

Order Diptera as a model in the studies of insect immunity: a review

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Abstract: Order Diptera is the most important group of animals when it comes to insect immunity research. The largest share of experimental data in the group falls on the genus *Drosophila* - a model species with a number of advantages. Other crucial representatives are those of the mosquito group, as they are vectors of a number of infectious diseases infecting higher vertebrates and humans. As representatives of the genera, *Anopheles*, *Aedes* and *Culex* are very significant model organisms. In total, more than 40 dipteran species are being actively studied as models in various aspects related to immunity. Together with the representatives of the order Lepidoptera, they are the major source of the knowledge gained so far on the defense mechanisms in insects. The current review demonstrates that the studies conducted on dipteran species concern all existing mechanisms of immune defense, namely antimicrobial peptides, signaling pathways, pathogen recognition, the different types of hemocytes, antiviral and other immune responses (phagocytosis, nodulation, melanization and encapsulation).

Key words: Dipterans, antimicrobial peptides, immune responses, signaling pathways, hemocytes

1. Introduction

While vertebrates have developed a second line of immune defense (acquired immunity) that is specific and contains adaptive mechanisms targeting particular threats in insects — the most common metazoa on Earth — so far, only the innate immune system is known to react effectively against various infectious agents (Beckage, 2008). The fat body and hemocytes play the main role in the production and secretion of antimicrobial agents and activators of the cellular and humoral response (Hoffmann, 2003; Rosales, 2017). While mammalian immunity has been the subject of detailed studies for decades and many of the molecular and cellular mechanisms have already been studied in comparatively minute detail (Murphy and Weaver, 2016), the insect immune system has been the subject of extensive studies only recently. Most commonly, human-relevant organisms such as *Bombyx mori* Linnaeus, *Aedes aegypti* Linnaeus and *Anopheles* mosquitoes, as well as *Drosophila melanogaster* Meigen, whose genome has been studied in detail, are used as models.

The review aims to summarize the list of dipteran species and the main directions of the research related to innate immunity, in insects - the largest group of animals on Earth.

2. Insect immunity investigations and *Drosophila melanogaster*

The studies of mechanisms of immune defense in insects have shown that the fat body is the largest organ in the hemocoel (insect body cavity) and is the major site for production and secretion of antimicrobial peptides (Hoffmann, 2003). Most of the proteins in the hemolymph are synthesized there, with the fat body also serving as a lipid, carbohydrate, and protein reservoir. It is a multifunctional organ and a target for all major insect hormones, such as neural hormones, juvenile hormones, and ecdysone (Zheng et al., 2016); it is the site of response to microbial infections. The characteristic genes associated with immunity in the fat body are induced by infectious agents and encode antimicrobial peptides that are released into the hemolymph after expression (Hanson et al., 2019; Hanson and Lemaitre, 2020). Immediately after septic damage, the insect fat body (mammalian liver homologue) and some hemolymph cells begin to produce antimicrobial peptides. It was shown that these peptides consisted of 20–40 amino acids in *D. melanogaster* (Lemaitre and Hoffmann, 2007; Hanson and Lemaitre, 2020). These molecules are released into the hemolymph, where they act synergistically to destroy the invading microorganisms.

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One of the most common pathways of natural exposure to infection is food. The response of the immune system to noninfectious microorganisms by oral infection has been thoroughly investigated.

The common fruit fly, *D. melanogaster*, has long been recognized as a model organism in genetics and studies of insect immunity. It was Thomas Hunt Morgan, who first pioneered in 1910 the use of *Drosophila* in genetics. As a model organism, *D. melanogaster* has several distinct advantages: 1) ease of culturing under laboratory conditions, 2) short maturation time, 3) high fertility and fecundity, and 4) having only 4 pairs of chromosomes, which have been completely sequenced in 2000 (Adams et al., 2000). The first studies on this organism were related with inheritance, and demonstrated the role of chromosomes in transmission of heritable traits, sex linkage and epistasis in genetics, which brought the Nobel Prize to Morgan in physiology and medicine in 1933.

With respect to studies of insect immunity, two of the main pathways of immune response initiation, the *Toll* and IMD pathways, were elucidated first in *Drosophila* (De Gregorio et al., 2002; Yamamoto-Hino and Goto, 2016). Experiments with *Drosophila* have demonstrated the essential roles of hemocytes in insect immunity, particularly the role of plasmatocytes, which generates 95% of the circulating hemocyte pool in fruit flies (Williams, 2007; Rosales, 2017; Salminen and Vale, 2020). Plasmatocytes were also found to induce the production of antimicrobial peptides by the fat body, an action that was determined to reduce infections by *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus* (Neyen et al., 2014; Rosales, 2017).

Regarding insect immune signaling, the *Drosophila* activation of *Toll* by Spätzle is required for developmental and immune signaling; the *Toll* protein is also crucial in the embryogenesis of fruit flies (Beckage, 2008; Parthier et al., 2014). In *Drosophila*, the *Toll* pathway is responsible for host defense against the entire range of fungal pathogens and gram-positive bacteria, with inactivation of either the *Toll* or Spätzle genes compromising immune responses (Lemaitre et al., 2007). Gram-negative bacteria, in contrast, activate the IMD pathway, leading to the activation of the Relish protein and the expression of dipterin and other specific antimicrobial peptides (Levashina et al., 1998; Myllymäki et al., 2014). The third main immune signaling pathway in insects, namely JAK/STAT signaling, seems to be largely inactive in *Drosophila* and plays a role in antiviral responses only (Lemaitre et al., 2007). Interestingly, JAK/STAT-deficient flies are similarly resistant to bacterial and fungal infections as they are wild-type flies, and they express a normal AMP profile. They are, however, sensitive to infection with the *Drosophila* C virus (DCV) (Dostert et al., 2005).

When it comes to immune effectors, *Drosophila* relies on seven main classes of antimicrobial peptides (Table 1).

Drosocin and drosomycins have been first identified in fruit flies, but are commonly found among other members of order Diptera (Lemaitre and Hoffmann, 2007; Beckage, 2008). Specific AMP expression profiles are determined by the host's pathogen-recognition systems, which include peptidoglycan-binding proteins (PGRPs) that subsequently activate the *Toll* and/or IMD pathways (Steiner, 2004). In *Drosophila*, the PGRP-SA group of proteins (PGRP-SA, SD, LA, LD, LE, and LF) recognizes gram-positive bacteria, while PGRP-LC and related proteins (PGRP-LCa, LCx, and LCy) recognize gram-negative bacteria (Royet et al., 2005). Another group of recognition proteins, GNBP, acts against gram-negative bacteria and fungi, and it activates the *Toll* pathway (Kim et al., 2000). GNBP-1 and GNBP-3 contain a N-terminal domain that specifically recognizes β (1,3)-glucan and a C-terminal domain that is homologous to the catalytic domain of β -glucanase, meaning that these proteins not only bind, but also degrade β (1,3)-glucans (Gottar et al., 2006).

Antiviral immunity is particularly crucial in *D. melanogaster*, since about 40% of all flies are infected with horizontally transmitted viruses, and vertical transmission of viruses is also common in this species (Ashburner et al., 2005). Over 25 distinct *Drosophila* viruses have been identified, all of them RNA (Habayeb et al., 2006). The only antiviral effector discovered so far is RNA interference (RNAi), where specific antisense RNA oligonucleotides bind to viral RNA, and double-stranded RNA fragments are then specifically degraded by enzymes such as Dicer (Wang et al., 2006). In *Drosophila* antiviral immunity is mediated by the JAK-STAT pathway (Mussabekova et al., 2017). Mutations in the genes encoding argonaute-2 or Dicer 2, which participate in the RNAi system, increase the susceptibility of flies to a large number of RNA viruses including DCV, flock house virus (FHV), *Drosophila* X virus and others (Galiana-Arnoux et al., 2006; van Rij et al., 2006).

Although it is far from certain that innate immunity in fruit flies has been thoroughly explored, *D. melanogaster* is still one of the best model organisms studied in insect genetics and immunology (Neyen et al., 2014). The tools and methods concerning *Drosophila* are developed and continue to be used in the studies of organisms (Levashina et al., 1998; Blandin et al., 2002; Mussabekova et al., 2017).

3. Insect immunity and other important dipteran species

While the genus *Drosophila* holds the lead in investigations on dipteran immunity, there are other significant representatives of the order that are of interest, such as mosquitoes (*Anopheles*, *Aedes*, *Culex*) and other diptera

that are vectors of disease and are of interest in terms of their immune processes. For example, *Aedes aegypti* can transmit arboviruses such as dengue virus, yellow fever, Chikungunya and Zika virus, which cause serious damage in human health. Immune pathways in mosquitoes are highly conserved with respect to evolution. Due to their complex life cycles, mosquitoes are exposed to a variety of pathogens and it is quite possible that they have acquired certain characteristics during evolution due to the effects of these pathogens. Studies of immune-related molecules, pattern recognition molecules, genes involved in the melanisation pathway, and effector molecules have shown significant expansion, indicating that *Aedes aegypti* and other species have a complex innate immune systems and defense mechanisms (Wang et al., 2018). *Anopheles gambiae* Giles is a well-studied species as the main vector in the transmission of malaria and O'nyong-nyong virus. In studies of immunocompetent cells in hemolymph similar to hemocytes, they can be used as a model to study the immune response to the relationship between a vector and a pathogen (Adang et al., 2020). Other organisms, which have been well characterized are leishmaniasis vectors (the sandflies *Phlebotomus duboscqi* Neveu-Lemaire and

P. papatasi Loew) (Boulanger et al., 2004; Rosetto et al., 2003). In general, insect immunity in dipterans other than *Drosophila* has been studied mostly in medically important insects. The list of study topics is presented in Table 2 and the list of investigated dipterans (including the authors and topics) in Table 3.

The current review shows that more than 40 model species of the order Diptera have been used in insect immunity studies. Results have been obtained on a number of mosquitoes and flies in all aspects of immune protection known to date – antimicrobial peptides, hemocytes, signaling, pathogen recognition, antiviral and immune responses. The list presents a large number of authors with significant contribution to the topic. In our opinion, the number of investigated dipteran species will expand due to their importance as vectors of a number of animal and human diseases, as well as due to the advantages of *Drosophila* as a classical model species for molecular and genetic research.

4. Conclusion

Dipterans are the most significant order when it comes to insect immunity research. The data collected in this review

Table 1. Main classes of antimicrobial peptides (AMPs) in *D. melanogaster*.

AMP classes	Class members	Mol. weight	Active against
Diptericins	2	9 kDa	Gram-negative bacteria
Attacins	4	20 kDa	Gram-negative bacteria
Drosocin	1	2 kDa	Gram-negative bacteria
Drosomycins	7	5 kDa	Fungal pathogens
Cecropins	4	3.8 kDa	Gram-negative bacteria
Defensin	1	3-4 kDa	Gram-positive bacteria
Metchnikov	1	26 kDa	Fungal pathogens

Table 2. Classification of different types of immune responses and pathways.

Studied topics	Abbreviation	Specification of the immune responses.
Antimicrobial effectors	AMP	Antimicrobial peptides: drosocin, diptericin, and others.
Signaling	S	Mechanisms, relating to immune signaling: IMD, Toll, and JAK/STAT The IMD pathway: Imd, Relish, RING, BIR, and others. The Toll pathway: Toll, Pelle, Spätzle, Tube, Cactus, Dorsal, DIF, etc. The JAK/STAT pathway: DOME, Domeless, Hopscotch, and others.
Pathogen recognition	PR	PGRPs (peptidoglycan recognition proteins), β -1,3-glucan recognition proteins, immunoreceptors, integrins.
Hemocytes	H	Lamellocytes, proleukocytes, plasmatocytes, and others.
Immune responses	IR	Phagocytosis, nodulation, melanisation, and encapsulation.
Antiviral responses	AR	RNA interference: the enzymes Argonaute, R2D2, Dicer, and others.

Table 3. List of other dipterans (without *Drosophila melanogaster*, reviewed above), authors who have investigated their immunity, and studied topics (according to Table 2).

Dipteran species	Abbreviation of the studied topics (Authors)
<i>Aedes aegypti</i>	H (Kaaya and Ratcliffe, 1982); AMP (Lowenberger, 2001); H (Hillyer and Christensen, 2002); H, S (Bartholomay et al., 2004); S (Bian et al., 2005); H (Castillo et al., 2006); S (Waterhouse et al., 2007); S (Ramirez et al., 2019); S (Wang et al., 2018); AMP (Zhang et al., 2017)
<i>Aedes albopictus</i> Skuse	S (Li et al., 2007); S (Beck et al., 2007)
<i>Aedes triseriatus</i> Skuse	PR (Ludwig et al., 1989, 1991); S (Blitvich et al., 2002); S (Beck et al., 2007)
<i>Aedes trivittatus</i> Coquillett	H, IR (Forton et al., 1985)
<i>Anopheles gambiae</i>	H (Foley, 1978); AR (Barillas-Mury et al., 1996); AMP, S (Dimopoulos et al., 1997); S (Barillas-Mury et al., 1999); IR (Levashina et al., 2001); S (Christophides et al., 2004); S (Osta et al., 2004); IR (Moita et al., 2005); H (Castillo et al., 2006); IR (Whitten et al., 2006); S (Riehle et al., 2006); S (Waterhouse et al., 2007); PR (Dong and Dimopoulos, 2009); AR (Fu et al., 2017); S (Zakovic and Levashina, 2017); H, IR (Adang et al., 2020); PR (Dong et al., 2020)
<i>Anopheles dirus</i> Peyton & Harrison	IR (Somboon et al., 1999)
<i>Anopheles quadrimaculatus</i> Say	AMP (Ham et al., 1994); IR (Liu et al., 1998)
<i>Anopheles stephensi</i> Liston	H (Foley, 1978); AMP (Luckhart et al., 1998, 2003)
<i>Armigeres subalbatus</i> Coquillett	IR (Ham et al., 1994); IR (Zhao et al., 1995); IR (Shiao et al., 2001); H (Bartholomay et al., 2004); PR (Wang et al., 2004); IR (Wang et al., 2005)
<i>Calliphora vomitoria</i> L.	H (Franchini et al., 1996); H, IR (Kind, 2008); H (Kind, 2010);
<i>Calliphora vicina</i> (erythrocephala) Robineau-Desvoidy	H (Zachary et al., 1973); S (Pau and Kelly, 1975); H (Zachary et al., 1975); H (Kind, 2012); AMP, IR (Crowley and Houck, 2002); AMP (Yakovlev, 2011)
<i>Ceratitis capitata</i> Wiedemann	IR, AMP (Postlethwait et al., 1998); H (Lavine and Strand, 2002); AMP (Rosetto et al., 2003a, b); AMP, S (Mura and Rui, 2018)
<i>Ceratitis rosa</i> Karsch	AMP (Rosetto et al., 2003)
<i>Chironomus luridus</i> Strenzke	IR (Götz and Vey, 1974); IR (Götz et al., 1987)
<i>Chironomus plumosus</i> L.	IR (Götz et al., 1987); AMP (Lauth et al., 1998)
<i>Chironomus riparius</i> Meigen	IR (Götz and Vey, 1974); IR (Götz et al., 1987)
<i>Chrysomya megacephala</i> Fabricius	IR (Zhao et al., 2009)
<i>Culicoides sonorensis</i> Wirth & Jones	AMP, S (Nayduch et al., 2014); AR (Drolet et al., 2005)
<i>Culex quinque fasciatus</i> Say	AMP (Domnas et al., 1974); H (Kaaya and Ratcliffe, 1982); AR (Girard et al., 2005, 2007)
<i>Culex pipiens</i> L.	IR (Poinar and Leutenegger, 1971); H (Drif and Brehelin, 1983); S (Beck et al., 2007)
<i>Dacus cucurbitae</i> Coquillett	IR (Postlethwait et al., 1998)
<i>Dacus dorsalis</i> Hendel	IR (Postlethwait et al., 1998)
<i>Drosophila algonquin</i> Sturtevant & Dobzhansky	IR (Nappi, 1975)
<i>Culex tarsalis</i> Coquillett	S (Beck et al., 2007)
<i>Glossina morsitans</i> Wiedemann	H (Kaaya and Ratcliffe, 1982); H (Kaaya et al., 1986); AMP (Kaaya et al., 1987); AMP (Hao et al., 2003)
<i>Glossina austeni</i> Newstead	H (Kaaya and Ratcliffe, 1982)
<i>Glossina</i> sp. Wiedemann	H (Kaaya et al., 1986)
<i>Hermetia illucens</i> L.	AMP (Park et al., 2014)

Table 3. (Continued).

<i>Lucilia sericata</i> Meigen	H (Kaaya and Ratcliffe, 1982)
<i>Lutzomyia longipalpis</i> Lutz & Neiva	S, IR (Heerman et al., 2015); AMP, S (da Silva Gonçalves et al., 2019)
<i>Musca domestica</i> L.	S (Tsukamoto et al., 1992); IR (Timothy et al., 2017)
<i>Phlebotomus duboscqi</i>	AMP (Boulanger et al., 2004)
<i>Phlebotomus papatasi</i>	AMP, IR (Rosetto et al., 2003)
<i>Phormia terraenovae</i> Robineau-Desvoidy	AMP (Dimarcq et al., 1986, 1988, 1990); AMP (Keppi et al., 1986, 1989); AMP (Lambert et al., 1989); AMP (Hoffmann and Hoffmann, 1990)
<i>Sarcophaga bullata</i> Parker	S (Chase et al., 2000); IR (Franssens et al., 2005); S (Xie et al., 2007)
<i>Sarcophaga peregrine</i> Robineau-Desvoidy	PR (Komano et al., 1980); AMP (Okada and Natori, 1983); S (Matsuyama and Natori, 1988); AMP (Kanai and Natori, 1989)
<i>Sarcophaga ruficornis</i> Fabricius	H (Pal and Kumar, 2014)
<i>Simulium damnosum</i> Theobald	IR (Ham et al., 1994)
<i>Simulium ornatum</i> Meigen	IR (Ham et al., 1994)
<i>Stomoxys calcitrans</i> L.	H (Kaaya and Ratcliffe, 1982)
<i>Tipula paludosa</i> Meigen	H (Carter and Green, 1987)
<i>Zaprionus indianus</i> Gupta	IR, H (Kacsoh et al., 2014)

summarize valuable information related to the species composition of the studied dipterans and the specific defense mechanisms reported by a number of authors in their studies. The current publication list is a convenient

roadmap for professionals in the field who can access in an expedient and fast way to a basic information on dipteran immunity studies in a synthesized presentation.

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