

**Impact of dietary protein content on the
immune response of *Drosophila*
*melanogaster***

Dissertation

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Summary

Out of the several factors that can impact the quality of an organism's immune reaction, like genetics, age, circadian rhythm, and commensal bacteria, diet is outstanding as it can modify the immune response at different levels. Though nutritional immunology is a widely studied field, the results for the impact of diet on the immune response are highly inconsistent. These discrepancies can be explained by the differences in the nature of the nutrients under study and the measured immune parameters. To overcome this issue, the central goal of this thesis was to study the impact of dietary protein content in a standardized fashion on different components of the immune system and obtain a complete mechanistic overview.

To this end, I first started by conducting a comparative transcriptomic analysis on *Drosophila melanogaster's* two professional immune cell types, hemocytes and fat body cells, upon a systemic infection. I additionally compared it to already available data for the response at the whole-body level. I found highly divergent transcript signatures in both cell types, which also differed from the whole-body response. The few commonly regulated genes encode mainly classical immune effectors from the antimicrobial peptide (AMPs) family. Both cell types showed functionally relevant specific reactions that were neither present in the other cell type nor the whole animal body.

Having comprehensively characterized the immune cells, I created a system of dietary regimes using a standardized exome-matched holidic diet to enable precise manipulations of macronutrient concentrations. I chose dietary treatments with control, reduced or no protein content (Normal Media, Dietary Restriction and Protein Malnutrition, respectively) for my investigation. Starting with holistic parameters of the immune response, infection with *Bacillus subtilis* results in clear segregation of the three diets in terms of immune outcome, with a higher dietary protein resulting in a higher probability of survival to infection and faster clearance of the bacterial load. To understand the mechanism behind this, I measured the transcript levels of immune effectors in the whole animal body but did not find any differences between the diets. The transcriptomic profiles of the fat body and hemocytes under these various treatments were compared, which could help to explain the differences in the immune response on various diets. In both cell types, the number of overall infection response genes declined with the dietary protein. This decline did not affect the expression of AMPs in the fat body on nutrient-poor diets. However, it impacted all the other processes expressed on top of the direct antibacterial response. On the other hand, hemocytes had a different response, where both

direct (AMPs) and indirect antimicrobial reactions were impacted by lowering the dietary protein. These resource allocation strategies were also different when an injury was introduced without any bacterial infection. Fat bodies highly reduced their injury response on low or no-protein diets, while hemocytes still maintain a significant portion of theirs.

Hence, I provide a possible mechanism for the differences in the immune outcome of animals on reduced dietary protein. I also provide evidence for resource allocation strategies within the immune system, which change with the intensity of the immune stress and dietary stress.

Zusammenfassung

Die Qualität der Immunreaktion eines Organismus hängt von unterschiedlichen Faktoren ab, unter denen die Ernährung eine herausgehobene Rolle einnimmt. Obwohl die Ernährungsimmunologie ein etabliertes Forschungsgebiet ist, sind die Ergebnisse zu den Auswirkungen der Ernährung auf die Immunreaktion sehr widersprüchlich. Diese Diskrepanzen lassen sich zum Teil durch die Unterschiede zwischen den untersuchten Nährstoffen und den gemessenen Immunparametern erklären. Um dieses Problem zu überwinden, war das zentrale Ziel dieser Arbeit, die Auswirkungen des Proteingehalts in der Nahrung auf verschiedene Komponenten des Immunsystems in möglichst standardisierter Weise zu untersuchen und auf diese Weise einen vollständigen Überblick dieser Abhängigkeiten zu erhalten.

Zu diesem Zweck führte ich zunächst eine vergleichende Transkriptom-Analyse der beiden professionellen Immunzelltypen von *Drosophila melanogaster*, den Hämözyten und dem Fettkörper, nach einer systemischen Infektion durch. Zusätzlich wurden diese Ergebnisse mit bereits verfügbaren Daten über die Transkriptom-Antwort von Fliegen nach unterschiedlichen Infektionen auf der Ganzkörperebene verglichen. Beide Zelltypen zeigten stark voneinander abweichende Transkript-Signaturen, die sich auch von denjenigen der Ganzkörperantworten unterschieden. Der überwiegende Teil der wenigen in beiden Geweben regulierten Gene kodiert für klassische Immuneffektoren aus der Familie der antimikrobiellen Peptide (AMPs). Beide Zelltypen zeigten darüber hinaus funktionell relevante spezifische Reaktionen, die weder im jeweils anderen Zelltyp noch in den Ganzkörperantworten aufzufinden waren.

Nachdem die Immunzellen umfassend charakterisiert wurden, entwickelte ich unterschiedliche Ernährungsregime unter Verwendung einer standardisierten holidischen Diät, in der alle Komponenten in definierter Weise zugesetzt werden. Für die Untersuchung wurde ausschließlich der Proteingehalt verändert (Normal Media, Dietary Restriction bzw. Protein Malnutrition). Ausgehend von ganzheitlichen Parametern der Immunreaktion stellte ich fest, dass eine Infektion mit *Bacillus subtilis* eine eindeutige Differenzierung der Immunantworten der drei Diäten ergab, wobei ein höherer Proteingehalt in der Nahrung zu einer höheren Überlebenswahrscheinlichkeit nach der Infektion und einer schnelleren Beseitigung der bakteriellen Belastung führt. Der Vergleich der Transkriptom-Profile des Fettkörpers und der Hämözyten nach einer Infektion bzw. einer sterilen Verwundung unter den verschiedenen Nahrungsbedingungen ergab wesentliche Unterschiede. In beiden Zelltypen nahm die Zahl der

regulierten Gene der allgemeinen Infektionsreaktion mit der Reduzierung des Proteingehalts ab. Dieser Rückgang hatte allerdings keinen Einfluss auf die Expression antimikrobieller Peptidgene (AMPs) im Fettkörper bei Protein-reduzierter Ernährung. Diese Intervention wirkte sich jedoch auf alle anderen Prozesse aus, die zusätzlich zu der direkten antibakteriellen Reaktion induziert wurden. Die Hämozyten hingegen reagierten anders: Hier wirkte sich die Senkung des Nahrungsproteins sowohl auf direkte (AMPs) als auch auf indirekte antimikrobielle Reaktionen aus. Diese Strategien der Ressourcenallokation unterschieden sich auch, wenn eine Verletzung ohne bakterielle Infektion erfolgte. Die Fettkörper reduzierten ihre Verletzungsreaktion bei eiweißarmer oder -freier Ernährung stark, während die Hämozyten immer noch einen erheblichen Teil ihrer Reaktion beibehielten.

Damit liefert diese Arbeit einen möglichen Mechanismus für die Unterschiede in der Immunantwort von Tieren, die mit einer proteinarmen Nahrung ernährt werden. Außerdem werden auch Strategien der Ressourcenzuteilung innerhalb des Immunsystems, die sich mit der Intensität des Immunstress und des Ernährungsstress ändern aufgeklärt.

General Abbreviations:

Abbreviation	Full form
AMP	Antimicrobial Peptides
Cec	Cecropin
DAPI	4,6-diamidine-2-phenylindole
Def	Defensin
Dpt	Diptericin
Dro	Drosocin
Drs	Drosomycin
Drs	Drosomycin
GAPDH1	GAPDH1somycin
GFP	Green Flourescent Protein
IMD	Immune Deficiency
LD	Lipid Droplet
Mtk	Metchnikowin
NBDG	2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-
p.i.	post infection
PAS	Periodic acid–Schiff stain
PBS	Phosphate buffered Saline
PBST	Phosphate buffered Saline - Tween 20
PFA	Paraformaldehyde
RpL13	Ribosomal protein L13
RpL132	Ribosomal protein L32
TAG	Triacylglycerol
UAS	Upstream Activating Sequence

Abbreviations for the treatments used in this thesis:

Abbreviation	Full form
C	Control
DR/DR1	Dietary Restriction
DR2	Dietary Restriction2
DRC	Dietary Restriction_Control
DRI	Dietary Restriction_Infection
DRS	Dietary Restriction_Injury
I	Bacterial infection
NM	Normal Media
NMC	Normal Media_Control
NMI	Normal Media_Infection
NMS	Normal Media_Injury
PM	Protein Malnutrition
PMC	Protein Malnutrition_Control
PMI	Protein Malnutrition_Infection
PMS	Protein Malnutrition_Injury
S	Sterile injury

1. Introduction

1.1. Immunity

The fight between host and pathogen is a long-running evolutionary 'arms race' where both opponents are under pressure to evolve and outrun the other [1]. This race has resulted in the evolution of an elaborate and robust immune system of the host and effective strategies of the pathogen to infect the host. Due to its scientific and medical implications, immunology has always intrigued scientists, resulting in a comprehensive knowledge available in this field.

Most organisms possess defense mechanisms or immune responses to fight the attacks by pathogens. In a broad sense, these immune responses can be classified in two categories - innate and adaptive immunity [2]. Innate immunity is the branch that is present in all metazoan animals. It is defined mainly by its non-specific nature and a lack of memory of an earlier attack [3]. It is entirely genetically inherited and shows a high degree of conservation in the signaling cascades triggered by the pathogens. Adaptive immunity, on the other hand, is classically defined as the branch of response that is highly specific to the pathogen and depends on the immunological history of an organism, as it shows a memory [3]. An example is the production of specific antibodies against a pathogen and a memory of this response, which shows by the rapid response on re-encounter. However, a growing number of studies now imply that these definitions are binary while immune system is much more complicated for that [4] [5].

Even though a significant portion of immunology studies concentrate on adaptive immunity, due to a focus on human relevance, the innate response has also grabbed attention in the last few decades. In this thesis, I will describe our study on the impacts of dietary protein content on the innate immune response, using *Drosophila* as a model.

1.2. *Drosophila* as a model

More than 100 years ago, when Thomas Hunt Morgan was inspired by C.W. Woodworth and first pursued the idea of working with fruit flies, his biggest motivation was how inexpensive and easy it is to rear these animals [6]. Today, a century later, after several Nobel wins and countless discoveries, his initiative led us to this tiny creature that is allowing us to understand the basics of life better every day.

Working with *Drosophila* has several advantages that make it a perfect model organism. It has a short generation time, where an egg can develop into an egg-laying animal within 9-10 days.

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Drosophila's life cycle consists of three larval instar stages, which are feeding stages of the animal, where they gain their size. This is followed by the non-feeding pupal stages and then metamorphosing into adulthood. Adults become reproductively active nearly 6 hours after eclosion, and females can lay up to 100 eggs daily. Depending upon the environmental conditions, they can survive nearly 60-100 days [6]. Due to their small size, they do not require much space and are very easy to culture and handle. This was presumably one of the reasons that fruit flies have even been cultured in a space shuttle to understand the effects of space travel on the immune system [7]. They are also relatively inexpensive to maintain in the lab for several generations. Recently there have been studies that could even cryopreserve the embryos which successfully developed into adults [8].

Genetic manipulations are convenient to perform in flies. Some of the first manipulations included using the transposable P-element, which later led to the discovery of the GAL4/UAS system [9]. The GAL4-UAS system is the most commonly used system to drive genetic changes [10]. A driver gene for the cells/tissue of interest can be tagged to GAL4, whose expression, in turn, activates UAS and further the downstream target. This downstream target can differ based on the aim of the study, for example, it can be a reporter like fluorescent tags (GFP, RFP, etc.) or Lac-Z to investigate the expression patterns of the gene of interest (GOI), or it could be an RNA interference (RNAi) signal to 'knock-down' the expression of GOI. Another common strategy to knock out a gene is the CRISPR/Cas9 system [9]. The guide RNA for this system can be modified according to the gene of interest, making the endonuclease cut the gene and produce a complete loss of function mutant. Thanks to all these genetic tools, thousands of mutants are now readily available in stock centers worldwide.

Drosophila is not only a convenient model but possesses substantial similarities to mammals. Nearly 60 percent of *Drosophila* genes can also be found in humans, and 75 percent of disease-causing human genes have direct orthologues in flies [11]. Due to all these reasons, *Drosophila* has been a powerful model organism for several biological processes, including genetics, inheritance, genomics, development, immunology, memory, behaviour and aging.

1.3. *Drosophila* immune system

As for many other fields, *Drosophila* has been a great model for investigating the innate immune system. It lacks an adaptive immune response, which not only decreases the complexity but also helps study innate immunity in isolation.

Introduction

Epithelia is the first line of defence in any immune system [12] [13] (Fig.1) . In flies, it includes the epidermal cells of the gut as well as the trachea, as they are the first targets for pathogens.

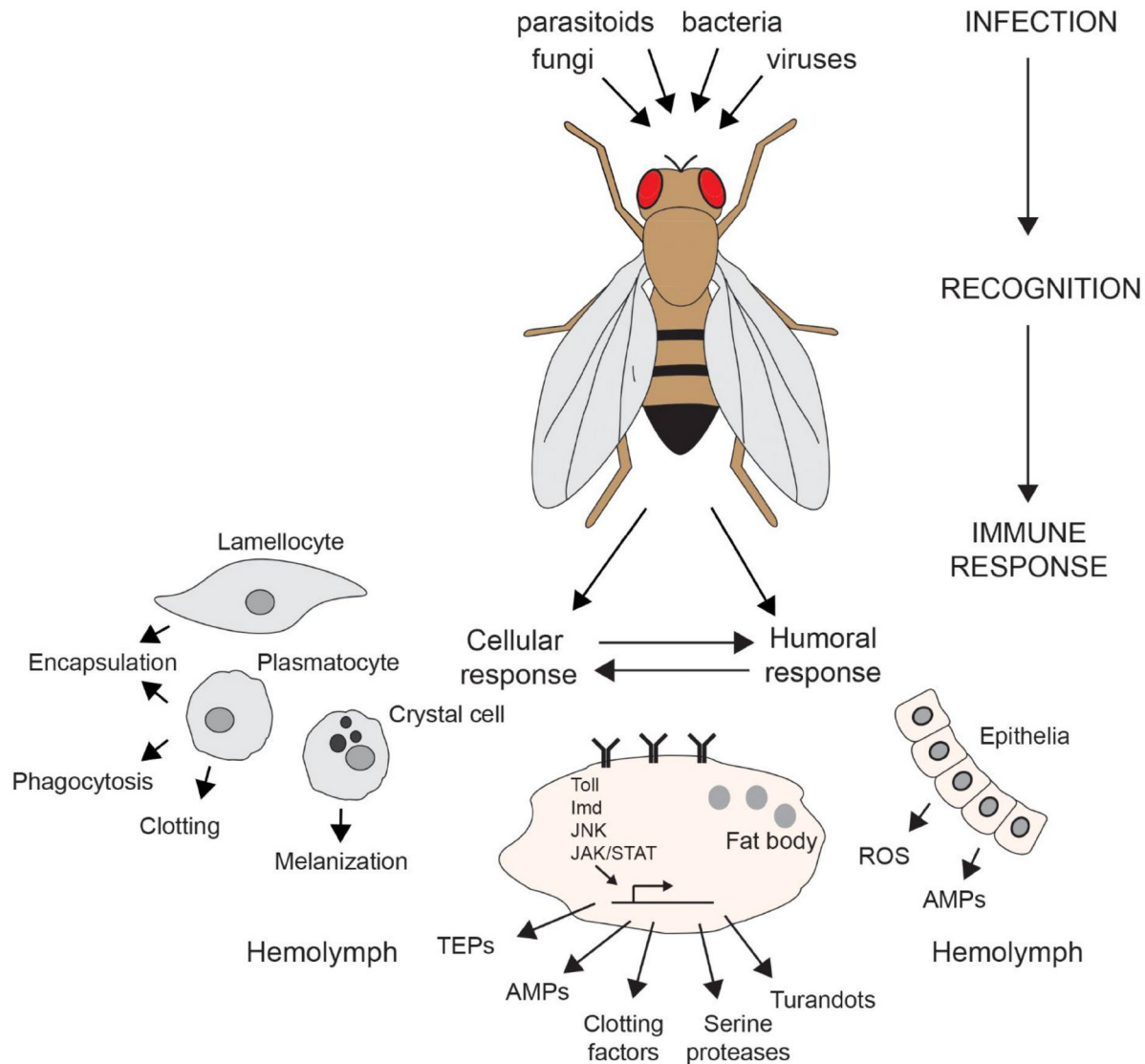


Figure 1: *Drosophila* immune system: The scheme represents the three main branches of the immune system of *Drosophila*: cellular, humoral and the epithelial. The cellular part mainly consists of the hemocytes which further have three types: Lamellocytes, plasmatocytes and crystal cells. The humoral part includes the peptides or compounds released into the hemolymph. The main source of this release is the fat body. The epithelial part includes the response blocking the entry of pathogen into the body. This image is taken from [13].

The primary strategies epithelia employs are the local release of antimicrobial peptides (AMPs) and Reactive Oxygen Species (ROS) for their bactericidal properties. The intestine is believed to be a major tissue attacked by pathogenic invaders and possesses a strong response. Many oral infections are entirely eliminated by the gut, which then never reach the systemic state. AMPs are mainly produced in the anterior midgut, where they eradicate the ingested pathogens [14]. Once inside the general body cavity of the animals, the microbes are encountered by two systemic responses: the humoral and the cellular response.

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The humoral response consists of the production and release of AMPs into the hemolymph of the animal in large amounts [15] (Fig.1). AMPs are small cationic molecules that can target the negatively charged membranes of microbes and lead to their destabilization and further death [16]. The fat body, the functional equivalent of a mammalian liver, is believed to be the primary source of production and release of these AMPs into the hemolymph. AMPs are produced by the two evolutionarily conserved NF- κ B signalling pathways - Toll and IMD. The Toll pathway mainly targets fungi and gram-positive bacteria with Lys-type peptidoglycan in their cell wall. The IMD pathway focuses on the gram-negative bacteria with DAP-type peptidoglycan. The antiviral immune response in *Drosophila* mainly consists of the Janus kinase/signal transducers and activators of transcription (JAK-STAT) pathway and release of antiviral effector molecules [12].

Cellular immune response consists of hemocytes, the equivalents of mammalian leukocytes, which are found circulating in the hemolymph or attached to different tissues [12] (Fig.1). Their functions involve phagocytosis of microbes, encapsulation of large pathogens, melanization and wound healing. There are three types of hemocytes in *Drosophila* - Plasmatocytes, Crystal cells and Lymphocytes. Plasmatocytes make up nearly 95% of the hemocyte number and are largely responsible for phagocytosis of microbes and small particles. They engulf the microbes and eliminate them with the help of AMPs or ROS. Crystal cells are low in number and help the melanization process by the prophenoloxidase cascade. Lamellocytes are large flat cells involved in encapsulating large pathogens or foreign materials. They are not present in adult stages or healthy larvae.

1.4. Fat body

The fat body is one of the tissues with a significant contribution to the systemic immune response [15]. Unlike many other organ systems, the fat body does not have a direct vertebrate organ equivalent, but it is functionally similar to the liver and adipocytes [17]. It is a large tissue and is present throughout the body of the organism. It is in contact with different tissues across the body and is generally also in direct contact with hemolymph. This provides it with the right opportunity to release the required peptides, enzymes or signalling molecules into the hemolymph.

The fat cells for the pre-adult stages are formed during embryogenesis, which stay constant in number during the larval stages and only increase in size [18]. They are present as hexagonal cells attached to each other forming lobes. The domains of fat body lobes present in different

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body parts are also shown to have distinct roles. During the pupal stages, they undergo significant morphological changes, losing their cell shape and becoming spherical. Further, they lose attachment to each other and become loose cells floating in the hemolymph. This

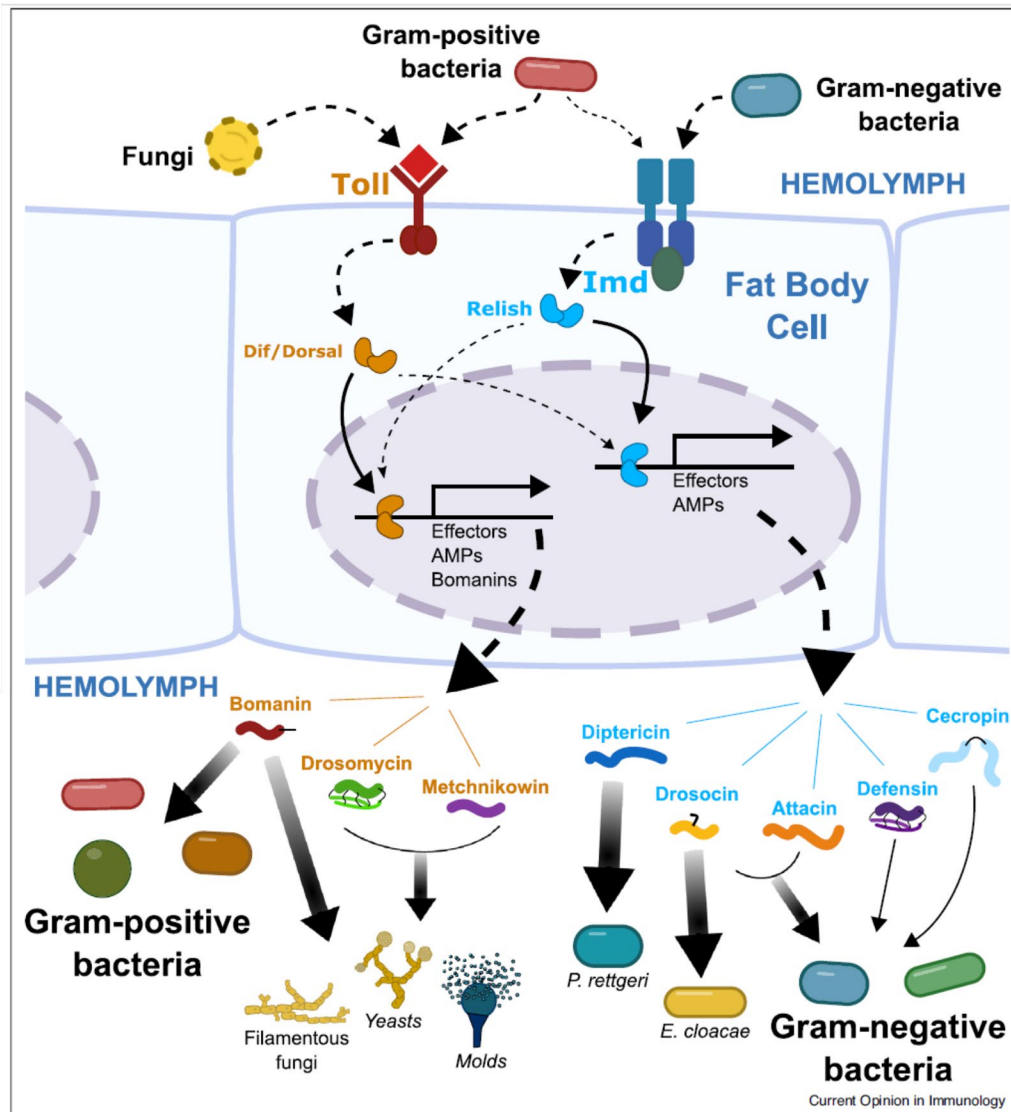


Figure 2 Antimicrobial peptides in *Drosophila*: The scheme shows the different antimicrobial peptides in *Drosophila* against various kinds of pathogens and the molecular pathway involved. While both gram positive and negative bacteria can induce both Toll and Imd pathway, the Imd pathway is mainly for Gram negative ones and Toll is mainly for Gram positive and fungi. Fat body is the major contributor of these AMPs in hemolymph. This image is taken from [16]

phenomenon is termed 'fat body remodeling' [19]. These lose fat cells help the animal in this non-feeding pupal stage as an energy reserve. In the first few days of the adult stage, they also utilize these cells for providing energy as feeding rates are not so high. However, in these days, these pupal fat cells start undergoing programmed cell death and are replaced by the adult fat body. Adult fat body cells are also present around various tissues across the body, including the gut and reproductive tissues. The adult fat body is also present as lobes of cells attached to

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each other rather than lose fat cells. This contact with different tissues, as well as direct access to the hemolymph, helps them communicate to different tissues and send these signals further to different parts of the body.

The fat body in insects is a critical organ for energy storage and controlling metabolism [20]. Most of the excess nutrients of the body are stored in the fat body in the form of lipid and glycogen. It is also a hub for the metabolic activities of the animal body, including protein synthesis for releasing into hemolymph, nitrogen and carbohydrate metabolism. As a robust metabolic junction, it also requires communication with various tissues in the body. The physiological presence in proximity to many tissues and release of signalling molecules in the hemolymph facilitate this role quite well. The signals from distant tissues can also be in the form of hormones. Some noteworthy examples of the functional capacity of the fat body as a storage and release reservoir include the release of several amino acids for morphogenetic changes in development, the release of vitellogenins for the maturation of eggs, and the release of circulating energy reserves like trehalose and lipids for energy-intensive functions like flight [17]. Some studies show the different roles of fat body cells present in different locations in the body. Though some essential functions stay the same in all cells, some roles change with the developmental stage or with the regional localization of the cells.

On top of the metabolic and storage roles, fat body cells' physiological and functional capabilities also put them in the proper position for nutrient sensing [20]. This role further helps it control the release of nutrients into the hemolymph or the storage of excess ones. Nutrient sensing is controlled by specific amino acid transporters in the fat body cells. With the help of insulin-producing cells, it can also harmonize nutrient sensing with systemic animal growth. As already mentioned, fat body cells have essential immune responsibilities [12]. These are discussed in different parts of the thesis in more detail. The most significant role in the immune repertoire is the release of AMPs into the hemolymph (Fig. 2) [21]. Additionally, in the pupal stages, they are also shown to migrate to wound sites for healing. This thesis provides a detailed transcriptional profile of the fat body cells under infection and injury states. These provide us with new information on their roles and the molecular underpinnings of their functions in an immune-challenged environment.

1.5. Hemocytes

Insect hemocytes are the mediators of cellular immunity [12]. They are functional equivalents of the leukocytes in mammalian systems. In different insect species, there is a difference in the

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types of hemocytes present. In *Drosophila*, there are three main lineages with different functions – plasmatocytes, crystal cells, and lamellocytes [22] [23] (Fig. 3). Collectively, their main functions include phagocytosis, melanization, and encapsulation. They are also reported to serve as a mediator to convey the information of a pathogenic attack to the fat body cells to

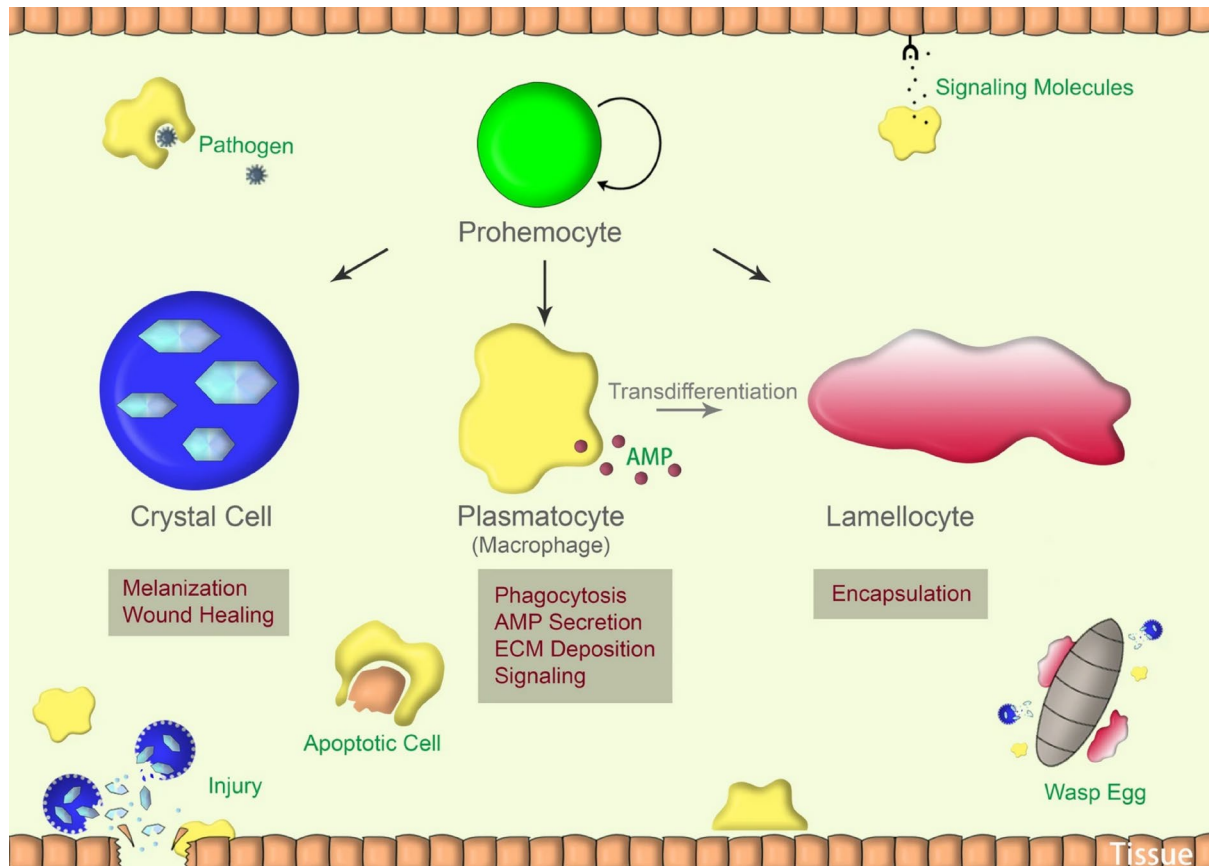


Figure 3 Types of hemocytes in *Drosophila*: Schematic representing the different types of hemocytes in *Drosophila* along with their functions. There are three main types of hemocytes: Crystal cells, which take care of melanisation and wound healing, plasmatocytes, which are the equivalent of macrophages and are responsible for phagocytosis and lamellocytes, which are responsible for encapsulation of bigger particles. The image is taken from [23]

induce a systemic humoral response.

Plasmatocytes are the most abundant hemocyte types, making up to 95% of the total hemocyte population in adults and healthy larvae [22], [24]. They are considered the professional phagocytes, which are functionally equivalent to the macrophages in mammals. They can produce antimicrobial peptides and encapsulate pathogens or foreign particles as well. A detailed description of phagocytosis is provided later. Crystal cells comprise the remaining population of hemocytes in adult stages and larvae not under a parasitic attack. Their central role is the melanization of the wound for repair and coagulation. Melanisation is characterized by the conversion of tyrosine to melanin, a black pigment. This is facilitated by a series of complex enzymatic cascades involving phenol oxidase [24]. Melanin production also generates

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free radicals as by-products with cytotoxic capabilities toward pathogens. Lamellocytes are the third category of hemocytes which are only present in larval stages and are usually just seen in animals under parasitic attack [22]. These cells are considerably larger than the other two cell types. They can surround the big parasite or foreign particles and encapsulate them.

The process of phagocytosis is evolutionarily conserved among different species. In *Drosophila*, the first step involves recognizing invaders in the hemocoel with the help of broad-range receptors. The detection of a foreign surface activates the resting plasmatocytes that firstly tether to the said substance. Further, the hemocytes 'spread' to capture the substance [25]. This spread induces cytoskeletal and morphogenetic changes in the cell. Then they engulf and internalize the particle, which is further destroyed with the help of lysozymes, reaction oxygen species, nitric oxide, or antimicrobial peptides locally produced by the cells [26]. In the larval stages, plasmatocytes initiate the detection of a foreign subject or a wound that further increases the number of crystal cells and lamellocytes and starts encapsulation and melanization.

The hematopoiesis in *Drosophila* occurs in waves during pre-adult developmental stages [27]. The first wave occurs during embryogenesis, which generates plasmatocytes and crystal cells. The plasmatocytes migrate and spread in the whole embryo while crystal cells differentiate and remain localized around a specific gut region. The second wave of hematopoiesis occurs during the larval stages in the lymph gland. The lymph gland contains undifferentiated prohemocytes in the posterior lobe and differentiated hemocytes in the anterior lobe that are released into circulation. Lamellocytes are generally not differentiated in these waves. They are differentiated in large amounts in the lymph glands when facing a parasitic attack [22]. As they attain pupal stages, the lymph glands release the macrophages into circulation as this gland disperses during metamorphosis. These are called 'active phagocytes' that also play a role in phagocytosis of larval structures and hence remodelling of the organism. Earlier it was believed that adult stages entirely lack any hematopoietic activity [27]. However, recently there have been certain disputing pieces of evidence. One study suggested the existence of a hematopoietic hub in the adult stages, which could result in plasmatocyte proliferation on bacterial infection [28]. In contrast, another study found no evidence of this proliferation or differentiation in adults [29]. Recently it was shown that while adult plasmatocytes are typically non-differentiating, there is a small population of cells with minimal hematopoietic capabilities in adults, which they believe is not very noteworthy [30].

1.6. Nutrition and immunity

The immune response is a complex multi-dimensional trait that is impacted by a range of factors encountered by an organism. Some of these include genetics [31], age [32], time of the day [33], stress levels [34], commensal bacteria [35], and diet [36]. Nutrition is one of the modifiable factors here and is known to be tightly linked to an organism's immune response.

For the host cells, nutrition is essential at different levels. The metabolic state of a cell is a dynamic factor that changes with the functional requirements or, in some cases, is needed to activate a cell functionally. Immune cells also follow such metabolic rewiring to 'activate' and functionally adapt to cope with the stress of an infection. Nutrition will be critical here, as metabolism depends highly on the dietary environment [37]. Another level of complexity is the nutritional demands of immune cells to respond to an infection functionally. Considering the case of a bacterial infection in *Drosophila*, it would require sensing the pathogen and a further release of high abundances of AMPs into the hemolymph by the fat body. Hemocytes would also be required to migrate to the infection site to melanise the wound and phagocytose the pathogen by releasing AMPs and ROS. Other tissues could also be contributing to systemic infection eradication or dealing with the infection if directly infected [12]. All these processes would require the cells to generate a lot of ATP for this energetically demanding process. Additionally, they would require building blocks for synthesizing macromolecules like proteins to generate the required products like AMPs [38] [39]. Both these processes are nutritionally dependent as well.

These cells can either fulfil their nutritional requirements from an endogenous source, i.e. stored form of energy in the cells, or an exogenous source of dietary intake. As not all cells have stored reserves, dietary intake is essential [39]. Additionally, undernutrition has been shown to be detrimental to the immune response. Lowered dietary nutrition can result in inhibition of the immune functions and make the host further susceptible to more infections [39]. Also, a nutritional deficiency decreases a host's chances of fighting an infection effectively. On the other hand, over nutrition can be equally damaging. Considering an infection from the pathogen's perspective, the host's nutrition would be vital for it. The pathogen has high nutritional demands to grow and replicate and depends on the host for these demands. Thus, it would be in its best interest to increase the input of the host [40]. Therefore, for the host, an optimal immune response requires an optimal dietary intake.

Introduction

1.7. Dietary Restriction (DR)

Dietary restriction is the reduction in food's nutrient content without causing a deficiency or malnutrition [41]. Reducing dietary nutrients increases the lifespan of a range of animals across the animal kingdom. This increased longevity has been shown in several animals, including yeast, flies, mice, and *C. elegans* [41]. There are a few studies on primates as well, where one showed a higher lifespan in rhesus monkeys [42], and another showed better health parameters or anti-ageing effects on DR [43]. In humans, DR can improve markers related to ageing [44]. Now DR is defined as the phase in the nutritional-lifespan landscape where a decrease in the dietary nutrients results in an increase in the lifespan of the animals. Many of these studies show a parallel decrease in the fecundity of the animals [41].

A big debate in the nutrition field is whether this increased lifespan is because of a reduction in calories or some macronutrients. Initially, it was shown that an imbalance in the amino acids could be responsible for the lifespan-extending impacts of DR [45]. However, a growing body of evidence now suggests that it is because of the ratio of protein to carbohydrate in the diet [46]. A study in *Drosophila* successfully showed that while keeping the calorie content of the food constant, a change in protein to carbohydrate ratio could successfully change the lifespan of animals, where essential amino acids were the major players. Following this evidence, the term Dietary Restriction will be used in this thesis.

1.8. Protein malnutrition (PM)

Malnutrition or starvation is the severe deficiency or complete removal of dietary macronutrients, alone or in combination with the micronutrients [47]. This drastic nutritional shortage results in the deficiency of energy in the animal body and further disruption of normal bodily functions. Malnutrition is considered a significant health concern across the globe. It is one of the leading causes of death in children under five years of age [48]. Early-life malnutrition has been evidenced to cause developmental defects, where immunological development can also be impacted. As protein is the building block of life and contributes significantly to providing energy to the body, I chose to study protein malnutrition. DR would also be enforced through protein content, and a further decline in the protein content can be studied as PM.

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1.9. Effect of dietary interventions on immunity

Considering the importance of nutrition in the immune response, the field of nutritional immunology is very well investigated. However, it is essential to point out that both these components are highly complex and multi-dimensional. On the one hand, we have the immune system, which consists of multiple layers of response. Even in the case of invertebrates, which lack an adaptive response, innate immunity alone has several components, responsible tissues, signaling pathways, and multiple interactions between the tissues [15]. There cannot be a single measurable read-out of the whole system that would show the output for all the components. On the other hand, we have nutritional intake, which can consist of several macro and micronutrients in various combinations. Though all the nutrients are essential for an organism's general well-being and maybe play a part in the proper functioning of the immune system, their contributions are certainly involved to different degrees. Numerous studies show the impact of several or single macro and micronutrients on different components of immunity in different animals [39]. Micronutrients like vitamins and minerals are known to be integral for a proper response, and both their deficiencies and overfeeding have been linked to an immune disruption [49]. Similarly, macronutrients like protein and carbohydrates get a lot of focus and show different results in different studies with varying models and measured parameters. To show the complexity of this problem, some examples are shown below.

1.10. Effect of DR and PM on immunity

Even though there is a widespread belief in the nutrition community that DR only has beneficial effects on the health of an organism, the evidence does not support it entirely [41]. While many studies point to it, there is a number of studies with opposite results.

Considering the mammalian model mice, the earliest studies showed that CR betters some immune functions when infected with non-intact pathogens [50]. In contrast, later studies investigating infections with an intact pathogen showed better survival in fully fed individuals [51]. Interestingly, the *in-vitro* analysis in this setting showed improved immune functions in CR mice [52]. Thus, even though CR-fed mice show an enhancement in their immune functions, they are still more susceptible to infection. For the protein to carbohydrate ratio, it has also been demonstrated that a higher protein content results in better survival of mice infected with *Salmonella typhimurium* [53]. However, in another study, lower protein content lowered the immunopotential when fed for a short time but increased it after more prolonged

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treatment [54]. Wound healing is another example where CR-treated mice fail to heal well compared to well-fed ones [51].

Similar discrepancies are also observed in the simpler innate immune systems of invertebrates. Locusts have been shown to have better survival to fungal infections if raised on a restricted diet [55]. African army work *Spodoptera exempta* also is more susceptible to infections on a diet with a lower protein to carbohydrate ratio [56]. In contrast, caterpillars have positive effects on immune performance and the ability to resist bacterial and viral infections by increasing dietary protein [57]. They are also seen to prefer higher protein diets on infection as a form of self-medication [56]. Mealworm beetles also show a decline in the immune effector system after short-term nutrient deprivation [58].

All these results show the number of discrepancies that are observed in the effect of DR on immunity. If we go to the end of the spectrum and consider malnutrition, the results are a bit more unanimous. Malnutrition, specifically protein malnutrition, is associated with a decrease in immune response and increased susceptibility to infections [47]. As mentioned earlier, immune responsiveness is an energetically demanding process. Undernourished individuals do not have the proper resources to meet these demands.

There are several studies on the effect of protein malnutrition on immunity in mice. On infection with *Mycobacterium tuberculosis*, malnourished mice show impaired granuloma formation and die sooner. Similarly, on infection with *Salmonella typhimurium*, malnourished mice show higher infection rates and more chances of worsened infection [59]. One reason could be that malnutrition leads to an overall energy deficit, and the animal needs to preserve this energy supply to survive. This results in an overall decline in investments in the whole body, including the costly immune response. This resulting decrease in immunity makes them a better ground for the attack, survival, and reproduction of pathogens inside the body.

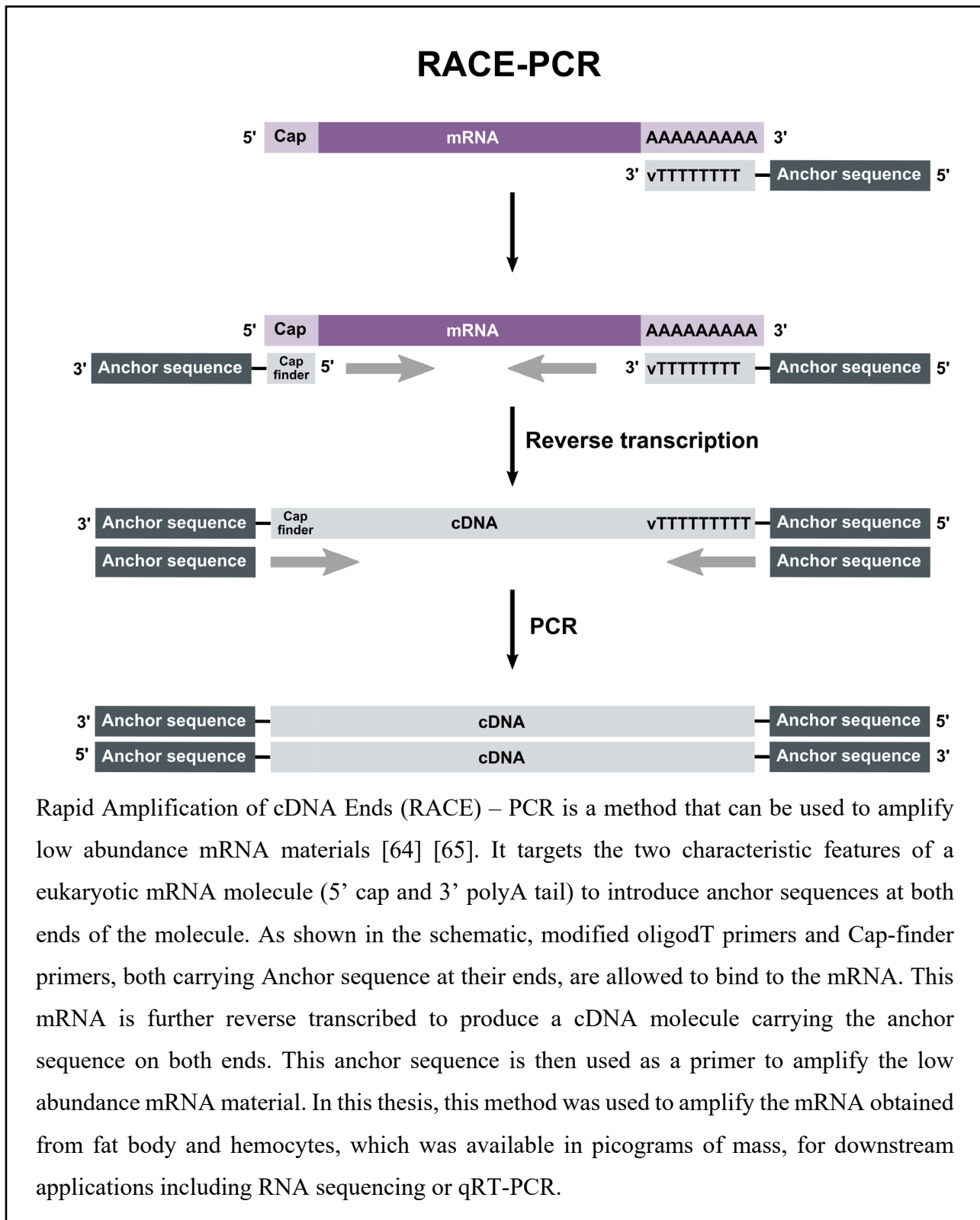
Interestingly, there are also studies that show that, in some cases, malnutrition can actually make organisms resistant to some kinds of infections. An example is the older studies where starved rabbits were seen to be more resistant to the vaccinia virus [60]. This could be because of the dependency of pathogen metabolism on host metabolism. For pathogens with specific metabolic requirements for replication, a malnourished host environment can cause them not to be able to establish themselves in the host.

1.11. Reasons for variability in the response

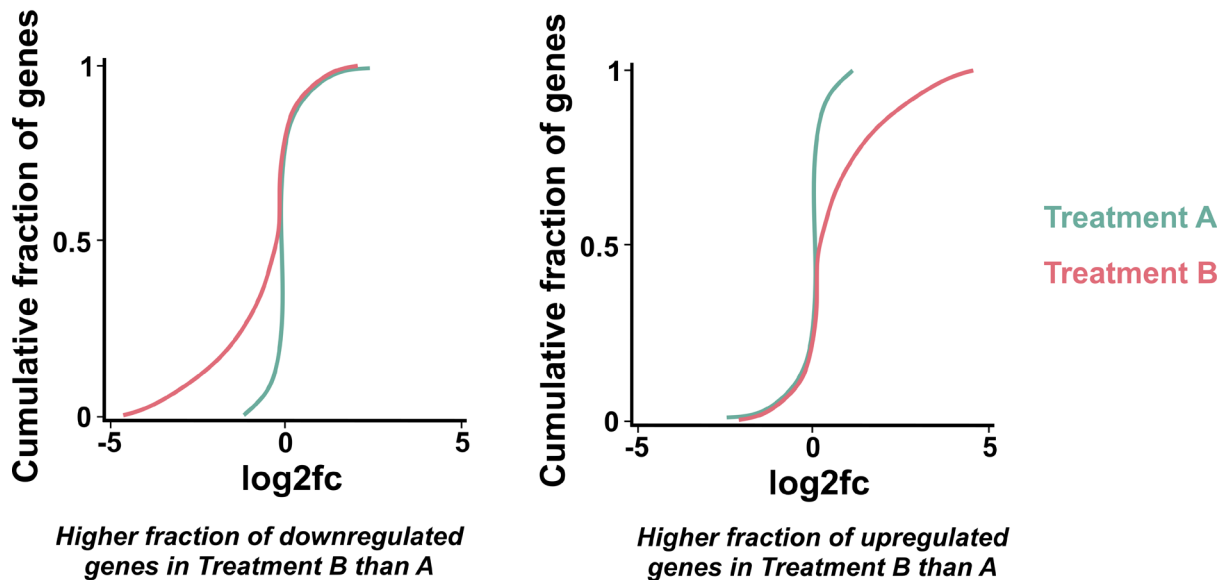
These differences in the results can be explained by several factors, like the type of diet, method and intensity of dietary restriction, duration of restriction, type of immune stress, type of pathogen, parameter of immunity measured, and many more. While some factors are unmodifiable, diet is a factor that can be controlled. However, the diets studied in different labs working on even the same animal can differ substantially. Even for the same diet, the restriction is enforced through different parameters and in different intensities and duration. All of these factors make the results from different studies incomparable. The added complexity of the parameter of immune response measured is also unavoidable. While most studies use the survival probability of infection as the most prominent parameter, it clearly is not a good proxy for other parameters [51]. Deeper navigation into the molecular basis can help understand the fundamental differences better.

Another unique approach to discuss here would be the Geometric Framework of Nutritional analysis to understand the effects on immune components [61]. A geometric framework (GF) is defined as measuring the required trait on different points in an n-dimensional space, with each dimension representing the amount of a different nutritional component. One study used this approach to study the patterns of different immune components in a geometric framework of various relative abundances of protein and carbohydrate [62]. They found that the different immune response components are not effectively maximized with higher quantities of nutrients. Instead, the ratio of components depicts the change. The various immune parameters reach their maximum values at different points in this GF. They found that no dietary composition could simultaneously maximize all the parameters. As most studies only take a few dietary regimes into account, they analyze a small part of this GF, where the optima can differ drastically, which they observe as a result when comparing different investigations. Additionally, they show a switch in animal dietary preferences when facing immune stress. This preference focuses on reaching an optimal point where different parameters perform well, if not maximized. In case the dietary input cannot be controlled, the internal investments in different components are altered. The investments also change with different infection challenges, as they would have different metabolic requirements and nutritional optima. An example of this is the DR-induced higher tolerance to *Salmonella typhimurium* and lower resistance to *Listeria monocytogenes* in *Drosophila* [63].

Two technical aspects are introduced here which were used in this thesis:



How to Interpret CDFs



Cumulative Distribution Functions (CDFs) were used in this thesis to visualise and compare the expression trend for a group of genes between different treatments [66]. A schematic description here is shown to represent how to interpret CDFs. The x-axis in the graph represents the \log_2 fold change and the y-axis represents the cumulative fraction of genes. The curve represents the fold change values in the treatment following an order of least to the greatest values along with the frequency for each \log_2 fc. Some observations that can be made from these graphs:

- For treatment B, the \log_2 fc values go from -5 to around 2 in the left graph and from 5 to -2 in the right one for this set of genes
- For Treatment B, 50% of the genes are downregulated in the left graph and 50% are upregulated in the right one
- When comparing multiple CDFs, one can compare the curves to understand the differences in the trend for multiple treatments. In the above schematic, Treatment A has no change in the expression for most genes in both left and right graph. In the left graph, Treatment B has more downregulated genes than A and in the right graph, Treatment B has more upregulated genes than A [67].

2.Objectives of the thesis

The quality of an organism's immune response is controlled by many aspects, like genetics, age, diet, circadian rhythm, and commensal bacteria. Some of these are unmodifiable, while diet is one of the factors that can be controlled for an optimal response. Malnutrition - the significant reduction in dietary nutrients to cause deficiency - is linked to a substantial health decline. However, Dietary Restriction - the reduction in dietary nutrients without causing malnutrition - is believed to have several benefits for the immune system. Nevertheless, this highly depends on the type of nutrient restricted and the kind of immune parameter measured. The central goal of this thesis was to provide a deeper understanding of the influence of nutrition on immunity. The impact of dietary restriction and malnutrition are highly studied in *Drosophila* and many other animals for synergistic immune parameters, like survival, bacteria clearance, and enzymatic outputs of different cascades. While these can serve as a useful holistic output, the information on different components and their mechanical underpinnings is lost. To this purpose, the molecular changes happening in hemocytes and fat bodies, the professional immune organs of *Drosophila*, were targeted. Investigating all the biological processes changing in these immune cells, facing infection and dietary interventions, can give us a more comprehensive overview of the changes in resource allocation. These basic molecular processes can be expected to be more constant with experimental differences than an integrated output like survival.

With the primary goal of the study being elucidation of the effects of dietary protein amount on different components of the immune system, the following primary questions were asked:

1. How do the holistic immune parameters differ in diets with different dietary protein content?
2. Can the biological processes changing in the fat body and hemocytes explain these differences?

With these questions in mind, the study followed this structure:

1. The basic transcriptional profiles of professional immune tissues, fat body and hemocytes were characterized after an infection
2. Holistic immune parameters, including survival to infection and bacteria clearance rate, were compared for different diets

Objectives of the thesis

3. The transcriptome of fat body and hemocytes of animals on different diets were compared in an uninduced state to see if there are differences
4. The transcriptional profiles of fat bodies and hemocytes of animals on different diets were compared after infection and injury to investigate the differences in resource allocation

3. Materials and Methods

3.1. Fly rearing

In all the experiments, animals were raised on a standard cornmeal yeast molasses medium at 25°C with 65% relative humidity and a 12h:12h L:D cycle. This was done by using big plant culture tubes where the bottom was removed and a plate containing media was attached to this end. Animals of desired genotypes were allowed to lay eggs in these tubes overnight and then the density of the eggs was set to 350 eggs per tube. When these eggs reached the pupal stage, the plate with standard media was replaced with a plate with a modified exome-matched holidic diet (described in detail later). Two days after hatching, the animals were further transferred to standard fly-rearing vials (nerbe plus GmbH (Winsen, Germany)) for variable times depending on the experiment. The media was changed every 2 days in the adult stage. For all experiments, female flies were used.

3.2. *Drosophila* culture media

3.2.1. Standard cornmeal yeast molasses media

For preparing 1L of standard cornmeal yeast molasses media the following ingredients were added:

Ingredient	Amount	Source of supply
Brewer's yeast	62.5g	Leiber (Bramsche, Germany)
Cornmeal	62.5g	Mühle Schlingemann (Waltrop, Germany)
Agar-agar	10g	Biolab products (Bebensee, Germany)
Glucose	20g	Carl Roth (Karlsruhe, Germany)
Molasses	30g	Biohof Heidelberg (Gerdau, Germany)
Sugar beat syrup	30g	Kanne Brottrunk (Selm-Bork, Germany)
Water	1L	
Cook for 10-15 minutes in water bath until the ingredients dissolve and a thick consistency is reached. Autoclave the media for 20 minutes. Cool it down to 60°C and add the following		
Propionic acid (10% in water)	10mL	Carl Roth (Karlsruhe, Germany)
Nipagin (10% in EtOH)	30mL	Carl Roth (Karlsruhe, Germany)

3.2.2. Modified exome-matched holidic media

The control holidic media (NM) described here was a modification of the recipe provided by Piper et al.[68]. Additionally, the composition of the Dietary restriction (DR1, DR2) and Protein Malnutrition (PM) are also mentioned here. The amount of protein in control diet (NM) was 22g/L (P:C – 1:0.8) which was reduced to 2g/L in DR1 (P:C – 0.125:1), 1g/L (P:C – 0.0625:1) in DR2 and 0g/L in PM (P:C – 0:1). Following are the recipes for 1L of media.

COMPONENTS	Stock Recipe	NM	DR1	DR2	PM
L-isoleucine (g)		1.12	0.112	0.056	0
L-leucin (g)		2.03	0.203	0.102	0
L-tyrosine (g)		0.93	0.093	0.0464	0
Water (mL)		565.1	804.1	817	830.6
Agar (g)		20	20	20	20
Sucrose (g)		17	17	17	17
CaCl ₂ stock (mL)	250g/L CaCl ₂ .2H ₂ O in H ₂ O	1	1	1	1
CuSO ₄ stock (mL)	2.5g/L CuSO ₄ *5H ₂ O in H ₂ O	1	1	1	1
FeSO ₄ stock (mL)	25g/L FeSO ₄ *7H ₂ O in H ₂ O	1	1	1	1
MgSO ₄ stock (mL)	250g/L MgSO ₄ .7H ₂ O in H ₂ O	1	1	1	1
MnCl ₂ stock (mL)	1g/L MnCl ₂ *4H ₂ O in H ₂ O	1	1	1	1
ZnSO ₄ stock (mL)	25g/L ZnSO ₄ *7H ₂ O in H ₂ O	1	1	1	1
Autoclave the above mentioned components and buffer base separately (120°C for 15 minutes)					
Buffer base (mL)	in 1L H ₂ O:	100	100	100	100
Cholesterol (ml)	20mg/mL in ethanol	7.5	7.5	7.5	7.5
Ergosterol (mL)	80mg/mL in Butanol (heated for 2 hours at 70°C to dissolve)	1.875	1.875	1.875	1.875
Put the beaker with autoclaved food on a stirrer. Add cholesterol and buffer base. Wait for it to reach 65°C and then add the following components					
Cobalamine (mL)	0.05g/L Vitamin B12 in H ₂ O	1	1	1	1
Molybdenum (mL)	0.1g/L Na ₂ MoO ₄ .2H ₂ O in H ₂ O	1	1	1	1
Ribonucleotides (g)		2	2	2	2
Cysteine (mL)	74g/L in H ₂ O	6	0.6	0.3	0

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Essential AA stock (mL)	in 1L H ₂ O: 13.48 g L-Arginine 5.4 g L-histidine 14.09 g L-lysine (HCl) 4.99 g L-methionine 8.34 g L-phenylalanine 9.15 g L-threonine 2.66 g L-tryptophan 9.93 g L-valine Adjust pH to 4.5 using NaOH and HCl	121.02	12.102	6.051	0
Non-essential AA stock (mL)	in 1L H ₂ O: 9.1g L-alanine 8.51 g L-asparagine 12.6 g L-aspartic acid 9.26 g L-glutamine 6.33 g Glycine 8.09 g L-proline 11.39 g L-serine Adjust pH to 4.5 using NaOH and HCl	121.02	12.102	6.051	0
Sodium glutamate	100g/L in H ₂ O	17.5	1.75	0.873	0
Cobalt (mL)	1g/L in H ₂ O	1	1	1	1
Vitamin solution stock (mL)	in 1L H ₂ O: 0.1 g thiamine (aneurin) 0.05 g riboflavin 0.6 g nicotinic acid 0.775 g Ca pantothenaten 0.125 g pyridoxine 0.01 g biotine	21	21	21	21
Sodium folate stock	0.5g Sodium folate in 1L H ₂ O.	1	1	1	1

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Other nutrients stock (mL)	in 1L H ₂ O: 6.25 g choline chloride 0.63 g myo-inositol 8.13 g inosine 7.50 uridine	8	8	8	8
Propionic acid 100%		6	6	6	6
Nipagin (mL)	10% v/v in EtOH	15	15	15	15

Following is the list of sources for holidic diet ingredients:

Chemicals	Vendor
L-isoleucine	Carl Roth (3922.2)
L-leucine	Carl Roth (3984.3)
L-tyrosine	Carl Roth (T207.2)
Agar-agar	Carl Roth (5210.2)
CaCl • 6H ₂ O	Carl Roth (5239.2)
Copper(II) sulfate pentahydrate	Sigma-Aldrich (C7631)
Iron(II) sulfate • 5H ₂ O	Sigma-Aldrich (F7002)
MgSO ₄ • 4H ₂ O	Carl Roth (P027.2)
Manganese(II) chlorid • 4H ₂ O	Sigma-Aldrich (M3634)
Zinc sulfate • 5H ₂ O	Sigma-Aldrich (Z0251)
KH ₂ PO ₄	Carl Roth (P018.2)
NaHCO ₃	Sigma-Aldrich (S5761)
Cholesterol	Sigma-Aldrich (C8667)
Ergosterol	Sigma-Aldrich (45480)
Cobalamin	Sigma-Aldrich (V6629)
Molybdenum	Carl Roth (0274.1)
L-cystein HCl	Carl Roth (3468.2)
L-arginine monohydrochloride	Sigma-Aldrich (A5131)
L-alanine	Sigma-Aldrich (A7627)
L-aspartic acid	Sigma-Aldrich (A6683)
L-asparagin	Sigma-Aldrich (A0884)
L-prolin	Carl Roth (T205.2)
L-glutamin	Carl Roth (3772.2)

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L-serin	Carl Roth (1714.2)
L-threonin	Carl Roth (T206.2)
L-methionine	Carl Roth (9359.1)
L-tryptophan	Carl Roth (4858.2)
L-glutamic acid monosodium salt hydrate	Sigma-Aldrich (G5889)
L-phenylalanine	Carl Roth (4491.1)
L-histidine	Carl Roth (3852.3)
L-lysin HCl	Carl Roth (9357.1)
L-valin	Carl Roth (4879.4)
Thiamine	Sigma-Aldrich (T4625)
Riboflavin	Carl Roth (9607.1)
D(+)-biotin	Carl Roth (3822.1)
Nicotinic acid	Carl Roth (3815.2)
Calcium-D(+)-pantothenat	Carl Roth (3812.2)
Pyridoxine HCl	Carl Roth (T914.1)
Folic acid	Carl Roth (T912.1)
Cholin chloride	Sigma-Aldrich (C1879)
Myo-inositol	Sigma-Aldrich (I7508)
Inosine	Carl Roth (6770.1)
Uridine	Carl Roth (0714.3)
Ribonucleic acid from torula yeast	Sigma-Aldrich (R6625)
Cobalt	Sigma-Aldrich (CDS004010)
Glacial acid	Carl Roth (3738.2)

3.3. Sterile injury, bacterial infections, and bacterial load measurement

The following strains of bacteria were used: *Bacillus subtilis* (ATCC6051), *Serratia marcescens* Db11, *Erwinia carotovora carotovora* (Ecc2046). Infections were performed using secondary cultures of the bacteria grown in LB media (Sigma Aldrich (Munich, Germany)) at 30°C. The bacteria were suspended in sterile PBS (8 g/l NaCl (Carl Roth), 0.2 g/l KCl (Carl Roth), 1.78 g/l NaH₂PO₄ (Carl Roth), 0.24 g/l KH₂PO₄ (Carl Roth) (pH = 7.4))

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and adjusted to the following OD₆₀₀ for the different bacteria: *S. marcescens* - 0.01, *Ecc2046* - 15 and *B. subtilis* - 1.5. Infections were performed by dipping a minuten needle in this bacterial solution and piercing the thorax of animals [69]. For sterile injury, the needle was dipped in sterile PBS and used to pierce the thorax. Bacterial load was also measured using the same protocol [69]. Briefly, one fly was taken per sample at the given time, homogenized in PBS, and dot-plated in serial dilutions. The colonies were counted after overnight growth.

3.4. Lifespan, fecundity, and egg volume

For measuring the lifespan, 8-10 animals were transferred to one vial and the number of dead individuals was checked every second day [70]. For counting the number of eggs, two females were kept in one vial with five males. The food vial was changed every 24 hours and the number of eggs laid in each vial were counted. To measure the egg volume, the eggs were imaged using the microscope. The images were analysed using FiJi [71] to measure the longest (c) and the shortest diameter of the eggs. Volume was calculated considering the egg as an ellipse and using the formula $\frac{\pi}{6}ca^2$.

3.5. Triglyceride and Protein content quantification

For measuring the body composition and weight protocols described in [72] . Briefly, five animals were taken for each biological replicate, weighed using microbalance (ABS 80-4, Kern & Sohn GmbH), and homogenized in 500µl 0.05% Tween-20 (Carl Roth (Karlsruhe, Germany)) in PBS (recipe in section 3.3) using a Bead ruptor (BioLab Products, Bebensee, Germany) [73]. Half of this volume was used to measure the body fat content by coupled colorimetric assay and the other half was used to measure the body protein content by Bicinchoninic acid assay. For body fat content, the solution was heat inactivated at 70°C for 5 minutes, centrifuged to remove the debris and incubated with 200 µl triglyceride solution (Thermo Fisher Scientific, TR22421) at 37°C for 30 minutes. Absorbance was measured at 540 nm using a Micoplate reader Synergy H1 (BioTek Instruments GmbH) and amount of stored fat was calculated using the standard curve prepared from glyceryl trioleate (Sigma Aldrich (Munich, Germany)). For body protein content, Peirce BCA protein assay kit (Thermo Fisher Scientific, Waltham, United States) using a microplate assay was used following their provided protocol. Briefly, the solution was centrifuged to remove the debris and incubated with the working reagent at 37°C for 30 minutes. Absorbance was measured at 562 nm using the

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Micoplate reader Synergy H1 (BioTek Instruments GmbH) and amount of protein was calculated using the standard solution provided was manufacturer.

3.6. RNA extraction and cDNA synthesis for whole animal

For RNA extraction from whole animal body, three individuals per biological replicate were directly transferred to RNA magic (Bio-Budget, #56-1000-100) and homogenized using a Bead ruptor (BioLab Products, Bebensee, Germany). Total RNA was extracted using Ambion Purelink RNA mini kit (Thermo Scientific (Waltham, USA)) using their provided protocol. The cDNA synthesis was performed using an oligo dT primer with the following reaction and procedure details [74]:

	Vendor	Concentration added	Volume added
To bind the OdT primer to RNA, the following were added to PCR tubes:			
OdT primer*	Thermofischer Scientific	10pmol	0.5µL
dNTP mix	Thermofischer Scientific	10mM	0.5 µL
RNA template			
Add upto 6.5 µL RNase-free water, vortex and incubate for 5 min at 65°C, followed by incubation on ice			
5X SSIV Buffer	Thermofischer Scientific		2 µL
DTT	Thermofischer Scientific	0.1M	0.5 µL
Ribolock RNase Inhibitor	Thermofischer Scientific	10U	0.5 µL
RT SSIV	Thermofischer Scientific	100U	0.5 µL

*OdT primer sequence:

GAGAGAGGATCCAAGTACTAATACGACTCACTATAGGGAGATTTTTTTTTTTTTTTTTTTTTTTTTT

The reaction tubes were put in the Thermocycler (SensoQuest GmbH (Göttingen, Germany)) using the following details:

- Incubate for 10 min at RT (25 °C)
- Synthesis for 20 min at 50-55 °C
- Stop Reaction for 10 min at 80°C

3.7. Fat body and hemocyte isolation and RNA extraction

Fat body was dissected from the dorsal abdominal region and directly transferred to RNA magic (Bio-Budget, #56-1000-100) as described earlier [75]. Hemocytes were extracted by removing the antenna and applying light pressure on the thorax of the animals to get a clear drop of hemolymph, which was further collected using a microcapillary [76] and directly suspended into RNA magic. For each replicate of fat body, one female was used, while for hemocytes, one replicate was made by pooling material from five females. The cells in RNA Magic were homogenized using a bead ruptor (BioLab Products, Bebensee, Germany), and total RNA was isolated using the phenol-chloroform-isopropanol method.

3.8. cDNA synthesis and amplification for fat body and hemocytes

cDNA synthesis and amplification for fat bodies and hemocytes were done using the two-step Smart-Seq3 protocol [77] [78]. This protocol can account for the low abundances of the material we have for these cell types.

Following are the steps for cDNA synthesis

	Vendor	Concentration added	Volume added
Add the following to a PCR tube			
PEG 8000	Sigma Aldrich	50%	1 μ l
Triton X-100	Sigma Aldrich	10%	0.1 μ l
OligodT Primer*	TaKaRa Bio	10 μ M	0.5 μ l
dNTPs	Sigma Aldrich	25 mM/each	0.2 μ l
H ₂ O			0.4 μ l
RNA Template			3.5 μ l
<i>Total</i>			<i>5.7 μl</i>
Incubate the above at 72°C for 10 min and then at 4°C			
Add 4.3 μ l of the following master mix to each reaction:			
RiboLock	ThermoFischer Scientific	40 U/ μ l	0.1 μ l
Tris-HCl Buffer pH 8.3	Sigma Aldrich	250 mM	1 μ l
NaCl	Thermo Fisher Scientific	300 mM	1 μ l
MgCl ₂	Thermo Fisher Scientific	25 mM	1 μ l

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GTP	Thermo Fisher Scientific	100 mM	0.1 µl
DTT	Thermo Fisher Scientific	100 mM	0.8 µl
CapFinder Primer*	TaKaRa Bio	100 µM	0.2 µl
Maxima H RT	Thermofischer Scientific	200 U/µl	0.1 µl

*Primer sequences:

Oligo dT: AAGCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT

Capfinder primer: AAGCAGTGGTATCAACGCAGAGTACAT666

The reaction tubes were put in the Thermocycler (SensoQuest GmbH (Göttingen, Germany)) using the following details:

Temperature	Time	Number of cycles
42°C	90 min	1x
50 °C	2 min	10x
42°C	2 min	
85°C	5min	1x

The synthesised cDNA was checked for the number of cycles required for amplification by amplifying for multiple cycles at an interval of three. Usually a 25 µl reaction was prepared for checking the cycle number. Here are the details for 100uL of such reaction:

	Vendor	Concentration added	Volume added
LA PCR Buffer	TaKaRa Bio	10X	10 µl
MgCl ₂	TaKaRa Bio	2.5mM	10 µl
dNTP Mix	TaKaRa Bio	25mM	1.6 µl
PCR Primer*	TaKaRa Bio	10 µM	2 µl
LA Taq Polymerase	TaKaRa Bio		0.5 µl
H ₂ O			x µl
cDNA			x µl
<i>Total</i>			<i>100 µl</i>

*Primer sequence:

PCR primer: AAGCAGTGGTATCAACGCAGAGT

The reaction tubes were put in the Thermocycler (SensoQuest GmbH (Göttingen, Germany)) using the following details with multiple cycle numbers:

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Temperature	Time	Number of cycles
94°C	1 min	1x
98°C	10 sec	x cycles
68°C	5 min	
72°C	10 min	
4 °C	∞	

The amplified cDNA was checked on a 1.5% agarose (Biolab products (Bebensee, Germany)) gel prepared in TE buffer (10 mM Tris·Cl, pH 8.0, 1 mM EDTA). A cycle number was chosen such that the product was still in exponential phase and not saturation phase of a PCR. The above amplification PCR was repeated choosing appropriate cycle number for each sample. The amplified cDNA was cleaned using the Monarch® PCR & DNA Cleanup Kit (New England Biolabs, Ipswich, United States) using the protocol provided from the manufacturer. The product was eluted in water and the concentration was measured using the Qubit dsDNA HS assay kit (ThermoFischer Scientific) and Qubit 3 (ThermoFischer Scientific) using manufacturer's protocol.

3.9. Quantitative real time PCR

The cDNA obtained from the whole body (section 3.6) or the tissues (section 3.8) was used for qRT-PCR using the following reactions:

	Vendor	Concentration added	Volume added
qPCRBIO SyGreen Hi-ROX	PCR Biosystems		5 µl
Forward primer		25 µM	0.5 µl
Reverse primer		5 µM	0.5 µl
cDNA		5 ng/µl	4 µl

The reactions were pipetted with two technical replicates for each sample into individual wells of a 48 or 96-well plate (ThermoFischer Scientific), sealed using an adhesive film and the following reaction conditions were used in the QuantStudio 3 qPCR Thermocycler (ThermoFischer Scientific):

Temperature	Time	Number of cycles
95°C	10 min	1x

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95°C	15 sec	40x
60°C	20 sec	
72°C	35 sec	
95°C	15 sec	1x
60°C	1 min	1x
95°C	15 sec	1x

The Ct values obtained and used for the calculation of relative expression with the help of $\Delta\Delta C_t$ calculations [79].

The following Primers were used for qPCR in different experiments in this thesis:

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Rpl32	CCAGTCGGATCGATATGCTAA	GTTTCGATCCGTAACCGATGT
Mtk	CCACCGAGCTAAGATGCAA	AATAAATTGGACCCGGTCTTG
Drs	GTA CTTGTTTCGCCCTCTTCG	AGCATCCTTCGCACCAGCAC
CrebA	AAGTCCGGAACACCTGTTTG	GTGGTAACCGTTGAGCTGGT
AttB	ACAATCTGGATGCCAAGGTC	TGTCCGTTGATGTGGGAGTA
Dro	GTTCAACCATCGTTTTCTGC	GGCAGCTTGAGTCAGGTGAT
CecC	AAGATCTTCGTTTTTCGTCGC	GTTGCGCAATTCCCAGTC
Bom	TCGTCACCGTTTTTGTGCTC	CCCTGCAATCGCCATTGATG
Dip	GATGGTTTTGGCTTTGCAGT	TTTCCAGCTCGGTTCTGAGT
Tret1-1	GGCTCCCTAGTGGTCGGATT	GCATCTTGGGTGACCTCAAACG
Rpl13	CAGGCATGCGAACCGCGTCAA	CAGGCATGCGAACCGCGTCAA
GAPDH1	CAGTGCTGGAGCCGAGTATGTGG	TGCACGAGGCGTTGGAGACC
Act42a	CTCCGTCCACCATGAAGATT	TCTGAAGGAGCGGAAGTGTT
Ptth	GGTGGTCACCAGATCCTCAT	ATTCAGTCCCAGCACTCCAC

3.10. Sequencing and Analysis

Library preparation was done using an adaption of the protocol described in Picelli et al. 2014 [80] and sequencing was done on the NextSeq 500 (Illumina, California, US) using the High Output Kit v2.5 (75 cycles). The quality of the reads was checked using fastqc (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). The trimming of low-quality reads, mapping to the reference genome (BDGP6) [81] and calculation of read counts for each

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gene were done using CLC workbench (Qiagen, Hilden, Germany). The differential expression of each gene was calculated using the BioConductor package DESeq2 [82, p. 2] (employing the shrinkage estimator apeglm [83]) and GO term analysis on differential expressed genes was performed using DAVID [84], flyenrichr [85] and ShinyGO [86]. Additional graphs were plotted using GraphPad Prism v7. For comparisons of the whole-body data set [87] to our dataset, their read counts for individual replicates were reanalysed using the same procedure as mentioned above. The expression levels for core genes were calculated as the average of the gene expression in all the bacterial treatments.

3.11. Imaging of fat body and hemocytes

Fat bodies were dissected from the control and bacteria infected individuals as described in section 3.7. The tissue was further fixed for 15 minutes in 4% (w/v) paraformaldehyde (Polysciences Inc. (Warrington, USA)) in PBS (recipe in section 3.3) at room temperature and mounted on a microscope slide using RotiMount Flour-Care DAPI (#HP20.1, Carl Roth GmbH, Karlsruhe, Baden-Württemberg, Germany). The hemocytes were collected using a microcapillary as described earlier and the hemolymph volume was released onto a microscope slide coated with Poly-L-Lysine (CellSystems, Troisdorf, Germany). This was immediately followed by putting a small drop of PBS over the hemolymph and incubating the slide in a moist chamber for 15 minutes, to let the cells adhere to the slide. The cells were further fixed using 4% Paraformaldehyde for 10 minutes, gently washed with 0.1% PBST (PBS containing 0.1% Tween 20) three times and mounted using Rotimount. Imaging was performed using fluorescence microscope Axio Imager Z1 (Carl Zeiss, Jena, Germany), equipped with Apotome (Carl Zeiss Image Axio Vision, Jena, Germany).

3.12. Bodipy staining of hemocytes

For staining the lipid droplets in hemocytes, the cells were fixed as described in section 3.11. The fixed cells were incubated with 1:500 dilution of Bodipy 493/503 (ThermoFischer Scientific) in PBS for 15 minutes in the moist chamber, followed by washing three times with 0.1% PBST (same as section 3.11). They were further stained with 1:50 dilution of Alexa Fluor™ 488 Phalloidin (ThermoFischer Scientific) for 15 minutes in the moist chamber and then washed three time. The cells were further stained with 1:1000 dilution of DAPI in PBS, washed and mounted using Mowiol (Carl Roth (Karlsruhe, Germany)) containing DABCO (Carl Roth (Karlsruhe, Germany)). The cells were imaged in the same day using Zeiss LSM880 confocal microscope (Carl Zeiss, Jena, Germany)

3.13. Hemocytes Lipid Droplet number and size quantification

For measuring the number and size of lipid droplets (LDs) in hemocytes, z-section images were obtained using Zeiss LSM880 confocal microscope (Carl Zeiss, Jena, Germany) with the protocol described in Section 3.12. The z-section images were taken for the whole width of the cell at a gap of 1µm. Image analysis was performed using FIJI [71]. To calculate the cell volume, the area of the cell was measured in the middle z-section and this measurement was further used to calculate the radius and hence the volume. To measure the LD size, maximum intensity projection was obtained for all the z-sections covering the cell for the GFP channel. The image was threshold and brightness were adjusted so that each LD could be segregated and the image was converted to binary. The ‘analyse elements’ tool of FIJI was employed to measure the area covered by each LD. These values were further used to calculate the diameter and hence volume of each LD.

3.14. Hemolymph trehalose and glucose level measurement

The hemolymph trehalose and glucose levels were measured using the protocols described in [72]. For hemolymph extraction, 12 animals per replicate were pricked on the thorax and transferred to the DNA extraction spin columns (after removing the DNA binding layer and only keeping the cell removal filter). The spin columns were centrifuged at 10,000g for 7 minutes at 4°C and 1µL of the hemolymph drop hence obtained was collected and released into 99µL Trehalose buffer (5 mM Tris pH 6.6, 137 mM NaCl, 2.7 mM KCl). The trehalase in this solution was heat inactivated by incubating at 70°C for 10 minutes. This solution was split into two parts, one of which was used to measure free glucose and other one for trehalose. The half to measure trehalose was treated with Trehalase solution (5 µl porcine trehalase (Sigma; T8778-1UN) / 1 ml Trehalose Buffer) at 37°C for 18-24 hours to digest the Trehalose into Glucose to calculate trehalose abundance. Glucose amounts in both parts were quantified using Glucose HK kit (Sigma Aldrich, St. Louis, United States) using the manufacturer’s protocol. The absorbance was measured at 340nm were performed using a Micoplate reader Synergy H1 (BioTek Instruments GmbH).

3.15. PAS staining of fat body

The stored glycogen in fat body was measured by quantifying the intensity of Periodic Acid-Schiff stain. The fat body of adult *Drosophila* was isolated and fixed as described in Section 3.7. It was further stained with 1:7 dilution of periodic acid in water for 2 minutes and then

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washed 3 times with 0.1% PBST (PBS containing 0.1% Tween 20). It was further stained with 1:7 dilution of Schiff's reagent in water for 7 minutes, washed with PBST, mounted using glycerol, and imaged using Stereo light microscope Leica S6 E (Leica Microsystems, Wetzlar, Germany). FIJI [71] was used to calculate the intensity of the stain by measuring the Mean Gray Values in small areas of equal sizes across the tissue. The inverse of Mean Gray Value was directly proportional to the intensity of the stain which was compared between the treatments.

3.16. 2-NBDG uptake measurement for fat body

The glucose uptake in the fat body was measured by a modification of the 2-NBDG uptake assay used by [88, p. 5] for the *Drosophila* gut. A single animal was pinned to the dissection plate at a time and a filial cut was made as described as in [89]. All organs on top of the fat body attached to the dorsal abdominal cuticle of the animal were removed to expose the fat body. The live fat body cells attached to the cuticle were incubated with a freshly prepared 300µm solution of 2-NBDG (Thermofischer) in PBS (section 3.3) for 15 minutes. The cells were then washed with PBS and fixed for 10 minutes with 4% (w/v) paraformaldehyde (Polysciences Inc. (Warrington, USA)) in PBS. The fixed fat body was dissected out on a microscope slide, washed with 0.1% PBST, mounted using RotiMount Flour-Care DAPI (#HP20.1, Carl Roth GmbH, Karlsruhe, Baden-Württemberg, Germany) and imaged using fluorescence microscope Axio Imager Z1 (Carl Zeiss, Jena, Germany), equipped with Apotome (Carl Zeiss Image Axio Vision, Jena, Germany).

4. Results

4.1. Hemocytes and fat body cells, the only professional immune cell types in *Drosophila*, show strikingly different responses to systemic infections

4.1.1. Experimental setup and quality control

To compare the transcriptome responses of the fat body and hemocytes, we subjected both tissue types to the identical sequencing protocol. However, because adult hemocytes cannot be isolated in such quantities in pure form to allow direct sequencing, we used an amplification protocol for both tissues. Moreover, we employed a cDNA synthesis protocol that accounts for low abundance and hence results in minimal loss [77] and amplified this material by Rapid amplification of cDNA ends PCR (RACE - PCR, [90]) (Fig4A) [77].

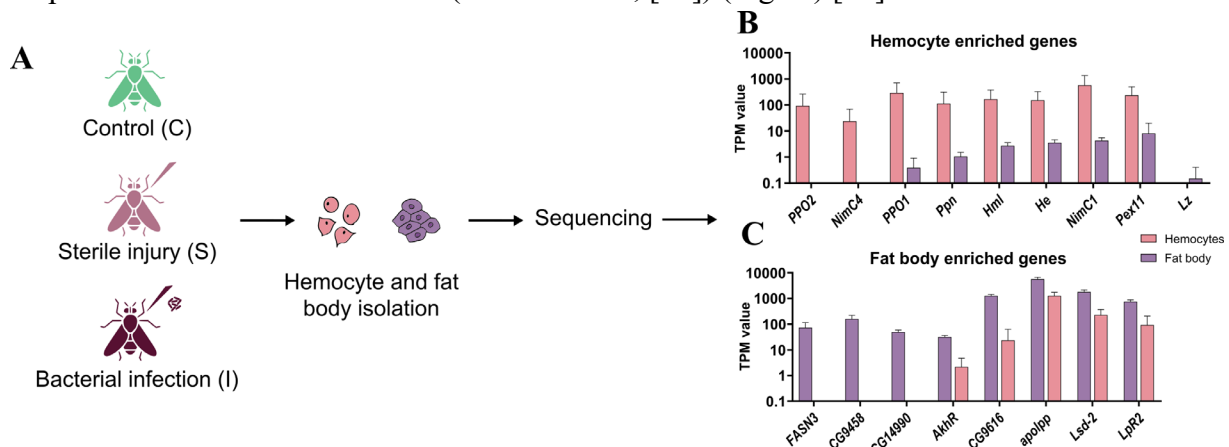


Figure 4: Experimental design of the systemic infection experiments. (A) Scheme showing the setup of the experiment. Samples were collected from control(C), sterile injury(S), and bacteria-infected (I) animals. Infections were performed using the bacterium *Bacillus subtilis*. Hemocytes and fat bodies were isolated 20 hours after infection, subjected to RNA extraction, cDNA synthesis, and amplification, and then sequenced. Three biological replicates were sequenced for fat body (one animal per replicate) samples and five for hemocytes (hemolymph pooled from 5 animals in each replicate). (B) TPM values for some known hemocyte-enriched genes in both hemocytes and fat bodies samples. Most genes show a 100-fold increase in the expression value in the hemocyte samples, indicating hemocytes are the main cell types in the volume. (C) TPM values for some of the most enriched genes in the adult fat body according to Flyatlas2 in both hemocytes and fat body samples. All genes show much higher expression in the fat body samples, indicating the sample mainly consists of fat body cells

I confirmed the purity of our hemocyte samples by looking at the hemocyte population obtained using the reporter lines *Hml-GFP* and *srpHemo-mcherry* and observed fluorescence in all cells obtained. Furthermore, I validated the amplification system by comparing the expression of well-known hemocyte marker genes in the hemocyte as well as fat body transcriptomes to confirm their enrichment in the former. These markers included *hemolentin*, *hemese* [91], which are known for all developmental stages and some larval hemocyte markers including

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Prophenoloxidase1, *Prophenoloxidase2*, *papilin*, *Nimrod C4*, *Nimrod C1* and *Peroxin 11*, which are also present in adult hemocytes [30] (Fig. 4B). Consistent with the previous larval and adult hemocyte studies, no expression of *glial cells missing* or *glial cells missing 2*, which are only expressed in the embryonic stage of hemocytes [91] or *lozenge*, which is required for crystal cell differentiation, could be detected [92] (Fig. 4B). Though there is no known marker for crystal cells in adults, the absence of *Lozenge* can indicate that our hemocyte population

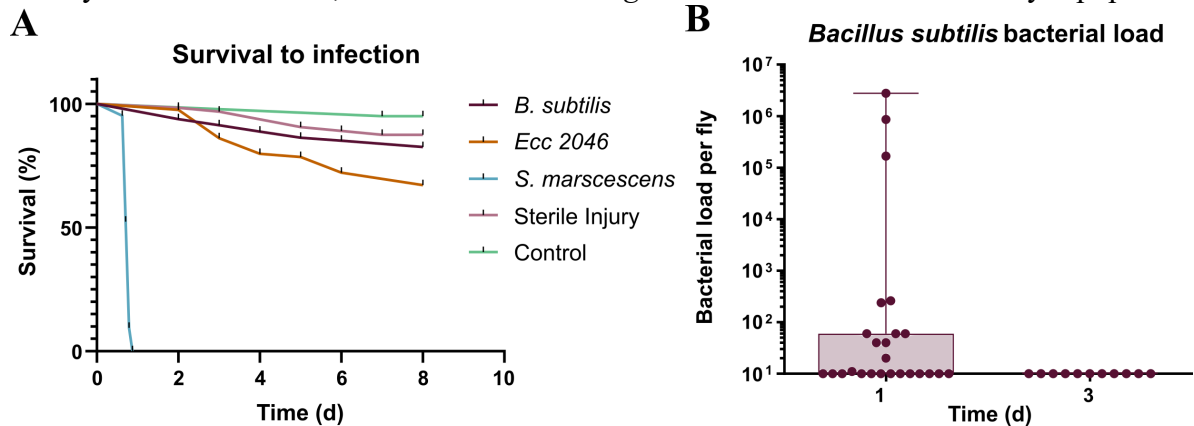


Figure 5 Experiment to choose the bacteria for infections and time point post infections (A) The survival curve of flies subjected to the following treatments: Untreated Control (n=40), Sterile Injury (n=64), *S. marcescens* systemic infection (OD₆₀₀ 0.01; n=30), *Ecc2046* systemic infection (OD₆₀₀ 15; n=82), *Bacillus subtilis* systemic infection (n=81). **(B)** Bacterial load of the flies 24 hours (n=25) and 72 hours (n=11) after infection with *Bacillus subtilis*. Each dot represents one animal, mean values \pm S.D..

mainly consists of plasmatocytes. Similarly, for the fat body, the purity of samples was confirmed by the expression of some of the most enriched genes in this tissue according to flyatlas2 including *Fatty acid synthase 3*, *apolipophorin*, *CG9458*, *CG14990* and *CG9616* (Fig. 4C). Additional highly enriched genes in the fat body with known functions in the tissue like *Adipokinetic hormone Receptor*, *Lipid storage droplet-2* and *Lipophorin receptor 2*, were also detected in high abundances (Fig. 4C) [93].

To identify the most suitable bacterium that generates a systemic infection and a corresponding response from the host, but at the same time causes low lethality and can be eliminated from the host after a few days, we performed preliminary tests with different bacteria. Here, *Bacillus subtilis*, *Erwinia carotovora* (*Ecc 2046*), *Serratia marcescens*, and sterile injury was tested (Fig. 5A). In contrast to infection with *Serratia marcescens* and *Erwinia carotovora* (*Ecc 2046*), infection with *Bacillus subtilis* met the expectations and it could be completely eradicated within three days after infection was initiated (Fig.5B).

Results

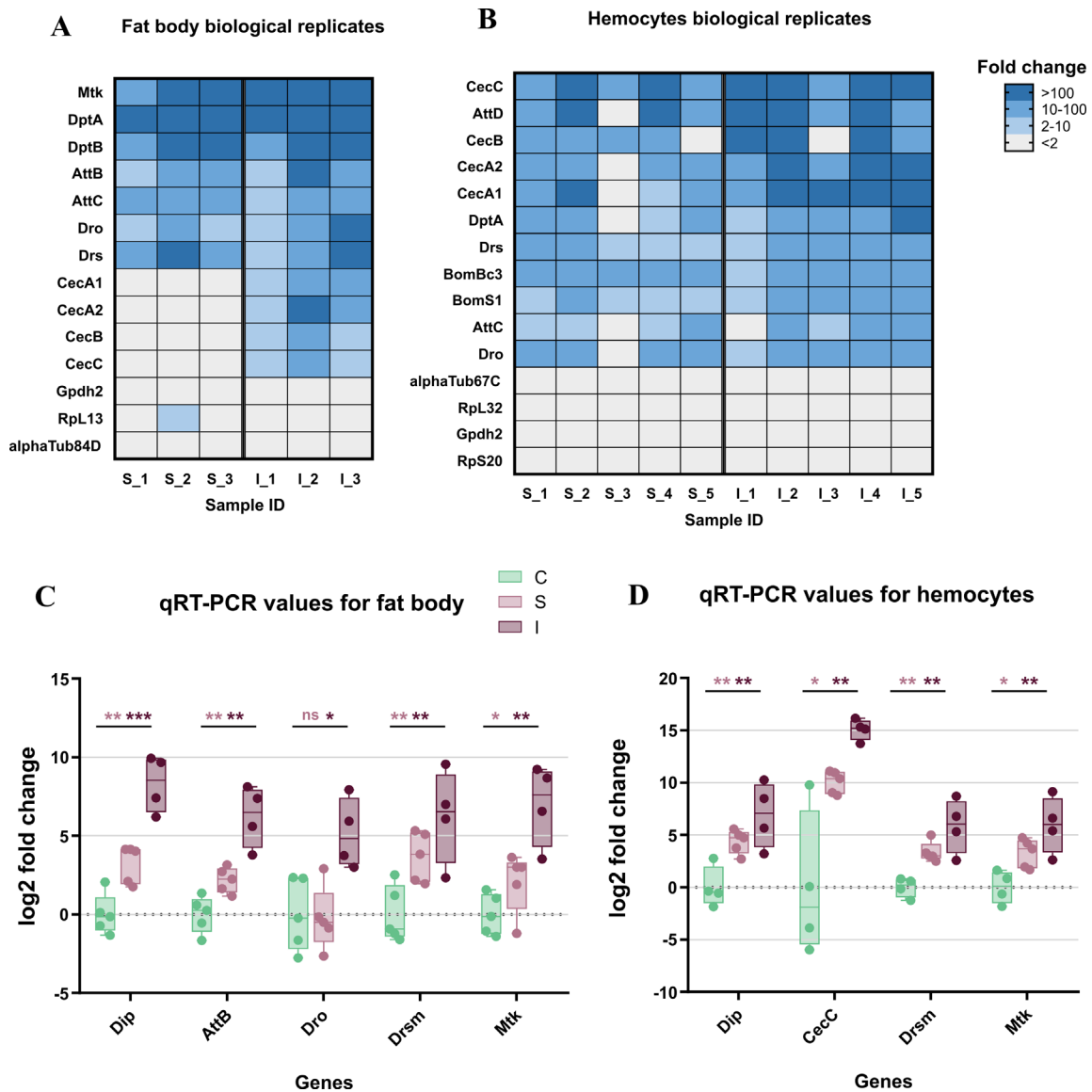


Figure 6 Validation of the amplification and sequencing setup: (A) Expression values for some AMPs and housekeeping genes in individual biological replicates for the injured and infected animals' fat bodies. A number at the end of the treatment name indicates the individual replicates: S_1, S_2 and S_3 are sterile injury and I_1, I_2 and I_3 are bacteria infected. Fold change was calculated relative to the average TPM value in the control fat body. (B) Expression values for some AMPs and housekeeping genes in individual biological replicates for the injured and infected animals' hemocytes. A number at the end of the treatment name indicates the individual replicates: S_1 - S_5 are sterile injury and I_1 - I_5 are bacteria infected. Fold change was calculated relative to the average TPM value in the control fat body. (C) The log2 fold changes for a few AMPs in the fat body upon infection and injury. 4-5 biological replicates were used for each treatment and Rpl13 was used as a housekeeper gene for normalization. The log2fc values were calculated using the $\Delta\Delta C_t$ method. Statistical significance was calculated by performing multiple t-tests and using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli to calculate the adjusted p-value. (D) The log2 fold changes for a few AMPs in the hemocytes upon infection and injury. 4 biological replicates were used for each treatment and Rpl32 was used as a housekeeper gene for normalization. The log2fc values were calculated using the $\Delta\Delta C_t$ method. Statistical significance was calculated by performing multiple t-tests and using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli to calculate the adjusted p-value.

To validate the functioning of the amplification and sequencing setup for the different treatments, we compared the expression values of a few antimicrobial peptides in the injured and infected animals' fat body and hemocytes (Fig. 6A, B). We compared the fold change in

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the TPM values of these AMPs in individual biological replicates relative to the average values for the respective tissue controls. All biological replicates show a consistent upregulation of most of the AMPs for both cell types indicating the replicability in the data (Fig. 6A,B). We additionally included the same analysis for a few housekeeping genes, which showed maintenance of the expression values in almost all biological replicates. To provide independent proof of the functionality of the setup, we performed qRT-PCR on the amplified cDNA from the different treatments for selected AMPs (Fig. 6C, D). For both the cell types, we observed a significant increase in the expression levels of all the genes upon infection and for some of them upon injury. These results were consistent with what we observed from the transcriptomic analysis.

4.1.2. Infection induced transcriptional changes in fat body are distinct from whole body responses

To demonstrate that the experimental infection elicited an immune response, we used a reporter line, where expression of GFP is under the transcriptional control of the *drosomycin* promotor [94]. Here, strong GFP signals were seen in fat body cells after infection, whereas no signal was seen in non-treated controls (Fig. 7A). To evaluate the fat body dataset, we first performed a principal component analysis for the three treatment groups (control – C, sterile injury – S, and Infection - I) (Fig. 7B) and observed very distinct separation and low variance in the samples of control and sterile injury. In contrast, bacteria-infected samples show very high variability. We also observe this high inter-replicate variability in bacteria-infected fat bodies in other experiments (unpublished work), which implies that this variance is an inherent feature of this complex biological treatment [95]. To test the validity of our sequencing strategy, we checked the expression of AMP genes known to be regulated in response to infection in infected fat bodies compared to non-infected controls and detected strong increases between isolated fat bodies from infected animals and matching controls. Here, a clear increase in the expression of these AMP genes was seen (Fig. 7C). Comparing the data of infected animals and those subjected to sterile injury with matching controls, we found 490 genes regulated in bacterial infection (447 upregulated and 43 downregulated) and 1580 regulated genes in response to sterile injury (1221 upregulated, 359 downregulated, Fig. 7D).

Results

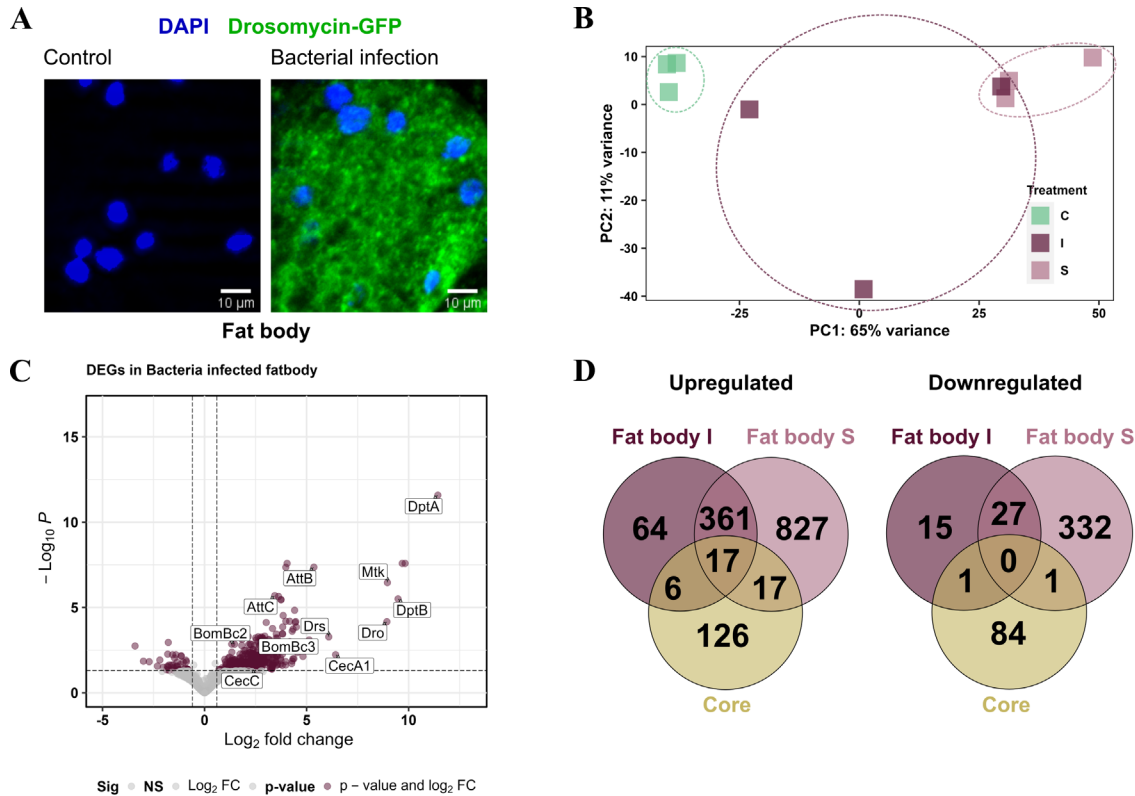


Figure 7 Systemic infection with *Bacillus subtilis* as well as sterile injury exert an immune response in the adult fat body. (A) The fat body from the reporter line *Drosomycin-GFP* in non-treated controls (left) and after infection with *B. subtilis* (right). The nuclei are stained with DAPI. (B) Principal Component Analysis plot showing the first two principal components for the RNA-seq analysis of control (C), sterile injury (S), and bacteria-infected (I) fat bodies. (C) Volcano plot depicting the differentially expressed genes in bacteria-infected fat body cells as compared to untreated control fat bodies. The genes passing the threshold (adjusted p-value < 0.05, log₂fold change > 0.6) were considered differentially expressed and are marked in maroon and the rest of the genes are marked in gray. (D) Venn diagram analysis of the common number of regulated genes in the bacteria infected (I) and sterile injury (S) fat body data sets and the ‘core’ genes from whole body infection experiments are shown separately for the upregulated and downregulated genes.

We next compared the immune-induced response of the fat body to the 252 ‘core’ genes found in the averaged-out whole-body reaction [87]. We observed only minimal overlap in the infection-induced upregulated genes in the fat body and the whole-body datasets and almost no commonality in the cohorts of downregulated genes. There were only 17 genes common in both S and I fat body and ‘core’, with most of them being AMP genes. Further, there were a few genes only common between infection (I) and ‘core’ which also mainly included AMPs, while in comparison to sterile injury (S) more immune-related genes were included (Fig. 8A). Looking at the 30 most abundantly expressed upregulated genes on infection, we found some important immune effectors including AMPs and IMPPP and many ribosomal protein genes (Fig. 8B).

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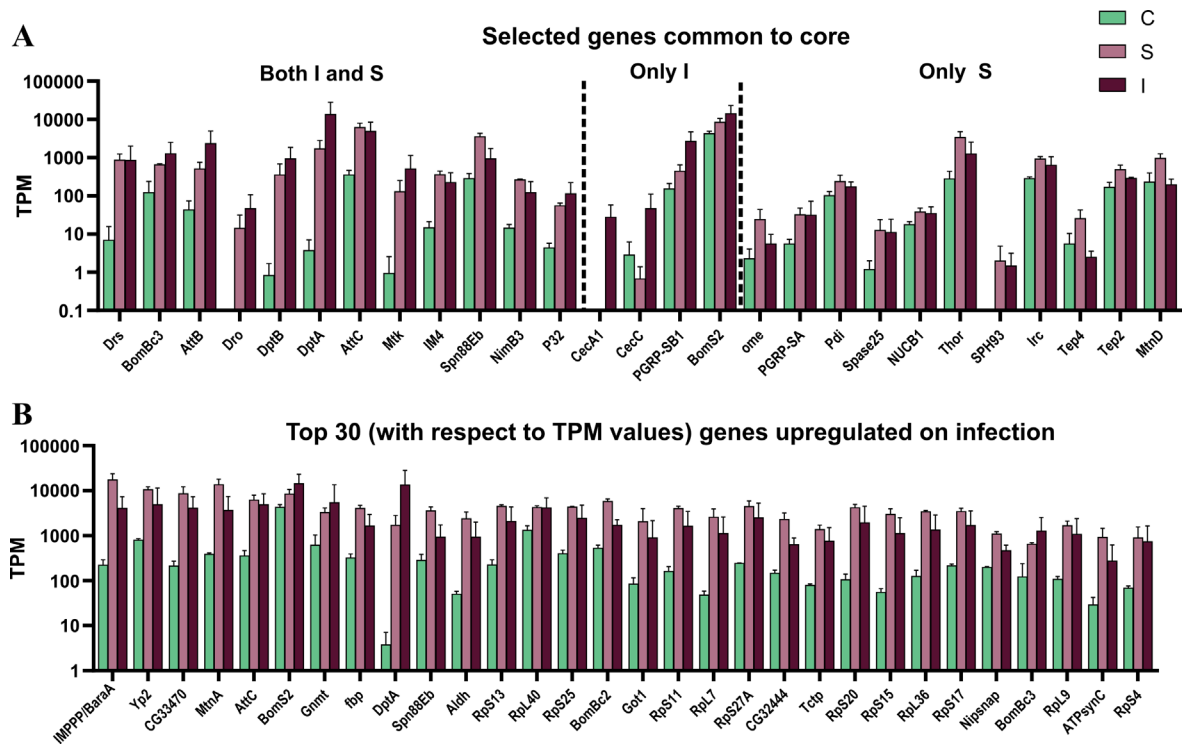


Figure 8 Expression values for important genes upregulated in the fat body upon infection: (A) Transcript per Million (TPM) values are shown for selected genes that were commonly regulated between the full body ‘core’ dataset and the bacteria infected (I) and sterile injury (S) fat body data set. **(B)** TPM values are shown for the top 30 upregulated genes (in terms of read counts) in bacteria-infected fat bodies.

This indicates the importance of a highly upregulated translation machinery for the functionality of the fat body against bacterial invaders. A considerable portion of regulated genes overlaps between both datasets from the fat body (sterile injury and infection, Fig. 7D). These include nearly 377 upregulated genes, which could represent the ‘core’ gene set induced in immune-related stress in the fat body. GO term analysis of these central genes, as well as those ones specifically regulated in either treatment, indicated most genes were associated with similar processes. We provide a schematic summary of the processes upregulated and the percentage of genes activated in each process in S and I fat bodies (Fig. 9A) and show the fold change in expression values for the most highly expressed genes for these processes (Fig. 9B). Apart from the classical innate immune response, the most prominent process is translation with nearly 70 regulated genes. Most regulated genes are upregulated, which implies higher transcriptional/translational activity. Interestingly, GO terms such as proteasomal machinery, ubiquitin-based protease activity, and protein export are also enriched. We also observed terms related to increased oxidation-reduction processes, which are already known to be crucial for fighting infection or any stress. Oxidative phosphorylation-related GO terms are also enhanced, which points to a metabolically active fat body that requires much energy. We also see a small number of metabolic genes included in processes like lipid homeostasis, sugar transport,

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glycogen metabolism, and fatty acid degradation. The downregulated genes did not yield significant GO terms.

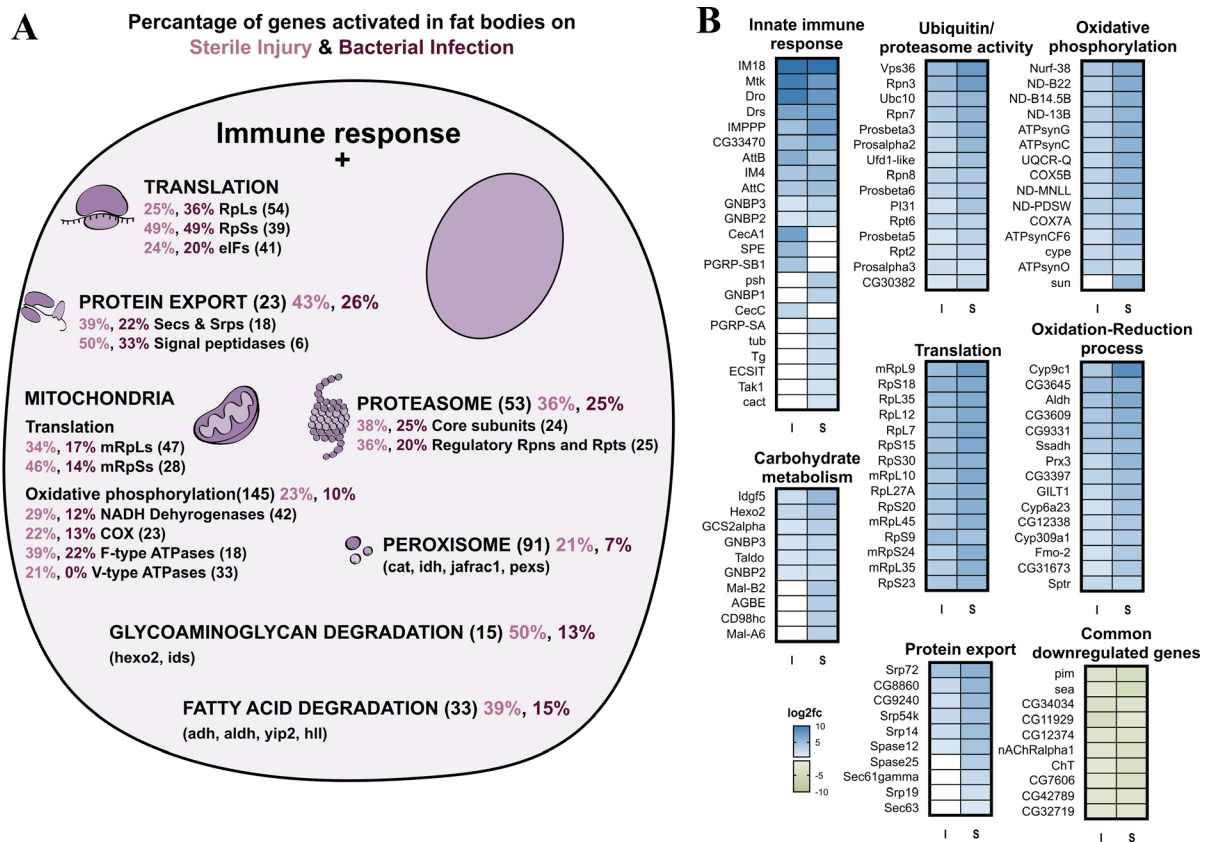


Figure 9 GO term analyses of genes regulated in the fat body in response to infection and sterile injury. **(A)** Summary of the processes activated on top of the classic antibacterial response in fat body cells on bacterial infection or sterile injury. For each process, the total number of genes associated with the process is shown in brackets next to it. Out of these genes, the percent activated in bacterial infection are shown in maroon and in sterile injury in pink color. For some processes, the subunits are shown (like the RpLs and RpSs for translation) and for some processes, some selected genes are shown (like hexo2 and ids for glycosaminoglycan degradation). **(B)** The log2 fold changes of selected genes from the processes shown in A are plotted as heatmaps for bacterial infection (I) and sterile injury(S). For the innate immune response all associated activated genes are shown, while for the rest, the top 10-15 genes were picked based on the magnitude of fold change expression. The top 10 commonly downregulated genes in I and S with the highest fold change in expression are also shown.

4.1.3. Infection induced transcriptional changes in hemocytes are distinct from those observed in whole body data sets

As for the fat bodies, we also could confirm the induced expression of AMPs in hemocytes upon infection using the AMP reporter lines (Fig. 10A) and further identified this in the transcriptomic analysis (Fig. 10C). The principal component analysis of the hemocyte samples showed a clustering based on their infection state (Fig. 10B). We observe that samples from sterile wounding and from bacterial infection are closer to each other than unhandled control

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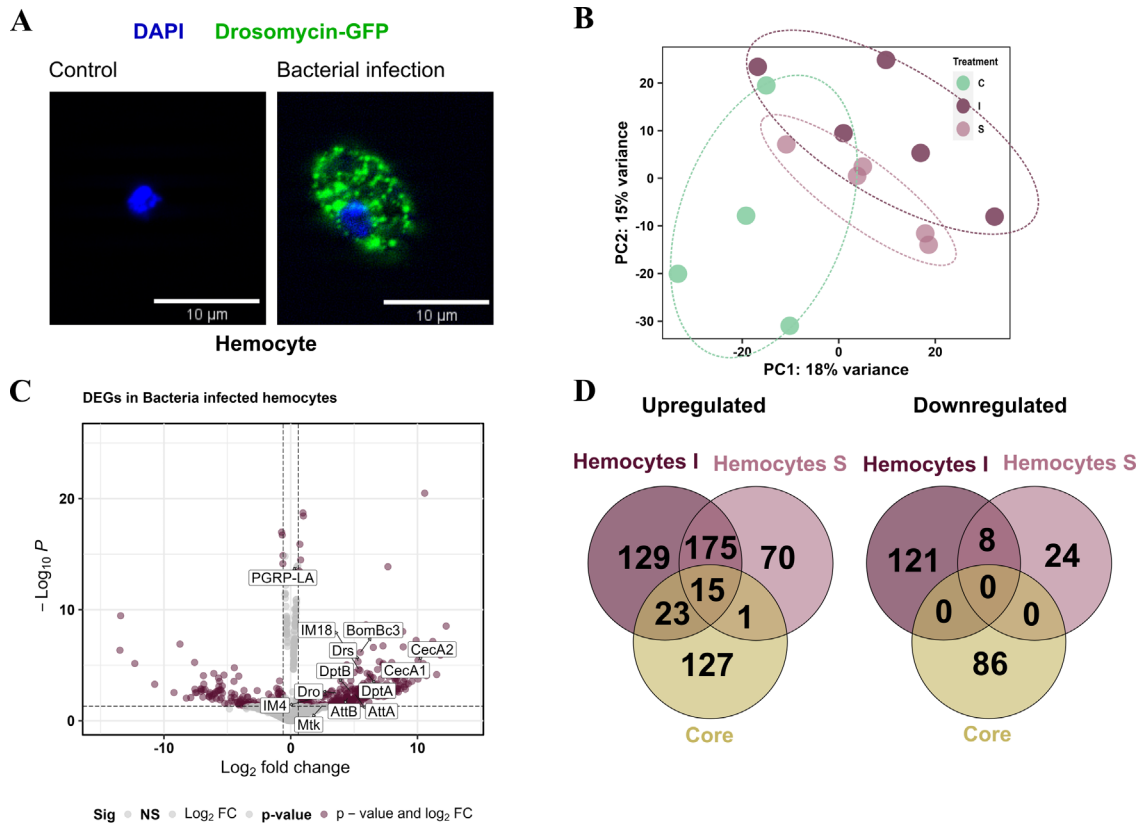


Figure 10 Responses of adult hemocytes to systemic infection and to sterile injury. (A) Hemocytes from the reporter line Drosomycin-GFP left untreated (left) and after infection with *B. subtilis* (right). The nuclei are stained with DAPI. (B) Principal Component Analysis plot showing the first two principal components for the RNA-seq analysis of control (C), sterile injury (S), and bacteria infected (I) adult hemocytes. (C) Volcano plot depicting the differentially expressed genes in bacteria infected hemocytes as compared to untreated control hemocytes. The genes passing the threshold (adjusted p-value < 0.05 and log₂fold change > 0.6) were considered differentially expressed and are marked in maroon and the rest of the genes are marked in gray. Some of the differentially expressed AMPs are labeled in the graph. (D) Venn diagram analysis of the common number of regulated genes in the bacteria infected (I) and sterile injury (S) hemocytes (each compared to untreated controls) and the ‘core’ genes from the whole body are shown separately for the upregulated and downregulated genes.

samples. Comparing treated hemocytes with controls, we identified 469 (341 upregulated, 128 downregulated) genes regulated in bacterial infection and 291 (260 upregulated and 31 downregulated) in sterile injury (Fig. 10D). We further compared the immune-stress induced response of hemocytes to the whole-body ‘core’ immune-related genes. There was, as seen for the fat body, no significant commonality in the downregulated genes, while for activated genes, the small number of common genes mainly included AMP genes (especially for the bacterial infection treatment) and, in addition, a few genes involved in immune response pathways (Fig11. A,B)

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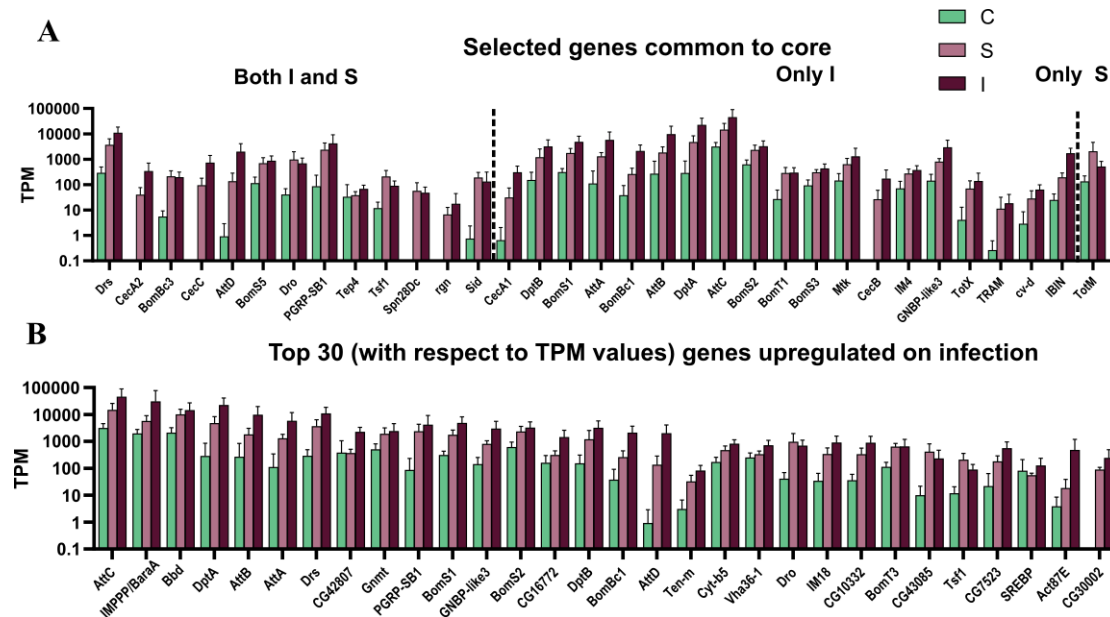


Figure 11 Expression values for important genes upregulated in fat body upon infection. (A) Transcript per Million (TPM) values are shown for selected genes that were commonly regulated between the full body ‘core’ dataset and the bacteria infected (I) and sterile injury (S) hemocyte data sets. (B) TPM values are shown for the top 30 upregulated genes, i.e., the DEGs with the highest abundance values in terms of read counts, in bacteria infected hemocytes.

4.1.4. Functionally relevant processes activated in hemocytes upon infection

We see a ‘central’ set of 190 genes commonly upregulated upon sterile injury and bacterial infection (Fig. 10D). Considering both treatments, we found that the GO term with the highest statistical support is “immune response”. Although the number of genes was not large enough for a statistically relevant GO term analysis, we found some genes associated with relevant processes (Fig. 12). Many genes were related to the oxidation-reduction process, as in the fat body, which might be essential for a highly active hemocyte to fulfil its tasks. Other genes I noticed included well-known and essential targets of the processes like actin cytoskeleton rearrangement, microtubule organization, cell morphogenesis, phagocytosis, cell migration, and adhesion. Some genes were also conspicuous that are related to metabolic changes, including lipid metabolism, Golgi organization, and proteolysis. The lipid homeostasis associated genes were also active in larval hemocytes on an infection or wound [91]. This points to an important role of lipid homeostasis in hemocytes facing immune stress. On top of that, the active hemocytes show increased expression of genes associated with an increased breakdown of proteins, which could provide energy and metabolites. The downregulated genes in infected hemocytes have, presumably due to the low number, no significantly enriched GO terms.

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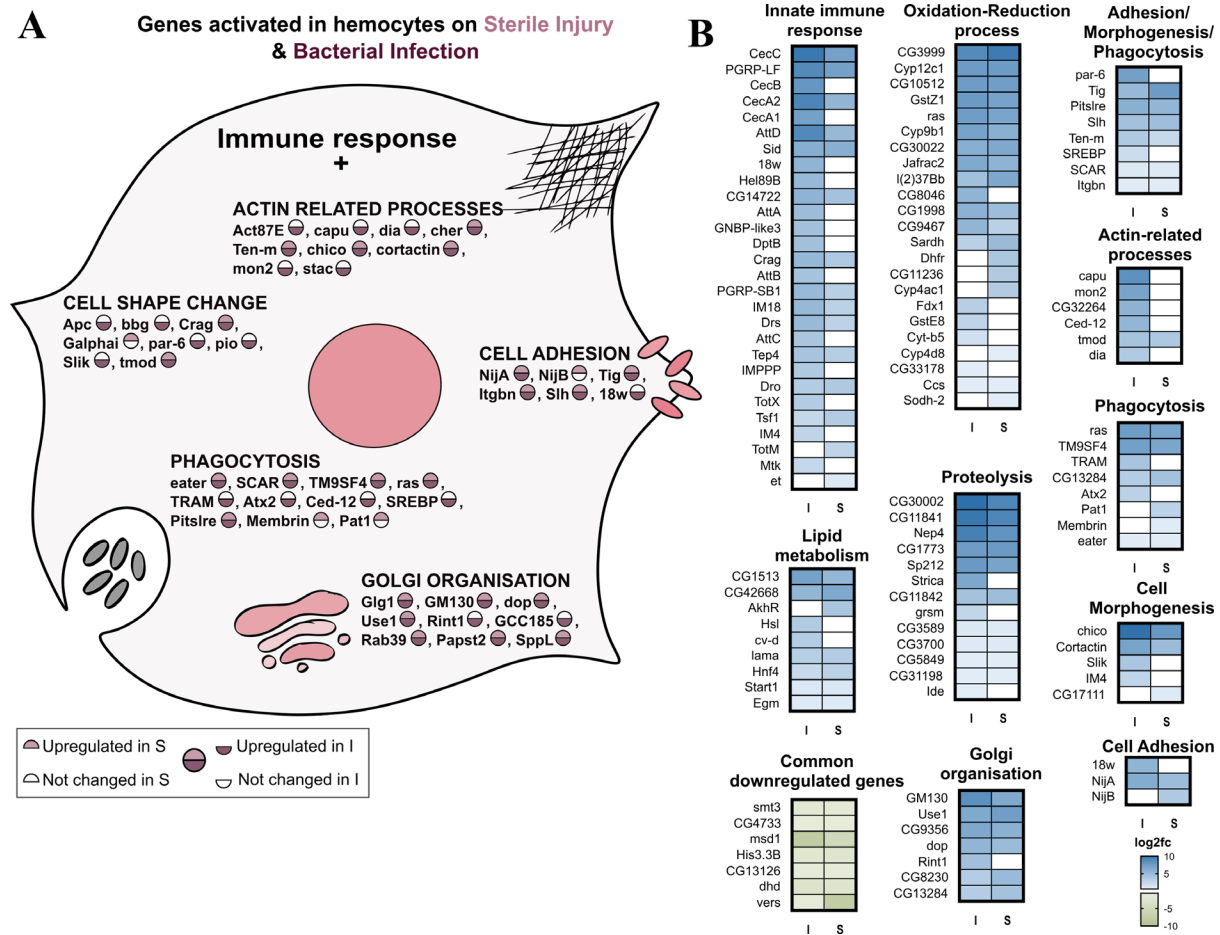


Figure 12: GO term analysis of the processes that are regulated in response to infection or sterile injury in adult hemocytes. (A) Summary of the processes activated in addition to the classic antibacterial response in hemocytes to bacterial infection (I) or sterile injury (S). For each process, selected genes are shown with the circle depicting their activation in I and S. As shown in the scale below, the upper half of the circle shows the upregulation in S, where no color means no change in expression while pink shows upregulation. The lower half shows the same information for I, with no color for no expression change and maroon for upregulation. (B) The log2 fold changes of genes associated with the GO terms of the processes shown A are plotted as heatmaps for bacterial infection (I) and sterile injury (S) hemocytes. The commonly downregulated genes in I and S are also shown

One aim of this study was to gain a deeper understanding of the mechanisms in adult hemocytes that are induced after infection. To this end, we analyzed in more detail the top 30 expressed genes, i.e., those with probably the greatest importance, among the upregulated ones (Fig. 11B). As expected, most of the induced antimicrobial peptides were among the most highly expressed genes in hemocytes after infection. *AttC* was the most highly expressed gene, followed by *baramicin A2* and *bombardier*. Moreover, among the most highly expressed genes were other *attacins*, *dipterocins*, and *bomanins*, as well as *drosomycin*. Of note are genes that are not primarily associated with an immune response, but which may be particularly important, for example, for digesting ingested bacteria (*Vha36-1*). In addition, several genes that play central roles in the control of metabolism should be mentioned, with *Gnmt* and *SREBP*

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being particularly prominent. Comparing this list of most highly expressed genes induced by infection with those following sterile injury reveals significant differences. It is striking that, those AMPs that were upregulated in response to infection did not do so to the same extent after sterile injury.

4.1.5. AMPs are the only commonly activated genes in the fat body, hemocytes, and the whole-body dataset on infection

Next, we compared the response in the two cell types following bacterial infection. Combining the samples from fat bodies and hemocytes in a PCA showed separate clusters for the two (Fig. 13A). Further, while PC1 mainly explained the differences between the two cell types, PC2 explained the differences between control and infected tissue. We further observed this by focusing on the top 50 genes associated with both treatments using PCA (Sup. Fig. 1).

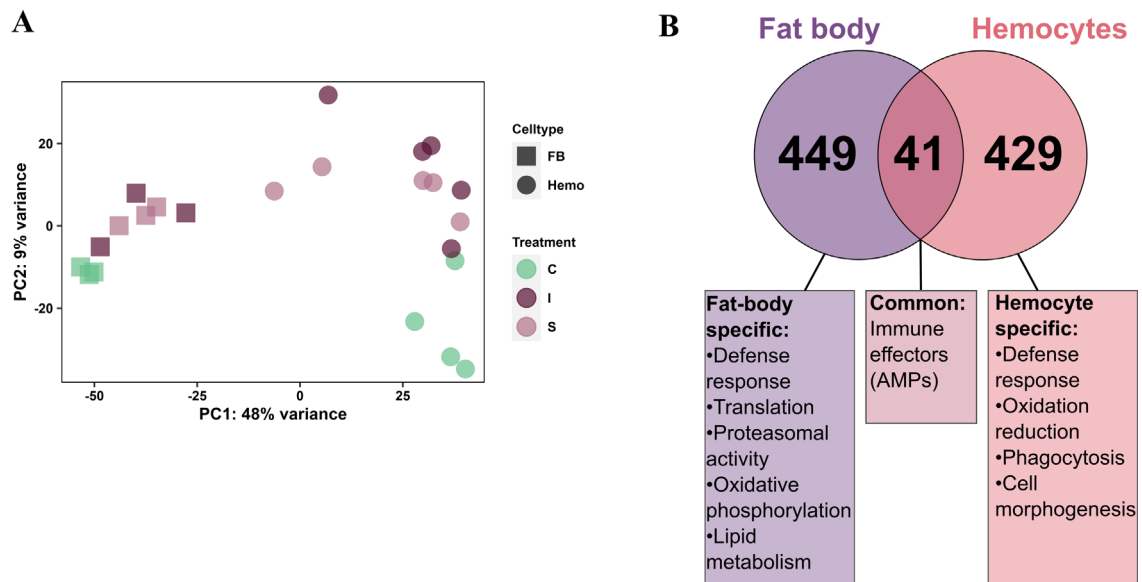


Figure 13 Comparison of the induced transcriptional changes in response to infection and sterile injury in fat body and hemocytes. (A) PCA plot showing the first two principle components for the RNA-seq data of control (C), sterile injury (S), and bacteria-infected (I) hemocytes and fat bodies. (B) The number of commonly regulated genes in fat bodies and hemocytes on bacterial infection is shown with a summary of the common and distinct processes activated in each cell type.

Comparing the lists of genes regulated in fat bodies and hemocytes from infected animals, we observed about 10% (41) of genes shared between both, and a major category in this group of commonly regulated genes are immune effector or AMP genes (Fig. 13B). Focusing on specific genes, we observed distinct processes regulated in the two cell types. A summary of the processes specific to each cell type is shown (Fig. 13B). While the fat body exhibit responses associated with metabolic and energetic changes like protein turnover in the form of translation, proteasomal activity and export, lipid metabolism and oxidative phosphorylation, hemocytes show responses associated with cell morphogenetic changes like actin-related processes, cell

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adhesion, and phagocytosis. One common process between the two is oxidation-reduction, which is functional through very different genes.

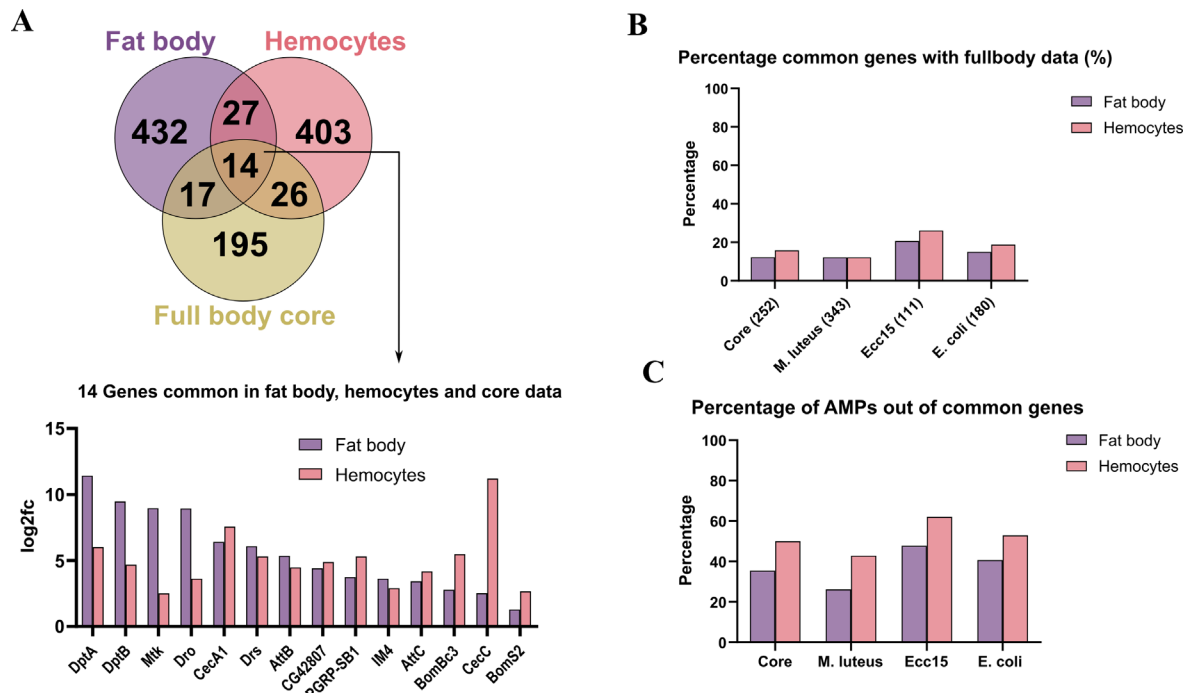


Figure 14 Comparison of the transcriptional response to infections in fat body, hemocytes and whole body: (A) Venn diagram showing the number of commonly regulated genes in bacteria infected fat bodies, hemocytes, and the 'core' from full body data [87]. The 14 genes commonly regulated in all three are shown at the bottom with the log2 fold changes in the expression in fat bodies and hemocytes. Most of these common genes are AMPs. (B) The percentage of commonly regulated genes in bacteria infected fat body and hemocyte samples to the different full body bacterial infection treatments. The number of genes regulated in each infection treatment are shown in brackets on the x-axis. (C) Percentage of AMPs out of the total common genes in B.

We next compared the bacterial infection-induced response of the fat body and hemocytes to the 'core' genes found in averaged-out whole-body responses to different bacterial infections [87]. Interestingly, we see only 14 genes common between the three, out of which 12 were AMP genes and one a PGRP gene (Fig. 14A). Subsequently, we investigated if the commonality between whole body and tissue-specific data changes if we look at information from infection experiments with specific bacteria instead. We picked *M. luteus*, *E. coli*, and *E. cc15* [87]. This is a collection of gram-positive and negative bacteria, where patterns of host survival and pathogen clearance were similar to the *Bacillus subtilis* (gram-positive bacterium with DAP-type peptidoglycan) infection used in the current study. Based on the presence or

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Figure 15 Comparison of the genes differentially expressed after infection and sterile injury, in fat bodies, hemocytes, and whole body data sets. Heatmap showing the clustering of expression data from I and S fat body and hemocytes and the whole body data for *E. coli* (Ecl), *Ecc15* (Ecc), *M. luteus* (MI), and the core. K-means clustering was performed on the genes differentially expressed in bacteria infected fat bodies, hemocytes, and full body data from Ecl, Ecc, MI, and core based on the absence or presence of regulation of a gene. The scaled values for the \log_2 fold change are plotted here for the different clusters. The data was further divided into biologically relevant sets of clusters. Set A consists of genes differentially expressed in all the treatments and consisted mainly of AMPs, while Set C is only differentially expressed in fat bodies and hemocytes. Set B, D, and E are the sample-type specific clusters for whole body, fat body, and hemocytes respectively with some relevant categories and example genes shown for each set.

absence of differential expression, we clustered the collection of genes differentially expressed in the animals infected with one of these three bacteria, the core or bacteria-infected fat body and hemocytes and plotted the scaled fold change values (Fig. 15). We observed a small cluster expressed in all of them, and it contained only AMPs (Fig. 15-SetA). Quantitatively, only 10-20% of the genes expressed in full-body data (either core or specific pathogen) are also regulated in the fat body or hemocyte samples (Fig. 14B), respectively. Here, 30-50% in the fat body and 40-60% in hemocytes data sets are AMP genes (Fig. 14C). Another small cluster

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is Set C in Fig. 15, with the genes shared between the fat body and hemocytes. However, the most prominent and highly distinct clusters are specific to the cell types (Set D and E).

4.2. The stored lipid droplets in hemocytes increase in size upon injury

Infections are known to induce metabolic rewiring in the hemocytes as well as other cell types to produce enough energy for this resource-demanding process [96]–[99]. Of these metabolic processes, I found a strong upregulation of many lipid homeostasis genes in the hemocyte transcriptome after infection or sterile injury (Fig. 12B). These included a range of genes across the metabolic landscape, including lipases like *Hsl* and *Lama*, predicted lipid transporters like *cv-d* and *start1*, and critical lipid homeostasis genes including *AkhR*, *amacr*, *enigma*, *pudgy*, *hnf4*, and *SREBP*. The expression values of some of these genes are shown in the figure 12B. Lipid metabolism was also shown to be regulated in larval hemocytes upon immune stress, but no further studies have been performed yet in this area [91]. The importance of lipid metabolism for the functionality of macrophages (which can be considered the functional

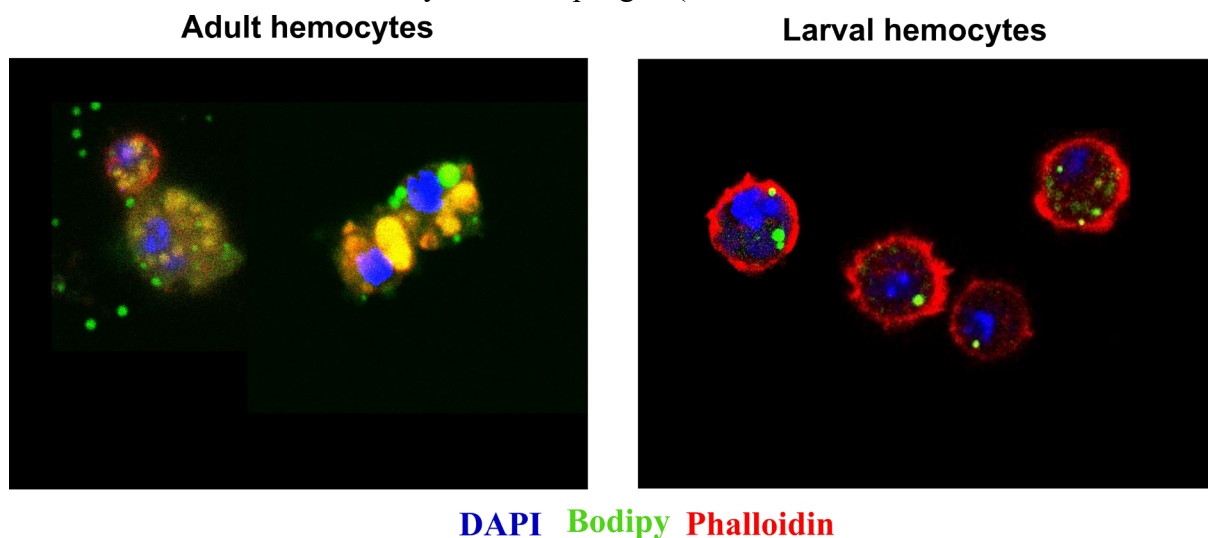


Figure 16: Adult and larval hemocytes with lipid droplets: The image shows adult and larval hemocytes stained with Bodipy to visualise the lipid droplets. The Bodipy is in green, Phalloidin in red and DAPI in blue, marking the lipid droplets, cell membrane and nuclei respectively. The larval hemocytes (right) show very clear distinct lipid droplets. The adult cells on the left also show lipid droplets, but they also show these yellow colored big spherical entities. These were autofluorescent and always visible in every channel of fluorescence microscope. They increased in size and number with age.

equivalent of plasmatocytes in the mammalian system) has been studied in great detail [100] and they are shown to accumulate Lipid Droplets on infection [101], [102].

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I aimed to check if the lipid metabolism change could be measured with the help of LDs in *Drosophila* hemocytes as well. As no studies to date have looked for the presence of LDs in hemocytes, I first started by investigating this in different developmental stages of *Drosophila*. Here, the presence of LDs in larval as well as young and older adult hemocytes was apparent (Fig.16). These droplets were sparse and on average 0.1-0.5 microns in radius. They were

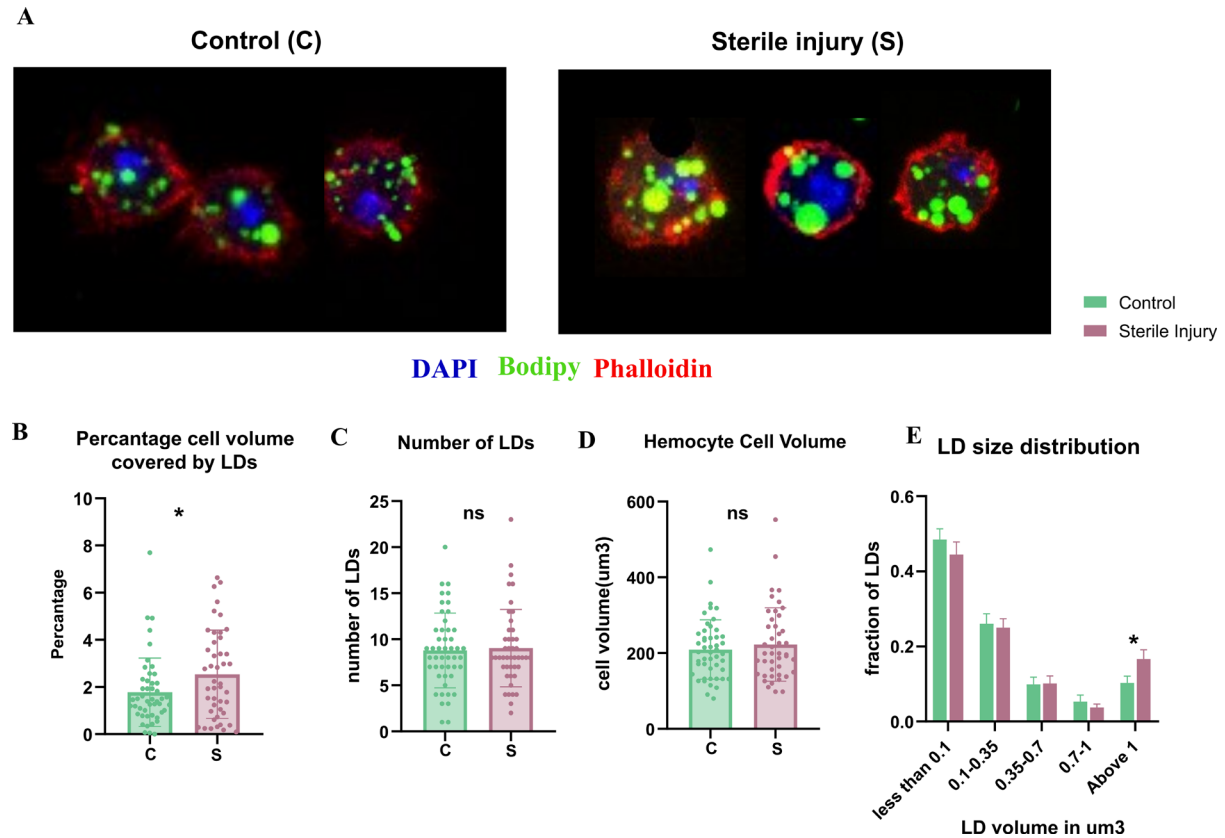


Figure 17 Increase in size of Lipid Droplet in hemocytes upon injury: (A) Images show larval hemocytes stained for bodipy (green), DAPI (blue) and Phalloidin(Red). Z-section images were obtained for the whole size of cell and a maximum intensity projection was created for a single cell, which is shown in the image. The Droplet size increases upon injury. (B) Percentage of volume covered by LDs out of the whole cell volume. Calculated by measuring the cell radius and radius of every LD in the cell. These values were used to calculate the volume. (C) Number of LDs in each cell compared between injured and control hemocytes. (D) Volume of hemocytes compared between control and injured animals. (E) Distribution of LD size compared between the two treatments. The fraction of LDs with size more than $1\mu\text{m}^3$ were significantly higher in injured hemocytes. 45-48 cells from 8 animals were sampled for each treatment. T-test were conducted to compare the treatments. ns - $p > 0.05$, * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$, **** - $p < 0.0001$

present in most but not every hemocyte in an animal. In adult hemocytes, I observed the presence of some highly autofluorescent material that was also round and increased in size and number with age (Fig.16). This made it very difficult to effectively quantify the lipid droplet size and number in adults after infection. As the upregulation of lipid metabolism was also seen in larvae [91], I checked the LDs of larval hemocytes 2 hours after sterile pricking. Staining the fixed hemocytes with bodipy and measuring the area covered by them was done. I saw a significant increase in the percentage volume of the cell covered by LDs (Fig17A, B). The

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volume of the cell itself as well as the total number of LDs stays the same while (Fig17C, D), if the size distribution of LDs was taken into account, a greater number of bigger LDs on pricking became apparent (Fig 17E)

4.3. Experiments to show a novel function of the *Drosophila* sugar transporter gene *Tret1-1* in maintaining infection-induced energy homeostasis in the fat body

On comparison of the transcriptome of injured or bacteria-infected individuals' fat body to control, I observed a strong upregulation in the abundance of *Tret1-1* (Fig. 18A). Trehalose transporter 1-1 (*Tret1-1*) is a bi-directional trehalose transporter, which can also facilitate

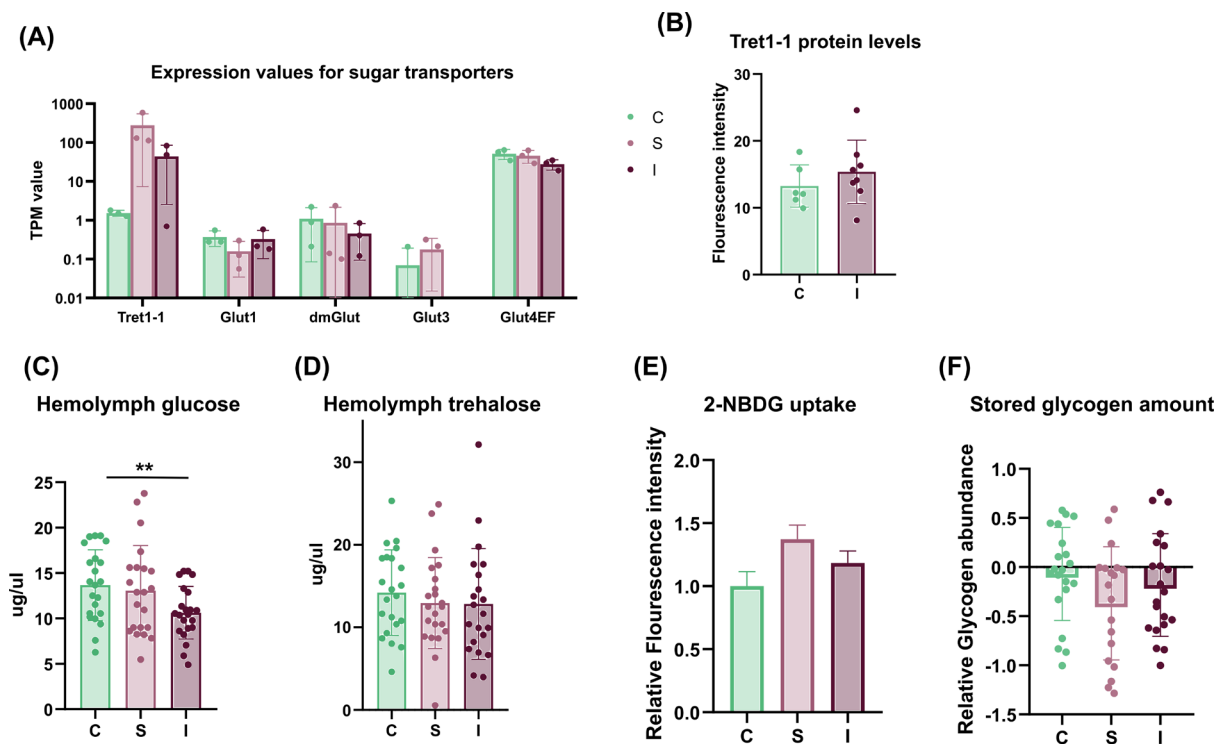


Figure 18: Tret1-1 mediated glucose transfer in infection facing fat body: (A) TPM values for the glucose transporters and Tret1-1 from the RNA sequencing analysis performed on control, injured and infected animals' fat bodies. (B) Tret1-1 protein abundance compared between control and infected fat bodies. The measurement was conducted by comparing the fluorescence intensity in the fat bodies of Tret1-1-GFP marked fly line. Preliminary experiment with 6-8 animals per treatment. (C) Hemolymph glucose measured by Glucose HK kit. Each dot represents one sample consisting of 5 animals. N=25. Significantly lower glucose in hemolymph upon infection. (D) Hemolymph trehalose measured for different treatments. Each dot represents one sample. No significant differences observed between the treatments. (E) The glucose uptake measured by 2-NBDG uptake assay. Fluorescence intensity was measured relative to the control treatment. The bars represent mean values with Standard deviation for 100 samples obtained from 6 animals for each treatment. (F) Glycogen abundance in fat body measured by PAS staining. The y axis represents the inverse of mean grey value of the image of stained fat body, thus directly proportional to the amount of glycogen. Each dot represents an average value for one animal. Injured individuals have slightly lower glycogen than control, however not statistically significant. (N=25). T-test were conducted to compare the treatments. ns - $p > 0.05$, * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$, **** - $p < 0.0001$

glucose transfer [103], [104]. To independently confirm the *Tret1-1* expression via a different

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method, the protein abundance was measured with the help of a reporter phenotype. I obtained animals carrying a GFP marker attached to *Tret1-1* by crossing the animals with *Tret1-1-Gal4* genotype to the ones with *UAS-GFP*. I further measured the fluorescence intensity in the fat bodies of animals with and without an infection to measure *Tret1-1* abundance (Fig. 18B). In a preliminary experiment with 6-8 animals, I observed a trend towards upregulation in the infected animals, which was not statistically significant as the sample size was low.

Next, it was tested if this upregulated *Tret1-1* is for the transfer of trehalose or glucose from the fat body to the hemolymph or *vice versa*. To this end, I compared the amount of circulating hemolymph trehalose and glucose in the control, injured and infected animals (Fig. 18C, D). The trehalose abundances were slightly lower in the injured and infected animals, however, not statistically significant (Fig. 18D). Glucose, on the other hand, was considerably lower in the infected animals as compared to the control but not in the injured animals (Fig. 18C). This indicates a transfer of glucose from the circulating hemolymph to the fat body upon infection. To confirm this, I compared the glucose uptake capabilities of fat bodies using the glucose fluorescent analog 2-NBDG (Fig. 18E). In a preliminary experiment using six animals for each treatment, I observe a slight upregulation in the glucose uptake capabilities of infected and injured animals' fat bodies. This upregulation was much more in injured as compared to infected animals (Fig. 18E). These results point towards an increased uptake of glucose from the hemolymph by the fat bodies on facing an immune stress. Furthermore, it was checked if the other stored energy sources in the fat body were also impacted. Thus, I measured the stored glycogen abundances by comparing the intensity of Periodic Acid and Schiff's reagent staining and observed a decrease in the amount of stored glycogen upon immune stress as well, which was higher upon injury than infection (Fig. 18F). This shows that the glycogen reserves are also being used up in the fat body under immune stress.

4.4. Resource allocation strategies in the two professional immune cell types of *Drosophila*, hemocytes and fat body, differ with the magnitude of dietary and immune stress

4.4.1. Criteria for choosing the dietary treatments

I first started by creating a dietary regime that could help us make measurable and repeatable nutritional changes. As it is seen that oligidic diets have unknown dietary compositions, and any changes lead to unrecognised macro/micronutrient changes as well [105], an enhanced

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exome-matched holidic diet was used [68]. The animals were raised on a standard cornmeal-molasses-yeast diet until pupal stages and then transferred them to the holidic diet to avoid any significant dietary changes in adulthood. After keeping the adults on nutrient-rich media for five days after eclosion, I switched them to the different nutritional treatments of interest. As a growing body of evidence suggests that the Protein: Carbohydrate ratio is the primary factor for the lifespan-extending impacts of Dietary Restriction (DR) [45], [106]–[109], DR was enforced by lowering dietary protein content while keeping everything else constant. I started with two different DR treatments with the P:C ratios dropping from 1.26:1 in the normal media (NM) (22g/L protein) to 1:8 in one diet (2g/L protein) and 1:16 in the second (1g/L protein). Additionally, to enable Protein Malnutrition, protein was removed altogether from the diet (PM) (0g/L protein). This dietary treatment would also provide additional information for distinguishing the impacts of restriction from malnutrition (Fig. 19A).

Next, I confirmed if our dietary treatments fulfil the two essential defining features of DR, i.e., increased lifespan and decreased fecundity [110]–[112]. While both DR treatments resulted in a higher median and maximum lifespan, the milder DR diet (DR 1:8) resulted in a better lifespan extension curve than the stronger DR (DR 1:16) (27% increased median lifespan in DR 1:8 and 12% in DR 1:16) (Fig. 19B). The daily eggs laid by individuals also go down from 20 eggs per day in NM to 1-2 eggs per day in both DR treatments (Fig 19C). As milder DR resulted in better lifespan extension parameters, it was used for most of the following experiments and is called ‘DR’ from now on. The malnutrition diet worked efficiently as well, as the median lifespan goes down by 41% in PM, and the daily number of eggs laid is nearly zero in 7-8 days (Fig 19B, C).

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Further, I aimed to identify a suitable duration of these dietary treatments that would induce stable systemic changes such that the immune parameters become more experimentally reproducible. To this end, different outputs of body composition and reproduction from the day

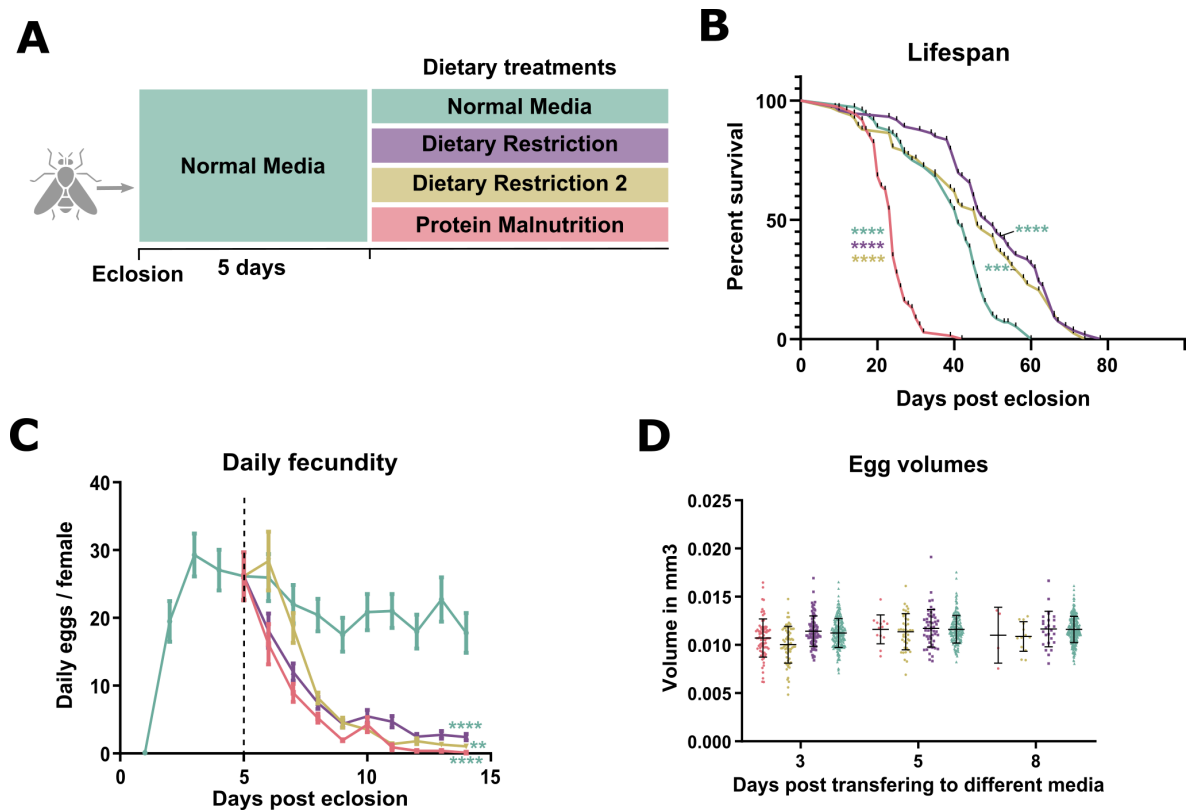


Figure 19: Reproduction and lifespan for the chosen DR and PM diets. (A) Schematic representing the experimental setup. Animals were transferred to exome-matched diet in the non-feeding pupal stage and adults were kept on this control diet for 5 days before transferring them to the different dietary treatments. The Protein to carbohydrate ratios in the three diets are 1.26:1 in Normal Media (NM), 1:8 in Dietary Restriction (DR), 1:16 in Dietary Restriction 2 (DR2) and 0:1 in Protein Malnutrition (PM). (B) Lifespan curves of the animals on the different diets. Both DR diets result in significant increase of median and maximum lifespan as compared to NM and PM results in much lower lifespan. Mantel-Cox test was used to compare the survival curves. N = 70 each. (C) Daily fecundity measure of animals since eclosion. The first five days all animals were on NM after which they were transferred to the different diets. The y-axis represents the number of eggs laid by a single female in 24 hours. The fecundity measures on the 14th day are significantly lower in DR (p-****, N=20), DR2 (p-**, N=10) and PM (p-****, N=20) as compared to NM (N=20). To test statistical differences multiple t-tests were done with Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli to correct for false discovery rate. (D) Volume of eggs were compared between the different diets after different duration of dietary treatments. Each dot represents a single egg. ns - p > 0.05, * - p < 0.05, ** - p < 0.01, *** - p < 0.001, **** - p < 0.0001: The statistical difference of a treatment with another is shown close to the curve of one in a color representing the treatment it is compared to.

of eclosion to 9 days after switching to different diets were measured. Here, the number of eggs laid and their volume was measured [113] (Fig 19 B, C), and for the reproductive output, the weight of animals, their stored protein and lipid as a proxy for the metabolic state was also quantified (Fig 20 A,B,C). For all traits - even though the changes start happening as soon as the diet is switched – the changes happen gradually for at least five days and become more stable after this. This also provided important information about the metabolic state of the

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animals. The weight and protein content of the body decrease on lowering dietary protein, while the TAG amounts start increasing (Fig 20 A, B, C). A presumable explanation for this could be the compensatory feeding observed in animals to reach a target protein level [114]. Here, it would result in higher food intake in the low protein diets, hence higher carbohydrate intake and storage.

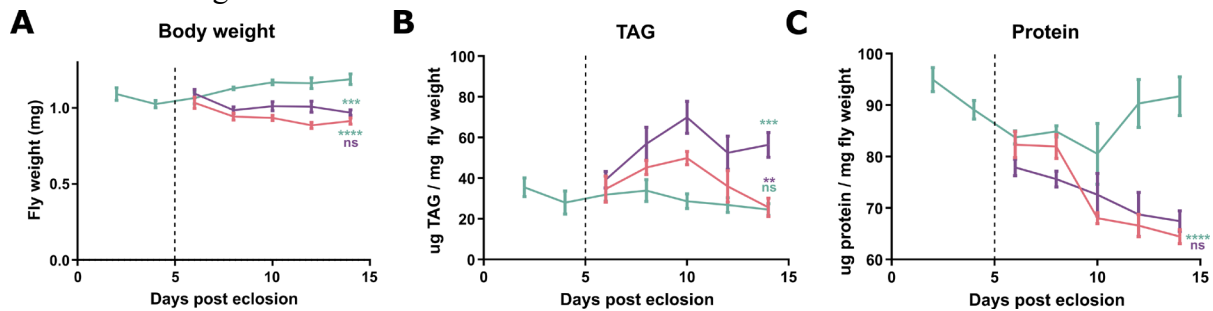


Figure 20 Body composition of animals with different duration of dietary treatments. Body weight (A), Triacylglyceride (B), and Protein (C) content of the flies were measured from the day of eclosion until 9 days of transferring to different diets. Measurement was taken every 2nd day. T-tests were done to compare the values between different diets on the 14th day (9th day of transferring to different diets). N=7 each time point each diet. ns - $p > 0.05$, * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$, **** - $p < 0.0001$. The statistical difference of a treatment with another is shown close to the curve of one in a color representing the treatment it is compared to.

These preliminary experiments helped to choose a proper dietary regime and a duration of seven days of the dietary treatment to measure the immune response.

4.4.2. On infection with *Bacillus subtilis* holistic immune parameters decline with decreasing dietary protein

I first started exploring the impact of dietary protein content on the immune response at a holistic level [115], [116]. For this purpose, the animals raised on different diets with different pathogens were infected and their survival rates compared. A systemic mode of bacterial infection was used to induce all branches of the immune system and minimise the expected changes in feeding patterns generated by oral infection. Interestingly, infections with different pathogens gave variable results. For *Serratia marcescens*, a highly virulent natural pathogen most animals died within the first 24 hours of infection, no substantial differences in the survival of animals raised on different diets were seen (Fig. 21A). This was also true for the less pathogenic *Ecc 2046* (Fig. 21B). On the other hand, for the bacteria *Bacillus subtilis*, the chances of surviving the infection were directly proportional to the amount of dietary protein (Fig. 21C). For *S. marcescens*, one could argue that the high virulence of the bacteria leaves no chance for the animals to fight the infection. However, *Ecc2046* and *B. subtilis* have similar survival rates in the control diet, but it changes drastically as dietary protein lowers.

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The bacterial load of animals on different diets was quantified to investigate if the pathogen's ability to grow inside the host or the host's ability to clear the pathogen are impacted [69]. For *Ecc2046*, even though there were no differences in survival, animals on NM had more bacterial

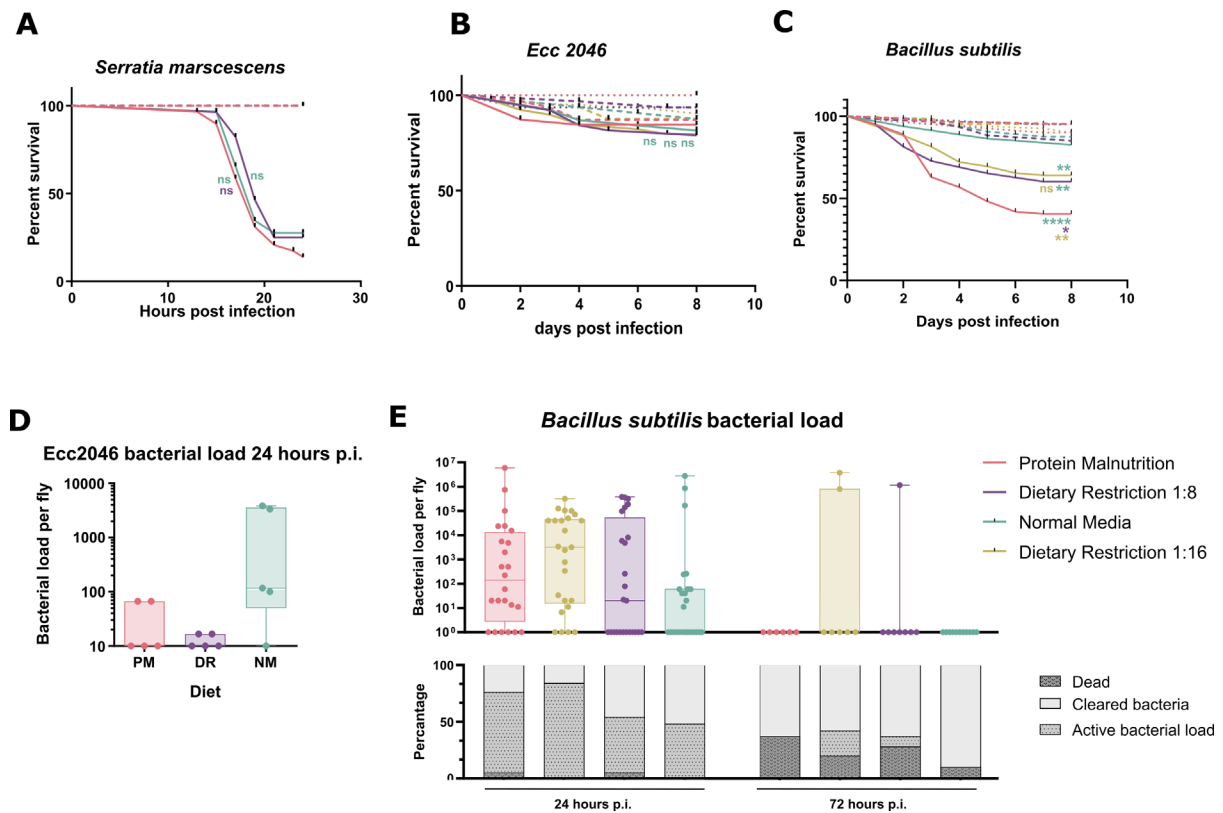


Figure 21 Infections after treatment with DR and PM. 7 days after treatment with the different diets, the animals were infected with the mentioned bacterium. Along with the bacteria infection treatment (Solid line), two controls were included in each experiment: sterile injury (dashed line) and unhandled control (dotted line). Survival curves for infections with (A) *S. marcescens* (OD₆₀₀ 0.01; n=30) (B) *Ecc2046* (OD₆₀₀ 15; n=40), (C) *B. subtilis* (OD₆₀₀ 1.5; n=76-80). Statistical significance was calculated using Mantel-Cox log rank test. The significance values for the infection treatments are shown on the graph. ns -p > 0.05, * - p<0.05, ** - p<0.01, *** - p<0.001, **** - p<0.0001 The statistical difference of a treatment with another is shown close to the curve of one in a color representing the treatment it is compared to. Bacterial load of flies infected with *Ecc2046* (N=5) (D) and *B. subtilis* (N=25 for 24 hours p.i., N=6-11 for 72 hours p.i.) (E) are shown. Each dot represents one animal. The bacterial load for *B. subtilis* also shows the percentage of animals dead, with clear bacteria (0 bacterial load) and active bacterial load (>0 bacterial load).

load than DR or PM (Fig. 21D), perhaps because a more nutritious diet also serves as a better environment for the growth and reproduction of these bacteria [63], [117]. However, for *Bacillus subtilis*, the bacterial clearance improved with the dietary protein content (Fig. 21E). Nearly 45-50% of the animals had eliminated the bacteria in DR and NM by 24 hours, while this number was only 24% for PM. I also investigated the DR1:16 diet in this experiment to see if the trend holds for the stronger DR. The mortality rate for both DR diets after infection with *Bacillus subtilis* were the same, i.e., lower than PM and higher than NM. This also shows the strength of this pattern. Further, the percentage of animals with totally cleared bacteria in the DR1:16 diet was only 16% by 24 hours (Fig. 21E). Thus, at 24 hours after infection, the

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chances of clearing the pathogen load increase as dietary protein increases. As most of the animals were alive in all diets one day after infection, the bacterial load was measured three days after infection, when some animals have already died (Fig. 21E). For NM and PM, at this time point, the animals had either eliminated the pathogen or were dead, with 37% dead individuals in PM and only 10% in NM. While for both the DR diets, a small percentage of animals still had an active bacterial load. Together from the time course bacterial load and survival percentages, it can be concluded that the animals fed a higher protein diet cleared the pathogen *Bacillus subtilis* faster from their system and hence have a higher chance of survival.

From these experiments, I found *Bacillus subtilis* to be an ideal candidate to understand further the molecular changes in the immune response following a dietary change. It neatly segregates the different diets in terms of survival and bacterial load. This resolution can be advantageous for looking at the molecular mechanisms and recognizing what components of the immune response change and which do not.

4.4.3. AMP abundance in the whole animal cannot explain the differences in holistic immune parameters

Having seen a divergence in the different diets in the ability to survive and clear *Bacillus subtilis* infection, the underlying molecular mechanisms were studied. First, the antimicrobial peptide genes were analysed (AMPs) (Fig. 22A), which are crucial immune effectors from the Toll and IMD pathway and are indispensable for clearing out the pathogen burden [12], [16], [118]. To quantify the differences in the activation of these AMPs, their transcript levels were measured using qRT-PCR. The expression levels of three classic AMPs were quantified [119], [120] - *Diptercin*, as an output of the *IMD* pathway, and *Drosomycin* and *Metchnikowin*, for the *Toll* pathway - and one member of the immune modulator *Bomanin* family - *Bomanin SI* [121]. Interestingly, following a bacterial infection, higher levels of all four in the DR-raised animals than in NM were apparent, statistically significant for *Mtk* and *Dpt* (Fig. 22). On the other hand, for PM animals the levels were significantly higher than NM for *Mtk* and not different for the others, however there is a trend of higher expression than NM for *Dpt* and lower for others (*BomSI* and *Dro*).

While on sterile injury, the expression levels of these peptides are the same for all diets, except for higher expression of *Mtk* in PM and DR ($p < 0.05$). This is interesting, as even though these animals raised on low protein diets express higher abundances of the bactericidal peptides and

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pattern recognition receptor, they are slower at clearing out bacteria and have fewer chances of survival.

Lastly, I measured the levels of the proposed tolerance factor *CrebA*, which has been reported to be activated on infections [87]. In the NM diet, no significant upregulation on infections was

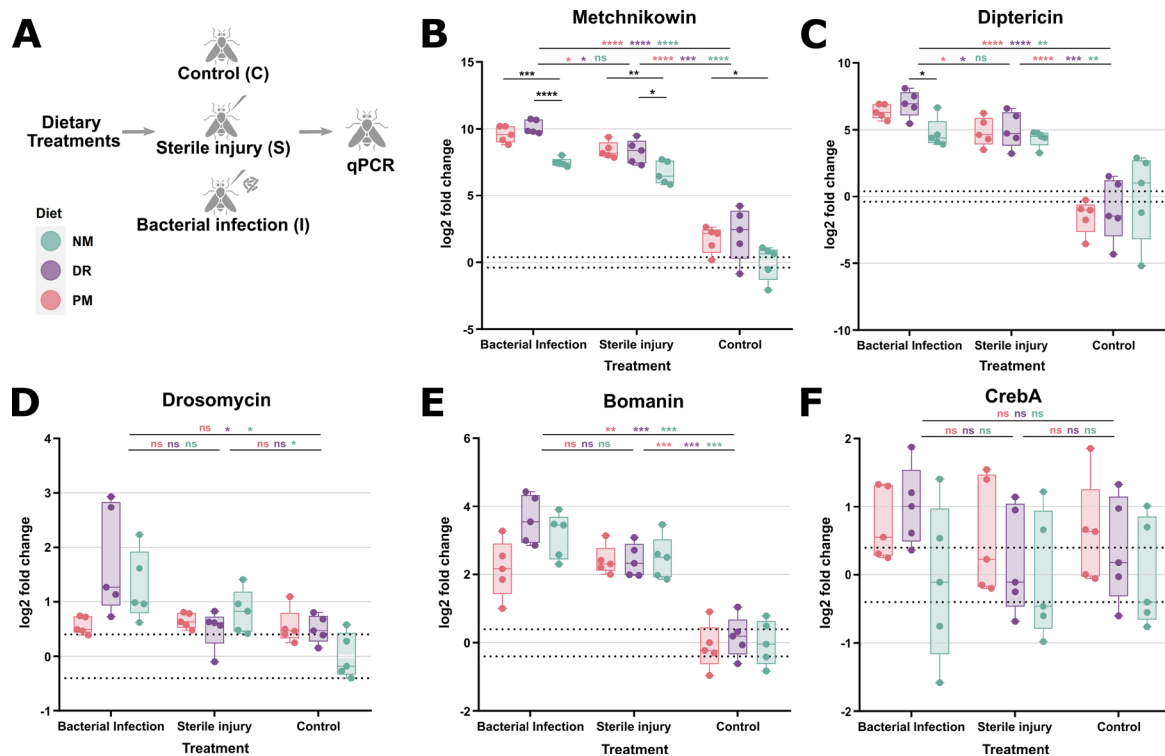


Figure 22 AMP measurement in the whole animal body after infections on DR and PM. (A) Schematic representing the setup of this experiment. After 7 days of the treatment with different diets, animals were treated with one of the three treatments: Bacterial infection (I), sterile injury (S) and Unhandled control (C). 6 hours after the respective treatment, RNA was extracted from these animals to perform qPCR for the AMPs Mtk (B), Dip(C), Dro(D), Bom(E) as well as the tolerance factor gene *CrebA* (F). The transcript values were normalized to Rpl32 in NMC. N=5. To test statistical differences multiple t-tests were done with Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli to correct for false discovery rate. ns - $p > 0.05$, * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$, **** - $p < 0.0001$

seen, only a higher variance in its expression, which could pertain to differences in the population expressing a toleration factor. When looking at DR and PM, the *CrebA* expression slightly increased on infection, though not statistically significant.

Thus, in the quest to shortlist processes that could explain the higher chances of survival of animals raised on high protein diets and facing a *Bacillus subtilis* infection, no solid patterns in the whole-body datasets were seen. Rather I found a higher abundance of immune effectors in the low protein diets, which die faster.

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4.4.4. Basal antibacterial response does not change in fat body and hemocytes upon lowering dietary protein

As the three diets differ in the expression of immune effectors, I next aimed to find how the other biological processes are impacted. Section 4.1 showed that the transcriptional changes in the two professional immune cell types of *Drosophila*, i.e., fat body and hemocytes, are very distinct and specific to their molecular functions. Additionally, this response drastically differed from what is seen in an averaged-out whole-body response. Thus, the transcriptional response in these two cell types via RNA sequencing to examine if this can explain the differences in the immune response of the three diets was studied. RNA sequencing of fat bodies and hemocytes of animals raised on different amounts of dietary protein without an infection or injury was started with (Fig. 23A).

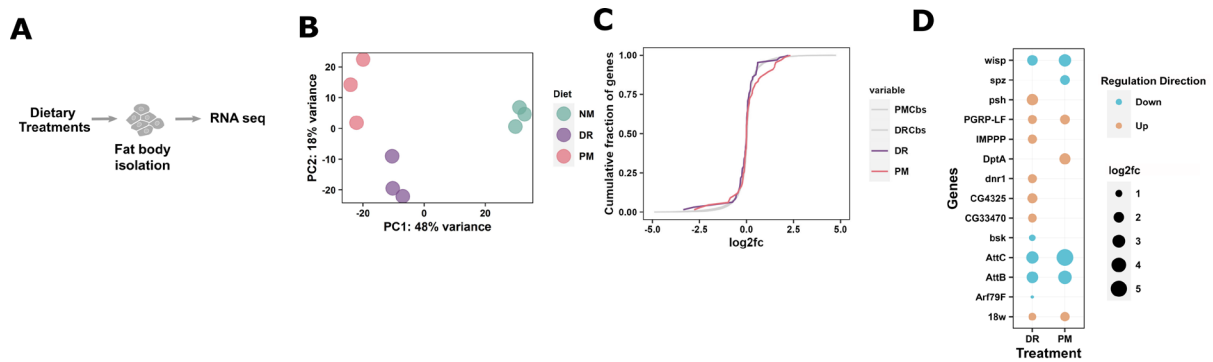


Figure 23 Transcriptomic analysis of fat bodies on different diets. (A) Experimental setup: RNA sequencing was performed on fat bodies isolated from animals kept on different diets. (B) Principle Component Analysis plot for fat body samples on different diets. (C) Cumulative distribution function for the 67 genes related to GO term ‘Antibacterial response’ in DR and PM in fat bodies. The expression values of all the expressed genes in each treatment are represented as baselines in gray color. (D) Expression values of the few genes related to GO term ‘Antibacterial response’ differentially expressed in DR and PM fat bodies

A substantial transcriptional rewiring of the fat body under both dietary interventions was seen (Fig. 23B). This would be expected of a tissue that is the most significant storage organ of flies and is crucial for handling dietary stress and maintaining metabolic homeostasis [122]–[124]. In DR, 1144 genes differentially expressed (504 upregulated and 640 downregulated), and in PM, 1209 genes were differentially expressed (548 up and 661 downregulated) (Fig. 24). Nearly 50 percent of the upregulated and 60 percent of the downregulated genes were shared between the two treatments, indicating there is a common response triggered by either lowering or removing proteins (Fig. 24). GO term analysis of these genes indicated that the most prominent molecular process in the common activated genes was translation and peptide synthesis. Further, DR increased the upregulation of more metabolic processes, like lipid

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homeostasis, fatty acid degradation and carbohydrate biosynthesis. While PM further upregulated more genes regulating peptide biosynthesis. GO term analysis of the commonly downregulated genes indicated significant repression of redox homeostasis processes like glutathione metabolism and Cyp-450 metabolism, some metabolic processes like lipid homeostasis and N-glycan biosynthesis, and the general vesicular transport system of the cell.

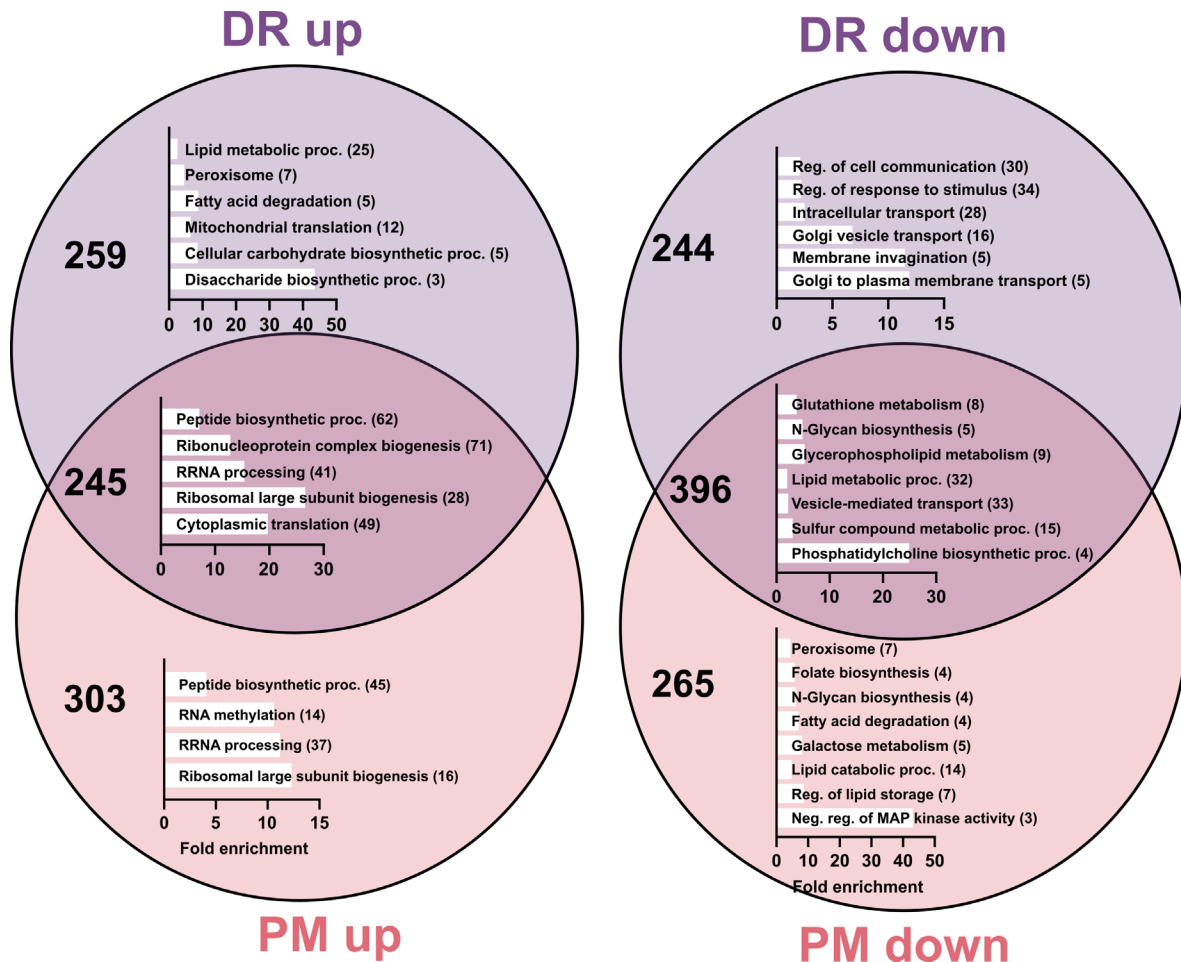


Figure 24 GO term analysis for genes differentially expressed in DR and PM fed fat bodies. The common and unique up and downregulated genes in DR and PM are shown as Venn diagram. The number of genes in each category along with some GO terms related to each category is shown. The x-axis on the GO term plot shows the fold enrichment for each GO term calculated by ShinyGO. Additionally, the number of genes related to each GO term is represented in brackets next to the name. (NM:Normal Media, DR:Dietary Restriction, PM:Protein Malnutrition, C:Control, S:Sterile injury, I:Bacterial Infection)

For DR, the specific downregulated processes included the general cell communication and response and transport machinery. While for PM, additional repressed processes include fatty acid degradation, galactose metabolism, lipid catabolism and storage, and folate biosynthesis. All of these indicate that in both dietary interventions, the peptide synthesis is highly heightened in the fat body while the general cell machinery and metabolism are downregulated, though to different degrees in DR and PM. A more detailed summary of the GO terms in both diets is provided in Fig. 24.

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Interestingly, no GO terms related to the immune response in the differentially expressed genes in fat body became apparent (Fig. 24). I further examined all the genes related to the GO-term “antibacterial immune response” in DR and PM fat bodies and plotted a cumulative density function (Fig. 23C). Both diets showed no meaningful changes in the process, and there was only a handful of genes related to immunity changing in the two, with some downregulated (*Attacins*) and some upregulated (*IMPP*, *DptA*) (Fig. 23D). Thus, in an uninduced state, the direct immune response of fat bodies undergoing different dietary stress does not change.

Examining the changes in hemocytes upon DR and PM, very few genes were differentially expressed (Fig. 25A, B). This could be because the primary roles of hemocytes are related to phagocytosis and wound repair [22], [125], and they do not directly play any role in the

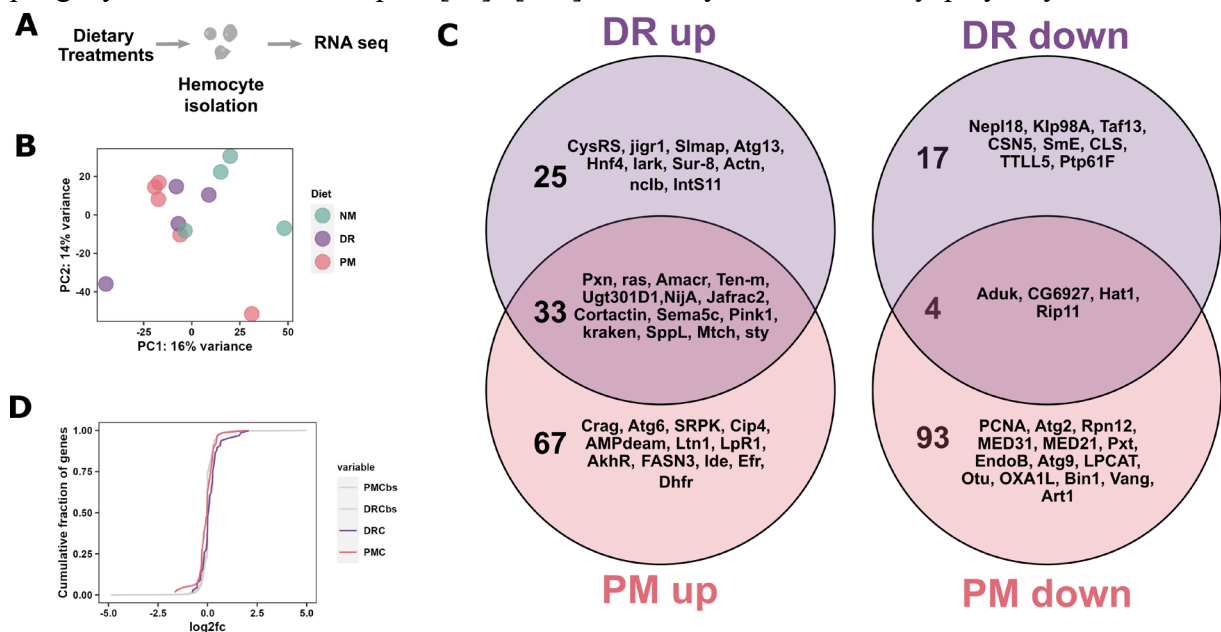


Figure 25 Transcriptomic analysis of hemocytes on different diets. (A) Experimental setup: RNA sequencing was performed on hemocytes isolated from animals kept on different diets. (B) Principle Component Analysis plot for hemocyte samples on different diets. (C) The number of common and unique up and downregulated genes in DR and PM are shown as Venn diagram. Selected genes from each category are also shown. (D) Cumulative distribution function for the 67 genes related to GO term ‘Antibacterial response’ in DR and PM in hemocytes. The expression values of all the expressed genes in each treatment are represented as baselines in gray color. (NM:Normal Media, DR:Dietary Restriction, PM:Protein Malnutrition, C:Control, S:Sterile injury, I:Bacterial Infection)

metabolic maintenance of the body. In DR, 79 genes were differentially expressed (58 up and 21 downregulated), and in PM, 197 genes were regulated (100 up and 97 down) (Fig. 25C). As these lists are quite small, no statistically significant GO terms were related to them. Some selected genes are shown in Fig. 25C for each category. Further, I tried to find if there was anything directly related to the immune system in the differentially expressed genes, but no genes related to the GO term “antibacterial immune response” were found. The eCDF for the two diets for this category of genes also shows the same result (Fig. 25D).

Results

This set of experiments showed us that in an uninduced state, fat bodies and hemocytes do not show any transcriptional changes to the direct antibacterial immune response after treatment with the DR and PM diets. Consequently, I decided to look at the response of the two tissues after induction of the immune system by a sterile injury and bacterial infection. The animals were raised on the three diets and then subjected to the three treatments – Unhandled control, Sterile injury, or Bacterial infection. mRNA was sequenced from these animals' fat bodies and hemocytes, as shown in the scheme. The following sections describe the results for each cell type.

4.4.5. The magnitude of infection response decreases proportional to the dietary protein content in fat body

Intending to identify the biological changes that follow an immune challenge on a nutrient-rich vs nutrient-deprived diet in the fat body, the tissue's transcriptomes after different treatments were compared (Fig. 26A).

I first focused on the bacterial infection dataset from the three diets and aimed to compare the transcriptomic profile of the fat body after a combination of dietary and immune stress to that of a single stress. For the initial investigation, hierarchical clustering of the expression values for all genes differentially expressed in NM infection (NMI) and PM and DR were performed with and without infections (PMC, DRC, PMI, and DRI), using NMC (NM unhandled control) as the control (Sup. Fig. 2A). The treatments with both nutritional and immune stress (DRI and PMI) had activated genes shared with both their respective dietary controls (DRC and PMC, respectively) and the infection control (NMI) (Sup. Fig. 2A). Thus, a combination of stressors produced a response that was common with some parts of the response to each of the individual stressors but not entirely common to any. Further, the Euclidean distance (used for hierarchical clustering of the samples, shown on top of graph) was less between DRI and NMI, while the PMI response was more similar to its own dietary control PMC (Sup. Fig. 2A). This indicates differences in the extent of similarity to a control infection response in the two nutritionally deprived diets, where the stronger PM stress induces more PM-coping changes even after infection. Interestingly, when repeating the same analysis on the sterile injury dataset, I

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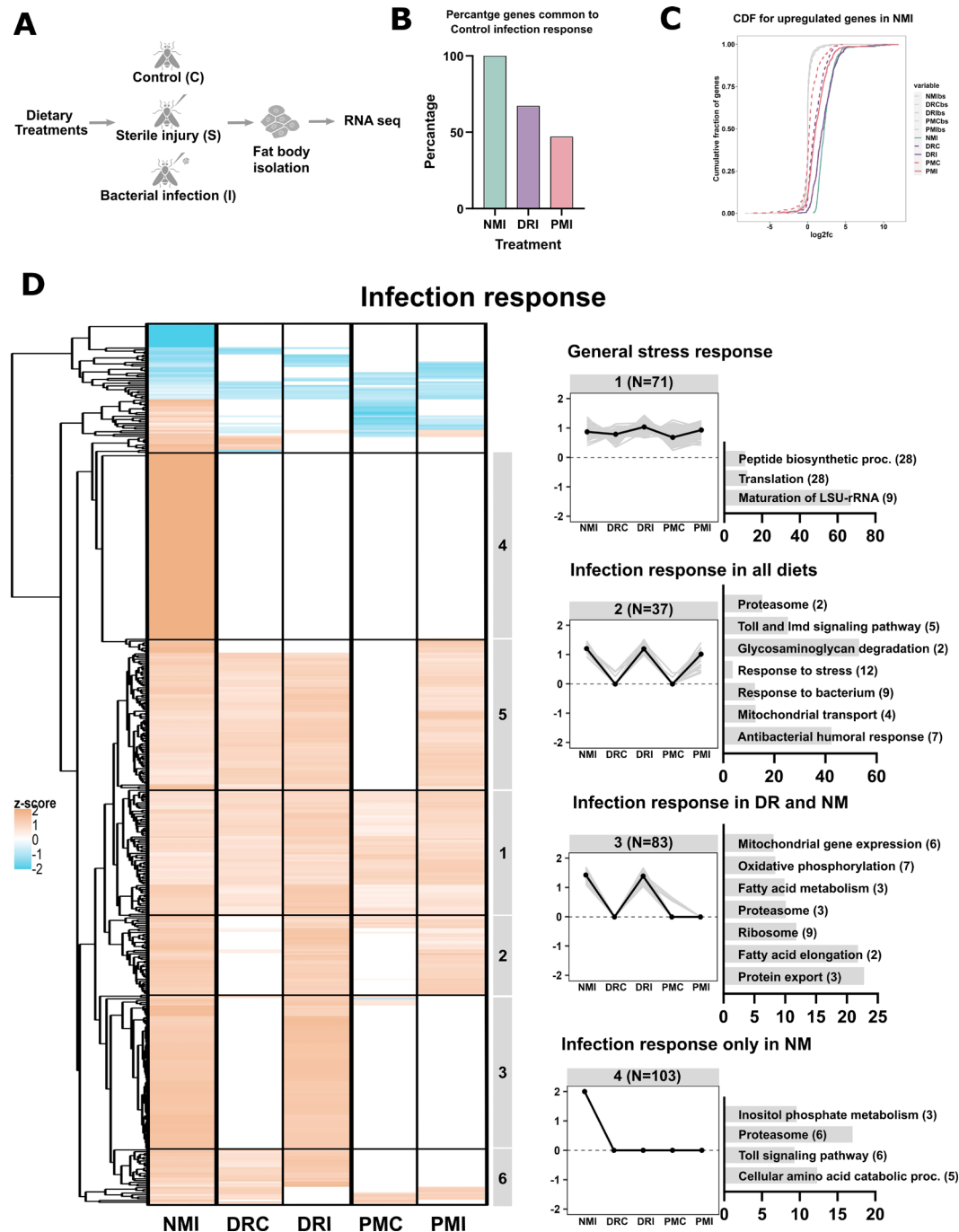


Figure 26 Infection response in the fat body on different diets. (A) Experimental setup: RNA sequencing was performed on fat bodies isolated from animals kept on different diets and further infected (I), injured (S) or control (C). (B) Percentage of 447 upregulated control infection response genes upregulated in DR and PM infection treatments. (C) Cumulative distribution function for the 447 control infection response genes in the different treatments for the fat body. The expression values of all the expressed genes in each treatment are represented as baselines in gray color. (D) Hierarchical clustering of the expression values of 489 control infection response genes in the different treatments. The scaled values are plotted for expression levels, i.e., the log2 fold change of a gene in the treatment relative to NMC. Relevant gene clusters are marked in gray with numbers and their expression patterns are shown on the right. The cluster number is followed by the number of genes in each cluster. GO term analysis of each cluster is represented on the right. The x-axis on the GO term plot shows the fold enrichment for each GO term calculated by ShinyGO. Additionally, the number of genes related to each GO term is represented in brackets next to the name. (NM:Normal Media, DR:Dietary Restriction, PM:Protein Malnutrition, C:Control, S:Sterile injury, I:Bacterial Infection) observed more overlap of the profiles of nutritionally deprived diets with injury (PMS and

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DRS) to their respective dietary controls (PMC and DRC), while the control injury response (NMS) was considerably different (Sup. Fig. 2B). For example, the Euclidean distance between DRC and DRS was less than that between NMS and DRS. This implied that when treated with a nutritionally deprived diet, the resource allocation in the fat body towards eliciting an immune response was kept to a minimum in mild injury stress, but was considerably elevated upon bacterial infection.

A noteworthy observation in this analysis was that particular genes being activated by both immune and dietary stress (Sup. Fig. 2A, Fig 26D). This hints toward a general stress response of this professional immuno-metabolic tissue. To further analyse the functional relevance of these genes, this 'stress' response as the genes upregulated in both infection and dietary controls – NMI, DRC, and PMC was defined. 80 genes were found in this category, for which translation was the most significant Gene Ontology term (Fig 26D). Nearly 30 of these genes contributed to the processes of translation and peptide biosynthesis. The fat body is actively involved in dealing with any immune or nutritional challenge and is essential in maintaining metabolic homeostasis. A higher peptide synthesis would support changing the proteome of the tissue to handle the stress at hand.

Having found that a combination of stressors induces a combination of reactions for the different stressors, specific changes to the 'immune' part of the reaction were analysed. I first defined a 'control infection response' as the 489 genes differentially expressed in NM on bacterial infection (NMI). Out of the 447 upregulated infection response genes, DRI shares 67% while PMI only shares 47% (Fig 26B). This indicates linearity in the magnitude of infection response (in terms of the number of genes) in the different diets, which is proportional to their dietary protein content. To examine this pattern for expression levels of genes, the CDF plot for these 447 upregulated genes was compared for the three diets (Fig 26C). Here, some percentage of these 'infection' genes were activated in the dietary controls as well (DRC and PMC), even though the cumulative expression level and the number of genes were higher in the infection treatments (PMI and DRI) (Fig 26C). Further, the linearity also holds true for the cumulative gene expression, as the levels for PMI were lower and DRI is intermediate between NMI and PMI (Fig 26C).

Subsequently, I focused on finding the specific gene groups shared and different between the three diets. To organize them into relevant groups, hierarchal clustering of the 489 'infection' genes was performed for their expression levels in DRI and PMI (Fig 26D). The respective dietary controls (PMC and DRC) were included in this setup to differentiate a general 'stress'

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response from a specific 'infection' response. Firstly, 71 out of the 80 classified 'stress response' genes of the fat body were also activated in the combined stress treatments PMI and DRI (Cluster 1) (Fig 26D), indicating the functional cruciality of these genes in the activity of this tissue. Additionally, there were genes activated in at least four of these five treatments (Cluster 5 and 6), also demonstrating the non-specific nature of these genes to stress. As discussed earlier, a big portion of these 'stress' genes was related to the process of biosynthesis of proteins in the cell. Identifying a specific 'infection' response as the gene groups exclusively activated

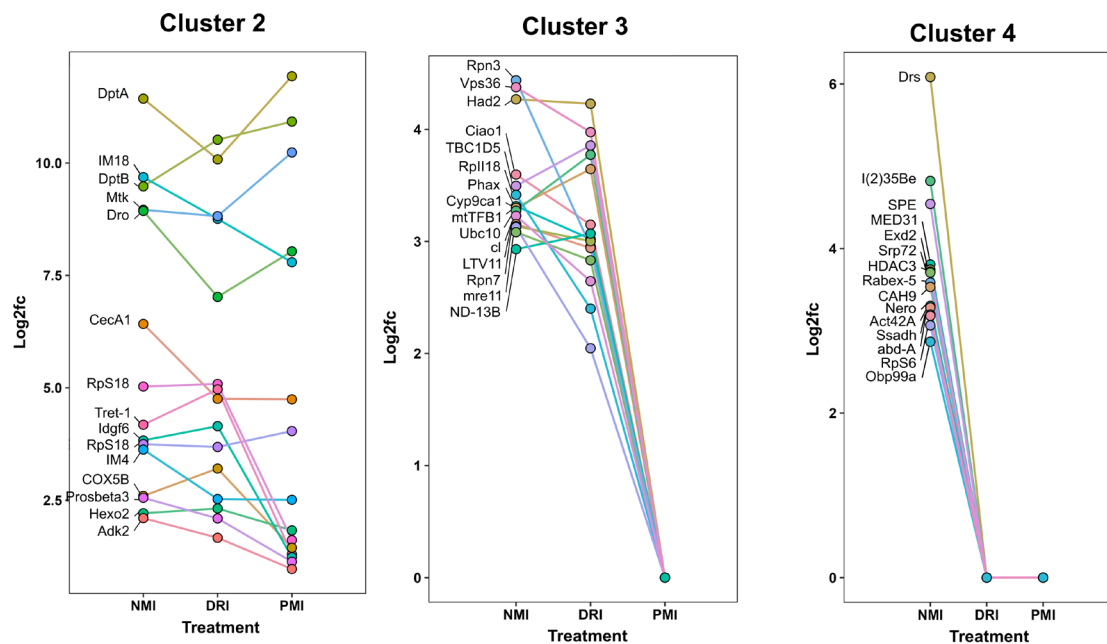


Figure 27 Selected genes for relevant clusters from Fig26D. Top15 genes from each category of cluster shown in Fig26D are represented with their expression values in the different treatments. Cluster 2: Genes upregulated in all three diets upon infection, Cluster 3: Genes upregulated on in NMI and DRI and Cluster4 : Genes upregulated only in NMI. (NM:Normal Media, DR:Dietary Restriction, PM:Protein Malnutrition, C:Control, S:Sterile injury, I:Bacterial Infection)

in the treatments facing infection, I detected division into very distinct three clusters: Cluster 2 – activated in all three diets only upon infection (NMI, DRI, and PMI), Cluster 3 – activated only in DR and NM on infection (NMI and DRI) and Cluster 4 – which is only activated in NMI (Fig 26D). This demonstrates the presence of a core in the 'infection' response, which is activated regardless of nutrient availability. In contrast, the rest of the response is dispensable and proportionally reduced upon nutrient reduction.

GO term analysis were performed to investigate the biological processes related to each cluster (Fig 26D). Interesting to note, I found that the 37 genes comprising the 'core' cluster (Cluster 2) were mostly connected to Antibacterial humoral response, Response to bacterium, Toll and Imd signalling pathways and some metabolic processes. The top 15 genes in this group

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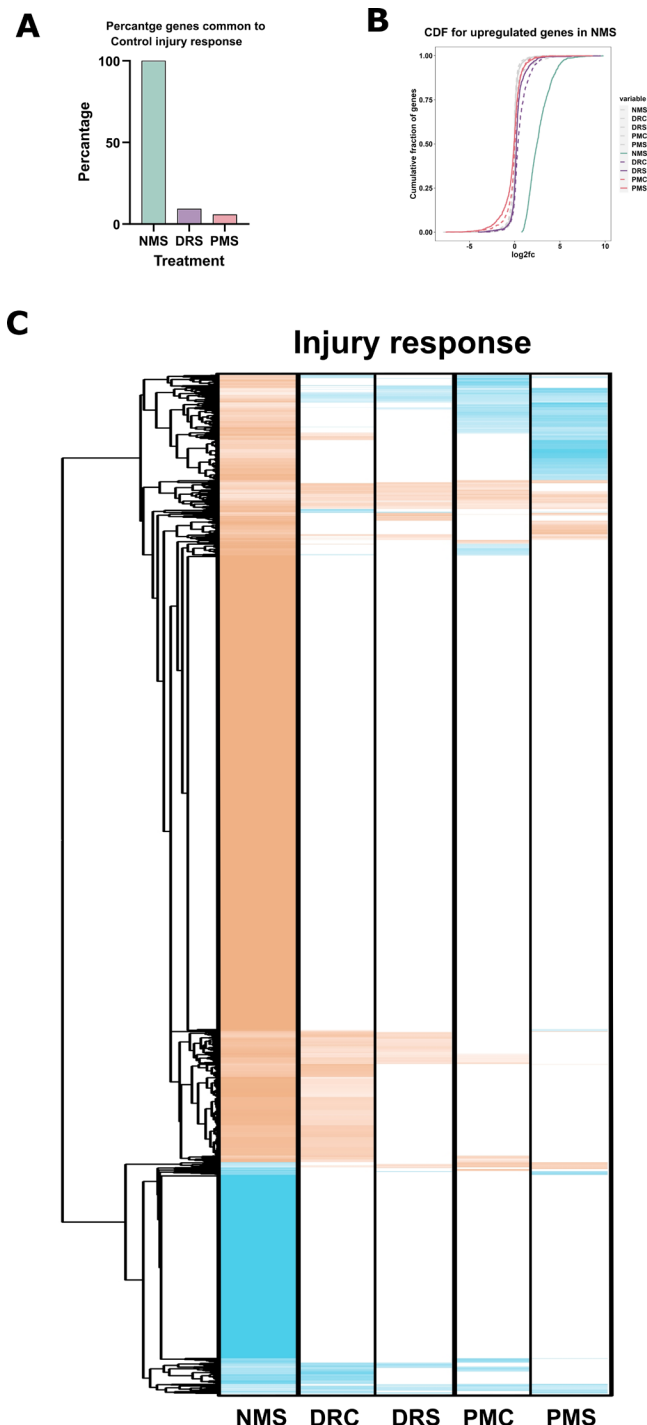


Figure 28: Injury response in the fat body on different diets. (A) Percentage of the 1221 control injury response genes upregulated in DR and PM injury treatments. (B) Cumulative distribution function for the 1221 control injury response genes in the different treatments for the fat body. The expression values of all the expressed genes in each treatment are represented as baselines in gray color. (C) Hierarchical clustering of the expression values of 1580 control injury response genes in the different treatments. The scaled values are plotted for expression levels, i.e, the log2 fold change of a gene in the treatment relative to NMC. (NM:Normal Media, DR:Dietary Restriction, PM:Protein Malnutrition, C:Control, S:Sterile injury, I:Bacterial Infection)

consisted mainly of the Toll and Imd pathway effectors - Antimicrobial peptides (Fig 26D, 27). Cluster 3, which is maintained in DR, included genes responsible for Oxidative Phosphorylation, proteasome, protein export, and translation machinery. Cluster 4, which was dispensable in the low-nutrient diets, included more genes for the proteasomal machinery and protein export, inositol phosphate metabolism, and some toll signalling pathway genes (Fig 26D, 27).

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As discussed earlier, the response to sterile injury showed a very different pattern than bacterial infection, with minimal similarities in the differentially expressed genes in the three diets. I checked this further by looking at the percentage of genes from the ‘control injury response’ (1580 genes regulated in NMS: 1221 upregulated) and found only 9% upregulated genes in DRS and 6% in PMS (Fig. 28A). Further comparing the expression values of these shared genes, the CDF plots for these genes were analysed (Fig. 28B). In contrast to the bacterial infection, the expression values for the low protein diets on injury were very similar to their own dietary controls. This is also demonstrated by the hierarchical clustering of the injury response genes for the different treatments, where little to no commonality is observed (Fig. 28C). There are a few genes activated in all the treatments, which are very similar to the earlier described ‘stress’ response. However, unlike the infection response comparing the genes activated in all three diets only upon injury, only four genes were in common, which are all

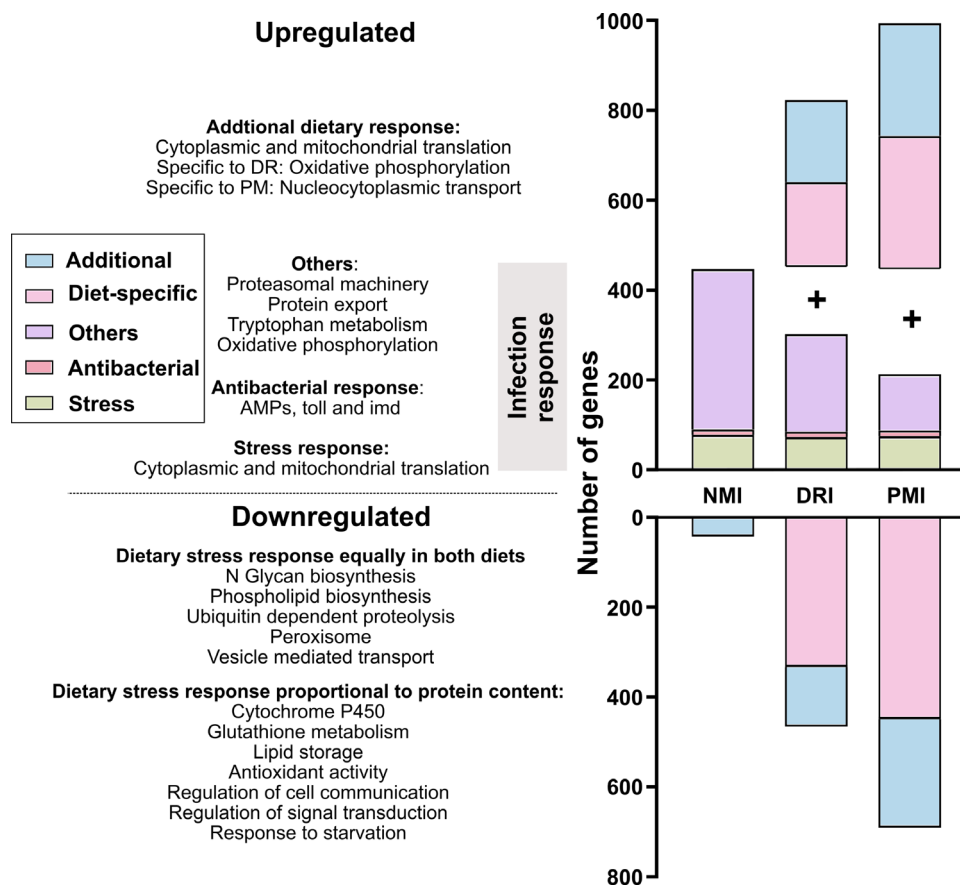


Figure 29 Number of genes to cope with dietary and infection part of the stress. The response in treatments with combination stress (PMI and DRI) was divided into genes common to the dietary control (Diet-specific), common to the infection control (Infection response) and anything on top (Additional). The infection response has three functional categories: Stress response, antibacterial response and Others. The right side shows the bar graph with number of up and down regulated genes from each category. The left side indicates some relevant GO terms for each category. (NM:Normal Media, DR:Dietary Restriction, PM:Protein Malnutrition, C:Control, S:Sterile injury, I:Bacterial Infection)

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immune effectors (Fig. 28C). Hereby I concluded that the magnitude of response to an injury decreases dramatically in the nutritionally deprived diets, while on bacterial infection this investment is resumed and is directly proportional to the dietary protein content.

4.4.6. The differences in infection response of fat body on low or no-protein diets are not due to the classic antibacterial response

Having found differentiation in the infection part of the response for the fat bodies on different diets, I next aimed to analyse and compare it to the dietary part of the response. To this end, the differentially expressed genes on the treatments with combination stress (DRI and PMI) into infection response (genes common to the infection control NMI), dietary response (genes common to the respective dietary controls DRC and PMC) and additional response (genes not common to any control) were analyzed (Fig. 29). The DRI and PMI treatments showed a significant number of up and downregulated genes common to their respective dietary controls.

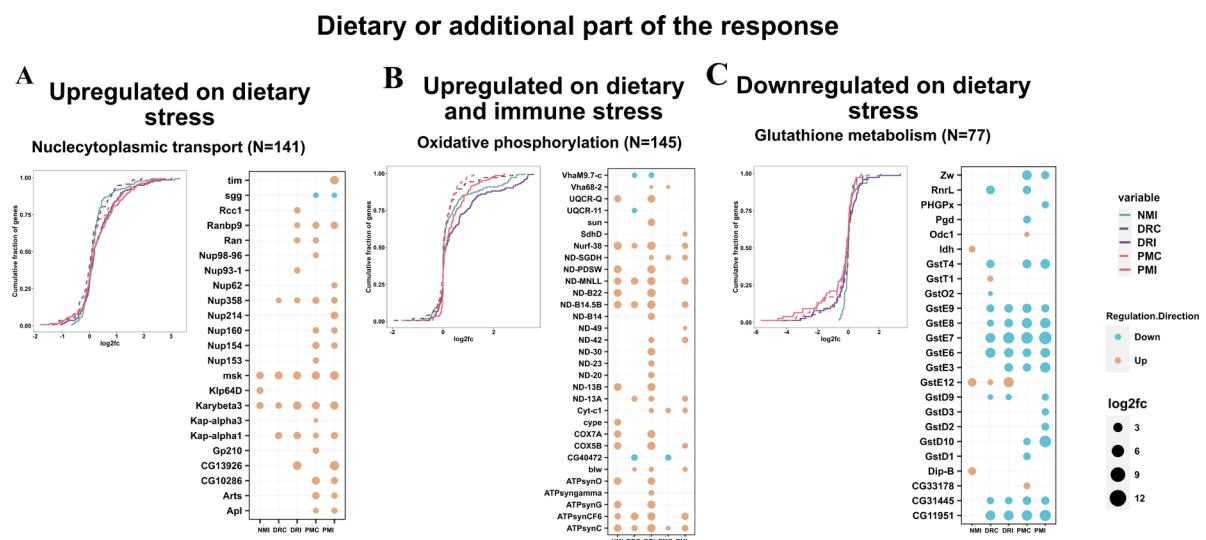


Figure 30 Some examples of processes from the dietary part of the response in Fig. 29. For different categories of genes, a GO term is shown with the Cumulative distribution function for all the genes related to that GO term to compare the expression trend between the treatments. Further the expression values of all differentially expressed genes for that term are shown for different treatments. (A) shows nucleocytoplasmic response which has 141 associated genes. It is a process which is activated in the low protein diets, especially in PM. (B) Oxidative phosphorylation is an example of a process regulated upon both dietary and immune stress. The combination stressors have then higher expression than their controls, specially DRI with much higher expression than its controls. (C) Glutathione metabolism with 77 associated genes is downregulated in both dietary treatments proportional to the dietary protein content. (NM:Normal Media, DR:Dietary Restriction, PM:Protein Malnutrition, C:Control, S:Sterile injury, I:Bacterial Infection)

Interestingly, there were also a similar amount of genes in the ‘additional response’ category. I performed a GO term analysis on the different categories of responses, a summary of which is shown in the Fig. 29. Here, particularly the ‘additional response’ category is of interest, as it depicts the extra activated or repressed genes specific to a combination of stressors. However,

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a GO term analysis for this category for both DRI and PMI had no unique functional processes and were remarkably similar to the processes observed in the respective dietary response category. Interestingly, the same was true for the genes differentially expressed in the dietary controls but not in combination stressors. This indicated that the combination treatments (DRI and PMI) and dietary controls (DRC and PMC) had the same processes being changed but with some different genes. For example, Fig. 30 shows the cumulative differential expression of a few GO terms from the different categories with the help of CDFs for all the genes related to

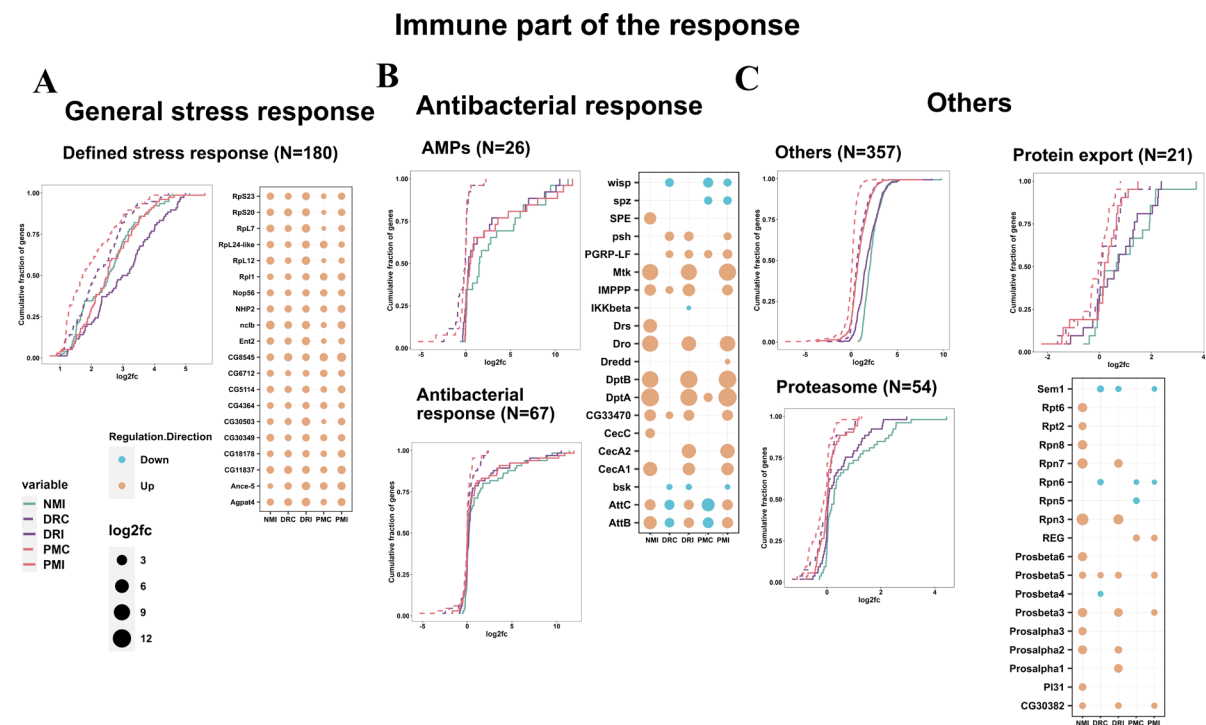


Figure 31 Three categories in the infection part of the response in Fig. 29. For different categories, the Cumulative distribution function for all the genes in that category are shown to compare the expression trend between the treatments. Further, the expression values of all or selected differentially expressed genes for that category are shown for different treatments. (A) shows CDF for the 80 defined stress response genes. These were the genes differentially expressed upon both dietary and immune stress. Expression values of selected genes from this category are shown next to it. Most of these genes were expressed in all treatments, to different extent (B) Antibacterial response GO term has 67 genes related to it, for which the CDFs are shown. An additional CDF for the 26 AMPs in *Drosophila* are also indicated. The expression values for all differentially expressed genes associated with the GO term Antibacterial response are shown next to it. The number and expression level of antibacterial genes in very similar in the three diets upon infection. (C) Others are all the genes on top of the stress or antibacterial response. These genes were different in expression pattern between the different diets. Examples of processes from this category include Proteasomal machinery and protein export. For both the processes, I can see a difference between the diets in the CDFs. The expression values for all differentially expressed genes related to Proteasomal machinery are shown. (NM:Normal Media, DR:Dietary Restriction, PM:Protein Malnutrition, C:Control, S:Sterile injury, I:Bacterial Infection)

that term, as well as fold changes for selected genes related to each process. An important upregulated process in the dietary response category is the nucleocytoplasmic transport, which is activated in both PMC and PMI as well as in DRI (Fig. 30A). Oxidative phosphorylation was another process, which was activated by both infection and DR controls to different

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extents, and showed even higher additive expression in the combination treatment DRI (Fig. 30B). For the downregulated genes, there were many processes in both DR and PM treatments, some of which were previously described. Out of these, some processes were equally repressed in both diets, while others were repressed proportional to the nutritional stress intensity (Fig. 29). The Fig. 29 summarises some GO terms from both types, and Fig. 30C shows the CDF for one such process, glutathione metabolism. Glutathione metabolism is downregulated in both diets, but the extent is greater in PM than DR, as indicated by the eCDF, as well as the gene expression values in this category (Fig. 30C).

In the infection part of the response, I detected earlier that the small cluster of genes activated in all three diets upon infection was mainly related to the antibacterial response (Fig. 26D). To compare the intensity of this immune response, I compared the number and expression levels of all differentially expressed genes related to the GO term antibacterial response in the three diets (Fig. 31B). Surprisingly, the number of antibacterial genes and their relative abundances were the same in the different diets after infection (NMI, DRI and PMI) (Fig. 31B). The eCDF plots of the antibacterial genes were also overlapping and non-significantly different. Most of the genes from the earlier defined 'stress' response were also differentially expressed in all the diets with and without infection (Fig. 31A). However, the expression levels were different in the various treatments. Thus, the decline in the net infection response with lowering dietary protein is contributed by the genes activated on top of an infection or stress response. The eCDF for these 358 genes also indicated this as it shows the same pattern between the different treatments as the whole 'infection' response. Functionally these genes were not responsible for eliciting a direct immune response but instead for metabolism or cell maintenance-related processes like proteasomal machinery, protein export, redox homeostasis and oxidative phosphorylation (Fig. 29). A clear example here was the proteasomal machinery, which was activated to a greater extent in NMI but decreased proportionally to the dietary protein amount in terms of the number of genes and their expression (Fig. 31C). Oxidative phosphorylation was another process activated in NMI and DRI but not to the same extent in PMI (Fig. 30B). Thus, even with no dietary protein intake, the fat bodies of infected animals still produce the same amount of antimicrobial proteins but decrease the production of the additional metabolic processes, which make up a big part of the infection response machinery.

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4.4.7. The magnitude of infection response decreases proportional to the dietary protein content in hemocytes as well

To identify biological changes in the second professional immune cells, the hemocytes, the transcriptomic profiles of the cells upon infection and injury on the different diets were compared. The experimental setup was the same as fat body as demonstrated in the Fig. 32A.

Starting with the bacterial infection response, I first began by comparing the response from a combination of dietary and immune stress to that of a single stress. For exploring the data, the

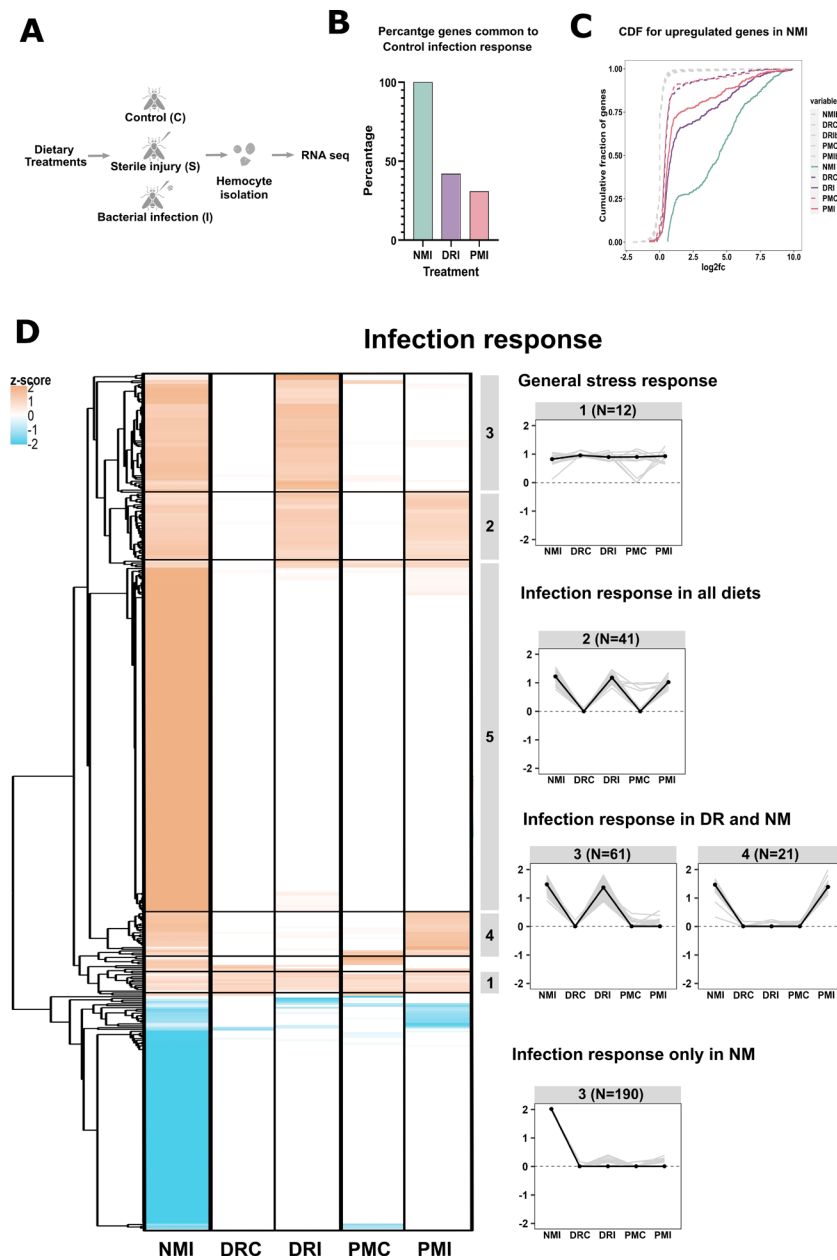


Figure 32 Infection response in the hemocytes on different diets. (A) Experimental setup: RNA sequencing was performed on hemocytes isolated from animals kept on different diets and further infected (I), injured(S) or control(C). (B) Percentage of 342 upregulated control infection response genes upregulated in DR and PM infection treatments. (C) Cumulative distribution function for the 342 upregulated control infection response genes in the different treatments for the hemocytes. The expression values of all the expressed genes in each treatment are represented as baselines in gray color. (D) Hierarchical clustering of the expression values of 470 control infection response genes in the different treatments. The scaled values are plotted for expression levels, i.e., the log2 fold change of a gene in the treatment relative to NMC. Relevant gene clusters are marked in gray with numbers and their expression patterns are shown on the right. The cluster number is followed by the number of genes in each cluster. (NM:Normal Media, DR:Dietary Restriction, PM:Protein Malnutrition, C:Control, S:Sterile injury, I:Bacterial Infection)

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hierarchical clustering of the expression values for all the genes differentially expressed in the combination stress treatments (DRI and PMI) and infection (NMI) and dietary controls (DRC and PMC) were compared (Sup. Fig. 3A). The heatmap shows the scaled values for the log₂ (fold change) for each gene compared to the control NMC. It is important to note here that the dietary controls didn't induce a strong reaction in the hemocytes as compared to the infection control. On combination of dietary and immune stress (DRI and PMI), more genes are differentially expressed than dietary controls (DRC and PMC) but not as much as infection control (NMI) (Sup. Fig. 3A). Similar to the fat body, there are some overlaps in the infection response in the three diets and there is also a small number of genes activated on both dietary and infection stress. This general 'stress' response was much less prominent in hemocytes with only 18 genes as compared to 80 genes in the fat body. This is probably because of less involvement of hemocytes in regulating dietary stress. However, the stress genes like *Jafrac2*, *eater*, *Ten-m* or *Cortactin* can have functionally relevant roles in hemocytes. On repeating the same analysis with the sterile injury dataset (Sup. Fig. 3B), the number of differentially expressed genes in the three diets upon injury were quite comparable and also had considerable overlap with each other.

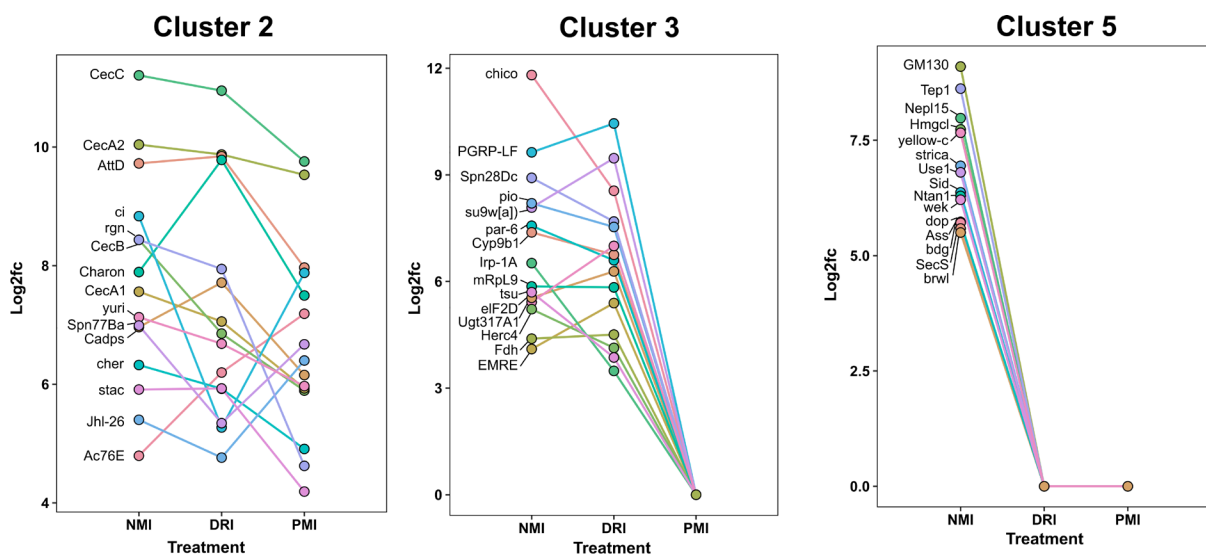


Figure 33 Selected genes for relevant clusters from Fig32D. Top15 genes from each category of cluster shown in Fig32D are represented with their expression values in the different treatments. Cluster 2: Genes upregulated in all three diets upon infection, Cluster 3: Genes upregulated on in NMI and DRI and Cluster 5: Genes upregulated only in NMI. (NM:Normal Media, DR:Dietary Restriction, PM:Protein Malnutrition, C:Control, S:Sterile injury, I:Bacterial Infection)

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In order to pinpoint the differences in the immune reaction of hemocytes from different diets, the response in nutrient deprived and the control diet were compared. I defined the 470 differentially expressed genes in NMI as the ‘Control infection response’. I found that DRI shares 42% of the 342 activated infection response genes while PMI only shares 31% (Fig. 32B). To further compare the expression level of these genes in the different treatments, the eCDF curves for this ‘control infection response’ (Fig. 32C). Similar to fat body, a linearity in not only the number but also the cumulative expression level of the infection response, which is directly proportional to the dietary protein content, became apparent. Subsequently, I aimed to find the gene groups still expressed in the nutrient deprived diets and the ones only in nutrient

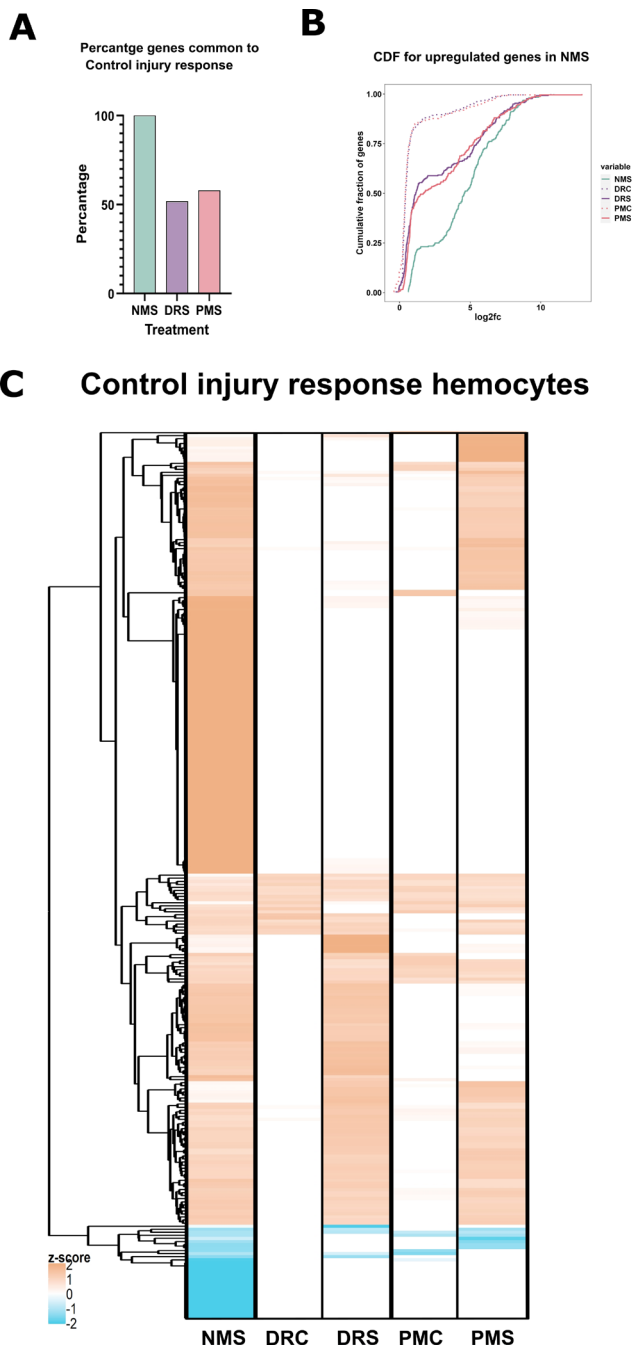


Figure 34 Injury response in the hemocytes on different diets. (A) Percentage of 260 upregulated control injury response genes in DR and PM injury treatments. (B) Cumulative distribution function for the 260 upregulated control injury response genes in the different treatments for the hemocytes. (C) Hierarchical clustering of the 291 expression values of control injury response genes in the different treatments. The scaled values are plotted for expression levels, i.e, the log2 fold change of a gene in the treatment relative to NMC. (NM:Normal Media, DR:Dietary Restriction, PM:Protein Malnutrition, C:Control, S:Sterile injury, I:Bacterial Infection)

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rich ones. To organize these infection response genes into relevant groups, a hierarchical clustering for the expression values of these genes in the different treatments was performed (Fig. 32D). Most of the earlier defined 'stress' response genes were also activated in the combination treatments PMI and DRI, which is shown as Cluster 1 (Fig. 32D). Like fat body, three distinct categories of clusters could be identified: Cluster 2 – genes activated in all three diets only upon infection (NMI, DRI and PMI), Cluster 3 and 4 – genes activated in the control and one of the nutrient deprived diets on infection (NMI and DRI or PMI), and Cluster 5 – genes only activated in the infection control NMI (Fig. 32D). It is noteworthy here that the genes only common between DRI and NMI are almost three times that between PMI and NMI. Thus, it can be concluded that there are certain core genes in the hemocytes as well, which are activated independent of the nutritional status of the animal while the others can be dispensed dependent on nutrient availability. These clusters did not provide any significant GO terms, however, top 15 genes from each cluster are shown in Fig. 33.

Next, a quantitative comparison of the injury response in the different diets was made. Defining the 291 genes differentially expressed in the NMS as the 'control injury response', revealed that out of the 260 upregulated injury response genes, DRS 52% and PMS shares 58% (Fig. 34A). I further compared the expression values of the injury response by comparing the eCDF curves in the different treatments (Fig. 34B). The curves for both DRS and PMS were overlapping. Thus, the injury response in hemocytes is lowered to nearly 50%-60% on decreasing or removing the dietary protein, both at the scale of number and the cumulative differential expression of those genes. This is different from the changes in the injury response in fat body, where the response was reduced to less than 10% upon a decrease in the dietary protein. However, one similarity between the two cell types was that the impact on both DR and PM was to similar extent, in contrast to bacterial infection, where the response was reduced proportional to the dietary protein content.

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4.4.8. Whole infection response, including the classic antibacterial response, is impacted in the hemocytes on low or no-protein diets

Having found differences in the whole infection response of hemocytes on restricted or malnourished diets, I next aimed to pinpoint which parts of the immune response are maintained and which ones are given up on. The control infection response from NMI can be grouped into the three following categories: Antibacterial response – all the gene associated with the GO term antibacterial response, Stress response – as defined in previous section, the genes activated by both dietary and immune stress and Others –genes other than the antibacterial and stress response (Fig. 35). The stress response was almost constant between the different diets facing infection, both in terms of number of genes as well as their expression

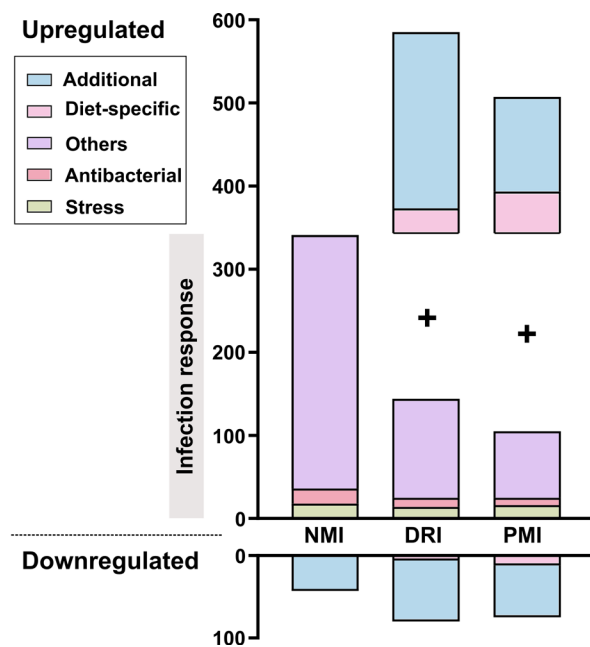


Figure 35 Number of genes to cope with dietary and infection part of the stress. The response in treatments with combination stress (PMI and DRI) was divided into genes common to the dietary control (Diet-specific), common to the infection control (Infection response) and anything on top (Additional). The infection response has three functional categories: Stress response, antibacterial response and Others. the bar graph shows number of up and down regulated genes from each category. (NM:Normal Media, DR:Dietary Restriction, PM:Protein Malnutrition, C:Control, S:Sterile injury, I:Bacterial Infection)

(Fig. 36A). This indicates the crucial nature of these genes for well-functioning hemocytes that can withstand different kinds of stress. Surprisingly, the antibacterial response was very different between the three diets (Fig. 36B). As pointed out earlier, the dietary controls do not show any expression of the antimicrobial proteins, while the infection control shows a high expression of almost all antimicrobial proteins. In the DR and PM diets upon infection, there were a few central antibacterial genes, which were expressed in high abundance, whereas the others were not (Fig. 36B). As we can see from the eCDF for AMPs, nearly 50% of the AMPs expressed in NMI are expressed in DRI and PMI (Fig. 36B). This is quite interesting as in the fat bodies antibacterial response was the core response which was almost the same in all the diets, both in terms of genes as well as their expression levels. Thus, on dietary nutrient scarcity, hemocytes try to maintain their functions via high expression of a set of antimicrobial proteins,

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instead of reducing the expression of all of them. In section 4.1, I tried to characterize the transcriptomic changes in hemocytes upon bacterial infection and sterile injury and found genes from functionally relevant processes. From the others category of genes, as the lists are too small for a proper GO term analysis, the genes from some of these processes were compared (Fig. 37). For all the processes, Phagocytosis, lipid metabolism, proteolysis and redox homeostasis, a decline in the number of these genes expressed in the PMI and DRI was observed. However, the maintenance of expression of a few genes with important phagocytosis related roles in hemocytes like *SCAR*, *Slik* and *Pitslre* was obvious to the same extent in the three diets (Fig. 37A). Further other important targets like *TRAM*, *par-6* and *chico* with possible roles in actin cytoskeleton rearrangement were maintained just in DR but not in PM (Fig. 37A). Similarly, *Hnf4* with important lipid metabolism roles, *Jafrac2*, and *ras*, which are essential for a redox homeostasis, were expressed in all three diets upon infection (Fig. 37B, C). This

Immune part of the response

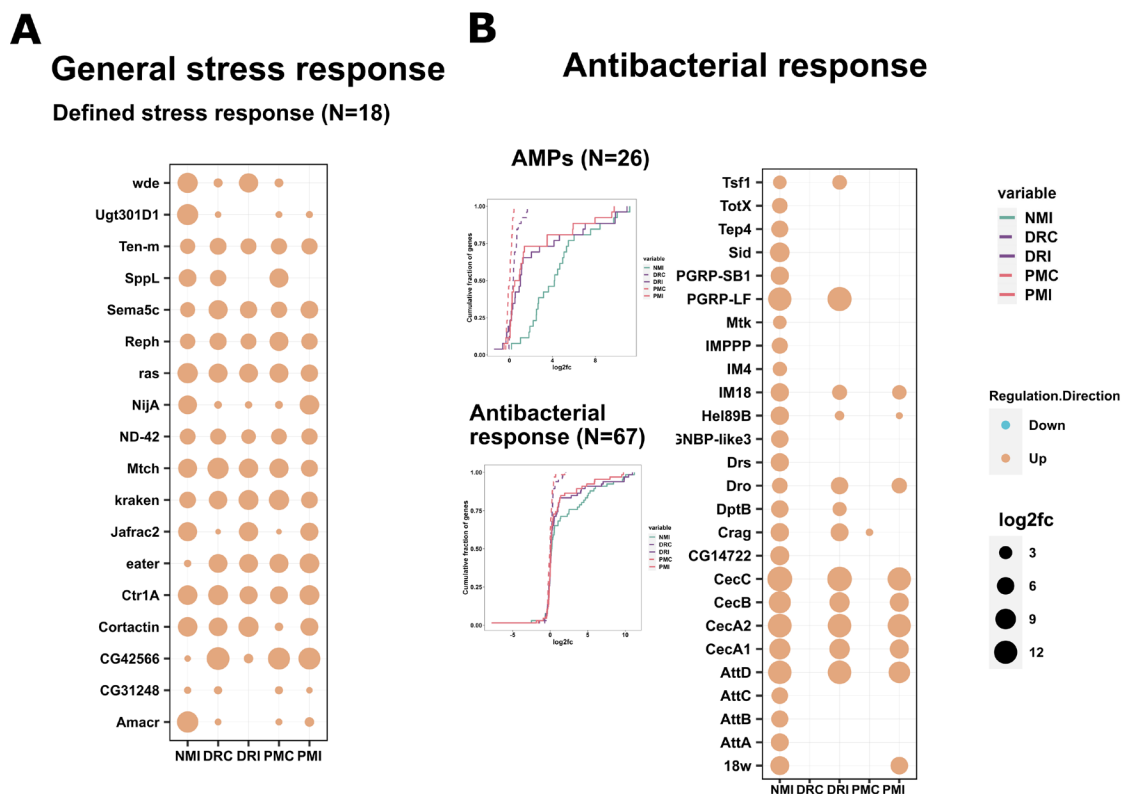


Figure 36 Two of the three categories in the infection part of the response in Fig. 35. (A) shows expression values for 18 defined stress response genes. These were the genes differentially expressed upon both dietary and immune stress. Most of these genes were expressed in all treatments, to different extent (B) Antibacterial response GO term has 67 genes related to it, for which the CDFs are shown. An additional CDF for the 26 AMPs in *Drosophila* are also indicated. The expression values for all differentially expressed genes associated with the GO term Antibacterial response are shown next to it. The number of differentially expressed antibacterial genes decreases immensely in DRI and PMI. (NM:Normal Media, DR:Dietary Restriction, PM:Protein Malnutrition, C:Control, S:Sterile injury, I:Bacterial Infection)

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also indicates that the vitality of these genes for proper functioning of the hemocytes in the event of an infection.

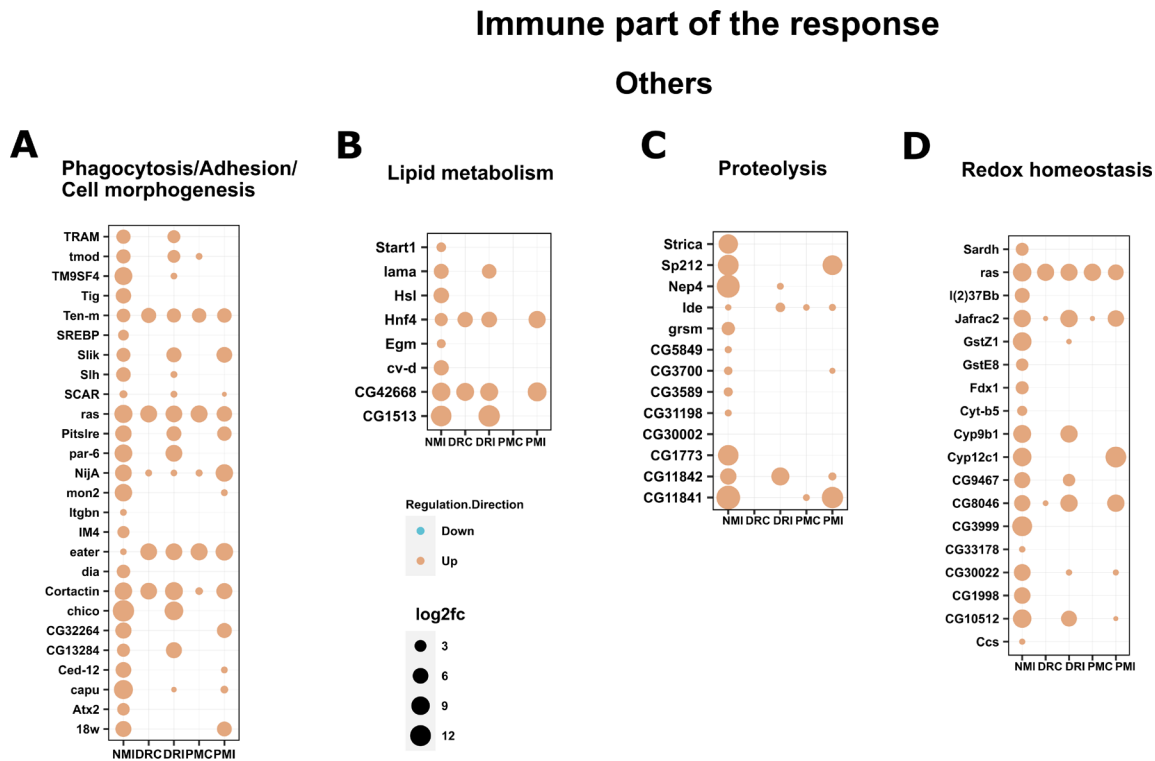


Figure 37 The ‘Others’ categories in the infection part of the response in Fig. 35. Others are defines as all the genes expressed on top of the stress or antibacterial response. These genes were different in expression pattern between the different diets. Selected genes are categorized according to their functions and their expression values for all treatments are shown for (A) Phagocytosis related pathways, (B) Lipid metabolism (C) Proteolysis and (D) Redox homeostasis. (NM:Normal Media, DR:Dietary Restriction, PM:Protein Malnutrition, C:Control, S:Sterile injury, I:Bacterial Infection)

4.4.9. Fat body and hemocytes have different resource allocation strategies on a combination of dietary and immune stress

At the end, I would like to point out the major differences between the two cell types in their response to an infection on a nutrient deprived vs nutrient rich diet. One of the most significant differences was the expression of antimicrobial peptides. In a nutrient-abundant environment, there are many more AMPs expressed in hemocytes as compared to fat body (Fig. 38A, B). However, on lowering or removing the dietary protein of the animals all the AMPs are still expressed to similar extent in the fat body (Fig. 38A), while in the hemocytes only a fraction of it are expressed but to the same level (Fig. 38B). Additionally, in the fat body, sterile injury on a control diet induced the expression of almost all the AMPs as bacterial infection, but it

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was reduced to a few genes in the other diets (Fig. 38A). While in hemocytes, a sterile injury didn't induce the expression of a lot of AMPs in the control diet, which further became almost negligible in the other two diets (Fig. 38B).

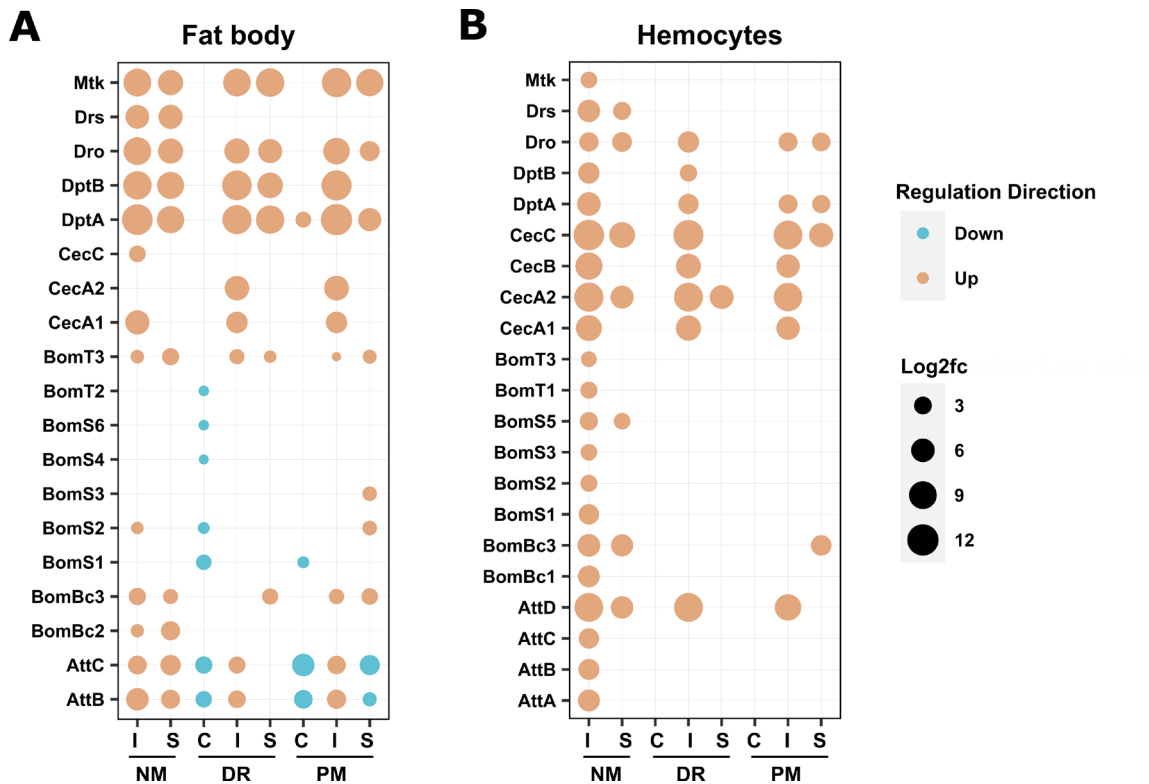


Figure 38 The expression levels of AMPs in different treatments in fat body and hemocytes. Expression values for all the AMPs differentially expressed in any of the treatments relative to NMC are shown for (A) fat body and (B) hemocytes. Fat body has less number of AMPs expressed in Infection control, however most of them are also expressed in the other diets upon infection. Hemocytes has many more AMPs expressed in the infection control, out of which only a few are expressed in the other diets upon infection. (NM:Normal Media, DR:Dietary Restriction, PM:Protein Malnutrition, C:Control, S:Sterile injury, I:Bacterial Infection)

Thus, on lowering the dietary protein intake, while both fat body and hemocytes reduce their investments in the immune response proportional to the protein content, the functional categories of these genes are quite different in the two. Fat body on one hand, maintains its antibacterial response, despite no dietary protein intake (Fig. 39). Hemocytes on the other hand, maintain the expression of only a few central antimicrobial genes. The genes expressed on both a dietary and an immune stress alone are expressed to the almost the same amounts in both the cell types on a combination of these stressors as well. The genes expressed on top of these two categories made up a major portion of an infection response and contained functionally relevant processes for both cell types, like phagocytosis or redox homeostasis for hemocytes and proteasomal machinery or protein export for fat bodies. These processes do not have a direct bacterial resistance role, but are probably essential for the proper functioning and maintenance

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of these cells. They would also be required to facilitate the functionality of the direct immune effectors. In both the cell types, these genes reduce proportionally to the dietary protein content.

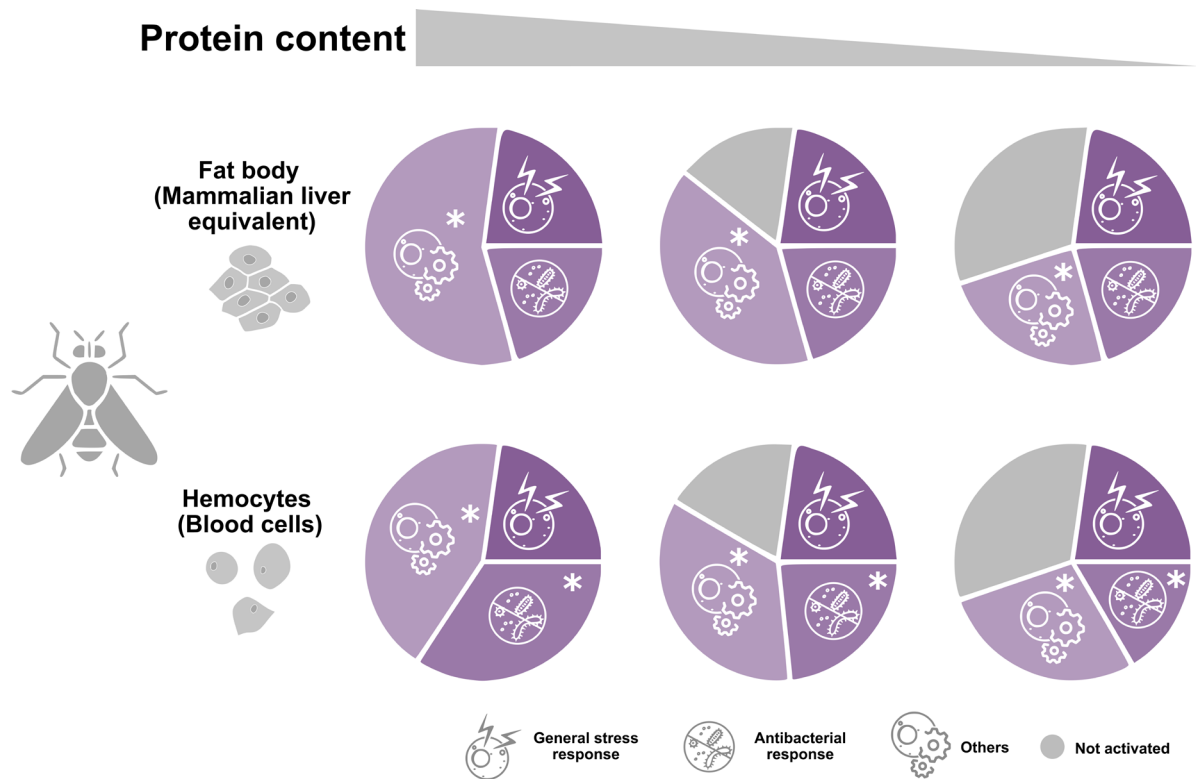


Figure 39 Graphical abstract showing the resource allocation strategies in the infection response of fat body and hemocytes on lowering dietary protein. Considering infection response for a tissue as all the genes differentially expressed upon infection in the control diet, relevant categories were defined in the response: Direct Antibacterial response – all the genes related to the GO term ‘Antibacterial response’, Stress response – genes expressed on both dietary and immune stress and Others – All the remaining genes in the infection response. I observed that this number of infection response genes activated on lowering the dietary protein declines proportionally to the dietary protein content in both the cell types. In fat body this decline in the total genes is only contributed by the ‘Others’ part of the response while in hemocytes both ‘Others’ and ‘Antibacterial response’ are impacted.

4.5. Possible roles of the *Drosophila* Prothoracicotropic hormone (*Ptth*) in dealing with nutritional stress.

On comparing the transcriptome of fat bodies on the nutrient-poor diets DR and PM, I observed a high upregulation for *Ptth*. *Prothoracicotropic hormone* (*Ptth*) is usually known for its roles in the development of individuals but not in adults [126]. I further confirmed this upregulation via qRT-PCR and observed a nearly 2-fold change in DR and 3-fold change in PM. These values are directly normalized by using the same amount of starting cDNA material, as no appropriate housekeeping gene were found. I tested for RpL32, RpL13, Act42a and Gapdh1 and observed different Ct values for all of them in the different diets.

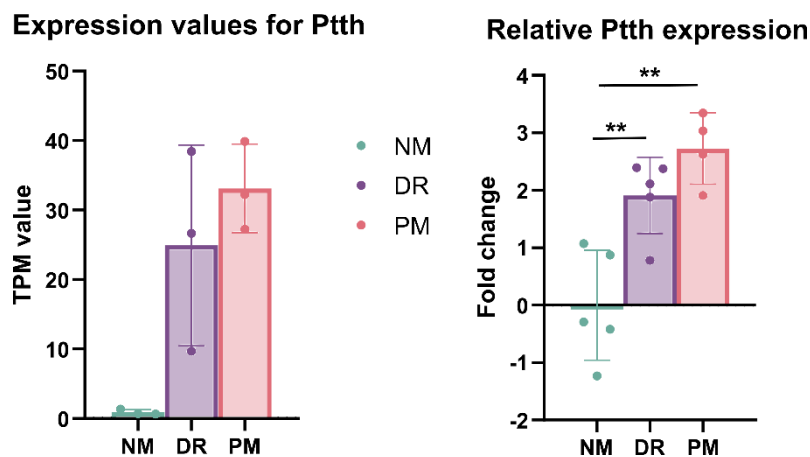


Figure 40 Increased *Ptth* transcript levels upon Dietary restriction and Protein Malnutrition. (A) TPM values for *Ptth* in the different diets in the fat body as obtained from RNA sequencing analysis. (B) Transcript level of *Ptth* measured in the different diets by qRT-PCR. With equimolar concentrations of cDNA, the Ct values were obtained for *Ptth*, which were used for normalisation using C as the control, as not stable housekeeping gene could be detected. N=5 T-test were conducted to compare the treatments. ns -p > 0.05, * - p<0.05, ** - p<0.01, *** - p<0.001, **** -p <0.0001

5. Discussions

5.1. Hemocytes and fat body cells show strikingly divergent transcriptomic responses to infection

The transcriptional responses of isolated *Drosophila* immune organs to systemic infection and to sterile injury was systematically studied. Comparing both professional immune organs, fat body, and hemocytes, revealed a surprisingly low concordance of transcriptional profiles. This strongly divergent transcriptional response of fat bodies and hemocytes to infection and sterile injury was surprising especially considering that both organs use the same signalling systems for pathogen recognition [12], [14]. The cohort of genes upregulated after infection in both fat body and hemocytes consisted mainly of immune effector-encoding genes. A closer look at those genes that were regulated in only one of the two organs shows that they represent immune-associated aspects that fit well with the specific tasks of humoral and cellular immunity, respectively. For hemocytes, these are mainly GO terms associated with phagocytosis of particles in the narrower and broader sense. While phagocytosis as a GO term directly fits the expectations, processes including cell adhesion, cell shape changes, actin-related processes, and Golgi organization are also linked to phagocytosis. While the mechanism of infection is not well studied for *Bacillus subtilis*, phagocytosis would serve as an essential process for the functionality of hemocytes against any invading bacterium. In contrast, responses of the fat body focus on the synthesis and export of antimicrobial peptides. Here, appropriate GO terms such as protein transport and translation are foremost. However, GO terms such as proteasome and fatty acid degradation represent secondary effects providing metabolites and energy, respectively. The increased mitochondrial activity, which enables more ATP production, could also be in the same context.

5.1.1. The whole body ‘core’ infection response does not extend to individual tissues

I observed minimal overlap when comparing the already available whole-body data [87] to the tissue-specific dataset from the two major immune-responsive cell types generated in this study. Several reasons might account for this. On the one hand, it is possible that there are significant differences in responses to infections with different pathogens, an aspect that is certainly important [127] [21]. On the other hand, these differences may also represent secondary effects that could be explained by different degrees of infection events and responses

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caused by them [78] [79]. I assume that the whole-body transcriptomic signatures in response to infection are neither dominated by the fat body responses nor by those of the hemocytes. Instead, other organs that show only secondary immune-related responses might dominate this response. The current results support this hypothesis because specific immune responses and sterile injury shared far more than 50% of commonly upregulated genes. This large overlap is seen both in the fat body and hemocytes implying that a large proportion of the infection-induced response in these professional immune organs is part of a core response launched in any infection of a certain intensity. Recent transcriptome studies focusing on the immune response of the *Drosophila* fat body further support this hypothesis [130]. In these studies, infections with gram-positive or gram-negative bacteria resulted in approximately 50% concordantly induced transcripts [130]. Larval hemocytes responded in a similar fashion to different types of infection [91]. Thus, it seems that the core response of the insect immune system is essentially limited to antimicrobial effectors, among which antimicrobial peptides are most important. The immune responses of individual immunocompetent organs reveal a more comprehensive core that considers the specific requirements of the organs in question. This organ-specific core can thus be identified as the overlap in induced transcript changes of sterile injury and specific immune responses. However, a technical problem arises in infection experiments with living microorganisms, often complicating such comparisons. Here, the variance in such experiments is higher than, for example, in sterile injury. This high variance in infection experiments might depend on inter-experimental variation in the infection procedure, but, and this is more likely, be due to stochastic processes inherent in the infection process [95].

5.1.2. High variance in infection experiments

In the bacteria-infected fat body samples, a high variability between the replicates was observed. One reason for this could be the infection procedure itself, as the needle pricking protocol used here is more practical, but it can introduce a variable amount of microbes into the host. Infections via the capillary method are more consistent in terms of the volume of bacterial solution introduced. However, they are more time consuming in experimental setups with a higher sample size and the number of live bacteria introduced can still be variable. Nevertheless, a major reason the variability might come from the biology of the process and the inherent stochastic variability associated with it. This is particularly evident from the expression values of AMPs in the different biological replicates of bacteria infected and injured individuals' fat bodies. All replicates show a consistently high expression of the AMPs, with

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many more of them expressed upon infection than injury. This could indicate the variability comes from biological variability in the response on top of the direct antibacterial effector response itself. I also observed that while the expression of AMPs was higher in bacteria-infected fat bodies as compared to sterile injury, the values for several other genes were higher upon sterile injury instead. This could indicate a strategic resource allocation differing in the two conditions. For a bacterial infection, the AMPs are an essential immediate requirement to facilitate quick removal of the microbes and survival of the organism. While this results in a very high AMP production, the limitation in the total energy reserve results in less investment in the other functionalities. In sterile injury, the main focus of the tissue is the proper healing of the wound and providing the body with appropriate metabolic environment to deal with the stress. Thus, they can invest less in the AMP production and more in rest of the processes.

5.1.3. Immuno-metabolic transcriptomic response of fat body upon infections

Looking at how the fat body reacts to an infection and comparing this with the reaction to a sterile injury, the overlaps are, as already described, extremely large. On the one hand, there is the activation of the classical immune response, with a focus on the expression of antimicrobial peptide genes. The fat body shows significant modifications in the metabolic, cellular, and gene expression machinery. The most prominent process observed along with the innate immune response is translation, which is highly active in all steps, including the basic setup of ribosomal proteins and initiation and elongation factors. Protein production is an energy-consuming process, though such high levels of translation can allow the fat body cells to reprogram their proteome and provide them with the plasticity to change and adapt to cope with the stress in this metabolically active phase. This protein production machinery could further help to deliver AMPs into the hemolymph, a known fat body function. An increased protein export also supplements this role. Additionally, a substantial activation of proteasome and ubiquitin processes was obvious, which degrade misfolded proteins and act as quality control. This could serve to ensure the quality of the highly active translational machinery or to hydrolyze the unused or unrequired proteins and augment the proteome reprogramming. The enhanced activity is a characteristic of cells with high secretory capacities such as insulin-producing cells [131], [132]. Another possibility could be the removal of oxidized, and hence misfolded or non-functional, proteins produced as a side effect of a high ROS environment, a role proteasomes perform in macrophages [133]. I also saw an enriched cellular oxidative phosphorylation, which could help with the energy requirements of translation and proteasome

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in the context of the immune response. All these processes, along with some carbohydrate metabolic functions including glycogen metabolism, sugar transport, and fatty acid beta-oxidation, indicate a highly active transcriptional landscape, which would be expected of this immuno-metabolic tissue. Thus, this usage of energy stores in fat body cells for the sake of immunity follows a well-accepted hypothesis of resource allocation during infection [134]–[136].

5.1.4. Antibacterial and phagocytosis-centric infection response in hemocytes

In contrast to the study situation regarding the immune response of the adult fat body, corresponding studies for adult hemocytes are not available so far. The main reason for the lack of comparative organ-focused studies of the immune response in adult *Drosophila* so far is technical. Hemocytes of adults are difficult to isolate in sufficient quantity making their analysis demanding [29]. To circumvent this problem, appropriate amplification steps had to be included in the procedure [77, p. 3] allowing to perform reliable studies with less than hundred cells per biological replicate. Hemocytes react to an infection with bacteria and they orchestrate parts of the immune response, but they show no expansion in response to an infection [29], a finding that contrasts earlier studies that described an adult hematopoietic organ in *Drosophila* [28]. A number of relevant genes were observed among the list of the top 30 genes with the highest transcript levels in response to infection. Besides the expected antimicrobial peptides, the high expression of bombardier is of note, as it has recently been identified as a hemolymph protein that enables delivery of bomanins and therewith enabling their anti-fungal activity [137]. I could therewith show that hemocytes are an important source of this highly important hemolymph protein. Moreover, the high expression of the vacuolar proton transporter gene Vha36-1 has to be mentioned. It is involved in the acidification of lysosomes, which is an essential task for the inactivation of ingested microbes [138]. Transferin1 (Tsf1) was also among the genes with the highest expression further highlighting this arm of the immune system as an iron scavenger thus preventing microbial growth [139]. Again, here, hemocytes are an important source of this highly important hemolymph protein. Finally, unexpected genes whose products are important regulators of metabolism were also found with Gnm1 and SREBP as being the most important ones that had yet not been associated with hemocytes. Gnm1 is a central regulator of the S-adenosyl-methionine (SAM) metabolism that is induced upon infection and damage, but which has up to now mostly been associated with the fat body [140]. SREBP on the other hand is a central regulator of fat and cholesterol

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metabolism and might be operative in fuelling the energy-demanding immune response [141]. Focusing on the most strongly expressed genes in response to infection seems justified especially for the comparison with sterile injury. The simple comparison of the up-regulated genes revealed large similarities, suggesting that the responses are very similar. However, focusing only on the most highly expressed genes it became apparent that the top expressed genes are not activated to the same extent following sterile injury. This means that this quantitative component can be particularly important for the classification of the biological significance of an immune or stress response.

Activation of hemocytes by an immune stressor induced the upregulation of many immune effectors at high levels, including most AMPs. The number of AMPs expressed, and, in some cases, the expression level is higher than in the fat body. It could indicate that adult hemocytes also contribute to the release of AMPs into the hemolymph, as speculated for larval plasmatocytes. However, it would be intriguing if hemocytes perform this role in adults, despite their small numbers. This local release of the AMPs can also possibly facilitate a transmission of 'stress' or 'infection' information from the hemocytes to the surrounding tissues. It is crucial to point out that the expression of a few genes is enhanced that are involved in phagocytotic processes and interact with the Actin-related protein (Arp) 2/3 complex [142], a significant regulator of actin nucleation, polymerization, and lamellipodia formation in different cell types. The most relevant of these is the SCAR gene which forms a complex with WAVE to control actin rearrangement [143]. Other relevant and upregulated genes include Cip4 [144] and Cortactin [142], both activators of the Arp2/3 pathway, and Diaphanous [145] that interacts antagonistically with Cip4 to maintain actin rearrangement homeostasis. This suggests that Wasp/WAVE-Arp2/3 pathway plays a critical role in cellular morphogenetic changes in hemocytes on infection. I also found many other genes known for these morphogenetic changes in other cell types or developmental stages in different roles, which could be functionally conserved in an activated hemocyte for similar processes.

5.1.5. Commonalities between infection response of fat body and hemocytes

One of the very few processes that are activated in both cell types in addition to the central immune responses is the regulation of redox homeostasis. Reactive oxygen species have different tasks, but the strategy of protecting one's own cells from damage is important in many contexts. In hemocytes, the production of ROS is a mechanism that is closely associated with the antibacterial function of these cells. ROS primarily serve to damage or kill bacteria [146]. Interestingly, there is also a regulation of ROS in the fat body, which further questions what

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roles it would perform in organs not directly infiltrated by bacteria. Here, it is more plausible that the massive increase in the fat cells' metabolism leads to the production of substantial amounts of ROS that have to be dealt with. Consequently, it would be a simple reaction to cope with the high energy demands.

Taking together, the response of hemocytes substantially differs from the fat body's response. In hemocytes, besides the core immune response, mainly processes associated with phagocytosis are activated. In contrast to the fat body, the hemocyte could not use internal energy stores to fuel its activities. Thus, the enhanced lipid metabolic processes in hemocytes are highly interesting and require future research efforts. In the fat body, I found a completely different situation, where this immunocompetent organ has massive energy resources and uses this treasure to enable an effective immune response. Moreover, the significant responses in the fat body focus on enabling the production and release of antimicrobial peptides. For the overall immune response seen in the whole-body experiments, both organs mainly contribute to the AMPs, but most of the other transcriptomic signatures might come from different organs as a secondary response to the infection in combination with the response to the intense immune reaction.

5.2. Hemocytes show the ability to store and increase lipid droplets

Immune stress is known to induce many metabolic changes across the body of animals, with different strategies in different tissues depending on its function [96]–[98], [147]–[149]. Hemocytes are also assumed to undergo these changes, an excellent example being the switch to aerobic glycolysis in hemocytes upon bacterial infection [99]. However, the studies on the metabolic remodelling of hemocytes upon infection are sparse. One such change is lipid metabolism, as both larval and adult hemocytes are seen to regulate lipid homeostasis genes upon infection [147]. Lipid metabolism is not well studied in *Drosophila* hemocytes but has been shown to play essential roles in gut immunity [150] and is vital for primary energy production in the fat body during the infection [141]. A recent study also showed the importance of peroxisomal lipids in effectively fighting an infection in larval plasmatocytes [151].

Exploring this area, I not only successfully showed the presence of lipid droplets in hemocytes in all the stages of *Drosophila* development but also showed an increase in the LD size upon injury. To my knowledge, this is the first study to show the presence of lipid droplets in *Drosophila* hemocytes *in vivo*. Interestingly mammalian macrophages have been shown to

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accumulate lipid droplets in case of infection or mitochondrial [101], [152] or redox stress. Recently it was shown that this lipid build up is crucial for the functional activation of these cells [102] [153]–[155].

This is quite interesting as, on the one hand, it shows the further evolutionary conservation between vertebrate and invertebrate immunity and more similarities in the two systems. It also sparks questions on the functional relevance of the increased stored lipid in *Drosophila* hemocytes.

5.3. Potential roles of Tret1-1 in aiding an infection response in fat body

As the fat body is a professional immune tissue, an immune stress would involve an active and strong participation from it [17]. It involves numerous changes in the metabolic landscape of the tissue including shift in the proteome and oxidative phosphorylation. While all these changes are vital for the proper functioning of the tissue, they are also energetically demanding. Thus, a major metabolic requirement of the tissue would be an increase in the energy providing glucose molecules. Tret1-1 is established for its role in the transport of trehalose [103], [104], [156], [157] and due to structural similarities, it is also speculated to regulate this transfer for glucose [104]. I observed an increase in the abundance of Tret1-1 in this active fat body while the usual glucose transporters, the Gluts, were not changing [158]. Furthermore, preliminary data for an increased glucose uptake capability for the fat body fighting an infection or injury as well as a transfer of the glucose pool from the hemolymph was provided. The stored nutrient pool of glycogen was also decreasing under this stress in the fat bodies. All this preliminary data collectively points to an increased glucose abundance in the fat body when facing an immune stress, which is also shown in earlier studies [159]. This would be expected from a tissue which is metabolically so active and in need for more energy substrate. I also provided evidence for the possible role of Tret1-1 in facilitating this transfer of glucose into the fat body and further helping with the energy homeostasis. Further experiments with an increased sample size can evidence this role in a much more robust fashion. Additional experiments to compare the immune outcome of individuals genetically modified to remove this gene from the fat body will also be necessary to further this hypothesis.

5.4. Experimental evidence for different resource allocation strategies upon dietary, immune or a combination of both stressors in fat body and hemocytes

In this study, the major aim was to provide a comprehensive analysis of the impact of dietary protein content on different components of the immune response of *Drosophila melanogaster*. First, holistic immune parameters like survival to different pathogens and speed of clearing the bacteria were measured. Having found a clear advantage in the immune capabilities of animals reared on a nutrient-rich media against the bacteria *Bacillus subtilis*, I looked for possible molecular mechanisms behind it. While the whole-body transcript levels of important immune effectors could not explain these differences, the transcriptomic landscape of the two professional immune cell types, fat body, and hemocytes, did provide a hint towards a possible mechanism. In both cell types, a decline in the overall investment made for dealing with an infection as the dietary protein content decreased was observed. However, the investment in the direct antibacterial response differed between the two cell types. Fat bodies, on one hand, maintained the high expression of almost all antimicrobial peptides, even with no dietary protein intake. Hemocytes, on the other hand, maintained the expression of a few antimicrobial peptides but not as many as in presence of a nutrient-rich diet. This resource allocation differed a lot in response to a sterile injury, where the investments were not proportional to dietary nutrition. In both the cell types, the response to an injury was made to the same extent in a restricted or malnourished diet. Further, the amount of investment was very different between the tissues, where hemocytes still have a substantial reaction but the fat bodies show a minimal response.

5.4.1. Lowered dietary protein does not impact basal immune response in fat body and hemocytes

The fat body is an important metabolic and storage tissue and is well known for its role in coping with nutritional stress [19], [20]. Several studies have shown the specific molecular changes it shows after dietary restriction [160]–[163] and one study also showed the whole transcriptomic changes [164]. I provided here an additional analysis of all the changes in the transcriptomic landscape of the fat body upon protein restriction and further compared it to complete protein removal to clearly distinguish the effects of restriction from possible malnutrition. These changes mainly included metabolic rewiring of the cells and included both

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an upregulation as well as the complete shutdown of an almost equal number of processes. Moreover, while both treatments have their own signatures, half of the activated genes and most of the repressed genes are common between the treatments. This shows the presence of some go-to processes that dietary stress induces regardless of the intensity and potentially indicates a degree of hormesis in the response to these dietary stressors [165], [166]. Hormesis is defined as the phenomenon where an organism's response to a chemical or biological entity is proportional to the intensity of the entity. This phenomenon is also believed to prime an individual under dietary restriction without malnutrition to the nutrient shortage and result in higher lifespan [165]. Interestingly, no changes were induced to the basal immune system including the *Toll* and *IMD* pathway members. The fat body is an immuno-metabolic tissue with major involvements in both processes, but even in the total absence of a dietary protein intake, their metabolic changes do not come at a cost of compromise in the immune system.

Hemocytes, on the other hand, are mainly known for their immune functions. Like other study fields, most of the analysis to understand the impact of nutritional stress on hemocytes is done using larval systems. Nutritional stress in larval stages has been linked with lower hemocyte number in adult stages in *Aedes aegypti* [167] and a lower hemocyte proliferation in the larval stages of *Drosophila* [168] as well as *Periplaneta americana* [169]. Selected carbohydrates have also been shown to change the attachment of certain pathogens to hemocytes in *Galleria mellonella* [170]. However, the impact of nutrition on adult hemocytes is highly understudied. Though there are contradictory studies on the existence of hematopoiesis in adults [28], [29], its absence is more accepted in the scientific community. With a constant number of nearly 200 hemocytes in an adult [29], the information from nutritional studies from larvae, with thousands of cells and the possibility for proliferation [171] [22], [172], can hardly be extended to adults. I provide an analysis of the transcriptomic changes that follow a restriction as well as malnutrition in the adult hemocytes. Not surprisingly, only a few genes were differentially expressed in the hemocytes. Most of the characterized genes among these were metabolism or redox homeostasis related. This could point out that under nutritional stress, hemocytes also face some amount of strain due to their dependence on hemolymph to fulfil their energy demands [173]. A few cytoskeletal genes, essential for the cell shape change and movement were among the activated genes as well, including *eater*, *NijA*, *Ten-m*, and *Cortactin*. They could indicate a potential function of hemocytes in transmitting information about nutritional stress to other tissues or in nutrient transport roles known in other species [174], [175]. Similar to the fat body, no change in the direct antibacterial response even on complete protein removal

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was apparent. For adult hemocytes, this would be necessary, due to their small numbers and indispensable roles. Thus, I concluded that a dietary impairment does not come at a cost of compromising the basal immune response.

5.4.2. Wound healing response in hemocytes and fat body facing nutritional stress

I further demonstrated that this resource allocation towards the immune response on a nutrient-deprived diet change when the immune system is induced with the help of a stress. Additionally, the strategy for resource allocation was dependent upon the intensity of dietary stress, the intensity of immune stress, and the cell type under investigation. Milder immune stress like an injury without any bacterial infestation induces numerous transcriptional changes in the fat body in a nutrient-rich environment. However, on lowering the dietary nutrient intake a mere 10% of these changes are maintained, while a major part of the response is towards dealing with nutrient scarcity. These investments are also quite similar between diets with low and no protein. This shows that an intense response to cope with an injury is only made in a healthy environment with enough energy sources. The fat body is known to have essential roles in helping the wound healing process [12]. While it was believed just to supplement the humoral release of clotting factors [12], [176], recent studies also show the capacity for pupal fat body cells to migrate to the wound site [177]. The fat body would not only help in closing the wound, but a release of AMPs can also help in preventing an infection [177], [178]. This response includes several safety mechanisms from sealing the wound to making sure a resistance towards any microbe from entering the hemolymph. However, in presence of another more pressing stress like restriction or absence of protein in the diet, this tissue with both immune and metabolic functions shifts most of its attention towards the later roles. This indicates that a major part of the injury response in the fat body is dispensable and is focused on reducing the probability of an infection. This would be more useful in a pathogen-ridden natural environment compared to a sterile lab environment with negligible chances of an infection. Though the fat body picks to invest in a more immediate nutrient problem, it works well in the sterile lab environment as almost no animals die from the injury in any of the diets. The second immune cells, hemocytes, have major involvement in both healing a wound and resisting an infection, but less contribution in dealing with nutritional stress. Hemocytes not only directly contribute to the wound healing process by the prophenoloxidase cascade [179], [180], they also transmit the signal of an injury to the fat body cells [180], [181]. On low or no-

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protein diets, nearly 50% of the injury response from a control diet is still maintained. Though the injury response on a control diet is not as high in hemocytes as in fat bodies, a much higher percentage of the total is maintained upon lowering dietary nutrients in hemocytes. This is explained by the fact that hemocytes have primary roles in dealing with a wound, including moving up to the site of injury and facilitating the melanization process [12]. Thus, one can speculate that the genes that are still maintained in the low protein diets upon injury must have crucial roles that other cell types can't make up for and hence can't be given up.

A noteworthy observation for both hemocytes and fat bodies is the expression of AMPs upon injury. The main function of AMPs is to kill the pathogen, however, they are also known to be involved in other secondary functions including promoting the healing of a wound, maintaining redox homeostasis, or acting as a signal molecule [182]–[186]. Upon an injury where no bacteria are introduced, expression of these peptides in high abundance can be futile and energy-draining in absence of enough resources. However, it can add the benefit of reducing the chances of secondary infection from an open wound. It's widely seen that AMPs are produced in the insect body even upon just an injury and sometimes to high abundances [187]. I observed that this was true for both our cell types individually as well, as a sterile wound in a healthy diet led to the expression of a few AMPs in high amounts. This expression was, however, reduced in the low or no-protein diets both in terms of the number of AMPs as well as their expression levels. This further shows that the expression of a few AMPs is dispensable in case of nutrient deficiency.

5.4.3. Different antibacterial resource allocation strategies in fat body and hemocytes upon protein deprivation

When the immune stress on top of the dietary stress is more severe, like infection with a virulent bacterium, the resource allocation scenario changes in both cell types. In fat bodies and hemocytes, the amount of resources invested in the infection response was proportional to the amount of dietary protein. However, there was a set of 'core' genes in both the cell types, which included nearly 40 genes, different for both cell types, which were activated in all the diets. The rest of the response was dispensable and was invested in depending upon the available nutrient abundance. For the fat body, this core consisted mainly of direct antibacterial responses like AMPs and other immune effectors, which were induced to the same level, irrespective of dietary protein content. This is essential as fat body is considered the main contributor of these peptides to the hemolymph stream [12], [188]. Thus, a decline in effector production in the fat

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body would result in an overall decline in the immune response of the animal. The main differences between the diets were contributed by the investment made in the indirect infection response including the general maintenance, proper functioning and metabolic processes to cope with this stress. The right metabolic rewiring of the cell is absolutely essential for shifting the functions towards the required goal, infection resistance in this case [130], [135], [141], [189]. Thus, even in the presence of all the immune effectors, an improper metabolic environment can have severe impact. It is noteworthy that several of the metabolic changes made to cope with the lower dietary protein levels are also present in these treatments with a combination of stress. Thus, along with the infection response-related metabolic processes, they also repress various processes as part of a dietary stress response. Thus these animals give up processes, but maintain a very high level of almost all AMPs. These peptides constitute a handful of genes but can be increased to thousands of folds [16], [21], indicating a high-energy requirement. As the fat body serves as a storage tissue for lipids and glycogen [135], it has autonomous nutrient availability and hence the opportunity to make energy investment even in the absence of a dietary nutrient source. Thus, even in the complete absence of dietary protein intake, this tissue is able to produce these peptides, which would be an important resource allocation to be made in this alarming situation for the survival of the animal.

Even though the net infection response is also proportional to the dietary protein content in the hemocytes, the functional portfolio for the ‘core’ looks different in these cells. Unlike the fat body, AMP production is not maintained to the same extent on nutrient deficiency. Instead, lowering the dietary protein abundance results in expression of only a set of central AMPs to high levels and no expression of the rest. Although AMPs are vital for the proper functioning of hemocytes, including the local or internal release of these peptides for bactericidal purposes [12], [22], the metabolic state of these cells is quite different. Hemocytes are not known to store nutrients in any form implying a certain level of dependency on the hemolymph for energy source. In a lower nutrient environment, they make an investment decision to invest in certain AMPs but not all. This might also point towards the possibility for specificity of these AMPs towards *Bacillus subtilis*, which has been hinted towards in some studies [190], [191]. Apart from AMPs, the additionally expressed genes for functional categories including phagocytosis, cell adhesion, movement, and some metabolic processes, especially lipid metabolism showed the same pattern, where some genes were expressed to similar extent as control while others were not activated at all.

5.4.4. Possible reasons for a lower pathogen clearance despite high AMP production in low/no-protein diets

Putting the information from the holistic and tissue-specific datasets for the immune system together gave a comprehensive overview of the impact of dietary protein content. Both at the level of the whole body and the fat body, the abundance of AMPs is independent of the dietary protein content. These peptides are responsible for the successful removal of the invading pathogen [187], however, even then the rate of bacteria removal is slower in diets with lower or no protein. Even when considering the bacterial clearance 24 hours after infection, which is also the time point when the tissue transcriptomes were samples, I observed an equal AMP production in the fat body but more bacterial load in the nutrient-deprived diets. One reason for this could be the smaller number of AMPs expressed in hemocytes in these diets. Though the exact mechanism of action of hemocytes against *Bacillus subtilis* is not known, a general antibacterial response for these cells involves a local release of AMPs or phagocytosis of the pathogen followed by the removal with the help of AMP produced inside the cell [22]. An impact on this AMP production would result in a lower functioning of the cells against a pathogen and further result in slower removal of the pathogen. It could also be a reason for the eventual death of a much bigger population on nutrient-deprived diets. Another possible reason for the difference in the immune outcome could be the exhaustion of energy in the low-protein diets. As most of the immune parameters were measured within one day of the infection, it is a possibility that the situation changes at a later time point, when the stored nutrients from the fat body run out and the animal can't produce the same amount of immune effectors. The fat body is shown to undergo apoptosis under energy depletion due to thermal stress [192]. Energy exhaustion in our study could also result in energy shortage for the normal functioning of the cells and eventual death. The fat body also shut down a lot of processes to cope with the lowered dietary protein, which would exacerbate the overall poor functioning of the cells. A major example of this is the redox homeostasis in the fat body, which suffered in both DR and PM in terms of several processes including the glutathione metabolism, CytochromeP-450 metabolism, hydrogen peroxide metabolism, and direct redox homeostasis genes. Various studies showed that the damage or a disrupted redox homeostasis impairs different cell types [193]–[196]. A shutdown of these processes, followed by an increase in the ROS, as a by-product of the infection response and all the related processes to cope with it, would result in a gradual increase in the harmful ROS species impacting the health of the individual.

5.4.5. Infection investments from the perspective of resistance and tolerance

A fascinating observation to make here is the impact on two major aspects of the immune response i.e., resistance and tolerance upon lowering the dietary protein. In broad terms, resistance is defined as the mechanisms employed to directly reduce the pathogen abundance within the host while tolerance is defined as the processes aimed at maintaining the fitness of the host regardless of the pathogen abundance [197]–[199]. In the insect immune system, AMPs can be considered the biggest contributor to resistance while the metabolic processes can be classified as tolerance. I observed here that investments towards resistance are maintained despite the dietary protein content at the levels of fat body and hemocytes. This comes at a cost of less investment in tolerance and resulting lower chances of survival of the animal. This shows us that while resistance, which is usually considered as a proxy for the whole immune system, is a major part of the immune response, it can't represent the whole system. A similar observation was made in an earlier study, where higher dietary protein resulted in lower survival of burying beetles against a pathogen, however, it was because of the impact of diet on tolerance mechanisms rather than resistance [200].

5.4.6. Technical aspects of the study: dietary regime and immune parameters

On infecting the flies with different bacteria, I observed differences in the relative immune outcomes of the animals reared on various diets. These discrepancies are a common occurrence in the field of nutritional immunology. A well explored example of this is the impact of Dietary Restriction on the immune response. DR is one of the most studied dietary interventions and is believed to have several health benefits like anti-tumour effects [201] and reduced susceptibility to infections [202]. However, DR is also reported to have detrimental health effects, depending on the nutrients targeted or the immune parameter measured. For example, several studies reported better immune functions in DR-fed mice when infected with non-intact pathogens [50]. However, on infections with intact pathogens, fully-fed mice showed higher chances of surviving [51]. Additionally, the same experiment in an *in-vitro* setting revealed better immune functions in DR mice [52]. These disparities are also seen in different species across the animal kingdom. For instance, locusts survive a fungal infection better on a restricted diet [55], while caterpillars show positive effects on immune performance to bacterial and viral infections on a protein-rich diet [57]. These discrepancies are explained not only by the diet but furthermore by the immune trait under consideration. Immunity is also a multi-dimensional

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trait with several components, which can interact differently with different nutritional elements. The most extensive study that accounted for these complexities has been an investigation in caterpillars where several immune parameters were measured on 20 diets varying in their protein-to-carbohydrate ratios and quantities [62]. Interestingly, the authors observed that various traits behave differently to different ratios, and no single dietary composition could maximize all immune traits. On a given dietary composition, the immune challenge depicts which immune parameters are optimized, representing a trade-off between different attributes. I believe that our study furthers this hypothesis, where we observed different resource allocation strategies on different types and intensity of stressors, maximising for different traits.

I would like to discuss the specific controlled dietary regime that I used in our study. In nutritional research, one reason for the low reproducibility of the experiments is the different dietary treatments used for experiments. The dietary regimes used in different studies, even for the same model organism, vary a lot. In addition, when manipulating the macronutrients, a food source majorly containing a macronutrient is changed (like yeast in fruit fly studies) [105]. This ignores the fact that other macro or micronutrients present, even if in small amounts, were also manipulated and affected the results. To overcome this issue, I controlled for all relevant factors in the experimental setup. I used the highly standardized exome-matched holidic diet, that is, a diet completely comprised of chemicals [68]. This also makes it easier to manipulate the dietary components in a very controlled fashion and eliminates the possibility of hidden micro or macronutrient changes. As this diet doesn't support the proper development of the flies, I switched the animals to this diet in the non-feeding pupal stages to avoid any big dietary changes during adulthood. Further, the diet was restricted in terms of the protein content of the food. Growing evidence suggests that the effect of dietary restriction is because of a relative abundance of different macronutrients in the food [53], [203], with the protein-to-carbohydrate ratio being the most important one [45], [46], [54]. I also added a dietary treatment with no protein to differentiate the effects of dietary restriction from malnutrition. Thus, I tried to implement as controlled dietary regime as possible to make the study reproducible.

5.4.7. Outlook

I performed an extensive multi-factorial analysis for the impact of dietary protein content on the immune response and observed several resource allocation strategies. The study also opened up a possibility for many factors that could be informative to explore. In our experimental setup, it would be interesting to study the tissues at a later time point in the infection phase of the bacteria, i.e., when only a part of the population is surviving. This could

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provide information on whether there is an energy exhaustion in the low protein diets at a later time points. It would be interesting to also know if the AMP production is still the same between the diets at a later time point. As I analysed the immune response towards *Bacillus subtilis*, it would be interesting to analyse and compare these changes with another pathogen, with different mode of action, virulence, mechanism of action and antibacterial response. This could provide us with more information on the resource allocation strategies depending on these factors. An analysis of the tissues not directly responsible for the immune response especially the gut would also be interesting, as it would be expected that the priorities, functions and hence the resource allocation strategies would be different for it.

5.5. Potential role of *Ptth* in dealing with nutritional stress:

Prothoracicotropic hormone (*Ptth*) is an ecdysone-regulating hormone, which is mainly responsible for the larval to pupal transitioning in *Drosophila melanogaster* [126], [204]–[207]. Though almost exclusively studied for its role during developmental processes, there are no studies discussing its role in adults. I found an increase in its abundance in the fat bodies when the animals are fed a low or no-protein diet and I successfully confirmed this at the transcript level via two independent methods of RNA sequencing as well as qRT-PCR. Further experiments to test its role could show us novel roles for *Ptth* in dealing with nutritional stress. In mammalian systems the role of hormone Fibroblast Growth Factor 21 (FGF21) are well studied [208, p. 21], [209, p. 21], [210]–[212]. It is a hormone released in the liver and pancreas, responsible for an adaptation to metabolic stress. It mediates an adaptive response to starvation including lipolysis, gluconeogenesis, ketogenesis and more [211]. In the *Drosophila* system, branchless is considered the equivalent of all Fgfs from the mammalian systems [213], however, it is not shown to have functions related to starvation resistance. I speculate that *Ptth* could be the functional equivalent of Fgf21 in *Drosophila*, especially related to roles in adaptation to metabolic stress.

6. Declaration

I declare that the dissertation titled “Impact of dietary protein content on the immune response of *Drosophila melanogaster*” apart from my supervisor Prof. Dr. Thomas Roeder’s guidance and the listed references, is my own literary property and represents my own work. This dissertation has not been submitted elsewhere in order to obtain an academic degree and was prepared according to the Rules of Good Scientific Practice of the German research Foundation. I hereby assure that I have not been revoked any of my academic degrees.

Ich erkläre, dass die Dissertation mit dem Titel "Impact of dietary protein content on the immune response of *Drosophila melanogaster*" abgesehen von der Betreuung durch meinen Doktorvater Prof. Dr. Thomas Roeder und den angegebenen Referenzen mein eigenes literarisches Eigentum ist und meine eigene Arbeit darstellt. Diese Dissertation wurde nicht zur Erlangung eines akademischen Grades anderweitig eingereicht und nach den Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft erstellt. Ich versichere hiermit, dass mir keiner meiner akademischen Grade entzogen wurde.

Kiel, November 2022



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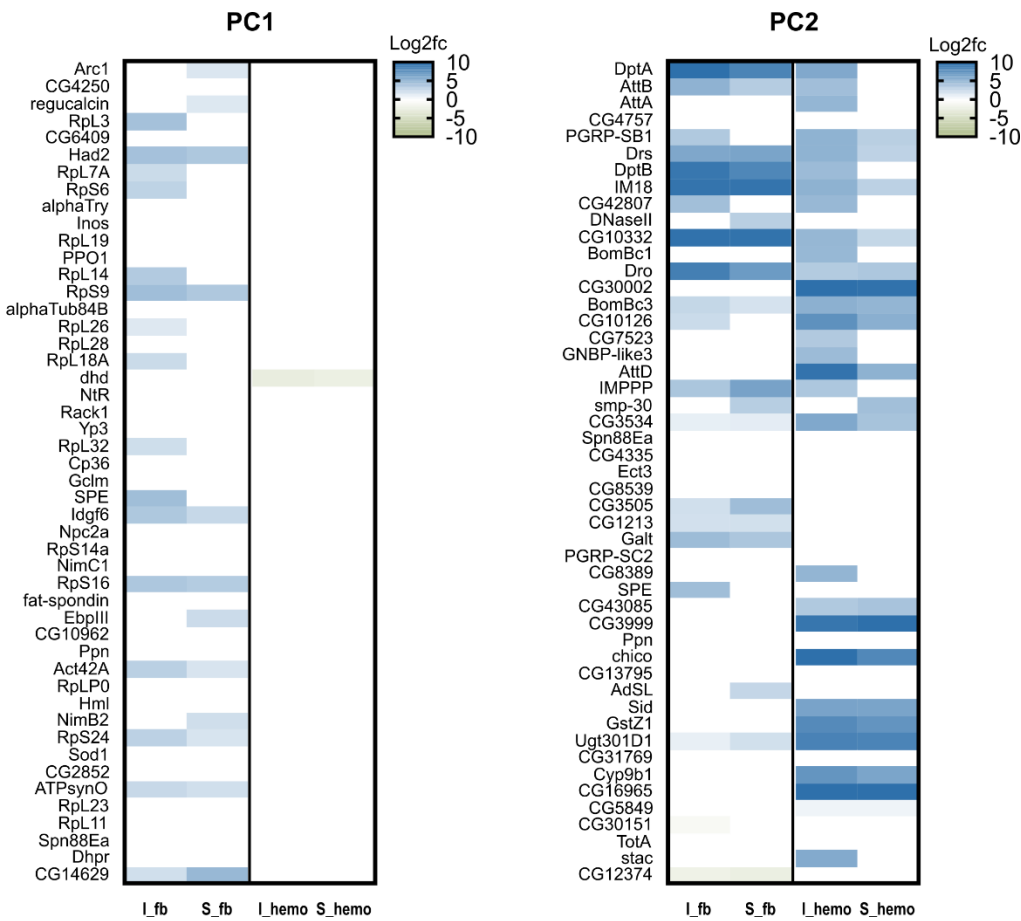
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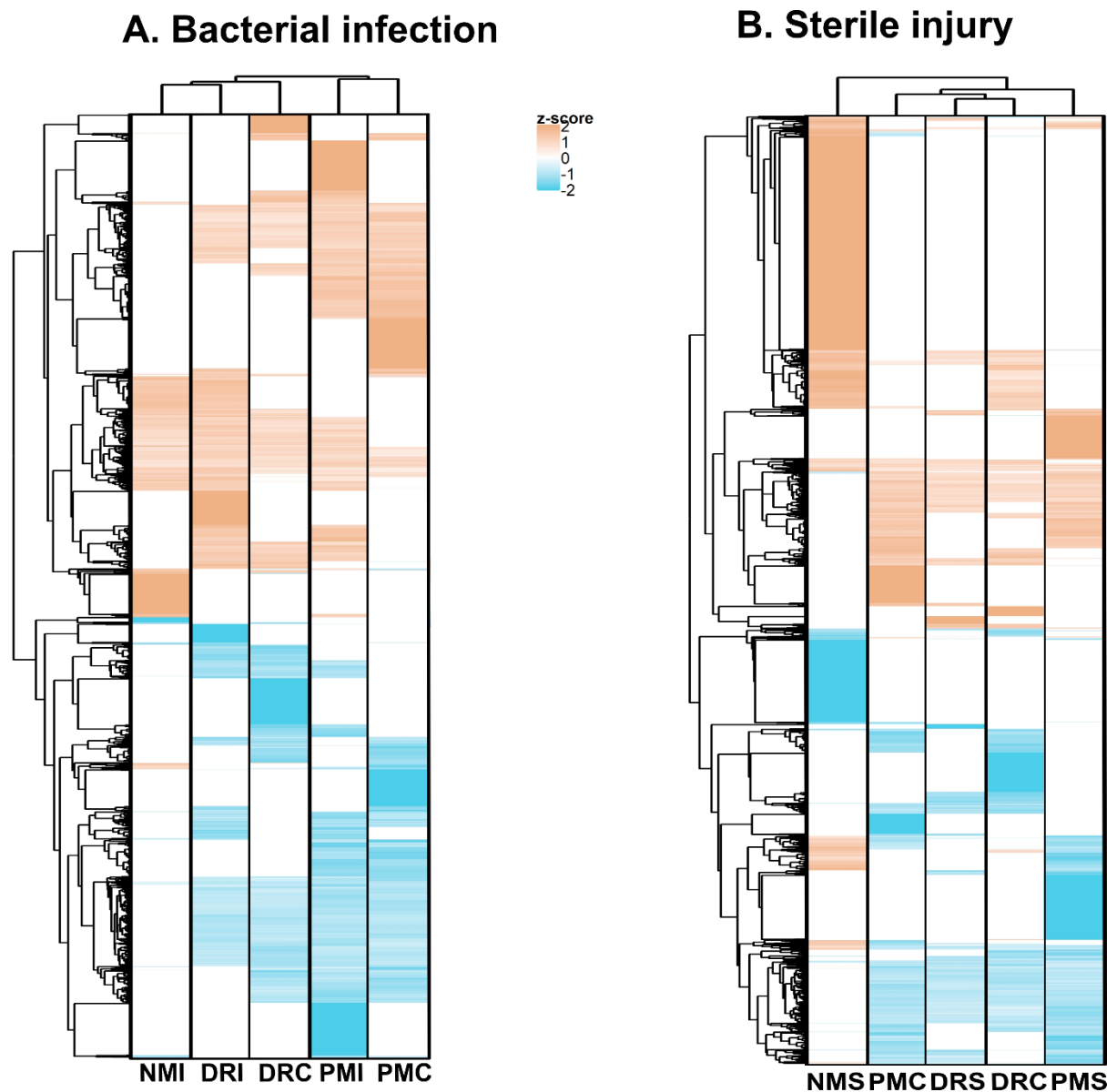
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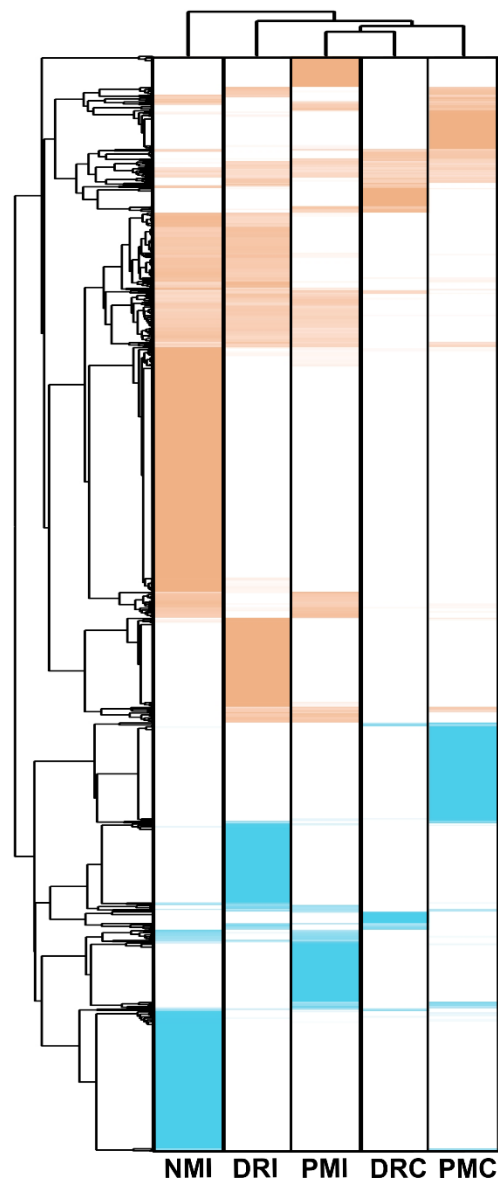
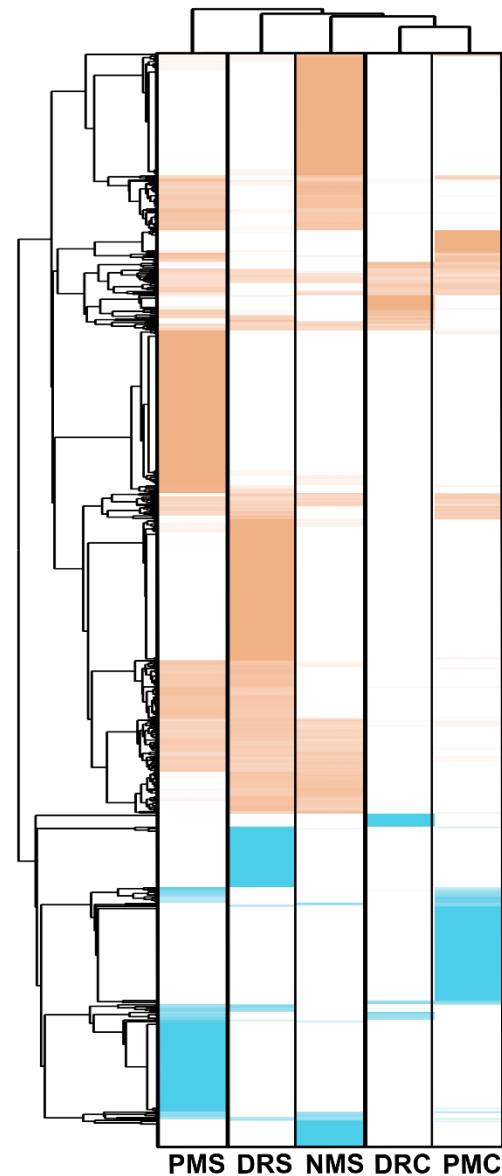
10. Supplementary material



Supplementary figure 1: The top 50 genes contributing to PC1 and PC2 for the PCA plot shown in Fig. 13A. I observed that PC1 explained more the differences between the two cell types and the top 50 genes contributing to PC1 are mainly the ones only expressed in fat body. PC2 explained more the differences between the different treatments and here the top 50 genes contain the ones expressed in both cell types with AMPs being a part of this list.



Supplementary figure 2: Explorative analysis of the infection and injury response of fat body (A) Hierarchical clustering of the expression values of all genes differentially expressed in NMI, DRI, DRC, PMC and PMI. The scaled values are plotted for expression levels, i.e, the log2 fold change of a gene in the treatment relative to NMC. The treatments are also clustered based on the Euclidian distance between them, which is shown on top of the heatmap as a tree (B) Hierarchical clustering of the expression values of all genes differentially expressed in NMS, DRS, DRC, PMC and PMS. The scaled values are plotted for expression levels, i.e, the log2 fold change of a gene in the treatment relative to NMC. The treatments are also clustered based on the Euclidian distance between them, which is shown on top of the heatmap as a tree (NM:Normal Media, DR:Dietary Restriction, PM:Protein Malnutrition, C:Control, S:Sterile injury, I:Bacterial Infection)

A. Bacterial infection**B. Sterile injury**

Supplementary figure 3: Explorative analysis of the infection and injury response of hemocytes (A) Hierarchical clustering of the expression values of all genes differentially expressed in NMI, DRI, DRC, PMC and PMI. The scaled values are plotted for expression levels, i.e, the log₂ fold change of a gene in the treatment relative to NMC. The treatments are also clustered based on the Euclidian distance between them, which is shown on top of the heatmap as a tree (B) Hierarchical clustering of the expression values of all genes differentially expressed in NMS, DRS, DRC, PMC and PMS. The scaled values are plotted for expression levels, i.e, the log₂ fold change of a gene in the treatment relative to NMC. The treatments are also clustered based on the Euclidian distance between them, which is shown on top of the heatmap as a tree (NM:Normal Media, DR:Dietary Restriction, PM:Protein Malnutrition, C:Control, S:Sterile injury, I:Bacterial Infection)

Supplementary tables: Differentially expressed genes in different treatments relative to NMC

The list of DEGs with their log2 fold change and adjusted p-values for the following treatments are in the attached CD:

File 1: DEGs fb.xls – DEGs in fat body

Subsheets: NMI, NMS, DRC, DRI, DRS, PMC, PMI, PMS

File 2: DEGs hemo.xls – DEGs in hemocytes

Subsheets: NMI, NMS, DRC, DRI, DRS, PMC, PMI, PMS