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Innate immune memory in invertebrates: Concept and potential mechanisms

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ABSTRACT

Invertebrates are the protagonists of a recent paradigm shift because they now show that vertebrates are not the only group with immune memory. This review discusses the concept of immune priming, its characteristics, and differences with trained immunity and immune enhancement. We include an update of the current status of immune priming within generations in different groups of invertebrates which now include work in 5 Phyla: Ctenophora, Cnidaria, Mollusca, Nematoda, and Arthropoda. Clearly, few Phyla have been studied. We also resume and discuss the effector mechanism related to immune memory, including integrating viral elements into the genome, endoreplication, and epigenetics. The roles of other elements are incorporated, such as hemocytes, immune pathways, and metabolisms. We conclude that taking care of the experimental procedure will discern if results provide or do not support the invertebrates' immune memory and that regarding mechanisms, indeed, there are no studies on the immune memory mechanisms, this is how specificity is reached, and how and where the immune memory is stored and how is recall upon subsequent encounters. Finally, we discuss the possibility of having more than one mechanism working in different groups of invertebrates depending on the environmental conditions.

1. Innate immune memory: a problem of concepts?

Immune memory is one of the central concepts in immunology. The paradigm was that only mandibulated vertebrates present immune memory through somatic rearrangements and clonal expansion of lymphocytes. However, in recent times, this concept has changed, and it has been considered that immune memory is widely distributed in animals. Given that the mechanisms behind the process of immune memory in invertebrates are not known, it is defined as an immune system's ability to store and recall information of a previously encountered pathogen or parasite upon a subsequent specific exposure (Milutinovic and Kurtz, 2016). Under this concept, immune memory in invertebrates should be specific at the level of species or strain (Contreras-Garduño et al., 2016). We agree with other researchers (Little and Kraaijeveld, 2004; Little et al., 2008; Milutinovic and Kurtz, 2016) to define innate memory from a phenomenological point of view without invoking mechanisms, because mechanisms may differ between taxonomic groups, and perhaps, even within insect groups (Kurtz and Armitage, 2017).

As a young research topic, there are potential confounding concepts in the literature that may impede fully developing the subject of innate immune memory (Lanz-Mendoza and Contreras-Garduño, 2018). Here, we define key concepts. Innate immune memory is similar to immune priming in invertebrates, and immune priming was the first formal concept proposed (Kurtz, 2005), so immune priming and innate immune memory are synonyms. First, priming is mainly defined as an immune challenge alone that does not include a subsequent immune activation (Contreras-Garduño et al., 2016; Ferro et al., 2019). Priming alone is induced both by biotic and abiotic environmental stimuli (Kelly and O'Neill, 2015) and is mandatory in the experimental procedure to test innate immune memory (Little and Kraaijeveld, 2004; Contreras-Garduño et al., 2016) and adaptive immune response. Second, it is essential to note that immune memory should be tested by comparing immune response, survival, and or parasite load after homologous (similar) challenge against heterologous (different) challenges at the level of parasite or pathogen strain or species (Little and Kraaijeveld, 2004). An increase of immune response and survival and decrease of parasite load in homologous challenge compared with heterologous

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challenges suggest evidence for immune memory. The lack of differences between homologous and heterologous challenges means immune enhancement but not immune memory.

The first formal study that tested the possibility of immune memory within generations in invertebrates used the crustacean *Macrocyclops albidus* against the Cestoda *Schistocephalus solidus* (Kurtz and Franz, 2003). The experimental procedure included homologous and heterologous challenges. A first infection was carried out with *S. solidus*, and two days later one group (homologous) was challenged with a genetically similar parasite (full sibs) or nongenetically (heterologous) similar parasite (nonfull sibs). Authors found a reduced reinfection success of *S. solidus* and less host intensity of reinfection in homologous than heterologous challenge (Kurtz and Franz, 2003). Since this pioneering study, researchers have been interested in testing innate immune memory within generations (Table 1). Although studies claim to test the innate immune memory, some of them did it, but not others. The fundamental difference is the experimental procedure. For example, in mosquitoes, a study compared resistance against dual homologous challenge with *Plasmodium berghei* (a lethal dose followed by a lethal one) or the first feed with non-infected blood followed by a lethal dose of *P. berghei*. Authors found evidence for immune priming: higher immune response and less parasite load in homologous but not heterologous challenges, and these results were not dependent on endosymbiotic bacteria (Contreras-Garduño et al., 2015). Another study in mosquitoes suggested support for innate immune memory. However, in this case, the parasite reduction was dependent on endosymbiotic bacteria (Rodrigues et al., 2010). In this case, it is not clear why and how endosymbiotic bacteria provide specific protection against parasites and pathogens and the protections seems not derive from immune response but bacterial interaction. Finally, another example of immune enhancement in mosquitoes was carried out in *Aedes aegypti*. In this study, authors found that infected larvae with *Bacillus thuringiensis* or *Enterobacter ludwigii* were better protected against Zika than Dengue viruses at adulthood (Carlson et al., 2020). In this case, the protection is due to a cross protection or immune training but not immune memory.

In vertebrates (Netea et al., 2011; Netea and van der Meer, 2017; Gourbal et al., 2018) and invertebrates (Yan et al., 2020; Kulkarni et al., 2021), immune memory and immune enhancement has been used as synonym and defined together as immune training or immune priming. However, on the one hand, it is critical to note that immune enhancement and immune training are, indeed, synonyms of a nonspecific response because immune training has been defined as improved immune protection without specifying that it should be specific (Netea et al., 2011; Netea and van der Meer, 2017). On the other hand, innate immune memory (mostly referred to as immune priming) provides long-lasting protection and is a specific immune resistance (for review, see Milutinovic and Kurtz, 2016; Contreras-Garduño et al., 2016). In the oyster *Crassostrea gigas*, it lasts for at least 5 months (Lafont et al., 2017) and in *Tribolium castaneun* (Thomas and Rudolf, 2010) and *Anopheles gambiae* (Brown et al., 2019), persists from larvae to adults. In addition, in *T. castaneun* (Roth et al., 2009) and *Tenebrio molitor* (Dhinaut et al., 2018), the immune protection was specific at the bacterial strain level.

Now, evidence for immune priming or innate immune memory has been reported in arthropods, ctenophores, mollusks, cnidarians and nematodes (Table 1). We propose only to use the term *memory* if the immune response is specific and long-lasting. Otherwise, if it is nonspecific, it should be termed immune enhancement or immune training (see a similar rationale in Boraschi and Italiani, 2018; Lanz--Mendoza and Contreras-Garduño, 2018). In invertebrates, immune priming is a synonym of immune memory (Kurtz, 2005; Milutinovic and Kurtz, 2016). In vertebrates and sometimes invertebrates, immune training has been used as a synonym for immune enhancement. For example, in mice, heterologous challenge (training immunity) favored the mice's immune response and survival compared with untrained mice (Ciarlo et al., 2020). Hence, immune memory and immune training seem to be the opposites of a continuum (Cooper, 2016; Pradeu and Du Pasquier, 2018): from unspecific immune training (i.e. Covián, 2020; Paris et al., 2020) to specific innate immune memory (i.e. at the level of strain such as in Kurtz and Franz, 2003; Roth et al., 2009). This confusion in the use of terms may explain why the immune memory seems to be specific or non-specific (but see also the importance of time lapse between the first and second encounter in Rowley and Powell, 2007). More attention should be paid on the experimental designs to include the authors results within the topic of immune memory or immune enhancement, and if it is tested the immune memory, whether this provide full, partial or no support for immune memory (Table 1).

The immune protection occurs not only within but across generations. In the pioneering study, the offspring of *Daphnia magna* were challenged with *Pasteuria ramose,* and its mothers were or were not confronted (Little et al., 2003). Results revealed offspring specific protection at a strain according to their mothers' immune challenge. This phenomenon is referred to as immune priming across generations, transgenerational immune priming (Little and Kraaijeveld, 2004), or innate memory across generations (for review, see Tetreau et al., 2019). Again, it is important to consider that this phenomenon should not be confused with immune enhancement across generations. The main difference between immune enhancement and innate immune memory across generations is the experimental procedure. Homologous and heterologous challenges should be used to test innate immune memory, and to test immune enhancement, only heterologous challenges could be used. A recent review explores the occurrence of immune memory across generations (for review see Milutinovic and Kurtz, 2016; Contreras-Garduño et al., 2016; Tetreau et al., 2019; Vilcinskas, 2021). Bellow, we will explore the potential mechanisms of immune memory within generations.

2. Mechanisms

The immune memory is central to the invertebrate capacity of surviving in diverse environments. Therefore, we must expect some characteristics in the presumed mechanism: a) there must be a system to keep the information of the previous encounter; b) there must a rapid recall response (Melillo et al., 2018); c) with some degree of specificity; d) long-lasting memory. Unfortunately, the precise mechanism (s) that allow the development of invertebrates' immune memory after encountering a pathogen is unknown. However, in recent years several exciting clues have been observed:

1) Integration of viral elements into the genome

The integration of viral components in the host cells can provide an RNA interference response amplification that prevents a second infection with the same virus. Tassetto et al. (2017) described a mechanism of RNAi amplification and dissemination in *Drosophila* to infection with Sindbis virus (SINV). Hemocytes take up dsRNA from infected cells and, using a reverse transposon transcriptase, produce virus-derived complementary DNAs (vDNA). These vDNAs provide a template of de novo synthesis of secondary viral siRNA (vsRNA). These molecules are secreted in exosome-like vesicles conferring passive protection against virus challenge in naïve animals. They found that hemocytes accumulate specific secondary vsRNAs in an AGO2- dependent manner in response to infection. Hemocytes isolated three weeks after infection still contained SINV-derived vDNAs, indicating that vDNAs not only allow for amplification of antiviral responses but also provide immunological memory and long-lasting response. The authors also suggest that vsRNAs might be packaged into exosome-like vesicles (ELVs). Indeed, *Drosophila* flies with hemocytes that cannot produce ELVs have higher viral titers after SINV infection than wild-type flies. ELVs isolated from the hemolymph of SINV-infected flies transferred passive antiviral immunity when injected into naive flies. A similar genomic structure has been observed in the *Ae. aegypti*-derived cell line Aag2 with endogenous viral elements (Whitfield et al., 2017).

Table 1
Experimental studies that tested the invertebrate immune memory within generations. We searched for studies published since 2003 by using the word "invertebrate immune priming" in Web of Science and found 1,291 papers. From this, 65 were reviews or comments and were excluded. From 1,226 research papers, we excluded those: (a) published in vertebrates; (b) about immune priming across generations; and (c) that did not include homologous and heterologous challenge, a requirement to test innate immune memory. We found only 85 papers that fit these criteria. Table shows, host Phylum, Subphylum and species, immune challenge, immune response (survival, parasite elimination and/or potential immune mechanism involved in parasite elimination), and if such research provide (Yes; 77.64%) or not (No; 7.05%) evidence for invertebrate immune memory. Some papers reported at the same time mixed evidence for immune priming (Yes/No; 15.31%), and this may be explained by host age, sex Garbutt et al. (2014) and/or pathogen species and host reproductive costs (Contreras-Garduño et al., 2016; Contreras-Garduño et al., 2019). The table shows that innate immune memory has been tested in hosts that belong to 5 Phyla: Ctenophora, Cnidaria, Mollusca, Nematoda, and Arthropoda, being this last one the more studied so far, and the subphylum Exapoda the most studied (50.58%) followed by Crustacea (25.88%) and Mollusca (17.64%). Given the enormous contribution of invertebrates to animal biodiversity (more than 90% of all animals; May, 1988; Mora et al., 2011), more Phyla should be considered. Regarding parasites, most living forms have been taken into account: Virus, Bacteria, Fungi, Protozoa, and Animalia (Nematoda). More research is needed to consider other organisms such as parasitoids (i. e., parasitoid wasps). Most papers have recorded survival (74.11%), followed by immune response markers and parasites, viruses, protozoa, or bacterial elimination. Very few papers have tested all components simultaneously (20%), and only 2.35% have tested if the immune response is biphasic (see Contreras-Garduño et al., 2016). None study has demonstrated where the immune memory is stored and how it is recalled after a first encounter.

Phylum (subphylum)	Host species	Challenge	Response	Evidence of innate memory	References
Mollusca	*Haliotis diversicolor*	*Vibrio harveyi*	Survival Differential gene expression (i.e. calmodulin, lysozyme, alpha-glucosidase, metabolism and Galactose-specific lectin nattecin)	Yes	Yao et al. (2021)
Arthropoda (Hexapoda)	*Tenebrio molitor*	*Rhabdits regina*	Survival Metabolic rate	No	Méndez-López et al. (2021)
	Anopheles albimanus	*Plasmodium berghei*	Parasite elimination Differential gene expression related with metabolism, immune response and epigenetics	Yes	Maya-Maldonado et al. (2021)
Arthropoda (Crustacea)	*Litopenaeus vannamei*	*Vibrio alginolyticus* *Vibrio harveyi* *Vibrio alginolyticus*	Survival Phagocytic activity Virus elimination	Yes	Hsu et al. (2021)
Arthropoda (Hexapoda)	*Anopheles gambiae*	*Serratia fonticola* *Enterobacter* sp.	Survival Differential gene expression (i.e. TEP2, proPhenoloxidase, FREPs and AGO2)	Yes	Kulkarni et al. (2021)
Arthropoda (Hexapoda)	*Aedes aegypti*	Dengue virus (DV)	Virus elimination DCR-2 AGO-2 R2D2 VAGO	Yes	Vargas et al. (2020)
Mollusca	*Crassostrea gigas*	*Vibrio splendidus*	Differential gene expression (i.e. MPK, TLR, cathepsin, MyD88, CgTIMP and CgPRTP)	Yes	Wang et al. (2020)
Arthropoda (Chelicerata)	*Lycosa cerrofloresiana* *Centruroides granosus*	*Escherichia coli*	Survival Bacterial elimination	No	Gálvez et al. (2020)
Arthropoda (Hexapoda)	*Tribolium castaneum*	*Bacillus thuringiensis bv tenebrionis and Bacillus thuringiensis 407*	lncRNAs involved for example in metabolism methyltransferases c-type lectin and toll-like receptors	Yes	Ali and Halim (2020)
Arthropoda (Hexapoda)	*Anopheles gambiae*	*Escherichia coli*	Survival Parasite elimination Hemocytes count Phagocytic capacity Differential gene expression (i.e. Nitric oxide synthase, proPhenoloxidase, TEP1, RPS17, CEC1, and LYSC1)	No	Powers et al. (2020)
Mollusca	*Crassostrea gigas*	Ostreid herpesvirus 1 (OsHV-1)	Survival Viral elimination Differential gene expression (i.e. metabolism, apoptosis, antiviral pathways, Jak/Stat, Toll, and antimicrobial peptides)	Yes	Lafont et al. (2020)
Arthropoda (Crustacea)	*Scylla paramamosain*	*Vibrio parahaemolyticus*	Survival Differential gene expression (i.e. TLR, Cactus, Dorsal, Pelle, Dscam, Myd88, Spaetzle, ALF, Crustin, Arasin and Hyastatin)	Yes	Zhang et al. (2020)
Arthropoda (Crustacea)	*Scylla paramamosain*	*Vibrio parahaemolyticus*	Survival Phagocytosis Hemocytes count Phenoloxidase activity Bacterial elimination	Yes	Yang et al. (2020)
Arthropoda (Crustacea)	*Armadillidium vulgare*	*Salmonella enterica* and Wolbachia	Survival	Yes/No	Prigot-Maurice et al. (2020)
Nematoda	*Caenorhabditis elegans*	*S. aureus,* *P. aeruginosa* and *S. typhimurium*	Survival Serotonin Dopamine Insulin-like signaling pathway DAF genes	Yes	Yan et al. (2020)
Mollusca	*Biomphalaria glabrata* and *Biomphalaria straminea*	*Schistosoma mansoni*	Hemocyte count Phenoloxidase activity	Yes	De Melo et al. (2020)

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Table 1 (*continued*)

Phylum (subphylum)	Host species	Challenge	Response	Evidence of innate memory	References
			Melanization FREP expression		
Arthropoda (Crustacea)	*Eriocheir sinensis*	*Aeromonas hydrophila*	Survival Total hemocyte count Phenoloxidase, proPhenoloxidase and lysozyme activities in hemolymph Phagocytic activity of hemocytes Crustin Anti-lipopolysaccharide factor	Yes	Wang et al. (2019)
Arthropoda (Hexapoda)	*Anopheles gambiae*	*Escherichia coli, Enterobacter* sp., *S. aureus and Plasmodium yoelii*	Survival Bacterial infection intensity Hemocytes Phagocytic activity Differential gene expression (i.e. cecropin A, Lysozyme C1, Signal transducer and activator of transcription-A, proPhenoloxidase, and Tep1)	Yes/No	Brown et al. (2019)
Arthropoda (Hexapoda)	*Galleria mellonella*	*Candida albicans*	Survival Differential Protein profiles Antimicrobial peptides (Galiomycin, hemolin, cecropin, moricin-like like peptide A, Lysozyme, antifungal peptide, gallerimycin and anionic peptide 2) Lysozyme type activity Apolipophorin III Yeast alimination	Yes	Vertyporokh et al. (2019)
Arthropoda (Hexapoda)	*Galleria mellonella*	*Bacillus thuringiensis*	Coagulation Phenoloxidase activity	Yes	Sułek et al. (2019)
Mollusca	*Mytilus galloprovincialis*	*Vibrio splendidus*	Differential gene expression (i.e. heat shock protein, control and inhibition of reactive oxygen species production, vitellogenin and lysozyme) Granulocytes Hyalinocites Hemocyte count	Yes	Rey-Campos et al. (2019)
Mollusca	*Biomphalaria glabrata*	*Schistosoma mansoni* and *Schistosoma rodhaini*	Survival Parasite elimination FREPs TEPs	Yes	Pinaud et al. (2019)
Arthropoda (Hexapoda)	*Tribolium castaneum*	*Bacillus thuringiensis*	Survival Antibacterial activity	Yes	Khan et al. (2019)
Arthropoda (Hexapoda)	*Bombyx mori*	*Photorhabdus luminescens*	Differential gene expression (i.e. metabolism, antioxidant activity, lipid transport, RNA processing and modification, chromatin structure and dynamics, etc.)	Yes	Yi et al. (2019)
Arthropoda (Crustacea)	*Cherax quadricarinatus*	White spot syndrome virus (WSSV)	Survival proPhenoloxidase Dscam Virus elimination Phagocytosis	Yes	Ng et al. (2019)
Arthropoda (Hexapoda)	*Tenebrio molitor*	*Metarhizium brunneum*	Survival CO_2	Yes	Contreras-Garduño et al. (2019)
Arthropoda (Hexapoda)	*Aedes aegypti*	Dengue Virus (DENV)	NS1 protein Hnt gene expression Virus elimination	Yes	Serrato-Salas et al. (2018b)
Arthropoda (Hexapoda)	*Anopheles albimanus*	*Plasmodium berghei*	DNA synthesis DNA concentration Cell viability Cell number TEP1, Hnt, LRIM1 and proPhenoloxidase expression		Cime-Castillo et al. (2018)
Arthropoda (Hexapoda)	*Drosophila melanogaster*	Drosophila C virus (DCV)	Survival Viral elimination	Yes	Mondotte et al. (2018)
Arthropoda (Hexapoda)	*Tenebrio molitor*	*Staphylococcus aureus, Bacillus thuringiensis, Escherichia coli and Serratia entomophila*	Survival Antimicrobial activity, ProPhenoloxidase, Phenoloxidase, Phagocytosis	Yes/No	Dhinaut et al. (2018)
Arthropoda (Hexapoda)	*Tenebrio molitor*	*Metarhizium bruneum* *Serratia marcescens, Bacillus thuringiensis*	Survival	Yes/No	Medina-Gómez et al. (2018a, b)
Arthropoda (Crustacea)	*Fenneropenaeus chinensis*	White Spot Syndrome Virus (WSSV)	Survival *Dscam*	Yes	Cao et al. (2018)
Arthropoda (Hexapoda)	*Tribolium castaneum*	*Bacillus thuringiensis*	Survival Super oxide dismutase	Yes	Ferro et al. (2017)
Arthropoda (Hexapoda)	*Galleria mellonella*	*Bacillus thuringiensis*	Survival CecropinB Apolipophorin III Blastopores Bacterial elimination	Yes	Taszlow et al. (2017)

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Table 1 (*continued*)

Phylum (subphylum)	Host species	Challenge	Response	Evidence of innate memory	References
Mollusca	*Haliotis tuberculata*	*Vibrio harveyi*	Survival Hemocyte count Phagocytosis Bacterial elimination	Yes	Dubief et al. (2017)
Arthropoda (Hexapoda)	*Tribolium castaneum*	*Bacillus thuringiensis*	Survival	Yes	Futo et al. (2017)
Arthropoda (Hexapoda)	*Galleria mellonella* and *Parasemia plantaginis*	*Serratia marcescens*	Survival Phenol oxidase Reactive oxygen species Lytic activity Extracelular proteases 6-Tox CecropinA CecropinB PGRP2 Defensin Bacterial elimination	Yes	Mikonranta et al. (2017)
Mollusca	*Crassostrea gigas*	Ostreid herpes virus (OSHV-1)	Survival Poly(I:C)	Yes	Lafont et al. (2017)
Arthropoda (Hexapoda)	*Tribolium castaneum*	*Bacillus thuringiensis*	Gene expression (i.e. IMD, JNK, Toll, Jak/Stat, Antimicrobial peptides, Super oxide dismutase, Reactive oxygen species, Lectin, Lysozyme)	Yes	Greenwood et al. (2017)
Arthropoda (Hexapoda)	*Tenebrio molitor*	*Metarhizium bruneum* *Micrococcus lysodeikticus*	Survival Methylation of DNA and RNA		Castro-Vargas et al. (2017)
Arthropoda (Crustacea)	*Daphnia magna*	*Pasteuria ramosa*	Parasite elimination	No	Duneau et al. (2016)
Mollusca	*Biomphalaria glabrata*	*Schistosoma mansoni*	Parasite elimination Encapsulation Pathogen recognition receptors Antimicrobial peptides Cellular and epithelial immune response 23 differential proteins	Yes	Pinaud et al. (2016)
Arthropoda (Hexapoda)	*Tribolium castaneum*	*Bacillus thuringiensis*	Survival	Yes/No	Khan et al. (2016)
Arthropoda (Hexapoda)	*Galleria mellonella*	*Photorhabdus luminescens*	Survival Hemocyte count Phagocytosis Encapsulation Cecropin Galiomycin Gallerimycin	Yes	Wu et al. (2016)
Arthropoda (Hexapoda)	*Tribolium castaneum*	*Bacillus thuringiensis*	Survival Bacterial elimination	Yes	Futo et al. (2016)
Mollusca	*Crassostrea gigas*	*Vibrio splendidus*	Extracellular superoxide dismutase Pathogens' molecular associated patterns	Yes	Liu (2016)
Arthropoda (Hexapoda)	*Aedes aegypti*	*Escherichia coli*	Survival Phenoloxidase Nitric oxide Cecropin Attacin Defensin Antibacterial activity Bacterial elimination	Yes	Moreno-García et al. (2015)
Cnidaria	*Exaiptasia pallida*	*Vibrio coralliilyticus*	Survival 32 differentially expressed proteins were identify	Yes	Brown and Rodriguez-Lannety (2015)
Arthropoda (Hexapoda)	*Anopheles albimanus*	*Plasmodium berghei*	Survival Antimicrobial peptides Endoreplication Parasite elimination	Yes	Contreras-Garduño et al. (2015)
Arthropoda (Hexapoda)	*Bombyx mori*	*Pseudomonas aeruginosa, Candida albicans, Staphylococcus aureus* and *Lactiplantibacillus plantarum*	Survival Cecropin A Moricin I kappa B kinase Cytokine paralytic peptide	Yes	Miyashita et al. (2015)
Arthropoda (Hexapoda)	*Bombyx mori*	*Photorhabdus luminescens TT01, Photorhabdus luminescens H06* and *Bacillus thuringiensis* HD-1	Survival Phagocytosis Hemocyte identification and characterization Antibacterial activity Phenoloxidase activity	Yes	Wu et al. (2015)
Arthropoda (Hexapoda)	*Bombyx mori*	*Photorhabdus luminescens TT01*	Survival Phagocytosis Encapsulation rate	Yes	Wu et al. (2014)

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Table 1 (*continued*)

Phylum (subphylum)	Host species	Challenge	Response	Evidence of innate memory	References
			Hemocyte density Granular density		
Mollusca	*Crassostrea gigas*	*Vibrio splendidus*	Hemocyte countphagocytosis, CgIntegrin, CgPI3K, CgRho J, CgMAPKK, CgRab32, CgNADPH, CgBMP7	Yes	Zhang et al. (2014)
Arthropoda (Hexapoda)	*Parasemia plantaginis*	*Serratia marcescens* *E. coli*	Survival Reactive oxygen species activity Lytic activity	Yes	Mikonrata et al. (2014)
Arthropoda (Hexapoda)	*Anopheles albimanus*	*Plasmodium berghei*	Survival Parasite elimination	Yes	Contreras-Garduño et al. (2014)
Arthropoda (Hexapoda)	*Bombyx mori*	*Escherichia coli* O-157:H7, *Staphylococcus aureus* NCTC8325-4 and *Serratia marcescens* 2170	Survival Antibacterial activity Cecropin Hemocyte count Phosphorylated c-Jun N-terminal kinase	Yes	Miyashita et al. (2014)
Arthropoda (Hexapoda)	*Lasius niger* *Formica selysi*	*Beauveria bassiana*	Survival	Yes/No	Gálvez and Chapuisat (2014)
Arthropoda (Crustacea)	*Litopenaeus vannamei*	*Bacillus subtilis* DB431, BB80 and White Spot Syndrome Virus (WSSV)	Survival Virus elimination	Yes	Valdez et al. (2014)
Arthropoda (Hexapoda)	*Tribolium castaneum*	*Bacillus thuringiensis*	Survival	Yes	Milutinović et al. (2014)
Arthropoda (Crustacea)	*Cherax quadricarinatus*	*White spot syndrome virus*	Survival Dscam Virus elimination	Yes	Ng et al. (2014)
Arthropoda (Crustacea)	*Daphnia magna*	*Pasteuria ramos*	Bacteria elimination	Yes/No	Garbutt et al. (2014)
Arthropoda (Hexapoda)	*Camponotus pennsylvanicus*	*Serratia marcescens*	Survival	No	Rosengaus et al. (2013)
Mollusca	*Chlamys farreri*	*Vibrio anguil larum* *Micrococcus luteus*	Survival C-lectin	Yes	Wang et al. (2013)
Ctenophora	*Mnemiopsis leidyi*	Listonella anguillarum Planococcus citreus	Differential gene expression (adenosylhomocysteinase, proPhenol oxidase, Superoxide Dismutase and Complement factor B1)	Yes	Bolte et al. (2013)
Mollusca	*Biomphalaria glabrata*	Schistosoma mansoni	Parasite elimination	Yes	Portela et al. (2013)
Arthropoda (Hexapoda)	*Drosophila melanogaster*	*Streptococcus pneumoniae*	Survival Toll and IMD pathways Phagocytosis	Yes	Christofi and Apidianakis (2013)
Arthropoda (Hexapoda)	*Drosophila melanogaster*	*Drosophila C Virus*	Survival Viral elimination	No	Longdon et al. (2013)
Arthropoda (Crustacea)	*Litopenaeus vannamei*	*Vibrio alginolyticus*	Survival Phagocytosis Parasite elimination Hemocyte count Phenoloxidase Superoxide dismutase Respiratory burst Lysozyme Cell Proliferation Mitotic index	Yes	Lin et al. (2013)
Arthropoda (Hexapoda)	*Formica selysi*	*Beauveria bassiana*	Survival	No	Reber and Chapuisat (2012)
Arthropoda (Crustacea)	*Daphnia magna*	*Pasteuria ramosa*	Parasite infection	Yes	McTaggart et al. (2012)
Arthropoda (Hexapoda)	*Plodia interpunctella*	Plodia interpunctella granulosis virus	Proportion of infection	Yes	Tidbury et al. (2011)
Arthropoda (Crustacea)	*Penaeus monodon*	White Spot Syndrome Virus	Survival, viral elimination,LGBP and STAT	Yes	Kwang (2011)
Arthropoda (Crustacea)	*Litopenaeus vannamei*	*Vibrio harveyi* and *Bacillus subtilis*	Survival Phagocytosis Antibacterial activity Hemocytes count	Yes	Pope et al. (2011)
Arthropoda (Crustacea)	*Litopenaeus vannamei*	*Vibrio harveyi, Vibrio alginolyticus* and *Vibrio anguillarum*	Phagocytosis Antibacterial activity	Yes/No	Powel et al. (2011)
Arthropoda (Hexapoda)	*Tribolium confusum*	*Gregarina minuta*	Survival Proportion of infection	Yes	Thomas and Rudolph (2010)
Arthropoda (Crustacea)	*Porcellio scaber*	*Bacillus thuringiensis*	Survival Phagocytosis	Yes	Roth and Kurtz (2009)
Arthropoda (Hexapoda)	*Tribolium castaneum*	*Escherichia coli*, *Bacillus thuringiensis* and *Bacillus subtilis*	Survival	Yes/No	Roth et al. (2009)
Mollusca	*Chlamys farreri*	*Listonella anguillarum*	Survival Phenoloxidase-like enzyme Superoxide dismutase Acid phosphatase Phagocytosis	Yes	Cong et al. (2009)
Arthropoda (Crustacea)	*Penaeus monodon*	Viral proteins and White spot syndrome virus	Survival Virus elimination	Yes	Sarathi et al. (2008)

(*continued on next page*)

Table 1 (*continued*)

Phylum (subphylum)	Host species	Challenge	Response	Evidence of innate memory	References
Mollusca	*Chlamys farreri*	*Listonella anguillarum*	Survival, phagocytosis,phenoloxidase-like enzyme, acid phosphatase and superoxide dismutase	Yes	Cong et al. (2008)
Arthropoda (Crustacea)	*Fenneropenaeus chinensis*	Viral proteins and White spot syndrome virus	Survival Phenoloxidase Superoxide dismutase	Yes	Kumar et al. (2008)
Arthropoda (Hexapoda)	*Drosophila melanogaster*	*Streptococcus pneumoniae* and *Beauveria bassiana*	Survival Toll Defensin Bacterial elimination	Yes/No	Pham et al. (2007)
Arthropoda (Crustacea)	*Procambarus clarkii*	Viral proteins and White spot syndrome virus	Survival	Yes	Jha et al. (2006)
Arthropoda (Crustacea)	*Penaeus monodon*	Viral proteins	Survival	Yes	Witteveldt et al. (2004)
Arthropoda (Crustacea)	*Macrocyclops albidus*	*Schistocephalus solidus*	Survival Parasite elimination	Yes	Kurtz and Franz (2003)

On the other hand, the sequences of endogenous viral elements (EVEs) derive from full or partial integrations of viral sequence into the host genome have been reported in several insects (ter Horst et al., 2019). Bonning and Saleh (2021) propose that the interaction of endogenous viral elements (EVEs) with exogenous cognate viruses could generate viral piRNAs with an antiviral role. These EVEs could originate from the integration of viral DNA forms of RNA viruses produced during viral infection in insects. The authors (Bonning and Saleh, 2021) argue that crosstalk may be between the two pathways to maintain host fitness during viral infection. Moreover, EVEs might provide the specificity determinants of a long-lasting and heritable nucleic acid-based silencing system. EVEs in *Ae. aegypti* and *Ae. albopictus* were found to be enriched within piRNA clusters and give rise to piRNAs (Palatini et al., 2017). Mondotte et al. (2020) observed transgenerational immune priming in *Drosophila* and *Ae. aegypti*. Progeny inherits a viral DNA form that is a partial copy of the RNA virus genome and is protected from infection with the same virus for several generations. These mechanisms are, in fact, interesting and deserve a more detailed analysis. First, however, it is necessary to characterize the mechanism to maintain and transfer the immune memory, its specificity with different viruses or pathogens, and determining its role on the immune memory in other species of insects and invertebrates.

2) Endoreplication.

Endoreplication is a variant of the normal replicative cell cycle, in which cells increase their genomic DNA content without division. Endoreplication can enclose different options of the cell cycle, such as endocycle, re-replication, and endomitosis. The first one consists of repeated successions of S–G phases of all genetic material, without cell or nuclei division. In re-replication, DNA synthesis is initiated multiple times at individual origins of replication within the same S phase, provoking site-specific replication of a unique sequence. In endomitosis, an entry into mitosis occurs. The cells condense the chromosomes but do not dissociate them to daughter cells. Instead, they re-enter a similar phase to G1, and the S phase starts again, resulting in multiple nuclei cells. (Lee et al., 2009). Numerous organisms employ endoreplication to provide nutrients and proteins needed to support the developing egg or embryo. Increasing DNA content by endoreplication is required to sustain the mass production of proteins and the high metabolic activity necessary for embryogenesis. Disrupting endoreplication in these cells often leads to embryonic lethality.

The characteristic illustration of endoreplication is the generation of polytene chromosomes in *Drosophila* salivary glands. For example, in the beetle *Tribolium castaneum* larval stages, intestinal stem cells (ISC) conduct endoreplication for adult midgut polyploidic epithelium formation (Parthasarathy and Palli, 2008). In the mosquito *An. albimanus's* midgut, an increase of DNA synthesis has been reported in the first few hours after emergence, including polypoid cell production (Maya--Maldonado et al., 2019). Also, in the flour moth *Ephestia kuehniella*, nuclei in Malpighian tubules and silk glands increase in size through larvae instars. Even in the last instar, larvae nuclei are polyploid with a high DNA content, provoking a branched nucleus. This polyploidy that results in branched nuclei, could be considered an adaptation because the distance between the nuclear area and the cytoplasmic zone is increased to permit traffic of molecules produced in high quantities (Buntrock et al., 2012). Gene amplification is used by follicle cells to increase the copy number of *Drospophila* chorion genes, which encode structural components of the eggshell. By quantifying genomic DNA hybridization to microarrays to assay gene copy number, Claycomb et al. (2004) identified two additional developmental amplicons in the follicle cells of the *Drosophila* ovary. Both amplicons contain genes that, following their amplification, are expressed in the follicle cells. The expression of three of these genes becomes restricted to specialized follicle cells late in differentiation.

One of the most important known pathways in endoreplication is activating the NOTCH pathway in *Drosophila* follicular cells. Oocytes express the Delta ligand, which activates the NOTCH receptor in follicular cells. Notch signaling in the follicle cells activates the transcription factor *Hindsight* (*Hnt*), which represses String/Cdc25 and the transcription factor *Cut* (Sun and Deng, 2007). Down-regulation of Cut allows Fzr/Cdh1 to accumulate. The shift to endoreplication occurs in two steps: 1, Hnt-mediated down-regulation of String/Cdc25 arresting the cell in G2 phase, 2) down-regulation of Cut and subsequent derepression of Fzr/Cdh1, allowing the cell to avoid mitosis and enter a G1-like state that allows PreRC formation (Sun and Deng, 2007).

The endoreplication has been explored in mosquitoes *An. albimanus* and *Ae. aegypti* during priming and recently in *Tenebrio molitor*. In mosquitoes, we have documented an increase in DNA synthesis in *An. albimanus* and *Ae. aegypti,* through Bromodeoxyuridine (BrDU) incorporation and DNA content, after immune challenge and priming in different tissues, including the midgut (Contreras-Garduño et al., 2015; Hernandez-Martínez et al., 2006, 2013). When DNA synthesis is blocked, the protection obtained with priming is abolished, suggesting that DNA synthesis participates in the priming mechanism (Serrato-Salas et al., 2018a; Maya-Maldonado et al., submitted). Also, during priming, the *Notch*, *hnt,* and *delta* are overexpressed, suggesting an endoreplication machinery activation (Contreras-Garduño et al., 2015; Serrato-Salas et al., 2018b). In *An. albimanus*, an up-regulation of cyclins (A, B, and E), Aurora kinase (*AurkA*), *Notch*, and *Hnt* has been observed at seven days post priming. After the second encounter with *Plasmodium* (24 h post-challenge with the parasite), Notch and Hnt were upregulated

in priming conditions, whereas CycA, CycE, and AurkA were down-regulated. The consistency in these data strengthens the idea that cell cycle regulation is crucial as insects' strategy for tissue homeostasis after immune challenge and supports a cell cycle switch to endoreplication (Maya-Maldonado et al., 2021, submitted).

The number of gene copies during endoreplication increases in *Drosophila*, and we have also observed it in the *An. albimanus* cell line LSB-AA695BB after treatment with *Plasmodium* parasites *in vitro*. The DNA cell content increased, and the cell cycle is arrested, and the *hnt* is overexpressed. The number of copies of the immune genes *TEP* (thioester protein) and *PPO* (prophenoloxidase) increases five to 10-fold, respectively. These genes are essential in the mosquito immune response against *Plasmodium*, while the number of other immune genes such as CTL4, CTL6, and DNMT2 did not change (Cime-Castillo et al., 2018). These observations suggest that increasing the copies of relevant genes against the pathogens might be a suitable strategy to respond in a second encounter rapidly. The endoreplication mechanisms can provide the substrate to keep the genetic information and to have a rapid response in a second encounter. Further work is necessary to outline the role of endoreplication in insects' "immune memory" and characterize the molecular mechanisms behind it and the effector molecules amplified.

3) Epigenetic.

Cavalli and Heard (2019) define epigenesist as "the study of molecules and mechanisms that can perpetuate alternative gene activity states in the context of the same DNA sequence." It has been proposed that epigenesis can have an essential role in invertebrate adaptive immune response, and it has been proposed as part of the immune priming mechanisms in insects. Epigenesis has become an exciting possibility to explain memory during priming. Epigenesis can occur through DNA or RNA methylation, histone acetylation or methylation, and noncoding RNAs (Glastad et al., 2019). Moreover, insects have the molecular tools to establish epigenetic modifications.

In the *Tenebrio molitor* beetle, Castro-Vargas et al. (2017) found a lower percentage of methylated cytosine entities in RNA (5 mC) within but not across generations in immune priming experiments adults against the bacteria *Micrococcus lysodeikticus* and larvae against the fungus *Metarhizium anisopliae*. In the transcriptome analysis of priming in *An. albimanus*, Maya-Maldonado et al. (2021) observed the up-regulation of the transcripts involved in epigenetics and chromatin regulation. Among them are METL-9 (Methyltransferase-like protein), Jumonji, and nucleosomal histone kinase 1. It is known that priming in insects induces changes in the susceptibility to pathogens. Recently it was published that *An. albimanus,* a susceptible strain became resistant to *Plasmodium*, erasing the DNA methylation. Diverse immune markers were also activated in response to DNA methylation changes (Claudio-Piedras et al., 2020). These results warrant further studies on epigenesis and induction of resistance through immune priming.

On the other hand, transgenerational memory (TGM) might also explain through epigenesis. Vilcinskas had shown the importance of epigenetics during transgenerational priming in *Galleria mellonella* and *Tribolium castaenum* (Vilcinskas, 2016) and *Manduca sexta* (Gegner et al., 2019). In *M. sexta*, they observed that TGM was mediated by the translocation of bacterial structures from the gut lumen to the eggs with the expression of immunity-related genes and enzymes involved in regulating histone acetylation and DNA methylation in larvae of the F1 generation. However, these studies need identification and characterization of the epigenetic modifications, the regulatory regions involved, and the genes affected.

We recommend that to have an overall picture of epigenetic in the insect immune memory; experiments can be done combining: 1) drugs affecting the epigenome, for instance, the use of azacitidine and decitabine to erase the DNA methylome (Claudio-Piedras et al., 2020) or affecting the Histone deacetylase (trichostatin a). 2) Silencing target genes using RNAi or CRISP-CAS 9. 3) DNA sequencing using Chip-seq and DNA-protein binding identification. Efficient tools depend on each insect species and pathogen interaction with their host. It will be fundamental to determine the extent to which epigenetic mechanisms influence immune memory and its transgenerationally.

2.1. Other relevant components

Besides the proposed mechanisms, it is essential to comment on other components that have shown to be part of the immune priming.

Hemocyte participation. Hemocytes are an exciting target of study during priming. It is known that depletion of hemocytes in *Drosophila* alters priming output (Pham et al., 2007). At the same time, several studies in larval stages in different species of insects have shown an increase in the number of hemocytes after priming (Wu et al., 2016). However, insects and invertebrates, in general, lack the mechanisms to develop selective clones, as shown in vertebrates through clonal selection (Hauton and Smith, 2007). It is fundamental to investigate if the rise of hemocyte numbers in the hemolymph is pathogen or antigen-specific and the hemocyte response is last-lasting.

As mentioned above, hemocytes also integrate viral information. On the other hand, it might be interesting to transfer hemocytes from primed and non-prime individuals to understand their role in the immune memory further. Edwin Cooper has successfully used this approach in a classic work transferring "coelomocytes" from a primed *Lumbricus terrestris* to a non-primed individual, conveying the memory response (Bailey et al., 1971). With this approach, hemocytes can also be collected to investigate molecules and genes involved in immune memory.

Metabolic modification during priming. In an extensive and interesting paper in the beetle *Tenebrio castaneum,* Ferro et al. (2019) observed down-regulated genes contain metabolism-associated genes, such as hexokinase type 2 and sedoheptulokinase, which have previously been implied in shifting the energy metabolism of immune cells in response to immune activation (Kelly and O'Neill, 2015; Nagy and Haschemi, 2013). Trained immunity in vertebrates is similarly based on changes in the energy metabolism of immune cells (Cheng et al., 2014). Several genes previously reported to be involved in the epigenetic reprogramming of immune cells during trained immunity were also upregulated in *Tenebrio castaneum*. The authors are indicating an evolutionarily conserved mechanism of innate immune memory. These data support similar results obtained by Tate et al. (2017) in *T. castaneum* upon transgenerational immune priming with Bt. In addition, the finding that a histone H3 gene is down-regulated in the unspecific treatment but upregulated in the specific treatment supports recent findings of a correlation between IFN memory in vertebrate trained immunity with histone H3.3 and H3K36me3 chromatin marks (Kamada et al., 2018).

In *An. albimanus* exciting changes were observed in the transcripts involved in carbohydrate metabolism, TCA cycle, lipid metabolism, and fatty acid synthesis. In general, a downregulation occurs after an immune challenge with *P. berghei* in Unprimed and Primed conditions. However, the trehalose transporter, GDP-D-glucose phosphorylase, and fatty acid hydroxylase transcripts are upregulated only in primed conditions (Maya-Maldonado et al., 2021). Several metabolic pathways as glycolysis, tricarboxylic acid (TCA) cycle, and lipid and amino acid metabolism are altered in trained immunity of innate immune cells of vertebrates (Domínguez-Andrés et al., 2019). These changes permit that immune cells can respond with a better capacity during a second stimulation. It will be essential to investigate and characterized the cellular pathway needed to get the energy during priming and immune memory.

2.2. Activation of immune pathways

It has recently been that some potential signaling pathways are known in the insect immune response and its relation to immune

memory. Pham et al. (2007) observed that the Toll pathway seems necessary but not sufficient to activate the immune memory. Toll mutants cannot generate priming, but activation of Toll using a mixture of inducers was not enough to protect flies. Pham et al. (2007) proposed a model in which the Toll pathway may be necessary for detecting microbes, and Toll activation becomes another critical path for the priming-specific response.

In the transcriptome analysis in *An. albimanus* primed mosquitoes (Maya-Maldonado et al., 2021), it has been found overexpression of Pellino which regulates the Toll pathway. In *Drosophila*, the absence of this protein provokes a decrease in Drosomycin expression, an essential peptide in Drosophila's immune response. Another overexpressed element is Serpin-5 (Maya-Maldonado et al., 2021). Serpins are serine proteinase inhibitors with a critical function in regulates innate immunity elements, including the Toll pathway. In the beetle *T. castaneum*, priming with B. *thuringiensis*, activated gram-positive responsive genes, such as a Toll-3-like receptor and Persephone (Ferro et al., 2019), indicative of Toll pathway activation. In *C. elegans,* immune priming was dependent on the insulin signaling (DAF-16) and p38 MAP kinase (PMK-1) pathways (Anyanful et al., 2009). It will be fundamental to establish the role of these pathways in the effector response in the priming response.

2.3. Is only one mechanism required during immune memory in insects?

It is not clear that only one mechanism is sufficient to support the immune memory in invertebrates. The enormous diversity of this group of animals and the different adaptive responses may require several immune memory mechanisms. It is necessary to discover the mechanisms of immune memory and characterize them with various pathogens and antigens to determine their limits and scope. We also need to consider the individual's life span and developmental stage, pathogen virulence, and resources to face the immune memory dare. One good example is the mosquitoes, which have very few hemocytes circulating in the hemolymph, but the midgut tissue maintains the information of the previous contact with pathogens.

3. Discussion

In this review, we highlight the number of papers on innate immune memory and it is shown a growing interest in this aspect of immunity. It is important to distinguish immune priming of the induction of immune response. The key points are the biphasic response, the long-lasting response (memory), and specificity. The last point is also under investigation. It isn't easy to consider that invertebrates require a very specific priming response. It is known that vertebrates develop specific immune responses based on clonal selection (Cooper, 2016). So far, this extent of specificity has not been evaluated in invertebrates. However, the specific adaptive response of vertebrates and mammals, in particular, is costly. This subject deserves detailed analysis in invertebrates to understand the decision made during priming and immune memory activation.

It is essential to consider that there may be different mechanisms to produce and conserve immune memory. But it is also crucial, the distinction between the memory mechanisms and effector mechanisms and their molecules. Therefore, It is necessary to use cellular, molecular, and population strategies to establish each group's general and particular mechanisms. Insects and mosquitoes can be an excellent group to address this problem as many are easy to maintain in the laboratory and can be challenged with natural pathogens. In addition to being the main vectors of some arboviruses such as DENV, CHIKV, ZIKA, and parasites such as malaria, it is of great medical importance to know the mechanisms of the immune response to develop alternative strategies to control these diseases. On the other hand, there is evidence of the possible pathways and molecules that could involve. The pathways Toll and IMD, the Dscam and FREPs receptors of lectin type C give us palpable ideas of a wide repertoire of receptors of the great diversity of the pathogen recognition (Pham et al., 2007; Yang et al., 2021). How it is that they are involved in immune memory, how they are selected, and how this information is stored is unknown. In addition to these possible mechanisms involved in immune memory, endoreplication and epigenetic modifications are in the process of development. They could shortly give us a broader explanation of the phenomenon of immune memory in invertebrates.

4. Conclusion

The immunological memory of invertebrates is attracting researchers from different fields. This review emphasized the importance of properly established procedures for assessing immunological memory and opened up some suggestions for determining the mechanism (s). We hope that molecular biology combined with evolutionary and ecological strategies will identify the limits of immune memory in invertebrates and its importance in their adaptation to the environment. It is important to note that so far, no study has successfully demonstrated the mechanisms of invertebrate immune memory: how organisms recognize a specific challenge and recall that memory in a future encounter, how memory is stored, and where it is stored. All of this information still warrants further investigation. Furthermore, a fertile field of research has been the application of the rationale for invertebrates 'vaccines', but this now only applies primarily to crustaceans (Table 1). An open question is whether pollinators or disease vectors such as triatomines possess immune memory (Rowley and Powell, 2007; Carmona et al., 2021) and a challenging project would be to know if immune memory is found in different Phyla of invertebrates and not only in the few groups analyzed so far.

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