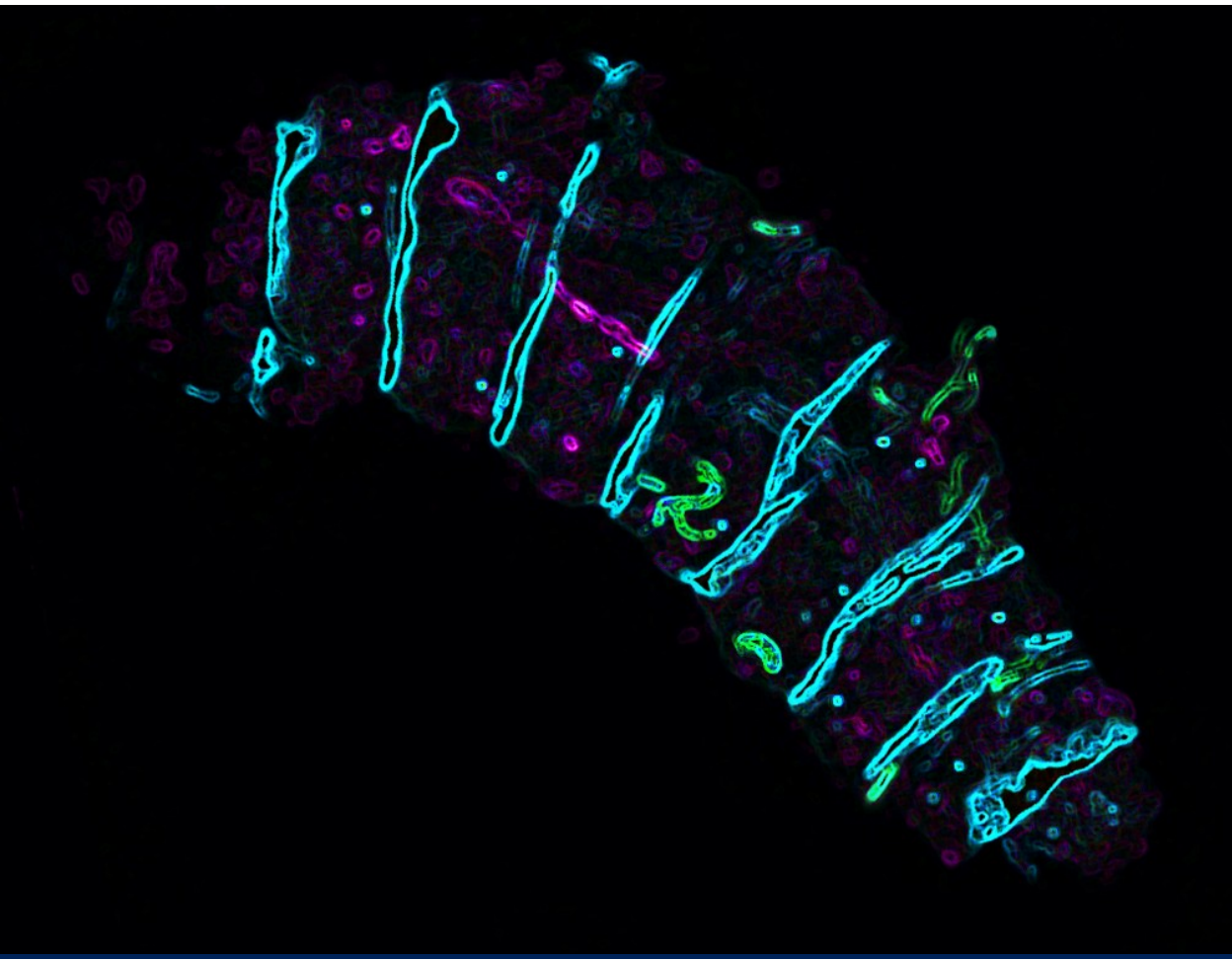
Stockholm University

Timing matters

Wounding and entomopathogenic nematode infection kinetics

Alexis Dziedziech



Doctoral Thesis in Molecular Bioscience at Stockholm University, Sweden 2021

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Abstract

Over time, insects have developed complex strategies to defend themselves against presenting threats. However, in the evolutionary arms race of survival, pathogens have adapted to quickly overcome the immune response mounted by the host. In this thesis, we assess how quickly entomopathogenic nematodes (EPNs) can overcome the host, *Drosophila melanogaster*. We then look at the clotting reaction at a hypothetical point of entry for the nematode and bring resolution to the order of protein interaction focusing on three proteins important in the anti-nematode defense. Finally, we look closer into detail at how crystal cells secrete one of those proteins, prophenoloxidase (PPOII) using a mode of programmed cell death.

(**Paper I**) In the course of EPN infection, little was known about how quickly the worms can overcome the host immune system. Here we found that after penetrating the host, EPNs cause septicemia within 4 to 6 hours. (**Paper II**) Three proteins, Glutactin (Glt), Transglutaminase (Tg), and PPOII have been found to be important in the anti-nematode response. Here we created GFP-tagged fly constructs to follow their role in clot formation. In early clot formation, Tg was immediately secreted from hemocytes though it was localized around the cell membrane, Glt then entered clot fibers followed by PPOII which acted in late clot formation. (**Paper III**) Here we looked closer into Tg and PPOII secretion variability. PPOII from immature, but not mature crystal cells colocalized with a membrane marker. Tg, when driven with a pan tissue driver, was found located in clotting fibers, in contrast with paper II. (**Paper IV**) In an *in vivo* immune scenario, crystal cells were recruited to the wound site and burst rapidly in a caspase-dependent manner. We demonstrate that the mode of programmed cell death, pyroptosis, exists in *Drosophila* by way of convergent evolution.

This thesis brings to light the variation found within the infection process for EPNs as well as the clotting response based on larval age, tissue type, and the maturity of a single cell type. Timing in each of these immune scenarios can give very different indications about the kind of immune response mounted and even the role of an individual cell.

Keywords: *Drosophila melanogaster, Heterorhabditis bacteriophora, Photorhabdus luminescens, entomopathogenic nematodes, worms, high-resolution microscopy, time-lapse, infection, kinetics, sepsis, septic wounding, injury, clotting, glutactin, transglutaminase, prophenoloxidase, cell death, pyroptosis, caspase.*

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Department of Molecular Biosciences, The Wenner-Gren Institute

Stockholm University, 106 91 Stockholm

Stockholm University

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Alexis Dziedziech

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"A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale."
~ Marie Curie

List of papers

I. **Dziedziech, A**., Shivankar, S., Theopold, U. (2020). High-Resolution Infection Kinetics of Entomopathogenic Nematodes Entering *Drosophila melanogaster*. Insects, 11, 60.

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III. **Dziedziech, A.**, Schmid, M., Arefin, B., Kienzle, T., Krautz, R., & Theopold, U. (2019). Data on *Drosophila* clots and hemocyte morphologies using GFP-tagged secretory proteins: Prophenoloxidase and Transglutaminase. Data in brief, 25, 104229.

IV. **Dziedziech, A.**, Theopold. U., (2021). Convergent evolution of pyroptosis, a caspase-dependent inflammatory cell death mechanism, in *Drosophila melanogaster*. (Manuscript).

Other publications

I. Zhao, Y., Duan, J., **Dziedziech, A.**, Büttner, S., & Engström, Y. (2020). Bab2 activates JNK signaling to reprogram *Drosophila* wing disc development. bioRxiv. Pre-print.

II. Theopold, U., **Dziedziech, A.**, & Hyrsl, P. (2020). Insects, Nematodes, and Their Symbiotic Bacteria. Insects, 11(9), 577. Editorial.

III. Chan, A., **Dziedziech, A**., Kirkman, L. A., Deitsch, K. W., & Ankarklev, J. (2020). A histone methyltransferase inhibitor can reverse epigenetically acquired drug resistance in the malaria parasite Plasmodium falciparum. Antimicrobial Agents and Chemotherapy, 64(6).

IV. **Dziedziech, A.**, Shivankar, S., Theopold, U. (2020). *Drosophila melanogaste*r Responses against Entomopathogenic Nematodes: Focus on Hemolymph Clots. Insects, 11, 62. Review.

V. **Dziedziech, A.***, Khalili, D.*, Theopold, U. (2018) Digging back in Evolution: Danger in Drosophila. Journal of Damage-Associated Molecular Patterns. JDAMP, 1(1): 1-8. (*shared first author). Review.

VI. Kirkman, L. A., Zhan, W., Visone, J., **Dziedziech, A.**, Singh, P. K., Fan, H., ... & Imaeda, T. (2018). Antimalarial proteasome inhibitor reveals collateral sensitivity from intersubunit interactions and fitness cost of resistance. Proceedings of the National Academy of Sciences, 201806109.

VII. Simon, M. S., Westblade, L. F., **Dziedziech, A.**, Visone, J. E., Furman, R. R., Jenkins, S. G., ... & Kirkman, L. A. (2017). Clinical and molecular evidence of atovaquone and azithromycin resistance in relapsed *Babesia microti* infection associated with rituximab and chronic lymphocytic leukemia. Clinical Infectious Diseases, 65(7), 1222-1225.

Thesis Abstract

Over time, insects have developed complex strategies to defend themselves against presenting threats. However, in the evolutionary arms race of survival, pathogens have adapted to quickly overcome the immune response mounted by the host. In this thesis, we assess how quickly entomopathogenic nematodes (EPNs) can overcome the host, *Drosophila melanogaster*. We then look at the clotting reaction at a hypothetical point of entry for the nematode and bring resolution to the order of protein interaction focusing on three proteins important in the anti-nematode defense. Finally, we look closer into detail at how crystal cells secrete one of those proteins, prophenoloxidase (PPOII) using a mode of programmed cell death.

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Keywords: *Drosophila melanogaster; Heterorhabditis bacteriophora; Photorhabdus luminescens; entomopathogenic nematodes; worms; high-resolution microscopy; time-lapse; infection; kinetics; sepsis; septic wounding; injury; clotting; glutactin; transglutaminase; prophenoloxidase; cell death; pyroptosis; caspase.*

Contents

Aims and objectives

Insects have evolved to evade infection from pathogens like Entomopathogenic nematodes (EPNs) and to quickly heal after septic injury. These processes are complex and time-dependent. To bring further resolution to the infection and wound healing process, this thesis aims to:

1. Determine the most probable time of when a host can overcome EPN infections.
2. Examine the clotting process using GFP-tagged proteins important in the anti-nematode defense
3. Explore the variability in the clotting reaction and secretion mechanisms of important wound healing proteins
4. Determine if and when crystal cells are recruited to the wound site and how they are activated

List of abbreviations

Abbreviation	Definition
AKH	Adipokinetic Hormone
AMP	Antimicrobial Peptide
ATP	Adenosine Triphosphate
DAMP	Danger-Associated Molecular Pattern
DAP	Diaminopimelic acid
DAR	DAMP-Associated Response
DCV	Drosophila C Virus
DDC	Dopa Decarboxylase
DIAP	Drosophila Inhibitor of Apoptosis Protein
DNA	Deoxyribonucleic acid
ECM	Extracellular Matrix
EPC	Entomopathogenic Complex
EPF	Entomopathogenic Fungi
EPN	Entompathogenic Nematodes
ERK	Extracellular signal-Regulated Kinases
FIM	FTIR Imaging Method
FTIR	Frustrated Total Internal Reflection
GAP	GTPase Activating Proteins
GDP	Guanosine Diphosphate
GEF	Guanine Nucleotide Exchange Factors
GFP	Green Fluorescent Protein
Glt	Glutactin
GNBP3	Gram-Negative Binding Protein 3
GTP	Guanosine Triphosphate
IAP	Inhibitor of Apoptosis Proteins
IDGF3	Imaginal Disc Growth Factor 3
IJ	Infectious Juveniles
IMD	Immune Deficiency

JAK/STAT	Janus Kinase protein and the Signal Transducer and Activator of Transcription
JNK	Jun-N-terminal Kinase
LPS	Lipopolysaccharides
LWR	Local Wound Response
MAMP	Microbe-Associated Molecular Patterns
NAC	N-Acetyl Cysteine
NETs	Neutrophil Extracellular Traps
NF-κB	Nuclear Factor Kappa B
PAS	PPO Activating System
PCD	Programmed Cell Death
PDGF	Platelet-Derived Growth Factor
PGN	Peptidoglycans
PGRP	Peptidoglycan Recognition Proteins
$PI(3,4,5)P_3$	Phosphatidylinositol (3,4,5)-Trisphosphate
PI3K	Phosphatidylinositol 3-Kinase
PO	Phenoloxidase
PPO	Prophenoloxidase
PPOAE	Prophenoloxidase-activating Enzyme
PRR	Pattern Recognition Receptors
Pvf	PDGF/VEGF-like factor
RNA	Ribonucleic Acid
RNAi	Ribonucleic Acid interference
ROS	Reactive Oxygen Species
Serpin	Serine Protease Inhibitor
Spp	Two or more species
SWR	Systemic Wound Response
TEP3	Thioester-containing Protein 3
TG	Transglutaminase
TH	Tyrosine Hydroxylase
TNF	Tumor Necrosis Factor
TNFR	Tumor Necrosis Factor Receptor
tTG	Tissue Transglutaminase
Vasp	Vasodilator-stimulated phosphoprotein
VEGF	Vascular Endothelial Growth Factor

Chapter 1. Host parasite-interactions

1.1 | Insect immunity. Insects are a group of organisms that likely evolved from crustaceans about 400 million years ago [1]. In this time, insects have been developing intricate immune responses in order to evade the threat of parasites and septic injury. To prevent a parasite from successfully entering the host, insects have developed strategies to evade invasion altogether, so-called, behavioral immunity. Some such strategies include using concealment, physical counter-attack, self-medicating, and spatial avoidance [2]. Social insects, like ants and honeybees, have also evolved strategies to avoid the spread of pathogens within a nest or hive, like the acts of grooming and removal of pests, which are common social immunity behaviors. Others include the removal of infested individuals, socially generated fevers, and interestingly, the spread of antifungal or antimicrobial substances in the community nest to stop pathogens from spreading [3–5]. At the individual level, the first layer of defense is the epidermal layer, a physical barrier separating one's self (internal organs) from non-self (external environment). Vulnerable entry points include the mouth and anal cavities, however, insects have developed defenses in these openings too. Microbes can induce the production of **r**eactive **o**xygen **s**pecies (ROS) in the gut triggering changes in gene expression, hormone levels, and cellular defense systems, as well as act as effectors themselves. Insects possess a robust innate immune system while lacking an adaptive immune system, specific to vertebrates. The innate immune system is non-specific and has both humoral and cellular arms which aid in defense. These defenses are carried out largely by the two major immune organs, the fat body and hemocytes.

The fat body, unique to insects but akin to the mammalian liver, is the largest insect organ and plays an essential role in metabolism, immunity, and secretion. This immunosecretory organ plays a major role in the humoral defense

system through the secretion of soluble effector molecules into the open circulatory system, necessary precursors in different signaling cascades. Some of these secretions are toxic to invading microbes such as some potent yet small molecules known as **a**nti**m**icrobial **p**eptides (AMPs). AMPs (often 12-40 amino acids in length) act through binding with specific bacteria or fungi leading to their eventual destruction. AMPs are secreted from both the fat body and hemocytes. Both immunosecretory organs are essential players in the melanization cascade, an important humoral defense strategy in wound healing that is catalyzed by the secretion of either **p**ro**p**henol**o**xidase (PPO) I, II, or III. Wound healing comprises the recognition of both intrinsic and extrinsic threats such as recognition of **D**anger-**a**ssociated **m**olecular **p**atterns, DAMPs, (loss of tissue integrity or damage) or **m**icrobe-**a**ssociated **m**olecular **p**atterns, MAMPs, (detection of foreign genetic material). It also involves the differentiation between a local, specific response, and a systemic, unspecific response. In a local response, *e.g.* a wound site, there exists orchestration for the release of soluble effector molecules, cell migration to the wound site, and coagulation reactions both enzymatically and chemically induced. In a systemic wound response, while potentially triggered by a local event, there is a long-range, global induction of an immune response through **red**uction/**ox**idation (redox) reactions [6]. This induction can affect all the internal tissues and includes the release of AMPs, cytokines, and/or other danger signals [6,7]. Both responses can lead to either controlled melanization which can be either protective against a pathogenic threat or uncontrolled melanization which can be lethal [6,8–11].

The cellular immune defense, largely employed by hemocytes, is comprised of many cell behaviors including encapsulation, nodulation, phagocytosis, cell migration, secretion, and cell death. In encapsulation and nodulation, the combined effort of multiple cells can lead to many cells surrounding and engulfing a large foreign body, like a wasp egg, or the entrapment of large numbers of bacteria, respectively [12]. Phagocytosis is the process of engulfing foreign invaders using the plasma membrane, an important cellular organelle also utilized and manipulated in cell migration, secretion, and cell death for the movement towards or against a stimulus, the secretion of particular proteins, or even the release of all its cytoplasmic contents [13–15]. When immune cells fail to recognize a threat, such as in the case of viral infections evading detection within an intracellular compartment of an infected cell, a cell can

undergo apoptosis and self-execute should it find itself working at suboptimal levels [16]. Once foreign genetic material is detected, it can be targeted by **r**ibo**n**ucleic **a**cid **i**nterference, or RNAi [17]. This process will degrade mRNA molecules to silence their expression. Finally, some long-lasting immunity may be transferred in the insect after its initial confrontation with a given bacteria, fungi, or virus, a process referred to as immune priming [18]. The genetic material can be recorded in an infected cell and neighboring immune cells can be alerted of the sequence via informational vesicles that aid in mounting a response against this threat in the future [19]. Information can even be passed transgenerationally [20].

1.2 | Insect pathogens. Just as insects come in many shapes and sizes, so do the pathogenic threats that ail them. Some such threats exist in the form of viruses, bacteria, protozoans, fungi, parasitoid wasps, and worms. Generally, pathogens are considered further along in the evolutionary process of overcoming the host immune system, largely because of their larger population sizes and their shorter generation times. Viruses in insects can be either double or single-stranded DNA or RNA viruses and are transmitted vertically within the population. They can be insect-specific and may well play important roles in manipulating phenotypic and behavioral traits in insects [21]. Most bacteria enter the insect hosts orally or through septic injury, though some enter through symbiotic invasion such as from inside the guts of infectious nematodes. Despite bacterial infection, genetic variation will confer resistance to the infection load depending on the bacterial species [22]. Further, some bacteria, like *Wolbachia*, may contextually either act as an endosymbiont or decrease host fitness [23]. Single-celled eukaryotic protozoans (e.g., malaria) also infect insects. One such group, Microsporidia are typically ingested and invade the host via gut epithelial cells which can lead to the devastation of insect nests. This is of particular concern when agriculturally favored insects, such as honey bees are afflicted or favorable against pests and used as a biological control agent [24,25]. Another group of eukaryotes, fungi, can be either symbiotic in the gut within insect diets or cause infection. These **e**ntomo**p**athogenic (insect infecting) **f**ungi (EPFs) invade through the integument, the outermost tissue of the insect, and extend hyphae which

reach the hemocoel, the blood, to establish infection. EPFs have been an asset in crop pest management and more EPF products are being developed based on the species *B. bassiana* and *M. anisopliae*, among others [26]. Further, parasitoid wasps can use insect larvae as a safe vehicle for egg development and will thus inject their eggs into the inner cavity of the larva. Polydnavirus, venom, or ovarian proteins may be injected with the egg to aid in successful embryo development [27]. The larval body is also the preferred hatching ground for another group of parasites, infectious worms.

1.3 | Entomopathogenic nematodes. A natural infection model.

Nematodes, a type of parasitic worm, are a threat to major agricultural productions and have an infection prevalence of up to 50% in all humans [28]. Two major nematode threats to humans are Elephantiasis, a filarial worm that infects the lymph system and Onchocerciasis, a worm that causes blindness and uses the Black Fly as a vector [29,30]. **E**ntomo**p**athogenic **n**ematodes (EPNs) are a natural threat to insect larvae and have been found to parasitize *Drosophila* larvae [31]. They use their hook-like tooth to breach the integument of the host and enter the hemocoel, or they can enter via the mouth or gut. Once inside, they regurgitate their symbiotic gut bacteria which subsequently proliferate in the cavity of the insect and release toxic proteins, such as “makes caterpillars floppy,” that break down the internal host tissues [32–34]. Degradation of the internal host structure signals to the infectious juveniles that it is time to sexually differentiate and proceed in the generational cycle until mass exodus from the insect cadaver occurs (Fig. 1).

In larval infection assays, there are two main genera of EPNs that are frequently used, *Steinernema* and *Heterorhabditis*. They form an infectious complex with their symbiotic gut bacteria which are gram-negative, proteogamma bacteria, such as *Photorhabdus spp*. and *Xenorhabdus spp*. in *Heterorhabditis spp*. and *Steinernema spp*. nematodes, respectively [35]. *Steinernema* is reported to be more pathogenic to some hosts (like *Drosophila* and *Galleria*) and gain access to the hemocoel by ambushing their hosts, an effective strategy against highly motile hosts. In contrast, *Heterorhabditis* tends to cruise, a better attack strategy against more stationary hosts [36]. An-

other theory to explain the different attack strategies and levels of pathogenicity pertains to their evolutionary history in that these two genera are not closely related and underwent divergent evolution, but results are not conclusive [37,38].

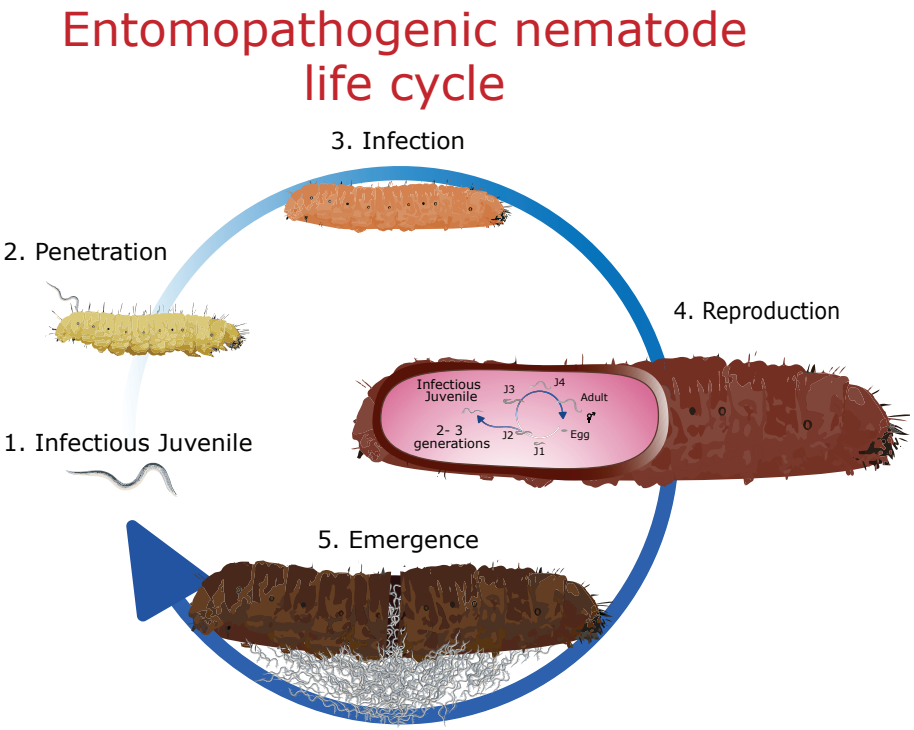

Figure 1: The Entomopathogenic nematode life cycle. First, the **i**nfectious **j**uvenile (IJs) finds a host. Second, it enters the host either through gut or epidermal penetration. Third, it infects the host through regurgitation of symbiotic gut bacteria and secretion of toxic effectors. Fourth, once internal conditions are ideal, IJs will sexually differentiate and begin to reproduce. Fifth, after 2-3 generations, newly hatched nematodes will emerge from the host.

In *Galleria* (Greater wax moth), it has been demonstrated that the epicuticle of an infectious worm mimics the host cells allowing the worm to invade undetected [39,40]. After entry, EPNs suppress the host immune system thus inactivating hemocytes and their ability to recognize a foreign threat and attack invading EPNs. With hemocytes subdued, there are decreased levels of

melanization making the internal environment more suitable to the pathogen [40,41]. In addition to *Galleria* studies, many studies pertaining to EPN wounding and infection have now been carried forward using *Drosophila melanogaster* and its wealth of tools.

1.4 | *Drosophila melanogaster*.

Drosophila and other insects have long been used as model systems to further our collective understanding of the innate immune system. As early as 1937, wounding kinetics were coming to light in *Rhodnius prolixus*, the vector for Chagas disease. It was observed that injured cells produced chemical substances which led to an activation of chemotaxis to the wound site [42]. Cellular immune responses to wounds and infection were further studied in *Drosophila spp*. through the use of *Pseudeucoila bochei*, a parasitic wasp [43]. Humoral aspects of wounding and infection in *Drosophila melanogaster* were discovered in a laboratory in Umeå, Sweden [44]. Shortly thereafter, the first AMPs, Cecropins A and B were discovered [45]. These studies led to the explosion of a new field, insect immunology.

Using the vast array of tools now available to *Drosophila* studies, ectopic expression of either knockdown or overexpression constructs in the fly has brought to light important molecular events in the nematode infection and wound healing process. Transcriptome profiling of *Drosophila* larvae infected with *Heterorhabditis* has determined that IDGF3, a key regulator of clotting and wound healing is necessary in the anti-nematode response [46]. Gene Ontology analysis determined that pathways associated with the formation of fibrotic lesions were downregulated in response to nematode infection including the Wnt, and JAK/STAT (**JA**nus **K**inase protein and the **S**ignal **T**ransducer and **A**ctivator of **T**ranscription) pathways in an IDGF3-dependent manner. Other clot and immune-associated genes were also identified to be important in the EPN response including, complement-like protein, TEP3, a basement membrane component, **Gl**u**t**actin (Glt), and a pathogen recognition protein, Gram Negative Binding Protein-like 3 [47,48]. Further, Drosomycin, an AMP was highly upregulated upon EPN infection though it appeared to be independent of the Toll pathway, in line with the findings that the Toll and

Imd (**Im**mune **d**eficiency), two immune pathways, seem dispensable in nematode infection [33]. In contrast, when infected with *Steinernema carpocapsae*, *idgf3* larvae induced drosomycin in a Toll-dependent manner [49].

Both the fat body and hemocytes, blood cells, are important in the EPN defense. These immunosecretory organs secrete proteins important in clotting like **t**rans**g**lutaminase (Tg), IDGF3, eicosanoids, and fondue, which are also anti-EPN [50–52]. One interesting phenotypic discovery is that lipid droplets, important in the storage and hydrolysis of neutral lipids, increase in size during infection with the EPN, *S. carpocapsae* [53]. In line with fat metabolism playing a potential role in infection, **a**dipo**k**inetic **h**ormone (AKH) important in energy metabolism and muscle locomotion, was found to have a negative effect on larval survival and locomotion when compared to wildtype, potentially due to the EPN taking advantage of the energy metabolism in normal functioning larvae [54]. Larval locomotion was also affected by AKH leading to increased numbers of larvae travelling further distances with implications for infection evasion. Interestingly, AKH has also been reported to interact with the **P**PO **A**ctivating **S**ystem (PAS) cascade which leads to increased numbers of nodules and activation of PPO in the locust, *Locusta migratoria* [55].

Differences in the genetic background of *Drosophila* laboratory reference strains could even elicit variable behavioral and immune responses as seen between W^{1118} and Canton S wildtype strains[56]. Kunc *et al.* found that different larval strains were more or less sensitive to the presence of nematodes, raising questions about how larvae can detect EPN infested areas. The larvae may use sensory neurons to help activate and recruit hemocytes to the right microenvironment in response to but also in preparation for a potential EPN attack [57]. Once hemocytes are recruited to a wound or fight active EPN infection, secretion of PPOs aid in the host defense, which is in contrast to *Galleria* where the melanization reaction is suppressed by EPN infection. *PPOI* and *PPOIII* are upregulated during axenic and symbiotic nematode infection with *S. carpocapsae* though PPOI, II, and III were all found to be protective [58]. The way in which *Drosophila* responds to wounds either through EPN infection or septic injury induced with a tungsten needle is the major focus of this thesis.

Chapter 2. Self vs non-self

2.1 | Wounding. In most organisms, the epithelial layer is the first line of defense against all infectious agents. When this layer is breached, it is vital for the injured party to quickly activate a response to reestablish the separation from one's self from its non-self. Through a series of cascades, the site of injury immediately begins to release and recruit factors to close the wound. In metazoans, and in particular *Drosophila melanogaster* larvae, there is a general kinetics to wound healing. Wound healing initiates with the epidermal cells in proximity to the wound gap sending out signals to induce cell migration and secretion at the site of injury. Then a plug or a soft clot occurs that aids in immediately separating the organism from its non-self (its external environment), followed by the formation of a scab, the hardening of the soft clot through the activation of PPOs (melanin-producing agents). Finally, wound healing is terminated by reepithelization [59–62]. However, the exact timing of molecular events is still not clearly understood. Wounding assays have been established in *Drosophila* for further understanding of the wound healing sequence of molecular events through pinching or sterile injury [63,64], pricking with a tungsten needle [59], laser injury [65], and infection techniques such as parasitic wasp infection [43], injection with microorganisms [66], or infection with EPNs [33]. Though wound healing has been described as stochastic [67–69], it is unclear if there is a more explicit order to clot formation and reepithelialization.

2.2 | Clot formation. After wounding, the wound must be plugged and sealed. The development of a clot involves the formation of a soft clot including recruitment of AMPs and hemocytes to the wound area, the formation of a hard clot, and the regeneration of cells at the wound. The detection of a breached barrier at the epithelial level and subsequent activation of the formation of a cytoskeleton ring, or 'purse strings' around the wound is

facilitated by the Toll pathway, which includes Spätzle, dif, and dorsal, two nuclear transcription factors that lead to the activation of AMPs and wound-healing genes [70]. In addition to Toll, a family of Ras, small guanosine triphosphates (GTPases), namely Rho are required for the purse strings that draw the wound to a close in *Drosophila* embryos [70,71]. In larvae, it was found that a **m**atrix **m**etallo**p**roteinase, Mmp1 was required for the reorganization of the actin cytoskeleton at wound sites and that mutants produced something akin to actin cables like in the embryos, perhaps a compensatory mechanism. Mmp1 was also required for cell elongation, repair of the basement membrane, promotion of ERK (**e**xtracellular signal-**r**egulated **k**inases) signaling, and reepithelization, with mutants having open wounds even 50 hours post-wounding [72]. In contrast, a pinch wounding experiment showed that within a few hours, epidermal cells were observed to detach from the surrounding wound gap and produce long cell protrusions, filopodia, and lamellipodia [73].

To plug the wound, epithelial cells at the wound site can fuse to form a syncytium, a multinucleated mass of cytoplasm, which occurs within hours. In order for cells to fuse, change shape, and be recruited to the wound site, reorganization of the cytoskeleton is required and is mediated through the activation of the **J**un-**N**-terminal **K**inase (JNK) pathway [59,74]. The soft clot includes cross-linking amino acids and major clotting substrates, like fondue, by Tg, a "glue" like protein [75,76]. The soft clot can harden through the activation of a secondary class of immune cells, crystal cells. Crystal cells contain crystalline deposits of PPO, for which they are named and rely on the JNK pathway, small Rho GTPases, and the **t**umor **n**ecrosis **f**actor (TNF) homolog Eiger (a JNK pathway ligand), for activation and the beginning of the PAS cascade. The crystal cells will then rupture leading to secretion of PPO and hard clot formation [77]. Other hemocyte secretions into the **e**xtra**c**ellular **m**atrix (ECM), such as collagen IV, Laminin A, Glt, Tiggrin, Papillin, and Peroxidasin are important in the clotting reaction and may also contribute to tissue regeneration [47,78–80]. Interestingly, retinoids, important in eye development and epithelial cell growth, also play a role in tissue regenerative growth with damaged eye discs delaying pupariation [81]. Lastly, IDGF3, **i**maginal **d**isc **g**rowth **f**actor **3**, a chitin-like protein, has been identified as a key component for regeneration, sealing of the wound, and the response against EPNs [48].

2.3 | Pathogen recognition. Once a wound occurs and the clotting reaction plugs the wound to separate self from non-self, the detection of a pathogen (or non-self) that may have entered upon injury is initiated through pathogen recognition or sensing atypical cellular activity/damage. Being that the identification of non-self is so important, many different pathogen detecting receptors and sensing cues have been developed by infected hosts. Depending on the kind of pathogen detected, the transcriptional response mounted is unique to the MAMP. MAMPs can be shared across microbes with many intruders being recognized by their foreign nature [82]. Bacteria for example contain foreign substances important to the formation of the cell wall called **p**eptido**g**lyca**n**s (PGNs) which are absent in eukaryotes. Specificity of response can even come down to the type of PGN found in the hemolymph with transcriptional differences existing between **Lys**(ine)-type (Lys) and meso-**D**i**a**mino**p**imelic acid-type (DAP) PGN [83]. Transcriptional differences largely validate previous findings that unique pathways are activated downstream of either DAP or Lys-type pathogens however not as categorically as previously believed. Thus, both Lys-type PGN from gram-positive bacteria and DAP-type PGN from gram-negative bacteria activate both the Toll and Imd pathways but the magnitude of activation varies in that Toll induces more transcriptional activation from Lys-type than DAP type bacteria [83,84]. Interestingly, stochastic effects of the initial infection can lead to differences in mortality and ability to clear the infection with some living with a chronic infection in the blood thereafter [85]. Further, the mode of entry for the pathogen can determine what kind of immune response is mounted and whether or not the microbe is pathogenic [86].

Both Toll and IMD pathways are stimulated by MAMPs such as PGN but also other signature foreign cell membrane components, like the fungal β-glucan [87]. To activate the Toll pathway, virulence factors, largely Lys-type PGN and β-glucan are recognized by specific **P**attern **R**ecognition **R**eceptors (PRRs). These receptors, **p**eptido**g**lycan **r**ecognition **p**roteins (PGRPs) can be specific to the type of immune threat posed. PGRP-SA and PGRP-SD are activated in response to gram-positive bacteria while fungi are recognized by their specific host recognizer protein, **G**ram-**n**egative **b**inding **p**rotein **3** (GNBP3) which binds to β-1-3-glucans in *Drosophila* [88]. In addition to the PRRs, Persephone, a serine protease, can induce a Toll signal cascade: a cytokine, pro-Spätzle is

cleaved by a hemolymph serine protease which in turn, is activated into processed Spätzle and binds to the Toll receptor located on the plasma membrane. After, Cactus, an NF-κB inhibitor, degrades and signals for the translocation of two transcription factors, Dif and Dorsal, to the nucleus, which then bind to the NF-κB-related factor, Relish. This induces transcription of antimicrobial genes and in particular, drosomycin [89]. Toll has been associated with AMP production and has been linked with wound healing genes [70]. Furthermore, Toll is important for activation of hemocytes important in immune defense and clotting [90]. The IMD pathway is largely activated in response to gram-negative bacteria and can respond to some gram-positive bacteria [91]. Like the Toll pathway, the main target of the IMD pathway is another NF-κB member, the Relish transcription factor. Further upstream, there are four PPRs, PGRP-LC, PGRP-LA, PGRP-LE, and PGRP-LF, whose most likely function is in sensing DAP-type microbial molecules which then signal the IMD pathway [92]. Three AMPs, cecropin, attacin, and defensin are associated with this pathway and may also be influenced by Ecdysone, a hormone regulator of development, which has been associated with PGRP regulation [93].

In response to fungi and viral infection, *Drosophila* typically responds with Toll though some viruses also trigger the IMD pathway [94,95]. Still further, particular viruses like **D**rosophila **C** **v**irus (DCV) and Invertebrate iridescent virus-6 can activate a different infection/stress pathway, JAK/STAT (see section 3.3) [96,97]. With up to 70% of larvae being attacked by the generalist wasp, *L. heterotoma* or the species-specific parasitoid, *L. boulardi*, the immune response against parasitoid wasps has evolved to become quite specific. Interestingly, the nutritional status of a host and environmental factors can affect the infected larva's ability to overcome infection [98]. Successful larvae have specialized cells that encapsulate the egg intrusion which specifically differentiates after wasp infection, the lamellocyte. This differentiation occurs after plasmatocytes detect the egg and send signals to the lymph gland to inhibit JAK/STAT in precursors cells, prohemocytes, in effect causing differentiation into this specialized cell [99]. Finally, the immune response against EPNs is still under investigation being that both the IMD and Toll pathways are dispensable in EPN infection [33]. Cellular defenses like encapsulation may be of necessity while protective effects of clotting and coagulation proteins may indicate the importance of the epithelial barrier from halting infection before it can either begin or challenge the larva systemically [31,33,52,100].

2.4 | Systemic vs local. Observing a wounded area, it appears that complex regulation creates melanin deposits or a hard scab precisely at the wound site and are not found throughout the cavity of the host. However, some injuries require a response from all remote tissues—how else does an organism outcompete a pathogen with a faster generation time? And how does the organism distinguish between an injury which requires a local response, a systemic response, or both? In the response to an injury, a specific response is required to close and heal the wound however, a complex **s**ystemic **w**ound **r**esponse (SWR) occurs congruently across plants and animals [101]. In the **l**ocal **w**ound **r**esponse (LWR), wounds trigger activation of a lymph serine protease, Hayan, an enzyme that cleaves the pro-peptide from PPO in the PAS. This system releases many ROS which leads to hemocyte recruitment as well as systemic activation of the JNK pathway [6]. Interestingly, in the adult fly, this response has been linked to the nervous system as a precursor to reestablishing host homoeostasis [102]. Remote tissues, such as the fat body and gut enterocytes, release AMPs and ROS to aid in the systemic immune defense against opposing pathogens as well as wound regeneration [103,104]. Eventually, the wound is sealed, a hard clot forms and reepithelialization occurs. Very tight regulation of a multiorgan response is necessary to avoid uncontrollable melanization, ROS, chronic inflammation, and/or a cytokine storm [105]. The defense against an EPN infection or injury is simultaneously local and systemic, especially due to the release of symbiotic gut bacteria into the cavity once penetration is successful [106]. Are the larvae always subject to mortality after infection in all immune scenarios? Immune priming from previous systemic infections has been linked with better disease outcomes or hormesis [107]. Priming *Drosophila* immune systems could lead to better survival rates of hosts infected with EPNs making the variables such as point of entry, pathogenic load, age of host, timing, and previous infection history important factors in immunity [85].

Chapter 3. Humoral immunity

3.1 | Danger signals. In aseptic injury, such as pinching, tissue malfunction, or tumor development, the host immune system can recognize that conditions are not homoeostatic based on the detection of danger signals. These danger signals occur in the absence of pathogens, however, pathogens can illicit MAMPs as well as DAMPs. When these danger signals are detected, such as the cellular release of cytokines, detection of intracellular content in the ECM, aberrant/damaged cells or tissue, or dysplastic development (tissue overgrowth), a cascade of immunoregulatory signals will be elicited to repair the damage [108,109]. The best-characterized DAMPs are Actin, ATP, Calreticulin, ROS, Eiger, and Spätzle [110]. Hemocyte recruitment and activation depend on these signals for both septic and aseptic injury. These DAMPs illicit a **D**AMP-**A**ssociated **R**esponse (DAR) in the fat body via the JAK/STAT pathway resulting in the release of cytokines [111,112]. DARs are a product of hemocyte signaling as well as a target leading to hemocyte recruitment and activation, confirmed with live imaging and laser wounding [113]. Endogenous DAMPs and exogenous MAMPs can simultaneously enhance signaling cascades associated with either danger or microbes to create a host appropriate immune responses to reestablish homoeostasis [114].

3.2 | Immune pathways. Four major immune pathways exist within *Drosophila melanogaster*: The Toll pathway, the IMD pathway [91], the JAK/STAT pathway [115], and the JNK pathway [59]. They are highly conserved in evolution and demonstrate striking similarity to mammalian pathways [116]. Three of the four pathways are major contributors to the production of AMPs, namely Toll, IMD, and JAK/STAT. Each one of these pathways is differentially induced based on the kind of bacterial cell wall components to which different PPRs react (see section 2.3 for Toll and IMD). JAK/STAT is highly conserved and is activated when the receptor Dome is bound to by one of

three cytokine-like proteins, **U**n**p**aire**d** (Upd) 1, 2, and 3 that trigger a response to many processes around the animal, including development, immune response, hematopoiesis, and cancers [117]. One important immune response occurs when negative regulation of JAK/STAT leads to the differentiation of lamellocytes for encapsulation of parasitoid wasp eggs via hemocyte release of cytokines, Upd 1,2 and 3 [118]. JAK/STAT has also been implicated in the response against tumors, viruses, and gut immunity [119]. Further, JAK/STAT can be activated through JNK signaling.

While the JNK pathway is not necessary for the expression of AMPs, it is a crucial player in hemocyte recruitment to the wound site as well as other immunological activations [120]. The JNK pathway, or Basket, is activated by Eiger, a transmembrane type II ligand [121] which binds to wgn, or in alternative activations, either Grindelwald or the TNFR (**t**umor **n**ecrosis **f**actor **r**eceptor) which in turn, both induce pro-apoptotic functions [122,123]. After this activation, production of the Upd 1, 2, and 3 ligands activates the JAK/STAT pathway [124]. JNK can also lead to Apoptosis induced Proliferation (AiP) via the initiator caspase, Dronc [125]. Proliferation of new cells is necessary at a wound site for regeneration of the wound gap. Cell migration to the wound site and subsequent closure are also dependent on JNK signaling [73,126]. JNK is activated in response to hemocyte recruitment, cell death, and invasion, oxidative stress resistance, tumor progression, migration, and fat body secretions [127]. Various danger signals, such as ROS, UV, inflammation, and heat attribute to this broad array of cellular activations [128]. Finally, the JNK pathway is associated with longevity and it has been postulated that hormetic effects may be seen when mild stresses, like fasting and cold, are experienced [129].

3.3 | Prophenoloxidase Activating System.

There two basic types of melanin which exist in a healthy larva or fly the most common being eumelanin (“good” melanin), important in black pigmentation and pheomelanin, which produces a reddish pigmentation [130]. Melanins have a range of biological functions including fortifying insect cuticles, creating free radicals in chemoprotection and, acting as an antibiotic through the formation of cytotoxic byproducts in the melanization cascade [130]. In development, melanogenesis occurs in an enzymatic and controlled fashion based

on genes such as *ebony* and *yellow* and enzymes such as TH (**T**yrosine **h**ydroxylase) and DDC (**D**opa **d**e**c**arboxylase) [131]. In the immune reaction, it is well-documented that melanin is synthesized from a cascade that starts with a zymogen called prophenoloxidase and proceeds chemically with some steps creating cytotoxic byproducts [132,133]. PPOs are an important part of the insect and crab immune response and are also found in other species but are named differently, such as tyrosinase in mammals and microbes, polyphenol oxidase in plants, and hemocyanin in arthropods [134,135]. PPOs have a highly reactive copper core that interacts with peroxide to form an intermediate catecholate, then finally, a quinone [136]. This process begins with the recognition of MAMPS such as **l**ipo**p**oly**s**accharides (LPS) and peptidoglycans like β-glucans and mannans in some species but may be dependent on DAMPs such as ROS in *Drosophila* [77,134,137]. *Drosophila* larvae contain both mono and diphenols and lack laccase activity in the hemolymph (laccase is a copper protein that oxidases only p-diphenol and o-diphenol and is found primarily in the cuticle) [138,139]. The activation of PPOAE (**p**ro**p**henol**o**xidase-**a**ctivating **e**nzyme) is usually inhibited by serpin27a (a **ser**ine **p**rotease **i**nhibitor) [140]. When activated, PPOAE, in turn, cleaves the pro-peptide from the PPO to allow the active PO (**p**henol**o**xidase) enzyme to oxidize mono and diphenols into L-tyrosine, then into the catechol L-Dopa and subsequently orthoquinones [138,141–143]. The production of quinones leads to toxic intermediates such as ROS including hydrogen peroxide and hydroxyl radical, both superoxide radicals. Finally, the quinones then polymerize to form melanin [144]. Being that the quinone interactions in the PAS are chemical and not enzymatic, there is no discrimination of self vs. non-self, thus surrounding tissues and cells can be destroyed in the reaction [145]. This redox reaction is highly regulated to avoid the spread of uncontrolled melanization which would be harmful to the animal. Furthermore, this degree of regulation has led to the hypothesis that *Drosophila* crystal cells have cleverly developed their crystals to keep the enzyme (PPO) and substrates separate until they are released in immune scenarios [146,147]. One study of note found that the moth, *Manduca sexta*, produced PPO molecules that are IL-1 like, linking PPO activity with invertebrate cytokine activity [133]. Another such link has been made in crayfish which found that PPO was negatively regulated by caspase-1 like activity [148]. These studies suggest that melanin production has long played a role in immunity. Interestingly, a quinone isolated from plant species found in Brazil called *Bignoniaceae* or 'ipê amarelo' has anticancer, antiviral, antimalarial, and anti-inflammatory

effects and has the therapeutic potential to be used as a kind of quinine (an antimicrobial used to treat malaria) [149–151].

Chapter 4. Cellular immunity

4.1 | The Hematopoietic system. In the *Drosophila* embryos, there are two waves of hematopoietic development that give rise to three different classes of hemocytes. The first wave occurs in late embryogenesis when differentiated hemocytes, plasmatocytes, embark to eliminate apoptotic cells throughout the embryo with the macrophage activity of phagocytosis, aided through the receptor *Croquemort*, a CD36 receptor designed to recognize apoptotic cells [152]. The second wave, around the same time in embryo development, leads to the development of the hemocyte subclass, crystal cells which are still not understood in terms of their function within the embryo [153]. Single-cell RNA sequencing in the embryo has unveiled at least 14 immune cell populations with unique roles, with two sub-classes of lamellocytes being specifically induced upon immune challenge [154]. A similar study looking at single-cell sequencing of immune cells in larvae found 16 subclusters of cells, also subject to shifts in cell population depending on specific immune challenge [155]. In the larval stage, the lymph glands are the main site of hematopoiesis [156]. In both the embryonic and larval stages, cell fate and restriction of lamellocyte progenitors are determined by Notch, a conserved pathway from insects to mammals [157,158]. A majority of the hemocytes produced are plasmatocytes with only five percent of the circulating hemocytes being crystal cells. Crystal cells are named after their large crystals there within [159]. They contain PPO which is cleaved after immune challenge and leads to the melanization cascade known as the **P**PO **A**ctivating **S**ystem (PAS) [160]. The release of PPO is not necessary in clot formation but in subsequent hardening of the clot and wound healing [61,132]. When there are decreased levels of Notch signaling as well as increased levels of ROS, as in the case of tissue damage or specifically parasitoid wasp infection, a third cell type differentiates from progenitors. These cells are called lamellocytes and have recently been observed after EPN infection as well. [58,146,154,155,158,161]. They are large, flat cells and aid in the animal's defense against wasp eggs and other

immune challenges by encapsulating large foreign objects that are too big to be phagocytosed [99].

Moreover, each of these cells produces crucial cell secretions in response to immune challenges and can migrate towards the wound site. Plasmatocyte, crystal cell, and lamellocyte migration is facilitated via actin and lamellipodia, activated by JNK (basket), and subsequently the Rho GTPase, Rac1, aids in the closing of the wound [15,162]. Interestingly, mutant larvae with ablated hemocytes, particularly plasmatocytes and crystal cells, were found to still be capable of wound healing and were resistant against nematode attack despite the absence of these hemocytes. Instead, induction of lamellocytes occurred, which signaled a shift in effector mechanisms [163]. Unveiling key regulators of cellular immune defense will be enlightening for understanding immunity given that effects, such as the distribution of subclasses of hemocytes and environmental factors all influence cell differentiation and immune defense.

4.2 | Cell migration and membrane interactions.

In order to seal wounds, epithelial cells produce filopodia to "zipper" the wound site and are highly reliant on Rho GTPases [164]. While it is unknown which Rho **g**uanine **n**ucleotide **e**xchange **f**actors (GEFs) and which **G**TPase **a**ctivating **p**roteins (GAPs) are involved in mediating the activation and subsequent deactivation after wound closure, for regulators of actin, Rho1, and Cdc42, it is known that the hemolymph components like the **P**DGF/**V**EGF-like (**p**latelet-**d**erived **g**rowth **f**actor/**v**ascular **e**ndothelial **g**rowth **f**actor-related) **f**actor (Pvf) can drive migration that is dependent on actin [73]. Similarly, hemocytes such as plasmatocytes and crystal cells produce membrane protrusions and require the small GTPase actin regulators Rho, Rac, and Cdc42 to migrate. Migration for plasmatocytes in embryos is dependent on Ena, a Vasp (**Va**sodilator-**s**timulated **p**hosphoprotein) family regulator of membrane protrusions [165] and on **p**hosphat**i**dylinositol **3**-**k**inase (PI3K), a downstream signaling molecule of G-Protein-Coupled Receptors [166], known to be chemokine receptors in mammalian cells and induce a distinct mode of migration in the case of wounding. Chemokines are still being discovered in *Drosophila* however

the ROS, hydrogen peroxide has been shown to act as a damage signal across metazoans at large [110,167,168].

In addition to the Rho GTPases described above, another branch of Ras small GTPases, Rabs, are known to be involved in membrane trafficking and in particular, endocytosis and exocytosis. They are a large family of at least 31 types in *Drosophila*, they are highly conserved amongst eukaryotic cells, frequently interact with organellar membranes, and contribute to migration, classical and non-classical secretion, as well as general immune function [169–171]. Rabs are thought to act as molecular switches as they have an inactive GDP-bound state and an active GTP-bound state, both mediated by GEFs and GAPs [172]. Phospholipids, such as PI(3,4,5)P_3 and membrane surface charges have been implicated in the spatio-temporal regulation of the correct Rab GTPase [173,174]. Rab5, Rab7, and Rab11 are well-documented early endosomal, late endosomal and recycling endosome vesicular trafficking markers, respectively. Early and late phagosomes formed from phagocytosis, a defensive endocytic process also includes the maturation of Rab5 to Rab7 as phagosomes prepare to become phagolysosomes [175]. However, it is still unclear exactly how Rabs substitute themselves one for another as vesicular membranes mature [176].
Interestingly, an endocytic-lysosomal response in macrophages is described when *Drosophila* are infected with *M. luteus* that consists of an increase in size and the number of Rab5 positive vesicles [171]. Further, more insight is being gained into how bacteria like *M. tuberculosis, C. burnetii,* and *H. pylori* can hijack host vesicles and essentially create parasitophorous vacuoles in humans [177].

4.3 | Classical vs non-classical secretion.

Classical secretion is a predominant function of all cells in development, homoeostatic activity, and immunity. Proteins are typically encoded with a signal peptide at the N-terminus of their sequence. This signal peptide indicates that the protein will be packaged into a vesicle and exported through the cell membrane via the endoplasmic reticulum and the Golgi apparatus. Several important ECM components are released in this manner that aid in the response to tissue damage and infection, like collagen IV and Glt, both important in the anti-EPN response [15,47,50,178]. Non-classical secretion is typically undertaken by proteins

lacking a signal peptide and can take shape in many different forms, including through membrane pores, vesicular secretion, compound secretion, or via cell death. One very important non-classically secreted protein is the highly conserved clotting protein, Tg, homologous to Factor XIII in mammals. Tg catalyzes the lysine-glutamine isopeptide bond in a Ca^{2+}-dependent manner. The protein acts as a "glue" at the wound site helping cellular debris and substrates to bind and seal the wound. This reaction in hemocytes may be associated with FYVE domain-containing proteins, which are important in the recycling endosome pathway found in mammals [179,180]. **T**issue **t**rans**g**lutaminase (tTG) interacts with β1 integrins to be encapsulated within the recycling endosome. A marker for recycling endosomes, Rab 11 GTPase was necessary for this process to occur. Once successfully bound to the membrane, interaction with phosphoinositides on endosomal membranes allowed externalization of the membrane [180]. More studies are needed to determine the conservation of tTG and Tg from mammals to insects. Tg is for clot formation and in the anti-EPN response [50,76]. Another non-classically secreted protein is PPO. Both PPOI and PPOII lack signal peptides and are primarily released from crystal cells (see sections 3.4 and 4.4).

4.4 | Cellular defense and behavior. Typically, cellular nomenclature has associated a cell type with a singular cellular function in effect limiting our understanding of the multiple roles a single cell could play, a phenomenon referred to in proteins as protein moonlighting [181]. One such case is the previously mentioned lamellocytes that specifically differentiate upon wasp infection to encapsulate the wasp egg (see section 3.3 or 3.4). Further, crystal cells known to release PPO are named after these crystalline enzymes (see section 3.4 or 4.4). Naming cells after these functions is helpful for remembering a single behavior they can exhibit, however it is also limiting in that we do not consider context-dependent activation of an array of cellular behaviors. Plasmatocytes in *Drosophila* makeup 90% of the blood cells and are typically described as macrophages, cells that eat up cellular debris and invading pathogens. They do this using phagocytic receptors on the cell membrane, such as Eater and NimC1, and scavenger receptors, Croquemort and Draper [159]. Once activated, particles are engulfed into phagosomes which fuse with lysosomes. The phagolysosome finally degrades the offending

agent providing the host with renewed cellular homoeostasis. Interestingly, phagocytic efficiency is dependent on the type of invading bacteria as well, with *E. coli* being more phagocytosable than *Staphylococcus aureus* [182]. Further, bacteria like *Photorhabdus luminescens* (found in the gut of the EPN) can suppress the phagocytic action of hemocytes to aid in their own host invasion [183]. Another tactic for killing large amounts of invading bacteria is through nodulation, or multi-cellular aggregates of hemocytes entrapping groups of bacteria [182]. However, recent studies have demonstrated that there are several different subtypes of *Drosophila* plasmatocytes, 13 in embryos and 12 in larvae, with untold functional significance [154,155]. Plastmatocytes and crystal cells are only now being characterized with divergent roles. Plasmatocytes and crystal cells are important secretory cells and both may serve as precursors for other blood cell types [184]. Plasmatocytes have also been observed to lyse at wound sites in a manner which may resemble NETosis, **n**eutrophil **e**xtracellular **t**raps (NETs) that create a network of extracellular fibers from the DNA of neutrophils (unpublished data). Other cell death behaviors include non-inflammatory modes such as apoptosis for development or aberrant cells, osmotic lysis or necrosis, or caspase-dependent cell death that is inflammation dependent.

4.5 | Cell death. Cell death is a process known to occur primarily in developmental processes, autophagy, and cellular immunity. **P**rogrammed **c**ell **d**eath (PCD) is dependent on activated caspases such as initiator caspases which in turn act on effector caspases. One well-documented form of PCD is apoptosis, a word of Greek origin meaning “falling off,” like petals from a flower, referring to the apoptotic bodies formed. Apoptosis is usually characterized by cell shrinkage, pyknosis (‘nucleus condensation’), karyorrhexis (‘nucleus bursting’), and the creation of apoptotic bodies [185].

Drosophila has three genes that are pro-apoptotic and post-translationally activated: *grim*, *reaper,* and *hid*. The proteins will then antagonize IAPs (**i**nhibitor of **a**poptosis **p**roteins), E3-ubiquitin ligase proteins which prevent unwanted cell death in *Drosophila*. In response to Grim, Reaper, or Hid, Diap1 (**D**rosophila **IAP**) can no longer inhibit cell death through the degradation of

the initiator procaspase [186]. Caspases are **c**ysteine **asp**artic acid specific prote**ases**. There are at least 7 caspases in *Drosophila* with more likely to be discovered. A well-established initiator enzyme, Dronc is necessary in cell death induction. With levels of Dronc increasing, the initiator caspase is activated through the endoproteolytic cleavage of the prodomain and it subsequently activates effector caspases such as Dcp-1, Drice, Decay, and Damm, all of which have small or no prodomains [187]. This series of molecular events eventually leads to cell death.

While insects have been described to employ PCD in immune scenarios including in the formation of extracellular traps made from the chromatin of immune cells [188], it is debated whether or not *Drosophila* contain the ability to engage in such cellular defenses. The activation of Rho GTPases and the JNK pathway can lead to *Drosophila* crystal cells undergoing a form of cell death through a yet unknown molecular mechanism. Morphologically, the cells have been described as bursting through a non-apoptotic form of cell death likely tightly regulated as to control the release of PPO and subsequent spread of melanization both spatially and temporally [77,189]. Recently, it has been shown that crystal cells are reliant on an increased production of intracellular ROS for activation, and when exposed to an antioxidant, NAC, lose their ability to activate fully via JNK and rupture [137]. Thus cell death in crystal cells is likely inflammatory-based in initiation with an inflammatory function after rupture. The release of cytosolic content becomes a protective mechanism for the host leading to the activation of a DAR important in wound healing and pathogenic threat.

Chapter 5. Summary of papers

5.1 | High-Resolution Infection Kinetics of Entomopathogenic Nematodes Entering *Drosophila melanogaster*

by Alexis Dziedziech, Sai Shivankar and Ulrich Theopold

Following the course of infection in an organism requires first understanding how some parameters, such as multiplicity of infection can affect the severity of infection and the response of the host. Using *Drosophila melanogaster*, we sought to bring higher resolution to the infection stages of pathogenic worm infection. *Heterorhabditis bacteriophora,* an EPN, has symbiotic gut bacteria, *Photorhabdus luminesces* sometimes referred to as an **e**nto-mo**p**athogenic **c**omplex (EPC). EPCs were first incubated with third instar larva for 30 mins to capture the very early stages of infection. Once the first layer of protection, the epidermal barrier was breached, and EPNs could be seen attached to the exterior of the larva, the "pre-infected" larvae were apprehended to a glass slide with super glue to capture the moment of entry. We documented EPNs breaching the epidermal layer and entering the interior of the larva after exsheathment. After entry, we found an additive effect of multiple EPNs infecting a single larva. Further, we used FIMtrack [190], a software developed to track small moving organisms, like insect larvae, to look for varying behaviors between infected and non-infected larvae in early infection stages. Infected larvae were found to move faster and bend more frequently than the non-infected larva. These behaviors may potentially serve as a mechanism for the larva to increase its individual immune response to the infection or serve as a behavioral alarm to alert nearby larvae that there is danger afoot. Finally, to identify a point of infection from which the host is overcome, we used the "Smurf Assay," a tool to measure tissue integrity of infected larvae. We determined that 6 hours after infection with a single EPN, loss of tissue integrity occurred. Larvae were deemed septic at

this point and were unlikely to be able to amount any immune response adequate to overcome EPN infection. Taken together, these results help us to understand the nuances in EPNs overcoming *Drosophila* immune responses. By enhancing our understanding of these events with spatio-temporal imaging of the tripartite system, host, worm, and gut bacteria, we can better understand which anti-nematode immune responses occur before septicemia.

5.2 | Insect hemolymph coagulation: Kinetics of classically and non-classically secreted clotting factors

by Martin R. Schmid[1], Alexis Dziedziech[1], Badrul Arefin[1], Thomas Kienzle, Zhi Wang, Munira Akhter, Jakub Berka, and Ulrich Theopold

The epidermal barrier is the first line of defense for many organisms, including *Drosophila*. The barrier can be broken through physical attack by a pathogen, like a nematode, or through abrasion. The wound healing reaction has been described as stochastic. Here we sought to bring more precision to the events that precipitate wound healing through the creation of three constructs, each important in the response against nematodes and septic injury. The three proteins of interest were Glt, Tg, and PPOII, one classically secreted basement membrane component and two non-classically secreted clotting/immune proteins, respectively. Each of these proteins was tagged with GFP and overexpressed within the blood cells, plasmatocytes, and crystal cells. Larvae were bled, a clotting reaction was stimulated *ex vivo* through the preparation of a hanging drop. These clotting reactions were allowed to proceed at biologically relevant time points to determine the kinetics of the wound healing reaction for these three proteins in relation to one another. Further, we characterized the manner in which the two non-classically secreted proteins, Tg and PPOII were transported out of the different immune cells. Using these three constructs, we were able to gain a better understanding of how the wound healing reaction is spatially regulated by a complex interaction of proteins and precipitating events. In particular, we found that Tg was secreted through recycling endosomes in a compound secretory manner immediately after clotting began. Of note, some of the Tg positive cells contained a bifurcation of their nucleus (akin to granulocytes) and secreted

Tg to their cellular membrane while others had no Tg secretion visible [15]. In Tg positive cells, Tg stayed adhered to the cell surface, and later Glt, secreted classically, was next incorporated into clotting fibers. Finally, PPOII, released from the crystal cell through a form of cell death, was not found to be in clotting fibers until later in the clotting reaction. Further work needs to be done to characterize the exact signals which elicit these cellular responses especially concerning septic wounding and nematode infection.

5.3 | Data on *Drosophila* clots and hemocyte morphologies using GFP-tagged secretory proteins: Prophenoloxidase and transglutaminase

by Alexis Dziedziech, Martin Schmid, Badrul Arefin, Thomas Kienzle, Robert Krautz, and Ulrich Theopold

In our previous article, we found that there was heterogeneity in the release of both Tg and the PPOII bringing further need for clarification to the specific circumstances under which each protein is released and the environmental cues that can elicit different responses. While PPOII is very strongly associated with hemocytes, Tg has been shown to be upregulated in other tissues in response to injury. In this paper, we employed the hanging drop assay to determine whether different membrane makers were associated with PPOII distribution about the crystal cell and whether Tg could behave in a tissue-dependent manner. Regarding further characterization of PPOII, we found that in mature crystal cells, there was no colocalization between PPOII and the general membrane marker mCD8::cherry. Interestingly immature crystals, distinguished through the cytoplasmic distribution of PPOII did portray colocalization between the zymogen and the membrane marker mCD8::cherry. This raises the question of whether early membrane interactions could aid in the aggregation of PPOII into the final crystalline form. Regarding Tg, we found that when using a pan tissue driver, Tg was secreted into the clot fibers, in contrast with our previous findings that, when secreted from hemocytes, Tg stays localized to the cell membrane. This demonstrates that the same protein can vary in its spatial allocation about a wound de-

pending on the tissue from which it is secreted. Together our data lay a foundation for further research into how PPOII matures within the cell as well as how tissue-specific secretion can change the role a protein plays in wound healing.

5.4 | Convergent evolution of pyroptosis, a caspase-dependent inflammatory cell death mechanism, in *Drosophila melanogaster*

by Alexis Dziedziech and Ulrich Theopold

We have previously described crystal cells to undergo a non-apoptotic form of cell death. Previous experiments have been done *ex vivo* and the question was raised if the observed cell death was an artifact from the experimental setup. Here, we used *in vivo* live cell imaging to demonstrate that crystal cells undergo a caspase-dependent, non-apoptotic form of cell death and describe a potential mechanism. Third instar larvae were apprehended to a glass slide using super glue and wounded superficially using a tungsten needle. Thereafter, live-cell time-lapse microscopy found that crystal cells are recruited to a wound and will quickly lose their cytoplasmic GFP signal which is atypical of apoptotic bodies. These rupturing cells release their cytosolic content locally into the wound area, a process that was inhibited with p35, a pan-caspase inhibitor. Next, we used a caspase reporter which fluoresces upon proteolytic cleavage to test which caspase was likely responsible for cell rupture. Crystal cells were found to lose caspase activity when p35, a pan-caspase inhibitor was expressed. Similarly, after overexpression of Diap1, no caspase activity was found. We further went on to characterize that the initiator caspase is Dronc and the executioner caspase is Dcp-1, most similar to caspase-7 in humans. Taken together, we describe a form of PCD which has never been described in an *in vivo* model of *Drosophila*. This kind of cell death, characterized through rapid loss of cell membrane integrity, the release of danger molecules important in the immune reaction, and the dependence on proteolytic activation through caspases rather than a passive, osmotic lysis are all evidence of convergent evolution of pyroptosis, a form of inflammatory cell death seen in mice and humans.

Chapter 6. Concluding remarks

To speak of a molecular event is incomplete, to better understand it, it is best contextualized within a system. In nematode infection, we see that EPNs can quickly overcome the *Drosophila* host immune response essentially rendering the larval attempts futile after 4 to 6 hours. Perhaps then larvae have developed dynamic clotting systems which mount a more protective response against EPNs. It may be more energetically effective to prevent an infection rather than fight it. Especially in an organism with an open circulatory system, mounting a strong clotting response is an important tool to keep from losing the hemolymph contained within. Looking closer at the clotting reaction, we see that there are general rules to the spatio-temporal organization of protein interactions necessary to ensure that the wound is plugged immediately and that threats are neutralized at the appropriate time. Perhaps it is more useful to think of these occurrences as patterns, events more likely to precipitate other events in a Bayesian ebb and flow of ECM components. While there may not be a single defined order, there may be a statistically significant good enough, or stochastic order. Looking more closely at the crystal cell, at least regarding the secretion of PPOII, there was an age-related morphology to PPOII distribution with mature cells containing fully-formed crystals. Not only does this support that differently aged larvae then employ different immune strategies, but it also implies that on the individual cellular level, cells vary in their own maturity and secretory mechanism. When crystal cells finally mature, they undergo a kind of PCD, most resembling pyroptosis, previously only described in mammals. Curiously, seeing this conservation of form brings some predictability back into our wound healing complex. Perhaps there are only so many kinds of secretion mechanisms that are best at treating different kinds of inflammatory conditions. And though so many kinds of mechanisms exist in nature, we may continuously find the same tools being employed if they are the best means to a preferred end. Life— finds a way [191].

Popular summary

Have you ever noticed that timing can affect the outcome of a situation? This is also true in our immune systems. The same sickness, for example, chicken pox, could be manageable as a child and deadly as an adult. A similar phenomenon can be found in the life cycle of a fruit fly—two hours may as well be two days! Developmentally, each day represents a new stage in the larval life cycle. Catching an infection or getting an injury at different stages can mean very different immune responses, for humans and larvae alike. In this thesis, I studied how quickly pathogenic worms (nematodes) can infect and take over the host, the fruit fly, at a stage in which they are more likely to have a mature immune cell called the crystal cell. Since worms can penetrate the skin, epidermis, I injured larvae and studied how quickly some important proteins in the immune response against disease-causing worms can be recruited to the wound site. This process, coagulation, can be very chaotic especially since the bleeding needs to stop quickly. To see if there is some order to coagulation, I looked at three proteins that help in early and late scab formation and found that first, the "glue" protein, Tg becomes active, then Glt, a protein which helps fortify the clot is found at the wound site. Lastly, prophenoloxidase helps to harden the clot to form a scab while adding a characteristic black color to the wound. Finally, I studied how one particular type of immune cell, the crystal cell, can explode to release the prophenoloxidase enzyme. This process has the explosive name, pyroptosis and is not random or accidental, instead mature crystal cells are specifically programmed to explode in immune scenarios. Taken together, my work contributes to understanding immune systems, which have been evolving for millions of years to become highly sophisticated. The difference between stopping an infection or stopping a wound can be age-, time- and cell-dependent as well as dependent on the threat or scenario. While the same exact sequence of events responsible for wound healing may not occur every single time, we can start to find patterns for which proteins are most important or which steps need to occur. Life—finds a way [191].

Populärvetenskaplig sammanfattning

Har du någonsin funderat på hur timing kan påverka resultatet av en situation? Detta gäller också i vårt immunsystem. Vissa sjukdomar, till exempel vattkoppor, kan vara hanterbar som barn men dödlig som vuxen. Ett liknande fenomen hittas i livscykeln hos en fruktfluga. Utvecklingsmässigt representerar varje dag ett nytt stadium i larvens livscykel. Att bli infekterad eller få en skada kan i olika stadier leda till mycket olika immunsvar, för både människor och larver. I den här avhandlingen studerade jag hur snabbt patogena maskar (nematoder) kan infektera och ta över dess värd, fruktflugan, i ett stadium där det var mer sannolikt att de hade en typ av ”mogna” immunceller, så kallade kristallceller. För att simulera nematodernas förmåga att tränga in genom huden (epidermis) så skadade jag larver och studerade hur snabbt ett antal proteiner som är viktiga i immunsvaret mot patogena maskar kan rekryteras till sårstället. Koagulation kan vara en mycket kaotisk process, särskilt eftersom blödningen måste stoppas snabbt. För att se om det finns någon ordning på koagulering tittade jag på tre proteiner som hjälper till vid tidig och sen bildning av sårskorpa och fann att Transglutaminas blir aktivt först, där det agerar som ett typ av lim. Näst aktivt var Glutactin, ett protein som hjälper till att stärka koagulatet vid såret. Slutligen hjälper Profenoloxidas, ett enzym som färgar koagulatet svart, till att härda blodproppen för att bilda en sårskorpa. Slutligen studerade jag hur en viss typ av immuncell, kristallcellen, kan explodera för att frigöra enzymet profenoloxidas, genom en process som kallas för pyroptosis. Ordningen på denna process är inte av misstag, i stället är mogna kristallceller specifikt programmerade för att explodera i immunscenarier. Sammantaget har immunförsvaret utvecklats i miljontals år för att bli mycket sofistikerat. Skillnaden mellan att stoppa en infektion eller stoppa ett sår kan vara åldersberoende, tidsberoende, cellberoende och naturligtvis beroende av hotet eller scenariot. Samma sekvens av händelser som ansvarar för sårläkning kanske inte inträffar varje gång, men vi kan hitta mönster för vilka proteiner som är viktigast eller vilka steg som behöver ske. Livet—det hittar en väg [191].

Resumen Publico

¿Has pensado como la temporalidad de las cosas puede afectar el resultado de una situación? Esto también ocurre en nuestro sistema inmune. Una misma enfermedad, como la varicela, la cual puede ser manejable en la niñez puede ser letal cuando adultos. ¡Un fenómeno similar puede ser observado en el ciclo de vida de la mosca de la fruta, donde la respuesta inmune varía enormemente en sólo dos días! Desde el punto de vista del desarrollo, cada día representa una nueva etapa en el ciclo de vida de las larvas. Tener una infección o sufrir una lesión en diferentes etapas del desarrollo también puede significar una respuesta inmune distinta, tanto para nosotros los humanos y las larvas de la mosca. En este trabajo de tesis, estudié cuan rápido los nematodos, gusanos patógenicos, pueden infectar y tomar control de la mosca de la fruta, el hospedero, en una etapa particular de su desarrollo en la cual presentan células cristal, un tipo de célula inmune. Después, y dado que los gusanos pueden penetrar por la piel o epidermis, generé heridas en las larvas de la mosca y estudié cuan rápido algunas proteínas importantes de la respuesta inmune son reclutadas al sitio de la herida. La coagulación puede ser un proceso caótico, especialmente cuando necesita ser detenido rápidamente. Para determinar si existe algún tipo de temporalidad durante la coagulación, observé tres proteínas las cuales ayudan en la formación de costras durante etapas tempranas y tardías. Así, encontré que primero la proteína “pegamento” transglutaminasa se vuelve activa y luego, la glutactina, proteína que ayuda en el establecimiento de las costras, se encuentra en el sitio de la herida. Finalmente, la enzima profenoloxidasa, la cual produce un color negro en la costra, ayuda en el endurecimiento y la formación de la costra. En ultimo lugar, estudié como las células cristal pueden explotar para así liberar la enzima profenoloxidasa durante la piroptosis. Este proceso no es azaroso ni accidental, las células cristal maduras están programadas para explotar en contextos inmunes. En su conjunto, los sistemas inmunes han evolucionado durante millones de años para volverse altamente sofisticados. La diferencia entre detener una infección o detener la coagulación de una herida depende de la edad, la temporalidad, el tipo celular y por supuesto el contexto o escenario. Si bien la misma secuencia de eventos responsables de la cicatrización no ocurra cada vez, existen patrones en la sucesión de los eventos que median este proceso. La vida se abre camino [191].

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