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Review

Pesticide immunotoxicity on insects – Are agroecosystems at risk?

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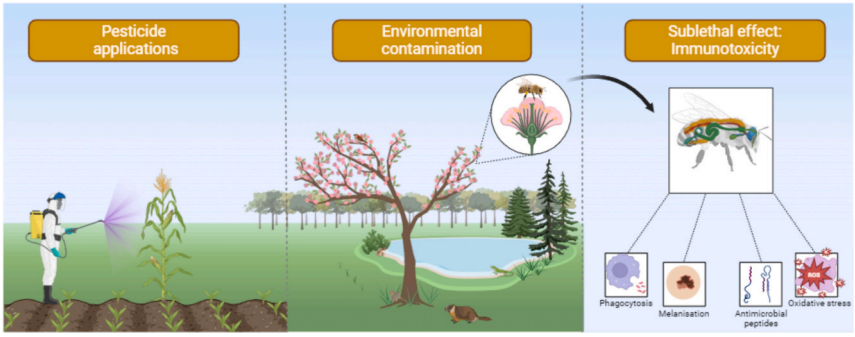
HIGHLIGHTS

- The effects of biological and synthetic pesticides at sublethal doses on insect immunity are well documented.
- Certain pesticides interfere with neuroendocrine-linked, cellular and humoral functions.
- Pesticides increase insect susceptibility to pathogens and parasites.
- Pesticides can either weaken or fortify insect immune response with potential fitness costs.
- Pesticide immunotoxicity poses risk to ecosystem services by jeopardizing beneficial insects.

GRAPHICAL ABSTRACT



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ABSTRACT

Recent years have witnessed heightened scrutiny of the non-target sublethal effects of pesticides on behavioural and physiological traits of insects. Traditionally, attention has focused on investigating pesticides' primary modes of action, often overlooking the potential secondary mechanisms. This review brings forth the nuanced impacts of sublethal pesticide exposure on the immune system of target and non-target insect species. Pesticides, such as for example neonicotinoids, suppress immune response, while others, like certain organophosphates and some insect growth regulators (IGRs), appear to bolster immunocompetence under certain circumstances. Beyond their individual impacts, the synergic effects of pesticide mixtures on insect immunity are garnering increasing interest. This review thus summarizes recent advances in the immunomodulatory effects of pesticides, detailing both mechanisms and consequences of such interactions. The implications of these effects for ecosystem preservation and viability of beneficial organisms, such as pollinators and natural enemies of pests, are discussed. The review also considers further research directions on pesticide secondary modes of action and explores potential implications for integrated pest management (IPM) programs, as several model organisms studied are

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crop pest species. While current data provide an expansive overview of how insect innate immunity is modulated, concrete endpoints remain elusive requiring further research into pesticide secondary modes of actions.

1. Agrochemicals and insect immunity

With over 400 million years of evolution, insects are among the oldest living and most adaptable organisms on Earth, surviving through intricate interaction with a plethora of organisms, including microbes and plants (Rasnitsyn and Quicke, 2007). However, the Anthropocene added another layer of complexity to these interactions, where the presence of toxic anthropogenic compounds is pervasive in both the rural and urban environments, resulting in a gradual and worldwide decline of insect biodiversity over time (Sánchez-Bayo and Wyckhuys, 2019; Wagner et al., 2021). Among these, agrochemicals represent one of the major environmental pollutants because of their worldwide applications to control plant diseases, weeds, pests and ensure food security (Devi et al., 2022; Guedes et al., 2016; Liess et al., 2021; Lima et al., 2016; Pesce et al., 2023; Sellare et al., 2020), despite shifts towards reducing chemical inputs in support of integrated management approaches (Desneux et al., 2021; Kogan, 1998; Lisi et al., 2023).

The repercussion of these contaminants extends beyond immediate human health and environmental impacts, encompassing a myriad of potential indirect effects, added by society concerns about their possible bioaccumulation, slow degradation, and secondary effects on living species (Fleeger et al., 2003; Gupta and Gupta, 2020; Tang et al., 2021). Particularly concerning are the sublethal effects, which manifest as behavioural and physiological alterations in both target and non-target organisms surviving to the pesticide exposure (Desneux et al., 2007; Guedes et al., 2022a), potentially cascading through stress response pathways to higher levels of biological organization, from the individual to the population and community (Guedes et al., 2016, 2022a, 2022b). In the context of physiological alterations, once an insect is exposed to pesticides its immune system can be triggered as an effect of the xenobiotic compounds (Bartling et al., 2021; James and Xu, 2012).

Indeed, the immune system provides the main lines of defense against biotic stressors and other foreign bodies in nature (Schmidt et al., 2008), which is activated when an exogenous agent enters the host and is recognized as infectious or non-self-structure (Hillyer, 2016; Tsakas and Marmaras, 2010). This recognition prompts a quick physiological response, either cellular or humoral (Fearon, 1997; Hillyer, 2016; Medzhitov and Janeway, 1997). Cellular immunity is mediated by hemocytes, which are blood cells circulating in insect hemolymph or attached to tissues and organs and mainly responsible for the processes of phagocytosis, nodulation and encapsulation. Humoral immunity is associated with the production of antimicrobial peptides (AMPs), melanisation response and reactive oxygen species (ROS) (James and Xu, 2012), and occurs not only in the insect hemocoel but also in the gut, where several commensal and symbiotic bacteria play a key role against pathogenic infections and influence the systemic immune response (Douglas, 2015; Alarcón et al., 2022; Lemaitre and Miguel-Aliaga, 2013).

The activation of these immune system is a metabolically costly phenomenon, finely cross-modulated by competing physiological needs, which can a have strong impact on host survival and fitness (Moret and Schmid-Hempel, 2000; Buchon et al., 2014). The optimal allocation of energy among different physiological processes during an immune response is a dynamic process influenced by a number of environmental stressors, including pesticides, which, by definition, are able to disrupt different physiological functions (Liu et al., 2017). These chemicals can destabilize the delicate interactions between immune system, nervous system, insect microbiota, symbionts, pathogens and parasites potentially leading to an altered immunocompetence, with consequences that, for the importance of the playing actors, can dramatically undermine insects at both the individual and population levels (Giordani et al., 2023; Annoscia et al., 2020; Chmiel et al., 2019; Di Prisco et al., 2016).

Toxicity of pesticides on insect immunity was reviewed and discussed one decade ago by James and Xu (2012). Since then, a substantial volume of research focused on this subject. Several model organisms, such as *Drosophila melanogaster* Meigen (Diptera: Drosophilidae), *Apis mellifera* L. (Hymenoptera: Apidae) and *Galleria mellonella* L. (Lepidoptera: Pyralidae), have been extensively studied to elucidate pesticide immunotoxicity (Zibaee and Malagoli, 2020), providing insights into potential effects on other animals, including humans (Jeibmann and Paulus, 2009). Increasing interest on pesticide immunotoxicity towards agricultural pests, including non-target pest insects and other phytophagous arthropods, has been recorded over the years (James and Xu, 2012). The study of the link between sublethal pesticide exposure and immunity is critical to understand the full spectrum of pesticide effects on both target and non-target organisms, so that we can predict not only their pest control efficacy but also any undesired negative effect they may have on providers of ecosystem services.

The aim of this review is to critically summarize and analyse the current knowledge on the effects and mechanisms of pesticide immunotoxicity towards insects and the potential consequences for individuals and their populations as part of a biotic community. An in-depth analysis of all these aspects aims at providing a comprehensive framework in which to place the development of sustainable integrated pest management (IPM) programmes, aware of the importance that immune interactions underlying resource competition have in the stability of trophic networks.

2. Materials and methods

A literature search was conducted in early 2023 to identify peer-reviewed and English-language publications from 1991 to 2022. The search involved the use of the scientific databases Web of Science (Clarivate) and Scopus (Elsevier), employing the following keyword combinations: “pesticides” OR “bioinsecticide” OR “entomopathogenic fungi” OR “*Bacillus thuringiensis*” OR “sublethal effect”, AND “immune response” OR “cellular immunity” OR “humoral immunity” OR “hemocyte” OR “encapsulation” OR “melanization” OR “immunotoxicity”, AND “insect” OR “pest” OR “honeybee” OR “predator” OR “parasitoid”. Then, this review exclusively included scientific research articles that investigated the pesticide sublethal effects on the cellular and humoral immune components of insects listed in the supplementary Table S1.

Table S1 provides a comprehensive overview of included studies, detailing for each study the pesticide characteristics (i.e., class, mode of action, compound and dose), target organism (i.e., ecological role, order and species) and main effects on immune system (i.e., cellular and humoral immunity). Papers evaluating the combined effects of pesticides and biotic stressors (e.g., arthropods and pathogens) on insect immunity were also considered to elucidate potential synergistic immunotoxicity. Four additional studies (i.e., Ghasemi et al., 2014a; Shaurub and Sabbour, 2017; Dubovskiy et al., 2010; Khanikor and Bora, 2012) were identified within the references of the analysed documents and they were included in this review due to their strong relevance to the topic.

3. Immunotoxicity of pesticides

Current understanding on pesticide immunotoxicity primarily focused on effects of sublethal concentrations of biopesticides and synthetic pesticides on pollinators and insect pests (Fig. 1). These studies have revealed that pesticides can induce either immunosuppression or increase immunocompetence in insects by altering their standard cellular and humoral responses (Table 1). Major cellular effects include

alterations of the total and differential hemocyte count (THC and DHC) in the hemolymph, alongside related immune processes such as phagocytosis, nodulation, and encapsulation. In contrast, effects on humoral immunity involve alteration of the production of antimicrobial peptides (AMPs), melanisation, reactive oxygen species (ROS) production and induction of oxidative stress.

3.1. Effects of synthetic pesticides on insect immunity

Early studies on pesticide immunotoxicology highlighted the non-target impacts of organochlorines (OCs) on a host-parasitoid interaction (Delpuech et al., 1996). For instance, dieldrin and endosulfan at LD_{30} significantly reduced the survival rate of *D. melanogaster* larvae parasitized by the larval parasitoid *Leptopilina boulardi* (Barbotin, Carton, Kelmer-Pillault) (Hymenoptera: Figitidae), as well as the host larvae's ability to encapsulate the parasitoid eggs (Delpuech et al., 1996). Additionally, chronic exposure of *Rhynocoris kumarii* (Ambrose and Livingstone) (Hemiptera: Reduviidae) adults to endosulfan led to significant alterations in hemocyte counts and composition (George and Ambrose, 2004).

Similar results were observed with pyrethroids, which mainly act as insecticides for domestic and agricultural purposes. Exposure of larvae of *Samia ricini* (Jones) (Lepidoptera: Saturniidae) to cypermethrin-contaminated castor leaves showed no impact on insect immunity 24 h post-exposure, but a significant decrease of THC 48–96 h post-exposure along with a significant reduction of prohemocytes, spherulocytes, oenocytes, and phenoloxidase activity. In contrast, plasmatocytes and granulocytes and the enzymatic lysozyme activity significantly increased (Kalita et al., 2017).

The pyrethroid flumethrin at sublethal doses strongly affected humoral immunity of honeybees by inducing oxidative stress, apoptosis of midgut cells, expression of phenoloxidase and antimicrobial peptides (Garrido et al., 2013; Qi et al., 2020), while tau-fluvalinate LC_{50} was only able to upregulate AMPs expression (Garrido et al., 2013). Despite of these results contributing to a better understanding of the unintended consequences of pesticides on insect immune system, research testing on pyrethroids and organochlorines has been limited because of a major interest in emphasizing the neonicotinoid impact on insect immunocompetence. Indeed, most toxicological studies have focused on the sublethal effects of neonicotinoids on the cellular and humoral immunity of pollinators (Fig. 1), as showed by Annoscia et al. (2020), who reported that clothianidin orally applied to *A. mellifera* larvae with field realistic doses and topically on adult bees with sublethal concentrations significantly reduced their encapsulation and melanisation immune responses (Annoscia et al., 2020). Similarly, clothianidin impaired *A. mellifera* queen immune responses at very low doses (Brandt et al., 2017), but affected worker bees immunocompetence only at higher than field realistic doses (Brandt et al., 2016). Multiple sublethal doses of thiacloprid and imidacloprid significantly reduced hemocyte number, encapsulation rate and antimicrobial activity of hemolymph in both bee castes (Brandt et al., 2017). At the same time, honeybees sublethally exposed to clothianidin and thiacloprid exhibited increased oxidative stress and inhibition of prophenoloxidase and phenoloxidase activity in hemolymph (Orčić et al., 2022), while phenoloxidase and antimicrobial activity of bumblebees was suppressed only at field realistic but not at sublethal doses of imidacloprid (Czerwinski and Sadd, 2017). Low concentrations of neonicotinoids acetamiprid and imidacloprid caused oxidative stress and interfered with the gut microbiota homeostasis of

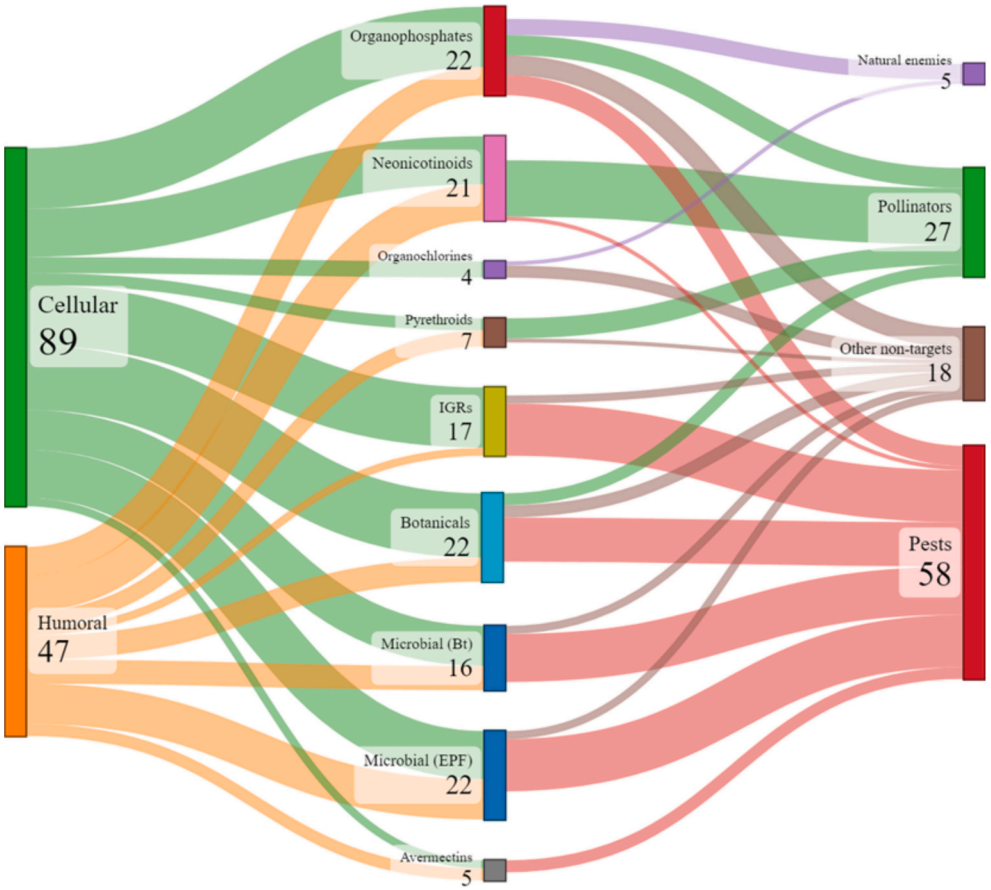

Fig. 1. Sankey diagram showing the interaction flows between the groups of studies focused on cellular or humoral immunotoxicity, different classes of pesticides and the tested insects according to their ecological functions. The values indicate the number of reports (n = 111) found from the analysed papers (n = 76). Figure was designed in the SankeyMATIC platform.

D. melanogaster and *B. mori* (Chmiel et al., 2019; Li et al., 2021a), resulting in increased larval susceptibility to pathogens, such as *Enterobacter cloacae* (Jordan) (Enterobacterales: Enterobacteriaceae) (Li et al., 2021a).

Low doses of imidacloprid or thiamethoxam caused oxidative stress and reduced the number and activity of hemocytes in larvae of *G. mellonella*. Moreover, induction of oxidative stress resulted from a decrease of superoxide dismutase (SOD) and catalase (CAT), which are important antioxidant enzymes involved in ROS excess removal (Kayis et al., 2019). On the other hand, a dose-dependent increase in SOD and CAT was reported under imidacloprid sublethal exposure on *G. mellonella* larvae; therefore, the pesticide effect on antioxidant enzymes likely depends on the type and exposure level of certain chemical compounds, as well as different susceptibility of exposed insects (Yucel and Kayis, 2019). Reports on pesticide immunosuppression prompted research to deepen the understanding of neonicotinoid role as drivers of bee susceptibility to pathogens and parasites, and the potential synergies between insecticides and biotic stressors on insect immunity (Brandt et al., 2016; Annoscia et al., 2020; Sánchez-Bayo, 2014). Accordingly, sublethal exposures of clothianidin inhibited honeybee immunocompetence and promoted both the replication of deformed wing virus (DWV) (Di Prisco et al., 2013) and fitness of the parasitic mite *Varroa destructor* (Anderson and Trueman) (Acarina: Parasitidae) (Annoscia et al., 2020). However, other studies showed that combined exposure to *V. destructor* and sublethal clothianidin altered honeybee cellular immunocompetence and weakened bee health, although the parasitic mite was the only factor increasing bee susceptibility to DWV (Morfin et al., 2020). Similarly, sublethal co-exposure of clothianidin and *Paenibacillus larvae* (Genersch) (Bacillales: Paenibacillaceae) spores suppressed *A. mellifera* larval immunity by decreasing the THC and DHC in hemolymph, while individual exposure triggered cellular defences (López et al., 2017).

However, controversial results were obtained by research on the interaction of microsporidic intestinal pathogen *Nosema* and neonicotinoids. For example, co-exposure of *Nosema apis* (Zander) (Dissociodihaplophasida: Nosematidae) and thiamethoxam significantly reduced the encapsulation rate of *A. mellifera* worker bees after 24 h (Grassl et al., 2018), but no cellular and humoral impairments were observed following a combined exposure of *Nosema* spp. and imidacloprid at sublethal concentrations on adult bees, except an increased susceptibility of the colony to the parasite (Alaux et al., 2010). Moreover, individual and combined applications of thiamethoxam and *N. cerane* (Fries) (Dissociodihaplophasida: Nosematidae) did not influence cellular immunity of *M. colimana*, but significantly reduced the pathogen replication in the combined treatment (Macías-Macías et al., 2020).

Organophosphates (OPs) showed an opposite trend on immunity than the previous molecules (Table 1) and current studies on this chemical group exhibited an equal focus on both target and non-target organisms (Fig. 1). Oxydemeton-methyl LD_{30} stimulated, albeit not significantly, the encapsulation rate in a non-reactive strain of *D. melanogaster* with no or low encapsulation ability of parasitoid eggs (Delpuech et al., 1996). At the same time, Delpuech and Tekinel-Ozalp (1991) showed that a contamination of the parasitoid L. *boulardi* with chlorpyrifos LD_{50} significantly increased the encapsulation rate of its host larvae. Enhancement of insect immune response has also been showed on *G. mellonella* larvae exposed to pirimiphos-methyl LC_{10} and LC_{50}, where both concentrations increased the encapsulation rate, hemocyte number and melanisation (Dubovskiy et al., 2013). However, exposure of the Colorado potato beetle larvae, *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae), to such organophosphate increased only the cellular immunity, showing that cellular and humoral components in insects are not always triggered together after the pesticide exposure (Dubovskiy et al., 2013).

Table 1
Overview of reviewed studies on pesticide immunotoxicity in insects. Studies demonstrating a significant sublethal impact on insect immunity were considered positive for side effects, and the percentage of positive studies is reported in table for each pesticide group. Based on the pesticide effects reported in each reviewed study (see supplementary Table S1), different colours represent overall pesticide impact on cellular and humoral immunocompetence of insects. When enhancement and impairment of immune system occur in the same manner within each chemical group, yellow is used to identify unclear effects (see supplementary Table S1 for detailed information). Green indicates a prevalence of beneficial effects, red negative effects, grey no effects.

Pesticide group	Key to targeted physiology	IRAC group - MoA	Number of studies	% positive on cellular immunity	% positive on humoral immunity
Organophosphates	Nerve and Muscle	1 - AChE inhibitors	12	58	42
Organochlorines	Nerve and Muscle	2 - GABA antagonists	2	100	0
Pyrethroids	Nerve and Muscle	3 - Sodium channel modulators	4	25	75
Neonicotinoids	Nerve and Muscle	4 - nAChr modulators	11	63	45
Avermectins	Nerve and Muscle	6 - GLUCL allosteric modulators	3	66	100
IGRs	Growth and development	7 - Juvenile hormone receptor modulators	5	100	0
		15 - Inhibitors of chitin biosynthesis	3	100	33
		18 - Ecdysone receptor agonists	3	100	33
Botanicals	Unknown	UNE - Unknown	13	85	38
Microbials (Bt)	Midgut	11 - Disruptors of insect midgut membranes	10	70	50
Microbials (EPF)	Unknown	UNF - Unknown	10	60	70

Hemogram analysis of *R. kumarii* topically exposed to sublethal concentrations of monocrotophos, dimethoate, methyl parathion or quinalphos revealed increased THC and granulocytes in hemolymph, but reduced prohemocytes and plasmatocytes (George and Ambrose, 2004). Low concentrations of acephate increased hemocyte abundance in *D. melanogaster* hemolymph, while higher exposure levels decreased the number of immunocompetent cells (Rajak et al., 2018). Similar studies showed an alteration of the DHC caused by acephate. This included a dose-dependent plasmatocyte decrease and crystal cell increase (1–6 μg/mL of acephate), while lamellocytes were significantly increased only by median concentrations (3–5 μg/mL) (Rajak et al., 2014, 2015).

Although organophosphates seem to enhance insect cellular immunity, inhibition of AMPs expression and midgut impairments were reported on treated insects. For example, sublethal exposure of *B. mori* larvae to phoxim inhibited the AMPs (e.g., *Bmdefensin1*, *BmcecA*, *Bmglv1*, *Bmglv2*, *Bmmoricin* and *BmmoricinB3*) gene expression and related signalling pathways (e.g., Toll, Imd and JAK/STAT), and increased the pathogenesis of *E. cloacae* and other non-dominant bacteria, such as *Staphylococcus*, to the detriment of dominant beneficial species (Li et al., 2020a, 2020b). At the same time, the OPs phoxim, malathion, methyl parathion and ethyl parathion compromise insect homeostasis and trigger oxidative stress in *G. mellonella* and *B. mori* larvae (İçen et al., 2005; Büyükgüzel, 2009; Yu et al., 2011), specifically by increasing the levels of the lipid oxidation marker malondialdehyde (MDA) and the detoxification enzyme glutathione S-transferases (GST), suggesting that they can also be used as biomarkers for OPs toxicity (Yu et al., 2011).

Immune stimulation was observed by the expression of the *hymenoptaecin* AMP in adult worker bees fed with contaminated sucrose solution containing combined sublethal doses of diazinon, malathion, profenofos and chlorpyrifos (Al Naggar et al., 2015). Like neonicotinoids, potential synergic effects with biotic stressors were also recorded for organophosphates. Encapsulation rate increased in the Colorado potato beetle larvae sublethally exposed to pyrimiphos-methyl, but significantly reduced when exposed larvae were infected with *Metharizium anisopliae* (Metsch.) Sorokin (Ascomycota: Clavicipitaceae) (Dubovskiy et al., 2010).

While previous studies indicated that synthetic molecules within the same chemical group show similar immunotoxic trend, insect growth regulators (IGRs) exhibited more controversial effects (Table 1) in studies focusing mainly on insect pests (Fig. 1). For example, sublethal applications of ecdysone receptor agonists, such as tebufenozide and methoxyfenozide, increased THC, nodulation and phenoloxidase activity in *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae), *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) and *Ostrinia furnacalis*, and larval resistance to microbial infections (Ghasemi et al., 2014a; Zibaee et al., 2011; Yu et al., 2018). Conversely, juvenile hormone mimics at sublethal exposure with IGRs, such as pyriproxyfen and fenoxycarb, inhibited the cellular and humoral immunity of several insects (Franssens et al., 2006; Ghasemi et al., 2014a; Mirhaghparast et al., 2015a; Nasr et al., 2010; Shaurub and Sabbour, 2017).

Pyriproxyfen shows a dose- and time-dependent decrease in hemocytes (Zibaee et al., 2011), and fenoxycarb interferes with midgut immunity and increases insect susceptibility to *Bacillus thuringiensis* Berliner (Bacillales: Bacillaceae) infections (Attarianfar et al., 2023). Among chitin biosynthesis inhibitors, buprofezin decreased nodulation and phenoloxidase (PO) activity of *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) larvae (Nasr et al., 2010). In contrast, *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) larvae sublethally exposed to hexaflumuron exhibited a dose-dependent increase of the THC and granulocytes 24 h post-exposure, followed by an inverse trend 96 h post-exposure (Zhu et al., 2012).

3.2. Biopesticide impact on insect immunocompetence

Research into the immunotoxicity of biopesticides has predominantly focused on microorganism and botanical formulations targeting insect pest species (Fig. 1), often using these organisms as model subjects. It is well known that the insect immune system is triggered in response to infections by microorganisms, such as bacteria, fungi and viruses (James and Xu, 2012). In this context, *B. thuringiensis*-based biopesticides were reported to stimulate the insect immune system, as recorded with cellular immune response of *Chironomus xanthus* Rempel (Diptera: Chironomidae) or *G. mellonella* larvae triggered by increased sublethal concentrations of *B. thuringiensis* subs. *kurstaki* (*Btk*) and *galleria* (*Btg*) (Dornelas et al., 2020; Dubovskiy et al., 2008). However, higher levels of exposure (e.g., LC_{50}) decreased the THC and inhibited the encapsulation response of exposed larvae (Grizanova et al., 2014).

Similarly, a LC_{50} exposure of *Btk* decreased cellular immunocompetence of *Agrotis ipsilon* (Hufnagel) (Lepidoptera: Noctuidae) larvae and *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Rhynchophoridae) larvae and adults (El-Aziz and Awad, 2010; Manachini et al., 2011; Celi et al., 2022). In particular, *Btk* exposure decreased all immune cells in females and increased granulocytes in males of *R. ferrugineus* adults (Celi et al., 2022). Remarkably, Ericsson et al. (2009) showed a decrease in THC, phenoloxidase and hemolymph protein concentration in susceptible *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae) larvae, but not on resistant specimens, after exposure to *Btk*.

Other microbials also exhibit toxicity towards the insect innate immune system, such as entomopathogenic fungi (EPF). Increased sublethal doses of a *B. bassiana* did not influence the cellular immunity of *C. xanthus*, but LC_{50} exposure significantly increased the THC, plasmatocytes, granulocytes, nodulation and phenoloxidase activity on *Glyphodes pyloalis* Walker (Lepidoptera: Pyralidae) (Dornelas et al., 2020; Pour et al., 2021). Moreover, sublethal concentrations of *Beauveria bassiana* (Bals.-Criv.) Vuill. (Hypocreales: Cordycipitaceae), *M. anisopliae, Isaria fumosoroseus* Wize (Deuteromycotina: Hyphomycetes) and *Lecanicilium lecanii* (Zimmerman) Zare and Gams (Hypocreales: Cordycipitaceae) enhanced insect immunity by increasing the THC, specifically plasmatocytes and granulocytes, and related immune reactions, such as phagocytosis and nodulation, in *E. integriceps*, *S. littoralis*, *Schistocerca gregaria* (Forsk.) (Orthoptera: Locustidae) and *Chilo suppressalis* Walker (Lepidoptera: Crambidae) (Gillespie et al., 2000; Mirhaghparast et al., 2013; Zibaee and Malagoli, 2014). However, immunosuppressive effects of entomopathogenic fungi, such as *B. bassiana,* were recorded reducing THC, DHC and phagocytosis of *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) larvae (Hung and Boucias, 1992). Also, non-lethal *B. bassiana* or *M. anisopliae* mycosis reduced the phagocytic activity of *G. mellonella* larvae hemocytes (Vilcinskas et al., 1997).

Besides cellular immunity alterations, microbial infections also play a key role in humoral immunomodulation (Table 1), which is mainly linked to abnormalities in immune gene expression. Transcriptome analyses of phytophagous moth larvae indicated that *Btk* cry proteins triggered different immune pathways and genes, especially those related to insect gut immunity (Wu et al., 2016; Rodríguez-Cabrera et al., 2008; Hernández-Martínez et al., 2010; Bel et al., 2013; Crava et al., 2015). Specifically, exposure to Cry1Ca and Cry1Ac toxins led to upregulation of 2962 and 4446 genes in the midgut of *S. exigua* and *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), respectively, most of them belonging to the expression of antimicrobial peptides, lysozymes and immune signalling pathways (Li et al., 2021b; Ren et al., 2020).

Higher transcriptomic stimulation of the AMPs cecropin, defensin, gloverin, spodoptericin, lebocin1, moricin, cobatoxin, diapausin and attacin was linked to the upregulation of *dorsal* and *cactus* genes involved in Toll pathways (Crava et al., 2015; Ren et al., 2020; Li et al., 2021b; Li et al., 2021b). Humoral alterations caused by *Btk* Cry proteins also involved change in the gene expression of serine protease, Repats (Response to Pathogen), lysozyme, protective enzymes, and induction of

detoxifying enzymes in the larval midgut related to Cry protein resistance (Van Munster et al., 2007; Nanoth Vellichirammal et al., 2015; Ren et al., 2020). Similarly, *Adelphocoris suturalis* Jakovlev (Hemiptera: Miridae) microinjected with the Gram-negative *E. cloacae* stimulated both *PPO4* gene related to melanisation and AMP defensin, while *B. bassiana* inhibited only the melanisation immune gene (Ma et al., 2021). Topical application of *B. bassiana* also inhibited the expression of AMPs in *Anopheles stephensi* Liston (Diptera: Culicidae). This led to a simultaneous increase in ROS promoting intestinal microbiota disruption and increasing proliferation of the opportunistic bacteria *Serratia marcescens* Bizio (Enterobacterales: Yersiniaceae), which acts as an entomopathogen when it moves from midgut to hemocoel and produce a septicemia (Wei et al., 2017). Opportunistic pathogenetic proliferation of *Serratia* and other enterobacteria was also observed in L. *decemlineata* larvae infected by *Metarhizium robertsii* (Metchnikoff) Sorokin (Ascomycota: Clavicipitaceae) (Kryukov et al., 2021).

Moreover, *M. robertsii* inhibited the detoxification enzymes GST and esterase (EST) in the larval midgut and increased insect susceptibility to avermectins, which are insecticides derived from fermentation of *Streptomyces avermitilis* (ex-Burg et al. 1979) Kim and Goodfellow 2002 (Streptomycetales: Streptomycetaceae). Such immune challenge resulted in major expression of transcription factors in the Toll and Imd pathways and the AMP attacin (Kryukov et al., 2021). Fungus-avermectins LC_{50} co-treatments suppressed granulocytes and increased larval susceptibility to the fungal infection in the hemolymph in L. *decemlineata* (Tomilova et al., 2016). Conversely, an individual exposure of abamectin LC_{30} on *S. litura* larvae significantly increased the THC from 2 up to 72 h post-treatment and enhanced prophenoloxidase activity (Vengateswari et al., 2018).

Lastly, current literature widely documented immunotoxicity of insects, in particular agricultural pests, sublethally exposed to plant extracts, essential oils (EOs) and azadirachtin (neem oil), commonly referred to botanical pesticides (Fig. 1). For instance, seed pod and leaf extracts of *Prosopis juliflora* (Sw.) (Fabales: Fabaceae) and *Manihot esculenta* Crantz (Euphorbiales: Euphorbiaceae) triggered cellular and humoral responses of *S. litura* and *S. ricini* by increasing prophenoloxidase, phenoloxidase and THC (Dhivya et al., 2018; Manjula et al., 2020). Among these, prohemocytes, plasmatocytes, granulocytes, oenocytoids and spherulocytes increased significantly after six hours of exposure (Dhivya et al., 2018; Manjula et al., 2020). Sublethal exposure to *Artemisa annua* L. (Asterales: Asteraceae) extracts led to dose-dependent suppression of cellular immunity, phagocytosis, nodulation and PO activity in *E. integriceps* adults (Zibaee and Bandani, 2010). When *S. littoralis* larvae were exposed to LC_{10} of castor or camphor EOs, hemogram analysis showed a decrease in the THC and DHC only 48 h post-exposure (Ali and Ibrahim, 2018). Similarly, sublethal doses of *Ocimum sanctum* L. (Lamiales: Lamiaceae) EOs increased THC of *Antheraea assama* Westwood (Lepidoptera: Saturniidae) larvae (Khanikor and Bora, 2012). Sadeghi et al. (2019) found a dose-dependent decrease of THC and DHC of *Sesamia cretica* Lederer (Lepidoptera: Noctuidae) exposed to *Ferula ovina* Boiss (Apiales: Apiaceae) EOs and *E. kuehniella* treated with sublethal doses of *Callistemon viminalis* Gaertn. (Myrtaceae: Myrtales) and *Ferula gummosa* L. (Apiales: Apiaceae) EOs after topical and fumigant applications (Ghasemi et al., 2014b). A LD_{20} exposure of *Cymbopogon martinii* (Poaceae: Poales) EOs and its major constituent, geraniol, did not influence the encapsulation response of *A. mellifera* (Santos et al., 2018).

Sublethal azadirachtin toxicity affects hemocyte numbers, phenoloxidase activity, and AMPs expression across a wide range of insects, including *G. mellonella*, *Sarcophaga barbata* (Robineau-Desvoidy) (Diptera: Sarcophagidae) and *Apis cerana cerana* Fabricius (Hymenoptera: Apidae) (Dorrah et al., 2019; Er et al., 2017; Zhao et al., 2022). Low hemocyte count and phagocytic activity, nodulation, AMP and lysozyme in hemolymph was observed in late instar nymphs of *R. prolixus* and lepidopteran larvae feeding on azadirachtin contaminated diet (Azambuja et al., 1991; Figueiredo et al., 2006; Pandey et al., 2008; Duarte et al., 2020). Also, Viana et al. (2021) showed that *Melipona quadrifasciata* Lep. (Hymenoptera: Apidae) infected by *Escherichia coli* K-12 exhibited increased cellular immunity. However, co-exposure with sublethal azadirachtin or spinosad did not affect hemocyte composition, showing the lack of synergic interaction between this bioinsecticide and microbials (Viana et al., 2021) and suggesting that the azadirachtin effects on insect immunity could be considered weaker in comparison to other molecules.

4. Trends and mechanisms of pesticide immunotoxicity

Although there is not always a sole effect in each pesticide group regarding immune activation or inhibition (Table S1), a peculiar toxicological trend emerges across most investigated groups (Table 1). Notably, cellular immunity appears to be more susceptible than humoral immunity to pesticide sublethal exposures (Table 1). This is supported by a higher number of studies documenting a significant sublethal impact on insect cellular responses, either positive or negative, than compared to the fewer studies reporting a prevalence of unclear or absent effects on humoral immunity.

Among the eleven pesticide classes reviewed, neonicotinoids were the only group simultaneously impairing the cellular and humoral immunity of insects. A strong variability occurred within the IGRs with the ecdysone receptor agonists (IGRs-18) and chitin biosynthesis inhibitors (IGRs-15) supporting insect immunocompetence. Juvenile hormone receptor modulators (IGRs-7) showed opposite trend, with no studies on humoral immunity. Similarly to the IGRs, the other pesticide groups did not show a consistent immunotoxic trend between the cellular and humoral components. In addition, while synthetic pesticides are frequently associated with a prevalence of negative effects and biopesticides appear safer, this expectation is not universally true. Surprisingly, organophosphates enhanced insect cellular immunocompetence, while botanicals inhibited this immune trait, although their effects on humoral immunity are still controversial (Table 1).

Among the other biopesticides, entomopathogenic fungi supported insect cellular immunity, while their secondary metabolites could induce immunodeficiency, with no discernible effects on humoral immunity. Conversely, *B. thuringensis* showed opposite trend than the EPF. Avermectins were the only group showing a clear and specific effect on humoral immunity, while their impact on cellular immunity is unclear and seems to be related to the level of exposure, despite few studies were performed on this pesticide group (Table 1). Organochlorine and pyrethroids strongly inhibited cellular immunity, with absent and uncertain reports on the humoral one, respectively (Table 1). Existing variations in insect immune response can be attributed to insecticide-related factors, such as origin, chemical group and level of exposure. This includes the relevance of the secondary modes of action of pesticides under a sublethal scenarios (Fig. 2) and the potential biphasic or divergent phases, such as immune system induction at low doses and suppression at higher concentrations or temporal transient increase of hemocytes. Overall, reported immune alterations would mainly depend on altered expression of genes involved in cellular and humoral defences, following induction of oxidative stress by sublethal exposures to biopesticides and conventional insecticides.

4.1. Synthetic pesticide mechanisms of immunotoxicity

Cellular immunosuppression (e.g., THC, DHC and encapsulation) induced by OCs (Fig. 2) is thought to depend on their potential interference with the monoamide oxidases, key enzymes responsible for melanisation and encapsulation of foreign bodies (Delpuech et al., 1996; Zibaee and Malagoli, 2020). Other reports suggest that these pesticides could alter the release of antidiuretic hormones leading to increased blood volume and dilution of hemocytes in hemolymph (George and Ambrose, 2004), causing a decrease in THC.

Because of their hydrophobic nature and low molecular weight,

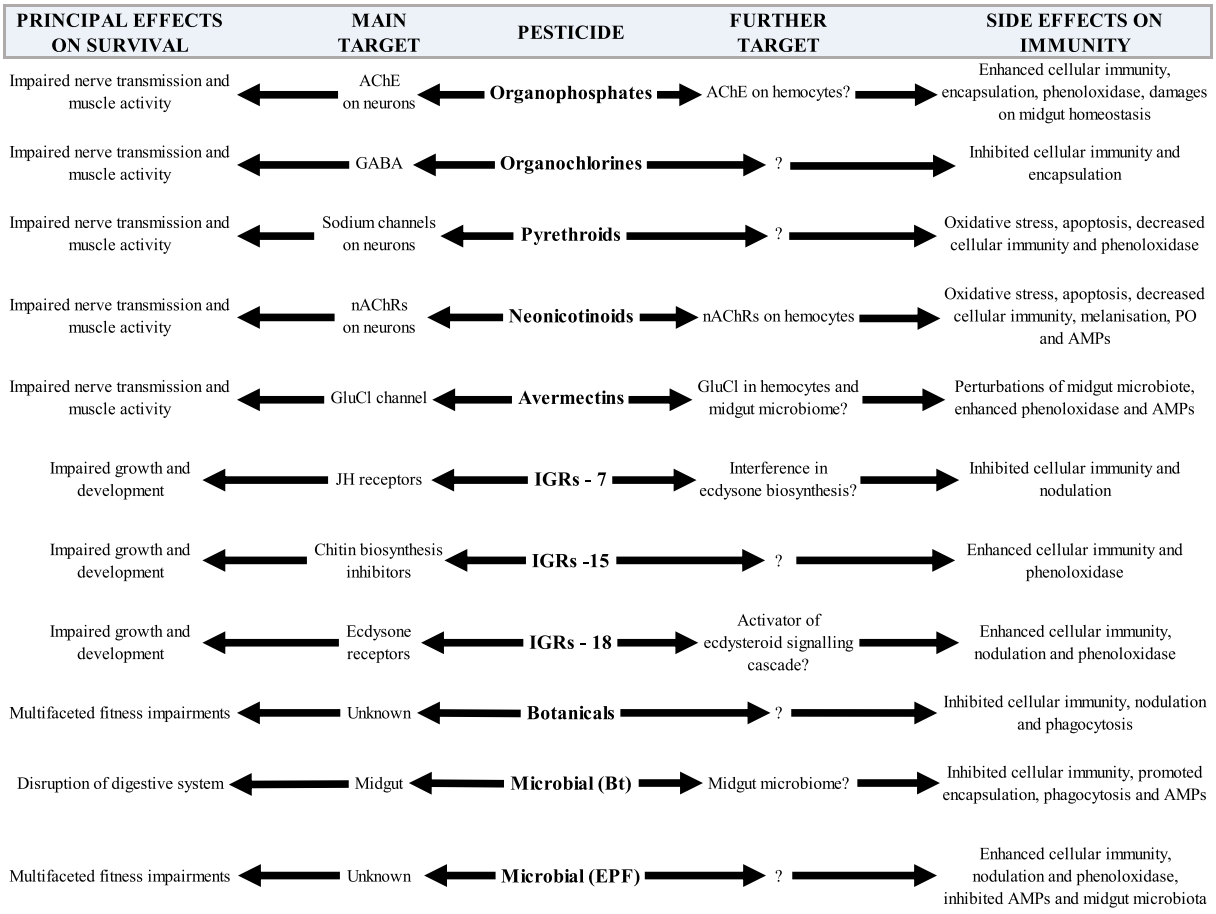

Fig. 2. Diagram showing effects, targets and potential secondary mechanisms of actions of pesticides, which can potentially explain their immunotoxic impact. Concrete conclusions have been achieved for neonicotinoids, while few hypotheses were made for the other pesticide groups.

pyrethroids seem to impair insect cellular immunity by directly crossing the hemocyte membranes and reaching the nucleus, where they cause DNA damage and apoptosis through strand breaks and activation of caspases (Kalita et al., 2017; Lindenboim et al., 2000). Consequently, cell death and inhibition of cellular division seem the primary causes of hemocyte and phenoloxidase decrease in hemolymph (Fig. 2) (Kalita et al., 2017). Pyrethroids cytotoxicity induces oxidative stress in insects, following the downregulation of antioxidant enzymes responsible for ROS elimination and the increase of MDA levels in organs and tissue (Qi et al., 2020). Although some evidence on organochlorine and pyrethroid immunotoxicity mechanisms exists, the lack of detailed studies on these molecules do not allow to draw firm conclusions on the real mechanisms behind the impairment of insect immunocompetence (Fig. 2). Conversely, in-depth research on neonicotinoids allowed to elaborate more concrete conclusions behind the insect immune susceptibility to these neurotoxicants. The most reasonable hypothesis suggested that neonicotinoids affect insect immunity due to the hemocyte connections with the neuroendocrine system (Makhijani et al., 2011; Makhijani and Brückner, 2012; Pamminger et al., 2017). Pamminger et al. (2018) reported that the innate immune system in insects can be considered an extension of the nervous system, considering that hemocytes may take part in neurogenesis and can react to signals coming from neurons, and neuronal cells can drive hemocyte maturation and hematopoiesis process (Malagoli et al., 2017; da Silva et al., 2015; Benton et al., 2014). Moreover, insect neurons express neuropeptides with antimicrobial activity upon immune challenges, and some genes take part in both neuronal and immune mechanisms. This evidence suggests that these physiological systems could “speak a common biochemical language” (Boulanger et al., 2001; Du Pasquier, 2005).

In turn, hemocytes can constitute themselves a neuronal-independent communication system by sending, receiving, and terminating acetylcholine (ACh) based signals (Giordani et al., 2023; Qi et al., 2016). Indeed, hemocytes can synthesize ACh and acetylcholine esterase (AChE) and produce functional nicotinic acetylcholine receptors (nAChRs) (Xu et al., 2017; Pamminger et al., 2018). The functional role of AChE and nAChR in immunity have been demonstrated (Bazzi et al., 2023; Giordani et al., 2023). Given that midgut and fat body also express nAChRs, there is evidence that insect immune defences are mediated by this ACh-based signal communication (Pamminger et al., 2017). Specifically, hemocytes would send signals to the fat body and midgut to coordinate a systemic immune response upon immune challenges.

However, this kind of communication is highly dependent on the presence and activity of AChE and functional nAChRs (Pamminger et al., 2017). As nAChRs are main target of neonicotinoids (Flatt et al., 2008), these pesticides would bind to these receptors, thus inhibiting the immune communication process mediated by nAChRs (Fig. 2) (Pamminger et al., 2017). Moreover, it is worth noting that there are different types of nAChRs exhibiting diverging sensitivity to neonicotinoids based on their subunit composition. Neuron nAChRs express mainly the subunit α7, and hemocytes express the subunits α2 and α7. These similitudes would help to better explain the hemocyte susceptibility to neonicotinoids and the different response to these chemicals according to the nAChRs subunit composition and binding probabilities (Pamminger et al., 2017). Overall, nAChRs binding would result in oxidative stress, apoptosis and cellular death (Fig. 2).

For example, acetamiprid binding with nAChRs caused receptor structural modifications by prompting higher absorption of Ca^{2+} in the cell (Jepson et al., 2006). In turn, high intracellular Ca^{2+} led to cell

oxidative stress (Yardimci et al., 2014) and potential damages to mitochondria structure and function (Paschen, 2000), as they are involved in growth and cellular division (McBride et al., 2006). Like other synthetic pesticides, thiamethoxam and imidacloprid would induce oxidative stress by directly entering in the redox cycle and inducing ROS and lipid peroxidation, with MDA release as end-product (Kayis et al., 2019; Yucel and Kayis, 2019). MDA causes loss of function and permeability of cellular membrane, apoptosis, necrosis and DNA damage (Kalita et al., 2017). Oxidative stress induced by neonicotinoids was also associated with impairment of Imd and Duox-ROS pathways, because of *duox* gene downregulation and higher production of ROS. Neonicotinoid toxicity can also result in gut damage and imbalance of gut microbiota with dramatic consequences for insect survival (Chmiel et al., 2019; Li et al., 2021a).

Although the neuroimmune interactions are poorly studied in insects, the tight hemocyte association with the neuronal pattern represent a primary candidate for neonicotinoid immunosuppression (Brandt et al., 2016; Pamminger et al., 2018). Simultaneously, another key mechanism of neonicotinoid immunosuppression arises from the impairment of the NF-κB pathway, which regulates expression of immune genes involved in AMPs production, melanisation, and clotting activity of hemocytes. Clothianidin upregulated the negative modulator *Amel\LRR* of the NF-κB pathway, which led to the downregulation of *Amel/102*, a specific immune gene regulating melanisation and encapsulation and other genes encoding AMPs expression (Di Prisco et al., 2013; Annoscia et al., 2020). In turn, melanisation and encapsulation reduce in a dose-dependent manner by sublethal doses of clothianidin (Annoscia et al., 2020). Interestingly, chlorpyriphos exposure did not impair any mechanism of the NF-κB pathway, suggesting that neonicotinoids affect insect immunity distinctly from other molecules (Di Prisco et al., 2013; James and Xu, 2012). Because the NF-κB pathway contributes to the overall fitness of honeybees, its impairment would explain the synergism of neonicotinoids in driving bee susceptibility to pathogens and parasites, such as DWV (Di Prisco et al., 2013) and *V. destructor* (Annoscia et al., 2020). Emerging evidence on their secondary effects indicates that OPs enhance cellular immunity and linked cellular process, such as encapsulation and melanisation, but impair insect midgut homeostasis (Fig. 2).

The stimulation of THC and DHC likely depends on hormetic effects or different molecular mechanisms. Rajak et al. (2018) hypothesized that the involvement of the apoptosis induced proliferation (AiP) mechanism, which consists in induced proliferation of surviving immune cells after organophosphate exposure, compensates the cell loss. However, enhanced cellular and humoral defences may be associated to standard activation of immune system to counteract the exogenous stress during detoxification, as reported for other molecules and microbial infections. Indeed, OPs exposure leads to gradual decrease of plasmatocytes and prohemocytes and increase of granulocytes, which are the immunocompetent cells involved in detoxification through phagocytosis (George and Ambrose, 2004).

Mechanisms of cellular immune stimulation can also depend on the level of OP exposure. Acute acephate treatments (2–8 μg/mL) on *D. melanogaster* larvae cause dose-dependent reduction of fly plasmatocytes and lamellocytes (Rajak et al., 2014), while chronic exposure increased these immunocompetent cells only at 3–5 μg/mL (Rajak et al., 2015). A similar chronic exposure with acephate caused a biphasic response in the THC of exposed fly larvae, consisting in increased hemocyte abundance at 1–4 μg/mL, followed by a decrease at higher concentrations. Because acephate and many other OPs, as profenofos and methyl parathion, induce cell death and inhibit the rate of mitosis (Tripathi et al., 2007; Ganguly et al., 2010), multiple OP concentrations under chronic or acute exposures can divergently affect plasmatocytes abundance and their differentiation in *D. melanogaster* larvae. Humoral impairments by organophosphates are linked to oxidative stress, decreased expression of antimicrobial peptides and midgut microbiota alterations. Phoxim actively damages midgut homeostasis by causing cell lysis and intestinal secretions (Gu et al., 2017), while oxidative stress is induced in a dose-dependent manner by increasing ROS, lipid peroxidation and MDA levels in the midgut and fat body (İçen et al., 2005; Büyükgüzel, 2009). Humoral alterations inhibit immune pathways regulating AMPs production (Zhang et al., 2018).

For example, phoxim upregulated *Cactus* gene in the Toll pathway, which inhibit the nuclear translocation of Dorsal and Dif transcription factors, responsible for AMPs gene expression (Gu et al., 2014; Li et al., 2020b). Insect growth regulators primarily affect endocrine system of insect juvenile stages and specifically influence the juvenile hormone (JH), ecdysone and chitin synthesis (Tunaz and Uygun, 2004). Evidence on relevant interconnections between the endocrine and immune systems are the bases to explain the cellular immunomodulatory effects of these molecules (Adamo, 2009).

Hemocyte differentiation in hemolymph is also an ecdysone-dependent process (Akai and Sato, 1973), thereby methoxyphenozide and tebufenozide were reported to increase insect THC due to their role as ecdysone mimic and activator of the ecdysteroid signalling cascade (Ghasemi et al., 2014a; Zibaee et al., 2011; Yu et al., 2018), while pyriproxyfen and fenoxycarb cause immunosuppression due to the ecdysone biosynthesis inhibition activity from prothoracic glands (Fig. 2) (Franssens et al., 2006; Ghasemi et al., 2014a; Mirhaghparast et al., 2015b; Nasr et al., 2010; Shaurub and Sabbour, 2017).

4.2. Biopesticide immunotoxicity mechanisms

Microbial-based biopesticides affect both cellular and humoral immunity of insects (Table 1), in particular pests and non-target species (Fig. 1). This can be explained by the assumption that microorganisms naturally elicit the innate immune responses of insect midgut, which is an immunologically active tissue that produces antimicrobial peptides and ROS (Wu et al., 2016). Specifically, *B. thuringiensis* infects insect orally and actively causes gut damage following the solubilization of δ-endotoxin in the intestinal tract and its transformation in active toxin (Siva-Jothy et al., 2005), which binds to gut epithelial receptors and cause cells lysis (Bravo et al., 2011). Under a sublethal exposure, *B. thuringiensis* cause minor but repairable damages to gut epithelium cells and the leakage of elicitors into the host hemocoel (Grizanova et al., 2014), which triggers plasmatocytes and granulocytes proliferation and related phagocytosis and encapsulation of non-self-particles (Lavine and Strand, 2002).

Increased phenoloxidase activity and melanisation occurs after sublethal *Bt* exposure to repair midgut damage and avoid the microorganism infection from spreading in the hemocoel (Dubovskiy et al., 2008; Grizanova et al., 2014). If bacteria do not infect insect hemolymph, only local (e.g., midgut) humoral response occurs, rather than systemic humoral stimulation (e.g., melanisation) (Crava et al., 2015). Because the main *Bt* target is the insect midgut, higher transcriptional activation of AMPs genes related to the Imd and Toll pathways (Crava et al., 2015; Bel et al., 2013; Ren et al., 2020), and alteration of native microbial community structure occur after sublethal *Bt* exposures (Fig. 2) (Li et al., 2021b). Indeed, AMPs are highly expressed to kill pathogens and maintain midgut homeostasis. In this context, commensal and symbiotic bacteria living in the intestinal tract are mainly in charge of digestion, but they are also known to mediate host defense responses (Wu et al., 2016).

Therefore, the loss of native midgut microbiota compromise insect immunocompetence and drive larval susceptibility to *Bt* and other stressors (Li et al., 2020). It is worth noting that increase if insect immunocompetence following *Bt* toxins seems to be a biphasic dose-depended process, as low concentrations triggered cellular and humoral immune responses (e.g., LC_5, LC_{15} and LC_{30}) (Dubovskiy et al., 2008; Crava et al., 2015; Ren et al., 2020; Li et al., 2021b), while higher doses (LC_{50}) cause immunosuppression (Grizanova et al., 2014; Li et al., 2018; El-Aziz and Awad, 2010; Manachini et al., 2011; Celi et al., 2022). Conversely to bacteria, fungi break insect integument to reach the

hemocoel, where innate insect immunity is triggered due to fungi spreading and production of extracellular toxins or proteins (Liu et al., 2017). Studies on entomopathogenic fungi consistently reported a transient increase of hemocytes in insect hemolymph, as mycosis at early infection stages trigger a major differentiation of plasmatocytes and granulocytes, which subsequently decrease to induce nodulation and phagocytosis of intruder fungi (Gillespie et al., 2000; Pour et al., 2021; Mirhaghparast et al., 2013; Zibaee and Malagoli, 2014).

The above hypothesis is supported by evidence of an inverse correlation between THC in the hemolymph and number of nodules during fungal infections (Gillespie et al., 2000; Zibaee et al., 2011). However, reports showing only immune cell decrease attributed such immunosuppression to the entomopathogenic fungi release of secondary metabolites with cytotoxic effects on hemocytes. Destruxins, cytochalasins and efrapeptins are some of the major secondary metabolites produced by the *B. bassiana*, *M. anisopliae* and *Tolypocladium cylindrosporum* (Hypocreales: Ophiocordycipitaceae). Specifically, they bind to membrane receptor molecules and alter the morphology and cytoskeleton of those hemocytes involved in immune defense, preventing ingestion or nodulation of fungal hyphae, and supporting fungus spread in the hemocoel (Huxham et al., 1989; Vilcinskas et al., 1997; Zibaee et al., 2011; Mazet et al., 1994).

In vitro studies demonstrate the inhibition of plasmatocytes, granulocytes, and phagocytosis by these fungal immunosuppressive agents, even at sublethal doses and in a dose-dependent manner (Vilcinskas et al., 1997; Hung and Boucias, 1992). Moreover, destruxins suppressed the overall melanisation response by downregulating phenoloxidase, PPO1, PPO2 and serine proteinase-like proteins expression, besides of antimicrobial peptides (Fan et al., 2014; Pal et al., 2007). *Beauveria bassiana* infection leads to immunosuppression of intestinal bacteria homeostasis by inhibiting AMPs and ROS (Wei et al., 2017); this last by the oosporein toxic effect on *Duox* gene (Feng et al., 2015). Like microbials, plant extracts affect insect immunity by inducing a prompt defense response during the early stages of contamination (Manjula et al., 2020). However, botanicals are rich in secondary metabolites, which exert insecticidal properties and likely affect insect immunity. For example, inhibition of the overall cellular immunity of *E. integriceps* following sublethal exposure to *A. annua* extracts produces artemisin, a secondary metabolite with notable insecticidal activity. Artemisin suppresses nodulation and phagocytosis by compromising hemocyte receptors in charge of detecting foreign bodies (e.g., fungal spores (Zibaee and Bandani, 2010). Similarly, sublethal doses of EOs induce a transient increase of hemocytes during intoxication, with a dose- and temporal-dependent initial rise, suggesting that cellular immunotoxicity would depend on the temporal activation of EOs bioactive compounds before affecting hemocytes, both by inhibiting their release from hemopoietic organs or their mitosis rate (Ghasemi et al., 2014b).

The route of exposure can influence the immune response to biopesticides, as topical application increases the penetration rate of these bioactive compounds rather than other application routes (Ghasemi et al., 2014b). Conversely, azadirachtin is widely reported to affect multiple physiological traits, along with its role as inhibitor of growth and neuroendocrine system (Mordue, 2004). Azadirachtin at non-lethal doses seem to reduce the THC and DHC in the hemolymph due to its powerful anti-ecdysis effects (Dorrah et al., 2019; Er et al., 2017; Figueiredo et al., 2006), as previously shown for some IGRs (Franssens et al., 2006; Ghasemi et al., 2014a). Indeed, sublethal exposure to azadirachtin inhibits ecdysone production in the prothoracic glands (Figueiredo et al., 2006; Azambuja et al., 1991).

Specifically, ecdysone and other ecdysteroids regulate hemolymph volume, differentiation of hemocytes and take part in the phagocytosis, establishing an immune-endocrine system connection (Dimarcq et al., 1997), as demonstrated by Figueiredo et al. (2006), which showed that the application of ecdysone in azadirachtin-exposed insects neutralized the immunosuppressive effects of the pesticide by restoring the number of hemocytes with phagocytosis function. In addition, the encapsulation of parasitized drosophila larvae was drastically compromised by low ecdysone levels and the ecdysone signalling pathway was blocked (Sorrentino et al., 2002). While Dorrah et al. (2019) and Azambuja et al. (1991) excluded potential genotoxicity and inhibition of melanisation, azadirachtin was reported to influence humoral immunity by downregulating AMPs and lysozyme gene expression (Zhao et al., 2022). Avermectins are associated with the ɣ-aminobutyric acid (GABA) receptor, affect the glutamate-gated chloride channel (GluCl), and disrupt intracellular Ca2+ homeostasis (Clark et al., 1995). Because of higher concentrations of glutamate-gated chloride channels in granulocytes membranes, higher concentrations of avermectins exert cytolytic activity on this immune cell (Kryukov et al., 2021). Avermectins can also promote fungal infections and cause perturbations on the midgut bacterial community, inducing a major production of AMPs from Toll and Imd pathways upregulation (Fig. 2) (Tomilova et al., 2016).

5. Implications of pesticide immunotoxicity on target and non-target insects

Understanding the relationship between pesticides and insect immunity is crucial for predicting the implications of agrochemical on non-target insects and some other model organisms (e.g., *G. mellonella*, *P. xylostella*, *S. litura*), which also play the role of polyphagous pests in agroecosystems (Takov et al., 2020). A major implication of pesticide immunotoxicity is the potential imbalance of insect ecological interactions with microorganisms and arthropods, both when they cause immunodeficiency or support insect immunocompetence increasing or decreasing their susceptibility to pathogens, parasites or natural enemies, impacting ecosystem services and pest control strategies (Fig. 3). Diseases and parasites alone are major drivers of honeybee colony losses

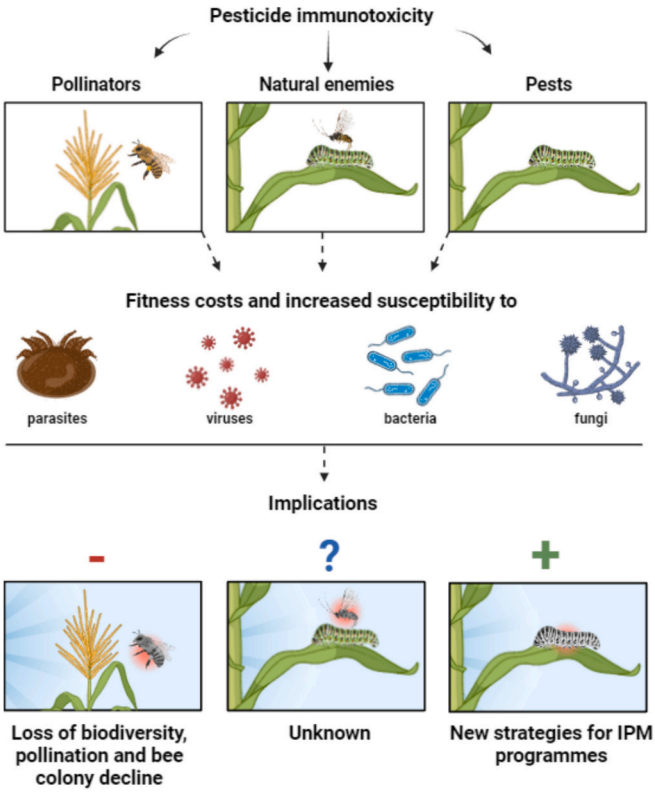

Fig. 3. Fitness costs and increased susceptibility to biotic stressors are the main implications of pesticide immunotoxicity on target and non-target organisms. This results in impairment of pollinator ecosystem service and potential support for integrated pest management strategies. Potential implications of pesticide immunotoxicity on insect natural enemies are still unknown. Figure was designed in the Biorender platform.

worldwide (Nazzi et al., 2012) and their infestations have become more and more prevalent in the last decades alongside the increasing use of pesticides (Sánchez-Bayo et al., 2016). The major consequences of pesticide sublethal exposure on pollinators are related to the increased susceptibility to *V. destructor* and viruses (Sánchez-Bayo et al., 2016). This effect seems to be specific to neonicotinoids (Annoscia et al., 2020), while organophosphates had a synergic effect with *P. larvae* in increasing honeybee mortality, which was not observed with pyrethroids (López et al., 2017). Novel crop protection strategies can be developed when immunosuppressive pesticides increase the susceptibility of insect pests to pathogen and parasites (Er et al., 2017; Kayis et al., 2019; Wu et al., 2016). Immunomodulation induced by organochlorine and organophosphate sublethal exposure shape host-parasitoid interactions (Delpuech and Tekinel-Ozalp, 1991; Delpuech et al., 1996), suggesting that pesticides have the potential to affect the biological control efforts when using parasitoids. Increased attention has been placed on the potential synergism between insecticides and entomopathogen candidates for integrated pest management. Microbial infections (e.g., *M. robertsii*) directly increase avermectins toxicity through inhibition of insect detoxication enzymes (Kryukov et al., 2021), while avermectins promote host susceptibility to pathogens by suppressing insect resistance mechanisms to microbials (Tomilova et al., 2016).

In contrast, when sublethal pesticide exposures enhance insect immunocompetence (Table 1), surviving insects may become more resistant to pathogens and parasites. The Asian corn borer larvae exposed to tebufenozide LC_{30} became more resistant to subsequent microbial infections due to the increased activity of phenoloxidase in the hemolymph, which helps to clear bacteria (Yu et al., 2018). However, stimulating and strengthening insect immunity implicates a higher investment of energy resources at the expense of other physiological and metabolic processes (Schmid-Hempel, 2005; Siva-Jothy et al., 2005; Dubovskiy et al., 2013). Indeed, the ability to defend must be maintained at some costs, and everything affecting insect immunity (e.g., conventional pesticides or microbial infections) imposes life-history changes with serious implications on the overall fitness of affected insects (Schmid-Hempel, 2005).

For example, while the activation of the phenoloxidase cascade induced by pesticides increases resistance to bacteria (Yu et al., 2018), it also produces cytotoxic secondary compounds that cause cell damage and death (Siva-Jothy et al., 2005). Behaviour and physiology in insects concurrently ensure the proper functioning of the living organism. Thus, pesticide sublethal effects on insect behaviour can have an indirect impact on immune response. For example, increased humoral immunity reported following avermectins sublethal exposure (Table 1) consisted in increased production of AMPs resulting from the pesticide impairment of feeding behaviour and gut bacterial community (Kryukov et al., 2021). Other pesticides disrupt insect chemical communication by affecting hemocytes (López et al., 2017), as oenocytoids takes part in PO production (Ribeiro and Brehélin, 2006), and are involved in bee social immunity by modulating behaviour and communication between nestmates (López et al., 2017). Particularly, these immune cells are involved in the production of cuticular hydrocarbons (CCH), which act as immunological cues for healthy honeybees to detect and exclude diseased nestmates from the colony and take hygienic measures to preserve the colony health. Also, when healthy bees detect CCH from diseased nestmates, their standard immune response is elicited (López et al., 2017). In this context, oenocytoids alteration by pesticides would strongly affect honeybee colony fitness and behaviour.

In conclusion, pesticides affect insect immunity and shape their interactions with microbials and arthropods, resulting in fitness loss and social immune alterations (Sánchez-Bayo et al., 2016), and compromising key insect ecological roles (Ellis et al., 2010; Barbosa et al., 2015; Lima et al., 2016; Bernardes et al., 2022). Pollinators are key drivers for the biodiversity preservation and crop pollination (Klein et al., 2007). As a key ecosystem service, honeybees are the most common pollinators in natural habitats (Hung et al., 2018) and Neotropical stingless bee species are paramount in the pollination of native and cultivated plant species in the Neotropics (Barbosa et al., 2015; Lima et al., 2016). Other non-target insect species, such chironomid flies, have a great ecological importance in freshwater ecosystems as decomposer and predominant components of aquatic food webs (Dornelas et al., 2020).

Insects are also key organisms for the human economy with honeybees generating significant products for the agri-food sector, such as honey, pollen, wax, propolis and royal jelly (Klein et al., 2007), while silkworms are primary source of natural fibre for the silk industry (Kalita et al., 2017). Exploiting pesticide immunotoxicity can have positive implications when developing IPM programs, but the potential impact on biological control agents (e.g., predators and parasitoids) remains unknown (Fig. 3). These effects can be potentially amplified towards an entire community with unpredictable long-term consequences (Guedes et al., 2017; Resende-Silva et al., 2023).

6. Knowledge gaps and future outlooks

While our review has shed light on the current understanding of pesticide side effects on insect immunity, several knowledge gaps remain, necessitating further research initiatives (Table 2). Although certain pesticide groups have been extensively studied for their immunotoxic effects, such as neonicotinoids (Annoscia et al., 2020), organochlorines, pyrethroids, avermectins (Tomilova et al., 2016), and IGRs (Franssens et al., 2006), others remain relatively unexplored (Table 1). For example, the impacts of diamides, spinosyns, fungicides, and herbicides on insect immunity require investigation. In support of this, different computational estimation methods are being used for predicting acute and chronic pesticide toxicity with the benefit to decrease the number and cost of animal testing (Roy, 2017).

For example, quantitative structure-activity relationship (QSAR) has been recognized as important research direction in the field of toxicology and environmental chemistry to predict the ecotoxicity of chemicals, although few studies have been developed for insects (Singh et al., 2014). Among the tested pesticide classes, the mechanisms of immunotoxicity have been explored in-depth only for neonicotinoids, while few hypotheses have been pointed out for other pesticide classes (Fig. 2). In particular, no studies reported specific sites of action of organophosphates on hemocytes, while data collected in this review could serve as a proxy to suggest that AChE expressed in hemocytes is a target for organophosphates, similarly to neonicotinoids affecting nAChRs in hemocytes (Fig. 2). Future research should address the need to investigate other potential effects and mechanisms by which insecticides can impair insect immunity. For example, several pesticides were reported

Table 2
Summary of the current knowledge gaps related to the pesticide immunotoxicity and potential outcomes that could be achieved by future research.

Knowledge gaps	Potential outcomes obtained by future research
Few or no studies for certain pesticide groups (see Table 1)	Broader and clearer understanding of pesticide immunotoxicity
Little knowledge on mechanisms of pesticide immunotoxicity	Improved understanding on secondary mode of action of pesticides on insects
Limited research focusing on arthropods natural enemies	Increased knowledge on pesticide non-target effects in support of IPM programs
Few studies on the DHC and its correlation with cellular defense processes	New approaches to provide a complete measurement of pesticide immunotoxicity
Uncertainty about immune priming induced by pesticides	Evaluation of insect adaptation towards pathogens, parasites and arthropods
Limited investigations under field and semi-field conditions	Interpretating the realistic impact of pesticides on target and non-target organisms

to increase pollinator susceptibility to a complex of viral infections against which insect immunity reacts through the RNA interference (RNAi) defense mechanism. Therefore, increased bee susceptibility to viruses might be associated with RNAi pathway impairment after sublethal pesticide exposure. Current research on pesticide immunotoxicity has primarily focused on pollinators and insect pests (Sánchez-Bayo et al., 2016), leaving a gap in understanding the effects on biological control agents, such as predators and parasitoids.

Additionally, other beneficial insects used in various industries, such as honey, wax, dye, and silk-producing species, warrant further investigation. Existing methods for assessing pesticide immunotoxicity often focus on total hemocyte count (THC) as a measure of cellular immunotoxicity. However, this approach alone provides limited information and may not accurately reflect the overall pesticide impact on insect immunity by overlooking the effects on different hemocyte subtypes and their specific immune functions. Future research should adopt a more comprehensive approach, considering differential hemocyte count (DHC) to assess pesticide sensitivity and immune defense capacity accurately.

It is also worth noting that current immunotoxicity assessments could be influenced by non-immune factors. For example, a large number of hemocytes normally rest on the surface of various organs of the hemocoel and only those freely circulating in hemolymph are subjected to the hematological count. Some pesticides can modulate insect blood volume determining a dilution effect on the hemolymph and consequently affecting the count of immune cells in a given volume. Furthermore, current knowledge of pesticide immunotoxicity suggests that hemocyte alterations are strongly influenced by the level of exposure, such as the route of exposure and the tested concentrations.

However, the timing of THC assessments can also impact results and conclusions on pesticide immunotoxicity, as most of the studies showed a transient increase or decrease of hemocyte abundance by stimulating the insect standard immune system, while others induce morphological and histological alterations of hemocytes (e.g., mitosis, vacuolization, nucleus deformation, and apoptosis), resulting in an overall decrease in their content in the hemolymph. There is evidence suggesting that pesticides at sublethal doses may prime insect immune responses, making them more resistant to subsequent stressors (Moret and Siva-Jothy, 2003; Pham et al., 2007). However, mechanisms underlying this phenomenon remain poorly understood. Future research should investigate the mechanisms of immune priming at sublethal doses to predict insect adaptation to pathogens, parasites, and other stressors more effectively. While laboratory studies have provided valuable insights, field evaluations are essential to understand the realistic impact of pesticides on insect immunity at the population and community levels (Guedes et al., 2016, 2022a, 2022b). Acute and chronic toxicity tests under field and semi-field conditions can help bridge the gap between laboratory findings and real-world scenarios. In summary, addressing these knowledge gaps and incorporating field evaluations will enhance our understanding of pesticide immunotoxicity, contributing to more effective and sustainable pest management practices.

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CRediT authorship contribution statement

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Declaration of competing interest

The authors declare that they have no competing interests.

Data availability

No data was used for the research described in the article.

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