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Health of the black soldier fly and house fly under mass-rearing conditions: innate immunity and the role of the microbiome

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REVIEW ARTICLE

Abstract

Rearing insects for food and feed is a rapidly growing industry, because it provides excellent opportunities for a sustainable approach to animal protein production. Two fly species, the black soldier fly (BSF) and the house fly (HF), naturally live in decaying organic matter (e.g. compost), and can thus be effectively reared on organic rest streams from the food and agricultural industry. The adoption of these insects as mini-livestock on microbially rich substrates, however, requires us to address how we can safeguard insect health under mass-rearing conditions. In this review, we discuss what is known about the innate immunity of insects in general, especially focusing on a comparative approach to current knowledge for the two dipteran species BSF and HF. We also discuss environmental factors that may affect innate immunity in mass-rearing settings, including temperature, insect densities and diet composition. Furthermore, we address the role of the microbiome in insect health and the associations of these fly species with detrimental or beneficial microbes. Finally, we present a perspective on important open scientific questions for optimizing the mass rearing of these insects with respect to their health and welfare.

Keywords: innate immunity, diet, density, temperature, microbiome

1. Introduction

Livestock rearing and domestication of animals, including insects, by humans started thousands of years ago. Beekeeping and sericulture are the two most characteristic examples of insect livestock – also called mini-livestock (Van Huis, 2013). Insects can be reared for services such as pollination or biological control, or for the products they deliver, such as silk, honey, or lac (Dicke, 2017). The practice of rearing insects for food and feed is not new, but has recently become more than an 'exotic activity' (from a western societal point of view). Producing insects as novel protein source to satisfy the amino acid requirements of humans and, especially, of livestock is a fast growing sector (Van Huis, 2019). It is forecasted to reach a global value of nearly USD 8 billion and a volume exceeding 730,000 tons in 2030 (Anonymous, 2019). A main advantage of insects as feed is that insects can be reared on substrates that are different from the resources that traditional livestock animals and humans consume, such as fish or soya; some species can even be reared on organic waste streams (Dicke, 2018; Hussein *et al.*, 2017; Sheppard *et al.*, 2002). The most common insects reared for feed are the mealworms *Tenebrio molitor* and *Alphitobius diaperinus* (both Coleoptera), the black soldier fly (BSF) *Hermetia illucens* and the common house fly (HF) *Musca domestica* (both Diptera). In particular, the two fly species receive increasing attention due to their capacity to develop on organic residual streams that are unsuitable as feed for livestock animals (Gold *et al.*, 2018).

The health of any livestock, i.e. being free of injury and diseases, is of paramount importance for production, as profit relies on it. Mass rearing of insects can lead to disease outbreaks in the production system (Maciel-Vergara *et al.*, 2021). Many insect pathogens are known, and the

diseases they cause have the potential to decimate insect populations (Maciel-Vergara *et al.*, 2021). Research on disease and immune responses has its roots in studies on insects (Box 1). Insects possess potent immune systems to survive in environments with high microbial loads. The cuticle is a first and very effective physical barrier against microorganisms. Behavioural adaptations, such as behavioural fever or grooming, are another way of fighting infection (Anderson *et al.*, 2013b; Zhukovskaya *et al.*, 2013). Innate immunity is the set of defences against pathogens initiated by tissues and cells such as the haemocytes (i.e. insect blood cells), the fat body and epithelia, meant to eliminate or control invading pathogens and parasites. Unlike vertebrates, insects do not have adaptive immunity in the sense of memory and specificity, resulting from somatic recombination of immunoglobulins. However, they do have so-called acquired immunity or immune priming, i.e. long lasting effects of immune activation that can sometimes offer protection against subsequent infections (Cooper and Eleftherianos, 2017). Finally, insects have numerous and diverse relationships with beneficial microorganisms that can also fortify their health and immunity (Engel and Moran, 2013).

Whereas the core pathways for innate immunity are highly conserved across insects, several components of these pathways evolved and diversified extensively (Lazzaro, 2008; Scott *et al.*, 2014; Stokes *et al.*, 2015; Viljakainen, 2015; Waterhouse *et al.*, 2007; Zhan *et al.*, 2020). Especially proteins that interact directly with the pathogens show considerable evolutionary dynamics in both numbers and diversity. For example, receptor proteins that are important in coagulation and recognition of non-self are more numerous in the HF compared to *Drosophila melanogaster* (Kurucz *et al.*, 2007), whereas the antifungal peptide drosomycin is not present in the HF (Scott *et al.*, 2014) and cecropins – one of the largest families of antibacterial peptides in Coleoptera, Diptera and Lepidoptera – are not found in Hymenoptera (Mylonakis *et al.*, 2016). Hence, the knowledge on innate immunity for a number of well-studied insect species is likely to be informative for other insects as well, while other aspects may be specific for the focal species that we are interested in.

Box 1. Brief history of insect immunity research.

The first time that a microorganism was identified as the causative agent of an animal disease was by Agostino Bassi, with the discovery that the entomopathogenic fungus *Beauveria bassiana* caused the white muscardine disease in silkworms (Steinhaus, 1956). This contributed significantly to the germ theory by Robert Koch (Steinhaus, 1956). Research on innate immunity in insects in its current form has largely been shaped in Boman's lab, using both the genetic model organisms *Drosophila melanogaster* and the saturniid moth *Hyalophora cecropia* (Boman *et al.*, 1972, 1974; Faye and Lindberg, 2016; Faye *et al.*, 1975). Later hallmark studies in Hoffmann's lab on the innate immunity of *D. melanogaster* included the discovery of Toll receptors as activators of innate immunity through the nuclear factor kappa-beta pathway (NF-kB) (Lemaitre *et al.*, 1996, 1997), for which Jules Hoffman received the Nobel Prize in Physiology or Medicine in 2011.

Apart from the intrinsic immunological differences among insect species, extrinsic factors can also affect health and immunity of insects. Although our knowledge on the role of extrinsic factors comes mostly from fundamental research, this may provide us with insights that can be implemented to optimise the mass rearing of insects. For example, temperature and relative humidity can alter pathogen growth and replication, while these factors may also enhance or impede insect immune responses. Unravelling the consequences of temperature variation on insect health and disease may thus aid in optimising rearing conditions. Similarly, understanding how diet composition or nutrient availability impact immune defences may provide a means to boost the insects' abilities to effectively defend themselves against various pathogens. Although one has to be cautious to generalise the findings from highly controlled laboratory experiments to the practicalities of mass production, providing an overview on the *status quo* of this substantial body of research on factors relevant for insect health may contribute to the optimization of mass-rearing conditions.

This review focuses on two insect species that are rapidly developing as mini-livestock, the BSF and the HF, and the factors and conditions that hamper or benefit their health. First, we will discuss the innate immunity of insects in general, with a special focus on the comparison among dipteran species, and examples of acquired immunity. Then, we will discuss environmental factors that, in mass-rearing settings, may affect innate immunity, such as temperature, insect density, food quality and/or availability. Finally, we will address the role of the microbiome in insect health. We discuss potential microorganisms and pathogens and their likelihood of becoming detrimental or beneficial for these two fly species. For this review, we will partly rely on literature from other dipteran species that have been more extensively studied, particularly *D. melanogaster,* as well as *Anopheles* and *Aedes* mosquitoes.

2. Insect innate immune system

Multiple reviews have been written on the immunity of *D. melanogaster* and the immunity of insects in general (Hoffmann, 1995; Kounatidis and Ligoxygakis, 2012; Lemaitre

and Hoffmann, 2007; Stokes *et al.*, 2015). Therefore, this review will not focus on detailed descriptions of the different components and the molecular mechanisms of insect immune systems. Instead, we will focus on a comparison of the immune systems of dipteran insects, in particular BSF and HF. It is worth mentioning that HF is phylogenetically closer to *D. melanogaster* than to BSF, whereas BSF is more closely related to mosquitoes (Wiegmann *et al.*, 2011).

Innate immunity can be divided into inducible and constitutive responses. Constitutive responses are always present and therefore fast acting, but they can incur a high cost (Chambers and Schneider, 2012; Johnston *et al.*, 2014; Poulsen *et al.*, 2002). These defences are only likely when they yield a significant fitness benefit, e.g. against target pathogens that the insect encounters regularly (Schmid-Hempel, 2005). In contrast, inducible defences start after the host recognises non-self, by various pattern recognition receptors or 'danger signals', such as the abundance of proteases in the host haemolymph (Gottar *et al.*, 2006; Krautz *et al.*, 2014). This triggers the production of effector molecules such as antimicrobial peptides (AMPs) or the proliferation, differentiation or activation of haemocytes (Kounatidis and Ligoxygakis, 2012; Lemaitre and Hoffmann, 2007). These effects can last long beyond the time of induction (Johnston *et al.*, 2014).

The large array of immune responses that insects have at their disposal to defend themselves against pathogens and parasites are regulated by different signal transduction pathways (Box 2, Figure 1). We will discuss the different immune defences, grouped by the tissues involved, i.e. the fat body, the haemocytes and epithelial cells. We finish this section with a brief discussion on acquired immunity.

Immune responses of the fat body

The fat body is where most AMPs are produced that are released into the haemolymph (Tzou *et al.*, 2000). There are several types of AMPs that have antimicrobial effects on

Box 2. Signal transduction pathways for innate immunity.

The best studied immunity signal-transduction pathways are the Toll and immunodeficiency (Imd) pathways that have strong homologies with the mammalian Toll-like receptor and tumour necrosis factor pathways – all belonging to the NF-kB pathways. The Toll and Imd pathways regulate the expression of various antimicrobial peptides (AMPs) that insects produce in the fat body (i.e. the homologue to the liver in vertebrates) in response to bacterial and fungal infections (Lemaitre and Hoffmann, 2007).

The immune involvement of the Janus kinase/signal transducers and activators of transcription pathway (JAK/STAT) was first described in mosquitoes (Barillas-Mury *et al.*, 1999). It is involved in immune defences against viruses (Dostert *et al.*, 2005; Kingsolver *et al.*, 2013), microbes and parasites (Agaisse and Perrimon, 2004; Bang, 2019; Theopold and Schmid, 2017) and regulates the production, proliferation and differentiation of haemocytes (Agaisse and Perrimon, 2004; Bang, 2019).

The prophenoloxidase-pathway (ProPO) is involved in the immune defence against Gram-positive and Gram-negative bacteria, fungi and parasitoids (González-Santoyo and Córdoba-Aguilar, 2012; Hillyer, 2016). This pathway consists of an enzymatic cascade of proteins, which produce the immune effectors through cleavage of a zymogen into its active form (Hillyer, 2016).

The most important antiviral defence is regulated by the small interfering RNA pathway (siRNA). Double stranded RNA is recognised as viral by this pathway, sequestered by RISC complexes and finally degraded (Kingsolver *et al.*, 2013; Mussabekova *et al.*, 2017).

The c-Jun-N-terminal kinase pathway (JNK) has various functions, including responses to stress, and has been suggested to play a role against infection by nematodes (Tafesh-Edwards and Eleftherianos, 2020). It is involved in the antibacterial immune response through AMP production (Kallio *et al.*, 2005) and through the shedding of intestinal epithelial cells, by which infected cells are expelled and replaced by new healthy epithelial cells (Zhai *et al.*, 2018).

In the intestinal epithelium of insects, the dual oxidase pathway (DUOX) regulates the generation of microbicidal reactive oxygen species (ROS) upon ingestion of pathogens. The generation of ROS in the gut is an important immune response, helping to protect against proliferation of the pathogens (Bae *et al.*, 2010; Ha *et al.*, 2005, 2009).

The target of rapamycin pathway (TOR) is involved in nutrient sensing, and coordinates growth, metabolism, development and lifespan (Katewa and Kapahi, 2011). It also modulates AMP expression in the fat body and the gut, in conjunction with the insulin signalling pathway and forkhead transcription factors, in response to nutritional and energy status of the organism (Lee and Lee, 2018; Varma *et al.*, 2014).

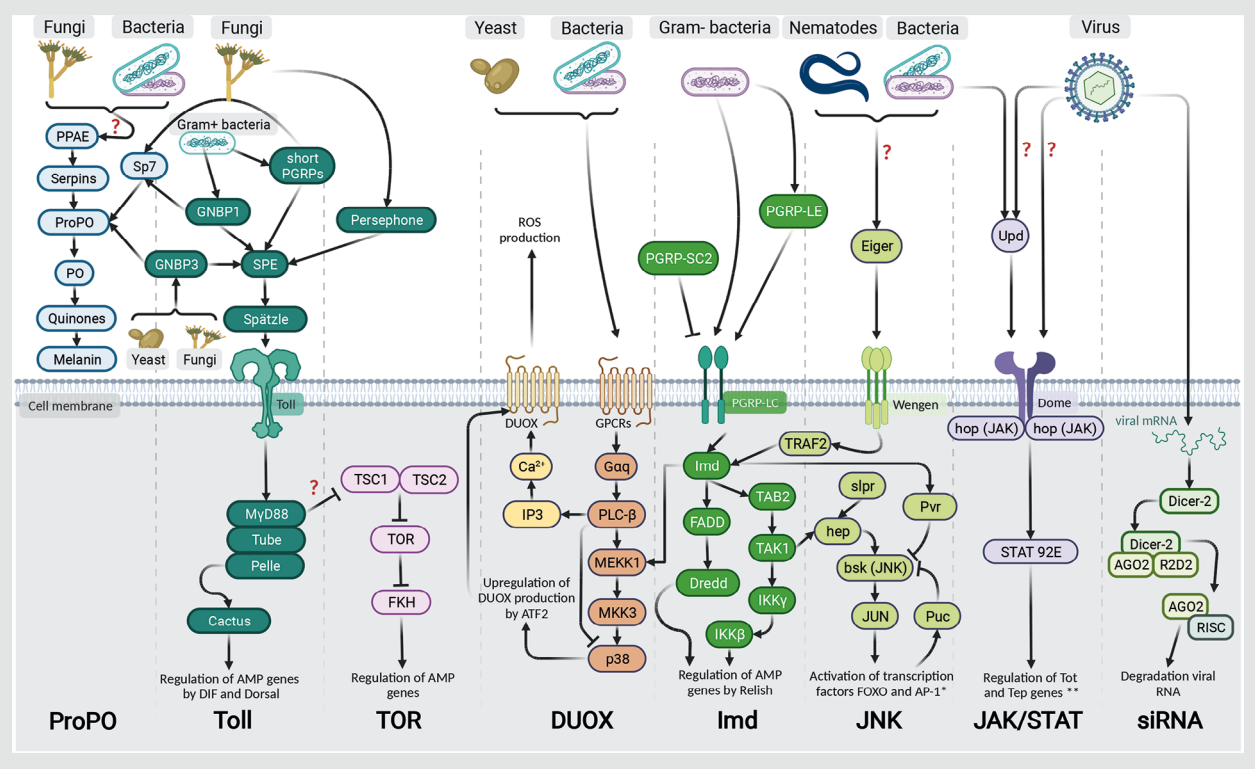

Figure 1. Schematic representation of the most important and best-studied immune pathways in insects. Not all known interactions between the pathways are included in the figure (such as the interactions between the Toll and Imd pathways (Tanji *et al.*, 2007)), to avoid cluttering of the figure. AGO2 = argonaute-2; AP-1 = Activator Protein 1; ATF2 = activating transcription factor 2; bsk = basket (JNK gene of *Drosophila melanogaster*); DIF = dorsal-related Immunity Factor; Dome = domeless; Dredd = death related ced-3/Nedd-2 like caspase; DUOX = dual oxidase; FADD = fas-associated death domain; FKH = forkhead; FOXO = forkheadbox O transcription factor; GNBP (1&3) = Gram-negative binding proteins; GPCRs = G-protein-coupled receptors; Gαq = G protein α q subunit; hep = hemipterous; hop = Hopscotch; IKK = IκB kinase; Imd = immunodeficiency; IP3 = inositol 1,4,5-triphosphate; JAK = Janus kinase; JNK = c-Jun-N-terminal kinase; MEKK1 = mitogen-activated protein kinase kinase kinase 1; MKK3 = mitogen-activated protein kinase kinase 3; MyD88 = Myeloid differentiation primary response 88; p38 = p38 mitogen-activated protein kinase; PGRPs (short and long) = peptidoglycan recognition proteins; PLC-β = phospholipase C-β; PO = phenoloxidase; PPAE = prophenoloxidase-activating enzyme; ProPO = prophenoloxidase; Puc = puckered; Pvr = platelet-derived growth factor- and vascular endothelial growth factor-receptor; RISC = RNA-induced silencing complex; ROS = Reactive Oxygen species; slpr = slipper; Sp7 = serine protease 7; SPE = spätzle processing enzyme; STAT 92E = signal-transducer and activator of transcription at 92E; TAB2 = TAK1-associated binding protein 2; TAK1 = transforming growth factor β-activating-kinase 1; Tep = thioesther-containing protein; TOR = target of rapamycin; Tot = Turandot stress genes; TRAF2 = tumour necrosis factor receptor-associated factor 2; TSC = tuberous sclerosis complex; Upd = unpaired.; *regulates: AMP expression, shedding of the intestinal epithelial cells to prevent colonisation; stress responses, e.g. mitigation oxidative stress, wound healing and apoptosis; **regulates: production, proliferation and differentiation of haemocytes; expression of AttC during viral infection; opsoinisation of microbes and parasites. Gram-negative bacterial cells in purple; gram-positive bacterial cells in light blue. Figure made in Biorender.

different types of pathogens, under the regulatory control of the Toll, immunodeficiency (Imd), Janus kinase/signal transducers and activators of transcription (JAK/STAT) and c-Jun-N-terminal kinase (JNK) pathways (Table 1). The literature for HF and BSF on the involvement of the JAK/STAT and JNK immune pathways in AMP production is very limited, only mentioning them as part of immunity (Gill *et al.*, 2017; Kariithi *et al.*, 2017) or in comparisons of immune genes across several insect species (Tang *et al.*, 2014; Zhan *et al.*, 2020).

Among the AMPs for the five dipteran species that we here compared (i.e. *Anopheles* and *Aedes* mosquitoes, *D. melanogaster*, BSF and HF), only four groups of AMPs were shared among all: attacins, defensins and cecropins, which have been reported in many insect species (Lemaitre and Hoffmann, 2007; Wu *et al.*, 2018), as well as diptericins that seem restricted to the Diptera. There are also several AMPs that are species-specific, including AMP17, crustin, eppin, MAF-1 (*Musca domestica* antifungal peptide-1), MDAP-2 (*Musca domestica* antimicrobial peptide-2), muscin, muslin and SVWC (secreted AMP, containing a single domain von Willebrand factor type C) in HF (Fu *et al.*, 2009; Guo *et al.*,

Table 1. Gene family numbers of immunity genes in various Diptera species and their functions. The exact numbers of genes for different classes of immune genes are largely unknown. When literature reports vary in the described numbers per class of immune genes, the range of reported gene numbers is provided. These ranges in gene family numbers can at least partially be explained by differing experimental approaches, although this does not explain all discrepancies in the literature. When a number of zero is reported, this means that this antimicrobial peptide (AMP) has at present not been described for this species. The descriptions of immune pathways and antipathogenic activity of immune genes described in the table is a general indication of the gene function in literature, not limited to studies of the five species discussed in the table.[1]

Immune genes		Dipteran species						
		Black soldier fly[2]	House fly[3]	*Drosophila melanogaster*[4]	*Aedes aegypti*[5]	*Anopheles gambiae*[6]	Involved immune pathways[7]	Antipathogenic activity[7]
AMPs	AMP 17	0	1	0	0	0	unknown	antifungal
	attacin	5-6	9-17	4	1	0-1	Imd and JAK/STAT	antibacterial (Gram-negative) and anti-viral
	bomanin	0	0	12	0	0	Toll	antifungal and antibacterial (Gram-positive and -negative)
	cecropin	7-36	3-16	4-5	9-10	4	Imd	antifungal and antibacterial (Gram-positive and -negative)
	crustin	0	4	0	0	0	unknown	antifungal and antibacterial (Gram-positive)
	defensin	3-26	1-21	1	4	2-4	Toll	antibacterial (Gram-positive)
	diptericin	5-10	2-6	2-3	0-1	0-1	Imd	antibacterial (Gram-positive and -negative)
	domesticin	0	1-2	1	0	0	unknown	antibacterial (Gram-positive and -negative)
	drosocin	0	0	1	0	0	Imd	antibacterial (Gram-positive and negative)
	drosomycin	0	0	7	0	0	Toll	antifungal
	edin	0	10	1	0	0	Imd	antibacterial (Gram-positive)
	eppin	0	43	0	0	0	unknown	antifungal and antibacterial (Gram-positive)
	gambicin	0	0	0	1	1	Imd, Toll and JAK/STAT	antifungal and antibacterial (Gram-positive and -negative) and active against plasmodium parasites
	holotricin	0	0	0	1	0	unknown	antibacterial (Gram-negative) and anti-viral
	MAF-1	0	1	0	0	0	unknown	antifungal
	MDAP-2	0	1	0	0	0	unknown	antibacterial (Gram-negative)
	metchnikowin	0	0	1	0	0	Toll	antifungal
	muscin	0	1	0	0	0	unknown	antibacterial (Gram-positive and -negative)
	muslin	0	37	0	1	0	unknown	antibacterial (Gram-positive)
	knottin-like peptides	0-4	0	0	0	0	unknown	antifungal and antibacterial (Gram-positive and -negative)
	sarcotoxin	4	6	0	0	0	unknown	antibacterial (Gram-negative)
	stomoxyn	2	0	0	0	0	unknown	antifungal and antibacterial (Gram-positive and -negative)
	SVWC	0	30	0	0	0	unknown	antifungal and active against *Trichobilharzia ocellata* parasites
	total	50-53	140-186	20-23	17	10	-	-
ProPOs	total	8	7-23	3-10	10-25	9-20	ProPO cascade	antifungal and antibacterial (Gram-positive and -negative) and active against parasitoids
lysozymes	total	13-36	13-34	11-13	7	8	unknown	antibacterial
GNBPs	total	16-23	3	3-7	7	7	Toll	pathogen recognition of fungi
PGRPs	total	31	16-20	13	8-10	7	Imd (long) and Toll (short)	pathogen recognition of bacteria (Gram-positive and Gram negative) by short and long respectively

[1] GNBP = Gram-negative binding proteins; Imd = immunodeficiency; JAK/STAT = Janus kinase/signal transducers and activators of transcription; MAF-1 = *Musca domestica* antifungal peptide-1; MDAP-2 = *Musca domestica* antimicrobial peptide-2; PGRP = peptidoglycan recognition proteins; ProPO = prophenoloxidase; SVWC = secreted AMP, containing a single domain von Willebrand factor type C.

[2] References: Elhag *et al.* (2017); Vogel *et al.* (2018); Zhan *et al.* (2020)

[3] References: Andoh *et al.* (2018); Fu *et al.* (2009); Guo *et al.* (2017); Pei *et al.* (2014); Qi *et al.*, (2021); Sackton *et al.* (2017); Scott *et al.* (2014); Tang *et al.* (2014); Zhan *et al.* (2020)

[4] References: Clemmons *et al.* (2015); Lemaitre and Hoffmann (2007); Lindsay *et al.* (2018); Qi *et al.*, (2021); Sackton *et al.* (2017); Tang *et al.* (2014); Vanha-aho *et al.* (2012); Zhan *et al.* (2020)

[5] References: Sackton *et al.* (2017); Waterhouse *et al.* (2007); Zhang *et al.*, (2017)

[6] References: Sackton *et al.* (2017); Tang *et al.* (2014); Vizioli *et al.* (2001); Waterhouse *et al.* (2007)

[7] References: González-Santoyo and Córdoba-Aguilar (2012); Hillyer (2016); Hwang *et al.* (2010a); Hwang *et al.* (2010b); Kim *et al.* (2013); Kingsolver *et al.* (2013); Lemaitre and Hoffmann (2007); Thomas *et al.* (2016); Ueda *et al.* (2005); Vizioli *et al.* (2001); Vanha-aho *et al.* (2012)

2017; Peng *et al.*, 2019; Qi *et al.*, 2021; Tang *et al.*, 2014) and knottin-like peptides and stomoxyn in BSF (Elhag *et al.*, 2017; Vogel *et al.*, 2018). Also, the number of AMP genes that these dipteran insects possess varies considerably.

Table 1 shows that expansion of AMP gene families differs substantially between Dipteran species. Furthermore, the enumerations of AMPs within the same species varies among different studies. This is partially due to the fact that insect immunity is a very active field of research and new AMPs are still being discovered, even in well-studied species like *D. melanogaster* (Clemmons *et al.*, 2015). Moreover, different experimental approaches in the studies may also contribute to these varying estimates. Studies focusing on cloning and purifying AMPs tend to only describe a restricted number of AMPs per study (Fu *et al.*, 2009; Guo *et al.*, 2017; Pei *et al.*, 2014; Vizioli *et al.*, 2001), while studies looking at the whole genome and/or transcriptome to describe AMPs tend to produce a more extensive list of (putative) AMPs (Elhag *et al.*, 2017; Sackton *et al.*, 2017; Scott *et al.*, 2014; Tang *et al.*, 2014; Vogel *et al.*, 2018; Zhan *et al.*, 2020). Using the predicted proteome to estimate AMP numbers can result in even higher numbers (Qi *et al.*, 2021). For example, for HF a total of 18-33 AMPs were described in studies using genomic and/ or transcriptomic approach (Sackton *et al.*, 2017; Scott *et al.*, 2014; Tang *et al.*, 2014) and 186 AMPs were reported by a study using the predicted proteome (Qi *et al.*, 2021). In BSF, a total of 50-53 AMPs were described, based on genomic and/ or transcriptomic studies (Vogel *et al.*, 2018; Zhan *et al.*, 2020). Even when studies use similar approaches within the same species, they can vary substantially in reported numbers of gene family members. For example, Vogel and colleagues (2018) and Zhan and colleagues (2020) used both a genomic and/or transcriptomic approach in BSF, and reported 7 cecropins and 26 defensins, and 36 cecropins and 3 defensins, respectively. More research, including functional characterization of each gene family member, will be needed to clarify these numbers.

The nomenclature that is used for AMPs can also complicate comparisons between different species. New AMPs are often times named after the species they were discovered in, regardless of whether they are part of an existing AMP family, even giving the same name to AMPs that belong to different families. For example, sarcotoxin I is described as part of the cecropin family and sarcotoxin II as part of the attacin family (Natori *et al.*, 1999). These AMPs might be grouped with their gene family in some studies, and not in others, providing a possible explanation for sarcotoxin only being identified in one of the three studies on BSF AMPs. This could be clarified by more extensive comparison of sequences, to identify homologous gene families in different studies and species.

Apart from producing AMPs, fat body cells in *D. melanogaster* also migrate to wound sites where they collaborate with haemocytes to clean cell debris around the wound, seal the cuticular epithelial wound gap and locally release AMPs to fight pathogens/foreign bodies (Franz *et al.*, 2018). Furthermore, the fat body functions in storage and metabolism of lipids and carbohydrates for energy reserves, hormonal regulation and participates in detoxification of waste products of nitrogen metabolism, e.g. uric acid (Arrese and Soulages, 2010). Although not directly related to immunity, these functions may still have an auxiliary role in the defences of insects against microbial infections.

Immune responses of the haemocytes

The haemocytes (blood cells) of insects play an important role in the immune response. They are responsible for phagocytosis of microbes, encapsulation of parasitoids, nodulation and wound healing. The types and numbers of haemocytes differ considerably among insect species (Strand, 2008). In response to infection, haemocytes can be produced in the lymph glands of insects or released from sessile haemocyte pools that form under the sub-epidermis (Lanot *et al.*, 2001; Leitao and Sucena, 2015; Márkus *et al.*, 2009). Under the regulatory control of the JAK/STAT and Notch pathways, prohemocytes can differentiate into various types of haemocytes, that differ in characteristics such as morphology (e.g. size, shape, appendages), phagocytotic capacity, adhesiveness, or contents (e.g. crystalline inclusions, melanin precursors).

In BSF, three types of differentiated haemocytes have been described: (1) crystal cells that produce and store prophenoloxidase (ProPO; i.e. a zymogen which catalyses melanin production once activated); (2) plasmatocytes, which look very similar to the lepidopteran plasmatocytes and thus are likely to form capsules and nodules; and (3) granulocytes, which are involved in phagocytosis and the initiation of encapsulation and nodulation (Ribeiro and Brehélin, 2006; Zdybicka-Barabas *et al.*, 2017). In HF, four types of haemocytes have been described, in addition to the undifferentiated prohaemocytes: (1) plasmatocytes; (2) granulocytes; (3) oenocytoids instead of crystal cells, which are involved in the phenoloxidase response; and (4) an abundant class of haemocytes with morphological characteristics intermediate between plasmatocytes and granulocytes (Borowska and Pyza, 2011). For both BSF and HF, the descriptions of haemocytes are based on morphology alone. Haemocytes are very sensitive to their environment, so fixating them for visual characterization can already influence their appearance, making the characterizations inaccurate (Borowska and Pyza, 2011; Fu *et al.*, 2020; Lavine and Strand, 2002). Descriptions of haemocytes using techniques like single-cell RNA sequencing seem to yield higher resolution and more reliable results, although it remains difficult to effectively

categorise entities on a continuous scale into discrete categories (Cattenoz *et al.*, 2021; Fu *et al.*, 2020; Tattikota *et al.*, 2020).

Comparing haemocytes between different insect species is further complicated by its nomenclature: haemocytes of the same name in different species can differ in appearance and/or function (Ribeiro and Brehélin, 2006). This is also the case within the order Diptera. Whereas they bear the same name, plasmatocytes in *D. melanogaster* are more equivalent in their role and appearance to the granulocytes of BSF and HF than to their plasmatocytes, since both are involved in phagocytosis, have a spherical shape and contain granules. The plasmatocytes in BSF and HF, in turn, are more comparable in function to the 'lamellocytes' of *D. melanogaster* that only appear in the larval haemolymph after an immune challenge by macro-pathogens. The plasmatocytes of BSF and HF differ in appearance from the lamellocytes of *D. melanogaster,* which are larger and flattened, whereas the plasmatocytes of BSF and HF have an ovoid shape of up to 20 µm long (Borowska and Pyza, 2011; Ribeiro and Brehélin, 2006; Zdybicka-Barabas *et al.*, 2017). Furthermore, the oenocytes in HF have similarities in form and function with the crystal cells in BSF and *D. melanogaster*. Both are rather big haemocytes with crystalline inclusions in the cytoplasm (Ribeiro and Brehélin, 2006).

In addition to the direct interaction of blood cells with pathogens (e.g. through phagocytosis or adhesion), another important immune function of haemocytes is the production and storage of ProPO, which catalyses melanin production once activated. Upon immune challenge, the zymogen ProPO is released into the haemolymph where it gets activated into phenoloxidase, which in turn activates melanisation in response to infection and wounding. Melanisation helps clear infections by oxidizing the pathogens and by isolating the pathogen from the haemolymph as part of the encapsulation and nodulation response (González-Santoyo and Córdoba-Aguilar, 2012; Hillyer, 2016).

Clade-specific expansions have been reported for ProPO genes, although most insect species possess only a moderate number of genes for ProPO. For example, *D. melanogaster* has three paralogs of ProPO, whereas several other *Drosophila* species have only two paralogs (Salazar-Jaramillo *et al.*, 2014). In mosquitoes, the number of ProPO genes is higher, with 9 in *Anopheles gambiae* and 10 in *Aedes aegypti* (González-Santoyo and Córdoba-Aguilar, 2012; Waterhouse *et al.*, 2007). In BSF eight ProPO genes and three phenoloxidase-related genes have been reported (Vogel *et al.*, 2018; Zhan *et al.*, 2020), and in HF seven ProPO genes (Tang *et al.*, 2014). Furthermore, ProPO levels in the haemolymph of BSF have been shown to increase up to twofold after immune challenge (Zdybicka-Barabas *et al.*, 2017).

Immune responses of the epithelial cells

Epithelial cells provide insects with multiple immune responses, which together may prevent infection by pathogens. Epithelial cells of the cuticle produce chitin and proteins that are the main components of the insect exoskeleton, which provides the physical barrier, both at the exterior body surface, and internally, lining the reproductive tract, respiratory system, foregut and hindgut (Moret and Moreau, 2012). Furthermore, epithelial cells can provide physiological and biochemical defences, such as extreme pH values, the secretion of lysozymes into the gut lumen, and melanin in the cuticle. The most extreme pH is usually found in the midgut. The pH of the midguts of BSF and HF is very low (pH 3.4-3.9 and 2.1, respectively), as is usual for flies with a bacteria-rich diet (Bonelli *et al.*, 2019; Douglas, 2015; Greenberg, 1968). Mosquitoes on the other hand have a more alkaline midgut milieu (Engel and Moran, 2013).

The midgut epithelium is not covered with a cuticle, but is physically protected by the peritrophic membrane, which is a tubular structure consisting of proteins, glycoproteins and chitin microfibrils. The type of peritrophic matrix that an insect possesses differs between species and depends on their diet. A type I peritrophic membrane is thin, gel-like and is often only produced after consumption of food. In contrast, a type II peritrophic membrane is generally tougher, thicker and is constantly being produced (Merzendorfer *et al.*, 2016; Terra, 2001). Larvae of mosquitoes, HF and *D. melanogaster* possess a type II peritrophic membrane (Lehane, 1997; Nayduch and Burrus, 2017; Rizki, 1956); for larvae of BSF this is not known. In *D. melanogaster* and HF, adults retain the same type of peritrophic membrane as the larvae. However, mosquitoes switch from a type II membrane in larvae to a type I peritrophic membrane in the adult females, which is better suited to their diet of intermittent bloodmeals; in adult male mosquitoes which only feed on plant nectar, the peritrophic membrane is absent (Lehane, 1997).

The border epithelium in the gut is in direct contact with the insect's surroundings. It produces tissue-specific AMPs, regulated by the Imd pathway (Tzou *et al.*, 2000). Tissue-specific expression of AMP's has been studied in both HF and *D. melanogaster* (Mura and Ruiu, 2017; Tzou *et al.*, 2000). Upon oral infection with *Brevibacilllus laterosporus*, HF larvae and adults significantly upregulate the expression of several AMPs in their gut. When fed a diet with *B. laterosporus,* the genes coding for attacin, cecropin, defensin and domesticin were upregulated in both adults and larvae, in comparison with individuals fed on control diet. In larvae, also diptricin, muscin and ProPO genes were upregulated (Mura and Ruiu, 2017). In adult *D. melanogaster*, all AMP genes were at least weakly expressed in the gut after immune challenge with *Erwinia carotovora*, but in larvae no drosocin or drosomycin were

recorded (Tzou *et al.*, 2000). This illustrates that these immune responses not only differ between species, but even within species substantial differences can be found between different developmental stages.

The JNK pathway is involved in the epithelial immune response through the shedding of intestinal epithelial cells, by which infected cells are expelled in order to prevent colonization and replaced by new healthy epithelial cells. The JNK pathway regulates this process in synergy with the Imd pathway (Tafesh-Edwards and Eleftherianos, 2020; Zhai *et al.*, 2018). To our knowledge there is no research into the involvement of the JNK pathways in epithelial immune responses in BSF and HF.

The dual oxidase (DUOX) pathway is part of the immune responses of the gut epithelia, where it regulates the generation of reactive oxygen species upon ingestion of pathogens. One recent study investigating the importance of the DUOX pathway in the immune response of BSF showed a significant link between the expression of *BsfDuox* and the bacterial load in BSF in response to oral infection (Huang *et al.*, 2020). We did not find any literature on the DUOX pathway in HF.

Acquired immunity – priming and immune specificity

Immune priming in some insects is somewhat analogous to the antibody-mediated adaptive immunity observed in vertebrates. It refers to the improved host immunological defence upon a second encounter with the same pathogen or parasitoid (Contreras-Garduño *et al.*, 2016). Immune priming can be highly specific, enabling the insect to distinguish specific enemies (Kurtz and Franz, 2003) with an enhanced immune response against certain microbial pathogens (Dhinaut *et al.*, 2018). The effectors produced upon pathogen re-encounter are not necessarily different from the initial exposure to the same pathogen. The difference lies within the more rapid pathogen recognition by the pre-activation of the immune response and in the amount of produced recognition and effector molecules (Contreras-Garduño *et al.*, 2016; Schulenburg *et al.*, 2007). The phenomenon is characterised by an increased density of circulating haemocytes, which arise from activated sessile haemocyte clusters, and/or increased levels of AMPs in the haemolymph (Fallon *et al.*, 2011). For example, in *Drosophila*, re-introduction of entomopathogenic fungi was shown to induce a response of antimicrobial peptide genes that was specific to a certain fungal infection (Lemaitre *et al.*, 1996), while repeated infection with *Streptococcus pneumoniae* did not prime the humoral response of *D. melanogaster,* but it rather activated plasmatocyte phagocytosis (Pham *et al.*, 2007). Immune priming can be metabolically costly (Ardia *et al.*, 2012; Zanchi *et al.*, 2011).

Retaining the memory of past infections has been connected with the development of a biphasic response upon pathogen re-exposure. In experiments with primed *Anopheles albimanus* mosquitoes, elevated levels of attacin, cecropin and gambicin were observed during re-encounter with *Plasmodium berghei*, despite the fact that AMP levels were left to return to basal levels and that the pathogen was eliminated after the first encounter (Contreras-Garduño *et al.*, 2016). This biphasic response might mean that some sort of memory mechanism is associated with subsequent exposures to the same pathogen. However, more studies are needed to study whether such a memory mechanism indeed exists in insects (Schmid-Hempel, 2011), because systematic experimental proof for such a claim is still lacking (Rowley and Powell, 2007).

Potential mediators for regulating the specificity in adaptive immunity are molecules that can display interaction diversity for numerous pathogen receptors. The Down syndrome cell adhesion molecule (Dscam) is such an example. It is a member of the immunoglobulin superfamily which contains three alternatively spliced immunoglobulin exon cassettes and a set of alternative transmembrane (tm)-domain exons in invertebrates (Kurtz and Armitage, 2006). This gene can produce a vast array of different transcripts (Watson *et al.*, 2005). In *An. gambiae*, the alternative splicing of Dscam is controlled by many different immune elicitors, which interfere with Dscam expression (Dong *et al.*, 2006). This alternative splicing could produce a diverse series of recognition elements that can directly bind particular microbes. The model, however, remains largely hypothetical and has not been directly associated with phagocytic stimulation (but see (Li *et al.*, 2019)).

Another form of immune priming in insects is the passing of information about the presence of a pathogen from the parent to the offspring (Moret, 2006). The conditioning mechanisms for transgenerational immune priming have not yet been fully revealed. Studies in red flour beetles (*Tribolium castaneum*) demonstrated that this priming can be achieved by laying eggs with fragments of bacterial cell wall material (Knorr *et al.*, 2015). Recently, it was observed that *D. melanogaster* and *Ae. aegypti* can transmit antiviral immunological memory to their progeny, which can lasts for several generations (Mondotte *et al.*, 2020).

3. Factors affecting insect health and immunity

The production of insects for food and feed is a growing sector, whereby insects are produced by both small- and large-scale companies, across both temperate and tropical regions, and fed various feed substrates (Chia *et al.*, 2018b; Van Huis, 2013). This section describes the effects of several conditions and factors on insect health and immunity.

Temperature

Insects are poikilotherms, whereby their internal temperature is variable and largely determined by external temperature. Fluctuations in rearing temperature not only impact the growth and development of insects, but also influences their metabolic rate, reproductive potential, immune function and longevity (Angilletta *et al.*, 2010). Furthermore, the rearing temperature affects the degree of susceptibility against pathogenic microorganisms. When *D. melanogaster* adults were injected with *Pseudomonas aeruginosa* (LD_{50} dosage), their mortality increased with increasing temperatures: while 60% and 54% of the infected adult population survived at 21.4 and 25 °C respectively, survival dropped quickly to zero at temperatures higher than 28 °C. In contrast, 100% of the uninfected adults survived at 35 °C (Fedorka *et al.*, 2016). The immune response of infected HF larvae was similarly influenced by temperature (Bahrndorff *et al.*, 2014). Third-instar larvae of HF were injected with *Campylobacter jejuni* (~10^9 cells/ml) and placed at 25 and 35 °C. After 20 h, all of the larvae maintained at 35 °C were dead whereas all larvae maintained at 25 °C were alive, indicating temperature-dependent influence on larval survival. Interestingly, some genes involved in temperature stress may also function in immune responses: in HF larvae, heat shock and septic injury induced the expression of *HSP70*, and knocking down the expression of this gene caused increased sensitivity to both heat shock and bacterial infection (Tang *et al.*, 2012).

The role of temperature on immunity is also reflected in temperature preferences of infected insects. When given the choice, *D. melanogaster* adults injected with *P. aeruginosa* consistently preferred slightly lower temperature environments than uninfected adults, which may be limiting the pathogen growth and allow the flies to mount a more effective immune response (Fedorka *et al.*, 2016). A similar temperature preference with beneficial effects on survival was observed during infection of *Drosophila* adults with the fungal pathogen *Metarhizium robertsii* (Hunt *et al.*, 2016). The infected flies preferred cooler temperatures (22 °C), which were non-optimal for pathogen growth and resulted in increased larval survival during fungal infections. Additionally, heat stress can also result in an increased bacterial load due to thermal mismatch between the fly and its endogenous microflora, rendering the fly more susceptible to bacterial pathogens (Telonis-Scott *et al.*, 2013).

Insects may also engage in behavioural fever when infected with a pathogen, either changing their own thermoregulatory behaviour, aggregating to form local hotspots, or by exploiting warmer microhabitats. The latter has been reported for HF infected by the fungal entomopathogen, *Beauveria bassiana* (Balsamo) Vuillemin. Fungal infected flies spent more time at higher temperatures when given the choice (Anderson *et al.*, 2013a). The higher temperature was unsuitable for pathogen growth and improved survival in infected flies compared to infected flies without access to a heated environment, although it came at the expense of a lower egg viability (Anderson *et al.*, 2013b). These studies focused on adult HF behaviour. It could be hypothesised that both HF and BSF larvae also show behavioural fever. Larvae of both species commonly aggregate within their feed substrates, and temperature can increase rapidly within larval aggregations – or maggot masses – of various dipteran species (Rivers *et al.*, 2011).

In several other insects, temperature affects melanisation, either the humoral melanisation responses, or by affecting cuticular melanisation. In adults of the mosquito *Anopheles stephensi,* temperature influences melanisation response to the presence of foreign bodies such as implants and Sephadex C-25 beads (Fedorka *et al.*, 2012; Murdock *et al.*, 2012). Maximum melanisation was observed in *An. stephensi* adults upon injection of Sephadex-25 beads at 18 °C, followed by decreasing degree of melanisation with an increase in temperature (Murdock *et al.*, 2012). Larvae of *D. melanogaster* reared at warm temperature (28.5 °C) develop into adults that possess lighter cuticles due to lower melanin secretion. These adults suffered an increased susceptibility to *P. aeruginosa* compared to darker adults that emerged from larvae reared at lower temperature (21.5 °C) (Kutch *et al.*, 2014).

Exposure of insects to different temperature regimes also influences their gene expression profile, especially the expression of AMP genes. The expression of *DEF1* (*Defensin1*) in adult *An. stephensi* mosquitoes decreased with an increase in temperature (Murdock *et al.*, 2013). *DEF1* expression in mosquitoes challenged with heat-killed *E. coli* was significantly higher at low temperature (i.e. at 18 °C compared to 26 and 32 °C). Furthermore, exposure of adults to a diurnal rhythm in their rearing temperature (26±6 °C) resulted in higher expression of *CEC1* (Cecropin1) compared to those exposed to a constant temperature of 26 °C.

Sub-zero temperatures can also evoke various immune responses, both in acute cold shock treatment and for sustained cold treatments (Salehipour-Shirazi *et al.*, 2017; Štětina *et al.*, 2019). Supercooling (i.e. the maintenance of body fluids in liquid state at sub-zero temperatures) leads to transcriptional upregulation of the innate immunity pathways Toll and Imd, as well as to activation of lysozyme-mediated degradation of bacterial cell walls in *D. melanogaster* (Štětina *et al.*, 2019). Freezing (i.e. the formation of ice crystals inside the body upon introduction to sub-zero temperatures) leads to degradation of macromolecules and induction of death-related processes such as autophagy and apoptosis (Štětina *et al.*, 2019).

An acute cold shock in *Drosophila* adults also leads to a greater melanisation response, compared to sustained cold treatment (Salehipour-Shirazi *et al.*, 2017). For BSF, prolonged exposure to chilling temperature treatments (4 °C) or freezing temperature (-12 °C) can cause mortality in eggs and larval stages (Chia *et al.*, 2018a; Raimondi *et al.*, 2020; Villazana and Alyokhin, 2019), but the effects of different temperatures on immunity/health of the larvae need to be further investigated.

Larval density

The effects of population density on immunity are multifaceted. High density increases the risk and spread of infectious diseases, also indirectly when it leads to increased wounding and subsequent infection. Furthermore, high densities can lead to severe resource competition or starvation. This, in turn, can result in poorer growth and nutrient reserves, which could entail that fewer resources would be available for immune defences. Female *Ae. aegypti* mosquitoes that developed at low density as larvae grew significantly larger, and had a stronger immune response when infected with Sindbis virus, compared to high-density reared females (Kim and Muturi, 2013; Price *et al.*, 2015). Cecropin expression of low-density reared females was 20-fold higher compared to larvae reared at higher densities (Kim and Muturi, 2013). High densities of *D. melanogaster* were also found to cause an adverse environment, through the build-up of waste products (e.g. ammonia) (Henry *et al.*, 2020), which could have detrimental effects on health and immunity. Moreover, at high larval densities, *D. melanogaster* larvae resort to cannibalism to fulfil their energy and protein requirements (Vijendravarma *et al.*, 2013).

High population density can also have beneficial effects such as improved defences towards biotic and abiotic stress factors. Rearing densities influence development time and egg-to-adult survival of various dipteran species, including HF, BSF and *Drosophila* (Horváth and Kalinka, 2016) with too low densities leading to small adults and/or high mortality. Larvae of *D. melanogaster* develop in high-density aggregations, which can suppress the invasion of harmful fungi, in particular when aggregating in large groups (Trienens and Rohlfs, 2020; Wertheim *et al.*, 2002).

Diet

The nutritional composition of the diet influences insect health and immunity (Table 2). High protein availability (increased yeast content) in the larval diet resulted in an increased constitutive transcription of *Diptericin A* and *Metchnikowin* in *Drosophila* adults, whereas larval gene expression remained unaffected (Fellous and Lazzaro, 2010). Also in BSF, a protein-rich diet upregulated the expression of various immunity genes (Vogel *et al.*, 2018). A dietary shift in protein and carbohydate content (P:C ratio from 1:4 to 1:10) in *D. melanogaster* adults after infection with Gram-positive *Micrococcus luteus* improved their post-infection survival (Ponton *et al.*, 2020). After infection, the flies modulated their nutritional intake to a low-protein and high-carbohydrate diet, indicating nutritional self-medication against infection. Interestingly, the expression levels of AMP genes were significantly influenced by the P:C ratio in the diet, but those of immune receptors or immune signalling were not (Ponton *et al.*, 2020). Additionally, upon infection of *D. melanogaster* with Gram-negative *P. aeruginosa*, the adults that fed on protein-rich diet had a higher fitness, i.e. a larger number of eggs laid, than adults fed with standard diet (Hudson *et al.*, 2020). Hence, increased protein content of diet can fortify the immune function in *D. melanogaster* adults against various bacteria. For the immune function against viral infections in various *Drosophila* species and HF, however, no effects of protein content in the diet were found (Roberts and Longdon, 2021; Schaler *et al.*, 2018).

Sugar levels in the diet can also influence immune responses and AMP secretion, including the overexpression of genes related with immunity and infections. When fed on high-sugar diet (HSD) of 1 M sucrose, *D. melanogaster* adults exhibited an upregulation of AMP genes (*Metchnikowin* and *Defensin)*, indicating activation of the Toll signalling pathway in haemocytes and the fat body (Yu *et al.*, 2018). Haemocytes of HSD-fed larvae exhibited higher nuclear p-JNK signals compared to controls, indicating the activation of the immune system via the Toll and JNK pathways. HSD-induced production of lamellocytes in the lymph glands, which are normally only induced during an immune challenge, indicate that HSD potentially induced an inflammatory response in the larvae. On the HSD, the immune function seems to become compromised rather than fortified. Provision of high-glucose diet increased susceptibility of *Drosophila* adults to the gram-negative bacteria *Providencia rettgeri* (Unckless *et al.*, 2015). The severity of bacterial infection in adults fed on HSD was correlated to glucose metabolism and conversion to/from glycogen, mediated by the *crinkled* gene.

A high-fat diet also led to increased expression of genes related to immunity and infection in *Drosophila* adults. During a microbial infection, when *Drosophila* adults were fed a high-fat diet, several genes associated with humoral immune challenge (i.e. AMPs including *CecA1, AttA, Dro, Drs, IM23, Def and Dpt*) were upregulated (Hemphill *et al.*, 2018; Ponton *et al.*, 2020). Similarly, when BSF was fed a diet enriched with plant oils, this resulted in upregulation of various attacins, cecropins, defensins and diptericins (Vogel *et al.*, 2018). Whether these changes in immune gene expression affected the immune function positively or negatively remains to be investigated.

Table 2. Effects of different diet factors on insect immunity.

Insect	Diet	Effect/result	Reference
Drosophila melanogaster	Different P:C ratios	Shift from 1:4 to 1:10 P:C ratio led to improved survival from infection against gram-positive bacteria, *Micrococcus luteus*	Ponton *et al.* (2020)
	High-protein content (31% versus 14% in standard Lewis diet)	Increased oviposition/egg-production upon infection with gram-negative bacteria, *P. aeruginosa*	Hudson *et al.* (2020)
	High yeast: sugar (4:1) ratio during larval stage	Increased constitutive expression of *Diptericin A* and *Metchnikowin* in the adult phase, but not in the larval stage	Fellous and Lazzaro (2010)
	High-glucose diet	Increased mortality in adults upon infection with gram-negative bacterium *Providencia rettgeri.* Immunity mediation by *crinkled* gene (encoding a myosin VIIa cytoskeletal ATPase)	Unckless *et al.* (2015)
	High-sugar level (1 M sucrose)	Activation of Toll pathway in haemocytes and fat body	Yu *et al.* (2018)
	High-sugar diet	Increased insulin signalling reduced expression of immune genes. Decreased insulin signalling increased immune gene expression and increased infection resistance against *Pseudomonas aeruginosa* strain *PA14*	Musselman *et al.* (2018)
	High-fat diet (addition of 20% w/v coconut oil compared to control diet)	Increased transcription of genes with ontology related to immunity and infection	Hemphill *et al.* (2018)
Aedes aegypti	High-sugar diet (10% sucrose)	Increased expression of anti-microbial peptides, cecropin (cecD) and defensin (defE)	Almire *et al.* (2021)
Hermetia illucens	Protein-rich and plant-oil diets	Upregulation of immunity-related genes	Vogel *et al.* (2018)

Starvation

Sufficient availability of nutrients can also influence immunity. As mentioned before, shortage of nutrients may lead to fewer reserves and resources for immunity. There is a clear distinction, however, between dietary restriction and starvation.

Dietary restriction involves reduced availability of one or multiple components or nutrients (e.g. yeast and/or sugar) in the insect diet, and typically results in an extended lifespan (Burger *et al.*, 2007; Katewa and Kapahi, 2011; Lee *et al.*, 2017). Dietary restriction is considered a low-intensity stressor that induces the insect to invest resources towards survival and stress resistance, often at the expense of resources for reproduction. Adult *D. melanogaster* flies fed with 7% Y:S (yeast:sugar) diet displayed a higher survival upon infection with Gram-negative bacteria (*P. aeruginosa*) compared to flies fed with 16% Y:S diet (Burger *et al.*, 2007). Exclusion of yeast from the diet of adult *D. melanogaster* influenced the regulation of the target of rapamycin (TOR) pathway, which is associated with survival against bacterial infections (Lee *et al.*, 2017). The TOR pathway modulates AMP expression, whereby the upregulation of TOR pathway in high yeast diets resulted in an overall reduction in expression of AMP genes, which in turn negatively influenced the post-infection survival of flies compared to flies on low yeast diets (Lee *et al.*, 2017; Varma *et al.*, 2014). Dietary restriction in *D. melanogaster* can also lead to different expression and delayed up-regulation of immune-related genes in uninfected flies (Pletcher *et al.*, 2005). The effect of dietary restriction on lifespan or immunity has not yet been studied in BSF or HF, but it is such an universal phenomenon in eukaryotes (Kapahi *et al.*, 2017) that it can be expected to operate similarly within these species.

Starvation of adult *D. melanogaster* flies resulted in down-regulation of genes involved in immunity and defence responses. The down-regulation can be attributed to the resource allocation by the adults to starvation resistance over protection against infection (Fujikawa *et al.*, 2009). For limited periods of starvation, it is possible that immune functions remain intact despite undergoing nutritional stress. Across different developmental stages of HF, exposure to starvation for 12 or 24 h did not bear any influence on the expression levels of its lysozyme *Mdlys* (Ren *et al.*, 2009). Lysozymes are utilised by insect larvae to hydrolyse bacteria and therefore can play an important role in the innate immunity of the insect larvae. The effect of starvation periods on the immunity of BSF and HF are not well characterised.

4. Microbiome

Insect-microbe associations

Early studies on the microorganisms associated with insects were focused on the capacity of insects to act as vectors for dangerous pathogens. In the case of the HF, the vectoring

ability of natural populations was investigated early on (Graham-Smith, 1910; Tebbutt, 1912), in relation to various pathogens (Greenberg *et al.*, 1970; Grübel *et al.*, 1997; Shane *et al.*, 1985; Zimmerman *et al.*, 1989). Also, in BSF reared on bio-organic waste streams, potential foodborne pathogens such as *Bacillus cereus* were detected in larvae (Wynants *et al.*, 2019) and prepupae (Raimondi *et al.*, 2020). Whether the discovered foodborne pathogens can also harm insect health and growth on these organic waste streams has not yet been clarified. However, there are studies indicating that *B. cereus* can indeed inhibit the growth of HF maggots in axenic conditions (Schmidtmann and Martin, 1992).

Nevertheless, the research of fly microbiota embraces a much larger scientific field than vectoring of potential harmful microorganisms, and includes the investigation of beneficial microorganisms linked to host health, fitness and their ability to produce offspring. It is now commonly accepted that the numerous and diverse relationships of insects with beneficial microorganisms largely contributed to their evolutionary success (Engel and Moran, 2013). The contribution of insect microbiota to the host functions has been characterised as highly relevant from several perspectives, particularly for the understanding of immunity and metabolic interactions (Lemaitre and Hoffmann, 2007). The gut microbiome aids in nutrient provision, which can indirectly contribute to immunity (Ayres and Schneider, 2009; Chambers and Schneider, 2012). Furthermore, studies with *D. melanogaster* larvae showed that microbiota perturbations influenced the host's resistance against natural parasites (Chaplinska *et al.*, 2016).

Early studies indicated the significance of bacteria for HF development as well, because larvae failed to grow in an axenic environment (Schmidtmann and Martin, 1992; Watson *et al.*, 1993). Later studies highlighted the presence of *Morganella morganii*, *Providencia* spp. and *Proteus* spp. and discussed their importance for HF larval development (Gupta *et al.*, 2012; Zhao *et al.*, 2017; Zurek *et al.*, 2000). Community analysis in maggots, pupae, and adult HF suggested a shift of the natural fly microbiota along developmental stages, possibly due to different host-bacterial interactions at each stage (Wei *et al.*, 2013). *M. morganii*, in particular, can remain in the fly gut after metamorphosis (Su *et al.*, 2010). The microbiota of the BSF revealed a similar core set of bacterial phylotypes, including *Morganella* sp., *Enterococcus* sp., *Pseudomonas* spp., *Providencia* sp. and members of the Bacillaceae (Raimondi *et al.*, 2020; Wynants *et al.*, 2019).

The existence of a set of recurrent, host-associated bacteria in both the HF and the BSF suggests this microbiome may be important for the host's biological function. Results of microbiological surveys both for HF and BSF populations from various habitats, showed that their internal bacterial community is very diverse, yet relatively consistent across geographic location and habitats (Bahrndorff *et al.*, 2017; Park *et al.*, 2019; Shelomi *et al.*, 2020; Wynants *et al.*, 2019), while it can be partly dependent on diet (Bruno *et al.*, 2019; Klammsteiner *et al.*, 2020; Varotto Boccazzi *et al.*, 2017). Furthermore, the composition and consistency of insect microbiota is more diverse in wild-caught flies than in laboratory flies, for *Drosophila* (Chandler *et al.*, 2011) and HF (Bahrndorff *et al.*, 2017; Park *et al.*, 2019), while diet has a strong effect on the diversity of the microbiota in BSF (Bruno *et al.*, 2019; Wynants *et al.*, 2019). Microbial diversity is suggested to be regulated through the host's immune system, which is an important filter for the gut microbial community (Ryu *et al.*, 2008). All in all, the microbiome and the immune system of the host function in an interactive cycle, the details of which are yet to be revealed.

The microbiome in insect immune responses

The colonization of the insect gut with symbiotic microbial communities plays a crucial role for insect health. More specifically, the gut microbiota aids in protecting the gut from pathogen invasions by niche occupation (Engel and Moran, 2013), although niche occupation can also trigger antagonistic interactions between beneficial bacteria and host pathogens (Cirimotich *et al.*, 2011). *Drosophila* adults with a regular gut microbiota were indeed less susceptible to oral infections compared to *Drosophila* with axenic gut environment (Blum *et al.*, 2013). Also, axenic *Drosophila* larvae died in the first instar when their diet was altered to contain a reduced content of yeast, while these detrimental effects on fly health were mitigated by the introduction of certain bacteria, such as *Lactobacillus plantarum* and *Acetobacter pomorum*, which can both regulate growth (Shin *et al.*, 2011) and antagonise microbial pathogens (Blum *et al.*, 2013). Furthermore, the gut microbiome is linked to local immunity of the insect intestinal epithelium, enabled mostly by the production of AMPs or the synthesis of reactive oxygen species in reaction to the gut microbes. This is modulated by feedback loops and components that tolerate the presence of the natural gut microbiota (Bischoff *et al.*, 2006; Ryu *et al.*, 2008; Zaidman-Rémy *et al.*, 2006). What is more, the prolonged interaction of the gut microbiota with the host immune system may have an impact on host physiology. Studies on *Drosophila* have shown that the gut microbiota can promote increased intestinal epithelial cell turnover compared with individuals with sterile guts (Buchon *et al.*, 2009, 2014).

Direct effects of the microbiome on immunity have also been shown. Experiments with *Drosophila neotestacea* showed the direct beneficial effects of a maternally transmitted bacterial symbiont of the genus *Spiroplasma*, which defends the fly by reversing the effect of the nematode parasite *Howardula*, which causes sterility in female flies (Jaenike *et al.*, 2010). It has also been hypothesised that

the insect microbiome may contribute to immune priming (Freitak *et al.*, 2014). Primed *Anopheles* mosquitoes which have been deprived of their natural gut microbiota, for instance, showed a lower phagocytic activity and a greater susceptibility to *Plasmodium*, when compared with primed mosquitoes with their natural gut microbiota (Rodrigues *et al.*, 2010). Other studies show that transgenerational immune priming may be supported by the vertical transmission of bacteria from mother to offspring (Freitak *et al.*, 2014; Hernández-Martínez *et al.*, 2010).

Effects of complex microbiome in waste streams

Insects survive and thrive within an immense range of ecological niches. Even when their diet is restricted to a poor nutrient content, there are numerous symbioses with microorganisms which can enhance dietary quality. Many bacteria can simply be digested in the gut and therefore enhance directly the insect diet. Lysozymes expressed in the gut of *Drosophila* flies, for instance, were proposed to relate to the digestion of microbes rather than to their immune defences (Daffre *et al.*, 1994). The *Drosophila* genome harbours genes that code for amino-acid transporters with high affinity for the D-amino acids of bacterial peptidoglycan, benefiting larval nutrition and development by acquiring bacterial and yeast fermentation products (Miller *et al.*, 2008). In mass rearing of insects, the use of organic waste streams as feeding substrate, teeming with microbes, could be a method of valorisation and biodegradation of waste (Diener *et al.*, 2009; Salomone *et al.*, 2017; Van Huis, 2013; Zhang *et al.*, 2012).

In the case of larvae of both the BSF and the HF, there is a proven capacity for the bio-conversion of the microbial biomass of organic waste streams into insect biomass (Miranda *et al.*, 2019; Zhang *et al.*, 2012). Rearing of HF on poultry manure reduced the bacterial content of the substrate and provided sufficient nutrients for the development of the flies (Fitches *et al.*, 2019). Similar results were found in BSF reared on three different manure types (swine, dairy, or poultry manure) (Miranda *et al.*, 2019). Furthermore, BSF larvae can also enhance the metabolic function of waste biodegradation through their gut microbiota (Jeon *et al.*, 2011; Jiang *et al.*, 2019). A series of studies suggests that BSF larvae are not only able to utilise the microbial biomass, but are also capable of reducing the load of pathogenic bacteria in their rearing substrate (Erickson *et al.*, 2004; Lalander *et al.*, 2015; Liu *et al.*, 2008; Salomone *et al.*, 2017). Some environmental microorganisms can synergistically contribute to the insects' antimicrobial capacity (Xiao *et al.*, 2018).

In these bio-conversion and biodegradation processes, the insects' microbiome could play a decisive role in facilitating insect metabolism and immunity. It has been suggested that the manipulation of microbiota for mass-cultured insects could enhance insect rearing, by exploiting and enhancing microbiota-related antimicrobial strategies (De Smet *et al.*, 2018). Manipulation could either focus on optimizing host-associated beneficial microbes, or on promoting the production of microbe-derived molecules that fortify their health and immunity. For this strategy to be exploited in mass rearing of BSF and HF, however, we would need more detailed information on the effect of the microbiome, or of particular microbiota, on the immune functioning of these insects.

5. Conclusion and future perspectives

When aiming to safeguard insect health under mass-rearing conditions, we will need to develop a more in-depth understanding of the functioning and diversity of the various components of the immune systems of the insects, as well as their specificity and regulation. Our basic understanding of the functioning of key immunity pathways in HF and BSF is limited. There is, for example, still substantial unclarity on the numbers of genes in the various AMP classes (Elhag *et al.*, 2017; Qi *et al.*, 2021; Sackton *et al.*, 2017; Scott *et al.*, 2014; Tang *et al.*, 2014; Vogel *et al.*, 2018; Zhan *et al.*, 2020), illustrating that more functional research is needed to better understand the role of these genes in combatting infections. Importantly, the differences in gene family numbers between HF and BSF, as well as with other species, suggest possible differences in importance of these immune system components for different species. Moreover, research on *Drosophila* shows that there are important differences in immunity between larvae and adults (Lemaitre and Hoffmann, 2007; Tzou *et al.*, 2000). Therefore, we need to characterise the induction of immune responses after various infections, in each of the different life stages, as well as their symptoms when diseased. This knowledge can then also be exploited for the design of diagnostic tools to monitor insect health and diseases in mass rearing systems.

Several environmental factors in a mass rearing can strengthen or impede health and immunity. Increased survival was found post-infection under relatively cool rearing conditions among several dipteran species, which may indicate enhanced immunity. In contrast, behavioural fever has been reported in HF to reduce fungal infections, which resulted in increased survival (Anderson *et al.*, 2013a,b). For animal production purposes, high rearing temperatures may be advantageous, as this speeds up developmental rate in the poikilotherm insects. However, it is important to note that thermal performance curves are usually asymmetric, declining sharply above the optimum temperature. In the case of an infection, adjusting the rearing temperature could be an intervention that may help to overcome the infection. This may involve an increase in temperature that may kill the pathogen and not the insect (Anderson *et al.*, 2013b), or a decrease of temperature to

fortify insect immune responses (Bahrndorff *et al.*, 2014). Other important factors in mass rearing are insect densities and diet composition. These factors have been well studied for effects on insect development and mortality (Barragan-Fonseca *et al.*, 2018), but the effects on insect immunity responses have received only limited attention. Both too high and too low larval densities may be detrimental for individual and group immunity, as well as for growth (Meunier, 2015; Trienens and Rohlfs, 2020; Wertheim *et al.*, 2002). Furthermore, the multivarious effects of diet composition on immune function in *Drosophila* indicate a possible overlap between nutrient processing and inflammation pathways that are induced during pathogen encounters (Hemphill *et al.*, 2018). Consequently, which diet composition will optimally boost immunity and resistance (Vogel *et al.*, 2018), without inducing an inflammatory response? Thus, there is a clear need for more research into optimisation of larval density in relation to nutrient availability and the effects on immunity, especially for the insect species reared for feed, HF and BSF.

Safeguarding insect health is a vital activity for the new sector that produces insects for feed. This relates to insect welfare, insect quality as feed, economic robustness for insect producers as well as the total sector, and consumer acceptance (Saatkamp *et al.*, in press). Insect health, the state of being free from disease or injury, is challenged when insects are exposed to harmful conditions. Assessing the activation of these immune responses provides a tool to assess the conditions that challenge insect health, even well before it leads to externally visible effects. In nature, larvae of many fly species feed in aggregations on decaying materials that are extensively colonised by microorganisms (Wertheim *et al.*, 2005). Fly larvae seem to be quite resistant to diseases (Joosten *et al.*, 2020). Yet, although this may be the case, it does not mean that this remains so under mass-rearing conditions. Therefore, we need to understand how the insects resist pathogens and how we can assess early phases of pathogenic challenge. When we gather knowledge on how mass-rearing conditions may mitigate pathogenic challenge, we can prevent such events or combat them upon early signs of pathogenic infection.

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Conflict of interest

The authors declare no conflict of interest.

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