 10^{-2}$, $df = 1$ and 63, $p = 0.75$), and fecundity (Gaussian GLM, $F = 0.677$, $df = 1$ and 45, $p = 0.42$). Female pupae were 37.7 mg larger than male pupae on average ($t = 3.83$, $p = 0.0003$)

and took 1.26 days on average longer to complete metamorphosis ($t = 4.28$, $p < 0.0001$). EPEC dose was a significant predictor of egg hatch rate (Quasi-binomial GLM, $F = 5.57$, $df = 1$ and 41, $p = 0.02$). EPEC dose was negatively associated with egg hatch rate ($\beta = -6.94 \times 10^{-8}$, $p = 0.02$) (Figure 2.7). Pupal mass was not a significant predictor of fecundity (Gaussian GLM, $F = 1.43$, $df = 1$ and 45, $p = 0.24$) or egg hatch rate (Quasi-binomial GLM, $F = 1.93$, $df = 1$ and 41, $p = 0.17$). No abnormal frass or diarrhea were observed post-injection.

2.3.4 EPEC virulence compared to other bacteria

The LD_{50} of EPEC was approximately 3 times lower than the LD_{50} of the Δ*escN* mutant in *G. mellonella* and more than 3 orders of magnitude lower than the LD_{50} of the DH5α strain (Table 2.1). The LD_{50} of EPEC was approximately 9 times higher than the LD_{50} of *P. rettgeri* and more than 2 orders of magnitude lower than the LD_{50} of *B. clausii* (Table 2.1).

2.3.5 Virulence of EPEC in a different insect species

The intrahemocoelic LD_{50} of EPEC in *B. mori* larvae was $3.89 \times 10^6 \pm 8.32 \times 10^5$ CFU (± 95% CI), which was more than 2 orders of magnitude higher than the LD_{50} in *G. mellonella* ($1.58 \times 10^4 \pm 1.26 \times 10^3$ CFU) (Table 2.1).

2.4 Discussion

2.4.1 EPEC and *G. mellonella* mortality

The dose-dependent increase in insect mortality (Figure 2.1) and decrease in survival time (Figure 2.2a) following EPEC injection demonstrates that EPEC causes disease in *G. mellonella* larvae when injected into the hemocoel. Ringer's alone did not kill any insects (Figure 2.1), indicating that injection trauma and blank inoculum do not cause insect mortality. The LD_{50} (1.58×10^4 CFU) of EPEC in *G. mellonella* larvae in this experiment at day 20 post-injection versus the LD_{50} (2.57×10^3 CFU) at 48 h post-injection determined by Leuko & Raivio

(2012) is likely due to the difference in the time of observation and differences in the physiological state of the larvae used in these experiments. Leuko & Raivio (2012) obtained the larvae directly from Recorp Inc., stored them at 4°C without diet for ≤ 7 days pre-injection and incubated them without diet post-injection. Cold shock (at 12°C or 4°C) of *G. mellonella* larvae pre-injection is known to increase resistance to intrahemocoelically injected *Bacillus thuringiensis* and *Candida albicans* by increasing both AMP expression and the number of circulating hemocytes (Mowlds & Kavanagh, 2008; Wojda *et al.*, 2014). Starvation, however, may reduce *G. mellonella* resistance to *C. albicans* by reducing AMP expression and the number of circulating hemocytes (Banville *et al.*, 2012). Though it is currently unknown how *G. mellonella* would respond to the combination of cold shock and starvation when faced with an immune challenge, the results obtained under such conditions would not be comparable to those obtained under optimal conditions in this study. The short observation time of 48h used by Leuko & Raivio (2012) to determine the LD_{50} would significantly underestimate true insect mortality by overlooking mortality that occurs at later times. Therefore, it is important to establish and follow a standardized protocol for the rearing and handling of *G. mellonella* as host for the evaluation of pathogens with the ability to compare results obtained from different laboratories. Recent reviews have identified and discussed this issue in more detail (Champion *et al.*, 2018; Cook & McArthur, 2013; Tsai *et al.*, 2016).

In this study, diseased larvae showed melanization (Figure 2.2c and 2.3), reduced survival time (Figure 2.2a), and delayed pupation (Figure 2.2b). Symptoms of moribund larvae included anorexia, lethargy, brachytosis, abnormal frass production, and diarrhea. The signs and symptoms of disease are likely due to a combination of EPEC replication and effector secretion in the hemolymph (septicemia) and collateral damage by the insect immune responses

(immunopathology). The mechanism of EPEC pathogenesis in *G. mellonella* is currently unknown but previous research suggested that EPEC virulence in the hemolymph is reduced by the inactivation of the T3SS and both inactivation and constitutive activation of the Cpx envelope stress response (Leuko & Raivio, 2012). In this study, melanization (Figure 2.3) was the first visible sign of an immune response against EPEC post-injection, indicating the activation of the PPO cascade. Melanization first occurred at the dorsal vessel, around which melanin can be seen through the cuticle (Figure 2.3b and 2.3c). This was hypothesized to be the result of melanized particle accumulation around the ostia of the dorsal vessel by hemolymph movement and subsequent phagocytosis by sessile periostial hemocytes (Sigle & Hillyer, 2016). Severity of melanization was dose-dependent (Figure 2.2c), indicating increasing activation of the PPO cascade with increasing EPEC presence in the hemocoel. Larval melanization could be used as a visual indicator for assessing EPEC virulence, since the degree of melanization was positively associated with mortality, negatively associated with survival time, and positively associated with time to pupation. Scoring of larval melanization, motor activity, cocoon formation, and insect survival have been used previously to construct a health index for *G. mellonella* to assess the virulence of group A *Streptococcus* (Loh *et al.*, 2013). A similar protocol could be used for EPEC and other pathogens in future studies. EPEC doses $\leq 5.0 \times 10^3$ CFU failed to kill any insects (Figure 2.1), indicating that *G. mellonella* immune responses were able to effectively control EPEC at lower doses. Activation of the PPO cascade produce ROS and cytotoxic quinones that damage the Malpighian tubules in *Tenebrio molitor* (Sadd & Siva-Jothy, 2006). Malpighian tubules are responsible for nitrogenous waste excretion and osmoregulation in insects, functionally analogous to the vertebrate kidney. Damage to the Malpighian tubules and/or the rectal complex (*i.e.* sites of water reabsorption) could potentially lead to excess water

excretion, resulting in diarrhea that were observed in moribund insects (Figure 2.4). Oxidative stress was known to increase mortality and development time in *G. mellonella* (Hyršl *et al.*, 2007). The dose-dependent increase in time to pupation (Figure 2.2b) could also be attributed, in part, to the immunopathology of melanization and indicate the presence of sublethal effects of EPEC on *G. mellonella* life history traits. Pupal mortality was not EPEC dose-dependent overall, but no pupal mortality was observed in control insects that were not injected with EPEC, implicating EPEC in pupal mortality. The proximate cause of pupal mortality could be reduced resistance to desiccation, since pharate adults can have difficulties eclosing from desiccated pupae (Tanada & Kaya, 1993a). Alternatively, it is possible that eclosion behaviors failed to initiate altogether, which could indicate the disruption of the insect endocrine system (*e.g.* insufficient levels of ecdysis triggering hormone and eclosion hormone) (Truman, 1978; Zitnan *et al.*, 1996). How EPEC presence is linked to pupal mortality remains unknown. Future experiments that examine pupal desiccation resistance and hormone levels in relation to EPEC injection could help unravel this mystery.

2.4.2 Sublethal effects of EPEC on *G. mellonella*

Increased time to pupation and reduced pupal mass were EPEC dose-dependent (Figure 2.6a and 2.6b), indicating that EPEC interfered with normal development in *G. mellonella*. This was likely mediated by insect cytokines of the ENF family, including plasmatocyte-spreading peptide (PSP), growth-blocking peptide (GBP), and paralytic peptide (PP), of which PSP and GBP are known to stimulate plasmatocyte-spreading, reduce larval growth rate, and induce temporary paralysis in *Pseudoplusia includens* and *Pseudaletia separata* (M. R. Strand *et al*., 2000). In *M. sexta* challenged with bacteria, a PSP precursor pro-PSP is expressed in the fat body and released into the hemolymph where cleavage by a protease produces the active PSP (I.

Eleftherianos *et al*., 2009). In *P. separata*, GBP inhibits juvenile hormone (JH) esterase activity in the hemolymph which delays larval development by reducing larval feeding and weight gain (Aizawa *et al.*, 2001; Hayakawa, 1990, 1991). It is likely that PSP and GBP share similar mechanisms of reducing larval growth and delaying development due to the structural and functional similarities between PSP and GBP (M. R. Strand *et al*., 2000). The lysis of oenocytoids are also induced by GBP, releasing stored PPO for melanization (Matsumoto *et al.*, 2003). As mentioned previously, the oxidative stress of melanization may also contribute to the developmental delay in *G. mellonella* (Hyršl *et al.*, 2007). An unidentified plasmatocyte depletion factor, likely an ENF cytokine, was found to be released from *G. mellonella* hemocytes following bacterial challenge in a dose-dependent manner (Chain & Anderson, 1983a). Future qPCR and RNAi experiments could determine the presence and roles of ENF cytokines in *G. mellonella* immunity. Cessation of feeding observed in moribund *G. mellonella* larvae injected with EPEC in the previous experiment indicates the possibility of a dose-dependent reduction in feeding of diseased larvae, though future experiments quantifying diet consumption is required to confirm this. Illness-induced anorexia is a common behavioral response to infection in insects (Adamo *et al.*, 2007). It was hypothesized to enhance immune responses in diseased insects since reduced lipid intake decreases hemolymph lipid concentration, which increases available apolipophorin III for pathogen recognition (Adamo *et al.*, 2010). In general, these results were consistent with a recent study, in which the recovery from *Serratia marcescens* infection increased larval development time, decreased pupal mass, and decreased adult eclosion rate in *Spodoptera frugiperda* (Zhang *et al.*, 2018). Thus, growth inhibition may be a common sublethal effect to bacterial infections in lepidopteran insects.

Complete metamorphosis in insects is a complex process involving extensive histolysis of larval tissue, histogenesis of adult tissue, and larval tissue remodeling into adult tissue (Rolff *et al.*, 2019). Melanin formed during melanization was not removed from the hemocoel post-infection and remain for the lifetime of the insect (Hillyer, 2016). Metamorphosis may be obstructed by the remnants of the immune response such as the presence of melanized nodules, capsules, and coagula, resulting in increased pupal duration. The injection of latex beads, which will be encapsulated and melanized in the hemocoel, could determine the effects of physical obstructions on metamorphosis.

In *G. mellonella*, fecundity and egg hatch rate are independent of EPEC dose (when injected intrahemocoelically) or pupal mass, indicating that resource allocation into reproduction takes higher priority than growth in order to maintain fitness. The results were surprising since positive correlations between the size of the female insect and fecundity has been the norm in insects (Honěk, 1993). Reduction in fecundity and egg hatch rate may manifest at higher doses of EPEC when resource allocation and *ad libitum* feeding can no longer compensate for it, which can be examined in future experiments with no artificial diet available to the larvae post-injection. Egg size was not measured in this experiment. It is also possible that the egg size was reduced while fecundity and hatch rate were maintained, though no such differences were noticed visually. The effect of EPEC on *G. mellonella* adult longevity was not examined in this experiment. It is possible that oxidative damage from melanization as a response to EPEC infection could result in reduced adult longevity.

EPEC does not affect the sexes differently. Males completed metamorphosis faster than females on average (protandry) (Figure 2.6c). Protandry is common in holometabolous insects and was hypothesized to increase male fitness by increasing potential mating opportunities

(Morbey & Ydenberg, 2001). Female pupae were larger than male pupae on average (Figure 2.6b). This female-biased sexual size dimorphism is known in *G. mellonella* (Kwadha *et al.*, 2017).

2.4.3 Route of infection and EPEC pathogenicity

The K-12 strain of *E. coli* could invade the hemocoel of *G. mellonella* from the gut, though the mechanism of the invasion is unknown (Freitak *et al.*, 2014). A previous experiment determined that EPEC could also invade the hemocoel of *G. mellonella* larvae from the gut (translocation) without compromising gut integrity (unpublished data). However, the translocation of EPEC was not efficient since the *per os* LD_{50} was greater than 10^4 times higher than the intrahemocoelic LD_{50} (Table 2.1). The foregut and hindgut possess a cuticular layer, a substantial barrier to bacterial penetration. The midgut, however, is not as protected by having a more porous peritrophic membrane (PM) which separates the gut lumen from the hemocoel by just a single layer of columnar epithelial cells attached to a basal lamina, making the midgut a likely route of EPEC entry into the hemocoel (Tanada & Kaya, 1993c). The PM and the presence of gut microbiota could potentially hinder EPEC translocation, while the release of AMPs and ROS into the lumen could limit EPEC growth in the midgut (K. Wu *et al*., 2016). The pore diameter of *G. mellonella* PM has not been determined to my knowledge but likely falls between 2 - 36 nm, which are too small for bacteria such as *E. coli* (0.25 - 1 μm in diameter) to pass through unassisted (Lehane, 1997). EPEC may be able to secrete a chitinase (*e.g.* ChiA found in *E. coli* K-12 strain) that can potentially damage the PM and allow EPEC access to the midgut epithelium (Francetic *et al.*, 2000). It is possible, but highly unlikely, that the injection process damaged the PM or the midgut epithelium allowing EPEC entry into the hemocoel despite the blunt needle tip and the care taken to avoid such damage. The mechanism of EPEC translocation

remains unknown and require ultrastructural and histological examination of the PM and the midgut epithelium to determine. The production of normal frass indicates that EPEC did not disrupt gut function when injected *per os*, but a histological examination of the midgut tissue is required to determine any pathological effect of EPEC on the gut epithelium. Insect survival time was not affected by EPEC. Insect growth and development (*i.e.* time to pupation, pupal mass, and pupal duration) were not affected by EPEC when introduced *per os*, in contrast to the previous experiment in which insect growth and development were stunted after intrahemocoelic injection of EPEC, indicate that EPEC is not pathogenic to *G. mellonella* by ingestion. However, survival time was only monitored for 20 days post-injection and did not account for adult longevity, which may be affected by EPEC. Taken together, the results indicate that EPEC must enter the hemocoel to become virulent but could not do so efficiently, which is typical for entomopathogenic members of Enterobacteriaceae in insects (Tanada & Kaya, 1993d). It is also possible that the gut transit time of *G. mellonella* larvae was too short for EPEC to invade the hemocoel or cause significant pathology in the gut. The examination of frass produced post-injection could help determine the fate of EPEC post-injection. Unexpectedly, egg hatch rate was marginally reduced with increasing EPEC dose injected *per os* (Figure 2.7) whereas egg hatch rate was unaffected when EPEC was injected intrahemocoelically in the sublethal experiment discussed above. The cause of reduced egg hatch rate is unknown and require histological examination to determine. How reduced hatch rate is linked to EPEC presence in the gut but not when EPEC was injected into the hemocoel is unknown and may involve gut-specific immune signaling and activation of transgenerational immune priming, which may be costly to eggs (Tetreau *et al.*, 2019). It is also possible that the injection wound suffered by intrahemocoelically injected insects was responsible for altering the physiological state of the insect, resulting in the

maintenance of egg hatch rate. Future experiments measuring *G. mellonella* fecundity, egg hatch rate, egg size, and egg AMP expression after *per os* and intrahemocoelic injection of EPEC with sham injected and untreated controls may be able to unravel this mystery. Pupal mass did not predict fecundity or egg hatch rate, which is consistent with the results from insects injected intrahemocoelically at the sublethal doses of EPEC, indicating that reproduction was prioritized over growth.

2.4.4 EPEC virulence compared to other bacteria

The Δ*escN* mutant of EPEC was approximately 3 times less virulent than the wild type (Table 2.1), indicating that the T3SS contributes to the virulence of EPEC in *G. mellonella*. This was consistent with the results of Leuko & Raivio (2012), who also found the Δ*escN* mutant to be less virulent than the wild type EPEC in *G. mellonella* but did not discuss it in detail. Some of the effectors secreted by the EPEC T3SS inhibit host immune responses. Macrophage phagocytosis is inhibited by EPEC effectors: EspB (*E. coli* secreted protein B), EspF, EspJ, and EspH (Dong *et al.*, 2010; Iizumi *et al.*, 2007; Marchès *et al.*, 2008; Quitard *et al.*, 2006). Phagocytosis is an evolutionarily conserved immune response in insects and vertebrates (Melcarne *et al.*, 2019). Phagocytosis of EPEC by *G. mellonella* hemocytes may similarly be inhibited by these effectors. The nuclear factor κB (NF-κB) transcription factors are inhibited by EPEC effectors: Tir (translocated intimin receptor), NleB (non-locus of enterocyte effacement encoded protein B), NleC, NleD, NleE, NleH in HeLa cells and mice (Baruch *et al.*, 2011; Gao *et al.*, 2009, 2013; Nadler *et al.*, 2010; Pearson *et al.*, 2011; Ruchaud-Sparagano *et al.*, 2011). In insects, the activation of NF-κB is responsible for inducing the expression of AMPs after upstream activation of the Toll and Imd pathways (Valanne *et al.*, 2011). NF-κB in *G. mellonella* may be inhibited by these effectors and result in reduced AMP expression and increased EPEC

survival. The inability of the Δ*escN* mutant to secrete these effectors may be responsible for the reduced virulence observed and can be tested in a future experiment by quantifying circulating bacteria over time and AMP expression post-injection. However, EPEC attachment and injection of effectors into insect cells have not yet been definitively demonstrated to date. Future experiments are required to determine which insect cell types are targeted by EPEC and the specific roles of EPEC effectors in *G. mellonella*.

The DH5α strain of *E. coli* lacks the locus of enterocyte effacement (LEE) that encodes the T3SS and some of its secreted effectors found in EPEC that are necessary for pathogenesis on the intestinal epithelial cells of vertebrates (Chart *et al.*, 2000; Croxen *et al.*, 2013). DH5α was 2.59×10^3 times less virulent in *G. mellonella* than the wild type EPEC and 8.69×10^2 times less virulent than the Δ*escN* mutant of EPEC (Table 2.1), which indicates that there are factors independent of the T3SS that were responsible for most of the virulence in *G. mellonella*. The mechanism behind the low virulence of DH5α compared to the wild type and mutant EPEC in *G. mellonella* remains unknown. DH5α does not express siderophores enterobactin and aerobactin typically found in pathogenic strains of *E. coli* including EPEC, which acquire iron that are essential for bacterial replication from host iron transport and storage molecules (Chart *et al.*, 2000; Law *et al.*, 1992). DH5α is vulnerable to attack by vertebrate complement proteins due to the inability to express long-chained LPS typically found on the surface of pathogenic *E. coli* that reduce the accessibility of the outer membrane (Chart *et al.*, 2000). Insect AMPs also require membrane access for lytic activities (Bulet *et al.*, 1999). It is possible that the lack of siderophores and long-chained LPS reduced the ability of DH5α to replicate and survive in *G. mellonella*, resulting in reduced virulence compared to EPEC. Future experiments could explore the roles of siderophores and long-chained LPS in EPEC virulence in *G. mellonella.*

In *G. mellonella*, EPEC was 8.88 times less virulent than the entomopathogen *P. rettgeri* but more than 2.36×10^2 times more virulent than the soil bacterium *B. clausii* (Table 2.1), indicating that EPEC could be considered a moderately virulent pathogen to *G. mellonella*. However, as the *per os* experiment indicated above, EPEC could not efficiently invade the hemocoel from the gut, making its pathogenicity low and opportunistic.

2.4.5 Virulence of EPEC in a different insect species

Virulence of EPEC in *G. mellonella* was approximately 2.46×10^2 times higher than in *B. mori* (Table 2.1), which is surprising since *B. mori* is generally considered to be very vulnerable to pathogens (Meng *et al.*, 2017). The incubation of *B. mori* larvae at 30°C for 72h post-injection aimed to be consistent with *G. mellonella* incubation temperature and to control for EPEC replication potential and virulence factor expression during this critical period without incurring mortality to *B. mori* due to prolonged heat stress. However, 30 - 35°C are considered non-lethal heat shock temperatures for *B. mori* (Manjunatha *et al.*, 2010). Heat shock is known to increase the expression of AMPs in *G. mellonella* associated with increased resistance to intrahemocoelically injected *B. thuringiensis* (Wojda & Taszłow, 2013). Increase in AMP expression may similarly be induced in *B. mori* post-injection and may contribute to the resistance to EPEC. The relatively high resistance to EPEC and the inability to be continuously reared at ≥ 30°C makes *B. mori* a less suitable host to study EPEC virulence than *G. mellonella*. However, different strains of *B. mori* have different thermotolerances (Joy & Gopinathan, 1995). Unfortunately, the race and strain information on the *B. mori* used in this experiment were unknown to the supplier Recorp. Inc. A more thermotolerant strain of *B. mori* could potentially be used as an additional insect host to *G. mellonella* for the study of EPEC and other human pathogens.

2.5 Conclusions

EPEC causes disease in *G. mellonella* when injected intrahemocoelically. Disease severity is dose-dependent and manifests as increased mortality, decreased survival time, delayed pupation, decreased pupal mass, and increased pupal duration. Disease symptoms can be used as metrics for the measure of EPEC virulence in *G. mellonella* and are likely due to a combination of septicemia and immunopathology. Symptoms were detectable at sublethal doses, possibly mediated by ENF family cytokines responding to EPEC infection. Larval feeding could have compensated for the costs of disease recovery and maintained fecundity and egg hatch rate. EPEC was not pathogenic *per os*, likely due to inefficient translocation from the insect gut to the hemocoel where it is virulent. However, reduced egg hatch rate after *per os* injection was EPEC dose-dependent but the mechanism remains unclear. The T3SS was partially responsible for EPEC virulence in *G. mellonella*, likely through the inhibition of phagocytosis and AMP expression by secreted effectors, allowing increased EPEC survival. There were unknown factors independent of the T3SS in EPEC responsible for most of the virulence, which may include siderophore and long-chained LPS expression. Overall, EPEC has low oral pathogenicity in *G. mellonella* and moderate virulence in the hemocoel, indicating that it cannot efficiently invade the hemocoel from the gut. *B. mori* was not a suitable insect host for EPEC compared to *G. mellonella* due to lower susceptibility to EPEC and lower tolerance to temperatures ≥ 30°C. This study provided insights into EPEC virulence and pathogenesis in *G. mellonella* and identified areas of future research on the subject.

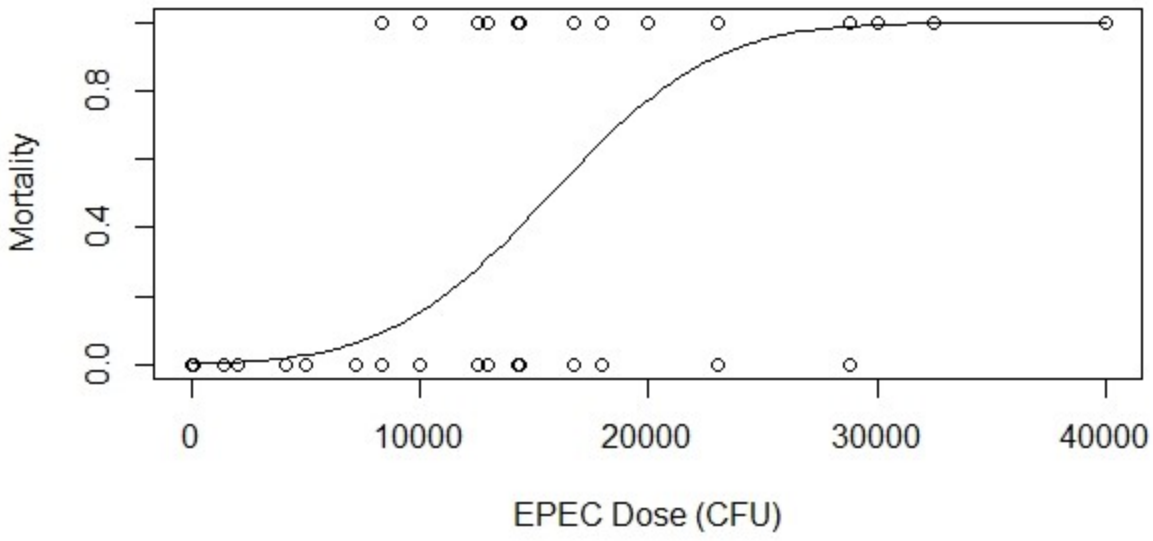

Figure 2.1 Mortality of *G. mellonella* larvae injected intrahemocoelically with various doses of EPEC (1 = dead, 0 = alive, n = 349). EPEC dose was positively associated with insect mortality ($\beta = 1.79 \times 10^{-4}$, $p < 0.0001$) The LD_5, LD_{50}, and LD_{95} (± 95% CI) were $6.56 \times 10^3 \pm 2.09 \times 10^3$ CFU, $1.58 \times 10^4 \pm 1.26 \times 10^3$ CFU, and $2.50 \times 10^4 \pm 2.56 \times 10^3$ CFU, respectively.

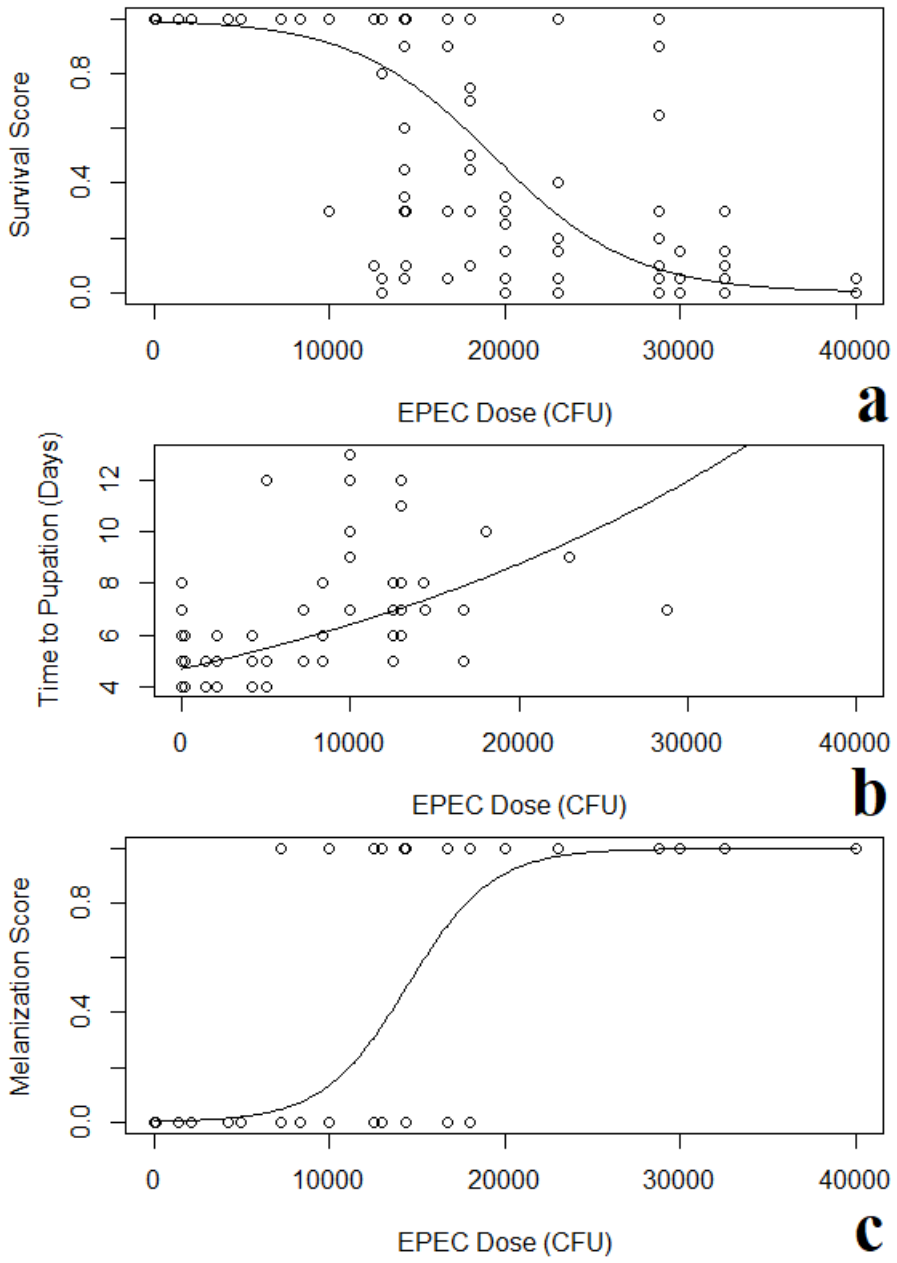

Figure 2.2 Survival score (a), time to pupation (b), and melanization score (c) of *G. mellonella* larvae injected intrahemocoelically with various doses of EPEC (n = 349). (a) EPEC dose was negatively associated with survival score ($\beta = -2.51 \times 10^{-4}$, $p < 0.0001$). (b) EPEC dose was positively associated with time to pupation ($\beta = 3.13 \times 10^{-5}$, $p < 0.0001$). (c) EPEC dose was positively associated with larval melanization ($\beta = 4.16 \times 10^{-4}$, $p < 0.0001$). The dose (± 95% CI) at which 50% of the insects are expected to display moderate to severe melanization is $1.45 \times 10^{4} \pm 1.28 \times 10^{3}$ CFU.

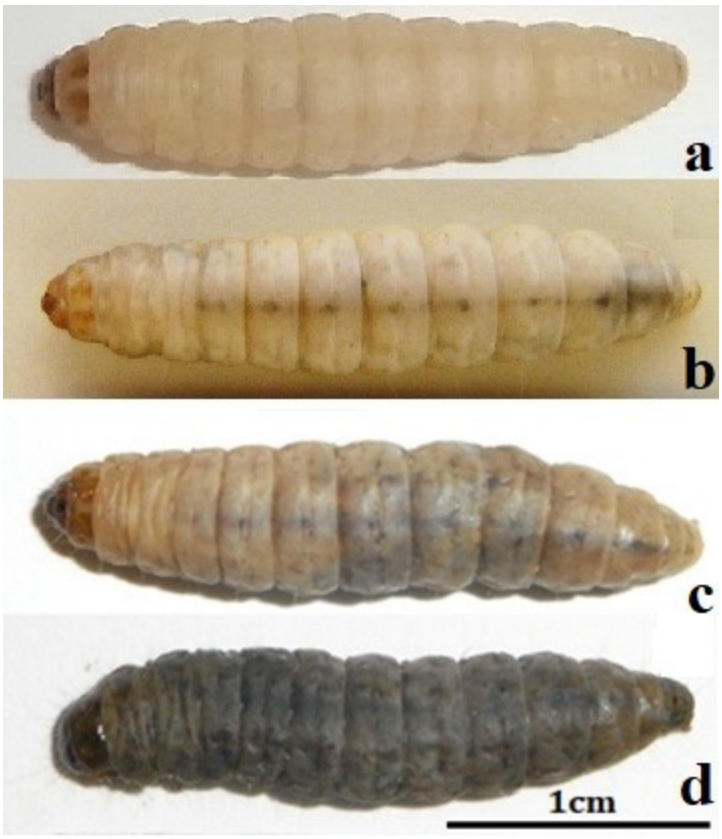

Figure 2.3 Colour patterns of *G. mellonella* larvae at 24h after intrahemocoelic injection. Untreated larvae and control larvae showed no melanization (a). Larvae injected with EPEC showed no (a), slight (b), moderate (c), to severe (d) melanization depending on EPEC dose. Larval appearance was photographed with a Ricoh R10 digital camera.

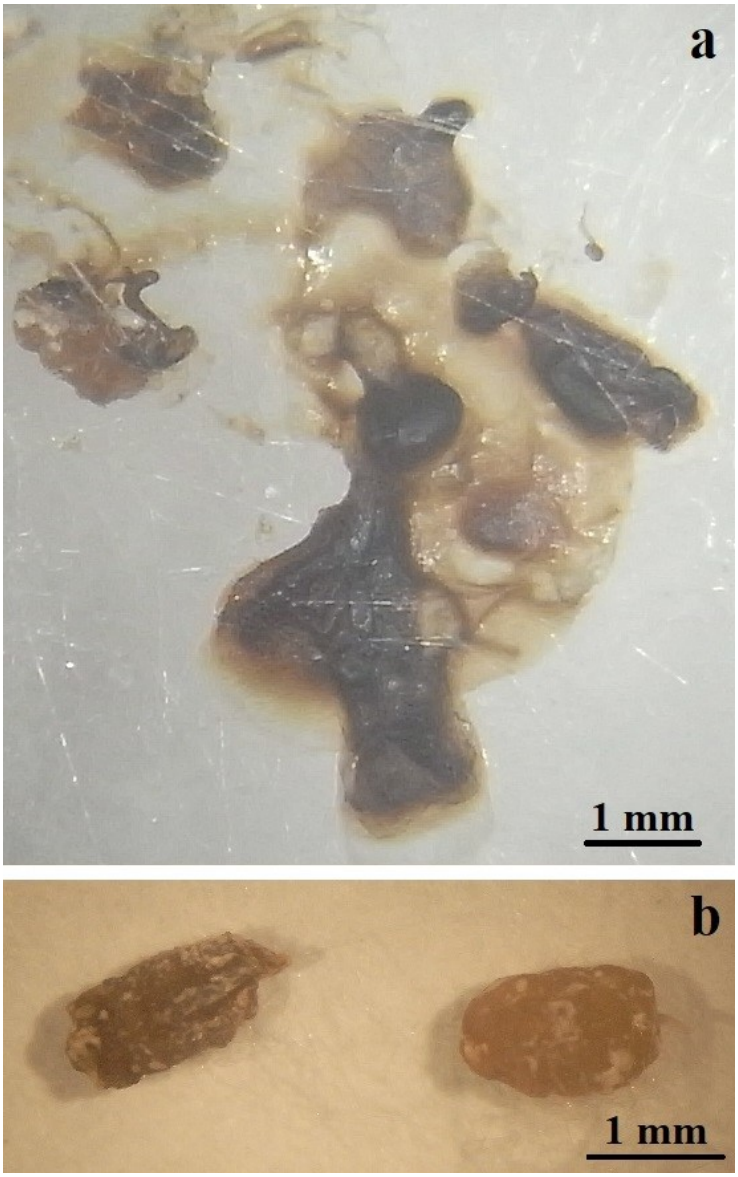

Figure 2.4 Frass produced by *G. mellonella* larvae 24h after intrahemocoelic injection. (a) Black frass and solidified diarrhea produced by larvae injected with EPEC (1.5×10^4 CFU). (b) Normal frass produced by larvae injected with Ringer's.

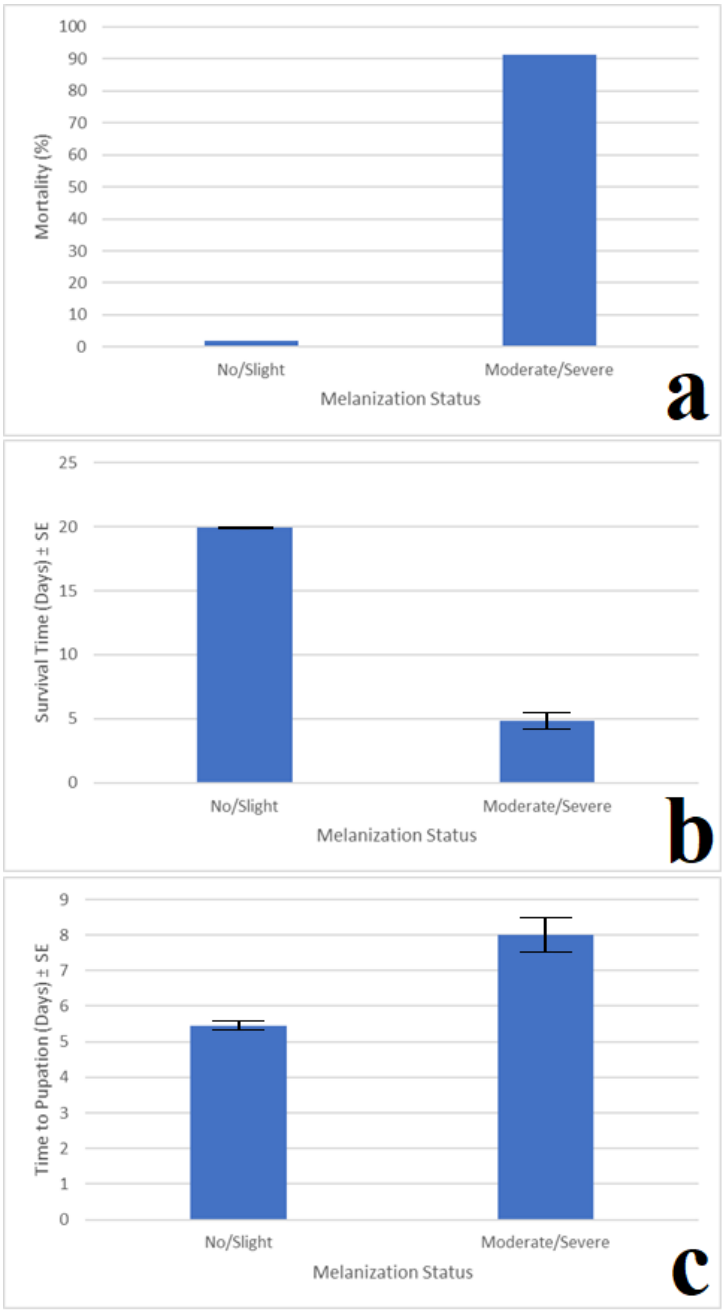

Figure 2.5 Mortality (a), survival time (b), and time to pupation (c) of *G. mellonella* larvae based on melanization at 24h after intrahemocoelic EPEC injection. Larvae that displayed moderate to severe melanization (Figure 2.3c and 2.3d) showed (a) significantly higher mortality (by 89%), (b) lower survival time (by 15 days on average), and (c) longer time to pupation (by 2.6 days on average) compared to insects that showed slight to no melanization (Figure 2.3a and 2.3b).

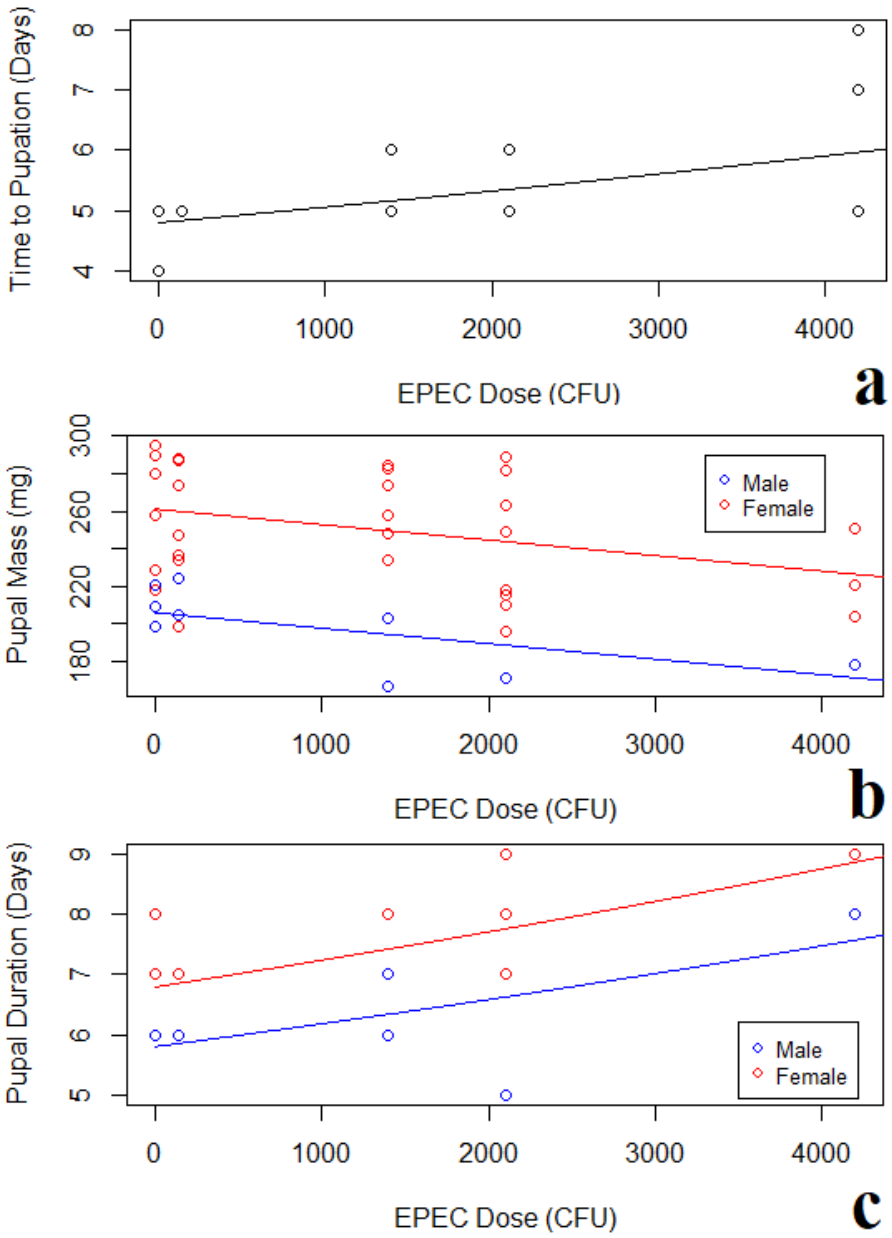

Figure 2.6 Time to pupation (a), pupal mass (b), and pupal duration (c) of *G. mellonella* larvae injected intrahemocoelically with sublethal doses of EPEC (n = 45). (a) Time to pupation post-injection increased as EPEC dose increased ($\beta = 5.20 \times 10^{-5}$, $p = 0.0001$). (b) Pupal mass decreased as EPEC dose increased ($\beta = -8.24 \times 10^{-3}$, $p = 0.02$), with female pupae being 55.1 mg larger on average than male pupae ($t = 5.27$, $p < 0.0001$). (c) Pupal duration increased as EPEC dose increased ($\beta = 6.34 \times 10^{-5}$, $p < 0.0001$), with female pupae taking 1.15 days longer on average than male pupae to complete metamorphosis ($t = 4.23$, $p = 0.0002$).

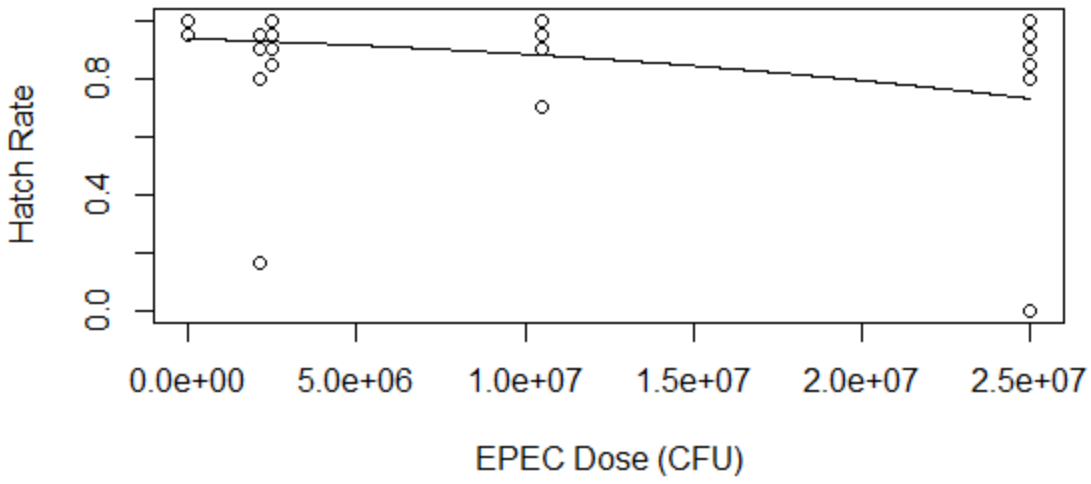

Figure 2.7 Egg hatch rate of *G. mellonella* larvae injected *per os* with various doses of EPEC (n = 70). Increase in EPEC dose was associated with a marginal decrease in egg hatch rate ($\beta = -6.94 \times 10^{-8}$, $p = 0.02$).

Table 2.1 The LD_{50} of different species and strains of bacteria in *G. mellonella* and *B. mori*.

Insect	**Bacteria**	**Route of injection**	**LD_{50} ± 95% CI (CFU)**	**n**
G. mellonella	*E. coli* (EPEC)	Intrahemocoelic	$1.58 \times 10^4 \pm 1.26 \times 10^3$	349
G. mellonella	*E. coli* (Δ*escN*)	Intrahemocoelic	$4.72 \times 10^4 \pm 5.41 \times 10^2$	110
G. mellonella	*E. coli* (DH5α)	Intrahemocoelic	$4.10 \times 10^7 \pm 1.53 \times 10^6$	85
G. mellonella	*P. rettgeri*	Intrahemocoelic	$1.78 \times 10^3 \pm 7.70 \times 10^2$	50
G. mellonella	*B. clausii*	Intrahemocoelic	$3.73 \times 10^6 \pm 1.08 \times 10^6$	55
G. mellonella	*E. coli* (EPEC)	*Per os*	$> 2.50 \times 10^7$	70
B. mori	*E. coli* (EPEC)	Intrahemocoelic	$3.89 \times 10^6 \pm 8.32 \times 10^5$	169

Chapter 3 The immune responses of *Galleria mellonella* against EPEC

3.1 Introduction

The immune responses of *Galleria mellonella* against bacteria typically involve melanization, hemolymph coagulation, AMP production, phagocytosis, and nodulation. These immune responses were reviewed in detail in Chapter 1. The immune responses induced by enteropathogenic *Escherichia coli* (EPEC) in *G. mellonella* are not well characterized, which to date only include melanization, nodulation, and the expression of the AMPs cecropin and gloverin (Leuko & Raivio, 2012). In addition, almost nothing is known about the events occurring inside the insect post-injection with EPEC, which are important in understanding both EPEC pathogenesis and *G. mellonella* immunity.

This study aims to (1) characterize the immune responses of *G. mellonella* against EPEC *in vivo* by hemolymph examination and larval dissection following intrahemocoelic EPEC injection; and (2) determine the temporal dynamics of circulating hemocytes, melanized particles, nodules, and EPEC replication/clearance during EPEC infection in *G. mellonella* by quantification using hemocyometer and the plate-count method following intrahemcoelic EPEC injection. If *G. mellonella* respond to EPEC in the hemocoel by mounting typical insect antibacterial immune responses, then melanization, hemolymph coagulation, phagocytosis, and nodulation should be observed by microscopy in the hemolymph and hemocoel post-injection. If EPEC activates humoral and cellular immune responses in *G. mellonella*, then the number of circulating hemocytes should decrease over time while the number of melanized particles and nodules should increase over time. If *G. mellonella* immune responses are effective in controlling the EPEC infection in the hemocoel, then the number of circulating EPEC should decrease over time. I found that, in addition to the typical insect immune responses (*i.e.*

melanization, hemolymph coagulation, phagocytosis, and nodulation), a novel insect immune response in the form of extracellular DNA release was present after intrahemocoelic EPEC injection in *G. mellonella*. The extracellular DNA immobilized EPEC and appeared to be hemocytic in origin. The origin and function of extracellular DNA in *G. mellonella* will be investigated further in Chapter 4. Hemocytopenia was temporarily induced in *G. mellonella* by EPEC between 3h - 6h post-injection but the circulating hemocyte count recovered by 48h post-injection. The immune responses of *G. mellonella* were unable to control EPEC replication at the LD_{50} in the early stage of infection (*i.e.* within 3h post-injection) but was eventually able to clear EPEC from the hemolymph by 48h post-injection. The clearance of circulating EPEC corresponded to the appearance of melanized particles and nodules, implicating these insect immune responses in EPEC clearance. Interestingly, EPEC clearance did not guarantee insect survival, likely due to irrecoverable damage from EPEC and the immune responses. This study opened a new frontier in insect immunology by the discovery of DNA extracellular traps in an insect *in vivo* and provided important background information on the *Galleria*-EPEC model system for future studies.

3.2 Materials and methods

3.2.1 Characterization of *G. mellonella* immune responses

The LD_{50} of EPEC (approximately 1.5×10^4 CFU) was injected intrahemocoelically into *G. mellonella* larvae as previously described. Larvae injected with Ringer's were used as control. Larvae were surface-sterilized (by immersion: 30s in 70% ethanol → 10s in sterile water → 60s in 10% bleach → 10s in sterile water) immediately prior to hemolymph collection. Hemolymph was collected at 24h post-injection aseptically from 42 insects (28 injected with EPEC, 14 injected with Ringer's) by micropipette after creating a small incision at the base of the right

anteriormost proleg (or, if not available, the left second-anteriormost proleg) with micro scissors and the application of gentle pressure to the insect until a droplet of hemolymph appears. Hemolymph (DNA) was stained with Hoechst 33342 (10 µg/mL, Riedel-de Haën) without fixing to minimize handling, since the stain is cell-permeable. Hemolymph collection and staining were performed aseptically in a biological safety cabinet (Model 1106, Forma). Larvae (25 injected with EPEC, 14 injected with Ringer's) were immersed in Ringer's and dissected at 24h post-injection under a stereo microscope using micro scissors. Melanized coagula attached to tissue surfaces were photographed *in situ* (Ricoh R10), then carefully removed from dissected insects using fine tip forceps (Dumont No.5), immersed in Ringer's, and stained with 10 µg/mL Hoechst 33342. Hemolymph and coagula were visualized by differential interference contrast (DIC) and fluorescent microscopy using a Reichert-Jung Polyvar microscope at 500x magnification and photographed using an Olympus E-420 digital camera.

3.2.2 Quantification of circulating hemocytes, nodules, melanized particles, and EPEC

G. mellonella larvae were injected intrahemocoelically with EPEC (1.5×10^4 CFU, n = 60) and heat-killed EPEC (HK-EPEC, 1.5×10^4 CFU prior to killing, n = 60). Insects injected with Ringer's (n = 60) and sham-injected insects (n = 60) were used as controls. HK-EPEC was prepared by heating the bacteria to 65°C for 30 minutes in a heating block (Isotemp Model 145, Fisher). Cell integrity was verified by DIC microscopy and complete killing was confirmed by plate-count. Sham injection was performed identically to standard intrahemocoelic injections except with no inoculum injected (*i.e.* wounding by needle insertion). Hemolymph was collected aseptically from 8 insects of each treatment, randomly without replacement, at 1h, 3h, 6h, 24h, and 48h post-injection. All insects were alive at the time of hemolymph collection. Hemolymph was immediately diluted ten-fold in an anticoagulant antimelanization solution (Appendix 2)

post-collection to prevent *ex vivo* hemolymph coagulation and melanization. Hemolymph samples from each insect was loaded onto an improved Neubauer hemocytometer to quantify circulating hemocytes, nodules, and melanized particles by brightfield and phase contrast microscopy using an Olympus CX41 microscope at 400x magnification. The proportion of hemocytes in clumps were calculated for each sample:

$Proportion\ of\ hemocytes\ in\ clumps = \frac{Number\ of\ hemocytes\ in\ clumps}{Total\ number\ of\ hemocytes}$ as proxies for hemocyte adhesiveness and activation. Only mature nodules with multiple layers of hemocytes surrounding a melanized mass of bacteria were counted (Ratcliffe & Gagen, 1976). The same hemolymph samples were also used to quantify circulating bacteria by plate-count. The remaining insects (20 of each treatment) were left undisturbed to monitor mortality and development. Hemolymph was collected aseptically from dead insects within 24h post-mortem to quantify circulating EPEC by plate-count on LB agar with 1 mg/mL chloramphenicol (courtesy of N. A. S. Hussain, University of Alberta) to control for potential bacterial contamination from loss of gut integrity. Insect rearing condition, bacteria culture condition, injection protocol, and incubation condition post-injection were as described in Chapter 2.

3.2.3 Statistical analyses

Statistical analyses in this chapter were conducted using R (R Core Team, 2019). GLMs were constructed to examine: (1) differences in hemocyte count and hemocyte clumping between different treatments, time points, and their interactions; (2) differences in melanized particle and bacteria counts between different time points in insects injected with EPEC; and (3) differences in the number of days to pupation post-injection between different treatments. The GLM family used in each model was determined by the type of data (*i.e.* Poisson family for count data and binomial family for proportion data). Overdispersion (dispersion parameter > 2.0) and

underdispersion (dispersion parameter < 0.5) were accounted for by using quasi-families. Model assumptions (*i.e.* absence of heteroscedasticity and non-normality of errors) were checked graphically. The minimum adequate models were obtained by stepwise deletion of non-significant factors and interactions when applicable. Model comparisons were conducted using F tests. Tukey contrasts (pairwise comparisons) were used to determine where significant differences occurred *post hoc* to GLMs.

3.3 Results

3.3.1 Characterization of *G. mellonella* immune responses

Melanization, hemolymph coagulation, nodulation, and phagocytosis were observed in the hemolymph of *G. mellonella* larvae at 24h post-injection with EPEC. The immune responses were absent in the control larvae injected with Ringer's (Figure 3.1a - c). The following were observed in the hemolymph of larvae injected with EPEC: (1) melanized particles (in the hemolymph of 89% of the larvae) free-floating in the hemolymph, internalized in hemocytes, embedded in coagula, and in the center of nodules (Figure 3.1d - g); (2) hemolymph coagulation around melanized particles (Figure 3.1f); (3) nodules containing melanized particles (Figure 3.1g); and (4) hemocytes containing EPEC, indicative of phagocytosis (Figure 3.1h - j). Dissection of larvae at 24h post-injection with EPEC revealed melanized coagula in the hemocoel attached to surfaces of various tissues and organs such as the fat body, trachea, and gut (Figure 3.2d and 3.2e). Melanized coagula were not found in control larvae (Figure 3.2b and 3.2c). Extracellular DNA was found within melanized coagula in 40% of EPEC-injected insects (Figure 3.3d and 3.2e). Extracellular DNA appeared as irregularly shaped intricate networks of intense blue fluorescence after Hoechst staining and were highly variable in size. EPEC was associated with extracellular DNA 90% of the time (Figure 3.3f). Extracellular DNA was

observed in the hemolymph as early as 1h post-injection (personal observation). No melanized coagulum or extracellular DNA were found in the control insects (Figure 3.3a - c). No discernible differences in hemolymph low molecular weight protein profile representing antimicrobial peptides (AMPs) were detected between *G. mellonella* injected with EPEC compared to the control (SDS-PAGE and silver staining of hemolymph protein extracted at 24h post-injection, data not shown).

3.3.2 Quantification of circulating hemocytes, nodules, melanized particles, and EPEC

Statistically significant differences in circulating hemocyte count between treatments (Quasi-Poisson GLM, $F = 14.8$, $df = 3$ and 156, $p < 0.0001$) and between time points ($F = 2.77$, $df = 4$ and 152, $p = 0.03$) were found (Figure 3.4). No differences were detected in hemocyte count between treatments over time (*i.e.* no significant interaction between treatment and time; $F = 1.32$, $df = 12$ and 140, $p = 0.21$). Larvae injected with EPEC showed significantly reduced number of circulating hemocytes compared to larvae injected with either Ringer's, HK-EPEC, or sham-injected larvae (Tukey contrasts, $p \leq 0.0001$ in all comparisons). No significant differences in circulating hemocyte count were detected between larvae injected with Ringer's, HK-EPEC, and sham-injected larvae ($p \geq 0.73$ in all comparisons). A significant difference in circulating hemocyte count was detected between 3h and 6h post-injection overall (Tukey contrasts, $p = 0.05$). No statistically significant differences were found in hemocyte clumping (proportion of hemocytes in clumps) between treatments (Quasi-binomial GLM, $F = 2.13$, $df = 3$ and 156, $p = 0.10$), between time points ($F = 0.920$, $df = 4$ and 152, $p = 0.45$), and between treatments over time ($F = 0.349$, $df = 12$ and 140, $p = 0.98$). No nodules were found in the hemolymph of larvae injected with Ringer's, HK-EPEC, and sham-injected larvae at any time point post-injection. Statistically significant differences in nodule count between time points were found in the

hemolymph of larvae injected with EPEC (Quasi-Poisson GLM, $F = 4.25$, $df = 4$ and 35, $p = 0.007$). Nodules began to appear in the hemolymph at 6h post-injection and were absent from the hemolymph by 48h post-injection (Figure 3.5). No melanized particles were found in the hemolymph of larvae injected with Ringer's and sham-injected larvae. Melanized particles (100/µL hemolymph at 6h post-injection) were found in 1 larva out of the 40 (2.5%) injected with HK-EPEC whereas 19 larvae out of the 40 (47.5%) injected with EPEC were found with melanized particles in the hemolymph. Statistically significant differences in the number of melanized particles between time points were found in the hemolymph of larvae injected with EPEC (Quasi-Poisson GLM, $F = 11.5$, $df = 4$ and 35, $p < 0.0001$). Melanized particles began to appear in the hemolymph at 6h post-injection and were also found at 24h and 48h post-injection (Figure 3.5). Melanization occurred at the injection wound in all insects. No bacteria were found in the hemolymph of larvae injected with Ringer's, HK-EPEC, and sham-injected larvae at any time point post-injection. Statistically significant differences in bacteria count between time points were found in the hemolymph of larvae injected with EPEC (Quasi-Poisson GLM, $F = 14.5$, $df = 4$ and 35, $p < 0.0001$). The number of bacteria increased from 1h to 3h post-injection (Tukey contrasts, $p < 0.0001$), decreased from 3h to 6h post-injection ($p = 0.002$), did not change between 6h to 24h post-injection ($p = 0.99$) and were cleared from the hemolymph by 48h post-injection ($p = 1.0$) (Figure 3.5). The reduction in bacteria count was followed by the appearances of melanized particles and nodules (Figure 3.5). All insects left undisturbed from Sham, Ringer's, and HK-EPEC treatments survived and eclosed as adults whereas 60% mortality (12 out of 20) was observed from the larvae injected with EPEC. EPEC was found in the hemolymph of only 1 larva (died at 48h post-injection with too many CFUs to count) out of the 12 that died. Statistically significant differences in the number of days to pupation between larvae of different

treatments were detected (Quasi-Poisson GLM, $F = 32.8$, $df = 3$ and 64, $p < 0.0001$). Pupation in larvae injected with EPEC were delayed by approximately 4 days on average compared to larvae injected with either Ringer's, HK-EPEC, or sham-injected larvae (Tukey contrasts, $p < 0.0001$ in all comparisons) (Figure 3.6). No statistically significant differences in the number of days to pupation were detected between larvae injected with Ringer's, HK-EPEC, and sham-injected larvae ($p \geq 0.72$ in all comparisons) (Figure 3.6).

3.4 Discussion

3.4.1 Characterization of *G. mellonella* immune responses

Typical insect immune responses against bacteria presence in the hemolymph include melanization, hemolymph coagulation, nodulation, phagocytosis, and AMP production (Wojda, 2017). Aside from AMP production, all of the above immune responses were observed in *G. mellonella* after intrahemocoelic EPEC injection whereas these immune responses were absent in the control larvae injected with Ringer's (Figure 3.1), indicating that *G. mellonella* is capable of recognizing EPEC as foreign and mount appropriate immune responses against it. Unfortunately, no discernable differences in AMP levels were detected by SDS-PAGE in a preliminary experiment, possibly due to interference from the presence of other low molecular weight proteins. The expression of AMPs in *G. mellonella* is known to increase after microbial challenge (including *E. coli*) and the repertoire of expressed AMPs differ depending on the type of microbe involved (Mak *et al.*, 2010). Future experiments using RT-qPCR could examine individual AMP expression specifically and may be able to detect EPEC-induced changes in AMP expression in *G. mellonella*. The melanized particles observed are likely formed by the deposition of melanin around EPEC, though this could not be confirmed since no green

fluorescence could be detected through the melanin. Sectioning of the melanized particles in future experiments are required to determine the presence of EPEC.

Hoechst 33342 staining, originally performed for the *in situ* visualization of hemocyte nuclei, also revealed extracellular DNA within melanized coagula that immobilized EPEC (Figure 3.3d - f). Extracellular DNA induces hemolymph coagulation in *G. mellonella* (Altincicek *et al.*, 2008). In vertebrates, neutrophils release DNA into the extracellular space after activation, termed neutrophil extracellular traps (NETs) that immobilize and kill microbes (Brinkmann *et al.*, 2004). A recent study found that *Periplaneta americana* hemocytes can also release DNA *ex vivo* that can immobilize bacteria (Nascimento *et al.*, 2018). Here it is likely that *G. mellonella* hemocytes released DNA upon activation by EPEC, since hemocytes were photographed in the process of DNA release and no DNA release were found in the control larvae (Figure 3.3b and 3.3e). Oenocytoids lyse upon activation to release PPO and the exposed nuclei may also rupture to release DNA (Altincicek *et al.*, 2008). Granulocytes may lyse upon contact with foreign objects and contribute to DNA release (Pech & Strand, 1996). Hemocyte nuclei were embedded within the coagulum, which may include granulocytes and oenocytoids (Figure 3.3e). The extracellular DNA appear to have trapped EPEC upon contact and induced hemolymph coagulation that also immobilized EPEC in the surrounding area, limiting EPEC spread in the hemocoel. The procoagulant activity of extracellular DNA likely synergizes with the degranulation of granulocytes and the lysis of oenocytoids, inducing hemolymph coagulation and melanization, contributing to the trapping and killing of EPEC. Hemolymph coagulation potentially enhanced EPEC killing by increasing local concentration of ROS and cytotoxic quinones produced by melanization and granule contents (*e.g.* lysozyme) released by the degranulation of granulocytes (Chain & Anderson, 1983b; Nappi *et al.*, 1995). The release of

DNA extracellular traps has not previously been documented *in vivo* in insects to my knowledge and may represent a novel form of insect immune response against microbial pathogens. The origin of the extracellular DNA and its effects *in vivo* will be examined further in Chapter 4.

3.4.2 Quantification of circulating hemocytes, nodules, melanized particles, and EPEC

The reduction in the number of circulating hemocytes (hemocytopenia) was only observed in *G. mellonella* larvae after the injection of viable EPEC, while Ringer's and HK-EPEC did not induce hemocytopenia relative to the sham-injected control (Figure 3.4), indicating that EPEC replication in the hemocoel is required to induce hemocytopenia. Hemocytopenia is a reaction to microbial infection in *G. mellonella* and is likely mediated by ENF family cytokines such as plasmatocyte-spreading peptide (PSP) and growth-blocking peptide (GBP) (Gagen & Ratcliffe, 1976). The cytokines were discussed in Chapter 2 in the context of larval growth inhibition following EPEC challenge. Another role of PSP and GBP in insect immunity is the stimulation of plasmatocyte spreading, which increases plasmatocyte adhesion and enhances phagocytosis, nodulation, and encapsulation (M. R. Strand *et al*., 2000). An unidentified plasmatocyte depletion factor, likely an ENF cytokine, was found to be released from *G. mellonella* hemocytes following bacterial challenge in a dose-dependent manner resulting in plasmatocyte-spreading and reduced number of circulating plasmatocytes (Chain & Anderson, 1983a). Plasmatocytes make up approximately 60 - 70% of the total number of circulating hemocytes in *G. mellonella* (G. Wu *et al*., 2016). Plasmatocyte-spreading mediated by ENF cytokines in response to EPEC replication in the hemolymph was likely responsible for most of the hemocytopenia observed. The sharp decline in hemocyte count between 3 - 6h post-injection with EPEC coincided with the sharp decline in the number of circulating EPEC, suggesting the removal of EPEC from the hemolymph by nodulation (Figure 3.4 and 3.5). Both plasmatocytes

and granulocytes participate in nodulation and most nodules in *G. mellonella* leave circulation and attach to the surfaces of tissues in the hemocoel (Ratcliffe & Gagen, 1976). Plasmatocytes not participating in nodulation could be attached to tissue surfaces in the hemocoel and out of circulation (sessile). Hemolymph coagulation could also contribute to hemocytopenia by immobilizing hemocytes along with EPEC (Figure 3.3d - f). Recovery from hemocytopenia between 6 - 48h post-injection with EPEC coincided with the clearance of EPEC from the hemolymph, indicating the restoration of homeostasis post-infection, likely by sessile plasmatocytes re-entering circulation and the production of new hemocytes (hematopoiesis). The similarity in circulating hemocyte clumping between treatments and between time points suggests that the hemocytes found in circulation were not different in their adhesion. In larvae injected with EPEC, hemocytes remaining in circulation likely represent un-activated plasmatocytes, granulocytes, and non-adhesive hemocytes (*i.e.* oenocytoids, spherulocytes, and prohemocytes) in the hemolymph. Nodulation was an immune response against EPEC, indicated by the appearance of nodules in the hemolymph (3 - 6h post-injection) that coincided with the sharp decline in the number of circulating EPEC (Figure 3.5). The majority of nodules were likely out of circulation and attached to the surfaces of tissues (Ratcliffe & Gagen, 1976). Nodule attachment to tissue surfaces may explain the absence of nodules in the hemolymph at 48h post-injection (Figure 3.5). The absence of nodules in the hemolymph and the absence of hemocytopenia in larvae injected with Ringer's, HK-EPEC, and sham-injected larvae indicate that nodulation was not significantly induced. Phagocytosis by hemocytes is likely sufficient in clearing HK-EPEC from the hemolymph without the involvement of nodulation since dead EPEC cannot replicate. Melanization was an immune response in the hemolymph against EPEC and HK-EPEC but not against Ringer's. This is expected since both viable and dead EPEC

present PAMPs and can be recognized by insect PRRs and induce melanization (*e.g.* LPS, which is heat-stable). Viable EPEC induced more melanization per larva and in more larvae than HK-EPEC due to EPEC replication. However, it is also possible that the melanized particles found in the single insect injected with HK-EPEC were, by chance, remnants of a prior infection, since melanin remain in the hemocoel permanently post-formation (Hillyer, 2016). In larvae injected with EPEC, the appearance of melanized particles (3 - 6h post-injection) coincided with the sharp decline in the number of circulating EPEC, suggesting that the melanized particles are likely the results of melanin deposition around EPEC (Figure 3.5). Melanization of the injection wound indicate that all insects are indeed capable of melanization. This was expected since melanization contributes to the hardening of the clot (Rowley & Ratcliffe, 1978).

At the early stage of EPEC infection (1 - 3h post-injection), the number of circulating EPEC in the hemolymph sharply increased by a factor of approximately 8 (Figure 3.5). The doubling time of EPEC in LB medium at 30°C, the same incubation temperature of *G. mellonella*, was determined to be 38 min (Appendix 4.1). Exponential increase by a factor of 8 took 114 min in LB medium but 180 min in *G. mellonella*, indicating that *G. mellonella* hemolymph is not optimal for EPEC growth. The number of circulating EPEC was likely reduced by early immune responses such as phagocytosis, nodulation, melanization, and hemolymph coagulation, all of which can be activated within minutes of microbial exposure (Gagen & Ratcliffe, 1976; Ratcliffe & Gagen, 1976). The immune responses between 1 - 3h post-injection were unable to control EPEC replication in the hemolymph, evident by the increasing number of circulating EPEC. The sharp decline in the number of circulating EPEC between 3 - 6h post-injection indicates that the rate of EPEC clearance exceeded the rate of EPEC replication. The immune responses at this point likely also involved the expression of

AMPs and other immune-related proteins in addition to the early cellular and humoral immune responses mentioned above. Cecropin-A, peptidoglycan recognition-like protein-B and prophenoloxidase-activating proteinase-1 (PAP-1) were reported to have increased expression in *G. mellonella* at 6h post-injection with *Candida albicans* (Sheehan & Kavanagh, 2018). Cecropin-A is an AMP that is effective against bacteria, including multidrug resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (E. Lee *et al*., 2015). Peptidoglycan recognition proteins (PGRPs) function as PRRs and induce AMP expression and melanization after pathogen detection (Iketani & Morishima, 1993; Yoshida & Ashida, 1986). PAP-1 converts the inactive prophenoloxidase (PPO) to active phenoloxidase (PO) during melanization (Zou *et al.*, 2005). The protein expression profile of *G. mellonella* post-injection with EPEC is unknown and require future experiments to investigate and is likely different from the expression profile post-injection with *C. albicans*, while similarly involve the expression of AMPs and other immune-related proteins enhancing EPEC clearance. The number of circulating EPEC did not change between 6 - 24h post-injection, indicating that the rate of EPEC replication matched the rate of EPEC clearance. Compared to between 3 - 6h post-injection, this could be due to either increased EPEC replication rate, decreased EPEC clearance rate, or a combination of both. The mechanism behind this is unknown and requires further experimentation to determine. Some EPEC may have re-entered the hemolymph after escaping from nodules and coagulum, increasing the apparent replication rate. It is possible that nodulation becomes less efficient in clearing bacteria at lower densities where the bacteria were scattered and do not form aggregates. Complete clearance of EPEC from the hemolymph occurred between 24 - 48h post-injection, possibly due to the expression of additional AMPs and immune-related proteins at this later stage of infection. The expression of PRRs (PGRP-LB and hemolin) and AMPs (gloverin, cecropin-D-

like peptide, and moricin-like peptide B) were increased in *G. mellonella* larvae at 24h post-injection with *C. albicans* (Sheehan & Kavanagh, 2018). Similar increase in PRR and AMP expressions may account for the final clearance of EPEC by 48h post-injection.

The intrahemocoelic injection of Ringer's, HK-EPEC, and the injection injury did not kill any *G. mellonella* or delay pupation. Interestingly, EPEC was cleared from the hemolymph by 48h post-injection at which point all insects were alive at the time of hemolymph collection, while 60% mortality (12 out of 20) was observed among the undisturbed insects by day 20 post-injection. Furthermore, out of the 12 dead insects, only 1 died from bacteremia whereas the remaining 11 died with no viable EPEC in the hemolymph. This indicates that there are at least 3 potential outcomes of EPEC infection in *G. mellonella* larvae at the approximate LD_{50}: (1) The larva clears all EPEC from the hemolymph and completes development into an adult. (2) The larva clears all EPEC from the hemolymph but dies anyway, likely due to irrecoverable damage to the larva by EPEC and the immune responses. (3) The larval immune responses fail to control EPEC replication and the larva succumbs to death by septicemia. Future experiments are required to determine the intricate dose-dependent effect of EPEC on the infection outcome. Outcome (2) likely represent moribund larvae discussed in the previous chapter that were able to remain alive for up to 20 days post-injection before finally succumbing to death. As EPEC dose increases beyond the LD_{50}, outcome (3) is expected to become increasingly prevalent and *vice versa* for outcome (1).

3.5 Conclusions

After intrahemocoelic injection of EPEC, *G. mellonella* larvae showed typical insect anti-bacterial immune responses including melanization, hemolymph coagulation, nodulation, and phagocytosis. The production of AMPs could not be determined by SDS-PAGE and require

future experiments. Extracellular DNA resembling vertebrate NETs was found in *G. mellonella* injected with EPEC. The DNA likely originated from hemocytes and synergized with hemolymph coagulation and melanization to immobilize and kill EPEC, representing a novel insect immune response against microbial pathogens. This work represents the first known documentation of DNA extracellular traps in insects *in vivo*.

Hemocytopenia was temporarily induced in *G. mellonella* by EPEC, likely through plasmatocyte-spreading and nodulation. The subsequent recovery from hemocytopenia was likely through the release of sessile hemocytes and hematopoiesis. Hemocytes in circulation did not differ in clumping between treatments and likely represent un-activated and non-adhesive hemocytes. The immune responses of *G. mellonella* were unable to control EPEC replication at the early stage of infection but was eventually able to clear EPEC from the hemolymph, likely through a combination of phagocytosis, nodulation, melanization, and increased AMP and immune-related protein expression. The clearance of EPEC did not guarantee insect survival, likely due to irrecoverable damage from EPEC and the immune responses.

Overall, this study provided important background information on the *Galleria*-EPEC system. The discovery of DNA extracellular trap *in vivo* in *G. mellonella* opened a new frontier in insect immunology, warranting further research.

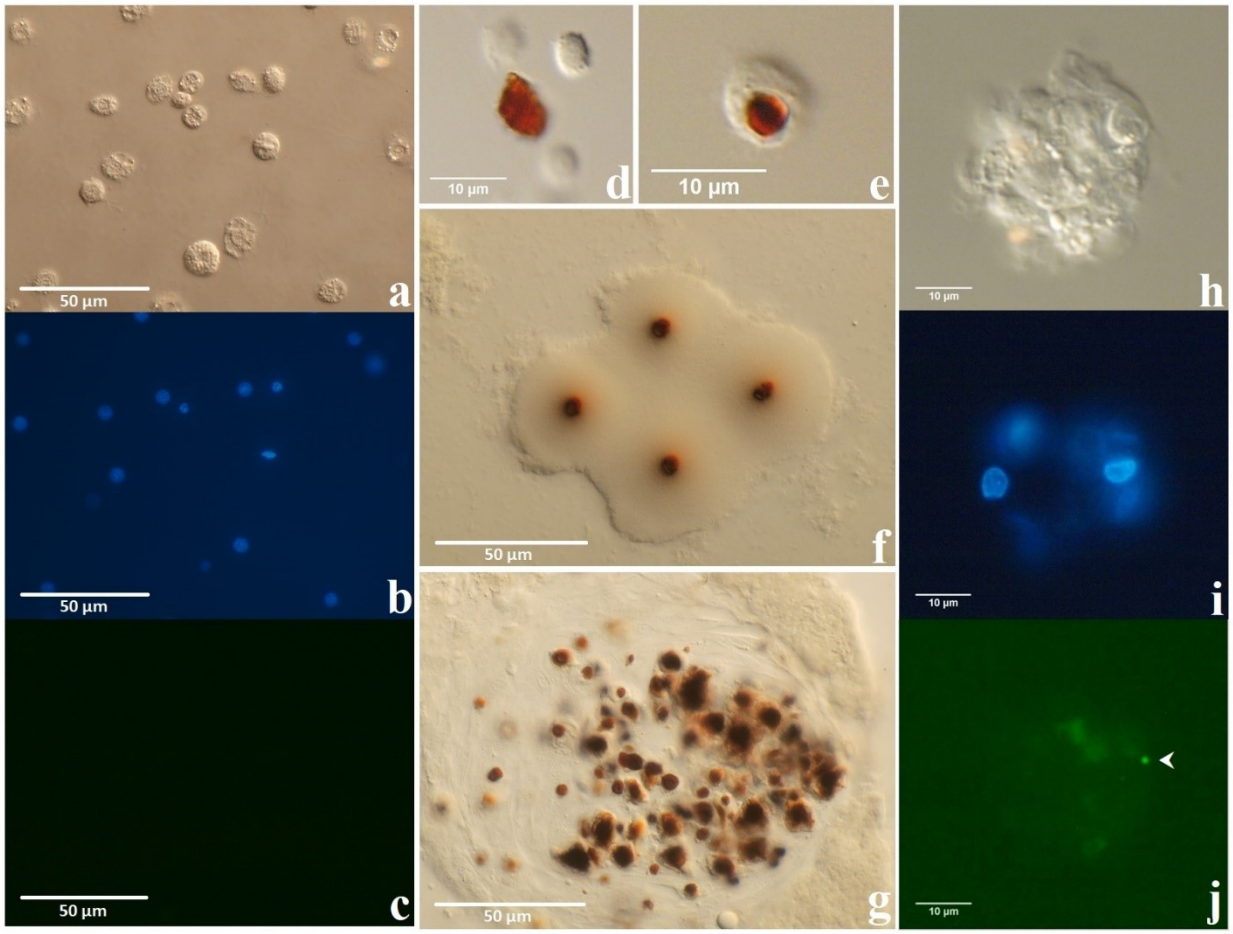

Figure 3.1 Typical immune responses observed in the hemolymph of *G. mellonella* larvae at 24h after intrahemocoelic injection with 1.5×10^4 EPEC. (a - c) Hemocytes from a control larva injected with Ringer's displaying: (a) typical hemocyte morphology, (b) typical hemocyte nuclei (blue fluorescence by Hoechst 33342 staining), and (c) absence of green fluorescence. (d) Extracelluar melanized particle. (e) Melanized particle internalized by a hemocyte. (f) Hemolymph coagulation around melanized particles. (g) Nodule containing numerous melanized particles. (h - j) Clump of hemocytes with internal green fluorescence (j, arrowhead) indicating phagocytosis of EPEC.

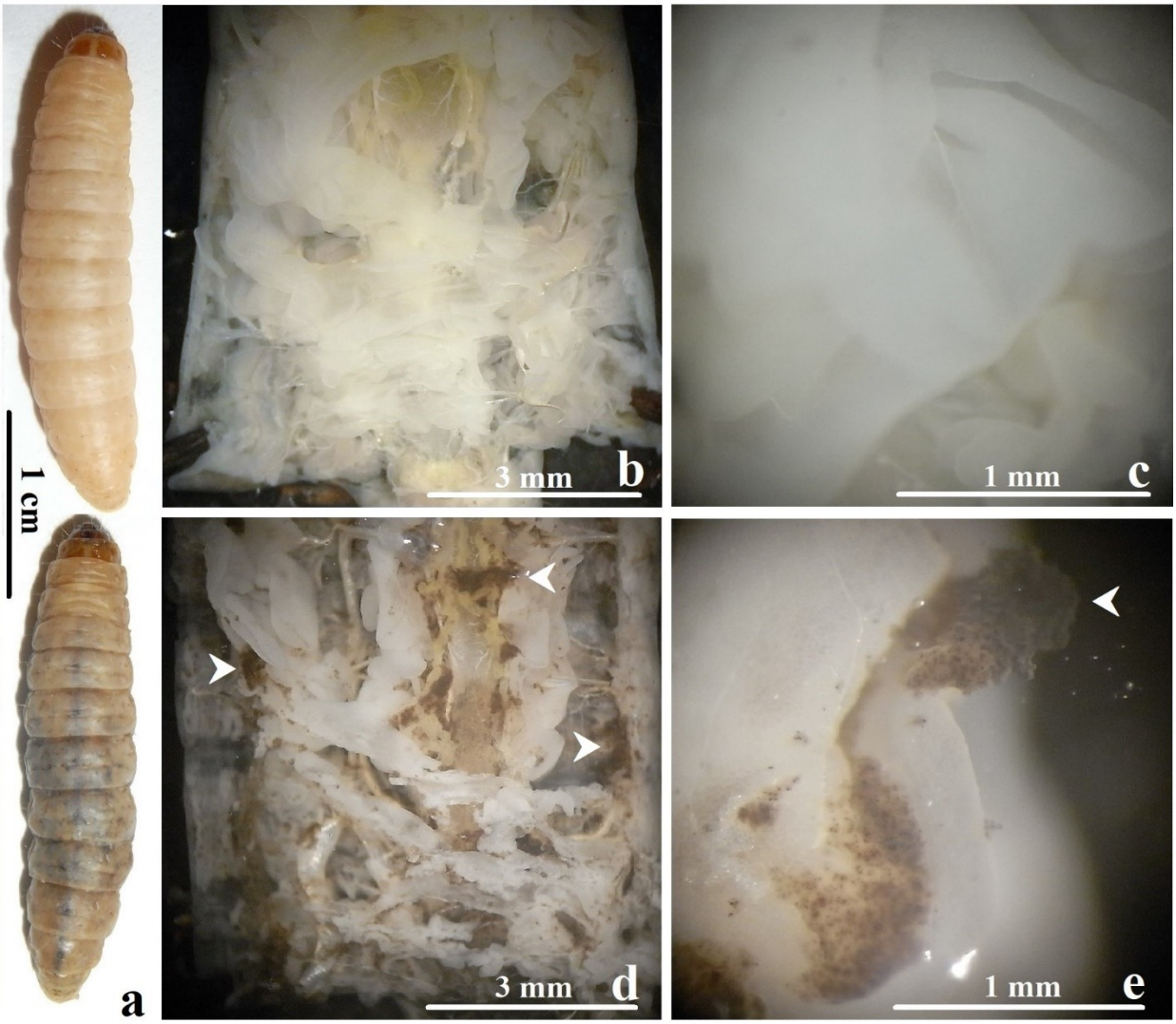

Figure 3.2 *G. mellonella* larvae at 24h after intrahemocoelic injection with Ringer's (top) and 1.5 × 10^4 EPEC (bottom). (a) Cuticular melanization was observed in larvae injected with EPEC but was absent in control larvae. (b - e) Upon dissection, melanized coagula (arrowheads) were seen attached to surfaces of various tissues and organs (*e.g.* fat body) in the hemocoel of diseased larvae (d, e) but were absent in the control larvae (b, c).

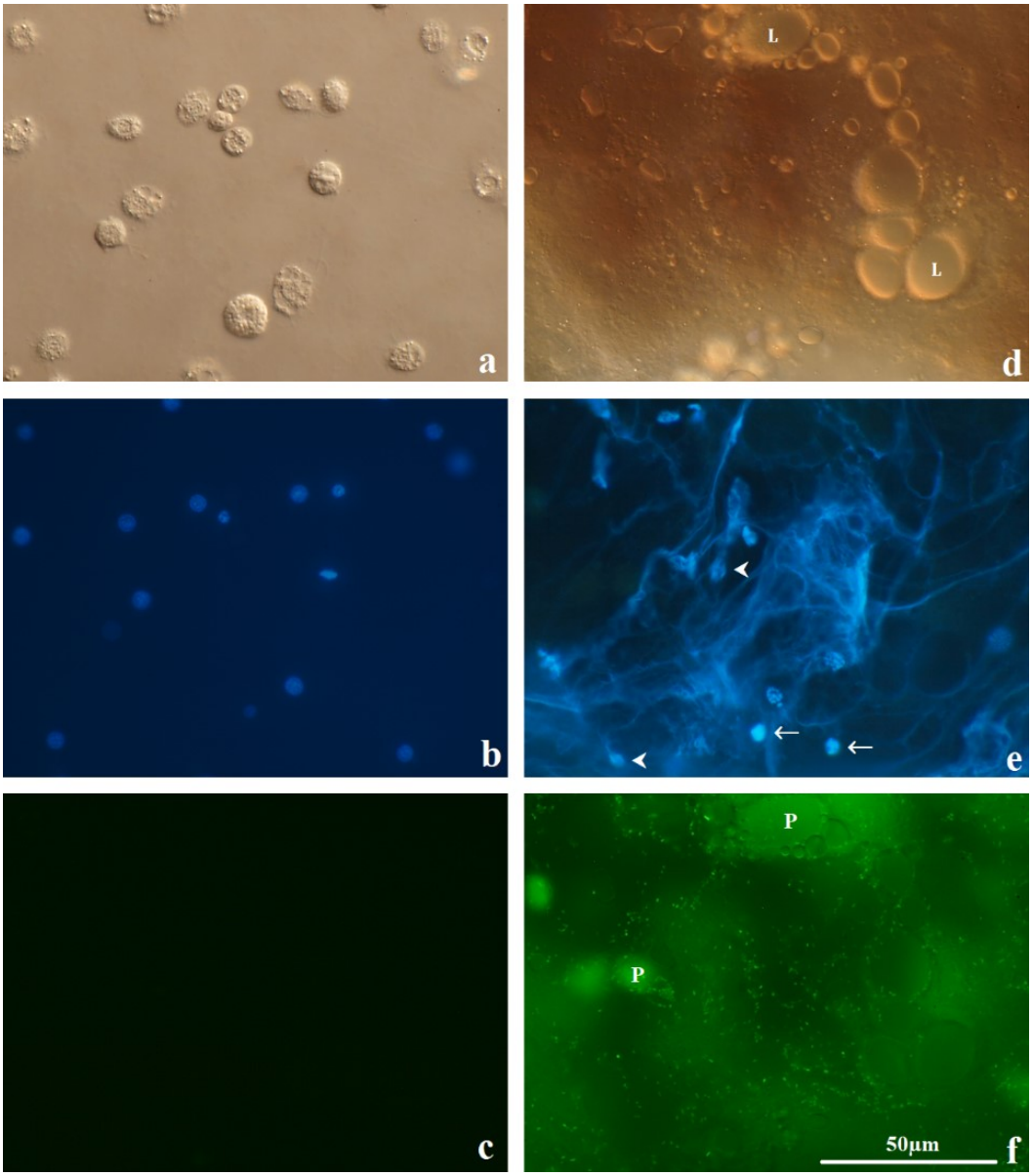

Figure 3.3 (a - c) Hemocytes from a *G. mellonella* larva at 24h post-injection with Ringer's displaying: (a) typical hemocyte morphology, (b) typical hemocyte nuclei (blue fluorescence by Hoechst 33342 staining), and (c) absence of green fluorescence. (d - f) Melanized coagulated mass excised from the surface of the fat body of a *G. mellonella* larva at 24h post-injection with 1.5×10^4 EPEC visualized with DIC and fluorescent microscopy. Both sets of images were taken at the same magnification, position, and focal plane. (d) Lipid droplets or gas pockets ("L") and hemocytes (arrows) were embedded in the coagulum. (e) DNA was seen projecting from some of the hemocytes (arrowheads) to form a net-like structure. (f) The network of DNA was co-localized with EPEC expressing GFP. Dense pockets of trapped EPEC ("P") were found on different focal planes within the coagulum.

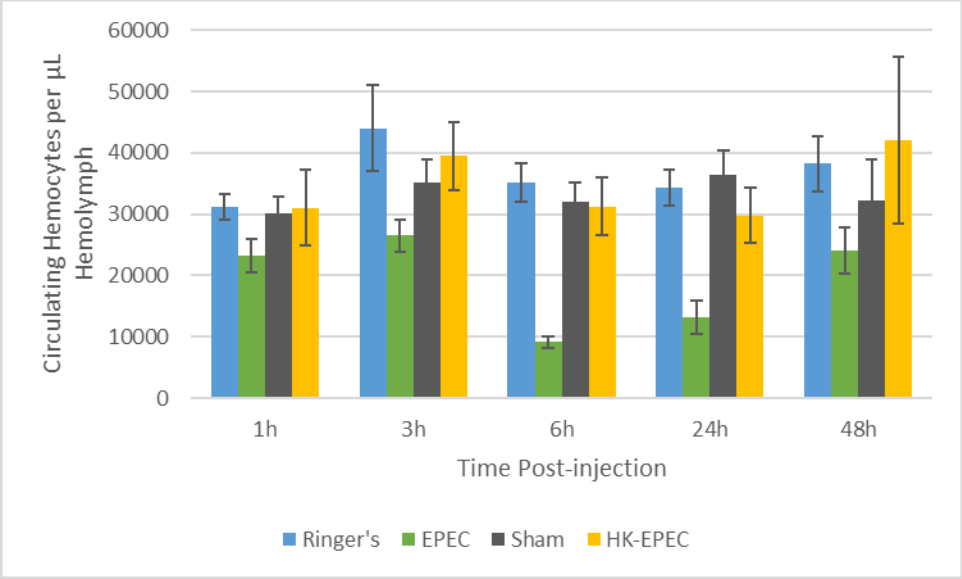

Figure 3.4 The average number of circulating hemocytes (± SE) per µL hemolymph at different time points in *G. mellonella* larvae injected intrahemocoelically with Ringer's, EPEC, HK-EPEC, or sham-injected (n = 160). Larvae injected with EPEC showed reduced hemocyte count compared to the other treatments, most prominently at 6h post-injection (74% reduction relative to Ringer's). Within the EPEC treatment, hemocyte count reduced by 66% between 3h and 6h post-injection but recovered between 24h and 48h post-injection. The hemocyte count for the other treatments remained relatively constant across all time points.

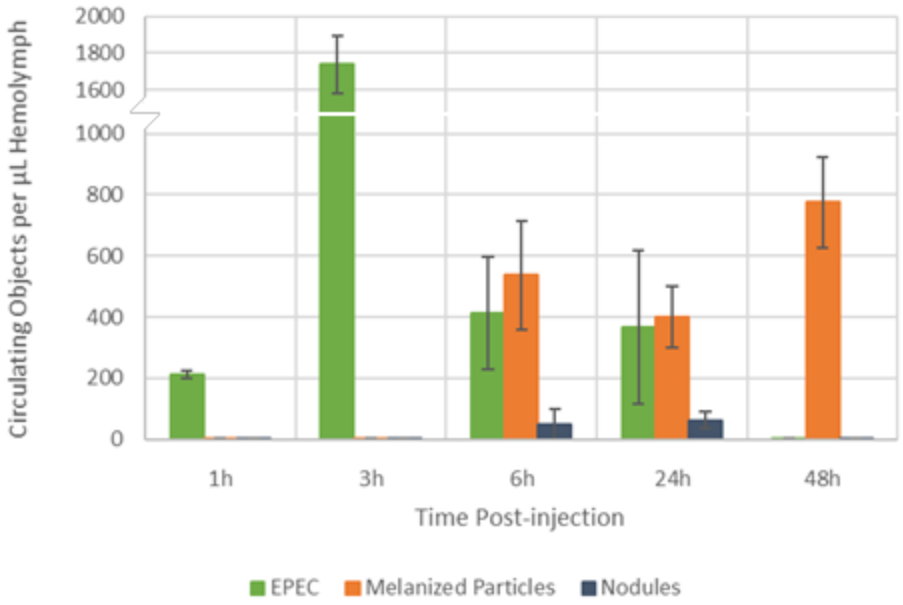

Figure 3.5 The average number of circulating EPEC, melanized particles, and nodules (± SE) per µL hemolymph at different time points in *G. mellonella* larvae injected intrahemocoelically with EPEC (n = 40). The reduction in the number of circulating EPEC is followed by the appearances of melanized particles and nodules in the hemolymph.

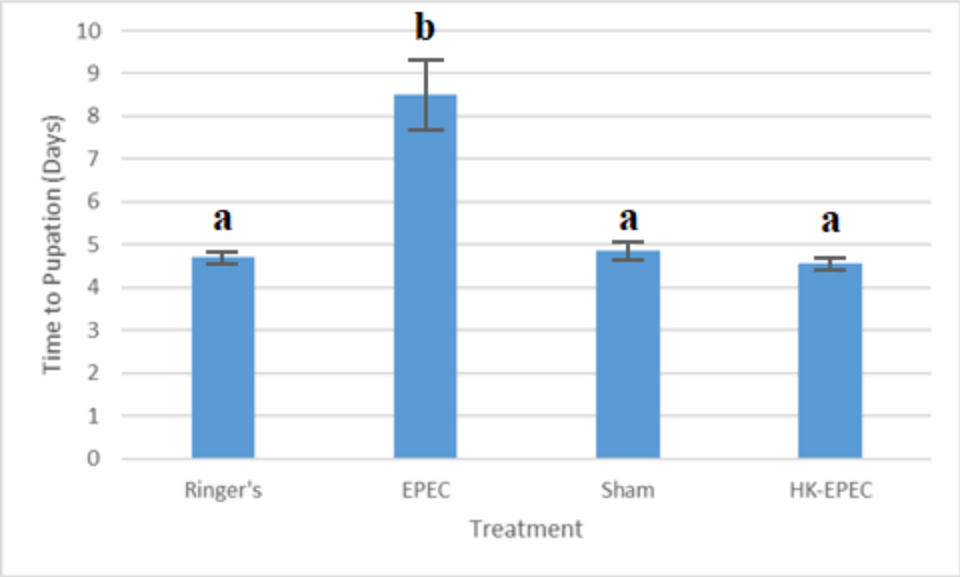

Figure 3.6 The average number of days to pupation (± SE) of *G. mellonella* larvae injected intrahemocoelically with Ringer's, EPEC, HK-EPEC, and sham-injected larvae (n = 80). Larvae injected with EPEC showed significant delay in pupation (by approximately 4 days) compared to larvae from the other treatments.

Chapter 4 Extracellular DNA and its role in insect immunity

4.1 Introduction

Neutrophil extracellular traps (NETs), first discovered in 2004, are extracellular networks of DNA, histones, and granule contents released by neutrophils that trap and kill microbes including bacteria and fungi (Brinkmann *et al*., 2004; C. F. Urban *et al*., 2006). The process of NET release is known as NETosis and is considered to be an active form of neutrophil death distinct from apoptosis and necrosis (Brinkmann, 2018). The immobilization of microbes by NETs prevent microbial dispersion to other locations within the host. The antimicrobial activity of NETs was attributed to the toxicity of neutrophil granule contents (*e.g.* neutrophil elastase, myeloperoxidase, lysozyme, and defensins), histones, and DNA (Brinkmann, 2018). The two currently recognized forms of NETosis result in either the lysis of neutrophils after NET release (suicidal NETosis) or the formation of anuclear neutrophils that remain intact and functional (vital NETosis) (Yipp & Kubes, 2013). Suicidal NETosis can be induced by pathogens through cell surface receptors (*e.g.* Toll-like receptors, Fc receptors, and complement receptors). Receptor activation stimulates the release of Ca^{2+} from the endoplasmic reticulum into the cytoplasm. Increased cytoplasmic Ca^{2+} levels activate protein kinase C (PKC) and result in downstream assembly and activation of the NADPH oxidase complex and the production of ROS. The ROS rupture cytoplasmic granules and the nuclear envelope, resulting in the mixture of granule contents with the nucleoplasm. The granule enzymes neutrophil elastase, myeloperoxidase, and peptidyl-arginine deiminase 4 enter the nucleus and collectively induce chromatin decondensation. Finally, the cell membrane ruptures and release NETs into the extracellular space (Brinkmann, 2018). Suicidal NETosis can also be induced chemically by the PKC activator phorbol 12-myristate 13-acetate (PMA) or microbial surface components (*e.g.*

LPS and β-glucans) (Brinkmann *et al.*, 2004). The biochemical mechanisms behind vital NETosis remains unclear to date. NETs are released by exocytosis through the budding of vesicles that transport DNA from the nucleus to the cell membrane. The process is ROS-independent and occur more rapidly than suicidal NETosis. Neutrophils that have undergone vital NETosis maintain cell membrane integrity and retain the abilities of adhesion, chemotaxis, degranulation, and phagocytosis (Pilsczek *et al.*, 2010). A different form of vital NETosis involving the release of mitochondrial DNA instead of nuclear DNA has also been documented (Yousefi *et al.*, 2009).

In addition to human neutrophils, the release of extracellular traps (ETosis) has also been observed in other vertebrates (*e.g.* avian heterophils and fish neutrophils), invertebrates (*e.g.* annelid coelomocytes, cnidarian mesogleal cells, and crustacean hemocytes), and even in plants (root border cells) (Chuammitri *et al.*, 2009; Hawes *et al.*, 2012; Homa, 2018; Ng *et al.*, 2013; Palić *et al.*, 2007; Robb *et al.*, 2014). As such, ETosis is considered to be an ancient and evolutionarily conserved immune response, at least within animals (Robb *et al.*, 2014). Evidence of ETosis in insects remain ambiguous. A recent study reported extracellular DNA release by *Periplaneta americana* hemocytes *ex vivo* after stimulation with delipidated LPS and DH5α *E. coli* (Nascimento *et al.*, 2018). However, the study lacked controls (*i.e.* blank stimulation) to account for potential experimental artifacts (*e.g.* mechanical lysis of hemocytes during handling and spontaneous DNA release) and the hemocyte type(s) involved remain unknown. An earlier study was unable to induce ETosis in *Galleria mellonella* hemocytes *ex vivo* using bacteria, but found that oenocytoid lysis occurs immediately upon collection and could potentially represent a source of extracellular DNA (Altincicek *et al.*, 2008).

In the previous chapter, extracellular DNA that immobilized enteropathogenic *Escherichia coli* (EPEC) was observed in the coagula of *G. mellonella* larvae 24h after intrahemocoelic EPEC injection, representing the first known record of ETosis in insects *in vivo*. Observations from the study indicate that hemocytes are most likely the source of the extracellular DNA. The present study aims to determine: (1) Do *G. mellonella* hemocytes release DNA after stimulation by known inducers of NETosis (*i.e.* bacteria and PMA)? If so, then extracellular DNA should be observed among the hemocytes post-stimulation. (2) Does extracellular DNA confer protection to *G. mellonella* against EPEC *in vivo*? If so, then *G. mellonella* injected with both EPEC and DNase I would show increased disease severity (*i.e.* increased mortality, decreased survival time, increased time to pupation, decreased pupal mass, decreased fecundity, and decreased egg hatch rate) compared to insects injected with EPEC alone due to the destruction of extracellular DNA by DNase I. This effect would be abolished with the injection of heat-inactivated DNase I (HI-DNase I) instead of active DNase I. Conversely, the injection of both EPEC and *G. mellonella* hemocyte DNA would have the opposite effect and result in reduced disease severity (*i.e.* decreased mortality, increased survival time, decreased time to pupation, and increased EPEC clearance rate) compared to insects injected with EPEC alone. My results confirmed the involvement of *G. mellonella* hemocytes (likely granulocytes and oenocytoids) in DNA release *ex vivo* and demonstrated that extracellular DNA confers protection (increased EPEC clearance rate and prolonged insect survival) to *G. mellonella* against EPEC infection of the hemocoel *in vivo*, providing support to the hypothesis that insect hemocytes release extracellular DNA that protects the insect against microbial infection in the hemocoel.

4.2 Materials and methods

4.2.1 Release of extracellular DNA *ex vivo*

Hemolymph from 2 *G. mellonella* larvae were collected aseptically in ice-cold anticoagulant antimelanization solution as previously described (Chapter 3). Hemolymph plasma was removed by micropipette after centrifugation (Eppendorf 5415L) at 200 g for 5 minutes. The hemocyte pellets were washed once with ice-cold Ringer's and resuspended in Grace's insect medium (Appendix 2.4) by gentle agitation. The hemocytes were loaded onto 6 sterile glass slides with approximately 1.5×10^5 hemocytes per slide, 1 slide per treatment, and 3 slides per insect. The hemocytes were allowed to adhere to the slides for 30 minutes and were stimulated for 1h with EPEC (5.0×10^4 CFU), PMA (50 µM), or Ringer's as control. The hemocytes were subsequently washed with Ringer's, fixed with 4% formaldehyde for 1h, and stained with 10 µg/mL Hoechst 33342. The slides were kept at 30°C in sterile glass petri dishes (PYREX, 90 mm diameter) humidified with wet sterile filter paper during all waiting steps to minimize evaporation and contamination. The hemocytes were examined by DIC and fluorescent microscopy using a Reichert-Jung Polyvar microscope at 500x magnification. Hemocytes releasing extracellular DNA and the total number of hemocytes were quantified in 5 random fields of views for each slide and were photographed using an Olympus E-420 digital camera. The hemocyte types responsible for DNA release were identified morphologically when possible based on the descriptions of Wu *et al.* (2016).

4.2.2 Removal of extracellular DNA *in vivo*

G. mellonella larvae were injected intrahemocoelically with various doses of EPEC in the presence of (1) 5U DNase I (Sigma-Aldrich, dissolved in Ringer's), (2) 5U heat-inactivated DNase I (HI-DNase I, dissolved in Ringer's), or (3) EPEC alone (Appendix 5.6). Larvae injected

with Ringer's, DNase I, or HI-DNase I in the absence of EPEC were used as controls. A total of 220 insects were used in this experiment with at least 10 insects per treatment (*i.e.* EPEC, EPEC + DNase I, and EPEC + HI-DNase I) at any dose of EPEC. Insect mortality, survival score, time to pupation, pupal mass, fecundity, and egg hatch rate were recorded or calculated as previously described (Chapter 2). DNase I was irreversibly heat-inactivated at 70°C for 15 minutes in a heating block (Isotemp Model 145, Fisher). DNase I activity and heat inactivation were confirmed by *in vitro* digestion of DNA extracted from *G. mellonella* hemocytes and quantification of DNA by a Qubit fluorometer (Invitrogen).

4.2.3 Addition of extracellular DNA *in vivo*

G. mellonella larvae were injected intrahemocoelically with two doses of EPEC (1.6×10^4 CFU or 2.2×10^4 CFU and a Ringer's control) in the presence or absence of *G. mellonella* hemocyte DNA (500 ng, dissolved and suspended in Ringer's) (Appendix 5.7). Larvae injected with Ringer's or DNA in the absence of EPEC were used as controls. A total of 240 insects were used in this experiment with at least 30 insects per treatment (*i.e.* EPEC and EPEC + DNA) at any dose of EPEC. Hemolymph was collected at 1h, 3h, 6h, 24h, and 48h post-injection aseptically from 16 insects per treatment at each time point without replacement to quantify circulating EPEC by plate-count. The remaining insects (40 of each treatment) were left undisturbed to obtain mortality, time to pupation, and survival score. The DNA used in this experiment was extracted from hemocytes of untreated *G. mellonella* larvae using a DNeasy Blood and Tissue Kit (Qiagen). DNA concentration was quantified by a Qubit fluorometer and DNA purity was assessed with a NanoDrop spectrophotometer (Thermo). DNA was pelleted and dried by a centrifugal evaporator (DyNA Vap, Labnet) and re-dissolved in Ringer's to reach the appropriate final concentration (verified by a Qubit fluorometer).

4.2.4 Statistical analyses

Statistical analyses in this chapter were conducted using R (R Core Team, 2019). GLMs were constructed to determine: (1) the effects of DNase I on insect mortality, survival score, time to pupation, pupal mass, fecundity, and egg hatch rate; and (2) the effects of additional extracellular DNA on EPEC clearance, insect mortality, survival score, and time to pupation. The GLM family used in each model was determined by the type of data: Gaussian family for continuous data such as pupal mass, Poisson family for count data such as time to pupation, and binomial family for proportion data such as egg hatch rate. Overdispersion (dispersion parameter > 2.0) and underdispersion (dispersion parameter < 0.5) were accounted for by using quasi-families. Model assumptions (*i.e.* absence of heteroscedasticity and non-normality of errors) were checked graphically. The minimum adequate models were obtained by stepwise deletion of non-significant factors and interactions when applicable. Model comparisons were conducted using F tests when dispersion parameters were estimated or χ^2 tests (deviance change) when dispersion parameters were fixed. Model coefficients (β) were used to infer relationships (*i.e.* positive or negative) between variables. Tukey contrasts (pairwise comparisons) were used to determine where significant differences occurred *post hoc* to GLMs.

4.3 Results

4.3.1 Release of extracellular DNA *ex vivo*

Extracellular DNA was released from 0.20% (12 out of 5977) of hemocytes stimulated with EPEC, 0.08% (5 out of 6572) of hemocytes stimulated with PMA, and 0.02% (1 out of 4562) of the hemocytes stimulated with Ringer's. Three patterns of DNA release were observed: Pattern I (Figure 4.1c, 4.1d, 4.2a, and 4.2c): the hemocyte produced fibrillar projections of DNA that originated from the nucleus. The nucleus appeared irregular in shape with diffuse

fluorescence compared to normal nuclei. The hemocyte appeared relatively intact. Pattern II (Figure 4.3c and 4.3d): the hemocyte produced fibrillar projections of DNA that appeared to originate from the cytoplasm. The nucleus appeared normal and the hemocyte appeared intact. Pattern III (Figure 4.2a, 4.2d, 4.4, and 4.5): the hemocyte lysed and the nucleus appeared diffuse or irregular in shape. Fibrillar DNA projected from the naked nucleus into the extracellular space. Hemocytes exhibiting patterns I and II of DNA release were tentatively identified as granulocytes due to the presence of numerous cytoplasmic granules. Lysed hemocytes were identified as granulocytes only when the exposed cytoplasm contained numerous granules, and those with few or no cytoplasmic granules could not be identified. No hemocyte lysis was observed after stimulation with Ringer's. The granulocyte that released DNA after stimulation with Ringer's was classified as pattern I. The hemocytes stimulated with EPEC displayed all three patterns of DNA release (patterns I - III from granulocytes and pattern III of unknown hemocyte) whereas only pattern III was found in the hemocytes stimulated with PMA. No EPEC was found to be associated with extracellular DNA. In general, hemocytes stimulated with PMA appeared more flattened compared to hemocytes stimulated with Ringer's or EPEC. Cellular debris were more abundant in the background of hemocytes stimulated with PMA or EPEC compared to the backgrounds of hemocytes stimulated with Ringer's.

4.3.2 Removal of extracellular DNA *in vivo*

Both EPEC dose (Binomial GLM, Deviance = 178, df = 1 and 218, $p < 0.0001$) and treatment (Deviance = 9.82, df = 2 and 216, $p = 0.007$) were significant predictors of insect mortality. Mortality was positively associated with EPEC dose ($\beta = 4.13 \times 10^{-4}$, $p < 0.0001$) in all treatments with no mortality observed in the controls (*i.e.* Ringer's alone, DNase I in Ringer's, and HI-DNase I in Ringer's). No significant differences in mortality were detected

between DNase I and HI-DNase I treatments (Tukey contrasts, $p = 0.97$) although insects from both DNase I and HI-DNase I treatments showed significantly higher mortality compared to insects injected with EPEC alone ($p = 0.02$ and $p = 0.01$, respectively) (Figure 4.6a). Both EPEC dose (Quasi-binomial GLM, $F = 265$, $df = 1$ and 211, $p < 0.0001$) and treatment ($F = 9.77$, $df = 2$ and 209, $p < 0.0001$) were significant predictors of insect survival score post-injection. Survival score was negatively associated with EPEC dose ($\beta = -4.36 \times 10^{-4}$, $p < 0.0001$) in all treatments. No significant differences in survival score were detected between larvae injected with DNase I and larvae injected with HI-DNase I (Tukey contrasts, $p = 0.90$). Insects from both DNase I and HI-DNase I treatments showed significantly reduced survival score compared to insects injected with EPEC alone ($p = 0.0005$ and $p = 0.0001$, respectively) (Figure 4.6b). EPEC dose (Quasi-Poisson GLM, $F = 247$, $df = 1$ and 147, $p < 0.0001$), but not treatment, was a significant predictor of time to pupation post-injection. Time to pupation was positively associated with EPEC dose ($\beta = 7.94 \times 10^{-5}$, $p < 0.0001$). EPEC dose (Gaussian GLM, $F = 22.8$, $df = 1$ and 128, $p < 0.0001$) and insect sex ($F = 17.2$, $df = 1$ and 127, $p < 0.0001$), but not treatment, were significant predictors of pupal mass. Pupal mass was negatively associated with EPEC dose ($\beta = -2.73 \times 10^{-3}$, $p < 0.0001$) regardless of treatment. Female pupae were on average 28.3 mg larger than male pupae. EPEC dose (Quasi-Poisson GLM, $F = 13.7$, $df = 1$ and 98, $p = 0.0004$), but not treatment, was a significant predictor of fecundity. Fecundity was negatively associated with EPEC dose ($\beta = -3.35 \times 10^{-2}$, $p = 0.0004$) regardless of treatment. Neither EPEC dose nor treatment were significant predictors of egg hatch rate.

4.3.3 Addition of extracellular DNA *in vivo*

Treatment (Quasi-Poisson GLM, $F = 11.9$, $df = 1$ and 157, $p = 0.0007$), time post-injection ($F = 11.1$, $df = 4$ and 153, $p < 0.0001$), and EPEC dose ($F = 67.8$, $df = 1$ and 158, $p <$

0.0001) were all significant predictors of circulating EPEC count. Statistically significant differences in circulating EPEC count among treatments over time were detected ($F = 3.15$, df = 4 and 149, $p = 0.02$). Overall, larvae injected with EPEC and DNA showed reduced number of circulating EPEC and earlier clearance compared to larvae injected with only EPEC (Figure 4.7). EPEC dose was positively associated with the number of circulating EPEC ($\beta = 1.65 \times 10^{-4}$, $p < 0.0001$). No bacteria were found in the hemolymph of larvae when no EPEC was injected in both treatments and at all time points. EPEC dose but not treatment was a significant predictor of insect mortality (Binomial GLM, deviance = 55.5, df = 1 and 78, $p < 0.0001$). No mortality was observed in the absence of EPEC (*i.e.* Ringer's alone and DNA in Ringer's). Mortality increased as EPEC dose increased ($\beta = 1.64 \times 10^{-4}$, $p < 0.0001$). Both treatment (Quasi-binomial GLM, $F = 6.39$, df = 1 and 76, $p = 0.01$) and EPEC dose ($F = 94.9$, df = 1 and 76, $p < 0.0001$) were significant predictors of insect survival score. Insects injected with EPEC and DNA survived approximately 1 day longer on average compared to insects injected with only EPEC (Figure 4.8). Survival score was negatively associated with EPEC dose ($\beta = -2.47 \times 10^{-4}$, $p < 0.0001$). EPEC dose but not treatment was a significant predictor of time to pupation post-injection (Quasi-Poisson GLM, $F = 90.2$, df = 1 and 51, $p < 0.0001$). Time to pupation was positively associated with EPEC dose ($\beta = 3.25 \times 10^{-5}$, $p < 0.0001$).

4.4 Discussion

4.4.1 Release of extracellular DNA *ex vivo*

The release of extracellular DNA from *G. mellonella* hemocytes was confirmed *ex vivo*, indicating that hemocytes were, at least in part, responsible for the extracellular DNA observed *in vivo* in the previous chapter. All three treatments resulted in extracellular DNA release. The granulocyte that released DNA after stimulation with Ringer's (Figure 4.1) was unexpected and

could have occurred spontaneously or as a result of activation against the un-coated glass slide. Slides coated in materials that mimic *G. mellonella* basal lamina (*e.g.* collagen IV, laminin, nidogen, and perlecan) could be used in future experiments to minimize undesired hemocyte activation. No hemocyte lysis was observed after stimulation with Ringer's, indicating that handling did not result in mechanical damage to the hemocytes. Oenocytoid lysis observed by Altincicek *et al.* (2008) after hemolymph collection was likely avoided in the present study by the use of ice-cold isotonic solutions and the removal of hemolymph plasma that contain damage signals from the wound created for hemolymph collection. The flattened appearance of the hemocytes after PMA stimulation was likely due to increased cell adhesion, a known effect of PMA on neutrophils (Webster *et al.*, 1986). Both EPEC and PMA stimulation increased the proportion of hemocytes releasing DNA compared to the control, indicating that EPEC and PMA were inducers of DNA release. Extracellular DNA was released by *G. mellonella* granulocytes stimulated with EPEC either from the nucleus (pattern I) or seemingly from the cytoplasm (pattern II). The diffuse fluorescence and the irregular shape of the nucleus (pattern I, Figure 4.2c) indicate, respectively, the decondensation of chromatin and the loss of nuclear envelope integrity, which are necessary steps of suicidal NETosis. The relatively intact appearance of the hemocyte (Figure 4.2a) suggests incomplete DNA release at the time of fixation and staining, which likely would culminate in the loss of cell membrane integrity and the release of the remaining DNA and cytoplasm into the extracellular space. Granulocyte lysis observed by Pech & Strand (1996) may be the end result of DNA release. Suicidal NETosis may take several hours to complete whereas vital NETosis only takes minutes (Yipp & Kubes, 2013). Time-lapse microscopy and longer stimulation times in future experiments are required to capture the entire process of DNA release and may result in a larger proportion of DNA-releasing hemocytes. The

intact nucleus and cell membrane with seemingly cytoplasmic origin of DNA release (pattern II, Figure 4.3) could indicate vital NETosis, in which the release of nuclear DNA by vesicular transport through the cytoplasm and/or the release of mitochondrial DNA is responsible. Anuclear hemocytes were not observed and the presence of condensed chromatin in the nucleus indicate that the release of mitochondrial DNA may be the case. Alternatively, a partial DNA release from the nucleus could also account for this pattern. Future experiments combining the fluorescent staining of DNA and histones could differentiate between nuclear and mitochondrial DNA. The complete lysis of hemocytes (pattern III; Figure 4.2d, 4.4, and 4.5) most closely resembles suicidal NETosis in that the decondensation of chromatin and the rupture of the nuclear envelope and the cell membrane were all present. The absence of granules in the cytoplasmic remains of some of the lysed hemocytes may indicate either suicidal NETosis of granulocytes that have already degranulated or the lysis of oenocytoids, plasmatocytes, or prohemocytes. The lysis of hemocytes was most likely responsible for the cellular debris observed in the background of hemocytes stimulated with EPEC or PMA. Fluorescent antibodies specific to each hemocyte type would enhance hemocyte identification where identification by morphology using DIC microscopy alone is insufficient. EPEC was able to induce all three patterns of DNA release whereas only pattern III was observed after PMA stimulation. This was expected as both suicidal (ROS-dependent) and vital (ROS-independent) NETosis are stimulated by microbes whereas PMA activates PKC and only stimulates suicidal NETosis through oxidative burst (Brinkmann *et al.*, 2004; Pilsczek *et al.*, 2010; Yipp & Kubes, 2013). Plasmatocytes and granulocytes of *G. mellonella* are capable of oxidative burst mediated by NADPH oxidase homologous to human neutrophils (Bergin *et al.*, 2005). Oenocytoid lysis in *Spodoptera exigua* is mediated by PKC through bacteria-induced eicosanoid signaling (Shrestha

& Kim, 2009). This provides additional support for the involvement of *G. mellonella* granulocytes and oenocytoids in the release of extracellular DNA after EPEC and PMA stimulation. The regulated nature of oenocytoid lysis may in fact be a form of ETosis and synergizes with PPO release and melanization to trap and kill microbes. The presence of exposed nuclei (Figure 4.4), likely from the lysis of oenocytoids after stimulation with EPEC or PMA, indicate the loss of cell membrane integrity before the loss of nuclear envelope integrity, which occurred in the opposite order compared to suicidal NETosis and may represent a novel form of ETosis unique to insects. Similarities and differences between NETosis and hemocyte extracellular DNA release observed in this study were summarized in Table 4.1.

No EPEC was trapped by the extracellular DNA observed in this study, which was likely due to the small number of DNA-releasing hemocytes that were only able to cover small areas and EPEC escape from the sparse extracellular DNA. A longer incubation time with a larger amount of EPEC could result in more extracellular DNA release and more frequent EPEC contact with the extracellular DNA. The formation of extensive networks of extracellular DNA observed in *G. mellonella in vivo* after intrahemocoelic EPEC injection (Chapter 3, Figure 3.3e) likely involved the coordinated release of DNA by numerous hemocytes followed by the coagulation of the surrounding hemolymph and the melanization of the coagulum. Coordinated DNA release was not observed *ex vivo*, possibly due to the removal of hemolymph plasma factors that may be involved and/or due to other factors from the artificial nature of the *ex vivo* environment such as the use of un-coated glass slides and cell culture medium. Removal of hemolymph plasma also prevented coagulation that could otherwise enhance EPEC immobilization and structurally reinforce the extracellular DNA. Conversely, the ability of DNA release by hemocytes *ex vivo* under plasma-free conditions indicate that hemolymph plasma

components are not necessary for DNA release. In a preliminary experiment, melanization, hemolymph coagulation, nodulation, but no extracellular DNA release were observed after *ex vivo* incubation of whole *G. mellonella* hemolymph with EPEC, similar to the lack of DNA release observed by Altincicek *et al.* (2008) (data not shown). The reason behind this is unknown but may be due to the presence of hemolymph proteins involved in hemocyte regulation and signaling that may have inhibited DNA release *ex vivo*. The antimicrobial activity of the extracellular DNA is unknown and requires microbial killing assays to establish. The granule contents of insect granulocytes remain poorly characterized to date. Granules of *G. mellonella* granulocytes contain lysosomal enzymes (*e.g.* lysozyme) but cannot be distinguished from lysosomes (Chain & Anderson, 1983b). The simultaneous fluorescent staining of DNA and lysozyme could help determine whether granule/lysosomal contents were associated with extracellular DNA released by hemocytes, similar to NETs. Though it is likely that the DNA release in insect granulocytes and NETosis are homologous, the biochemical mechanism in insects remains unknown and require extensive research to characterize. Future experiments that examine histone citrullination and the association of granulocyte granule contents with the extracellular DNA are required to definitively determine whether ETosis or necrosis is the cause of DNA release (Yipp & Kubes, 2013). My preliminary experiments revealed: (1) Intrahemocoelic injection of EPEC induced extracellular DNA release in *Bombyx mori in vivo* (data not shown). (2) Intrahemocoelic injection of *Candida rugosa* induced extracellular DNA release in *G. mellonella in vivo* (data not shown). (3) Microbial surface components (LPS and β-glucan) induced extracellular DNA release in *G. mellonella* hemocytes *ex vivo* (data not shown). These findings indicate that extracellular DNA release may be common in insects and can be

induced by different types of microbes (*e.g.* bacteria and fungi). Future studies are required to determine the prevalence and specificity of extracellular DNA release in insects.

In consideration of the multiple potential hemocytic origins and pathways of the release of extracellular DNA, I propose the term insect hemocyte extracellular traps (IHETs) be used to collectively describe the extracellular traps released by insect hemocytes that immobilize and potentially kill microbes. The naming scheme avoids confusion with heterophil extracellular traps (HETs) and hemocyte extracellular traps from other invertebrates.

4.4.2 Removal of extracellular DNA *in vivo*

Treatment with either DNase I or HI-DNase I increased EPEC virulence in *G. mellonella* leading to increased insect mortality and decreased insect survival time compared to insects injected with EPEC alone (Figure 4.6). The effects of HI-DNase I were unexpected since the injection of DNase I was hypothesized to increase EPEC virulence by destroying IHETs while the heat-inactivation of DNase I removes this effect. Several possible explanations exist: (1) The heat-inactivation of DNase I was reversed *in vivo* and the increase in EPEC virulence was due to the destruction of IHETs, which enhanced EPEC spread and survival. (2) DNase I enhanced EPEC virulence by unknown means independent of IHET destruction while IHETs played no significant role in controlling EPEC. (3) DNase I was inhibited *in vivo* while enhancing EPEC virulence by unknown means that were unaffected by heat-inactivation. Subsequent troubleshooting experiments revealed that DNase I was unable to digest *G. mellonella* hemocyte DNA *ex vivo* in the presence of *G. mellonella* hemolymph plasma and that EPEC replicated in Ringer's containing DNase I but was unable to replicate in Ringer's alone (data not shown). Taken together, the available evidence indicates possibilities (1) and (2) were unlikely due to DNase I inhibition by *G. mellonella* hemolymph plasma, leaving possibility (3) as the most

likely candidate. However, the effects of IHETs, the mechanisms of DNase I inhibition *in vivo*, and the mechanism behind the enhancement of EPEC virulence by DNase I remain unknown.

Actin is an endogenous inhibitor of DNase I and is found in the hemolymph of immune challenged *Anopheles gambiae* where it binds and kill pathogens and enhance phagocytosis (Lacks, 1981; Sandiford *et al.*, 2015). A similar release of actin may have occurred in *G. mellonella*, resulting in the inhibition of DNase I *in vivo*. The release of IHETs in *G. mellonella* could also release cytoplasmic actin that may contribute to both DNase I inhibition and EPEC killing. Group A *Streptococcus* release extracellular DNases that destroy NETs (Sumby *et al.*, 2005). The release of cytoplasmic actin may also protect IHETs from destruction by bacterial DNases and prevent bacterial escape. The injected DNase I (5U at 400U/mg) is equivalent to 12.5 μg of protein, which is only approximately 0.1% of total hemolymph protein by mass in a *G. mellonella* larva, assuming average hemolymph volume of 56.7 μL/larva and average hemolymph plasma protein concentration of 189.2 mg/mL (Jones, 1967; Sak *et al.*, 2011). This indicates that even though EPEC could replicate in Ringer's containing DNase I, the utilization of DNase I as a protein source may be inconsequential *in vivo*. Furthermore, the delay in pupation and reduction in pupal mass depended on EPEC dose but were independent of the presence of DNase I or HI-DNase I, indicating that EPEC replication may not affected by DNase I or HI-DNase I *in vivo*. However, direct quantification of circulating EPEC is required to confirm this. Insects from the control groups (*i.e.* larvae injected with Ringer's, DNase I in Ringer's, or HI-DNase I in Ringer's) did not differ from each other in mortality, survival time, time to pupation, pupal mass, fecundity, or egg hatch rate. This indicate that DNase I and HI-DNase I are not directly toxic to *G. mellonella* at the dose of 5U/larva and that other unknown factors are responsible for the EPEC dose-dependent increase in mortality and decrease in

survival time in the presence of DNase I and HI-DNase I. Future experiments investigating this phenomenon may uncover novel mechanisms of EPEC pathogenesis in *G. mellonella*.

The use of HI-DNase I as a control group was critical in this experiment. Without this control, the increased mortality and decreased survival time would almost certainly be falsely attributed to the *in vivo* destruction of IHETs by DNase I. Intrahemocoelic injection of *E. coli* into *P. americana* in the presence and absence of DNase I was used by Nascimento *et al.* (2018) to evaluate the effect IHET destruction on bacteria spread *in vivo*. However, no HI-DNase I control was used in that experiment, casting doubt upon the validity of their interpretations of the results. Future studies must first confirm the endonuclease activity of DNase I *in vivo* before conducting further experiments. Activity of other DNases (*e.g.* DNase II) could be tested in *G. mellonella* to identify a working DNase *in vivo* with which to investigate the effects of IHETs.

The overall EPEC dose-dependent increase in mortality, decrease in survival time, delay in pupation, decrease in pupal mass, and the maintenance of egg hatch rate were consistent with the results obtained in Chapter 2 of this thesis, providing additional support for the presence and manifestation of EPEC-induced lethal and sublethal effects in *G. mellonella*. However, insect fecundity was independent of EPEC dose in Chapter 2 whereas a negative association between EPEC dose and insect fecundity was found in this study, indicating that the situation may be more complex than previously thought and require additional experiments with larger sample sizes to conclusively determine the effect of EPEC infection on *G. mellonella* fecundity.

4.4.3 Addition of extracellular DNA *in vivo*

The effects of IHETs *in vivo* against EPEC infection in *G. mellonella* could not be determined by the previous experiment due to the inhibition of DNase I. The present experiment used an alternative approach by the injection of EPEC in the presence or absence of *G.*

mellonella hemocyte DNA. Larvae injected with EPEC and DNA cleared EPEC faster and survived longer compared to larvae injected with EPEC alone, indicating that extracellular DNA confers protection to *G. mellonella* against EPEC. The cation chelation property of DNA destabilizes bacterial cell membrane on contact, resulting in the lysis of the bacterium (Halverson *et al.*, 2015). DNA also induce hemolymph coagulation in *G. mellonella* (Altincicek *et al.*, 2008). The amount of injected hemocyte DNA (500 ng) is equivalent to the complete DNA release from approximately 27% of total circulating hemocytes in a last instar *G. mellonella* larva, assuming genome size of 578 Mbp, average base pair mass of 650 Da, diploid hemocytes, and 1.46×10^6 circulating hemocytes per larva (Jones, 1967; Lange *et al.*, 2018). The procoagulant and antimicrobial activity of the injected DNA are likely responsible for enhanced trapping and killing of EPEC in *G. mellonella*, resulting in increased EPEC clearance rate and prolonged survival in the larvae injected with EPEC and DNA compared to the larvae injected with only EPEC. However, the injection of DNA did not reduce insect mortality. This is likely due to the absence of granule contents that are normally associated with NETs (and potentially associated with IHETs) reducing the efficacy of the injected DNA in trapping and killing EPEC. The injection of pure DNA may severely underrepresent the true antimicrobial capabilities of IHETs despite the increased cation chelation ability of pure DNA compared to chromatin due to the absence of histones. The injection of complete IHETs (*i.e.* decondensed chromatin mixed with granule contents) equivalent to 27% of *G. mellonella* hemocytes, if such an inoculum is possible to prepare, may be able to reduce insect mortality.

Insects from the control groups (*i.e.* larvae injected with Ringer's or DNA in Ringer's) did not differ from each other in mortality, survival time, or time to pupation, indicating that DNA is not toxic to *G. mellonella* at the dose of 500 ng/larva. The overall EPEC dose-dependent

increase in mortality, decrease in survival time, and delay in pupation were consistent with the results obtained in Chapter 2 of this thesis, providing additional support for the presence and manifestation of EPEC-induced lethal and sublethal effects in *G. mellonella*.

4.5 Conclusions

IHETs were observed from *G. mellonella* hemocytes *ex vivo* likely involving both granulocytes and oenocytoids. The release of IHETs were induced by EPEC or PMA under plasma-free conditions, with features resembling suicidal or vital NETosis. The lysis of oenocytoids may represent a novel form of ETosis unique to insects. However, additional research is needed to confirm hemocyte identity, characterize the processes and mechanisms of IHET release, and determine the antimicrobial activities of IHETs. The injection of DNase I, either active or heat-inactivated, were detrimental to *G. mellonella* in the presence of EPEC. The effect was independent of the destruction of IHETs due to DNase I inhibition *in vivo*, highlighting the importance of the use of appropriate experimental controls and proof-of-concept experiments. The injection of hemocyte DNA conferred limited protection to *G. mellonella* against EPEC by increasing EPEC clearance rate and insect survival time but did not reduce insect mortality. However, the injection of DNA likely underrepresented the true antimicrobial effects of IHETs due to the absence of other potential IHET components. Overall, the results from these experiments indicate that IHETs are released by *G. mellonella* hemocytes and confer protection to the insect against EPEC infection of the hemocoel, supporting the hypothesis that insect hemocytes release extracellular traps as a novel insect immune response that protects the insect against microbial infection of the hemocoel and making the *Galleria*-EPEC system a novel model for the study of extracellular traps.

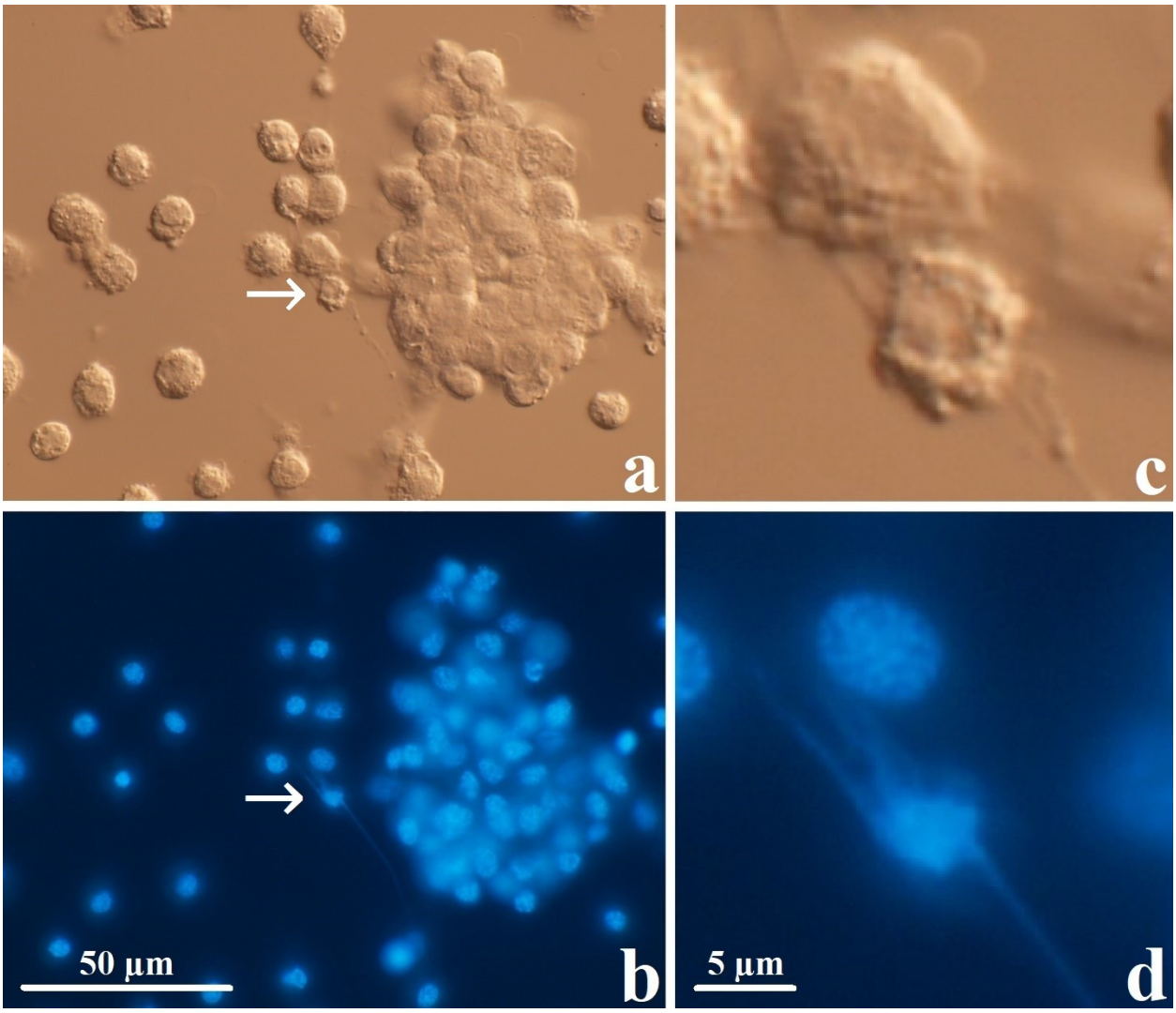

Figure 4.1 *G. mellonella* hemocytes stimulated for 1h with Ringer's, stained with Hoechst 33342, and visualized with DIC (a, c) and fluorescent microscopy (b, d). (a, b) Extracellular DNA was released by a granulocyte (arrow). (d) The nucleus of the granulocyte releasing DNA (d, bottom) was irregular in shaped and showed diffuse staining compared to an adjacent granulocyte (d, top). The extracellular DNA was visible by DIC microscopy as fibrillar structures.

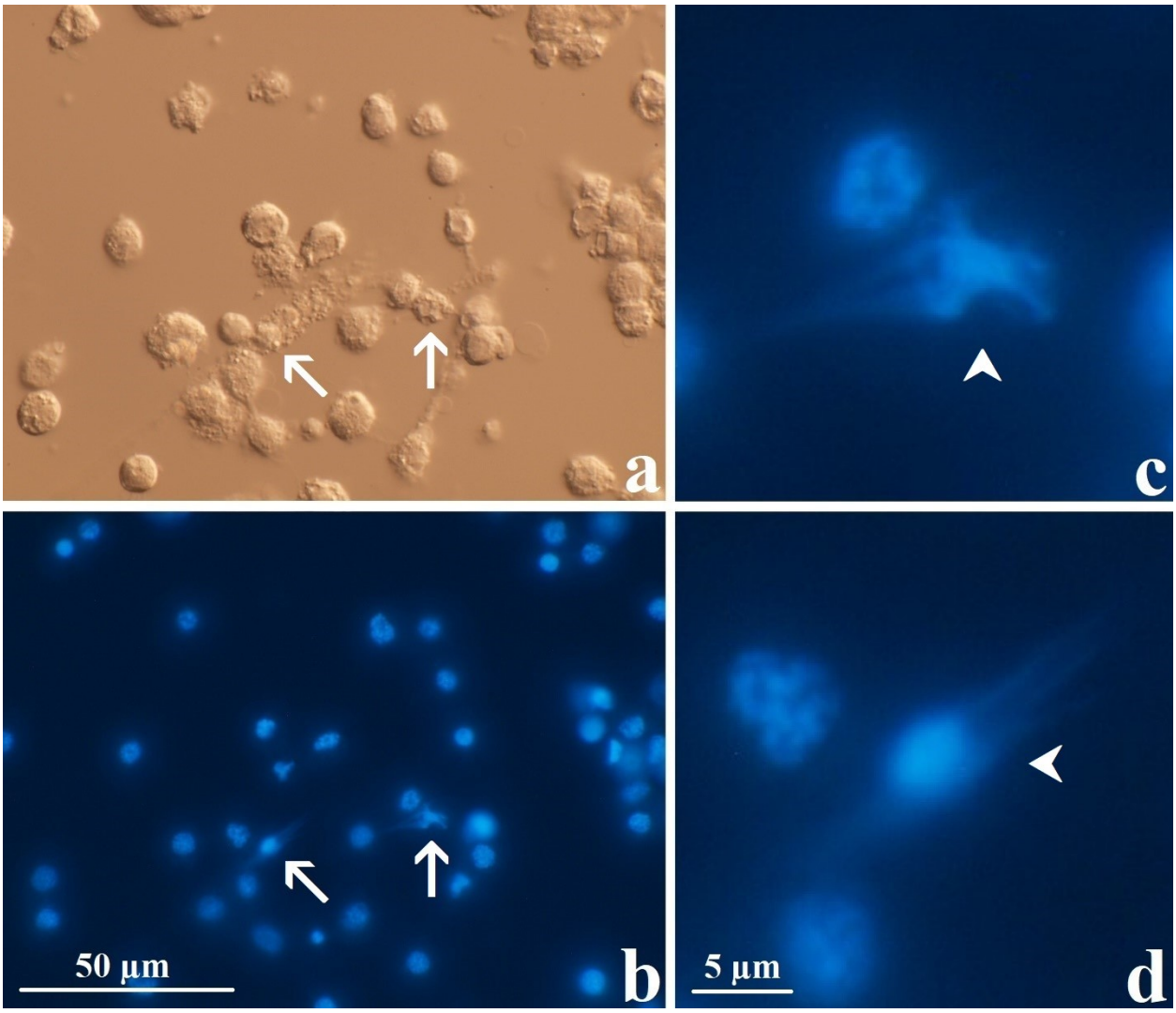

Figure 4.2 *G. mellonella* hemocytes stimulated for 1h with EPEC (5.0×10^4 CFU), stained with Hoechst 33342, and visualized with DIC (a) and fluorescent microscopy (b - d). (a, b) Extracellular DNA was released by an intact granulocyte (arrow, right) and a lysed granulocyte (arrow, left). (c, d) The nuclei of the granulocytes releasing DNA (arrowheads) were irregular in shape and showed diffuse staining compared to adjacent hemocytes.

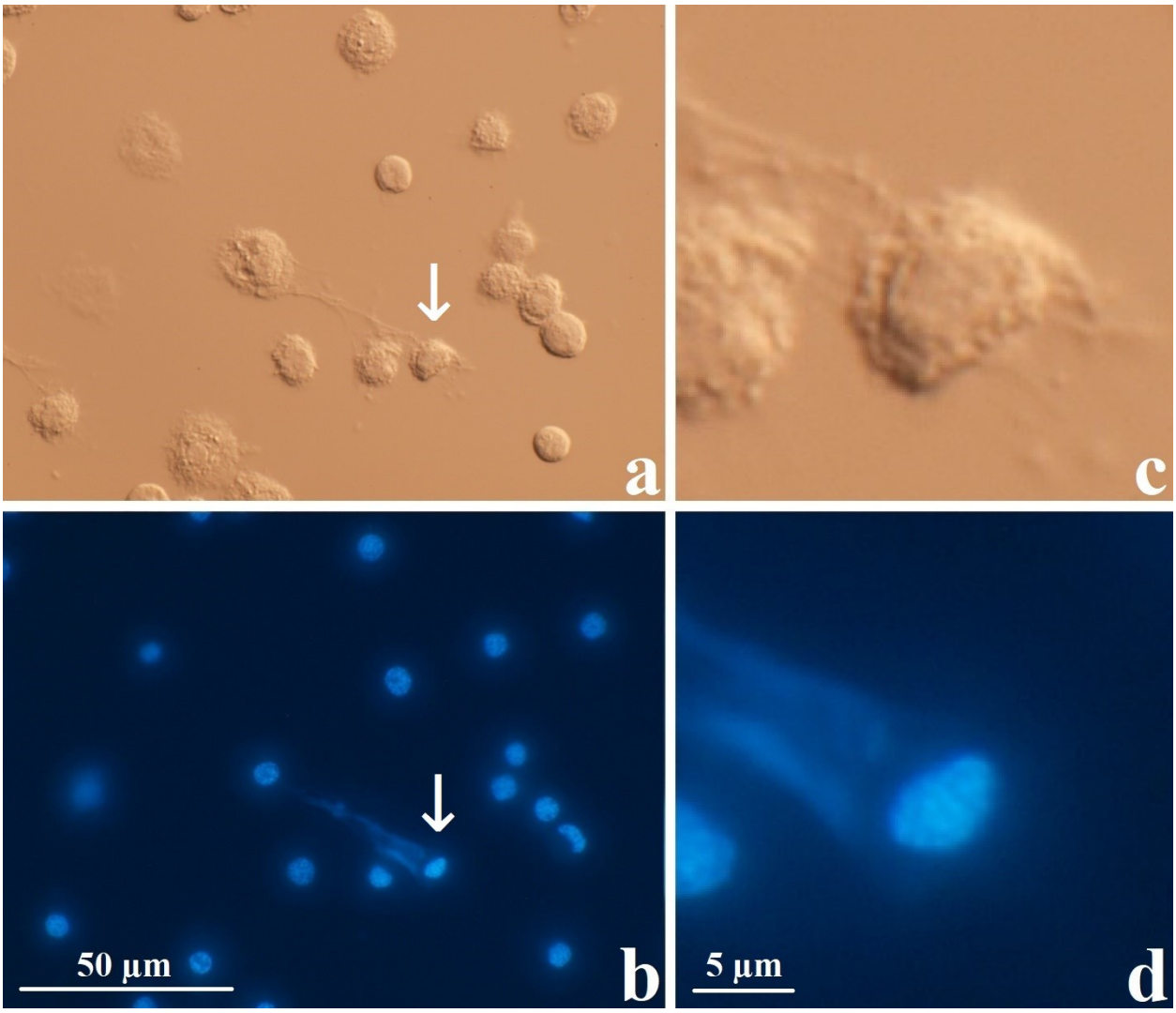

Figure 4.3 *G. mellonella* hemocytes stimulated for 1h with EPEC (5.0×10^4 CFU), stained with Hoechst 33342, and visualized with DIC (a, c) and fluorescent microscopy (b, d). (a, b) Extracellular DNA was release by a granulocyte (arrow) connecting to two nearby granulocytes by fibrillar extracellular structures. (c, d) The cell membrane and the nuclear envelope of the granulocyte appeared intact. The extracellular DNA appeared to have originated from the cytoplasm.

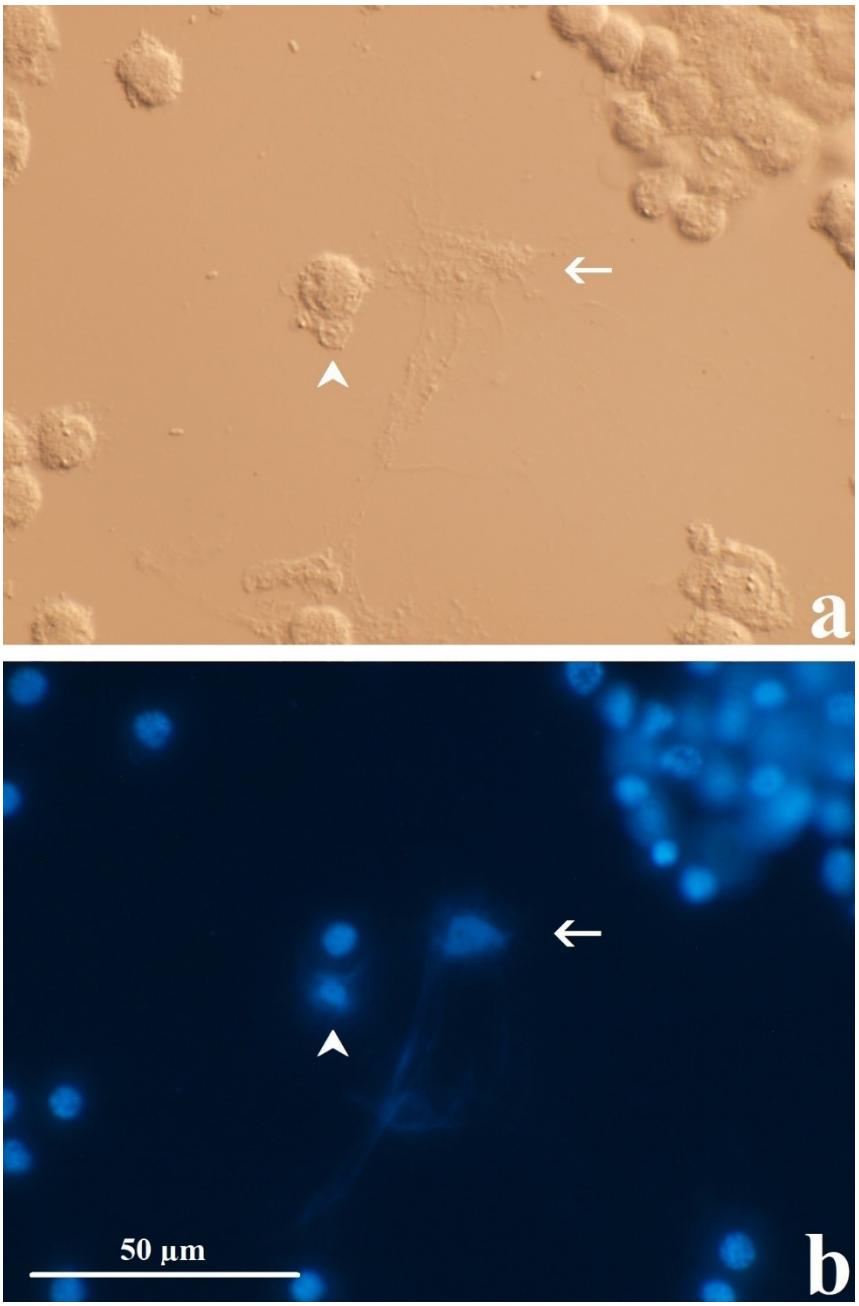

Figure 4.4 *G. mellonella* hemocytes stimulated for 1h with EPEC (5.0×10^4 CFU), stained with Hoechst 33342, and visualized with DIC (a) and fluorescent microscopy (b). Extracellular DNA was released by a lysed hemocyte (arrow). An exposed hemocyte nucleus (arrowhead) may also be releasing DNA.

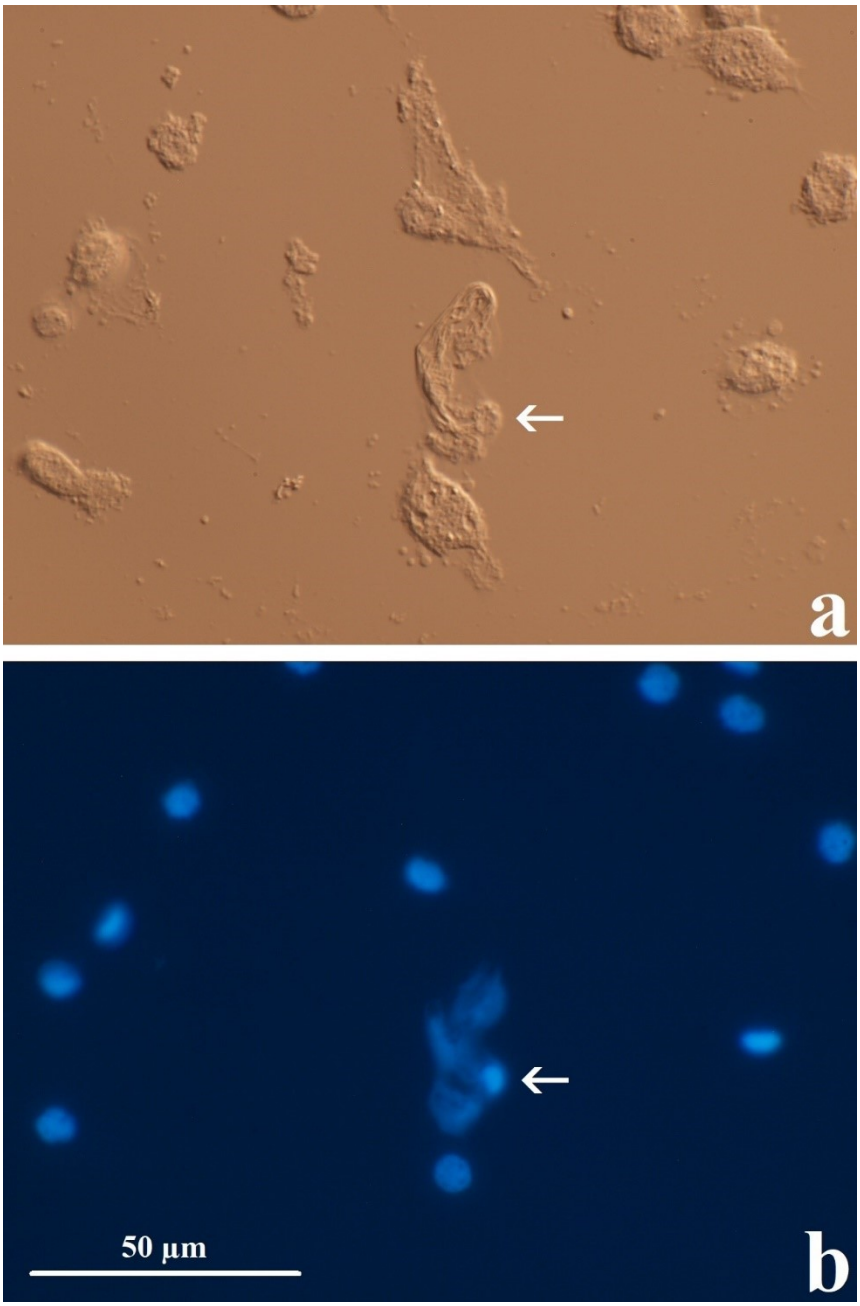

Figure 4.5 *G. mellonella* hemocytes stimulated for 1h with PMA (10 µg/mL), stained with Hoechst 33342, and visualized with DIC (a) and fluorescent microscopy (b). Extracellular DNA was released by a lysed hemocyte.

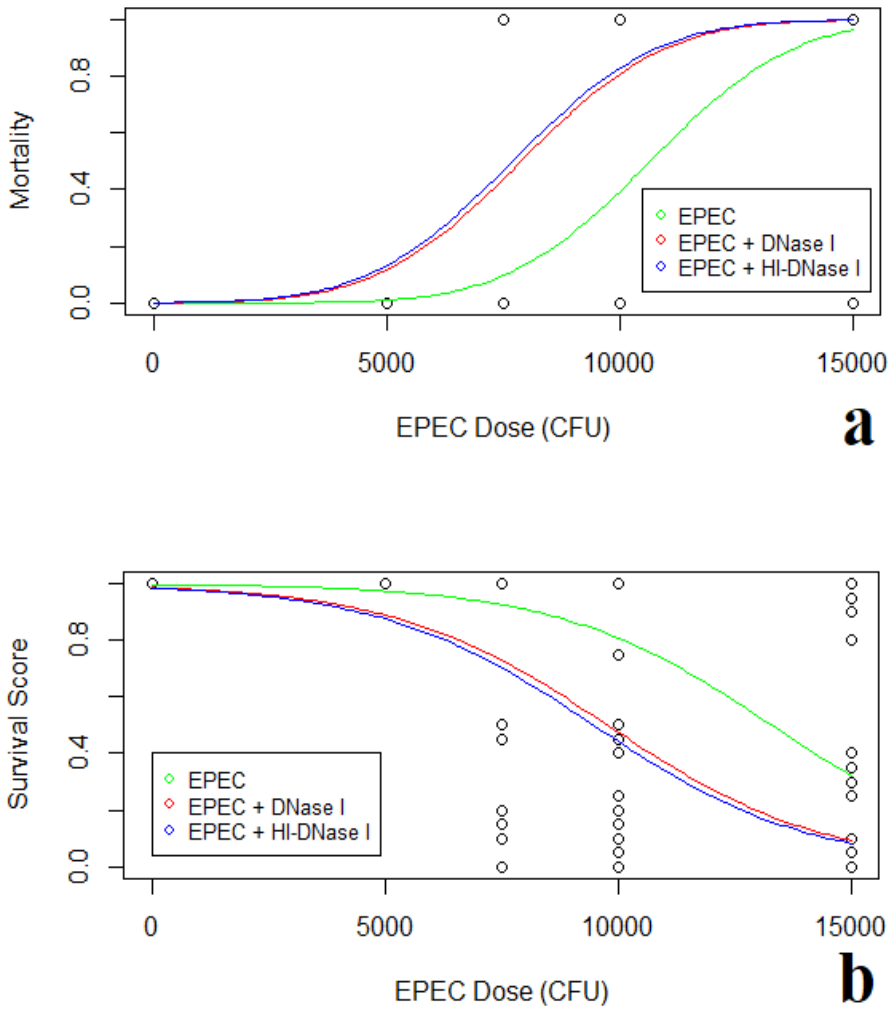

Figure 4.6 Mortality (a) and survival score (b) of *G. mellonella* larvae injected intrahemocoelically with various doses of EPEC in the presence and absence of 5U DNase I and HI-DNase I (n = 220). (a) Insect mortality was recorded as: 0 = alive and 1 = dead. Insects injected with EPEC + DNase I (LD_{50} = 7.88 × 10^3 CFU) and insects injected with EPEC + HI-DNase I (LD_{50} = 7.70 × 10^3 CFU) showed higher mortality compared to insects injected with EPEC alone (LD_{50} = 1.07 × 10^4 CFU). (b) Insects injected with EPEC + DNase I and insects injected with EPEC + HI-DNase I showed lower survival scores compared to insects injected with EPEC alone.

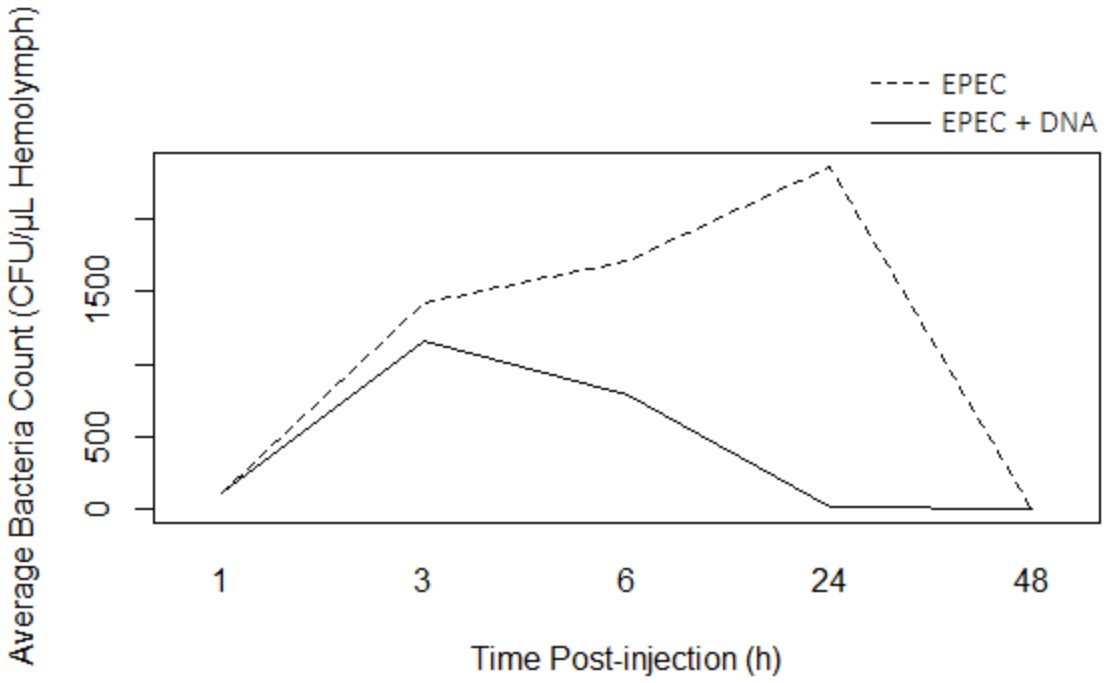

Figure 4.7 Average number of circulating EPEC per µL hemolymph at various time points post-injection in *G. mellonella* larvae injected intrahemocoelically with EPEC only and larvae injected with EPEC and 500 ng of DNA. EPEC was cleared faster and earlier in larvae injected with EPEC and DNA (24h post-injection) compared to larvae injected with EPEC alone (48h post-injection).

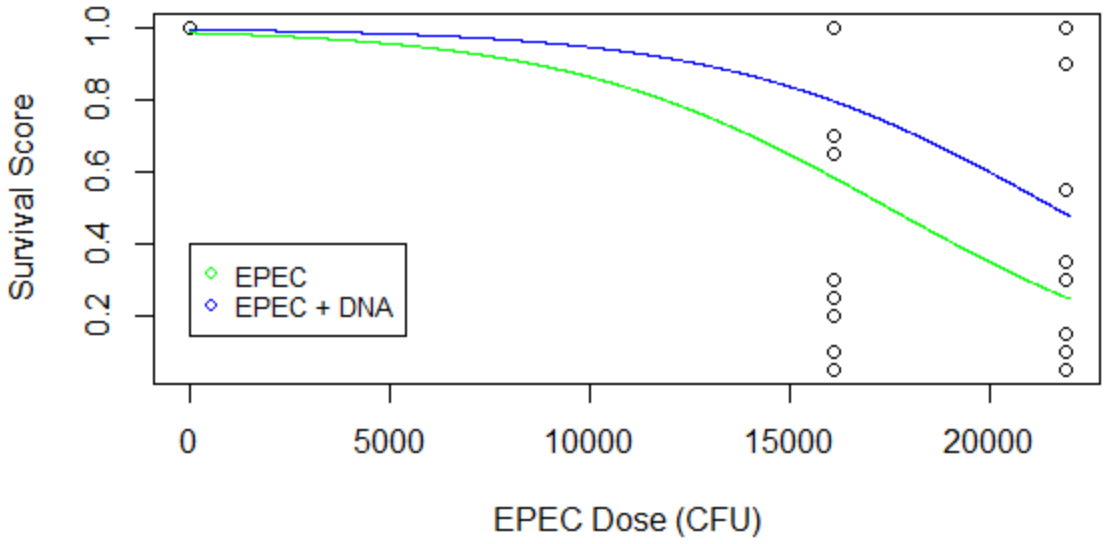

Figure 4.8 Survival score of *G. mellonella* larvae injected intrahemocoelically with EPEC only and larvae injected with EPEC and 500 ng of DNA (n = 80). Insects injected with EPEC and DNA showed higher survival scores (*i.e.* survived approximately 1 day longer on average) compared to insects injected with EPEC alone.

Table 4.1 Similarities and differences between NETosis and hemocyte extracellular DNA release observed in this study.

Cellular events	Suicidal NETosis	Vital NETosis (nuclear)	Vital NETosis (mitochondrial)	Pattern I (granulocytes)	Pattern II (granulocytes)	Pattern III (granulocytes and oenocytoids)
Chromatin decondensation	Yes	Yes	Unknown	Yes	No	Yes
Nuclear envelope rupture	Yes	No	No	Yes	No	Yes
Cell lysis	Yes	No	No	No	No	Yes

Chapter 5 The future of the *Galleria*-EPEC model system

This study began with the evaluation of EPEC virulence and pathogenicity in *G. mellonella* (Chapter 2), progressed to the characterization of *G. mellonella* immune responses against EPEC during which IHETs were discovered *in vivo* (Chapter 3), and ended with the investigations into the origin and function of IHETs (Chapter 4). Major findings in this study include: (1) EPEC is moderately virulent in the hemocoel of *G. mellonella* in a dose-dependent manner. EPEC virulence manifests as increased insect mortality, decreased survival time, delayed pupation, decreased pupal mass, and increased pupal duration. These life history traits could be used as novel metrics for the measurement of EPEC virulence in *G. mellonella* and may be applicable for other insect-pathogen models. (2) EPEC has low *per os* pathogenicity in *G. mellonella*, making EPEC pathogenesis in *G. mellonella* different from that of humans. However, the *Galleria*-EPEC model remains valuable for the ability to provide an inexpensive and efficient alternative to vertebrate models for the identification of potential virulence factors that may be involved in EPEC pathogenesis in humans. (3) The T3SS is only responsible for a minor portion of EPEC virulence in *G. mellonella*. The sources of the majority of the virulence are unknown and could lead to the discovery of novel virulence factors in EPEC pathogenesis in humans. (4) EPEC elicits typical insect antibacterial immune responses in the hemocoel of *G. mellonella*, including melanization, hemolymph coagulation, nodulation, and phagocytosis. Future studies can use this information to determine the effects of EPEC virulence factors on *G. mellonella* immunity. (5) The release of IHETs, a novel insect immune response, was documented for the first time *in vivo* using the *Galleria*-EPEC model system. (6) The release of IHETs (insect hemocyte extracellular traps) can be stimulated in *G. mellonella* hemocytes *ex vivo* using EPEC and PMA. (7) Extracellular DNA conferred protection to *G. mellonella* against

EPEC by increasing insect survival time and EPEC clearance rate in the hemolymph. These findings support the hypothesis that insect hemocytes release extracellular traps that protects the insect against microbial infection in the hemocoel and adds *G. mellonella* to the growing list of invertebrates capable of extracellular trap release.

This thesis provided extensive background knowledge on the *Galleria*-EPEC model system and identified numerous areas of future research. The most important topics of future study include: (1) The development of a comprehensive protocol to evaluate pathogen virulence in insect models that includes both lethal and sublethal effects, since the common practice of using only acute mortality in the current literature are insufficient in the determination of pathogen virulence factors in insects that contribute to sublethal pathology and delayed mortality which may also be involved in pathogen virulence in humans. (2) The identification of factors contributing to EPEC virulence in *G. mellonella* that were independent of the T3SS. These factors may also be important to EPEC virulence in humans. The virulence evaluation of different EPEC mutants in *G. mellonella* could be a starting point. (3) The characterization of immunopathology in *G. mellonella*, which is important in unraveling the contribution of the insect immune responses in the pathology of the *Galleria*-EPEC model system. Immunopathology may be investigated by monitoring *G. mellonella* life history after the intrahemocoelic injection of heat-killed EPEC at appropriate intervals and doses to achieve immune activation comparable to live EPEC infection without the involvement of secreted effectors. (4) The detailed characterization of IHET release using the *Galleria*-EPEC model system, including the conclusive identification of IHET-releasing hemocytes by fluorescent antibodies, the stepwise characterization of IHET release by time-lapse fluorescent microscopy, the determination of IHET composition by fluorescent microscopy (*e.g.* DNA, histone, and

lysozyme staining), the evaluation of antimicrobial activity of IHETs by microbial killing assays, the evaluation of the prevalence of IHET release in insects (*e.g.* members of different insect orders), the evaluation of the specificity of IHET release against different pathogens (*e.g.* bacteria, fungi, and protozoans), and the characterization of the biochemical mechanisms behind IHET release, possibly by insect mutagenesis and genetic screening.

Preliminary experiments revealed some interesting findings: (1) IHET release is not limited to the *Galleria*-EPEC system. *G. mellonella* also release IHETs *in vivo* against *C. rugosa* while *B. mori* release IHETs *in vivo* against EPEC. (2) Microbial surface components (LPS and β-glucan) induce IHET release by *G. mellonella* hemocytes *ex vivo*. Future experiments with higher sample sizes are required to verify these findings. These results, if repeatable, would support the idea of IHET release as a common insect immune response against microbes in the hemocoel.

The *Galleria*-EPEC model system has a bright future ahead in both the study of EPEC virulence and insect immunity. The serendipitous discovery of IHETs in *G. mellonella* marks the opening of an exciting new frontier in insect immunology, adding to its existing complexity and similarity to vertebrate innate immunity, and making *G. mellonella* a novel model organism for the study of extracellular traps.

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Appendix 1: Insect artificial diet

Appendix 1.1 *G. mellonella* artificial diet

Diet ingredient:	% by mass:
Wheat germ	28.2
Brewer's yeast	14.1
Beeswax (Shredded)	22.4
Glycerol (Sigma-Aldrich)	14.1
Honey	14.1
Water *	7.1
Glycerol, honey, and water were added to an Erlenmeyer flask and heated (without boiling) on a hotplate until the honey is fully dissolved using a magnetic stir bar. Wheat germ, brewer's yeast, and beeswax were mixed thoroughly with the solution in a mixing bowl. * Water used for all artificial diets, media, and solutions in this thesis was purified to approximately 15 MΩ-cm by a research grade ultrafiltration water polishing system (Modulab Model LBPUU 10 1002, Continental Water Systems).	

Appendix 1.2 *B. mori* artificial diet

Diet ingredient:	% by mass
Powdered Silkworm Chow (Recorp Inc.)	24.8
Bacto agar (BD)	0.7
Water	74.5
Agar and water were added to an Erlenmeyer flask and heated on a hotplate until fully dissolved using a magnetic stir bar. The solution was thoroughly mixed with the Powdered Silkworm Chow in a mixing bowl and allowed to congeal.	

Appendix 2: Solutions and media

Appendix 2.1 Insect Ringer's solution

Modified from Ephrussi & Beadle (1936)

Component:	Concentration (mM):
NaCl (Fisher)	128 (for *B. mori*) or 191.6 (for *G. mellonella*)*
$CaCl_2$ (Fisher)	18
KCl (Fisher)	1.3
$NaHCO_3 \cdot 2H_2O$ (Sigma-Aldrich)	2.3
pH: 6.0 (Measured) Osmolality: 314 mOs/kg (Calculated) Filter-sterilized (0.2 µm) * Osmolality of Ringer's solution was adjusted to 440 mOs/kg with NaCl to match *G. mellonella* hemolymph osmolality (Mead *et al.*, 1986).	

Appendix 2.2 Anticoagulant antimelanization solution

Modified from Haine *et al.* (2007)

Component:	Concentration (mM):
NaOH (Merck)	98
NaCl (Fisher)	145
Ethylenediaminetetraacetic acid (EDTA) (Fisher)	17
Citric acid (Fisher)	41
L-glutathione reduced (Sigma-Aldrich)	10
pH: 5.0 (Measured) Osmolality: 444 mOs/kg (Calculated) Filter-sterilized (0.2 µm)	

Appendix 2.3 Luria-Bertani medium

Modified from Bertani (1951)

Component:	Mass (g/500 mL medium):
Bacto tryptone (BD)	5
Bacto yeast extract (BD)	2.5
NaCl (Fisher)	5
Bacto agar (BD) *	7.5
Water	475
pH: 6.6 (Measured) Sterilized by autoclaving (Liquid cycle, 15 minutes) Final volume was adjusted to 500 mL * Bacto agar was used to make LB agar plates only.	

Appendix 2.4 Grace's insect medium

Modified from Grace (1962)

Category:	Component:	Mass (mg/L medium):
Salts	KCl (Fisher)	4100
	$CaCl_2 \cdot 2H_2O$ (Sigma-Aldrich)	1320
	$MgCl_2 \cdot 6H_2O$ (BDH)	2280
	$MgSO_4$ (BDH)	1360
	NaH_2PO_4 (Sigma-Aldrich)	866
	$NaHCO_3$ (Fisher)	350
Soluble Amino Acids (Sigma-Aldrich)	L-Arginine HCl	700
	L-Aspartic Acid	350
	L-Asparagine	350
	L-Alanine	225
	B-Alanine	200
	L-Glutamic Acid	600
	L-Glutamine	600
	Glycine	650
	L-Histidine	2500
	L-Isoleucine	50
	L-Leucine	75
	L-Lysine HCl	625
	L-Methionine	50
	L-Proline	350
	L-Phenylalanine	150
	DL-Serine	1100
	L-Threonine	175
	L-Valine	100
Insoluble Amino Acids	2N HCl	2 mL
	L-Cystine	44

(Sigma-Aldrich)	L-Tryptophan	200
	L-Tyrosine	100
Carbohydrates (Sigma-Aldrich)	α-Ketoglutaric Acid	370
	Fructose	400
	Fumaric Acid	55
	D-Glucose	700
	Malic Acid	670
	Succinic Acid	60
	Sucrose	26680
Vitamins (Sigma-Aldrich)	Thiamine HCl	0.02
	Riboflavin	0.02
	D-Ca Pantothenic Acid	0.02
	Pyridoxine HCl	0.02
	P-Aminobenzoic Acid	0.02
	Folic Acid	0.02
	Nicotinic Acid	0.02
	i-Inositol	0.02
	Biotin	0.01
	Choline Chloride	0.2
Water		927 mL
pH: 6.1 (Adjusted with 10N KOH) Final volume was adjusted to 1L Filter-sterilized (0.2 μm) Medium was prepared and provided by G. J. Hilchie (University of Alberta).		

Appendix 2.5 Literature cited

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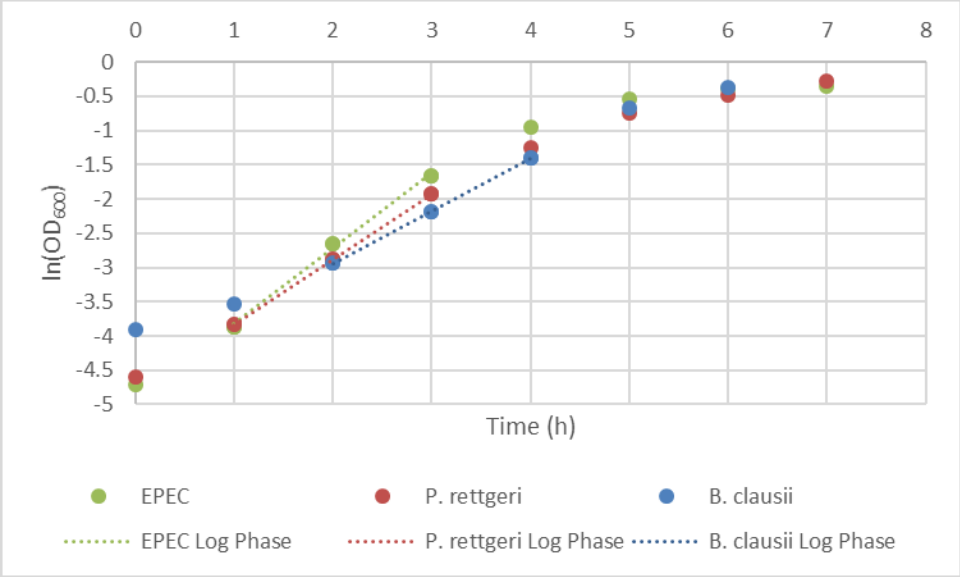
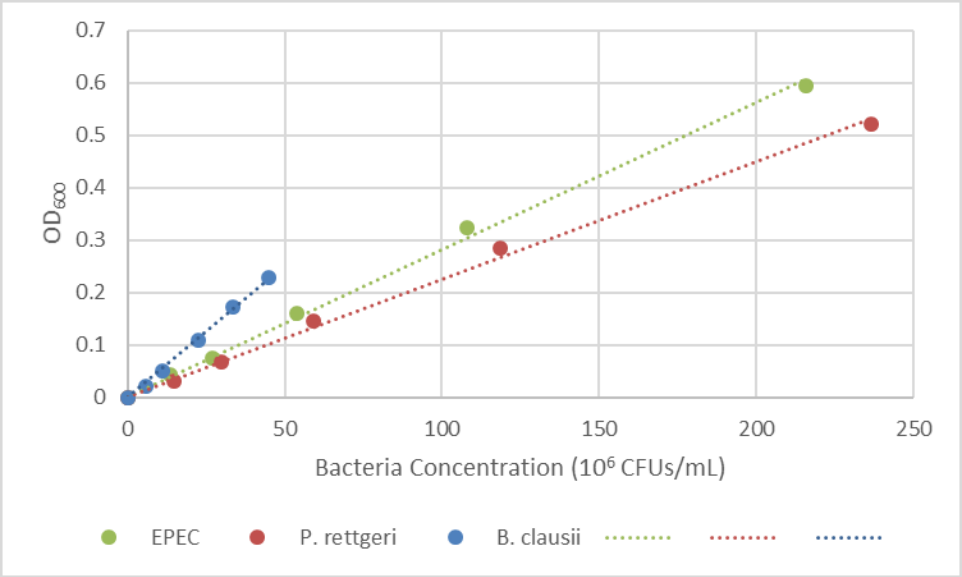
Appendix 3: Insect life cycles and development times

Insect	Temperature	Egg to larva	Larva to pupa	Pupa to adult
G. mellonella	30°C	~2 weeks	~6 weeks	~1 week
B. mori	27°C	~2 weeks	~4 weeks	~2 weeks

Appendix 4: Bacterial growth curves and standard curves



Appendix 4.1 The growth curve of EPEC, *P. rettgeri*, and *B. clausii* in LB medium at 30°C. Overnight culture of bacteria were used to inoculate LB medium and the absorbance values of the culture at 600 nm (OD_{600}) were measured hourly for 6 or 7 hours. The natural logarithms of the OD_{600} values over time during log phase growth (dotted lines) were used to calculate the generation times of the bacteria. The generation time of EPEC, *P. rettgeri*, and *B. clausii* under these culture conditions are 38 min, 43 min, and 55 min, respectively.



Appendix 4.2 The standard curve of EPEC, *P. rettgeri*, and *B. clausii* in Ringer's. Log phase bacteria harvested by centrifugation (Eppendorf 5415L) at 5000 rpm (2040 g) for 5 minutes. The pellet was washed and resuspended in ice-cold Ringer's then diluted on ice: 100% (Undiluted), 50%, 25%, 12.5%, 6.25%, 0% (Ringer's). The absorbance values at 600 nm for each dilution were measured. Bacteria were quantified by plate count (LB agar). Linear regression was performed in Microsoft Office 365 to establish the relationship between absorbance values and bacteria concentration: [EPEC](CFU/mL) = $OD_{600} \times 2.81 \times 10^9$, $R^2 = 0.998$; [*P. rettgeri*](CFU/mL) = $OD_{600} \times 2.25 \times 10^9$, $R^2 = 0.997$; [*B. clausii*](CFU/mL) = $OD_{600} \times 5.02 \times 10^9$, $R^2 = 0.998$. A similar protocol (pellet by centrifugation → wash with Ringer's → resuspend in Ringer's → measure OD_{600} → dilute to appropriate concentration) was used to prepare inoculum for injections.

Appendix 5: Experimental designs

Appendix 5.1 Experimental design for Chapter 2.2.4

(*G. mellonella*, intrahemocoelic)

EPEC dose (CFU)	Number of larvae injected
0 (Ringer's Control)	91
$1.0 \times 10^2 – 2.5 \times 10^3$	30
$4.0 \times 10^3 – 5.0 \times 10^3$	20
$7.0 \times 10^3 – 1.0 \times 10^4$	38
$1.3 \times 10^4 – 1.7 \times 10^4$	60
$1.8 \times 10^4 – 2.3 \times 10^4$	47
$2.9 \times 10^4 – 3.3 \times 10^4$	43
4.0×10^4	20

Appendix 5.2 Experimental design for Chapter 2.2.5

(*G. mellonella*, intrahemocoelic)

EPEC dose (CFU)	Number of larvae injected
0 (Ringer's Control)	10
1.4×10^2	10
1.4×10^3	10
2.1×10^3	10
4.2×10^3	5

Appendix 5.3 Experimental design for Chapter 2.2.6

(*G. mellonella*, *per os*)

EPEC dose (CFU)	Number of larvae injected
0 (Ringer's Control)	10
2.2×10^6	10
2.5×10^6	20
1.1×10^7	10
2.5×10^7	20

Appendix 5.4 Experimental design for Chapter 2.2.7

(*G. mellonella*, intrahemocoelic)

Δ*escN* dose (CFU)	Number of larvae injected
0 (Ringer's Control)	20
4.5×10^3	10
4.5×10^4	10
4.9×10^4	10
7.4×10^4	10
9.7×10^4	10
1.5×10^5	10
2.9×10^5	10
4.5×10^5	10
9.0×10^6	10

DH5α dose (CFU)	Number of larvae injected
0 (Ringer's Control)	5
2.0×10^1	10
2.0×10^2	10
2.0×10^3	10
2.0×10^4	20
1.9×10^5	10
7.8×10^6	10
4.1×10^7	10

P. rettgeri dose (CFU)	Number of larvae injected
0 (Ringer's Control)	10
1.8×10^2	10
1.8×10^3	10
1.8×10^4	10
2.6×10^4	10

B. clausii dose (CFU)	Number of larvae injected
0 (Ringer's Control)	25
3.5×10^2	10
3.5×10^4	10
4.6×10^6	10

Appendix 5.5 Experimental design for Chapter 2.2.8

(*B. mori*, intrahemocoelic)

EPEC dose (CFU)	Number of larvae injected
0 (Ringer's Control)	57
2.0×10^2	10
2.0×10^4	10
1.0×10^6	20
2.0×10^6	42
5.0×10^6	30

Appendix 5.6 Experimental design for Chapter 4.2.2

(*G. mellonella*, intrahemocoelic)

Treatment	EPEC dose (CFU)	DNase I (5U)	Larvae injected
EPEC	0	Absent	10
EPEC + DNaseI	0	Active	55
EPEC + HI-DNase I	0	Heat-inactivated	25
EPEC	5.0×10^3	Absent	10
EPEC + DNaseI	5.0×10^3	Active	10
EPEC + HI-DNase I	5.0×10^3	Heat-inactivated	10
EPEC + DNaseI	7.5×10^3	Active	10
EPEC + HI-DNase I	7.5×10^3	Heat-inactivated	10
EPEC	1.0×10^4	Absent	10
EPEC + DNaseI	1.0×10^4	Active	20
EPEC + HI-DNase I	1.0×10^4	Heat-inactivated	20
EPEC	1.5×10^4	Absent	10
EPEC + DNaseI	1.5×10^4	Active	10
EPEC + HI-DNase I	1.5×10^4	Heat-inactivated	10

Appendix 5.7 Experimental design for Chapter 4.2.3

(*G. mellonella*, intrahemocoelic)

Treatment	EPEC dose (CFU)	DNA (ng)	Larvae injected	Larvae bled
EPEC	0	0	60	40
EPEC + DNA	0	500	60	40
EPEC	1.6×10^4	0	30	20
EPEC + DNA	1.6×10^4	500	30	20
EPEC	2.2×10^4	0	30	20
EPEC + DNA	2.2×10^4	500	30	20