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Dani Sukkar

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THESIS

To obtain the title

Doctor of the University of Lorraine

Mention: Ecotoxicology, Biodiversity, Ecosystems

Defended by:

Dani SUKKAR

Role of *Nosema cerenae* and pesticides on the decline of bees:

Studies using a multifactorial approach

“Tipping the scale of honeybee immune responses: The effect of pesticides on immune-stimulation mimicking *Nosema* spp.”

July 6th 2023

Jury members

President	Pr. Christophe BERTSCH	Université de Haute-Alsace
Reviewer		
Reviewer	Pr. Angélique VÉTILLARD	INU Jean-François Champollion
Examiner	Dr. Sabine AZOURY	Université Libanaise
Supervisor	Pr. Jairo FALLA-ANGEL	Université de Lorraine, IUT de Thionville-Yutz
Co-director	Dr. Ali KANSO	Université Libanaise-Université de Lorraine

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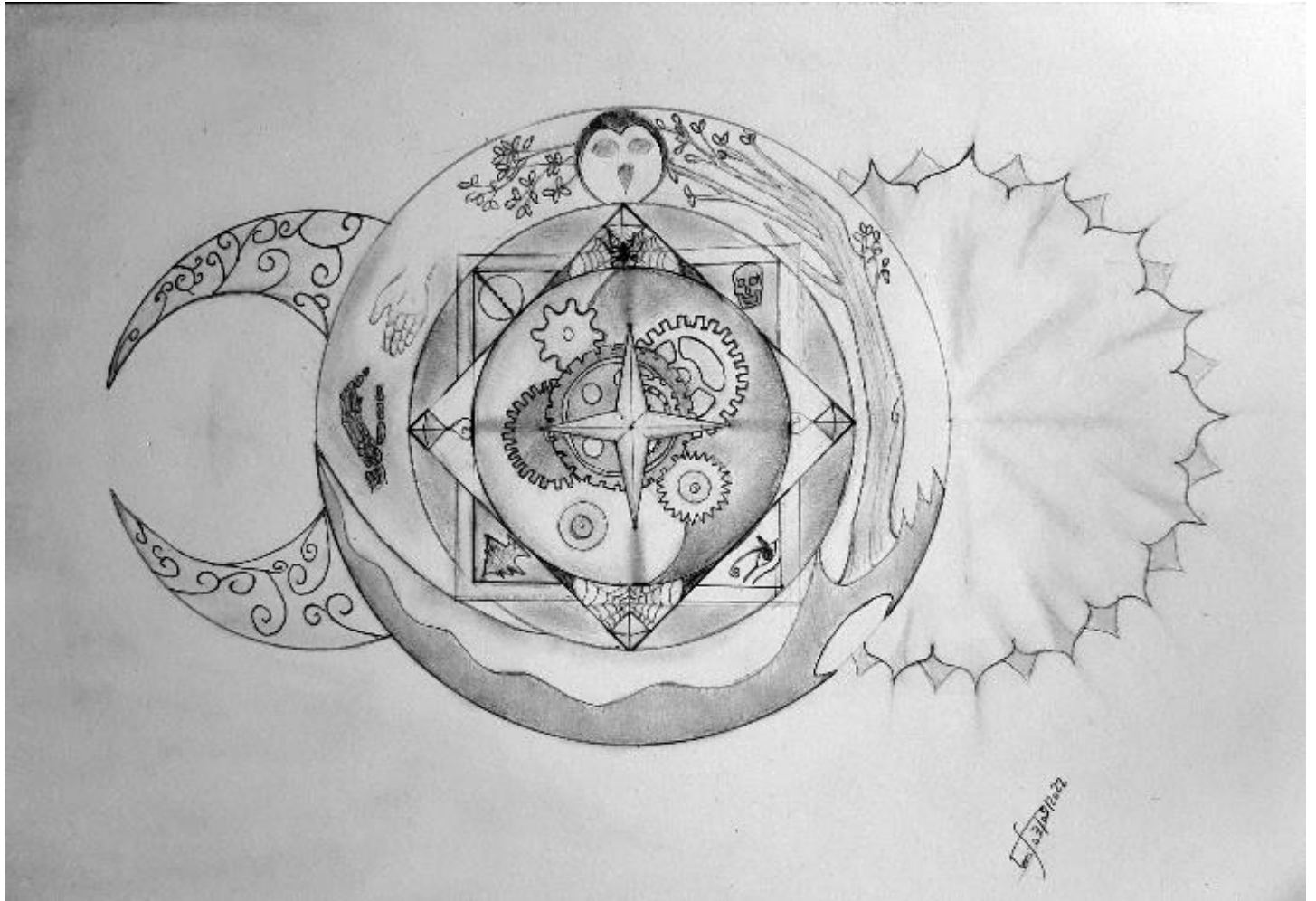
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List of abbreviation

Abs	Absorbance
AChE	Acetylcholine esterase
AGO	Argonaute
AMPs	Antimicrobial peptides
BYV	Beetroot yellow virus
CAT	Catalase
CCD	Colony collapse disorder
cDNA	Complementary deoxyribonucleic acid
DAMP	Damage associated molecular pattern
DDT	Dichloro-diphenyl-trichloroethane
DMFP	N ² -(2,4-Dimethylphenyl)-N ¹ -methyformamidine
DWV	Deformed wing virus
EFSA	European food safety authority
EGF	Epidermal growth factor
GNBP	gram-negative binding protein
GST	Glutathione-S-transferase
H ₂ O ₂	Hydrogen peroxide
IAPV	Israeli acute paralysis virus
IMD	Immune-deficiency
JAK	Janus Kinases
L-DOPA	Levodopa; l-3,4-dihydroxyphenylalanine

MAMP	Mitogen associated molecular pattern
MyD88	Myeloid differentiation factor 88
nAChR	Nicotinic acetylcholine receptor
NAG	N-acetylglucosamine
NAM	N-acetylmuramic acid
Neonics	Neonicotinoids
NIM	Nimrods
NO	Nitric oxide
PAMP	Pathogen associated molecular pattern
PGRP	Peptidoglycan recognition protein
PI	Propidium iodide
PO	Phenoloxidase
PO-AS	Phenoloxidase activation system
ppb	part per billion
ProPO	Pro-phenoloxidase
PTU	Phenylthiourea
qPCR	Real-time polymerase chain reaction
rcf	relative centrifugal force
RISK	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
SBV	Sacbrood virus

SOD	Superoxide dismutase
SPZ	Spaetzle; Spatzle
STAT	Signal transducer and activator of transcription proteins
USA	United States of America
VAMP	Virus associated molecular pattern
ZYM	Zymosan A

Résumé General

Les abeilles mellifères sont des pollinisateurs ayant une grande valeur économique grâce à leur production de miel. Les services de pollinisation assurés par les insectes représentent jusqu'à 84 % de la production agricole mondiale (Allsopp et al., 2008). Cependant, les abeilles mellifères font face à de nombreux facteurs de stress biotiques et abiotiques qui contribuent à leur déclin mondial, phénomène connu sous le nom de syndrome d'effondrement des colonies (CCD) (Oldroyd, 2007 ; vanEngelsdorp et al., 2009). Ce phénomène de mort soudaine des colonies est attribué à plusieurs facteurs interagissant les uns avec les autres, notamment les parasites, les maladies, le changement climatique et l'activité anthropique, en particulier l'utilisation de pesticides (Cox-Foster et al., 2007).

En 2016, la France a mis en place une interdiction d'utilisation des néonicotinoïdes en extérieur après une évaluation des risques qui indique les dangers de leur utilisation sur la santé des colonies d'abeilles ainsi que sur d'autres organismes non ciblés (Loi sur la biodiversité, 2016). Cependant, comme il n'y avait pas d'alternative aux néonicotinoïdes pour lutter contre le vecteur de propagation du virus jaune de la betterave qui affectait la culture de la betterave en France, une autorisation d'urgence a été en 2021 pour permettre l'utilisation d'imidaclopride et de thiaméthoxame (EFSA, 2021). L'imidaclopride est le néonicotinoïde le plus utilisé dans le monde et même avec une utilisation réglementée, il présente encore un risque pour les colonies d'abeilles (Jeschke et al., 2011). Au niveau mondial, parmi les résidus de néocotinoïdes, ce sont les résidus d'imidaclopride qui ont été trouvés avec une présence plus marquée dans le miel (Mitchell et al., 2017). Bien qu'il ne soit pas le néonicotinoïde le plus présent dans le miel européen, l'imidaclopride est le plus élevé dans les mélanges de pesticides dosés dans le miel, après l'Amérique du Nord, en 2017 (Mitchell et al., 2017).

Cox-Foster et al. ont réalisé une analyse métagénomique en 2007 afin de déterminer l'implication de différents agents pathogènes et leur degré de contribution au CCD. Le virus IABV a montré la corrélation la plus élevée et a été considéré comme le principal contributeur au CCD, mais le virus était déjà présent aux États-Unis avant la manifestation du CCD. Fait intéressant, toutes les ruches étudiées aux États-Unis et en Australie étaient infectées par *Nosema* spp. (Cox-Foster et al., 2007).

De nombreux autres agents pathogènes infectent les ruches d'abeilles, notamment des bactéries telles que la loque américaine ou la loque européenne (Martin & Allsopp, 2021). Les parasites attaquent également les ruches d'abeilles pour se nourrir de leurs produits ou se nourrir de l'hémolymphe de leur couvain. Le *Varroa destructor* est un ectoparasite qui se nourrit de l'hémolymphe, en particulier du tissu adipeux des larves d'abeilles mellifères (Ramsey et al., 2019). Dans les pratiques courantes de l'apiculture, les apiculteurs appliquent généralement l'acaricide amitraz pour lutter contre les infections par *Varroa* (Rosenkranz et al., 2010). L'amitraz est un agoniste des récepteurs de l'octopamine qui stimule continuellement le système nerveux des insectes, entraînant paralysie et mort (Ostiguy et al., 2019). Son effet toxique est attribué à son métabolite DMFP (Guo et al., 2021). La sélectivité de l'amitraz envers les tiques et les acariens, ainsi que sa faible métabolisation chez les abeilles mellifères, en font un acaricide approprié. Cependant, on a constaté que l'amitraz était un synergiste établi, augmentant les effets négatifs d'autres pesticides et réduisant la tolérance aux infections (Johnson et al., 2013 ; O'Neal et al., 2017), sans oublier l'effet proposé sur la fonction olfactive et l'effet observé sur la fonction cardiaque, la ponte des œufs et les performances de la ruche (Boncristiani et al., 2012 ; O'Neal et al., 2017).

Différents pesticides et agents pathogènes cohabitent avec les ruches d'abeilles et peuvent interagir, ce qui finalement affaiblit la vitalité de la ruche. Les pesticides peuvent affaiblir le système immunitaire des abeilles mellifères, entraînant une faible tolérance aux infections. L'effet des expositions individuelles à des pesticides tels que l'imidaclopride et l'amitraz a peut-être été évalué à certains niveaux, mais pas lorsqu'ils sont combinés. L'ajout de différents niveaux de stimulation immunitaire pourrait fournir des informations supplémentaires sur les interactions entre les pesticides et les agents pathogènes dans la réponse immunitaire et la santé des abeilles mellifères.

Le système immunitaire des invertébrés repose sur une immunité innée et ne possède pas la complexité du système immunitaire adaptatif que l'on retrouve chez les vertébrés, c'est-à-dire que les invertébrés ne possèdent pas de lymphocytes véritables (Larsen et al., 2019). Cependant, on a découvert l'existence d'une mémoire immunitaire associée au système

immunitaire inné des invertébrés (Melillo et al., 2018). Les réponses immunitaires cellulaires comprennent la phagocytose des particules étrangères et des microorganismes. La phagocytose est un mécanisme crucial de défense contre les infections pathogènes (Cosson & Soldati, 2008). Ce processus est conservé de manière évolutive depuis l'émergence des premiers eucaryotes jusqu'à aujourd'hui (Yutin et al., 2009). D'autres réponses immunitaires cellulaires comprennent l'encapsulation et la mélanisation. Les bourdons et les abeilles ont montré une phagocytose réduite lorsqu'ils étaient exposés à l'imidaclopride. Quant au bras humoral du système immunitaire inné, la défense contre les pathogènes est assurée par des peptides antimicrobiens et la production d'espèces réactives de l'oxygène telles que le peroxyde d'hydrogène et des espèces réactives de l'azote telles que le monoxyde d'azote (Eleftherianos et al., 2021). L'imidaclopride peut altérer la fonction mitochondriale et affecter les niveaux de production de peroxyde d'hydrogène. On a observé que l'imidaclopride réduisait la production de peroxyde d'hydrogène et de monoxyde d'azote chez les abeilles, même lorsque les cellules étaient exposées à un activateur immunitaire tel que les lipopolysaccharides (Walderdorff et al., 2018).

Les voies Toll, IMD et JAK/STAT sont les principales voies impliquées dans les réponses immunitaires chez les invertébrés. La voie JAK/STAT induit une activité phagocytaire lorsqu'elle est activée, en plus de sa réponse antivirale (Brutscher et al., 2015 ; McMenamin et al., 2018). Cependant, Eater est l'un des principaux composants de la phagocytose et est impliqué dans l'adhésion et la mobilité, ce qui peut être associé aux réponses immunitaires contre les agents infectieux (Melcarne, Lemaitre et Kurant, 2019). La voie IMD reconnaît les PAMPs (motifs moléculaires associés aux pathogènes) fongiques et bactériens et conduit à la production de peptides antimicrobiens pour se défendre contre les microbes (Aymeric et al., 2010 ; Evans et al., 2006). Relish est un facteur de transcription qui entraîne la production de peptides antimicrobiens via la voie IMD et joue un rôle clé en plus de son rôle dans les interactions croisées entre différentes voies (Evans et al., 2006). Plus important encore, la voie Toll joue un rôle dans la réponse immunitaire et le développement chez les invertébrés, possédant de nombreux éléments qui répondent à l'infection (Steward & Govind, 1993 ; Wilson et al., 2014). La voie Toll peut reconnaître principalement les PAMPs des bactéries Gram-positives et des champignons via des récepteurs de reconnaissance des pathogènes. Spaetzle est un récepteur de reconnaissance des

pathogènes extracellulaire qui conduit à la reconnaissance des PAMPs. En aval se trouve le récepteur Toll transmembranaire qui se lie à Spaetzle activé, et une cascade de signalisation se poursuit via MyD88, une protéine conservée chez les vertébrés et les invertébrés, ce qui entraîne l'activation des facteurs de transcription NF- κ B et la production de peptides antimicrobiens (Steward & Govind, 1993).

Un autre élément de l'immunité des abeilles est la vitellogénine. La vitellogénine est une protéine du vitellus liée à la reproduction chez les animaux ovipares, mais chez les abeilles mellifères, elle a une activité antioxydante et est liée au vieillissement (Seehuus et al., 2006). La vitellogénine est également importante pour la priming immunitaire transgénérationnelle (TGIP), qui correspond au transfert d'immunité des adultes aux larves. La TGIP confère aux larves naïves la capacité de mieux répondre aux pathogènes précédemment rencontrés par les adultes.

Les abeilles mellifères ne sont pas les seuls insectes exposés à l'imidaclopride et à l'amitraz. La teigne de la cire est un prédateur des abeilles mellifères et leurs différentes réponses aux pesticides peuvent donner un avantage à l'un par rapport à l'autre, notamment du fait que les ruches affaiblies sont une cible privilégiée de la teigne de la cire. Ainsi, évaluer au moins les niveaux immédiats de réponses immunitaires sur des cellules similaires à celles du ravageur est important pour avoir une vue plus complète des événements pouvant conduire au CCD. Étant donné que la mouche des fruits est l'organisme modèle pour le système immunitaire des insectes et est également utilisée pour évaluer l'effet des pesticides, il serait intéressant d'ajouter des cellules de *Drosophila* à cette approche comparative.

La co-exposition de l'imidaclopride et de l'amitraz dans différentes concentrations et leur effet sur les réponses immunitaires reste à évaluer. L'évaluation de l'effet des pesticides sur le système immunitaire nécessite de déclencher la réponse immunitaire sans les dommages cellulaires associés à une infection. Une approche pratique consiste à utiliser des activateurs/stimulateurs immunitaires tels que le zymosan A. Dérivé de la paroi cellulaire de la levure *Saccharomyces cerevisiae*, le zymosan A peut imiter une infection fongique similaire à celle de l'agent microsporidien *Nosema*.

Dans cette étude, nous avons évalué l'effet de l'imidaclopride et de l'amitraz en exposition unique ou en combinaison dans différentes proportions sur les hémocytes des abeilles mellifères à différentes concentrations de zymosan A, un activateur immunitaire. Les effets ont été observés à trois niveaux : le premier niveau concerne les produits cellulaires lors de la réponse immédiate, comprenant la production d'oxyde nitrique de 15 à 120 minutes après exposition aux pesticides et au zymosan, ainsi que l'hydrogène peroxyde et la teneur en protéines à 3 heures après exposition. Les réponses des hémocytes des abeilles mellifères ont été comparées à celles des cellules Schneider-2 représentant la mouche des fruits (système modèle) et aux cellules MB-L2 du papillon du chou représentant les insectes lépidoptères tels que la teigne de la cire. Le deuxième niveau concerne les réponses cellulaires, notamment la phagocytose et la cytotoxicité après 18 heures d'exposition aux pesticides et au zymosan A par cytométrie en flux. La cytotoxicité a également été évaluée par la méthode d'exclusion de colorant avant l'application de toute expérience pour évaluer l'applicabilité des concentrations sélectionnées. Le troisième niveau consiste à étudier l'expression des gènes des voies immunitaires telles que *spaetzle*, *toll* et *myD88* de la voie *Toll*, *relish* de la voie IMD, ainsi que *eater* et *vitellogenine* (*vg*) après 18 heures d'exposition.

L'imidaclopride et l'amitraz ont un effet spécifique sur le système immunitaire des abeilles. L'imidaclopride et l'amitraz réduisent la production d'oxyde nitrique et de peroxyde d'hydrogène par les hémocytes des abeilles au moment de l'exposition. Le zymosan A amplifie l'effet des pesticides, que ce soit seuls ou en combinaison, sur la production d'oxyde nitrique à une concentration de 1 µg/ml de zymosan A. Cependant, à une concentration de 10 µg/ml de zymosan A, l'effet négatif des pesticides sur les hémocytes des abeilles est atténué, ce qui laisse supposer qu'une stimulation immunitaire plus forte masque l'effet des pesticides sur la production d'oxyde nitrique. Comparés aux cellules MB-L2 et Schneider-2, les hémocytes des abeilles sont plus fortement affectés en ce qui concerne la production d'oxyde nitrique. En effet, les cellules Schneider-2 peuvent avoir un mécanisme régulateur interne qui les rend moins sensibles à l'imidaclopride et l'amitraz, car la production d'oxyde nitrique ne change pas aussi radicalement que celle des hémocytes des abeilles ou des cellules MB-L2. Cela est vrai non seulement à différents moments, mais aussi pendant la période de production allant de 15 minutes à 2 heures après

l'exposition. La production de peroxyde d'hydrogène diminue chez les hémocytes des abeilles exposées à l'imidaclopride et à l'amitraz, que ce soit sans zymosan ou avec 1 µg/ml de zymosan, mais aucun changement significatif n'a été observé avec une concentration plus élevée de zymosan, un schéma similaire à celui de la production d'oxyde nitrique.

L'imidaclopride et l'amitraz réduisent la production de peroxyde d'hydrogène dans les cellules Schneider-2, mais seulement en présence de zymosan. Dans l'ensemble, l'imidaclopride et l'amitraz semblent avoir un effet plus important sur la production de RNS et de ROS dans les hémocytes des abeilles que sur les lignées cellulaires de la teigne du chou (MB-L2) et de la drosophile (Schneider-2). Cela implique que les ruches d'abeilles pourraient être désavantagées dans les sites exposés à l'amitraz et à l'imidaclopride, et qu'elles sont plus vulnérables sur le plan immunitaire. De plus, la teneur en protéines totales dans toutes les espèces étudiées n'a pas été affectée par rapport à leurs témoins respectifs, sauf lorsque les cellules MB-L2 ont été exposées aux combinaisons de pesticides à une concentration de 10 µg/ml de zymosan. Ainsi, l'effet des pesticides sur les produits cellulaires n'est pas lié à la production de protéines. En tout cas, toute altération de la teneur en protéines est trop faible pour être détectée par le test de Bradford.

L'imidaclopride et l'amitraz réduisent le taux de phagocytose dans les cellules des abeilles, mais seulement lorsque le système immunitaire est stimulé par le zymosan, quelles que soient les concentrations utilisées. La phagocytose n'est pas affectée lorsque les cellules ne sont pas traitées avec le zymosan A. Cependant, lorsqu'elles sont traitées avec le zymosan, l'imidaclopride et l'amitraz montrent une diminution de la phagocytose de manière dose-dépendante. Les combinaisons de pesticides ont l'effet le plus fort sur la diminution de la phagocytose. La phagocytose est négativement corrélée à la viabilité des hémocytes telle que détectée par cytométrie en flux. La corrélation négative est présente uniquement lorsque les cellules sont activées immunitairement par le zymosan, suggérant la présence d'un compromis entre le taux de phagocytose et la viabilité cellulaire. Plus précisément, une phagocytose réduite est corrélée à une réponse cytoprotectrice qui augmente la viabilité cellulaire, même par rapport au témoin. L'exposition conjointe à l'imidaclopride et à l'amitraz induit cette réponse protectrice lorsqu'elle est activée immunitairement. Les hémocytes semblent prévenir l'effet cytotoxique des pesticides peut-être par un mécanisme

qui altère la membrane cellulaire. Comme la phagocytose est associée à l'activité membranaire, il est possible que la réponse protectrice entraîne une réduction de la phagocytose. L'augmentation de la viabilité des hémocytes des abeilles exposées aux mélanges de pesticides a également été observée avec la méthode d'exclusion des colorants. La corrélation négative entre la phagocytose et la viabilité suggère que les hémocytes exposés aux pesticides sont incapables de compter sur la phagocytose pour se défendre contre les agents pathogènes, ce qui rend l'organisme plus susceptible aux infections.

Au niveau moléculaire, que ce soit en exposition unique, en mélange, avec exposition au zymosan A ou non, l'imidaclopride et l'amitraz ont diminué l'expression des gènes *spaeztle* et *toll* de la voie Toll. L'expression de ces deux gènes semble fortement corrélée et, étant donné qu'ils se trouvent au premier niveau de reconnaissance des pathogènes, cela pourrait entraîner une diminution des réponses immunitaires résultant de la voie Toll, comme la production de AMPs (peptides antimicrobiens). Cependant, avec une exposition au zymosan, l'effet des pesticides est moins sévère mais toujours significatif. L'expression de *myD88* augmente lorsque les cellules sont exposées à l'amitraz sans défi immunitaire et avec 10 µg/ml d'imidaclopride. L'exposition au zymosan A semble atténuer dans une certaine mesure l'effet des pesticides sur la variation de l'expression des gènes. Ainsi, l'exposition à l'amitraz pourrait affecter les processus cellulaires dépendant de *myD88*.

L'expression de *relish* avec les pesticides sans zymosan est variable, mais avec une diminution prononcée dans les mélanges de pesticides contenant une concentration plus élevée d'imidaclopride ou d'amitraz. Cependant, lorsque des cellules sont exposées au zymosan, les variations entre les traitements diminuent. Le zymosan A semble réguler l'expression de *relish* à un niveau homéostatique.

Eater, en tant qu'élément crucial dans la phagocytose, n'a montré aucune association avec la lecture de phagocytose des perles fluorescentes. En fait, l'expression de *eater* a augmenté avec les combinaisons de pesticides, contrairement à la diminution de la phagocytose avec les mêmes combinaisons. Ainsi, l'action des pesticides entravant la phagocytose dans le contexte de la stimulation immunitaire n'est pas nécessairement liée à l'expression de *eater*. Les résultats suggèrent plutôt que la phagocytose est réduite par un mécanisme différent qui pourrait être lié à l'activité membranaire.

Les niveaux de vitellogénine ne sont affectés par aucun des traitements aux pesticides, que ce soit en exposition unique ou en combinaisons, à l'exception de la combinaison 10I-10A sans zymosan où l'expression génique augmente. Ainsi, l'imidaclopride et l'amitraz peuvent agir comme de la reproduction des conditions très spécifiques. Cela pourrait avoir des effets drastiques sur la reproduction et le développement des abeilles, altérant la cohérence normale de la colonie et de la couvée.

À partir de nos résultats, nous pouvons affirmer que l'imidaclopride et l'amitraz entravent la compétence immunitaire des hémocytes des abeilles au niveau de la réponse immunitaire et de la reconnaissance des pathogènes, principalement dans la voie du Toll. De plus, la combinaison de pesticides réduit la phagocytose, qui est un processus crucial de l'immunité innée. Le synergisme/antagonisme entre l'amitraz et l'imidaclopride est observé dans nos résultats.

Zymosan A pourrait constituer une bonne application pour les ruches d'abeilles en normalisant l'expression des gènes immunitaires, mais dans des conditions très strictes où la ruche n'est pas exposée simultanément à l'imidaclopride et à l'amitraz, car le zymosan semble induire une phagocytose réduite avec les mélanges de pesticides. Des recherches supplémentaires sont nécessaires au niveau des enzymes antioxydantes et de leur activité, ainsi que de l'expression des produits géniques associés, ce qui pourrait mieux expliquer les observations dans la production de RNS et de ROS. Cela pourrait également permettre une meilleure compréhension de la réponse immunitaire et de l'interaction intrinsèque avec les pesticides et les stimulateurs immunitaires, en fournissant une carte immunologique détaillée comprenant le mode d'action. Le compromis entre la phagocytose et la réponse cytoprotectrice est également un aspect intéressant à étudier, principalement les composants de l'action de la membrane cellulaire et les composants de l'action phagocytaire dans les cellules immunitaires.

L'amitraz et l'imidaclopride entravent la réponse immunitaire chez les abeilles et peuvent contribuer au CCD (Colony Collapse Disorder, ou syndrome d'effondrement des colonies). L'évaluation de la co-présence de différents facteurs doit être prise en compte dans les études futures lors de l'évaluation des risques liés à l'utilisation des pesticides, car les effets sous-jacents peuvent ne pas être directement apparents ou se produire dans un contexte

simple. L'ordre d'exposition aux pesticides ou l'activation immunitaire peuvent également influencer la réponse des hémocytes des abeilles. L'activation immunitaire avant l'exposition aux pesticides peut réguler les hémocytes de manière à ce qu'ils ne soient pas affectés de manière drastique par les pesticides, comme nos résultats l'ont démontré avec l'effet du zymosan dans certaines conditions en ce qui concerne les concentrations et les paramètres étudiés.

D'autre part, l'activation immunitaire peut entraîner un état hyper-inflammatoire qui peut rendre les abeilles plus susceptibles aux infections. Cela devrait être évalué avec des infections qui déclenchent les mêmes voies immunitaires ou des voies immunitaires différentes. Des recherches approfondies sont nécessaires pour comprendre pleinement l'occurrence du CCD dans différentes conditions et dans une approche globale.

Ce travail pourrait poser des bases solides pour évaluer des interactions complexes, en plus de mettre en lumière la présence de mécanismes du système immunitaire et de signalisation cellulaire qui restent à décrypter.

General Introduction

1. General Introduction

Honeybees are pollinators with high economic value by their honey production. The pollination services of insects reach up 84% of global agricultural crop production (Allsopp et al., 2008). In 2005, the economic value of pollinators was estimated to reach 153 billion dollars globally (Gallai et al., 2009). However, honeybees are facing many biotic and abiotic stressors leading to their global decline by a phenomenon termed colony collapse disorder (CCD) (Oldroyd, 2007; vanEngelsdorp et al., 2009). This phenomenon of sudden colony death is attributed not to one but several interplaying factors including pests, diseases, climate change, and anthropogenic activity especially pesticide application (Cox-Foster et al., 2007).

In 2016, France implemented a ban on using neonicotinoids outdoors after a risk assessment that states the dangers of their usage on the health on honeybee colonies in addition to other non-target organisms (*Biodiversity Act*, 2016). However, there was no alternative to neonicotinoids when it came to fending off against the vector spreading the beet yellow virus that affected the cultivation of beetroot in France, thus, France issued an emergency authorization in 2021 to allow the usage of imidacloprid and thiamethoxam on beetroot (EFSA, 2021). Imidacloprid is the most used neonicotinoids worldwide and even with regulate use, it still poses a risk to honeybee colonies (Jeschke et al., 2011).. Imidacloprid residues were found to be with the highest presence in neonicotinoid residues in honey globally (Mitchell et al., 2017). Though it is not the most present neonicotinoid in honey in Europe, it is detected with highest percentage of pesticides mixtures in honey second only to North America in 2017 (Mitchell et al., 2017).

Cox-foster et al. performed a meta-genomic analysis in 2007 to determine the implication of different pathogens and their degree of contribution to CCD. The Israeli acute paralysis virus (IAPV) showed the highest correlation and was thought to be the main contributor to CCD, but the virus was already present in the USA before the realization of CCD. Interestingly, all hives studies in USA and Australia were infected with *Nosema spp.* (Cox-Foster et al., 2007). Hence, *Nosema* infection poses a factor not to be taken lightly when it comes to CDD and its presence with pesticide application.

Many other pathogens infect honeybee hives including bacteria like the American foulbrood or the European foulbrood (Martin & Allsopp, 2021). Pests also attack honeybee hives to thrive on their hive products or feed on the hemolymph of their brood. The *Varroa destructor* is an ectoparasitic that feeds on the hemolymph and primarily the fat body tissue of honeybee larvae (Ramsey et al., 2019). In common beekeeping practices, beekeepers normally apply the acaricide amitraz to fend-off *Varroa* infections (Rosenkranz et al., 2010). Amitraz is an octopamine receptor agonist continuously stimulating the nervous system of insects leading to paralysis and death (Ostiguy et al., 2019). Its toxic effect is attributed to its metabolite DMFP (L. Guo et al., 2021). The selectivity of amitraz to ticks and mites in addition to its low metabolism in honeybees made it a suitable application. However, amitraz was found to be an established synergist increasing the negative effects of other pesticides and lowering tolerance to infection (Johnson et al., 2013; O'Neal et al., 2017).. Not to mention the proposed effect on olfactory function and observed effect on cardiac function, egg laying and hive performance (Boncristiani et al., 2012; O'Neal et al., 2017).

Different pesticides and pathogens are co-present with honeybee hives may interact and ultimately dismantle the fitness of the hive. Pesticides may weaken the honeybee immune system resulting in low tolerance to infection. The effect of pesticide single exposures like imidacloprid and amitraz may have been evaluated at certain levels but not when they are combined. Adding different levels of immune stimulation may give more insight on the inner workings of pesticide pathogen interaction in honeybee immune response and health.

The immune system of invertebrates on innate immunity and lacks the complexity of the adaptive immune system as found in vertebrates, i.e., invertebrates lack true lymphocytes (Larsen et al., 2019). However, immune memory was found in association with the innate immune system of invertebrates (Melillo et al., 2018). Cellular immune responses included phagocytosis of foreign particles and microbes. Phagocytosis is a crucial mechanism to defend against pathogen infections (Cosson & Soldati, 2008). This process evolutionary conserved from the emergence of the early eukaryotes until now (Yutin et al., 2009). Other cellular mediated immune responses include encapsulations and melanization. Bumblebees and honeybees showed reduced phagocytosis when exposed to imidacloprid. While in the

humoral arm of the innate immune system, Defense against pathogens is mediated via antimicrobial peptides and the production of reactive oxygen species like hydrogen peroxide and reactive nitrogen species such as nitric oxide (Eleftherianos et al., 2021). Imidacloprid can alter mitochondrial function and affect hydrogen peroxide production levels (Xu et al., 2022). Imidacloprid was observed to decrease the production of hydrogen peroxide and nitric oxide in bees even when cells were exposed to lipopolysaccharide as an immune activator (Walderdorff et al., 2018).

Toll, IMD and JAK/STAT are the main pathways involved in immune responses in invertebrates. The JAK/STAT pathway induces phagocytic activity upon activation in addition to its antiviral response (Brutscher et al., 2015; McMenamin et al., 2018).. However, Eater is one of the main components of phagocytosis and it is implicated in adhesion and mobility which intern, may be associated with immune responses against agents of infection (Melcarne, Lemaitre, and Kurant 2019). The IMD pathway recognizes fungal and bacterial PAMPS and leads to the production of AMPs to fend against microbes (Aymeric et al., 2010; Evans et al., 2006). Relish is a transcription factor leading to AMPs production via the Imd pathway and is a key regulator in addition to its posed role in cross-talk between different pathways (Evans et al. 2006). More importantly, the Toll pathway that plays a role in immune response and development in invertebrates possessing many elements that respond to infection (Steward & Govind, 1993; Wilson et al., 2014). The Toll pathway can recognize mainly the PAMPS of gram-positive bacteria and fungi via pathogen recognition receptors. Spaetzle is an extracellular PRR that leads to the recognition of PAMPS. Downstream is the transmembrane Toll receptor which binds to the activated Spaetzle and a signaling cascade is in continued via MyD88; a conserved protein in vertebrates and invertebrates which leads to the activation of NF- κ B transcription factors and production of AMPs (Steward & Govind, 1993).

An additional intriguing element in honeybee immunity is vitellogenin. Vitellogenin is a yolk protein related to reproduction in oviparous animals but in honeybees it has an antioxidant activity and is related to aging (Seehuus et al., 2006).. Vitellogenin is also important for trans-generational immune priming (TGIP) which is the transfer of immunity

from adults to larvae. TGIP gives naïve larvae the potential to have a stronger response to previously encountered pathogens by adults.

Honeybees are not the only insects exposed to imidacloprid and amitraz. The wax moth is a predator of honeybees and their different responses to pesticides may give an advantage of one over the other especially with the fact that weakened hives are a preferred target of the wax moth. Thus, evaluating at least the immediate levels of immune responses on cells similar to that of the pest is important to have a more comprehensive view of the occurrences that may lead to CCD. Since the fruit fly is the model organism for insect immune system and also used to evaluating the effect of pesticides it would be interesting to add *Drosophila* cells to the comparative approach.

The co-exposure of imidacloprid and amitraz in different concentration ratios and their effect on immune responses are yet to be assessed. Evaluation of the effect of pesticides on the immune system requires triggering the immune response without the cellular damage associated with infection. A practical approach in the use of immune activators/stimulators like zymosan A. Derived from the cell wall of the yeast *Saccharomyces cerevisia*; zymosan A can mimic fungal infection similar to that of the microsporidian *Nosema*.

In this study we have assessed the effect of imidacloprid and amitraz in single exposure or their combination in different ratios on honeybee hemocytes at different concentrations of the immune-stimulator, zymosan A. The effects were observed at 3 levels; the first being the cellular products at the duration of the immediate response which include the production of nitric oxide from 15 to 120 minutes post exposure to pesticides and zymosan, hydrogen peroxide and protein content at 3 hours post-exposure. Honeybee hemocytes responses were compared to that of Schneider-2 cells representing the fruit fly (model system) and to MB-L2 cells of the cabbage moth which represent Lepidoptiran insects like the wax moth. The second level is the cellular responses which include phagocytosis and cytotoxicity after 18 hours of exposure to pesticides and zymosan A by flowcytometry. Cytotoxicity was also asses by dye exclusion method before the application of any experiment to assessed the applicability of the selected concentrations. The third level is studying the expression of genes of the immune pathways like *spaetzle*, *toll* and *myD88*

from the Toll pathway, *relish* from the IMD pathway in addition to *eater* and *vitellogenin* (*vg*) after 18 hours of exposure.

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2.1. Colony collapse disorder

Colony collapse disorder (CCD) or colony depopulation syndrome (CDS) is an abnormal phenomenon characterized by a sudden decline of worker bees' population. In some cases, the queen and a small number of brood unattended may remain in the colony leading finally to their loss (Oldroyd, 2007; vanEngelsdorp et al., 2009). The spread of CCD is not well understood as it may be associated with several stress factors affecting the hives health and function (e.g. pesticides) or it may be affected by different contagious agents (e.g. pathogens) (vanEngelsdorp et al., 2009). In a metagenomic analysis conducted by (Cox-Foster et al., 2007) the analysis revealed that *N. ceranae* may be a contributing factor to CDD occurrence along with other factors that require further investigation. The impact of diverse stress factors on colony loss is confirmed by an epizootiological analysis where colony fitness is affected by these factors leading to higher pathogen susceptibility (VanEngelsdorp et al., 2010). In the USA, the estimated annual colony losses for 2014-2015 reached approximately 40% of total colony (Seitz et al., 2015). The major loss was observed during the summer. Loss of honeybee colonies will have a negative impact on pollination services that contribute to biodiversity and agricultural production (Klein et al., 2007) with around 84% of the world's crops requiring pollinators (Allsopp et al., 2008) in addition to honey production.

2.2. Honeybee pests and pathogens

With respect to pests, *Varroa destructor* mites are one of the major concerns and have resulted in significant economic drawbacks related to honeybees (Martin & Allsopp, 2021). The mites feed mainly on larvae's fat body tissues in addition to the hemolymph (Ramsey et al., 2019). *Varroa* mites act as a vector for viral transmission further weakening the hives (Francis et al., 2013). The development of a resistance to amitraz in *Varroa* mites is a growing concern for their strong spread (Maggi et al., 2010; Rodríguez-Dehaibes et al., 2005). Amitraz is one of the highly-effective acaricide used for mite treatment (Rosenkranz et al., 2010).

Honeybee pests include the greater wax moth (*Galleria mellonella*) and the lesser wax

(*Achoria grisella*) moth that target bee larvae after they infiltrate the hive (Ellis et al., 2015; Kwadha et al., 2017). The wax moth targets weakened hives, thus any alterations in fitness of honeybees, including immune responses may shift the scale towards more susceptibility to wax moth infestations.

Honeybees are a target to many diseases caused by different microbes. Microbes include viruses that are mainly positive-sense RNA mainly including deformed wing virus (DWF), sacbrood virus (SBV), and Israeli acute paralysis virus (IAPV). In addition to various positive-sense RNA viruses that infect honeybees, DNA viruses and viruses with unclassified RNA types have also been reported (Evans & Schwarz, 2011). As for bacterial infections, American and European foulbrood are two diseases caused by *Paenibacillus larvae* and *Melissococcus plutonius* respectively (Martin & Allsopp, 2021). Besides that, bees are also a target to other diseases caused by protozoans like trypanosomes and gregarines or by fungi like *Ascophæra apis* that causes chalkbrood disease (Martin & Allsopp, 2021).

A significant disease in honeybees is nosemosis which is known to be caused by two species of microsporidia, *Nosema apis* and *Nosema ceranae* (Fries, 1993, 2010). The contribution of *Nosema spp.* infection to the global colony collapse disorder (CCD) remains unclear with the presence of contradictory studies including those of Cox-Foster et al., (2007) and vanEngelsdorp et al., (2009) as they did not find any strong correlation between honeybee colony decline and nosemosis. However, other studies state that *N. ceranae* is particularly implicated in honeybee decline in Spain as a key factor in CCD (Higes et al., 2008; Martín-Hernández et al., 2007). Many factors may be involved in determining the impact of nosemosis on colonies, including pesticides, climate, inoculum and simultaneous infection with other diseases (Evans & Schwarz, 2011).

2.2.1. Nosemosis

Nosemosis is a honeybee disease characterized by gradual decrease of worker bees and forager bees leading to poor honey production and to intensive colony loss with severe levels of infection. Nosemosis is caused by *Nosema spp.*. The first species discovered is *Nosema apis* with the European honeybee (*Apis mellifera*) as its host (Zander, 1909). Another interesting discovery is that of *Nosema ceranae* and its characterization by Fries

et al., (1996) as a parasite of the Asian honeybee (*Apis ceranae*). *Nosema apis* and *Nosema ceranae* are both unicellular microsporidians (specialized parasitic fungi) (Fries, 1993; Fries et al., 1996). Microsporidians are ubiquitous obligate intracellular parasites that must infect the host cells to complete their life cycle (Han & Weiss, 2017).

Although *N. ceranae* is a pathogen that originally infects the Asian honeybee (*A. ceranae*), cases of infection in *A. mellifera* colonies were reported in Taiwan (Huang et al., 2007) and Spain (Higes et al., 2006) in 2005. *N. ceranae* is replacing *N. apis* as a cause of nosemosis in *A. mellifera*. Even in cases of co-infection, *N. ceranae* presence dominate that of *N. apis* (Y. Chen et al., 2009; Pettis et al., 2012). This is interesting since no competitive virulence is observed in-vivo between the two species regarding multiplication and mortality (Forsgren & Fries, 2010). Furthermore, *N. ceranae* is detected on all continents except Antarctica (Klee et al., 2007; Paxton, 2010).

Nosema ceranae infects the intestinal epithelial cells of honeybee ventriculus (digestive cavity) (Fries et al., 1996). In fact, most microsporidia infect the digestive tract of their host (Han & Weiss, 2017). However, some studies claim that *N. ceranae* infection is not tissue specific infecting hypopharyngeal glands, salivary glands, Malpighian tubules, and fat body (Y. P. Chen et al., 2009; Copley & Jabaji, 2012). At first, *N. ceranae* was thought to only infect adult honey bees (Smart & Sheppard, 2012) but recent studies were able to detect larval infections (Benvau & Nieh, 2017).

Nosemosis caused by *N. ceranae* is referred to as “Type C nosemosis”. This distinction is attained not just by the different causal agents of nosemosis but mainly by the symptoms that accompany the infection. In addition to poor honey production and colony depopulation, *N. ceranae* decreases the immune response in *A. mellifera* and causes senescence (Antúnez et al., 2009). Furthermore, *N. ceranae* shows no outward clinical signs as dysentery, crawling behavior or whitish gut coloration as with honey bees infected with *N. apis* (Y. P. Chen et al., 2009).

The prevalence of *N. ceranae* over *N. apis* may be attributed to the suggested eurythermal nature (tolerance to a wide range of temperature) of the former and the stenothermal nature (tolerance to a narrow range of temperature) of the latter. In other words, *N. ceranae* can

remain active throughout the year, unlike *N. apis* infection that gradually declines in the summer (Higes et al., 2010).

2.2.1.1. *Nosema ceranae* transmission and life cycle

Nosema infection is usually transmitted horizontally through the fecal-oral route by ingestion of food or water, and grooming activity (Fries, 2010) with the possibility of transmission through the oral-oral route with *N. ceranae* infections (Y. P. Chen et al., 2009; Smith, 2012). Recent studies revealed another mode of transmission of *N. ceranae* spores by air (Sulborska et al., 2019). Thus, seasonal variations must be considered in further studies.

A sustainable cell line model has not been developed for honey bees but the life cycle of *Nosema ceranae* is described by (Gisder et al., 2011) using a Lepidopteran cell-line model (IPL-LD-65Y) originating from *Lymantria dispar* as follows:

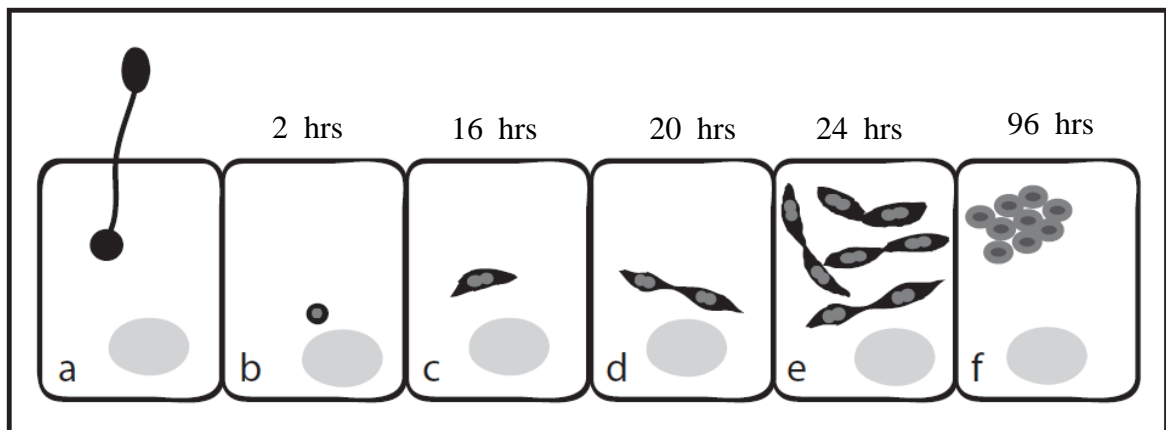


Figure 1. Schematic representation of *N. ceranae* life cycle.

The different developmental stages of *N. ceranae* are shown in Figure 1. The first stage is the extrusion of the polar tube (Figure 1. a). The polar tube is an organelle unique to microsporidia. It is an infection apparatus that enables the microsporidium to infected host cells from the lumen (Han & Weiss, 2017). The spore injects the sporoplasm into the host cell through the polar tube. The second stage is merogony (proliferative phase) characterized by meront formation and multiplication followed by formation of mature spores. Spores are released into the gut lumen “environmental spores” and may re-infect

other ventricular epithelial cells or are excreted with feces (Y. P. Chen et al., 2009) and the cycle is re-initiated through the fecal-oral route (Fries, 2010).

2.2.1.2. *Nosema spp.* spore morphology and structure

Nosema ceranae spores are oval or rod shaped with an average length of 4.4 μm varying between 3.9 μm and 5.3 μm and an average width of 2.2 μm varying between 2 μm and 2.5 μm (Y. P. Chen et al., 2009; Fries et al., 1996) while *N. apis* spores measure approximately 6 μm in length and 3 μm in width (Zander & Böttcher, 1984). *N. apis* and *N. ceranae* have different outer structure morphology visible by SEM (scanning electron microscopy) (Ptaszyńska et al., 2014) shown in Figure 2.

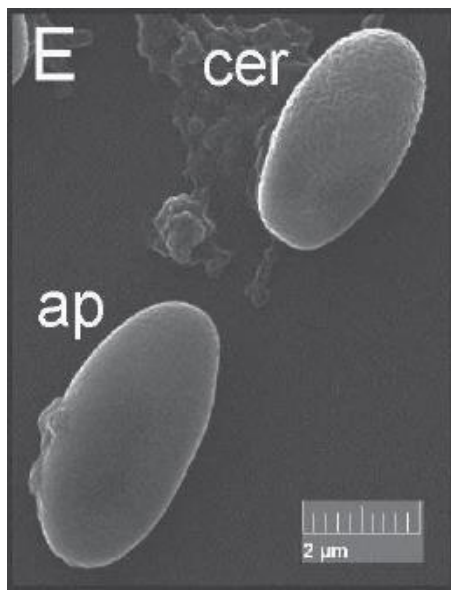


Figure 2. SEM (Scanning electron microscope) image of *N. ceranae* and *N. apis* spores. Cer (*N. ceranae*), ap (*N. apis*) (Ptaszyńska et al., 2014).

Spores of *N. ceranae* are found to have a more sculptured ornamentation than those of *N. apis*. Several characteristics that contribute to the difference in the outer structure are listed in Table 1 below:

Table 1. Comparison of different characteristics of *N. ceranae* spores and *N. apis* spores. Comparisons and values are mean averages. different letters signify statistical significance between species (Ptaszyńska et al., 2014).

Spore Characteristic	<i>N. ceranae</i>	<i>N. apis</i>
Furrows	Deep	Shallow
Furrow edges	Narrow	wide
Distance between furrows	Short (83.62 ^a nm)	Large (114.54 ^b nm)
Furrow width	Short (28.06 ^a nm)	Large (35.5 ^b nm)

2.3. Pesticides

Pesticides are chemical compounds that are used to kill pests, including insects, rodents, fungi, and unwanted plants (weeds) (WHO, 2018). The term also includes substances used to control vectors and diseases. A wide range of compounds are listed as sub-categories of pesticides including insecticides, fungicides, herbicides, rodenticides, molluscicides, nematocides, plant growth regulators and others (Aktar et al., 2009). Pesticides are composed of at least one active ingredient. Active ingredients are the chemicals in pesticides used to control pests and they can be categorized as antimicrobial (substances/mixtures used to kill or control microbes), biopesticide (originating from natural products) or conventional (anything other than biopesticides and antimicrobial pesticides) (WHO, 2018).

The use of pesticides dates back to ancient times. Dating back to 3000 BC, farmers in China used a mixture of lime, wood ash and chalk to kill insects that damage their crops (Matolcsy et al., 1988). In the 19th century, pesticides became a subject of intensive research with the development of organochloride pesticide DDT (dichloro-diphenyl-trichloroethane) against malaria and typhus (Hodgson, 2004). However, the dangers of DDT came to light with the publication of Rachel Carson's book "Silent Spring" in 1962 (Rachel Carson, 2000) which ultimately resulted in its ban. After the drawback of DDT, other less persistent organic pesticides dominated the market like organophosphates, carbamates and pyrethroids. That is, until the appearance of neonicotinoid pesticides which are now the most commonly used pesticides worldwide (McAfee, 2017).

Agrochemical pesticides are a relatively new threat to bee keepers demanding that they cope with the increasing use of pesticides as it was obvious that chemical insecticides would pose a threat to bee keeping. In the United States, a marked effect on the European honey bee (*A. mellifera*) came to surface with the use of DDT, decreasing the bee population from 6 million in 1947 to 3 million in 2010 (Sanchez-Bayo & Goka, 2016).

2.3.1. Neonicotinoids

Neonicotinoids are systemic insecticides which include imidacloprid, clothianidin, thiamethoxam, acetamiprid, nitenpyram and thiacloprid. They have high water solubility (610 mg / L for imidacloprid in water at 20 ° C and at pH 7) (Bonmatin et al., 2015). Neonicotinoids act by binding to nicotinic acetylcholine receptors (nAChR) in the central nervous system of insects and are fatal by disruption of the nervous system (Bonmatin et al., 2015; Tomizawa & Casida, 2005). This systemic function is what distinguishes neonicotinoids from other pesticides (Bonmatin et al., 2015). High concentrations of neonicotinoids cause receptor blockage, paralysis and ultimately insect mortality though low concentration stimulate the nervous system (Tomizawa & Casida, 2005). The chemical structures of different neonicotinoids is depicted in Figure 3 (Kasiotis & Machera, 2015).

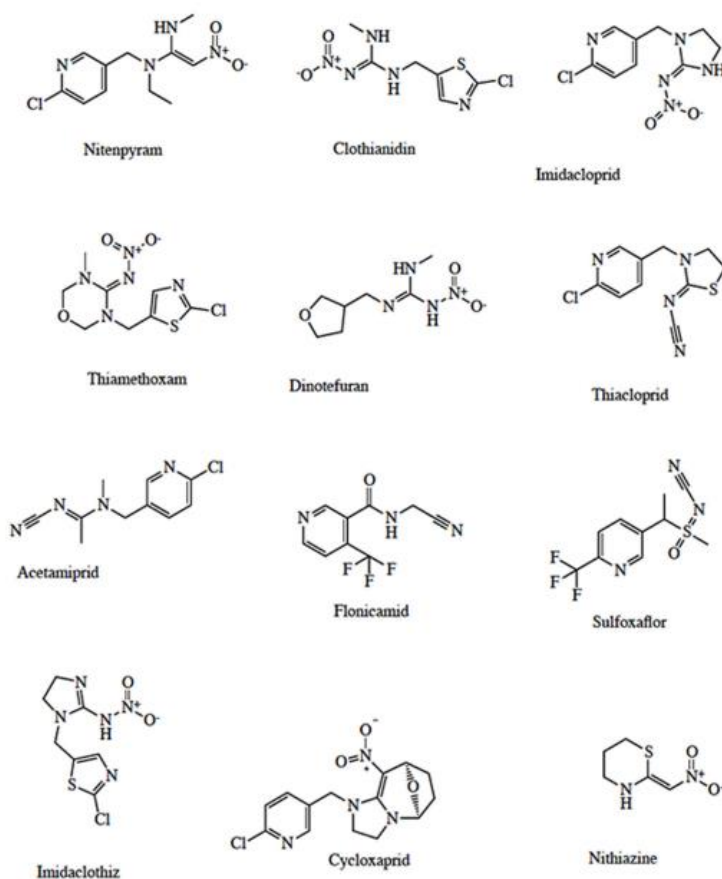


Figure 3. 2D chemical structures of neonicotinoids (Kasiotis & Machera, 2015)

Neonicotinoids dominated the pesticide market representing more than 25% of the market in 2010 (Jeschke et al., 2011) due to their effectiveness and low persistence and the developing resistance to organophosphates, carbamates and pyrethroids (Simon-Delso et al., 2015).

The most used neonicotinoid; imidacloprid, is the second most sold pesticide globally after glyphosate (Jeschke et al., 2011). Imidacloprid gains the focus in research of neonicotinoid-bee interaction since effects on bee colonies caused by pesticides may trace back to its usage. Behavioral anomalies are also observed in *A. mellifera* exposed to imidacloprid such as reduced associative learning at 12 ng/bee and decreased foraging activity at 24 $\mu\text{g}\cdot\text{kg}^{-1}$ in syrup (Jeschke et al., 2011).

Five neonicotinoids including imidacloprid were banned in France in 2016 (*Biodiversity Act*, 2016) due to their toxic and sub-lethal effects on honeybee colonies. Alternative chemicals (pyrethroids) were found as a promising replacement to neonics as well as non-chemical alternatives (Jactel et al., 2019). In 2021, an emergency authorization for usage of imidacloprid and thiamethoxam was issued in France to protect from the vector-transmitted beet yellow virus which no alternative method seemed to prevent the viral transmission in beetroot (EFSA, 2021).

Neonicotinoid residues may deposit on pollen and nectar of plants exposing European honeybees *A. mellifera* to these insecticides (Dively & Kamel, 2012). A 3-year survey initiated in 2002 in France found that 69% of studies honey samples contained residues of imidacloprid (Chauzat et al., 2006). In fact, imidacloprid is one of the main neonicotinoids found in honey in most continents (Figure 4) which is concerning since exports may contain residues of the pesticide spreading and distributing the exposure. This also implies that the effect of imidacloprid is indeed on a global scale and that limiting of its usage in Europe is still not sufficient to limit its impact on honeybee colonies.

The presence of neonicotinoids in the environment along with other factors may negatively affect bee colonies as imidacloprid was shown to decrease the immune response on honey bee larval hemocytes with an intensified impact when cells were immune activated with

lipopolysaccharide (LPS) (Walderdorff et al., 2018). Sub-lethal levels of imidacloprid (5 ppb) resulted in increased *Nosema* spore production in lab conditions but not at the colony level in field conditions. (Pettis et al., 2012). This demands more study on the interaction of pesticides, pathogens and the target and comparison between different methods of *Nosema* spp. detection other than spore count.

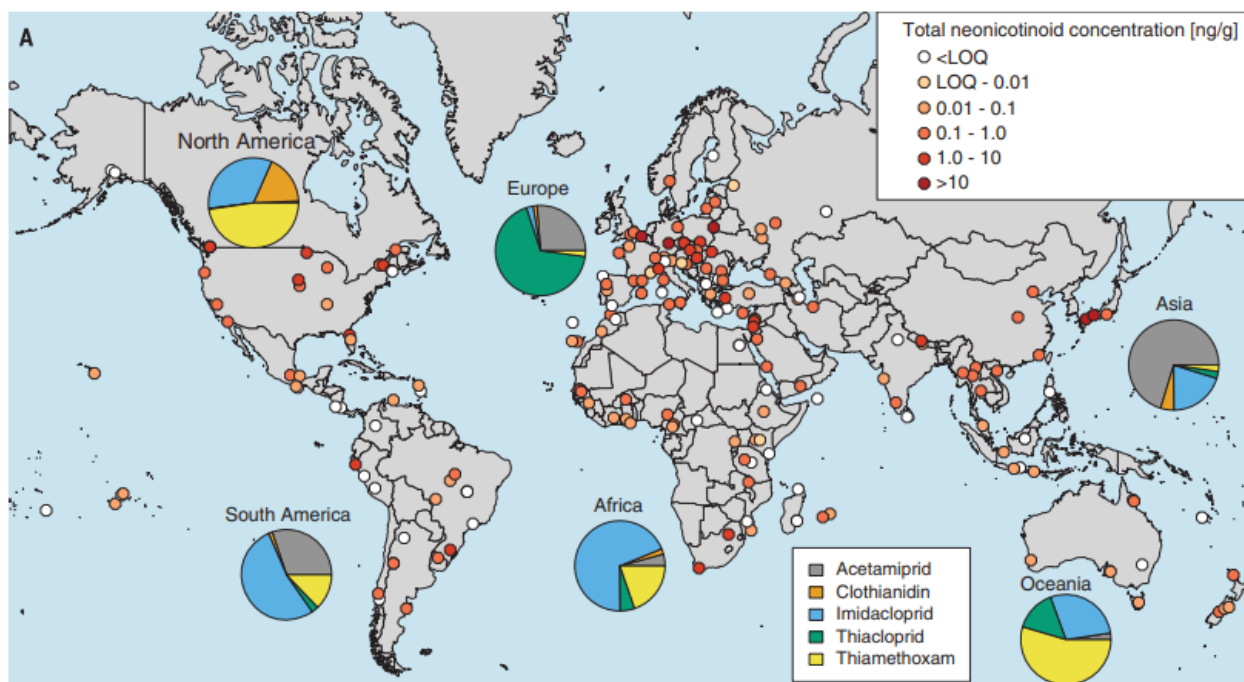


Figure 4. Global neonicotinoid pesticide honey contamination (Mitchelle et al. 2017)

When multiple pesticides are detected in the same environment, it is wise to assess their interplay and to establish a background of their usage. The order of exposure, for instance, is an important factor to consider since it has been shown that the toxicity of different pesticides may or may not be altered by the order of exposure. Indeed, the latter was demonstrated when exposure to sub-lethal doses of amitraz after exposure to tau-fluvalinate or coumaphos did not affect the toxicity of amitraz while exposure to tau-fluvalinate or coumaphos after amitraz increased the former's toxicity to honeybees (Johnson et al., 2013). Furthermore, pesticide residues vary by month and years, thus to describe the risk of pesticide exposure on honeybees, a long-term assessment with different time intervals is needed to provide more details (Ostiguy et al., 2019). North America has

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the highest percentage of neonicotinoid mixtures residues in honey followed by Europe (Mitchell et al., 2017). This necessitates the evaluation of pesticide mixtures and their impact on honeybees and the environment in these continents.

2.3.2. Amitraz

Amitraz (BTS 27419) is a non-systemic formamidine pesticide and acaricide used to treat mite and tick infection in cattle, fruit trees and honeybee hives (Proudfoot, 2003). It was first introduced into the market in 1974 (Bonsall & Turnbull, 1983). Figure 5 represents the chemical structure of amitraz as a tertiary amino compound where positions 1 and 5 hold 2,4-dimethylphenyl groups.

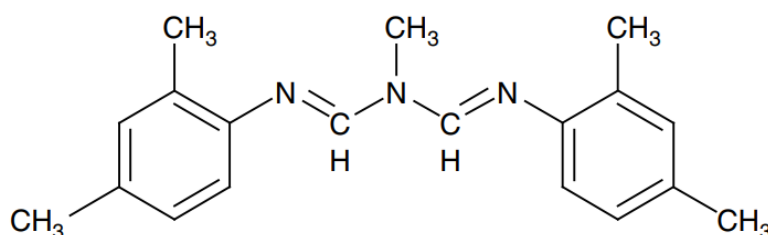


Figure 5. Chemical structure of amitraz (Proudfoot, 2003)

Amitraz acts as an octopamine receptor agonist resulting in the inhibition of monoamine oxidase and prostaglandin synthesis finally leading to the over-excitation of the nervous system (Ostiguy et al., 2019). The end-result would be paralysis and death of the target insects.

In beekeeping, it is used to treat *Varroa destructor*, an ectoparasitic mite that target honeybee hives (Rosenkranz et al., 2010). Although amitraz is continued in use, *V. destructor* is reported to develop resistance to amitraz (Maggi et al., 2010; Rodríguez-Dehaibes et al., 2005). Other target insects like cattle ticks were also reported to develop resistance (A. C. Chen et al., 2007), which raises a question on the efficiency and outcomes of continued use of amitraz-based pesticides. Chemicals like fenazaquin and etoxazole are being considered as a replacement to amitraz in beekeeping due to their high *Varroa* mortality rate and safety to honeybees in the same regard though other evaluations are required (Bahreini et al., 2020).

To add, amitraz is used in beekeeping because it's low metabolism in honeybees limiting the production of its more toxic metabolite DMFP (L. Guo et al., 2021). However, amitraz was observed to affect honeybee health not to mention its synergistic effect with other risk

factors including different pesticides. In some cases, the observed synergy resulted in the increased toxicity of other pesticides but not amitraz itself (Johnson et al., 2013). Amitraz was also reported to alter cardiac function in honeybees and decrease viral tolerance (O'Neal et al., 2017). The effect of acaricides was evident in alterations in the expression of detoxifying enzymes (Boncristiani et al., 2012) which may affect the response and tolerance of honeybees to other pesticides and induced stress. Direct lethal outcomes must not be the only parameters when assessing the impact of pesticide on non-target organisms as amitraz decreases honeybee performance in honey production and in oviposition in addition to neuropeptide gene expression implying an impact on olfactory function (Ilyasov et al., 2021).

When it comes to exposure, acaricides including amitraz are the most common pesticides found in honey (Ostiguy et al., 2019). Amitraz was observed to completely hydrolyze in honey from 2 to 4 weeks (Corta et al., 1999). However, during the *Varroa* treatment, hives are continuously exposed to amitraz. The fact that long-term evaluation of amitraz with other pesticides in the context of immune stimulation after infection is still lacking to define its implication in CCD poses a demand for more research in this direction.

2.4. Invertebrate immune system

The invertebrate immune system relies mainly on innate responses that lack the antibody-mediated specificity of the adaptive immune system in vertebrates. Innate immunity acts in defense against pathogens directly after the recognition of structures shared by non-related microbial groups (Larsen et al., 2019). The innate immune system comprises three components, the first being the physical and chemical barriers, the second component is humoral and the third is cellular (Negri et al., 2019). Innate immunity branches into two arms, the afferent (sensing arm) linked to the perception of the infection and the efferent (effector) arm related to the response to eliminate the infection. Both arms are constituted of the cellular and humoral elements (Beutler, 2004; Hultmark, 1993). Acting in synergy, physical and chemical barriers, humoral components, cellular components provide a robust defense against pathogens. Both vertebrates and invertebrates are dependent on innate immunity as the first line of defense against pathogens and microbes (Akira et al., 2006).

Humoral responses include the production of reactive oxygen species or reactive nitrogen species as a defensive response against pathogens (Eleftherianos et al., 2021). Production of antimicrobial peptides is also involved in this humoral response but they are more diverse as they are under the influence of genetic variability. Melanization is another defensive response seen in arthropods, mainly insects and it includes the production of melanin. Cellular responses include phagocytosis, encapsulation, and nodulation. The invertebrate immune system comprises 4 main pathways; The Toll pathway, Imd pathway, JNK pathway and the JAK/STAT pathway.

Physical and chemical barriers are preventive against pathogens from entering the organism. Examples of physical barriers are the skin in humans, cuticle in insects and cell walls in plants (Menezes & Jared, 2002). However, these barriers can be bypassed as in the case of viruses that enter *A. mellifera* using the *V. destructor* mite as a vector (Larsen et al., 2019). If these barriers are breached, complex immune reactions are activated by the insect (Feldhaar & Gross, 2008). Thus, the humoral and cellular processes of the innate immune system come into play after the physical and chemical barriers fail to prevent the infection.

The key difference between the vertebrate and the invertebrate immune system is the production of immunoglobulins and the presence of specific recognition of foreign particles or microbes after previous exposure in vertebrates (Rowley & Powell, 2007). The ability of the immune system to recognize previously encountered pathogens or foreign particles to elicit a specific immune response is termed “adaptive” immunity. Invertebrates were thought to lack specificity to pathogens they encounter regularly (Menezes & Jared, 2002), however, evidence of immune memory was found in invertebrates (Cooper & Eleftherianos, 2017; Menezes & Jared, 2002; Milutinović & Kurtz, 2016). This is realized by the clonal expansion of immune cells and the more robust response to the same pathogens after constitutive encounters (Milutinović & Kurtz, 2016) or through regulation of RNA expression of the pathogen via RNA interference (RNAi) (López et al., 2014; Rimer et al., 2014; Vilcinskas, 2021).

2.4.1. Social immunity

It is important to distinguish between “social immunity” and “individual immunity” in honeybees. Social immunity is on the colony level displayed by certain behaviors while individual immunity is characterized by internal processes and includes no collective behavior (Cremer et al., 2007). Honeybees are social organisms where the colony members collaborate to ensure the survival of their hive and to prevent establishment or spread of infections. This highly complex collective immunity in honeybees is termed social immunity or behavioral immunity (Cremer et al., 2007). Several mechanisms at different levels are part of the social immunity. They can be prophylactic or activated and they include social fever, which is a process that involved raising the nest temperature to fight pathogens and intruders. It is costly to healthy individuals of the colony but it is important for protecting the colony as a whole. This kind of social behavior is observed in Japanese honeybees (*Apis ceranae japonica*) against the giant hornet (*Vespa mandarinia japonica*). About 500 bees engulf the hornet in a ball raising its temperature to 47 C, a temperature that is lethal to the hornet but not to the bees (Ono et al., 1995). Raising the temperature is also observed to control the infection of the pathogenic fungus *Ascosphaera apis* (Starks et al., 2000). Grooming activity is another type of social immunity and it is characterized by the usage of mandibles and legs to remove external parasites and it can be of two forms, self-grooming (autogrooming) or social grooming (allogrooming) (De Roode & Lefèvre, 2012). In addition, honeybee have a hygienic behavior. This two-step behavior comprises (a) detection of diseased and/or parasitized individuals from the colony and (b) their removal from the hive (De Roode & Lefèvre, 2012). This defensive behavior allows the control of several pathogens including the fungus *Ascosphaera apis*, the bacterium *Paenibacillus larvae* (American foulbrood) and *Varroa destructor* mites (Larsen et al., 2019).

A form of social immunity is resin collection. Honeybees collect resin from plants that have antimicrobial properties. The resin is integrated into the nest wax along with glandular secretions and is termed propolis (De Roode & Lefèvre, 2012; Simone-Finstrom et al., 2017). Propolis is observed to have antimicrobial activity against *P. larvae* and *A. apis* (Simone-Finstrom et al., 2017).

In intense conditions like lack of food or extreme temperatures, honeybees may devour their brood to prevent loss of nutrients or the spread of pathogens like *A. apis*. In addition, honeybees apply decreased contact between congeners by moving from the nest to prevent spread of the disease (Rueppell et al., 2010).

2.4.2. Pathogen recognition in invertebrates

Before an immune response is triggered, pathogen recognition must be established. Recognition is facilitated via specialized structures termed PRRs (pathogen recognition receptors). These receptors are able to bind and recognize conserved structures known as pattern-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs) originating from damages microbial structures, and microbe-associated molecular patterns (MAMPs) expressed on microbial cell walls and include viral-associated molecular patterns (VAMPs) originating from viruses (Brutscher et al., 2015). PAMPs include lipopolysaccharides (LPS), lipoteichoic acid, zymosan, glycolipids, glycoproteins or double-stranded RNA (Murphy et al., 2017). Peptidoglycans and glucans are PAMPs present in bacterial and fungal cell walls respectively (Feldhaar & Gross, 2008; Smits et al., 1999). PRRs are able to recognize if peptidoglycans are from Gram-positive or Gram-negative bacteria despite variability between bacteria of the same cell wall type. Gram-negative bacteria generally possess peptidoglycans containing diamino acids such as diaminopimelic acid (DAP) while peptidoglycans of Gram-positive bacteria contain lysine (with a few exceptions). Peptidoglycan recognition proteins (PGRPs) and gram-negative binding proteins (GNBP) recognize these PAMPs. A downstream signaling cascade triggering the immune response is initiated following pathogen perception.

2.4.3. Cellular immune responses

The immune cells of insects that defend against pathogens are called hemocytes or haemocytes (Lavine & Strand, 2002). These cells have different roles within the immune system depending on their type. Their functions include phagocytosis, nodulation, encapsulation and production of AMPs.

2.4.3.1. Hemocytes: insect immune cells

Insects can have different types of hemocytes depending on the species including lamellocytes, oenocytoid cells, pro-hemocytes (in embryos), spherule cells, coagulocytes, adipohemocytes, plasmatocytes and granulocytes (Jones, 1962). Pro-hemocytes are precursors to different types of hemocytes having basophilic cytoplasm and enlarged nucleus (Lavine & Strand, 2002) while granulocytes function in phagocytosis, encapsulation, and nodulation of pathogens in addition to nitric oxide production as found in bees (Negri, Maggi, Correa-Aragunde, Brasesco, Eguaras, Lamattina, et al., 2013). Plasmatocytes are the most common hemocytes and they possess a phagocytic function. Oenocytoid are non-phagocytic hemocytes that were found to have a potential relation to melanization by their phenoloxidase activity while the adhesive lamellocytes are present during larval development and show phagocytic activity against pathogens (Eleftherianos et al., 2021; Jones, 1962). In *Drosophila*, however, only 4 types of hemocytes were identified including pro-hemocytes, plasmatocytes, lamellocytes and crystal cells (Tattikota et al., 2020). Crystal cells are granular hemocytes that are only found in *Drosophila* until now (Ribeiro & Brehélin, 2006). In honeybee, plasmatocytes and granulocytes were observed under the microscope. (Negri, Maggi, Correa-Aragunde, Brasesco, Eguaras, Lamattina, et al., 2013; Richardson et al., 2018) while 3 subsets were observed in flow cytometry analysis (Marringa et al., 2014). The third type was characterized as oenocytoid (Gábor et al., 2020). Pro-hemocytes and coagulocytes were described in honeybees by El Mohandes, Nafea, and Fawzy (2010). In addition, a rare type of larval hemocytes (L5-2) at the fifth instar stage was observed by (Negri et al., 2015). A characteristic of this hemocyte is the presence of extreme pseudopodia during locomotion. The characterized hemocytes of honeybees are illustrated in (Figure 6).

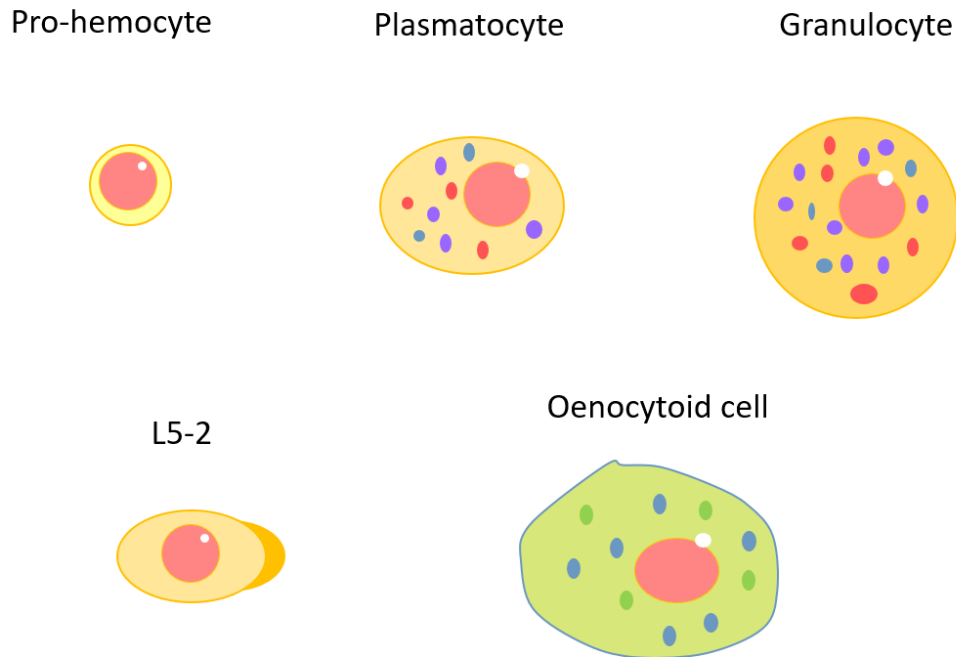


Figure 6. Illustration of different types of hemocytes in honeybee hemolymph.

2.4.3.2. Phagocytosis

Phagocytosis is an ancient process characterized by receptor-mediated engulfment of foreign particles or microbes. Phagocytosis is a feeding mechanism for unicellular metazoans and a key mechanism in fending against infection in complex organisms (Cosson & Soldati, 2008). This process has been conserved since the emergence of eukaryotes and is proclaimed to be implicated in eukaryogenesis (Yutin et al., 2009). Phagocytosis is promoted by the JAK/STAT immune pathway in *Drosophila* and honeybees (Evans et al. 2006) The process includes the engulfment of pathogens and intracellular destruction of apoptotic cells upon recognition of pathogen molecules by receptors on individual phagocytic hemocytes (plasmatocytes and granulocytes). Granulocytes are responsible for phagocytosis of dead cells while plasmatocytes are responsible for phagocytosis of pathogens and foreign particles (Marmaras & Lampropoulou, 2009). The phagocytized material become trapped in a phagosome consisting of about 600 proteins in *Drosophila* with about 70% mammal orthologs (Feldhaar & Gross, 2008). Thus, there is a resemblance between the phagosome of *Drosophila* and the mammalian phagosome.

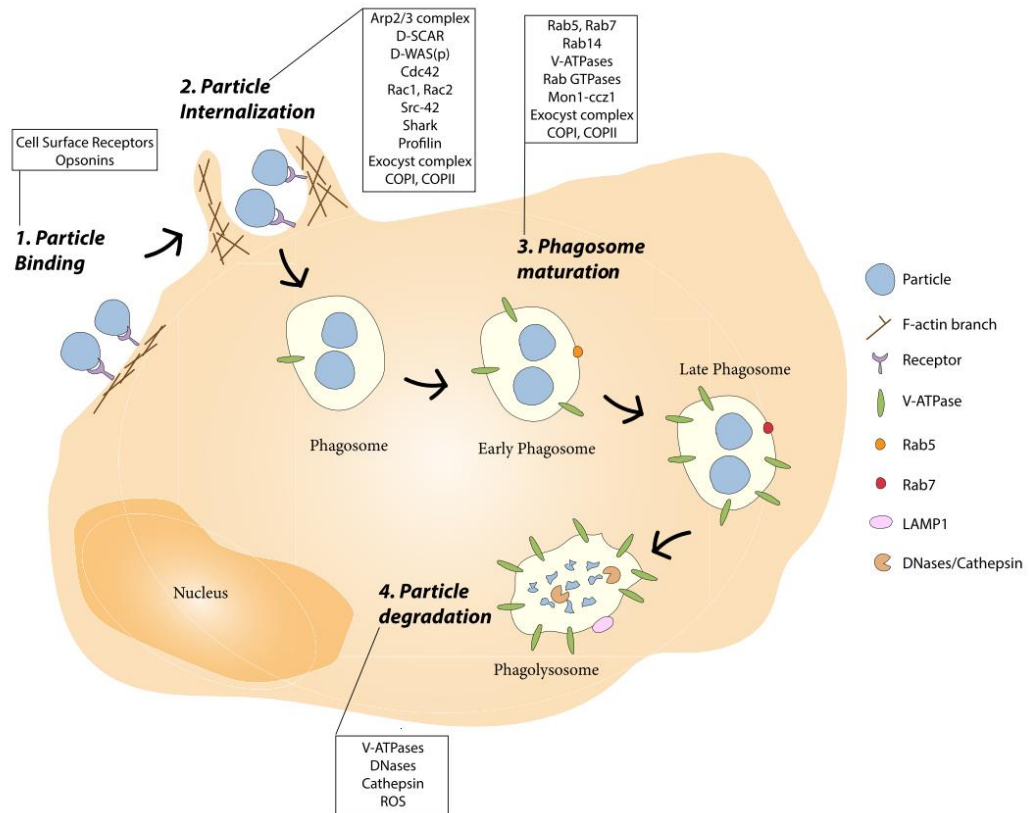


Figure 7. The process of phagocytosis in the insect model, *Drosophila*. The process comprises the steps and the occurrence with each step. The particle binds to cell surface receptors followed by internalization and early phagosome formation. The phagosome matures into a mature phagosome at which the phagocytosed particles are digested (Melcarne, Lemaitre, and Kurant 2019).

The steps of phagocytosis (Figure 7) are comprised of moving towards the site of infection by chemotaxis followed by recognition of the foreign particle or pathogen. The phagocytes attach to the recognized particle or pathogen via receptors or opsonins on the phagocyte cell surface. The plasma membrane extends around the pathogen or foreign particle to form a phagosome, this is termed engulfment. The phagosome is then internalized followed by fusion with a lysosome. This fusion results in the breakdown of the engulfed material by enzymes introduced from the lysosome. A phagolysosome is formed and the material is digested within it releasing the digested products to the cytoplasm for to be utilized by the cell. The remaining waste material of the digested products are then expelled from the cell through exocytosis (Melcarne, Lemaitre, et al., 2019; Uribe-Querol & Rosales, 2020).

In addition to PGRPs and other receptors, Nimrods (NIMs) are a type of phagocytic receptors discovered in *Drosophila* as a subtype of epidermal growth factor (EGF) and contain Nimrod repeats (NIM) (Bork et al., 1996).

2.4.3.2.1. NIMs: Eater

Eater, along with NimC1 are NIMs (Nimrods) and main components in phagocytosis (Melcarne, Lemaitre, and Kurant 2019). Eater, in particular, is involved in the uptake of gram-positive bacteria by direct binding (Kocks et al., 2005). Eater was shown to be involved in mobility and adhesion of hemocytes (Bretscher et al., 2015) thus may affect the response to pathogenic infections. However, the implication of Eater in adhesion is still unexplored in terms of mechanism and interactions. Eater appears to compensate for the function of NimC1 in *NimC1* null *Drosophila* mutants implying the crucial need for Eater activity in phagocytosis. In addition, NimC1 appears to be dispensable in the phagocytosis of gram-positive and gram-negative bacteria (Melcarne et al. 2019).

2.4.3.3. Nodulation

Nodulation is the entrapment of bacterial clusters by hemocytic aggregates (Browne et al., 2013) via lectin mediated nodule (melanized or non-melanized) formation in response to different pathogens (Larsen et al., 2019). Granulocytes bind together and excrete their contents after forming a layer followed by plasmatocytes adherence (Hillyer, 2016).

2.4.3.4. Encapsulation

When large pathogens like nematodes, protozoa and parasites enter the host, hemocytes bind to the target in multilayers and ultimately undergo melanization. The encapsulated target is degraded by ROS and RNS (Marmaras & Lampropoulou, 2009).

2.4.3.5. Melanization

In invertebrates, melanization is a response to injury or exposure to microbes and/or parasites. It is the production of melanin deposited to form a physical barrier around the injury site or around the foreign particles or microbes (Cerenius & Söderhäll, 2004). Melanin is implicated in cuticle hardening, egg chorion tanning, wound healing in insects.

An example, is melanization of encapsulated parasitic wasp eggs (Hillyer, 2016). Melanization is an enzymatic process mediated by prophenoloxidase (proPO) that produced by oenocytoids in insects (Hillyer, 2016). In the fruit fly, however, crystal cells are mainly responsible for melanin production. ProPO is the inactive form of phenoloxidase (PO) which is activated by serine proteases when secreted into the hemolymph. The active PO produces quinones by oxidation of phenols which in turn polymerize to form melanin (Binggeli et al., 2014).

The phenoloxidase system responsible for the production of melanin can be triggered by components present in the cell wall of microbes including LPS, β -glucans and PGNs (Amparyup et al., 2013; González-Santoyo & Córdoba-Aguilar, 2012) in addition to chemical activators like α -chymotrypsin, ethanol, or L-DOPA (K. Wu et al., 2018). Usually inhibition of melanization in assays is achieved by phenylthiourea (PTU) (Clark & Strand, 2013; Ryazanova et al., 2012). The phenol oxidase system shares serine proteases of the Toll pathway as illustrated in Figure 8.

In *Drosophila*, two distinct pathways induced melanization; one is the Toll pathway while the other includes a reaction to wounding and the activity of the Haya serine protease which is also present in the Toll pathway. The end-result of melanization in the two pathways is anti-microbial activity and blackening reaction respectively (Dudzic et al., 2019).

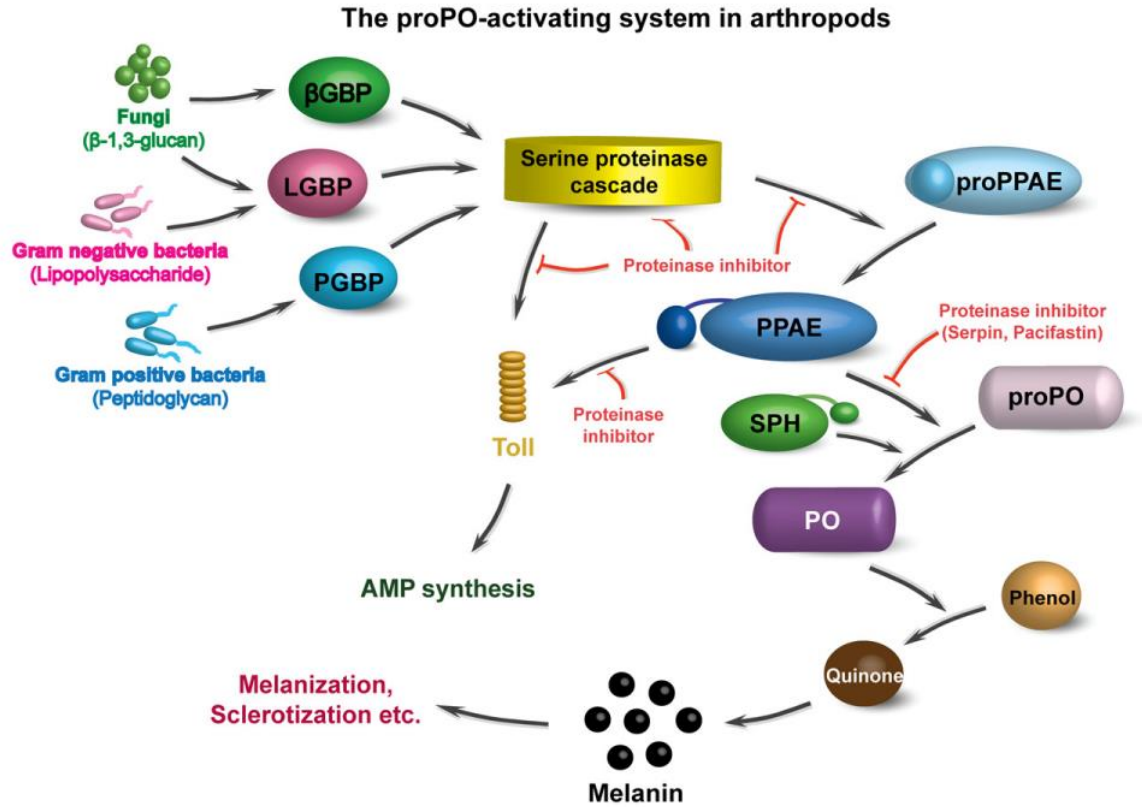


Figure 8. Phenoloxidase activating system (PO-AS) in arthropods

Melanization is a crucial immediate immune response in insects that even mature honeybees retain their PO-mediated immune responses even after reducing hemocyte-based immunity (Schmid et al., 2008). It is important to consider that melanization is deleterious to the cell (Eleftherianos et al., 2021) and continuous activation will ultimately lead to the death of the insect. Thus, it could be interesting to study the effect of pesticides on the melanization reaction which could give insight on the interplay of risk factor and their interplay in honeybee pathogen susceptibility and resistance.

2.4.4. Humoral immune responses: reactive molecules

2.4.4.1. RNS

Nitric oxide (NO) is an ancient biomolecule that precedes the divergence of vertebrates and invertebrates with diverse functions across different phyla noting that it emerged independently in each (Jacklet, 1997). NO was found to be produced at the beginning of the immune response in honeybee granulocytes (Negri, Maggi, Correa-Aragunde, Brasesco, Eguaras, Lamattina, et al., 2013). as a crucial signaling molecule in the immune system of honeybees in addition to its role in development (Negri et al., 2019).

Nitric oxide is produced by the conversion of L-arginine to L-citrulline by an evolutionary conserved hem-containing enzyme family (Figure 9), the nitric oxide synthases (NOS) (Al-Shehri, 2021). NOS activity requires binding to calmodulin is calcium-dependent in its constitutive isoforms (cNOS) unlike its inducible isoform (iNOS) which is already bound to calmodulin (Jacklet, 1997). Calcium dependence of NOS isoforms also seems to be similar between vertebrates and invertebrates. The NO produced through the enzymatic activity of iNOS has a pathogen destruction function as a non-specific response to infections (Colasanti et al., 2002; Zeidler et al., 2004). The high toxicity of inducible NO goes back to its production in high concentration reaching 1000 times more than NO produced via cNOS (Rivero, 2006).

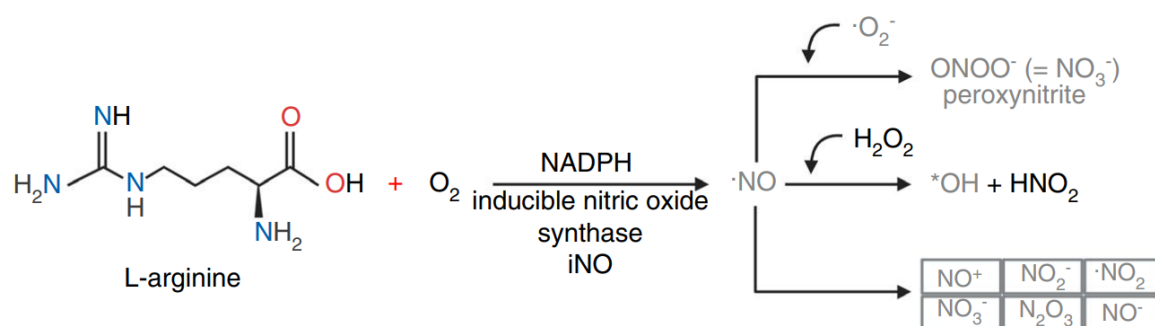


Figure 9. Nitric oxide production pathway (Eleftherianos et al., 2021).

Peroxynitrite, a by-product of the RNS production pathway, was also reported to have potent antimicrobial properties (Carreras et al., 1994). Peroxynitrite is produced as a result of the interaction between ROS and RNS at low oxygen tension (Radi, 2018).

2.4.4.2. ROS

Reactive oxygen species (ROS) like hydrogen peroxide are produced to fight pathogens causing damage to their membranes and during the normal aerobic process of the cell via the electron transport chain as the main source of intracellular ROS (Balaban et al., 2005; Eleftherianos et al., 2021). ROS can be found in the extracellular humor (Marmaras & Lampropoulou, 2009) and can limit the infection in honeybees with its antibiotic properties when present in larvae food (Bucekova et al., 2014). However, alteration in the production of hydrogen peroxide can have drastic effects on the organism. An increase of H_2O_2 production may induce oxidative stress while a decrease may cause susceptibility to pathogens.

ROS production is initiated by several oxidases such as nicotinamide adenine dinucleotide phosphate oxidase (NADPH), xanthine oxidase, cytochrome P450 oxidase, lipoxygenase or cyclooxygenases to produce a superoxide anion molecule ($\cdot O_2^-$) from univalent O_2 (Beutler, 2004; Eleftherianos et al., 2021). The superoxide radical is either dismutated by the superoxide dismutase (SOD) enzyme or spontaneously dismutated to hydrogen peroxide (H_2O_2) which is then hydrolyzed into H_2O via catalases or peroxidases (Eleftherianos et al., 2021). Superoxide is considered as the major precursor of ROS (Al-Shehri, 2021). The pathway of ROS production is illustrated in Figure 10.

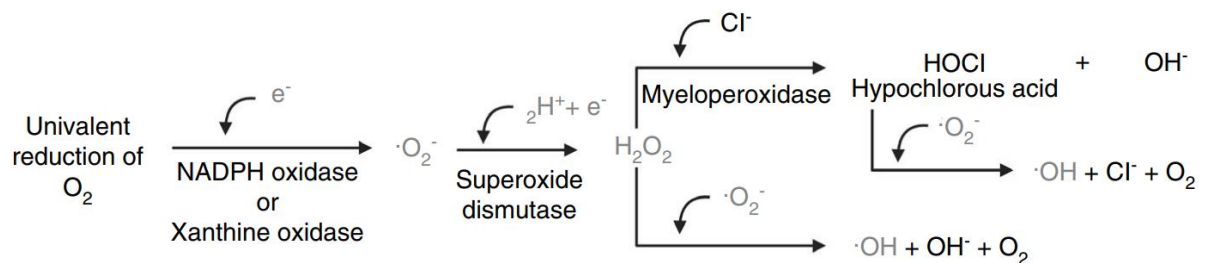


Figure 10. Pathway of ROS production in insects (Eleftherianos et al., 2021).

ROS production can take place by the mitochondria, peroxisomes and xanthine oxidase but in phagocytes, ROS is the main enzymatic product produced by NADPH oxidases and dual oxidases (DUOX) (Al-Shehri, 2021).

2.4.5. Signaling pathways in insect immune system

2.4.5.1. The Toll signaling pathway

Toll and Toll-like receptors (TLR) are important in ontogenic development and immunity. Regarding development, the Toll pathway is implicated in patterning during embryogenesis in insects including *Apis mellifera* and *Drosophila* (Steward & Govind, 1993; Wilson et al., 2014). So far, five Toll-related genes have been identified in honeybees (Toll-1, -6, -2/7, -8, -10) (Hillyer, 2016) which are also found in other orders of insects including Diptera, Lepidoptera and Coleoptera with a few exceptions (Brutscher et al., 2015). Orthologs of Toll-like receptors are found in mammals and in addition to honeybee orthologs to all members of the Toll pathway (Evans et al. 2006). The Toll signaling pathway is also implicated in controlling hemocyte density and proliferation with other pathways in *Drosophila* (Sorrentino et al., 2004; Zettervall et al., 2004).

2.4.5.1.1. Pathogen recognition via the Toll pathway

Recognition of pathogens in the Toll pathway (Figure 11) occurs after the interaction of microbial PAMPS by different PRRs. GGBP1, GGBP3, PGRP-SA, and PGRP-SD recognize gram-positive bacteria (Evans et al. 2006). GGBP3 also recognizes β -glucan of fungal cell walls while PGRP-SD additionally recognizes gram-negative bacteria that have diaminopimelic acid type peptidoglycan (DAM-type PGN).

Interaction of PAMPS with GGBPs and PGRP-SA leads to the activation of Grass, via a modular serine protease (ModSP) in *Drosophila* (Buchon et al., 2009). Grass is a gram-positive specific-serine protease that involved in recognition of gram-positive bacteria and fungus (Kambris et al., 2006). Grass forms a cascade with Spätzle-processing-enzyme (SPE) and Spätzle where SPE cleaves Spätzle resulting in its activation (Buchon et al., 2009). In addition, Persephone (PSH) is a proteolytic enzyme that recognizes bacterial and fungal virulence factors and activates SPE.

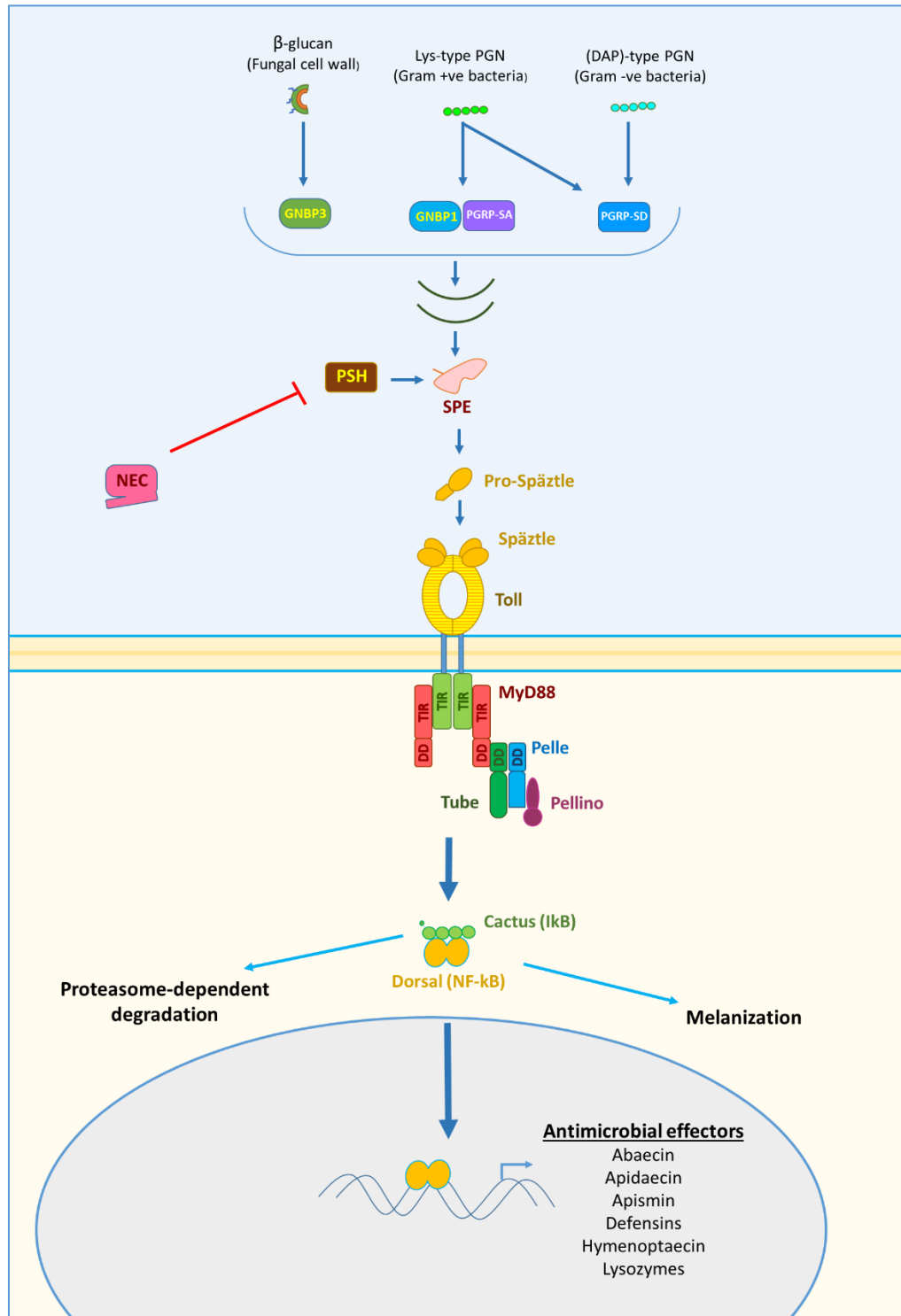


Figure 11. Activation of Toll signaling pathway in honeybees. PRRs recognize particles from bacterial and fungal cell walls to activate a signaling cascade via the transmembrane receptor, Toll. The signal transduction leads to the activation of the dorsal transcription factor resulting in the production of antimicrobial effectors.

2.4.5.1.2. Spätzle

Spätzle (Spaetzle, Spz) is an extracellular cytokine that circulates in the blood of both vertebrates and invertebrates (Weber et al., 2003) and plays a role in innate immunity and embryonic development of *Drosophila* (Steward & Govind, 1993). It is present in its inactive form and when activated, it directly binds to the Toll receptors activating the Toll pathway. The inactivity of Spätzle pro-protein is attributed to its natively unstructured pro-domain that prevents binding to Toll. It is activated with proteolytic cleavage that induced conformational change (Valanne et al., 2011) after the microbial recognition upstream the signaling cascade.

2.4.5.1.3. Toll

Toll is a transmembrane receptor. It is activated when a dimer of two Toll molecules is cross-linked via direct binding of Spätzle dimer to each Toll receptor's extracellular domain in the most recent model of interaction (Gangloff et al., 2008).

2.4.5.1.4. MyD88 and downstream signaling

The myeloid differentiation factor 88 (MyD88) is an adaptor protein of the Toll pathway conserved in vertebrates and invertebrates and it is a key modulator required in downstream signaling by activating NF- κ B transcription factors and production of antimicrobial effectors in insects such as *Drosophila* (Valanne et al., 2011) and *A. mellifera* (Brutscher et al., 2015). In addition, MyD88 plays a role in phagosome maturation after its interaction with the intercellular domain of recognition receptor during early phagocytosis (Benjamin et al., 2021). Phagocytosis can either be dependent on MyD88 or independent with regard to the pathogens seen in murine macrophage exposed to the pathogenic yeast *Candida albicans* and the mold *Aspergillus fumigatus* (Marr et al., 2003). However, it was seen to enhance phagocytosis nonetheless (Zou et al., 2015). MyD88 and Pelle (IL-1R associated kinase, IRAK) interact indirectly via Tube, an adaptor protein with a death domain (DD). The formed complex interacts with Pellino which is a positive regulator protein of the Toll pathway (Haghighyeghi et al., 2010; H. Sun et al., 2002).

Cactus is an I κ B that binds the Dorsal transcription factor and prevents its activity and nuclear translocation (L. P. Wu & Anderson, 1998). The activation of the Toll pathway leads to the phosphorylation of Cactus by Pelle leading to its degradation thus dissociating from Dorsal allowing its translocation to the nucleus. Once the Dorsal protein enters the nucleus, it induces the production of antimicrobial peptides (AMPs) /effectors (Valanne, Wang, and Rämét 2011; Evans et al. 2006).

2.4.5.2. The IMD, JNK and JAK/STAT pathways

Unlike the Toll pathway, the immune deficiency (IMD) pathway is not implicated in development (Hultmark, 2003) and plays a role in the immune response to mainly gram-negative bacteria and gram-positive bacteria that possess a DAP-type peptidoglycan cell wall (Aymeric et al., 2010; Evans et al., 2006). Other types of PGNs and fungi were also observed to elicit an immune response but to a weaker extent than gram-negative bacteria (Akira et al., 2006). Although *Drosophila* has 3 times more diversity than honeybees in genetic immune components, the IMD pathway is well preserved in honeybees (Figure 12) (Evans et al. 2006).

Recognition of PRRs occurs by PGRP-LC which has many peptidoglycan recognition domains and in turn activates a downstream signaling cascade that leads to the production of AMPs. Relish is a major component in the IMD pathways that leads to AMP production but interestingly it appears to be involved in the JNK pathway as well facilitating a cross-talk between different pathways. Relish is also implicated in the feedback mechanism of the IMD and JNK immune pathways. Both IMD and JNK pathways may lead to melanization. However, the activation of the JNK pathway is implicated in apoptosis (Evans et al. 2006).

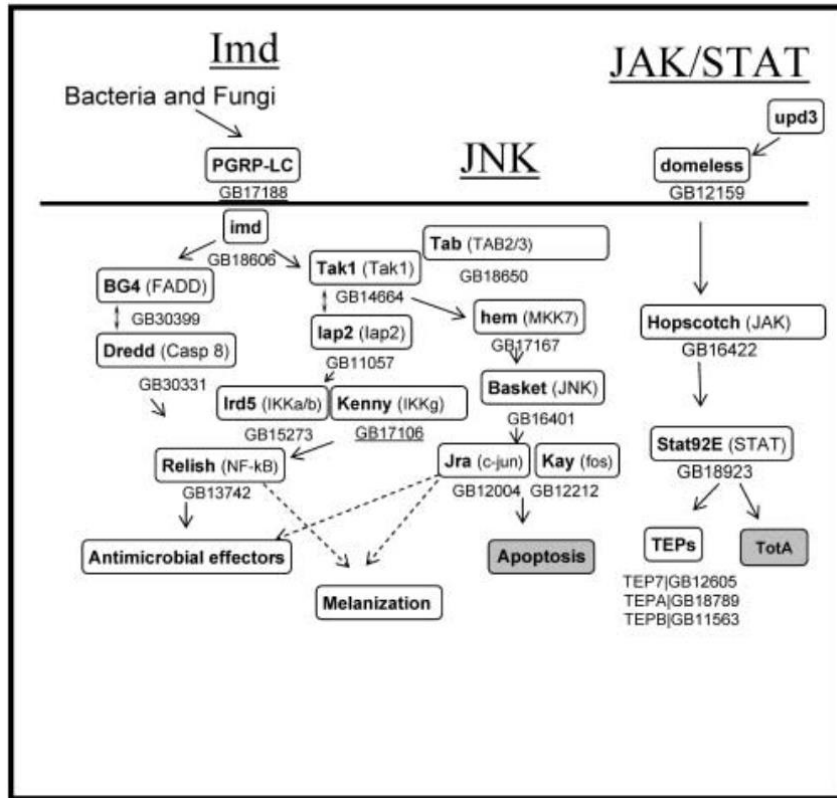


Figure 12. Candidate members of IMD (Imd), JNK and JAK/STAT pathways in honeybees. Vertebrate equivalents are between brackets while *Drosophila* component names are stated below each candidate member (Evans et al. 2006).

As for the JAK/STAT pathway, its activation results in the activation of phagocytosis in addition to the over-proliferation of hemocytes. Activation of the pathway is initiated via the extracellular ligand, Upd which results in phagocytosis. Upd binds to the cytokine-like receptor, domeless and continues to downstream signaling which leads to the activation of Hopscotch, Stat92E (honeybee homologs), and ultimately to the production of Thiolester-containing proteins (TEPs) which were detected across insect species including honeybees and *Drosophila*. A component found in *Drosophila* and not detected in honeybees are the Turandot (Tot) genes that are expressed in conditions of severe stress. The JAK/STAT pathway is involved in antiviral immune response in *Drosophila* (Boutros et al., 2002) and bees (Brutscher et al., 2015; McMenamin et al., 2018).

2.4.6. RNAi

RNA interference (RNAi; Figure 13) a process of post-transcriptional gene silencing and defense mechanism against viral infections found spanning several phyla including plants (Rosa et al., 2018), invertebrates (Nayak et al., 2013; Wang & He, 2019) and vertebrates (Anobile & Poirier, 2023). RNAi is confirmed as a defense mechanism against viral infections in honeybees (Brutscher et al., 2015; Galbraith et al., 2015). In insects, RNAi acts as a defense mechanism against viruses by recognizing VAMPs and silencing the viral cycle. An example is the deformed wing virus (DWV) (Galbraith et al., 2015) and the Israeli acute paralysis virus (IAPV) (Maori et al., 2009).

Viruses that infect honeybees are mainly of positive sense having ssRNA which generate dsRNA intermediates as a secondary structure during replication (Y. Chen, 2011; De Miranda et al., 2015). The double-stranded RNA (dsRNA) or the secondary structure within the viral genome act as a substrate and are recognized by a special sensor, the Dicer-like protein which cuts the dsRNA into shorter segments to become either small-interfering RNA (siRNA), piwiRNA (piRNA) or microRNA (miRNA) representing 3 distinct pathways. The siRNA pathway is the main dsRNA pathway that is included in antiviral defense in plant and invertebrates while the involvement of the other pathways in antiviral defense is debated in mammals. In honeybees, the miRNA and piRNA pathways are not well characterized and the focus is mainly on siRNA-mediated RNAi (Brutscher et al., 2015). Infact, the siRNA pathway is stated to be the first line of defense against viral infections in insects (Gammon & Mello, 2015).

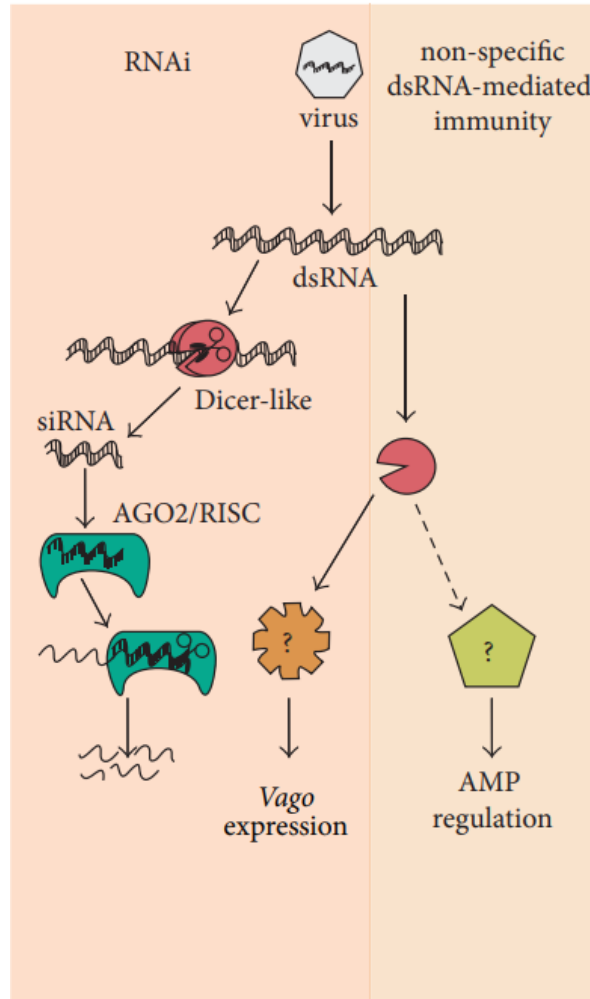


Figure 13. Schema representing the RNAi-mediated antiviral defense in bees (Brutscher & Flenniken, 2015).

This produced siRNA is then incorporated into AGO2 (argonaut-2) protein to form RNA induced silencing (RISK) complex containing a ssRNA that binds to complementary sequences (Galbraith et al., 2015) which degrades the RNA. In honeybees, RNAi was found to be triggered by the introduction of dsRNA, which also trigger signaling cascades against viruses even in a non-sequence-specific manner (Brutscher & Flenniken, 2015).

RNAi poses an important tool in beeking as potential applications of exogenous dsRNA to induce RNAi in honeybees for pest control and increasing resistance to pathogens are considered.

2.4.7. Vitellogenin

Vitellogenin (Vg) is a hemolymph protein produced by fat bodies in insects (Arrese & Soulages, 2010). It is usually produced in oviparous mammals but it is also found in sterile female worker bees (López et al., 2014). Vg contributes to development and was found to be linked to RNA-mediated gene knockdown in honeybees (Guidugli et al., 2005). Taking into consideration the mutualistic suppressive effect between juvenile hormone and (JH) and Vg (Guidugli et al., 2005), the outcome would be a positive feedback loop that states that low Vg will result in further low Vg levels.

Vg is found to be connected to aging in honeybees via antioxidant activity (Seehuus et al., 2006). Thus, any alterations in Vg will lead to abnormalities in development and longevity rates in honeybees. Vg levels in affect social behavior (Amdam et al., 2003) and foraging in worker honeybees where RNAi-mediated Vg silencing resulted in premature long-flight onset with more precocious workers (Marco Antonio et al., 2008). Vg plays a role in innate immunity by promoting phagocytosis and acting as opsonins as found in fish in addition to its antioxidant protective function (C. Sun & Zhang, 2015).

Vitellogenin is shown to facilitate trans-generational immune priming (TGIP) (Harwood et al., 2021; Salmela et al., 2015). TGIP is the transfer of immune experience from the bee queen to larvae (through eggs) increasing their resistance to pathogens including bacteria (López et al., 2014) and viruses (Lang et al., 2022). The transfer of vitellogenin is also observed in nurse bees to larvae via the hypopharyngeal glands (Amdam et al., 2003). The utilization of Vg in such a manner is categorized as a form of social immunity though Vg itself does not act on the immune system. This indicates that social immunity and individual immunity are connected, and that social immunity is more complex than just observable behavior.

2.5. Immune activators

There are different types of immune activators used in immunological studies to assess the response and implications on the immune pathways. These immune activators are derived from the cell walls of microbial organisms that usually stimulate and induce an immune response in the host.

2.5.1. Lipopolysaccharide

Lipopolysaccharide (LPS) is an outer cell wall component and crucial macromolecule of gram-negative bacteria like *Escherichia coli*. It functions as an endotoxin contributing to the microbial pathogenicity (Rietschel et al., 1994). LPS is mainly composed of 4 components including a repeated O-polysaccharide chain, an outer core, an inner core and a highly conserved Lipid A that is the definitive factor of endotoxicity (Figure 14) (Caroff & Novikov, 2019; Erridge et al., 2002).

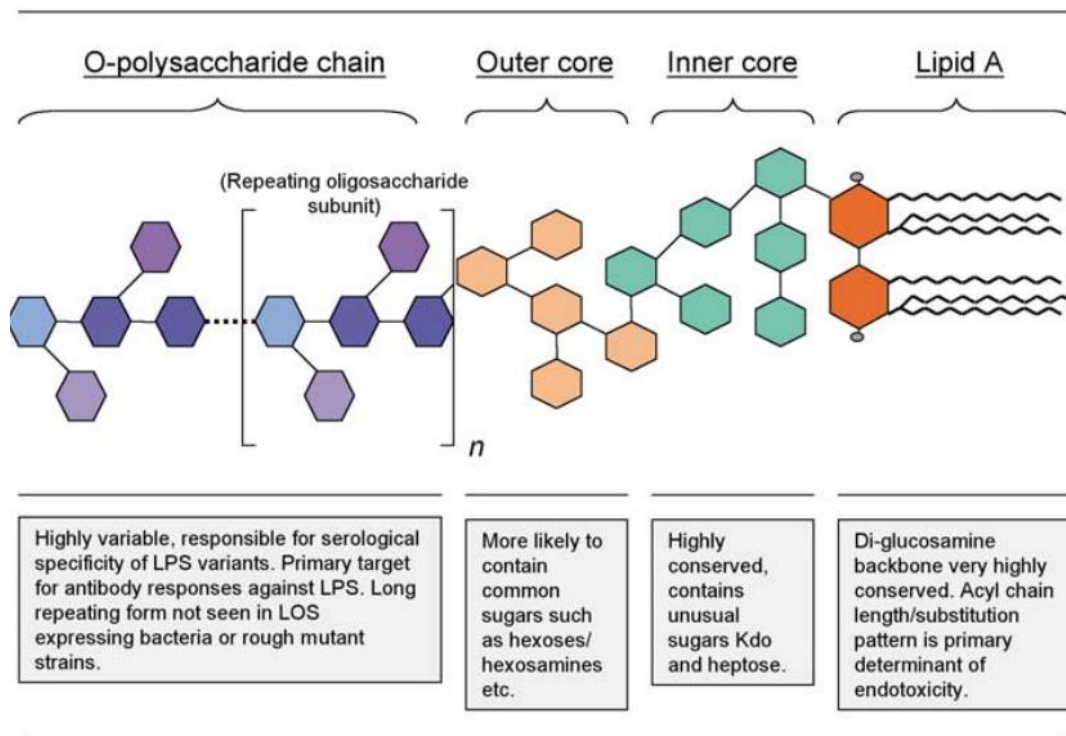


Figure 14. The general components of lipopolysaccharide (Erridge et al., 2002)

LPS has significance in the fields of microbiology and immunity as it is used as an immune stimulator. LPS is a PAMP that facilitates the recognition of gram-negative bacteria

activating both the Toll signaling immune pathway in vertebrates and invertebrates and the IMD pathway as well (Evans et al., 2006; Mogensen, 2009; Murphy et al., 2017).

In insects, LPS was observed to trigger the Toll signaling pathway resulting in the production of AMPs and it induces phenoloxidase activity (Evans et al. 2006).

2.5.2. Peptidoglycan

Peptidoglycan (PGN) is a component of bacteria cell walls with a structural function. In gram-positive bacteria, PGN is multi-layered and cross-linked alternating N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) and it is in the outer member. In gram-negative bacteria, PGN is situated as a middle layer not exposed to the surface (Vollmer et al., 2008). Gram-positive bacteria have relatively higher levels of PGN than gram-negative bacteria and thus high PGN levels is a determinant characteristic of gram-positive bacteria (Figure 15) (Liu et al., 2015).

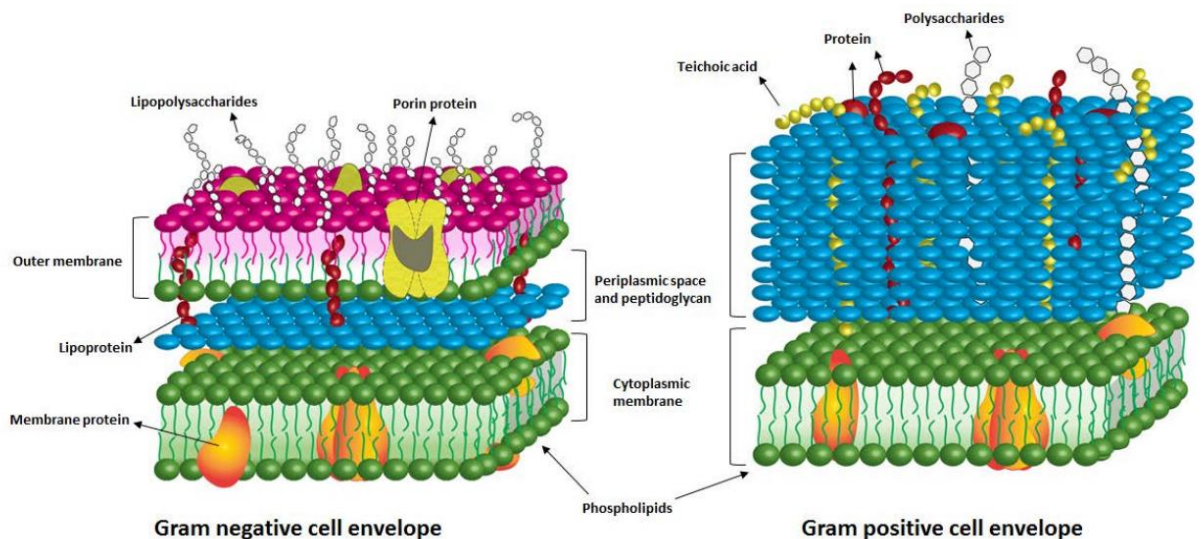


Figure 15. Comparison between cellular envelopes of gram-negative and gram-positive bacteria (Liu et al., 2015).

Like LPS, PGN serves as a PAMP in microbial recognition by PRRs of the immune system leading to the initiation of a signaling cascade that results in the production of AMPs and antimicrobial effectors via the Toll pathway. Gram-positive bacteria with DAP-type PGNs may activate the IMD pathway in insects as well (Aymeric et al., 2010; Evans et al., 2006).

2.6. Effect of pesticides on the immune system: Pro-inflammatory and immunosuppressive effects

Pesticides may exert a pro-inflammatory response by modulating cytokines (Gangemi et al., 2016). Organophosphates for instance, inhibit the action of AChE causing dysregulation in the cholinergic system and the production of pro-inflammatory cytokines in vertebrates and invertebrates since they share similar cholinergic systems (Camacho-Pérez et al., 2022).

Imidacloprid was observed to decrease the gene expression of AChE in honeybees in both field and laboratory conditions (De Smet et al., 2017) possibly modulating the cholinergic system and pro-inflammatory cytokine release.

PAMPs like LPS can induce an inflammatory response as well via the Toll signaling pathway (Ngkelo et al., 2012). 1,3- β glucans may also induce a proinflammatory response as seen in rainbow trout cell lines (Ordás et al., 2021). In a way, PAMPs and pesticides that cause inflammatory responses may act on the same signaling pathway possibly realizing synergism or antagonism if there is a competition for acting on a certain level of a given pathway.

As for the immune suppressive effect, imidacloprid had a significant impact on honeybee immune-related genes decreasing the expression of *relish*, *cactus*, *dorsal*, *domeless*, *apidaecin*, *defensin 1* and *PGRPs* but the effect was dependent on the development stage where brown-eyes pupae were most affected (Tesovnik et al., 2019). Imidacloprid was also reported to significantly decrease the expression of antioxidant enzymes in honeybees (Aufauvre et al., 2014).

The combination of pro-inflammation on one hand and immune-suppression of certain immune related genes may render the organism less fit for survival, function or diseases resistance.

2.7. Objective

Colony collapse disorder poses a threat to beekeepers affecting the economic income from honey production and the agricultural services of pollination by honeybees. In addition to their contribution to biodiversity and plant species continuation since many plants are pollinator dependent. Many factors in interplay may contribute to this phenomenon.

The application of neonicotinoid pesticides and their persistence in the environment may be one of the main factors that result in honeybee decline and CCD. Imidacloprid, the mostly used neonicotinoid pesticide globally, is the target of interest in this study. In addition, amitraz is a pesticide in direct contact with honeybee hives. Although amitraz has low metabolism in honeybees that limit the effect of its toxic metabolites, the effect of co-exposure with imidacloprid is still to be assessed since synergistic effects of amitraz were already documented.

Pesticide application may hinder the immune competence of honeybees to fend against pathogens and diseases. As many pests and microbes target bees we are interested in the *Nosema spp.*, particularly *N. ceranae* which seems to have an advantage over *N. apis*.

We aim to study the effect of imidacloprid and amitraz in single concentrations or in combinations on the immune cells of honeybee larvae in the context of immune stimulation, which activates the same immune responses as *Nosema spp.* We chose the in-vitro system approach to have a distinct observation of the effect on immune cells in particular in a highly controlled setting reducing any variations that may be present in-vivo.

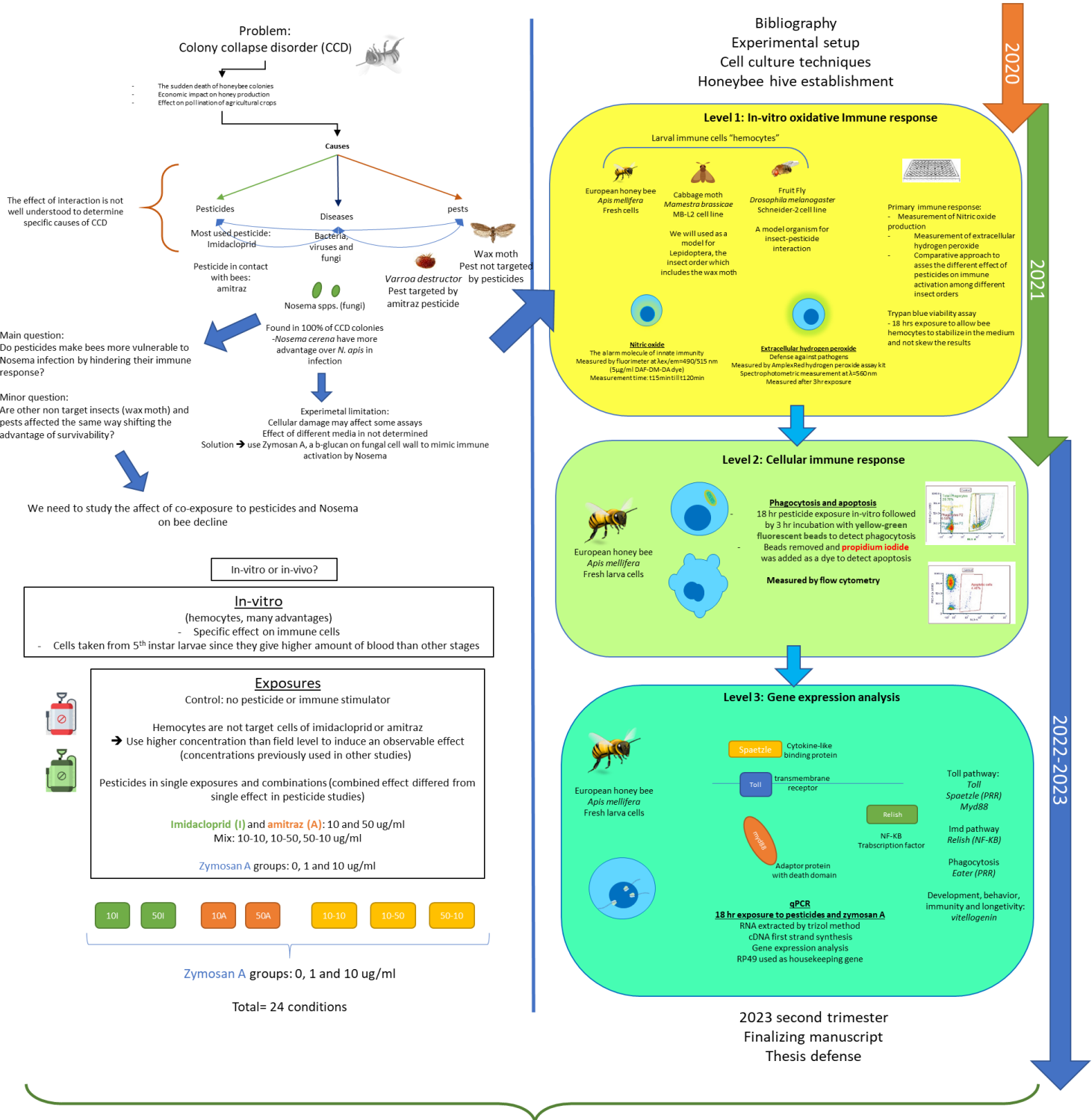
To mimic the immune stimulation induced by *Nosema spp.*, we will apply zymosan A, a fungal β -glucan to induce the activation of the same immune pathways as in the presence of nosemosis without any associated pathogenic damage that may skew the results on the immune stimulation.

We will study the effect of imidacloprid and amitraz at different concentrations of zymosan A on 3 fronts. The first is the humoral and cell-product levels assessing the production of total soluble proteins, nitric oxide and hydrogen peroxide at the first stages of immune activation in the larval hemocytes of honeybees (Hymenoptera; *Apis mellifera*) compared to the Dipteran fruit fly (*Drosophila melanogaster*) cell line Schneider-2 cell line and the

Lepidopteran Cabbage moth (*Mamestra brassicae*) larval hemocyte cell line, MB-L2. The aim of this comparison is to assess the any differential response between honeybees and the *Drosophila* since it is a model organism for pesticide toxicological studies and to compare the effect of exposure to the same conditions on Lepidoptera. This is gain some insight into potential advantage or disadvantage between species since multiple species exist within the same ecological niche and are exposed to the same risk factors. Honeybee pests such as the Lepidopteran wax moth may have an advantage if it is less affected by the same pesticides on the immune level. The next step is to assess the cellular response that includes cytotoxicity and phagocytosis. Cytotoxicity will be evaluated by microscopy and flow cytometry. Phagocytosis is a key immune response in organisms that rely on innate immunity for defending against pathogens hence it is a main target in our study in the presence of imidacloprid and/or amitraz. The final approach will be to assess the effect of imidacloprid and amitraz on the gene expression of immune genes present in the Toll pathway (*toll*, *spatzle*, *myD88*) and IMD pathway (*relish*) in addition to *eater* and *vitellogenin* genes.

The information obtained from this research would set a ground for better understanding of pesticides' modulatory mode of action on the immune system. In addition, the obtained data may be comparable to other studies on honeybees or different insect allowing a comprehensive assessment of the impact of pesticides and the immune system. Indeed, much information is needed on different parameters when studying honeybees and the implication of risk factors in CCD.

2.8. Project design



Role of *Nosema cerenae* and pesticides on the decline of bees: Studies using a multifactorial approach
"Tipping the scale of honeybee immune responses: The effect of pesticides on immune-stimulation mimicking *Nosema spp.*"

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3. Materials and Methods

3.1. Cell culture

3.1.1. Honeybee larvae hemocytes

Fifth instar larvae combs of the European honeybee (*A. mellifera*) were extracted from hives established at IUT Thionville-Yutz site in France and used as a source of hemocytes. The combs were incubated overnight at 32 °C with high moisture to simulate hive conditions. Hemolymph was extracted under a sterile laminar flow hood. Each larva was held by the non-dominant hand exposing the dorsal posterior part which was sterilized with 70% ethanol applied via a cotton swab. A sterile needle was used to puncture the dorsal section and the hemolymph was quickly collected and transferred to a Falcon tube via micropipette. Depending on the experiment, phosphate buffer saline (PBS; P2272 Sigma-Aldrich™) or WH2 medium was added to the extracted hemolymph.

Table 2. Composition of WH2 medium

Component	
Schneider's insect medium (21720-024, Gibco™)	35.55 %
0.06 M L-Histidine (H5659, Sigma Aldrich™)	47.39 %
De-complemented fetal bovine serum (FBS; F7524, Sigma Aldrich™)	11.85 %
CMRL 1066 (11530037, Gibco™)	3.55 %
Hank's salt solution (H6648, Sigma Aldrich™)	1.18 %
Insect medium supplement 10x (I7267, Sigma Aldrich™)	0.47 %
Gentamicin (G1397, Sigma Aldrich™)	50 µg/ml

WH2 medium was prepared according to Hunter (2010) with the composition mentioned in Table 2. The pH of the WH2 medium was set to 6.35-6.5 using 2N NaOH solution then

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sterile filtered with a 0.2 μm low protein-binding syringe filter (Acrodisk™ 4312, Pall corp.™).

Honeybee hemocytes are displayed in Figure 17 from a microscopic observation. Hemocytes showed adherence to the surface with variable morphologies ranging from round-oval to elongated appearance indicating the presence of different types of hemocytes.



Figure 17. Honeybee hemocytes in WH2 medium observed under an inverse microscope at 40x magnification.

3.1.2. Schneider-2 cell line

The Schneider-2 cell line (ACC 130, Leibniz Institute DSMZ TM) is a cell line developed from *D. melanogaster* embryonic hemocytes (Schneider, 1972). Cells were purchased and stored in liquid nitrogen. For culture initiation, tubes were thawed in a water bath set at 20 °C then transferred to a tube containing 5 ml of Schneider's insect medium with 20% de-complemented FBS. The tube was then centrifuged at 3000 x g, the supernatant removed and the pellet re-suspended in 39.5% Schneider's insect medium, 39.5% TC-100 insect medium (T3160, Sigma AldrichTM), 20% de-complemented FBS and 1% penicillin-streptomycin (P4458, Sigma AldrichTM). Hemocytes were incubated at 20 °C with no CO₂ for 2 weeks before subculture. The following subcultures were transferred bi-weekly in a culture medium composed of 44.5% Schneider's insect medium, 44.5% TC-100 insect medium 10% de-complemented FBS, and 1% penicillin-streptomycin 20 °C with no CO₂ until needed.

3.1.3. MB-L2 cell line

MB-L2 is a cell line established from the larval hemocytes of the cabbage moth (*M. brassicae*). A frozen stock of MB-L2 cell line was purchased from Leibniz Institute DSMZ TM (ACC. 76) and stored in liquid nitrogen until culture initiation. The stock was thawed at 20°C and transferred to a Falcon tube containing 5ml of 80% Grace's insect medium (G8142, Sigma-AldrichTM) and 20% de-complemented FBS. The tube was then centrifuged at 3000 rcf, the supernatant removed and the pellet re-suspended in Grace's insect medium supplemented with 20% de-complemented FBS and 1% penicillin-streptomycin for 2 weeks in 25 cm² cell culture flasks. Then the culture flask was sub-cultured on a weekly basis in Grace's insect medium with 10% de-complemented FBS and 1% penicillin-streptomycin until use.

3.2. Exposures

3.2.1. Stock solutions

Imidacloprid (37894-100 mg, Sigma-Aldrich™) and zymosan A (Z4250, Sigma-Aldrich™) were purchased in powder state and suspended in sterile PBS at 10 mg/ml and 1 mg/ml respectively. Amitraz (45323, Sigma-Aldrich™) was dissolved in hexane at 10 mg/ml. The solutions were diluted in PBS or culture medium and sonicated for 30 minutes before preparing cell exposures. After dilution and before preparation of pesticide mixtures, amitraz diluted in PBS or culture medium was left for 1 hr under a laminar flow hood to allow evaporation of hexane from the solution.

3.2.2. Treatments

Pesticide concentrations were chosen based on similar experiments in lab conditions (Dai et al., 2018; Walderdorff et al., 2018; Young et al., 2005) and considering the fact that hemocytes are not the main target of these pesticides, the concentrations were above field concentrations in order to produce an observable effect. Young et al., (2005) used amitraz concentrations of 1, 10, 50, and 100 µg/ml to treat human luteinized granulosa cells while (Mangia et al., 2018) used 25, 50, 100 and 150 µM equivalent to 7.325, 14.65, 29.3 and 58.6 µg/ml respectively for treatment of the sheep tick (*Ixodes ricinus*) cell line. The concentrations of amitraz used were within the experimental range used in cell-based assays. In addition to the control with no pesticides, the single exposures of imidacloprid (I) and amitraz (A) were at 10 or 50 µg/ml while the co-exposures consisted of 10I-10A, 10I-50A, and 50I-10A µg/ml of imidacloprid and amitraz respectively. All pesticide exposures either were without zymosan or treated with 1 or 10 µg/ml zymosan A. A schema of the exposures is represented in Figure 18.

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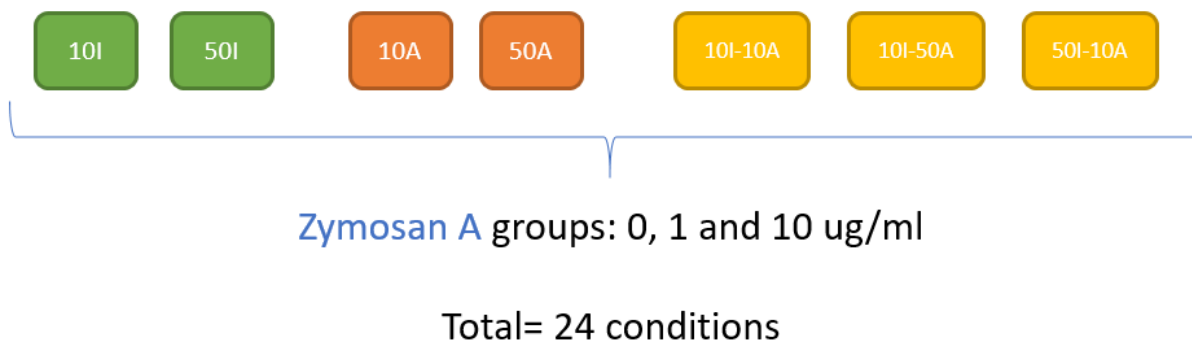


Figure 18. Schematic representation of imidacloprid (I) and amitraz (A) exposures in different zymosan treatment groups. All concentrations are in $\mu\text{g/ml}$.

3.3. Cytotoxicity assay

A viability assay was performed for Honeybee hemocytes, Schneider-2, and MB-L2 cell lines to assess the feasibility of performing assays with the used concentrations and allow stabilization of honeybee hemocytes after extraction. Schneider-2 and MB-L2 cultures were detached by gentle pipetting to prevent skewing of the viability results. Hemocytes were seeded in 96-well TPP™ tissue culture plates containing the exposures with the respective culture medium for each species. The plates were incubated with the treatments for 18 hrs at 20°C after being sealed with sealing tape. Trypan blue solution (T8154 sigma-Aldrich™) was used to measure viability via dye exclusion assay (Tolnai, 1975). Trypan blue was added to a 1:1 volumetric ratio in each well. At least 200 cells were counted under an inverted microscope in each well after 3 min incubation time. Hemocytes that retained the dye within them were considered dead. Each treatment was done in triplicates. The percentage of viable cells was calculated using the following equation for each well:

$$\begin{aligned} & \text{percentage of viable cells \%} \\ & = \frac{\text{total number of counted cells} - \text{total number of dead cells}}{\text{total number of counted cells}} \times 100 \end{aligned}$$

3.4. Oxidative response and cell products

3.4.1. Protein content quantification

We measured the protein content in pooled hemocytes of honeybees, MB-L2, Schneider-2 cell lines using Bradford's assay (Bradford, 1976) adapted to 96-well plates. Bradford solution was prepared by dissolving 10 mg of Coomassie Brilliant Blue G-250 (MFCD00078482, VWR™) in 5 ml of 95% ethanol. Then 10 ml of 85% phosphoric acid was added to the solution to be diluted to 100 ml with distilled water.

Hemocytes were centrifuged at 1000 rcf in 15ml Falcon tubes for 5 min then washed twice with PBS before seeding. Seeding was at a concentration of 150,000 cells/ml in a total volume of 120 μ l in each well. Hemocyte exposure to pesticides and zymosan A was done in PBS at 20 °C for 3 hrs in 96-well tissue culture plates. The plates were then frozen at -80 °C for later analysis. Prior to protein quantification, the plates were thawed, 20 μ l of the each well was pipetted and transferred to a fresh plate then 180 μ l of Bradford solution was added to each well. The plate was then read by a BioTek™ PowerWave XS2 spectrophotometer at $\lambda=595$ nm and 450 nm. A standard curve was obtained by using bovine serum albumin (BSA; A9576, Sigma-Aldrich™) as a standard. BSA concentrations ranged from 0 to 20 μ g/ml in PBS. The obtained data was linearized according to Ernst & Zor (2010). A ratio 590 nm/450nm was made before normalizing the absorbance to the protein concentration of the standard curve. All conditions were tested with 6 replicates for honeybee and Schneider-2 cells and 9 replicates for MB-L2 cells.

3.4.2. Nitric oxide production

Nitric oxide (NO) production was measured as done granulocytes (Negri, Maggi, Correa-Aragunde, Brasesco, Eguaras, Lamattina, et al., 2013) using DAF-FM DA fluorescent dye (D1946, Sigma-Aldrich™). Exposure solutions of imidacloprid, amitraz, and zymosan in PBS were added to 96-well Half Area High Content Imaging Glass Bottom Microplate (4850, Corning®). A stock solution of 50 mM DAF-FM DA was dissolved in DMSO. Honeybee hemocytes, Schneider-2 and MB-L2 cell lines were centrifuged at 1000 rcf for 5 min and washed with PBS twice before the addition of the DAF-FM DA to the hemocytes at a concentration of 20 μ M just before plate seeding. The end concentration of DAF-FM

DA was set to be 5 μM in each well while the that of of pesticides were 10 and 50 $\mu\text{g/ml}$ for imidacloprid and amitraz single exposures and 10+10 $\mu\text{g/ml}$, 10+50 $\mu\text{g/ml}$ or 50+10 $\mu\text{g/ml}$ of imidacloprid and amitraz respectively for mixtures. All pesticide exposures were either done with 1 or 10 $\mu\text{g/ml}$ zymosan A or without zymosan A. Treatments were made in 6 replicates. Nitric oxide production was measured at 15 min exposure and continues until 120 min by fluorescence detection using Fluostar Galaxy (BMG Labtech™) fluorimeter set at excitation/emission of $\lambda=490/515$ nm. The following equations were used for data presentation:

$$\begin{aligned} & \text{Nitric oxide production at 15 min (\%)} \\ & = \frac{\text{fluorescence of sample at 15 min}}{\text{Avg. fluorescence of the control group without zymosan at 15 min}} \times 100 \end{aligned}$$

$$\begin{aligned} & \text{Nitric oxide production at 120 min (\%)} \\ & = \frac{\text{fluorescence of sample at 120 min}}{\text{Avg. fluorescence of the control group without zymosan at 120 min}} \times 100 \end{aligned}$$

The rate of nitric oxide production from 15 minutes to 120 minutes was calculated by the using the equations:

$$\text{Sample ratio} = \frac{\text{fluorescence of sample at 120 min}}{\text{fluorescence of sample at 15 min}} \dots \dots \dots \text{Eqn(1)}$$

$$\text{Control group ratio} = \frac{\text{Avg. fluorescence of the control group without zymosan at 120 min}}{\text{Avg. fluorescence of the control group without zymosan at 15 min}} \dots \dots \dots \text{Eqn(2)}$$

$$\text{Change in nitric oxide production rate (\%)} = 100 \times \left[\frac{\text{Eqn(1)}}{\text{Eqn(2)}} - 1 \right]$$

3.4.3. Extracellular hydrogen peroxide

Hemocytes were seeded in in their respective culture mediums in a total volume of 70-100 μl in each well of 96-well plates containing different concentrations of imidacloprid, amitraz and zymosan. Each treatment was made in 5 replicates. The plates were incubated

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at 20 °C for 3 hrs without CO₂ exposure. We quantified extracellular hydrogen peroxide by Amplex™ Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen™, catalog no. A22188) following the manufacturer's protocol. We prepared 100 μM Amplex® Red reagent and 0.2 U/mL HRP working solution in 1x reaction buffer. Hydrogen peroxide standards of concentrations ranging from 0 and 10 μM were prepared in 1x reaction buffer by serial dilution. Plates were centrifuged using ThermoScientific™ Megafuge 16R centrifuge at 1000 rcf for 3 minutes. The working solution was transferred to a new 96-well plate in 50 μL volume to each well containing 50 μL of supernatant or the hydrogen peroxide standards. The plate was covered in aluminum foil to prevent light exposure and incubated 30 minutes. Absorbance was measured at $\lambda=560$ nm using BioTek™ PowerWave XS2 spectrophotometer. Results were normalized to the absorbance of the culture medium and expressed relative to the control group without pesticide exposures.

3.5. Cellular immune response and activity in honeybee hemocytes

Honeybee hemolymph was extracted from fifth instar larvae as described in 3.1.1. and pooled in a tube containing WH2 medium. Hemolymph from 40 larvae were pooled in a tube for each 2 ml of WH2 medium. One hundred microliters of the pooled hemolymph were then seeded in each well of 24-well TPP® Tissue Culture Plates. After seeding the hemocytes, 300 µl of treatment solution was added to each well. In addition to the control, the final pesticide concentrations were 10, 50 µg/ml of imidacloprid or amitraz in single exposures and 10 µg/ml+ 10 µg/ml, 10 µg/ml + 50 µg/ml or 50 µg/ml +10 µg/ml of imidacloprid and amitraz respectively in co-exposures. All treatments were carried out with 0, 1, or 10 µg/ml zymosan A. All conditions were prepared in triplicates and incubated at 20 °C for 18 hrs without CO₂ exposure. Subsequent analysis of phagocytosis and cytotoxicity was done via flow cytometry.

3.5.1. Phagocytosis and membrane integrity indicators

To analyze phagocytosis, we used fluorescent yellow-green amine-modified latex beads (L1030, Sigma-Aldrich™) diluted 1000x in PBS. After 18 hrs exposure, the culture medium was removed and replaced by 400 µl of diluted fluorescent beads. Plated were then incubated at 20 °C for 3 hrs. The plates were then removed from the incubator and wells were washed 3 times with 3% BSA diluted in PBS. After the BSA was removed from the last wash, hemocytes were detached by 200 µl of 0.25 % trypsin added to each well and then incubated at 30 °C for 30 minutes. Trypsin reaction was stopped by WH2 medium supplemented with 10 % FBS and 50 µg/ml of propidium iodide (PI) for indication of cell with compromised membranes. Propidium iodide is a fluorescent dye that binds to nucleic acid. It only penetrates cells with damaged or permeabilized membranes. The cells were left in PI for 15 min before measurement.

3.5.2. Flow cytometry measurement

We used the Attune™ NxT flow cytometer from Thermofisher™ to measure phagocytosis and PI in the cells. Blue laser ($\lambda=488$ nm) was used for excitation. Parameters were regulated and optimized for the application of PI and fluorescent beads with honeybee hemocytes. Hemocytes permeabilized and stained with 0.1% Triton X-100 and 25 µg/ml

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PI respectively were used as a positive control for dead cells while free fluorescent beads were used to optimize signal detection for phagocytosis. The threshold was set at 25 mV to remove debris from the reading. Forward scatter (FSC) and side scatter (SSC) were set at 250 mV and 375 mV respectively. The emission filter to detect phagocytosis was bandpass (BP) =530/30 and that to detect PI was BP=695/40 both set at 240 mV. The acquisition speed was set at 200 μ l/min and the sample volume was set at 200 μ l. Treatments were read in triplicates. Hemocyte singlets were gated and any remaining free beads were gated out from the reading. The data presentation and gating strategy were applied in FCS Express 7 Research Edition™ (De Novo Software™).

3.6. Gene expression analysis in honeybee hemocytes

Honeybee hemocytes were extracted and pooled as described in 3.5. Extracted hemocytes were seeded in 24-well plates that included the treatments with a total volume of 400 μ l in each well. The treatments were performed in triplicates. Plates were sealed with sterilized sealing tape and then incubated at 20 °C for 18 hrs.

3.6.1. RNA extraction

The supernatant of each well was transferred to corresponding Eppendorf tubes that were centrifuged at 5000 rcf for 5 min. Meanwhile, 400 μ l of TRIzol reagent (15596018, ThermoFisher™) was added to each well following the user protocol (MAN0001271) for RNA extraction. The supernatant was removed from the centrifuged tubes and the lysates were pipetted several times in the wells before transferring them to their corresponding tubes. The tubes were frozen at -80 C before continuation of the extraction. Upon thawing, a 80 μ l volume of chloroform was added to each tube, inverted several times and incubated for 2-3 minutes followed by centrifugation at 12,000 rcf for 15 minutes at 4 °C. Most of the upper phase was transferred to a new tube and 400 μ l of isopropanol was added. The samples were incubated for 10 minutes at 4 °C then centrifuged at 12,000 rcf for 10 minutes at 4 °C. The supernatant was discarded. 400 μ l of 75% ethanol was added to each sample and vortexed briefly. Tubes were then centrifuged at 7,500 rcf for 5 minutes at 4 °C. Supernatant was discarded and each sample was suspended in 40 μ l in RNase-free water. Sample concentration and purity were quantified by BioSpec Nano spectrophotometer (Shimadzu corps™).

3.6.2. cDNA synthesis

RNA of each sample was reverse transcribed to cDNA using RevertAid H Minus First Strand cDNA Synthesis Kit (K1632, ThermoFisher™). On ice, 500 ng RNA of each sample were added to a PCR tube followed by 1 μ l of oligo (dT)₁₈ primer, 4 μ l of 5X reaction buffer, 1 μ l RiboLock RNase inhibitor (20 U/ μ l), 2 μ l of 10 mM dNTP mix, 1 μ l of RevertAid H minus M-Mul V reverse transcriptase (200 U/ μ l). RNase-free ultra-pure water was added to reach 20 μ l total volume per tube. Sample tubes were incubated in ICycler thermocycler (Bio-Rad™) at 60 °C for 42 min then heated to 70 °C for 5 minutes.

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The samples were held at 4 °C before removal from the thermocycler. Sample concentration and purity were quantified by BioSpec Nano spectrophotometer (Shimadzu corps™).

3.6.3. T_m gradient analysis for primers

A temperature gradient analysis was set for honeybee primers between 50 and 63 °C. Analysis was performed with Hard-Shell High-Profile Semi-Skirted 96-Well PCR Plates (Bio-Rad™). Each well contained:

- 10 µl SsoAdvanced™ Universal SYBR® Green Supermix (#172-5271, Bio-Rad™)
- 2 µl honeybee cDNA
- 0.5 µM of forward and reverse primers
- Ultra-pure H₂O added to reach 20 µl

Forward and reverse primer sequences were chosen for *spätzle*, *relish*, *toll*, *myD88*, *eater*, *vgmc* (*vg*), and *rp49* genes as indicated in Table 3.

Table 3. Honeybee primer sequences for real-time PCR

Gene target	Forward primer	Reverse primer	Gene ID	Source
<i>spätzle</i>	5'-TGCACAAATTGTTTTTCCTGA-3'	5'-GTCGTCCATGAAATCGATCC-3'	GB15688	(Evans et al. 2006)
<i>relish</i>	5-GCAGTGTTGAAGGAGCTGAA-3'	5-CCAATTCTGAAAAGCGTCCA-3	GB13742	(Evans et al. 2006)
<i>toll</i>	5'-TAGAGTGGCGCATTGTCAAG-3'	5'-ATCGCAATTTGTCCCAAAAC-3'	GB18520	(Evans et al. 2006)
<i>myD88</i>	5'-TCACATCCAGATCCAACCTGC-3'	5'-CAGCTGACGTTTGAGATTTTG-3'	GB12344	(Evans et al. 2006)
<i>eater</i>	5'-CATTTGCCAACCTGTTTGT-3'	5'-ATCCATTGGTGCAATTTGG-3'	XP_001120277	(Simone et al., 2009)
<i>vgmc</i> (<i>vg</i>)	5'-AGTTCCGACCGACGACGA-3'	5'-TTCCCTCCACGGAGTCC-3	NP_001011578	(Simone et al., 2009)
<i>rp49</i>	5'-CGTCATATGTTGCCAACCTGGT-3'	5'-TTGAGCACGTTCAACAATGG-3'	AF441189	(Lourenço et al., 2008)

The reactions were carried out using the iCycler MyiQ™2 Two-color Real-Time Detection System with IQ5 software from Bio-Rad™. Reaction cycles set: 1x (3 min at 94 °C); 35x (30 sec 94 °C, 30 sec T_m gradient, 45 sec 72 °C) then samples were held at 4 °C.

3.6.4. Real-time PCR (qPCR)

Gene expression analysis of for *spätzle*, *relish*, *toll*, *myD88*, *eater*, *vgtc*, was performed by iCycler MyiQ™2 Two-color Real-Time Detection System (Bio-Rad™) in Hard-Shell High-Profile Semi-Skirted 96-Well PCR Plates with *rp49* as a housekeeping gene . Reaction mixtures contained:

- 10 µl SsoAdvanced™ Universal SYBR® Green Supermix
- 0.5 µM of forward and reverse primers (Final concentration)
- 300 ng cDNA
- Ultra-pure H₂O added to reach 20 µl

Reaction cycles set: 1x (30 sec at 95 °C); 45x (10 sec 95 °C, 30 sec 58 °C, 30 sec 72 °C) followed by melt curve analysis increasing temperature from 55 to 95 °C. Each sample was done with two technical replicates to check for repeatability.

3.7. Statistical analysis

Statistical analysis was done using the Addinsoft™ XlSTAT® 2019 software. Shapiro-Wilk test was used to test normality for each group of zymosan A treatments and homogeneity of variances testes with Bartlett's test. Significance between pesticide treatments within the same zymosan group was measured by ANOVA (Dunnett's test or Tukey HSD) for normally distributed samples. Groups with no normal distribution or non-homogenous variances were tested for significance using Kruskal-Wallis and Dunn pairwise comparison.

Gene relative expression calculation was carried out by Livak's method and analysis was done by 2-way ANOVA coupled with Duncan's test post-hoc to indicate significance between treatments in all the data set at 95% confidence interval with $n=3$. Non-normal data was transformed and normalized before analysis.

A principal component analysis (PCA) was carried out to determine the correlation between phagocytosis and viability measured by flow cytometry. The correlation was checked for each individual treatment within zymosan groups or for the whole groups by Pearson (n) test at 95% confidence interval. A PCA for gene expression was done for all the studied genes simultaneously.

Results and Discussion

4. Results and Discussion

4.1. Differential production of cellular products of immune activated hemocytes exposed to Imidacloprid and amitraz

4.1.1. Total protein content is context-dependent after pesticide exposure

The total protein content in honeybee hemocytes showed no significant variation with any of the pesticide treatments in the group without zymosan exposure (Figure 19A). The hemocytes produced the lowest concentration of 2.65 $\mu\text{g/ml}$ when treated with 10 $\mu\text{g/ml}$ imidacloprid without immune stimulation by zymosan while the highest concentration reached 3.76 $\mu\text{g/ml}$ with 50I-10A mixture of 50 $\mu\text{g/ml}$ imidacloprid and 10 $\mu\text{g/ml}$ amitraz respectively (Figure 19A). Similarly, the 50I-10A mixture gave the highest protein concentration (3.78 $\mu\text{g/ml}$) when co-exposed to 1 $\mu\text{g/ml}$ zymosan (Figure 19B) while the lowest protein concentration was obtained in the treatment with 50 $\mu\text{g/ml}$ amitraz at 2.55 $\mu\text{g/ml}$. However, there was no significant difference between the treatments in the 1 and 10 $\mu\text{g/ml}$ zymosan treatment groups. In the 10 $\mu\text{g/ml}$ zymosan group (Figure 19C), the average protein concentration was relatively lower than the other zymosan groups with 2.99 $\mu\text{g/ml}$ being the maximum concentration with the 50 $\mu\text{g/ml}$ imidacloprid single exposure.

Schneider-2 cells displayed a more consistent presentation of protein concentration in all treatment groups (Figure 19D, E and F). The variation between treatments was not only non-significant but also showed less variation and a narrower range of standard deviations. This could imply that the total protein content of Schneider-2 cells is not affected by pesticide treatments with any of the used zymosan concentrations. An internal regulatory mechanism could also be present.

The protein concentration in MB-L2 cells without zymosan exposure (Figure 19G) was the lowest when treated with 10 and 50 $\mu\text{g/ml}$ imidacloprid at 6.62 $\mu\text{g/ml}$ and 6.67 $\mu\text{g/ml}$ protein respectively. A significant difference was observed between 10 $\mu\text{g/ml}$ imidacloprid and 50 $\mu\text{g/ml}$ amitraz, which had the highest protein concentration in the group at 7.22

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$\mu\text{g/ml}$. This indicates that imidacloprid and amitraz act inversely on protein production, imidacloprid decreasing protein content while amitraz increasing it. The effect appear to be cancelled out when MB-L2 cells are exposed to mixtures of imidacloprid and amitraz.

When MB-L2 cells were exposed to 1 $\mu\text{g/ml}$ zymosan (Figure 19H), there was no significant difference among any of the pesticide treatments and the control.

However, when exposed to 10 $\mu\text{g/ml}$ zymosan (Figure 19I), the control without pesticide exposure had the highest protein concentration of 8.04 $\mu\text{g/ml}$ which was significantly different from pesticide mixtures 10I-10A, 10I-50A and 50I-10A in the same group. From these results we can infer that the effect of immune stimulation is altered by co-exposure to imidacloprid and amitraz. To add, the control of the 10 $\mu\text{g/ml}$ zymosan group has a significantly higher concentration than that of the 10 $\mu\text{g/ml}$ imidacloprid treatment from the group without zymosan exposure (Figure 19G), and from the control, 50I, 10A and 10I-50A mixture in the 1 $\mu\text{g/ml}$ treatment group (Figure 19H) (data not shown). This indicates that zymosan increases the total protein production but its effect is limited when exposed to multiple pesticides.

The fact that significance only appeared with MB-L2 cells suggests that pesticides variably affect cells of the different insect species. In addition, the effect of imidacloprid and amitraz was different with different concentrations of the immune stimulator, zymosan A. This suggests that the effect of pesticides is context specified and slight changes in the exposure condition may demonstrate different end-results.

Results and Discussion

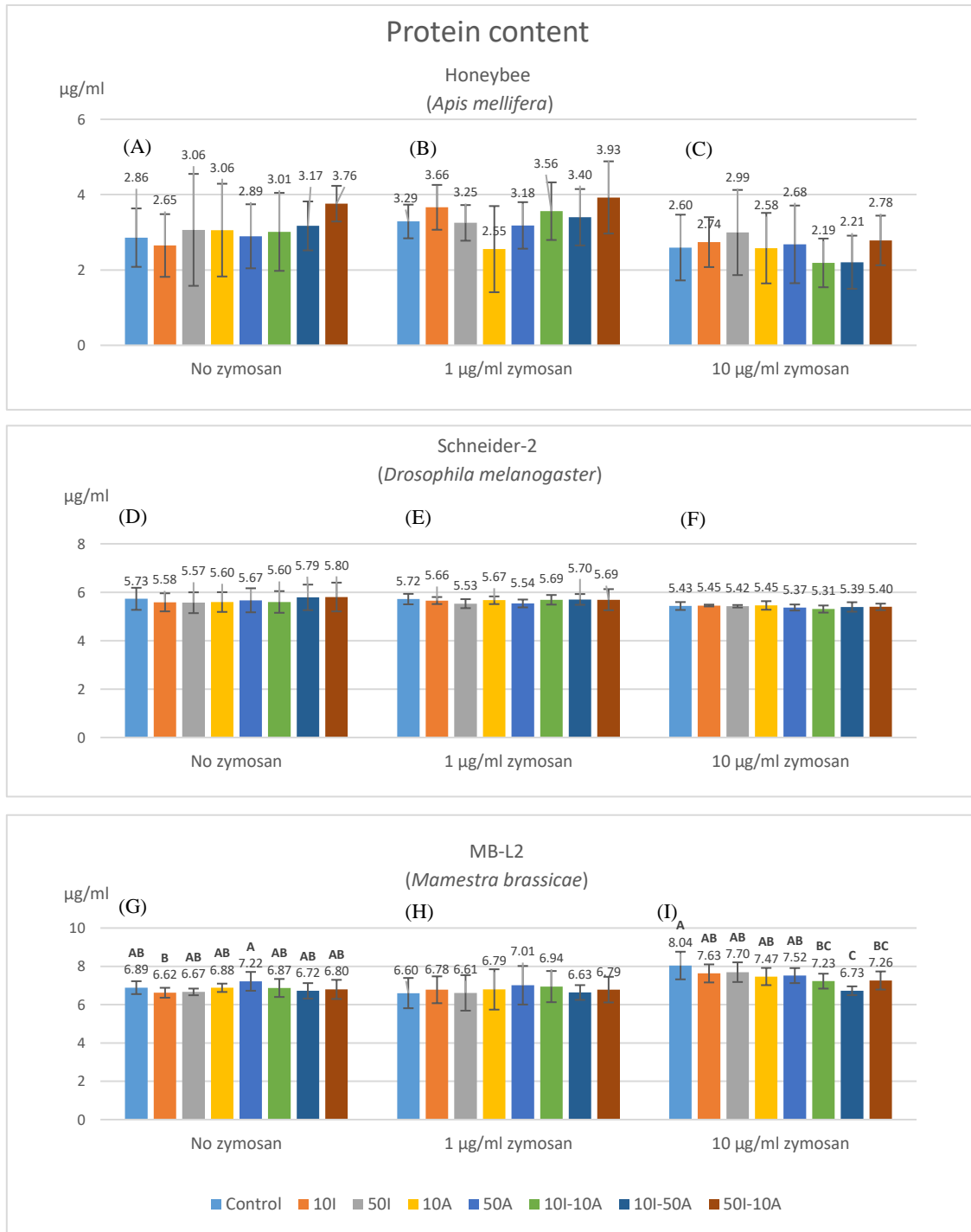


Figure 19. Soluble protein content in hemocytes of honeybees (*A. mellifera*; A, B, and C), Schneider-2 cell line (*D. melanogaster*; D, E, and F) and MB-L2 cell line (*M. brassicae*; G, H, and I) after pesticide treatments in groups of different zymosan concentrations. Significant difference displayed by different letters within zymosan groups at 95% confidence interval ($n=6$ for honeybees and Schneider-2 and $n=9$ for MB-L2 cells).

4.1.2. Immune activation by zymosan A alleviates the effect of pesticides on hemocyte viability

There was no observable decrease in viability of Schneider-2 and MB-L2 cells via the trypan blue exclusion method (data not shown). In fact, the viability was 100% in all condition in spite of any pesticide or zymosan A exposure. While for *A. mellifera* hemocytes, the results are shown in (Figure 20). The viability for control groups for no zymosan exposure, 1 µg/ml and 10 µg/ml zymosan 97.55%, 97.60% and 97.68% (the statistical significance between groups is not shown). Without zymosan exposure (Figure 20), 10 µg/ml of imidacloprid or the pesticide mixtures of either higher imidacloprid or higher amitraz combinations resulted in significantly higher viability of 99.25% compared to the control. Except for the control treatment, the exposure to amitraz without zymosan resulted in a significant decrease in viability to 95.91% compared to all pesticide exposures. All conditions that included zymosan A at 1 and 10 µg/ml showed no significant difference in viability by trypan blue method.

Increased production of enzymes that hydrolyze amitraz to the more metabolite DMFP might explain the lower viability in honeybee hemocytes since the metabolite is more potent (Yu, 2014). The Oct β 2R octopamine receptor is the main target of amitraz and is less sensitive to amitraz in honeybees than in target species but the amitraz metabolite DMFP can still bind to the octopamine receptor (L. Guo et al., 2021). We observed an antagonistic relationship between imidacloprid and amitraz on viability on the mixture treatments. It could be that imidacloprid affected the activity responsible for amitraz hydrolysis lowering the production of the more toxic DMFP metabolite or limiting the production of those enzymes when hemocytes are co-exposed to both amitraz and imidacloprid.

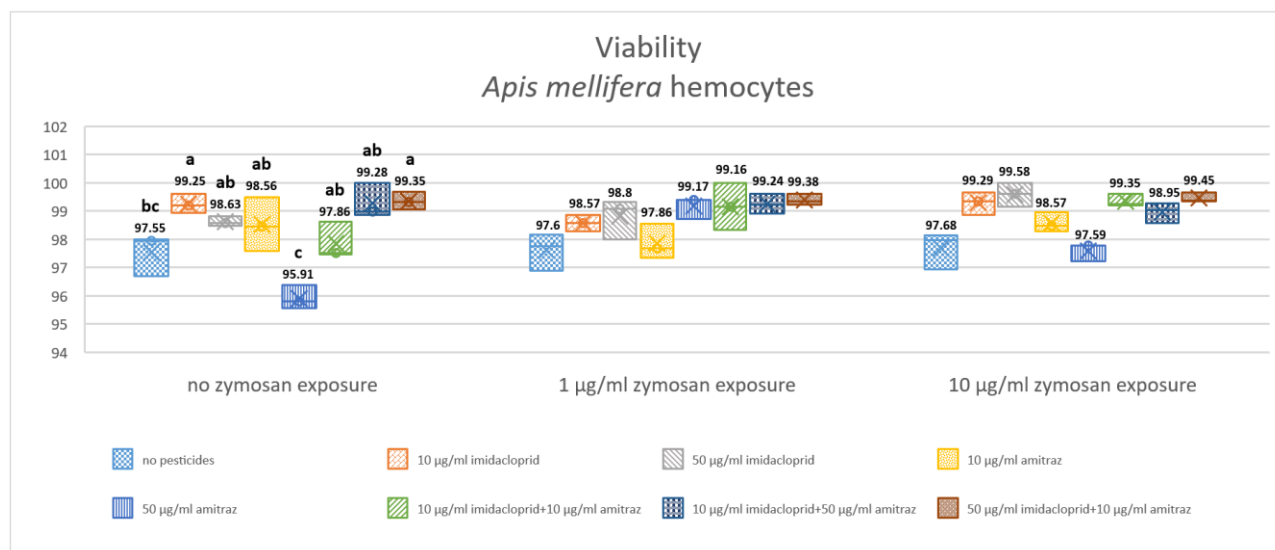


Figure 20. Effect of pesticide exposure on viability of honeybee hemocytes after 18 hrs of exposure by trypan blue method. Three groups of immune activation by zymosan A were exposed to different pesticides in single exposures or combinations. Different letters indicate significant differences within zymosan groups ($p < 0.05$, $n = 3$).

Schneider-2 and MB-L2 cells did not present a similar response as honeybee hemocytes which could indicate that the pesticides and their combinations present a different impact on pesticide metabolism. The production of cytochrome P450 detoxifying enzymes rather than their diversity was the driving factor behind imidacloprid toxicity (Casida, 2018). The toxicity of amitraz is also affected by its metabolism by cytochrome P450 (Moyano et al., 2019). Thus that the effect of simultaneous exposure of imidacloprid and amitraz may be intrinsically different from the single exposures in terms of effect on viability.

4.1.3. Nitric oxide endpoint production

Nitric oxide was measured at 15 min (Figure 21) and 120 min (Figure 22) by relative fluorescence intensity. Hemocytes of honeybee larvae (*A. mellifera*) produced significantly less nitric oxide reaching 73.55, 73.13, and 74.17% after 15 min incubation in the group without zymosan (Figure 21A) when exposed to 10 and 50 µg/ml amitraz or the 50I-10A mixture respectively. A significant higher decrease to 69.85% and 67.06% was observed when exposed to 50 µg/ml imidacloprid or the 10I-10A mixture. When honeybee

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hemocytes were immune-activated with 1 µg/ml zymosan A (Figure 21B), all pesticide treatments revealed a decrease in nitric oxide production after 15 min compared to the control of the group that was not exposed to pesticides. Only with 50 µg/ml imidacloprid, 10 and 50 µg/ml amitraz, and the 10I-10I mixture was significance apparent at 79.52, 81.49, 78.53, and 80.2% NO production respectively. Interestingly, exposure to 10 µg/ml zymosan A showed no significant difference with any of the treatments (Figure 21C). There was no significant effect on nitric oxide production in any of the zymosan A groups for the Schneider-2 (Figure 21D, E, and F). The MB-L2 cells, however, showed a significant decrease to 92.27, 89.88 and 89.54% in nitric oxide production at 15 minutes post-exposure in the 10I-10A, 10I-50A, and 50I-10A pesticide mixtures respectively with 10 µg/ml zymosan A (Figure 21I). No significant variation was detected without zymosan A exposure or with 1 µg/ml zymosan A (Figure 21G and H). It appears that the effect of imidacloprid and amitraz on NO production at 15 minutes is mitigated when exposed to zymosan A. The effect is completely masked in *A. mellifera* with 10ug/ml zymosan A

The effect of the exposures on the production of nitric oxide was more pronounced after 120 min post-exposure (Figure 22). Nitric oxide production in honeybee (*A. mellifera*) hemocytes decreased significantly in all the pesticide treatments compared to the control in the 0 zymosan A group (Figure 22A) with a 62.87% decrease when hemocytes were exposed to the 10I-50A mixture. Within the same group, the maximal decrease was at 46.99% with exposure to 50 µg/ml imidacloprid. This pattern is even more observable when honeybee cells were treated with 1 µg/ml zymosan A along with pesticides (Figure 22B). The least decrease was to 70.56% with 10 µg/ml imidacloprid reaching 56.03%, 55.94%, and 56.21% with 50 µg/ml imidacloprid, 50 µg/ml amitraz and 10I-10A mixtures respectively.

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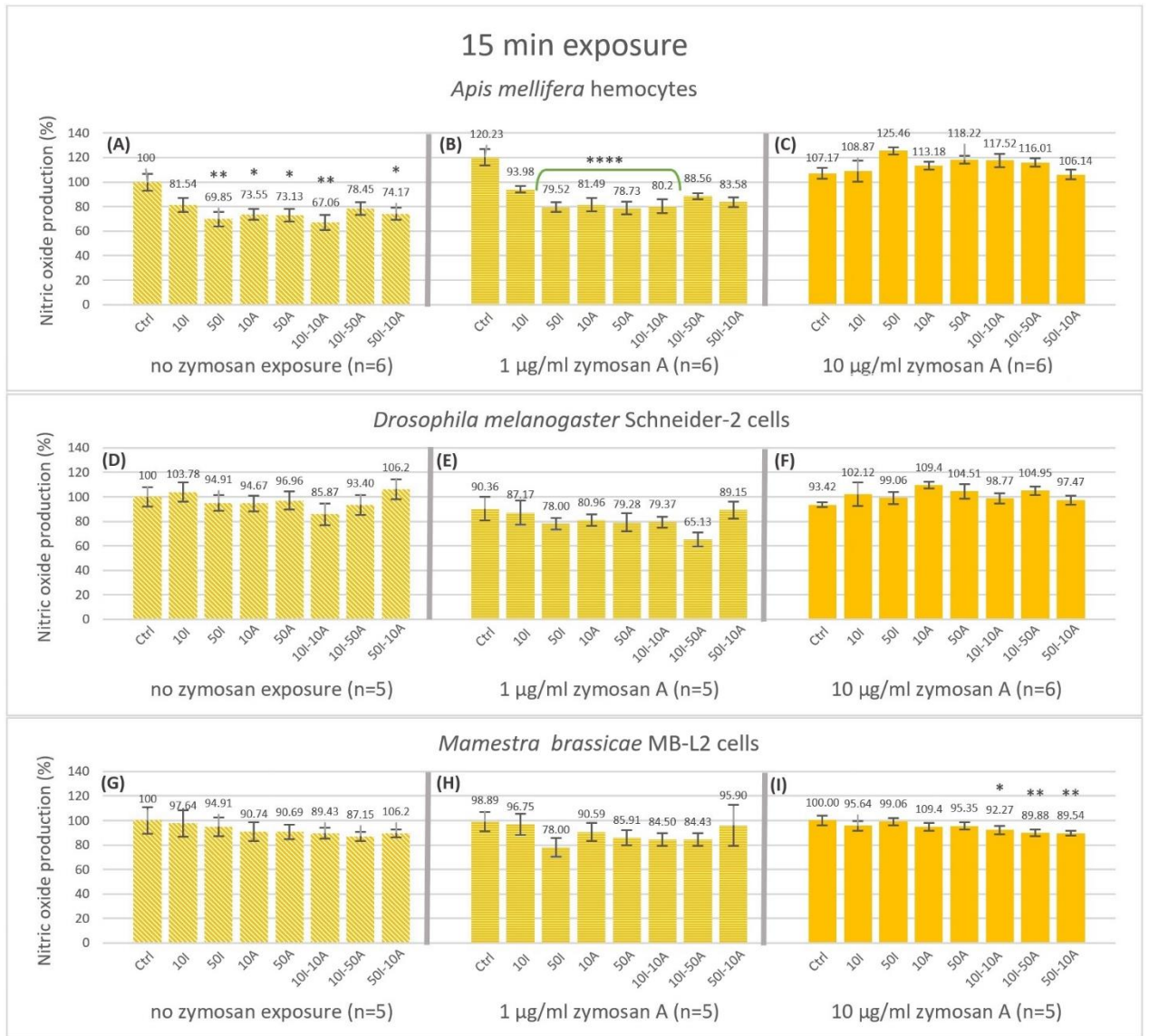


Figure 21. Nitric oxide production of hemocytes (*A. mellifera* larval hemocytes, Schneider-2 cell line, and MB-L2 cell line) after exposure to imidacloprid and amitraz. In addition to the control (Ctrl) without pesticide exposures, hemocytes were exposed to concentrations of 10 or 50 µg/ml of imidacloprid (10I, 50I respectively) or amitraz (10A, 50A respectively). Three pesticide mixtures of 10 µg/ml Imidacloprid+10 µg/ml amitraz (10I-10A), 10 µg/ml Imidacloprid+50 µg/ml amitraz (10I-50A), or 50 µg/ml Imidacloprid+10 µg/ml amitraz (50I-10A). All pesticide exposure conditions included immune activation with two concentrations of zymosan A (1 and 10 µg/ml) or none. Results were expressed as normalized percentages relative to the control group without pesticides or immune activation. Significant differences were tested by Dunnett's test within zymosan treatment

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groups relative to the respective controls and designated by an asterisk (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, $n=6$). Standard errors (SE) are represented by error bars.

However, in honeybee hemocytes of the 10 $\mu\text{g/ml}$ zymosan A group (Figure 22C), the highest significant decrease in nitric oxide production was observable with the pesticide mixtures followed by 10A, 50A, and 10I exposures while 50I exposure showed no significant change after 120 minutes of exposure. Inversely to honeybee hemocytes, Schneider-2 cells showed no significant change in the production of nitric oxide after 120 minutes in the group without zymosan exposure except for the 16.78% increase with the 50I-10A pesticide mixture (Figure 22D). When treated with 1 $\mu\text{g/ml}$ zymosan A, Schneider-2 cells produced significantly less nitric oxide with 10I-50A combination and more nitric oxide with the 50I-10A combination (Figure 22E). In the 10 $\mu\text{g/ml}$ zymosan A group, an increase of NO production in all pesticide treatments compared to the control was observed (Figure 22F). Significance was highest with amitraz single exposures ($p < 0.01$) while exposures that included imidacloprid in 10I, 50I, and 10I-50A showed an increase in NO with less significance ($p < 0.05$). The mixtures 10I-10A, and 50I-10A were not significantly different when compared to the control of Schneider-2 cells after 120 minutes of exposure.

There was significant decrease in the production of NO with MB-L2 cells reaching 32.13, 36.9, and 35.7% after 120 minutes without zymosan when exposed to pesticide combinations 10I-10A ($p < 0.05$), 10I-50A, and 50A-10I ($p < 0.01$) (Figure 22G). When immune stimulated by 1 $\mu\text{g/ml}$ zymosan A (Figure 22H), MB-L2 cells showed a similar pattern of response compared to the group without zymosan when exposed to imidacloprid and amitraz. In higher concentrations of amitraz and imidacloprid, NO production was lower and even lower in pesticide mixtures but only significant with the 10I-50A with a decrease to 65.65% at 120 minutes post-exposure. Exposure to 10 $\mu\text{g/ml}$ zymosan A did not result in any variation in NO production by MB-L2 cells at 120 minutes post-exposure (Figure 22I).

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Figure 22. Nitric oxide production 120 min post-exposure of non-activated and activated hemocytes (*Apis mellifera* larval hemocytes, Schneider-2 cell line, and MB-L2 cell line) to single or mixed exposure to imidacloprid and amitraz. Hemocytes of *A. mellifera* larvae (A, B, C), Schneider-2 cells (D, E, F), and MB-L2 cells (G, H, I) were exposed to 10 or 50 µg/ml concentrations of imidacloprid (10I, 50I respectively) or amitraz (10A, 50A respectively) in single exposures and in combinations of 10 µg/ml imidacloprid+10 µg/ml amitraz (10I-10A), 10 µg/ml imidacloprid+50 µg/ml amitraz (10I-50A), or 50 µg/ml imidacloprid+10 µg/ml amitraz (50I-10A). All pesticide exposure conditions included a

control with no pesticide treatment and immune activation with two concentrations of zymosan A (1 and 10 $\mu\text{g/ml}$) or none. Results were expressed as normalized percentages relative to the control group without pesticide or immune activation. Significant differences within zymosan treatment groups relative to the respective controls were designated by an asterisk (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$, $n = 5$ or 6). Error bars represent standard errors (SE).

4.1.4. Nitric oxide production rate

Honeybee hemocytes showed a significant decrease in NO production rate from 15 until 120 minutes in all treatments without immune activation relative to the control condition except 50I-10A (-14.03%) (Figure 23A). The decrease is by 21.43, 30.84, 25.72, 31.51, 24.15, 18.47 % for 10I, 50I, 10A, 50A, 10I-10A, and 10I-50A respectively. In the 1 $\mu\text{g/ml}$ zymosan A treatment group (Figure 23B), all treatments of imidacloprid and/or amitraz were highly significant ($p < 0.0001$) compared to the control ranging from 21.29% decrease with 50I-10A to 30.2% with 50 $\mu\text{g/ml}$ imidacloprid (50I). With 10 $\mu\text{g/ml}$ zymosan A (Figure 23C), a significant decrease resulted from amitraz exposures and pesticide mixtures 10I-10A and 10I-50A.

The longer exposure of imidacloprid and amitraz affected NO production even further than at 15 minutes. At 120 minutes, all pesticide treatments decreased NO production regardless of the concentration of immune-activator with the exception of 50 $\mu\text{g/ml}$ of imidacloprid with 10 $\mu\text{g/ml}$ of zymosan A with honeybee hemocytes (Figure 22C). Ultimately, the effect of imidacloprid and amitraz extended to the NO production rate between the set time points. The cut-down of NO production with exposure to imidacloprid and amitraz, honeybees would be more susceptible to infection not just at the instant of infection but post-infection as well, especially when considering the downstream signaling of NO and its involvement in the immune response (Bogdan, 2001; Wink et al., 2011). Exposure to 1 $\mu\text{g/ml}$ zymosan A resulted in a synergistic effect with imidacloprid and amitraz. However, When hemocytes are exposed to 10 $\mu\text{g/ml}$ zymosan A, the cut-down effect on the rate of NO production is concealed. However, the higher concentration of zymosan A might be

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equivalent to the immune stimulation of severe nosemosis but without the associated damage or it could be equivalent to the presence of gut bacteria in honeybees which play a role in resistance to *Nosema* infection (J. H. Li et al., 2019; Rubanov et al., 2019).

After 120 minutes of exposure, Schneider-2 cells showed an increase in NO production with the 50I-10A combination with 0 or 1 µg/ml zymosan A and a decrease with the 10I-50A combination at 1 µg/ml zymosan A immune activation. Imidacloprid and amitraz showed no effect on NO production in Schneider-2 cells at 15 min exposure times with no change in the production rate of NO despite any concentration of zymosan in all treatments. At 10 µg/ml zymosan A, all single pesticide exposures and 10I-50A showed an increase in NO production. The production rate of NO is did not change significantly in all conditions. This indicates that *Drosophila* responds to stressors in a complex manner at given time points and that Schneider-2 cells are overall less affected by pesticides regarding NO alternations with a strong internal regulation mechanism compared to honeybees.

As for the MB-L2 cells, the effect of imidacloprid and amitraz was restricted to mixture exposures but not single exposures. It seems that the effect of pesticides on the primary immune response is time-dependent taking into consideration the level of immune activation. With no zymosan A, the decrease in NO appeared after 120 min and in the production rate between the set time points but not at the first time point of measurement. Similarly, exposure to 1 µg/ml zymosan A decreased NO production but only in the 10I-50A combination at 120 minutes. The production rate was not significantly changed with immune activation. This could posit that *M. brassicae* becomes more resistant to the used pesticides' effects on oxidative response when immunologically challenged and it takes multiple stressors/stimulants to have an observable effect at the same level of immune activation.

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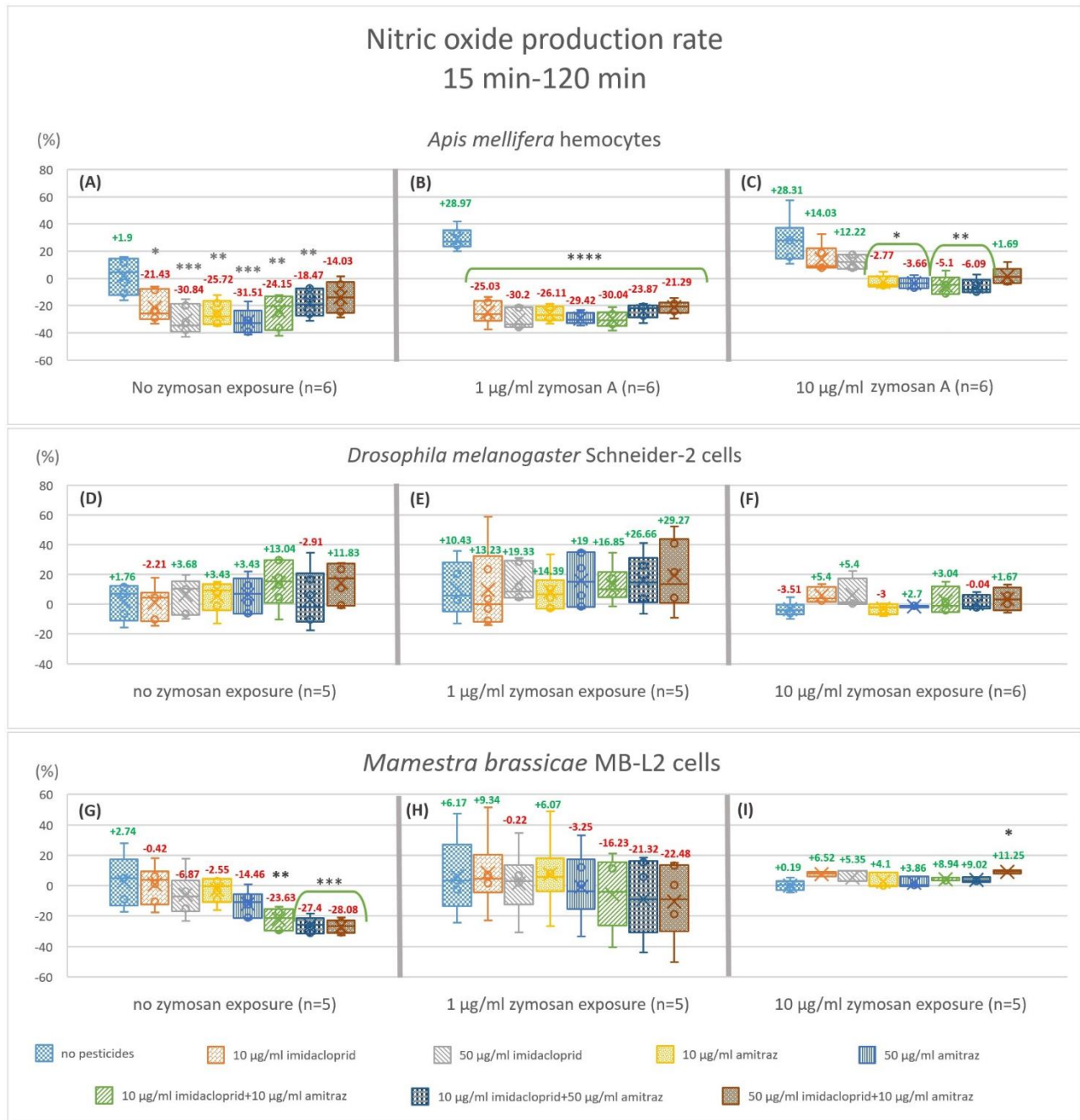


Figure 23. Nitric oxide production fold changes from 15 min to 120 min exposure. The box plots represent the ratio of relative nitric oxide percentage of endpoint fluorescence measurements at 120 min over 15 min in hemocytes of *Apis mellifera* (A, B, C), Schneider-2 cells (D, E, F), and MB-L2 cells (G, H, I). All results were normalized to the no treatment control (A). Statistical analysis was carried out within zymosan A treatment groups.

Significant differences within zymosan treatment groups relative to the respective controls were designated by an asterisk (* $p < 0.05$ and ** $p < 0.01$, *** $p < 0.001$ **** $p < 0.0001$, $n = 5$ or 6)

Schneider-2 cells showed no change in NO production rate in all conditions regardless of zymosan concentration or pesticide treatment (Figure 23D, E, and F). MB-L2 cells showed a significant decrease in NO production rate without immune activation (Figure 23G) when exposed to pesticide mixtures 10I-10A, 10I-50A, and 50I-10A. No significant change in NO production rate was visible when MB-L2 cells were challenged with 1 or 10 $\mu\text{g/ml}$ zymosan A (Figure 23H and I) except for the 50I-10A mixture with 10 $\mu\text{g/ml}$ zymosan A (Figure 23I) that showed an 11.25% increase.

4.1.5. Extracellular hydrogen peroxide production

The production of hydrogen peroxide in *A. mellifera* hemocytes significantly decreased compared to the control with 10I-50A (41.87%) and 50I-10A (49.73%) when no zymosan A was applied (Figure 24A). When exposed to 1 $\mu\text{g/ml}$ zymosan A (Figure 24B), all treatments showed a significant decrease compared to the control with the exception of 10A pesticide treatment. As for 10 $\mu\text{g/ml}$ zymosan A group (Figure 24C), there was no statistically significant change in hydrogen peroxide production after 3 hours. However, concentration-dependent change was visible graphically.

A significant decrease to 55.58% in hydrogen peroxide production by Schneider-2 cells was observed in 50I-10A exposure with 1 $\mu\text{g/ml}$ zymosan A (Figure 24E), and in all pesticide mixtures 10I-10A (62.14%), 10I-50A (66.15%) and 50I-10A (63.47%) but not in single exposures when exposed to 10 $\mu\text{g/ml}$ zymosan A (Figure 24F) compared to the control within the group. Contrary to *A. mellifera* and Schneider-2 cells, the results from MB-L2 cells showed a significant increase in hydrogen peroxide production with no immune stimulation (Figure 24G). The higher concentration of amitraz (50A), mixtures 10I-50A and 50I-10A have significantly higher H_2O_2 production by 27.7, 38.64 and 27.7%

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respectively. No significant change of H₂O₂ production by MB-L2 cells with immune activation at either 1 and 10 µg/ml zymosan A (Figure 24H and I).

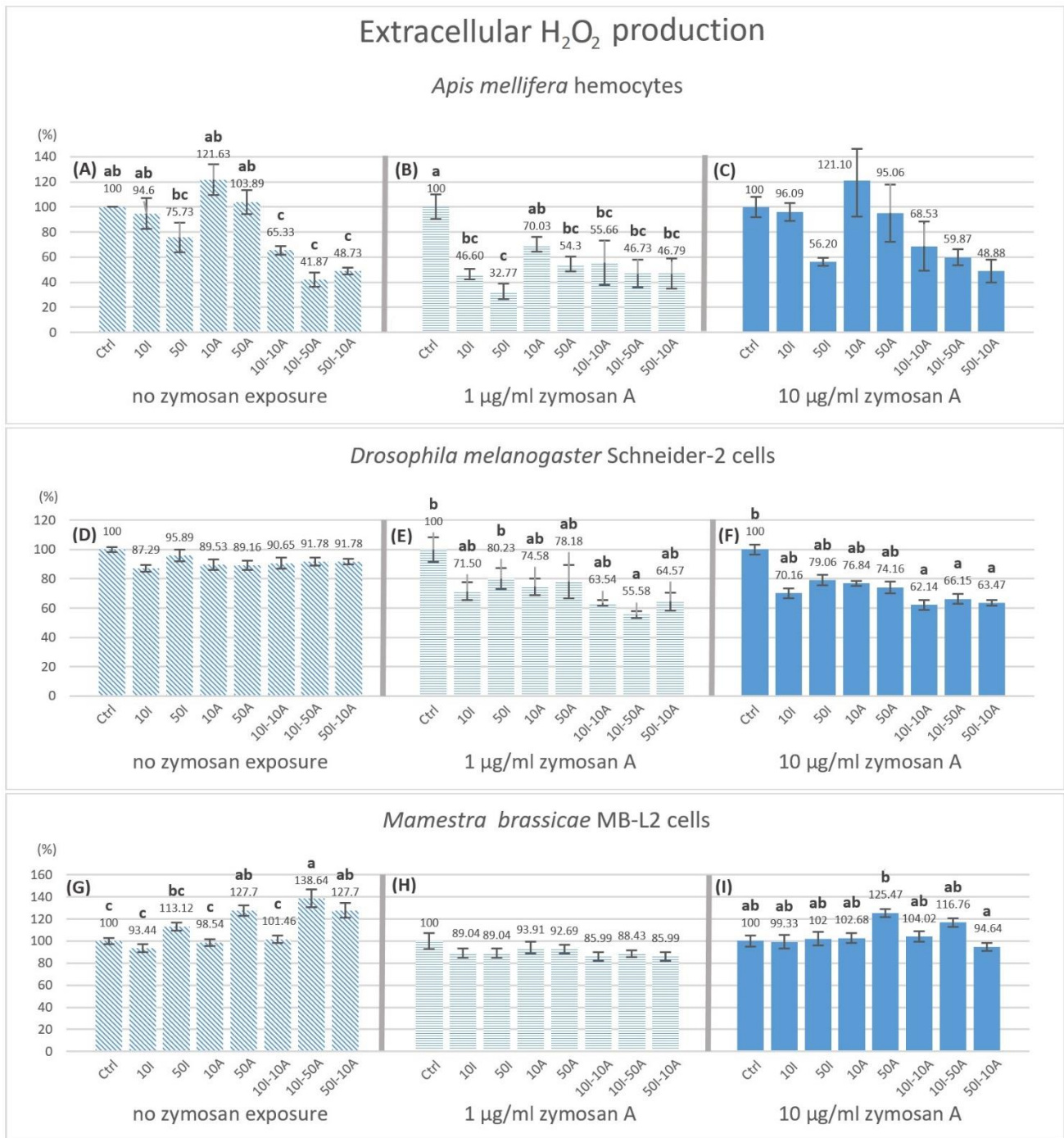


Figure 24. Hydrogen peroxide production by hemocytes after 3 hours of pesticide exposure with different levels of immune stimulation. Bar graphs represent the relative hydrogen

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peroxide concentration produced hemocytes of *Apis mellifera* (A, B, C), Schneider-2 cells (D, E, F), and MB-L2 cells (G, H, I) after 3 hours of exposure to pesticides. All concentrations are expressed in $\mu\text{g/ml}$ of imidacloprid (I) or amitraz (A) in legends. Statistical analysis was done with in zymosan A treatment groups. ANOVA (Tukey HSD) or Kurskal-Wallis were used to test for significant differences at $p < 0.05$ with $n = 5$. Different letters signify significant differences. The absence of indication refers to no significant differences. Error bars represent standard errors.

NO functions independently from the ROS system yet it plays a role in limiting the reactivity of hydrogen peroxide and oxygen radicals to specific cellular sites (Wink et al., 2011). This is in consensus with our results concerning honeybee hemocytes. However, other studies suggest that H_2O_2 can modulate NOS activity via targeting NF- κB gene sites (Herrera-Ortiz et al., 2011) suggesting a relationship between ROS and RNS production. In our results, the relative hydrogen peroxide levels were the highest when hemocytes were treated with amitraz without immune activation. This may be due to the ability of amitraz and its metabolites to limit ROS elimination (Moyano et al., 2019). Lower concentrations of amitraz resulted in a higher level of H_2O_2 when compared to higher amitraz concentrations. If H_2O_2 was observed alone, the expected effect would be that lower amitraz concentrations produce more ROS-mediated cellular damage in honeybees. However, when considering the production of NO at different time points, it is noticeable that a higher amitraz concentration results in significantly lower NO levels. This may infer that the production of NO may be more crucial in determining the susceptibility of hemocytes to ROS-mediated cellular damage than increased levels of H_2O_2 in honeybees (*A. mellifera*) when not immunologically challenged. In addition, when amitraz was present with imidacloprid, immune activation with zymosan A was not required to observe a change in extracellular hydrogen peroxide production in honeybee cells but it was required for Schneider-2 cells. The lowest concentrations of H_2O_2 were detected with the pesticide combinations in immune-activated honeybee hemocytes and Schneider-2 cells. The MB-L2 cell line was affected only with no immune activation. In contrast to honeybee and Schneider-2, H_2O_2 production increased in MB-L2 cells when exposed to 50 $\mu\text{g/ml}$ amitraz and combinations 10I-50A and 50I-10A but no effect was observed with immune activation.

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Cell line cultures may have different cell type constitution than freshly extracted hemocytes and this is important when addressing the results because there is variability of NO production between different cell types within a population as seen in honeybees where granulocytes showed higher production of NO than other hemocytes granulocytes (Negri, Maggi, Correa-Aragunde, Brasesco, Egvaras, Lamattina, et al., 2013).

Nitric oxide production is an ancient trait originating before the divergence of vertebrates and invertebrates (Cristino et al., 2008), thus it is possible that the utilization of such a molecule is variable and diverse between different species with its multiple roles in immune responses, neural responses, development, and oxidative stress modulation. This variability of responses between different orders may obligate the use of the exact organism toxicological studies. Honeybee sensitivity to risk factors contributing to colony collapse disorder is evidently dependent on the combination of specialized characteristics. Characteristics include genetic diversity of immune genes and detoxifying enzymes, social immunity, the effect of behavioral alteration and the temperature-dependent effect of pesticides on survivability are all considered when studying insects. In this study, we shed lights on the highly variable oxidative response of hemocytes between Hymenoptera, Diptera and Lepidoptera candidates.

The oxidative immune response of the European honeybee (*A. mellifera*) is more altered by amitraz and imidacloprid exposure than *D. melanogaster* and *M. brassicae*. Considering that the overall significant effect was apparent with the combinations that included both pesticides, amitraz may indeed prove to decrease the fitness of honeybee colonies in response to diseases. The synergistic effect of amitraz with pesticides was shown before and the order of their exposure was important in elucidating this effect (Johnson et al., 2013). Thus, the ability of honeybees to resist diseases such as *Nosema spp*s and pests such as the wax moth may be hindered by pesticide exposure even if the used concentrations are sub-lethal.

Indeed, our findings suggest a differential effect of risk factor exposure on the immune response in different species representing different orders. In addition, it is recommended to re-evaluate the risks of pesticide exposure to honeybees on all fronts in a comparable manner with the specific conditions. Cox-foster et.al (Cox-Foster et al., 2007) already presented a meta-genomic survey to assess the degree of contribution of different risk

factors to colony collapse disorder. However, temperature changes (Butolo et al., 2021), the order of exposure to risk factors, and its duration are also required to have a comprehensive view. Since CYP450 production affects imidacloprid toxicity world (Casida, 2018; Macaulay et al., 2021). and metabolism affects amitraz toxicity (Moyano et al., 2019), it is implied that the effect of imidacloprid and amitraz co-exposure may be different from the effect of single exposures as found in this study. The presented differential effect of pesticides between different insects, and extrapolation of experimentation on “model” organisms may be rendered inadequate depending on the basis of the design and the factors implicated, especially when compared to the honeybee system. The *in-vitro* system is advantageous where effects and mechanisms concerning pesticide exposure and immune response can be studied in parts that may illustrate the effects observed at the whole organism level. Another advantage is that cell lines are regularly maintained and available when needed but when it comes to honeybees, limitations must be considered. Though freshly collected hemocytes pose a more realistic approach for *in-vitro* assays, extracting hemocytes from honeybee larvae is limited to the seasonal availability of larvae, the physical effort that comes with hemocyte extraction also that extracting hemolymph from larvae has a risk of contamination with each larva and it is time-consuming. Not to mention that adult honeybees do not make a preferred alternative to larvae as only a small amount of hemolymph can be extracted from adults compared to larvae (Borsuk et al., 2017; Walderdorff et al., 2018) limiting experimental implementation.

Considering all the previously mentioned points, factors such as co-exposure to different biotic and abiotic factors require extensive studies on honeybees at immunologic, neural, behavioral, and developmental levels. Thus, cell based-system methodologies must be continued by *in-vivo* application and field applications. The social construct must also be taken to account when deriving the effects of pesticides on the colony level with the inclusion of honeybee drones whose haploid genome may be more sensitive to these risk factors leading to a weak colony (Bruckner et al., 2021).

The obtained results present an observable effect of imidacloprid and amitraz on immune activation of hemocytes of Hymenoptera, Diptera and Lepidoptera representatives. A complex interaction between these pesticides was also observed depending on the ratio of

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exposure and the duration that poses a synergistic or antagonistic effect depending on the parameter studied which requires an in-depth understanding of the mode of action in each species. In addition, pesticide exposure indeed altered the immune response and immune activation in insect hemocytes, a point to be taken in earnest when considering the impact of pesticide usage on insects and ultimately biodiversity. In spite of any comparative approaches, the effect of pesticides on honeybee immunity is prominent and requires further evaluation of pesticide usage and its implication in causing CCD, to preserve the economic and environmental benefits of honeybees.

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4.2. Immune stimulation is associated with a correlation between phagocytosis and cell survivability after pesticide exposure

4.2.1. Phagocytosis affected by pesticides when exposed to zymosan A

When not exposed to zymosan A, hemocytes showed no significant difference in phagocytic activity with any of the pesticide treatments (Figure 25A). The lowest percentage was observed in the control at 7.77% while the highest percentage was observed when exposed to 50 $\mu\text{g}/\text{ml}$ imidacloprid at 12.69%.

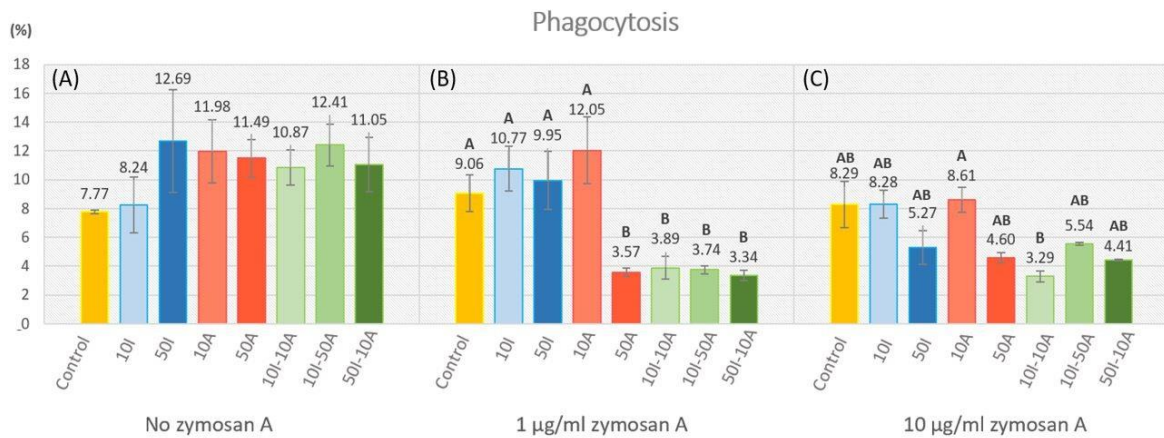


Figure 25. Honeybee hemocytes phagocytosis rate after pesticide exposure. Three groups of immune stimulant concentrations were treated with different pesticide conditions. Groups were categorized into a group without zymosan exposure (A), exposure to 1 $\mu\text{g}/\text{ml}$ zymosan (B) and 10 $\mu\text{g}/\text{ml}$ zymosan (C), Each group included a control, 10 $\mu\text{g}/\text{ml}$ imidacloprid (10I), 50 $\mu\text{g}/\text{ml}$ imidacloprid (50I), 10 $\mu\text{g}/\text{ml}$ amitraz (10A), 50 $\mu\text{g}/\text{ml}$ amitraz (50A) and double exposure combinations designated as 10I-10A, 10I-50A, and 50I-10A. Error bars represent standard deviation and different letters designate significant differences within a group ($p < 0.05$, $n = 3$).

When exposed to 1 $\mu\text{g}/\text{ml}$ zymosan A (Figure 25B), neither single exposures of imidacloprid nor 10 $\mu\text{g}/\text{ml}$ of amitraz induced a significant change in phagocytosis. In contrary, 50 $\mu\text{g}/\text{ml}$ amitraz and pesticide mixtures 10I-10A, 10I-50A and 50I-10A significantly lowered phagocytosis to at least 3.89% with 10I-50A. Phagocytosis showed no significant decrease with 10 $\mu\text{g}/\text{ml}$ imidacloprid or amitraz compared to the control in

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the 10 $\mu\text{g/ml}$ zymosan A group (Figure 25C). The 50 $\mu\text{g/ml}$ imidacloprid and amitraz single treatments showed lower phagocytosis compared to the group control and their respective 10 $\mu\text{g/ml}$ concentrations. Similarly, pesticide mixtures also showed a decrease in phagocytosis compared to the control and the single pesticide exposures of 10 $\mu\text{g/ml}$. Significant variation was observed between 10A and 10I-10A treatments having the highest and lowest phagocytosis of 8.61% and 3.29% respectively.

4.2.2. Immune stimulation by zymosan A results in higher viability with pesticide treatments when detected by propidium iodide.

There was no significant difference in viability between pesticide treatments in the group without zymosan A exposure (Figure 26A). However, with exposure to 1 $\mu\text{g/ml}$ zymosan A (Figure 26B), viability was the lowest with 10 $\mu\text{g/ml}$ amitraz at 86.38% with a significant difference compared to all treatments except the treatment with 10 $\mu\text{g/ml}$ imidacloprid. Compared to the control within the 1 $\mu\text{g/ml}$ zymosan A group, 50 $\mu\text{g/ml}$ amitraz and all pesticide mixtures were significantly higher. This pattern was inversely apparent with phagocytosis measurement with the same zymosan concentration (Figure 25B).

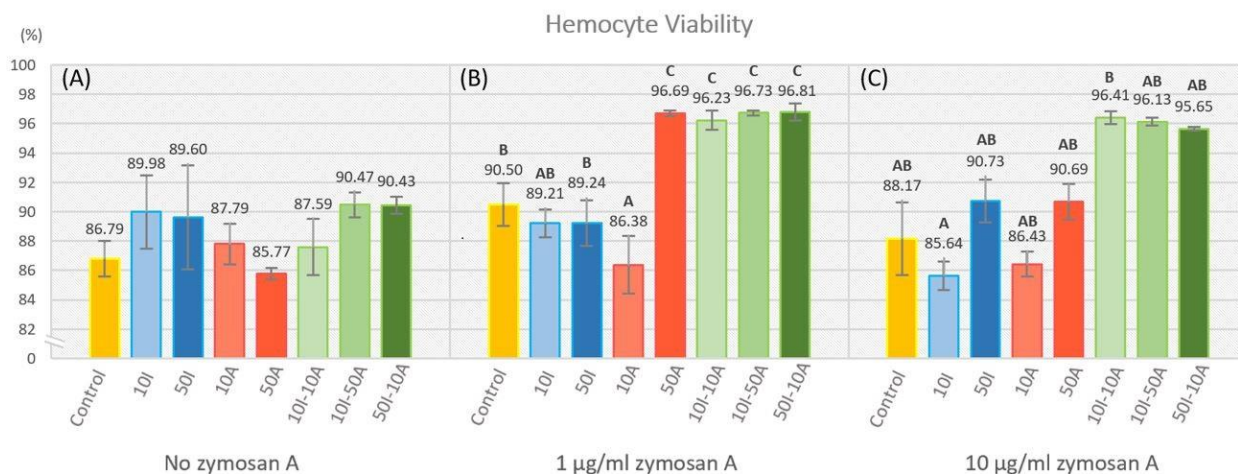


Figure 26. Viability in honeybee hemocytes after pesticide treatments. Three groups of different zymosan exposures are represented in the figure as a group without zymosan A (A) or either exposed to 1 (B) or 10 $\mu\text{g/ml}$ zymosan A (C). Each group included a control, 10 $\mu\text{g/ml}$ imidacloprid (10I), 50 $\mu\text{g/ml}$ imidacloprid (50I), 10 $\mu\text{g/ml}$ amitraz (10A), 50 $\mu\text{g/ml}$ amitraz (50A) and double exposure combinations designated as 10I-10A, 10I-50A,

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and 50I-10A. Error bars represent standard deviation and different letters designate significant differences within a group ($p < 0.05$, $n = 3$).

When exposed to 10 $\mu\text{g/ml}$ zymosan A (Figure 26C), the 10 $\mu\text{g/ml}$ imidacloprid and amitraz treatments had the lowest viability of 85.64% and 86.43% respectively except when compared to the control of the group. However, only the 10I treatment was significantly from the 10I-10A co-exposure which had the highest viability of 96.41% followed by non-significant 96.13% and 95.65% viability with 10i-50A and 50I-10A respectively (Figure 26C). The higher concentration of imidacloprid and amitraz single exposures (50I and 50A respectively) were not significantly different from the control but higher than 10I and 10A treatments.

4.2.3. Correlation between pesticide application, phagocytosis and cell viability

We analyzed the correlation between treatments, phagocytosis, and viability for all conditions (Figure 27). Axis 1 explains 88.4% of the variation. This axis separates the hemocyte of honeybees of two groups (Figure 27A). The first one consists of 50A-1Z treatment and of the hemocyte treated with both imidacloprid and amitraz (10 I -10 A, 10 I -50 A and 50 I -10 A) and exposed to Zymosan A (1 or 10 $\mu\text{g/ml}$). This group was positively correlated to cell viability and negatively correlated to phagocytosis. However, the following treatments form group 2: 1Z, 10 I-1Z, 10I-50A, 50I, 50I-1Z, 50I-10A, 10A and 10A-1Z had high phagocytosis and low cell viability. The other pesticide treatments do not have a strong correlation with any of the parameters studied. In Figure 27B, we observed a significant negative correlation between phagocytosis and viability at $r = -0.796$ ($p < 0.0001$).

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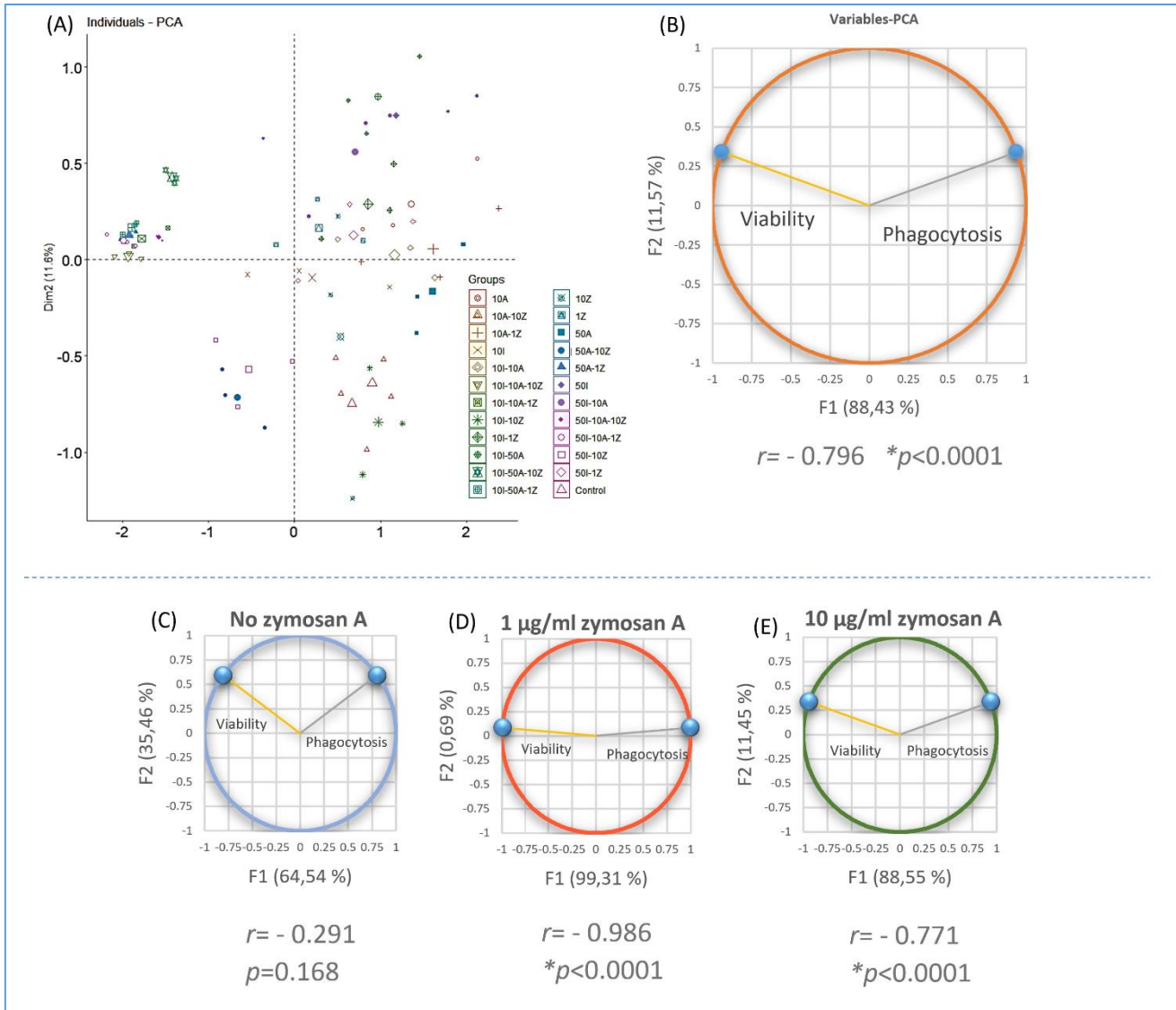


Figure 27. Principal component analysis (PCA) of phagocytosis and cell viability in honeybee hemocytes parameter after exposure to different concentrations of imidacloprid, amitraz, and zymosan A. (A) The individual treatments ($n=3$) factor map was tested for correlation with phagocytosis and viability while (B) represents the correlation (r) between variables using Pearson (n) test with 95% confidence. Different treatments are abbreviated as the following: I=imidacloprid, A=amitraz, and Z= zymosan A. All treatments are in $\mu\text{g/ml}$. Groups were also categorized into no zymosan exposure (C), exposure to 1 $\mu\text{g/ml}$ zymosan (D), and exposure to 10 $\mu\text{g/ml}$ zymosan (E).

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Interestingly, when zymosan groups were analyzed separately, the group with no zymosan treatment (Figure 27C) showed no significant correlation at $r = -0.291$ ($p = 0.168$). A significant negative correlation was apparent with 1 $\mu\text{g/ml}$ (Figure 27D) and 10 $\mu\text{g/ml}$ (Figure 27E) zymosan A groups at $r = -0.986$ ($p < 0.0001$) and $r = -0.771$ ($p < 0.0001$) respectively.

There was no effect of imidacloprid or amitraz on the rate of phagocytosis when hemocytes were without exposure to the immune stimulator, zymosan A. However, the effect was apparent with 50 $\mu\text{g/ml}$ amitraz and the pesticide combinations when exposed to 1 $\mu\text{g/ml}$ zymosan A. The higher concentration of zymosan A (10 $\mu\text{g/ml}$) illustrated a dose-dependent decrease of phagocytosis with pesticide exposures. A clear synergistic effect between imidacloprid and amitraz is evident on the decrease of phagocytosis after immune stimulation. All these observations highlighted the presence of a complex interaction not just between imidacloprid and amitraz but also between the latter and zymosan A. The synergism of amitraz with other pesticides or pathogens was previously reported in other studies including increased bee mortality and alteration of cardiac function. (Johnson et al., 2013; O'Neal et al., 2017). Acaricides altered the expression of genes related to immunity, detoxification and development (Boncristiani et al., 2012). Imidacloprid was shown to increase the levels of *Nosema* in honeybee colonies (Pettis et al., 2012). Hence, with our results can infer that the activation of the immune system weakens immune responses like phagocytosis when exposed to pesticides.

It also could be that the immune stimulation by zymosan A redirects the response of the immune system as it itself may not affect phagocytosis (Harshbarger & Heimpel, 1968). The redirection may be towards a detoxification mechanism of pesticides. However, it is important to note that amitraz toxicity is mainly by its metabolite (Moyano et al., 2019) and honeybees have a low capacity to metabolize amitraz (O'Neal et al., 2017). This requires further investigation into the effect of immune stimulation on its metabolism. Previous studies that show a differential effect of pesticides on honeybees only when immunologically challenged or when pesticides are present simultaneously are in consensus with our findings (Garrido et al., 2016).

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Further in-depth studies of the underlying mechanisms are necessary to comprehend the impact of pesticides on honeybee immunity causal possibilities within the system. The metabolism and detoxification process of amitraz and its metabolites after immune stimulation and/or pesticide exposure are needed. As for imidacloprid, previous studies have shown its effect on the immune system. Some studies suggested that neonicotinoids act differently depending on the degree of expression of different nicotinic receptors at certain doses of exposure taking into consideration the developmental stage (Guez et al., 2001, 2003; Shi et al., 2019, 2020; Tesovnik et al., 2017).

As for cell viability, our study showed an inverse pattern to that of phagocytosis at the same conditions, more apparent when exposed to zymosan A. In fact, exposure to pesticides without immune stimulation did not result in any variation in viability, as is the case with phagocytosis. Contrastingly, exposure to zymosan A resulted in increased viability with imidacloprid and amitraz mixtures. In addition, a dose-dependent response was visible in the 10 µg/ml zymosan A exposure group since increasing concentrations and mixtures resulted in higher viability, suggesting the presence of a process that protects honeybee hemocytes from cellular damage. The increased viability could be related to decreased production of reactive oxygen species (ROS) limiting cellular damage resulting from oxidative stress as observed in honeybee and *Drosophila* hemocytes being more pronounced with co-exposures than single exposures of imidacloprid and amitraz (Chmiel et al., 2019; Walderdorff et al., 2018). The cytoprotective outcome of reduced ROS was previously observed in starved skin fibroblasts of human in addition to an anti-apoptotic effect (Kumar et al., 2020).

An inverse pattern between phagocytosis and hemocyte viability was graphically clear. We observed a significant negative correlation between phagocytosis and viability. Interestingly, the correlation between phagocytosis and hemocyte viability was only significant when hemocytes were exposed to zymosan A. This indicates that immune stimulation by zymosan A may result in a protective response in hemocytes at the cost of phagocytic capacity referring to a trade-off between phagocytosis “offense” and the protective state “defense” from the effect of pesticides. Even with a small population, data collected from flow cytometry contains readings from thousands of individual cells that

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offer robustness to the approach. However, further expansion in assessment and exploration of the trade-off in different conditions is still needed in addition to understating the underlying mechanism that lead to this observation.

The primary interpretation would imply that honeybee hemocytes are protected from oxidative stress-mediated cellular damage, however, other studies have revealed a synergistic effect when honeybee hemocytes were exposed to pathogens and pesticides (Grassl et al., 2018; Parekh et al., 2021; Pettis et al., 2012; J. Y. Wu et al., 2012) suggesting that cellular immune responses like phagocytosis are more crucial than the reduction of oxidative stress in diseases resistance. Particular cases suggest that higher pesticide, concentrations may render the host unsuitable for infection thus reducing infection (Parekh et al., 2021) but it should be considered that infection is dependent on the mechanism and requirements of each causal agent.

To better understand observations and effects of pesticides on the immune system of honeybees, comprehensive studies are needed to complete the view on a more intrinsic scale. The latter approach not only could demonstrate the exact implications of pesticides in colony collapse but also give insight into the inner workings of the innate immune system of social insects allowing comparability with other insect species, invertebrates in general, or with vertebrates in response to stress factors.

4.3. Context specific action of pesticides on immune gene expression in honeybees.

4.3.1. Imidacloprid and amitraz affect the toll pathway on different levels of the immune pathway.

The gene expression of *spaetzle* is represented in Figure 28. All imidacloprid and amitraz exposures decreased the expression of *spaetzle* whether in single exposure or in co-exposures. This is also true for both groups of zymosan exposition (Zym 0 and Zym 1). Our results for the decrease of *spaetzle* expression is in consensus with laboratory application of imidacloprid on brown-eyed pupae (Tesovnik et al., 2019) confirming the negative impact of imidacloprid on pathogen recognition by *spaetzle*. In Zym 0, the 10I treatment was not significantly different from the control but 50I, 10A, 10I-10A and 10-50A showed a significant decrease compared to the control of the group with 10I-50A having the most decrease.

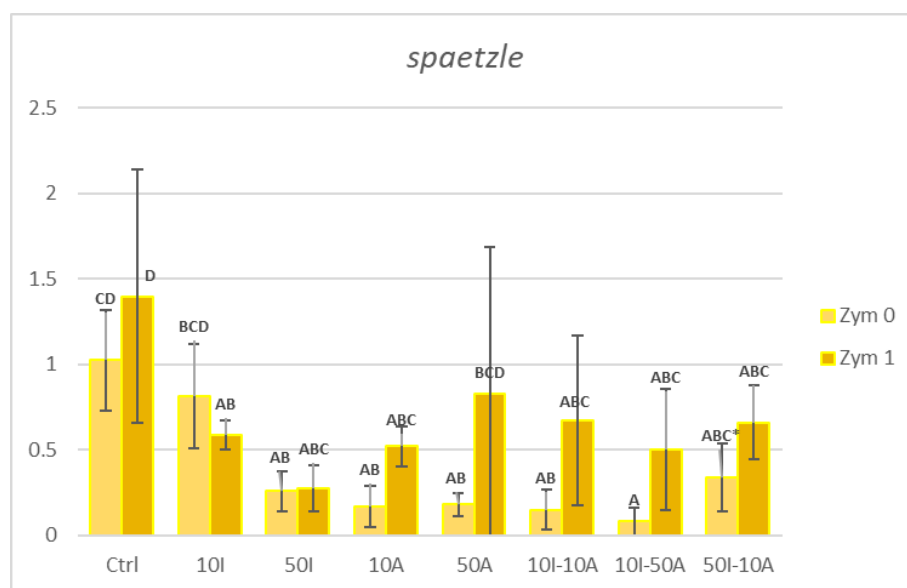


Figure 28. Gene expression analysis of *spaetzle* gene in honeybee hemocytes after pesticide treatment. Honeybee hemocytes exposed to imidacloprid (10I or 50I), amitraz (10A or 50A) or mixtures of both. All pesticide treatments were done without zymosan (Zym 0) or with 1 μ g/ml zymosan (Zym 1). All concentrations are in μ g/ml. Different letters indicate

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significant differences ($n=3$, confidence=95%) and the asterisk indicates significant differences from the control when only the zymosan group is considered. Standard errors (SE) are represented the error bars.

The 50I-10A mixture is only significantly different from the control with mean difference in considered only within the same zymosan group and not all the treatments. With 1 $\mu\text{g/ml}$ zymosan A, the same effect is observed with significant decrease in gene expression in all treatments compared to the control except for 10I.

When comparing each treatment with or without an immune activator, it is observable that zymosan A increases the expression of *spatzle* in all treatments except the single exposures with imidacloprid. Thus, zymosan induces the expression of *spatzle* and imidacloprid hinders this induction. In contrast, when amitraz is present with imidacloprid, the induction of gene expression by zymosan is not hindered by the presence of imidacloprid. We can infer that there is a competition between zymosan and imidacloprid on the signaling pathway that leads to the induction of *spatzle* expression. Hence, imidacloprid may neutralize the effect of zymosan but amitraz seems to be antagonistic to imidacloprid and allows the induction of *spatzle* by zymosan A even in the presence of imidacloprid in different imidacloprid-to-amitraz ratios.

Imidacloprid and amitraz significantly lowered the expression of Toll receptor gene in all treatments when not immunologically challenged by zymosan A (Figure 29). In fact, *toll* expression was decreased by at least 60% compared to the control when exposed to 10 $\mu\text{g/ml}$ imidacloprid. The significant decrease is observed in single exposure but the impact is even more significant in double pesticide exposures especially in mixtures that have 50 $\mu\text{g/ml}$ of either imidacloprid or amitraz.

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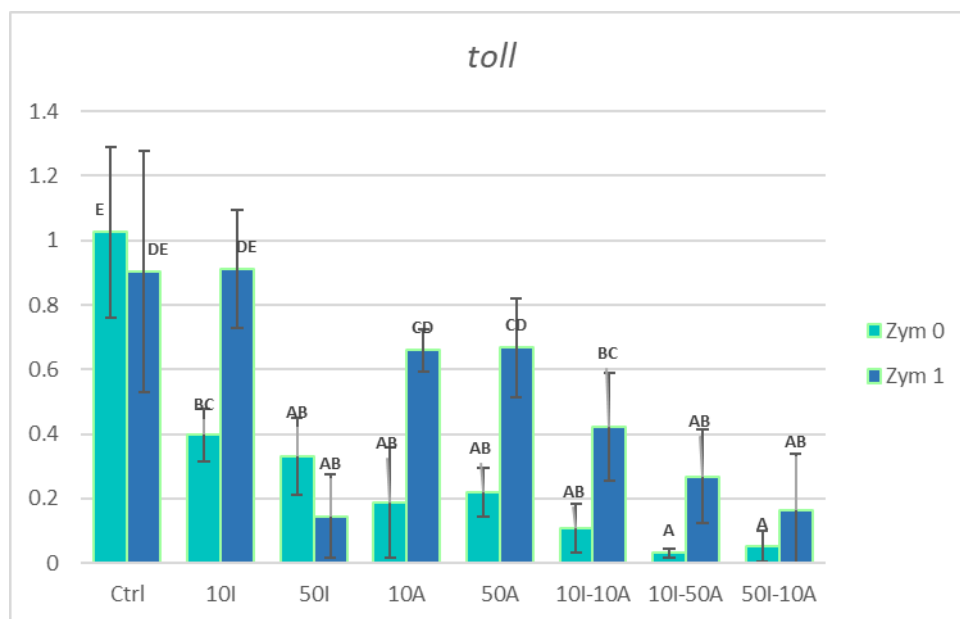


Figure 29. Gene expression analysis of *toll* gene in honeybee hemocytes after pesticide treatment. Honeybee hemocytes exposed to imidacloprid (10I or 50I), amitraz (10A or 50A) or mixtures of both. All pesticide treatments were done without zymosan (Zym 0) or with 1 $\mu\text{g/ml}$ zymosan (Zym 1). All concentrations are in $\mu\text{g/ml}$. Different letters indicate significant differences ($n=3$, confidence=95%). Error bars represent standard errors (SE).

In the presence of zymosan A, there was no effect of 10 $\mu\text{g/ml}$ imidacloprid or amitraz single exposures on *toll* expression compared to the zymosan control. The decrease was significantly present with the pesticide mixtures and 50 $\mu\text{g/ml}$ imidacloprid.

Thus, in the context of immune activation, the effect of imidacloprid on *toll* expression is dependent on the concentration and though amitraz does not seem to have an effect when hemocytes are immunologically challenged with zymosan, the combination of imidacloprid and amitraz appear to have synergism regarding the decrease of the *toll* expression. Comparing each treatment with zymosan absence (Zym 0) or presence (Zym 1), we can deduce that zymosan itself does not increase the expression of *toll* but attenuates the decrease of gene expression resulting from pesticide exposure. The results indicate that zymosan A has a regulatory role in maintaining the expression of Toll receptor and limiting the negative effects on its production. Zymosan may have an antagonistic effect to imidacloprid and zymosan at this level.

Comparing the two controls with and without zymosan, the myeloid differentiation factor 88 (MyD88) expression significantly increases when hemocytes are treated with zymosan (Figure 30). When not exposed to zymosan, myD88 expression remains the same with the imidacloprid and amitraz mixtures in addition to the 50µg/ml imidacloprid treatment. However, both amitraz exposures significantly increased the expression compare to the control and the 10 µg/ml imidacloprid exposure even increased the expression further and more significantly than the amitraz exposure. This could pose that the presence of an effect by imidacloprid on immune cells is dependent on the concentration of the pesticide. Hemocytes challenged with zymosan A has no significant change in *myD88* expression except for the 50I-10A mixture which was significantly lower than all treatments in the Zym 1 group including the zymosan control except for the treatment of 50 µg/ml imidacloprid. Again, the concentration dependent effect of imidacloprid is apparent and is synergistic with amitraz when imidacloprid is in a higher ratio. The 10I treatment is significantly higher than its corresponding treatment with zymosan. The case is inverted in most other treatments where pesticide treatments that included zymosan were higher in terms of *myD88* expression than without zymosan. However, the effect of zymosan in a significant increase is only observed in the control and the 10I-50A treatment. Hence, zymosan is implicated in increasing the expression of *myD88* acting on the Toll pathway.

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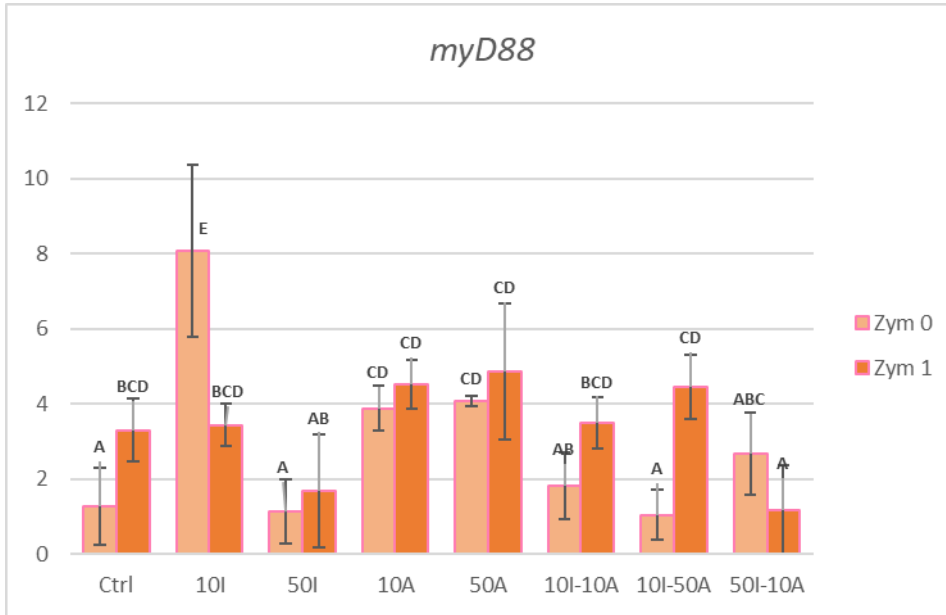


Figure 30. Gene expression analysis of *myD88* gene in honeybee hemocytes after pesticide treatment.

In the IMD immune pathway, Relish is a key component leading to the production of AMPs (Evans et al., 2006). In Figure 31, pesticide treatments that included 10 $\mu\text{g/ml}$ imidacloprid either alone or in a mixture with amitraz induced a significant increase in the expression of *relish* when hemocytes are not exposed to zymosan. A significant decrease in gene expression was observed with 10A, 10I-50A and 50I-10A treatments without zymosan. However, in the Zym 1 group, no treatment was significantly different from the zymosan control implying that zymosan act on sustaining the normal expression of *relish* in the IMD pathway. Fungal infections were already observed to activate the IMD pathway (Ramirez et al., 2019) but our results confirm that zymosan is also involved not just in the Toll pathway but the IMD pathway as well. Thus, the immune response to fungal infections comprises the activates of at least Toll and IMD pathways referring to the complexity of the honeybee immune system in specific. The importance of MyD88 in the immune response was previously demonstrated in MyD88-deficient mice which were unresponsive to stimulation by LPS (Kawai et al., 1999) and loss of bacterial resistance (Scanga et al., 2004). Thus, a decrease in Myd88 production caused by pesticide exposure may lead to immunosuppression resulting in increased infection rate and ultimately risking the survivability of hives.

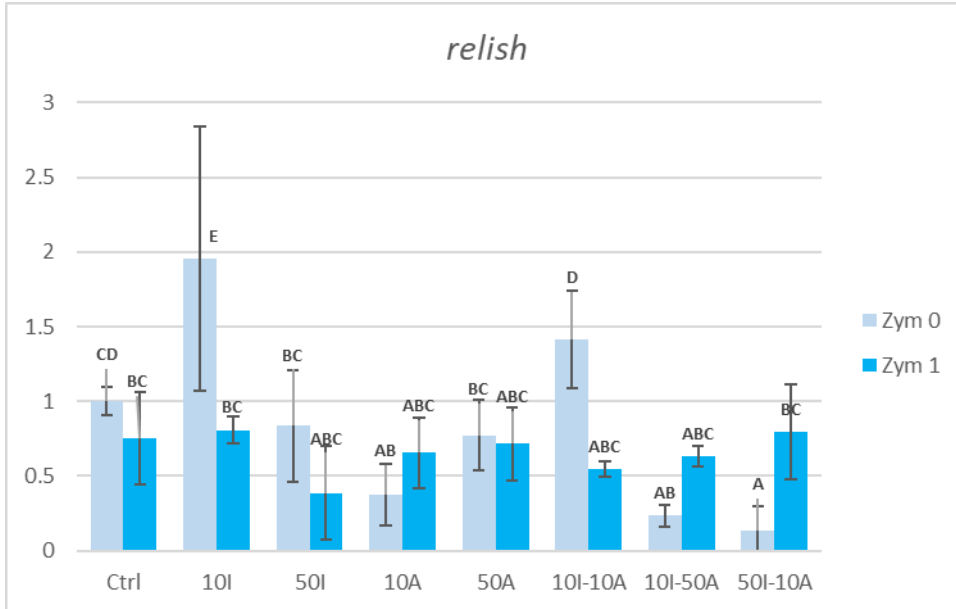


Figure 31. Gene expression analysis of *relish* gene in honeybee hemocytes after pesticide treatment.

Similarly, to the effect on *relish* and *myD88* when hemocytes were not exposed to zymosan, 10 $\mu\text{g/ml}$ imidacloprid resulted in an increase in the expression of the *eater* gene implicated in phagocytosis (Figure 32). A significant decrease was observed with the mixtures 10I-50A and 50I-10A compared to the Zym 0 control. As for the pesticide treatments that included zymosan exposure, no significant change was observed regarding expression of *eater* except with the 10I-50A mixture compared to the Zym 1 control. In addition, when comparing treatments with and without zymosan we only observe a significant decrease with 10I when treated with zymosan and the inverse in the 10I-50A treatment.

Amitraz may antagonize the effect of imidacloprid when it is either at the same concentration as seen with *relish* expression or when amitraz concentration exceed that of imidacloprid as seen with *eater* expression in the context of immune activation. Keeping to note that that concentration ratios between imidacloprid and amitraz may be very disruptive when concentrations are high as already observed. In laboratory conditions, the effect of amitraz on *relish* was strictly dependent on the developmental stage were the more developed the bee, the more the negative impact on *relish* (Tesovnik et al., 2019).

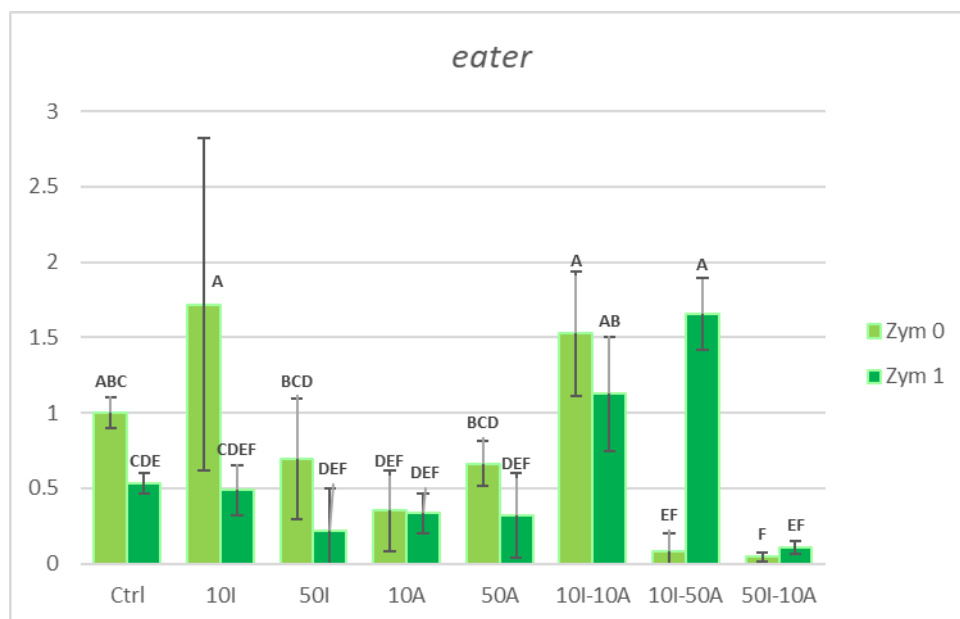


Figure 32. Gene expression analysis of *eater* gene in honeybee hemocytes after pesticide treatment. Error bars represent standard errors (SE). Different letters indicate significant differences between treatment groups ($n=3$, confidence interval=95%).

Eater production was reported to increase in honeybees when challenged with *Varroa*/DWS infection (Abbo et al., 2017). Yet, in our study we can observe that there is no significant difference on *eater* expression between treatments when comparing in absence and presence of zymosan expect for 10I and 10I-50A, which are significantly lower and higher after immune activation respectively (Figure 32). With zymosan A, the expression of *eater* was not significantly different in single exposures and 50I-10A mixture compared to the control. Intriguingly, the 10I-10A and 10I-50A treatments showed a significant increase when compared to the control with zymosan exposure. Furthermore, the 10I-50A treatment illustrated a contrasting result on gene expression with or without zymosan A. When not immunologically challenged, the *eater* gene expression with 10-50A treatment decreases significantly but increases significantly when zymosan is included. The two factors from the treatments that may have resulted in this observation could well be the high concentration of amitraz and zymosan. Hence, zymosan appears to alter the immunosuppressive effect of 10I-50A into a stimulatory response regarding *eater* though in all

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mixtures phagocytosis itself was reduced in our study suggesting that imidacloprid and amitraz affect phagocytosis by other components that Eater.

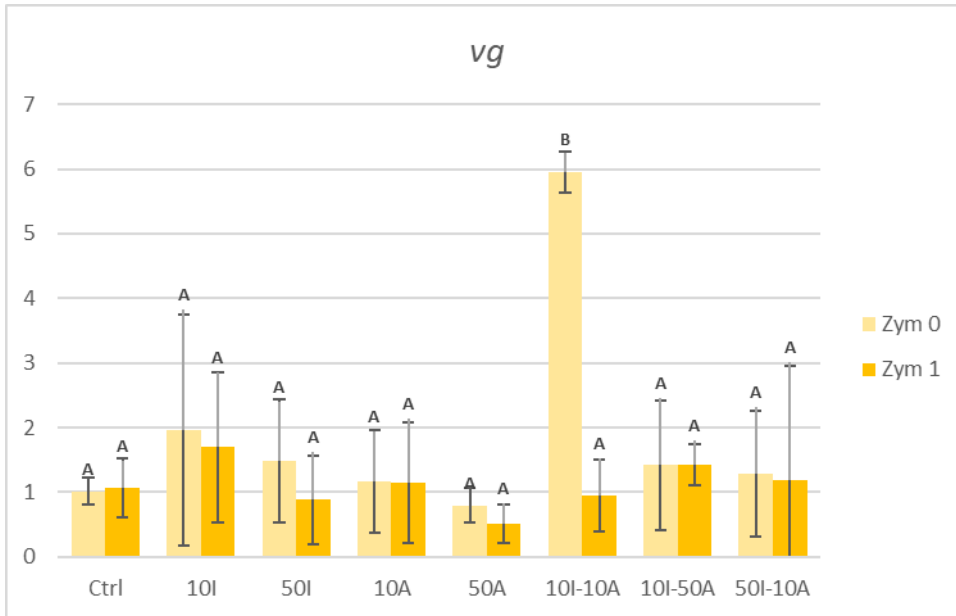


Figure 33. Gene expression analysis of *vg* gene in honeybee hemocytes after pesticide treatment. Error bars represent standard errors (SE). Different letters indicate significant differences between treatment groups ($n=3$, confidence interval=95%)

Imidacloprid and amitraz appear to have no effect on the production of vitellogenin gene regardless of exposure to zymosan with the exception of 10I-10A (Figure 33). This peculiar case infers that at given concentrations, imidacloprid and amitraz may act as reproductive disruptors when present simultaneously but not with single exposure or with mixtures of higher concentrations. A regulatory response may be activated to prevent alteration in *vg* levels. This could be connected to the observed trade-off between phagocytosis and the cyto-protective response. However, oral administration of sub-lethal doses of imidacloprid decreased *Vg* expression levels in honeybees (Abbo et al., 2017) suggesting that if there is an effect on *Vg* from combinations and imidacloprid or amitraz it may be more visible at the level of the whole organism or other types of cells. Variable degrees of *Vg* expression were also reported between caged bees and bees in the field when exposed to 5 and 200 ppb imidacloprid from 1 to 2 days (De Smet et al., 2017).

4.3.2. Zymosan A and honeybee gene expression interaction under pesticides application

The first 2 axes of PCA analysis express 67.7% of the total inertia of variability of the obtained data. The graph of individuals (Figure 34A) on the factorial design shows that axis 1 pits the treatment without Zymosan A application against the control and the honeybees treated with Zymosan A. This axis 1 explains 37.8% of the variation. The separation of two groups on this axis revealed that control and honeybee hemocytes treated with zymosan A were positively correlated to *myD88*, *spatzle* and *toll* gene expression. Regarding the representation of the variables on the factorial plane (Figure 34B), axis 1 shows groups of strongly contributing variables. The variables *eater*, *relish* and *myD88* are characterized by a strongly positive coordinate on the axis. These three variables all belong to the group of immune response to pesticides in bees. Axis 2 contrasts two groups of strongly contributing variables. On the one hand, the variables *toll* and *spatzle* are characterized by a strongly negative coordinate on the axis and, on the other hand, the variable VG are characterized by a strongly positive coordinate on the axis. With the exception of VG, which has functions of protecting bees against oxidative stress, the other two variables belong to immune response groups. Honeybees exposed to Zymosan A showed upregulation of Toll and SPZ immune pathway genes, but a downregulation of vitellogenin (VG). Zymosan A exposure therefore induces Toll and SPZ transcription on contrast to that of vitellogenin.

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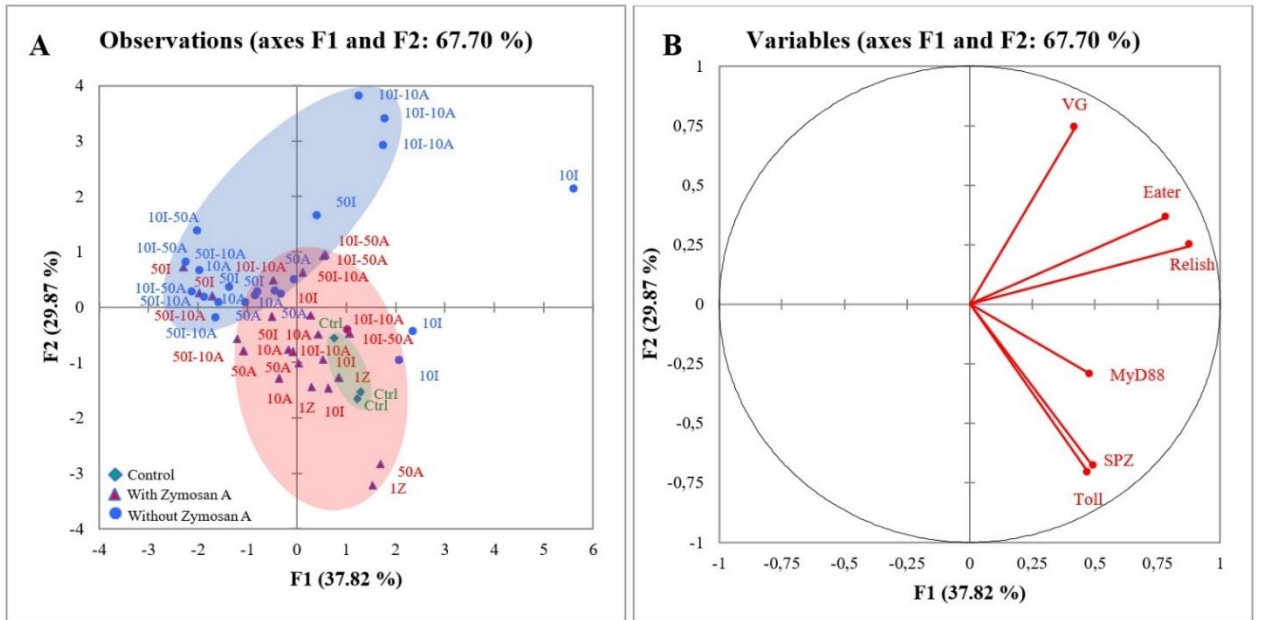


Figure 34. Principal Component Analysis (PCA) generated from genes expression after zymosan A application in honeybee exposed to different concentrations of imidacloprid and amitraz. (A) Individuals factor map according to the treatment ($n = 3$). Individuals are colored according to their membership in modalities of the variables. (B) Factor map of gene expression involved in the discrimination of different treatments.

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The viability of honeybee hemocytes increases with pesticide exposure as demonstrated by the trypan blue exclusion test and with propidium iodide staining. In cases where imidacloprid and amitraz did show an effect on viability, the combinations of both pesticides always demonstrated a synergistic effect on viability. An explanation for this observation could be the increase in antioxidant enzyme activity or a decrease in the production of oxidative molecules. This leads to low oxidative damage. Imidacloprid resulted in an increase of SOD, CAT, and MDA concentrations when applied to the larvae of the greater wax moth, *Galleria mellonella* (Lepidoptera) with doses reaching 1 µg/larva (Yucel & Kayis, 2019). In honeybees, 20 ppb of imidacloprid was also observed to up-regulate *catalase* gene expression but downregulate *sod2* expression when supplied with food to newly emerged bees (Gregorc et al., 2018). In addition, imidacloprid at 8.6 ng/bee elevated the GST activity after 48 hours of exposure (Z. Li et al., 2017). Thus, it is highly plausible that imidacloprid and amitraz synergize and increase the production of antioxidant enzymes, which reduce the concentrations of hydrogen peroxide and nitric oxide as observed in our results after pesticide exposure. An interesting enzyme is CYP450. This enzyme is not just responsible for detoxifying imidacloprid but also it is related to the toxicity of amitraz (Casida, 2018; Moyano et al., 2019). Thus, CYP450 determines if toxicity is mediated via amitraz or imidacloprid since it has an opposite effect on their toxicity after metabolism.

Since mitochondria are the main source of ROS production, any alteration in the electron transport chain leads to an alteration in hydrogen peroxide production. Change in calcium flux could be a reason for reduced ROS production. Imidacloprid was already observed to increase calcium flux, change mitochondrial morphology and affect the calcium signaling pathway in the midgut of the aquatic insect, *Chironomus dilutes* with a maximal exposure concentration of 100 µg/L of imidacloprid (Wei et al., 2020). Calcium flux may indeed play a role in the production of hydrogen peroxide. However, the production of nitric oxide via the inducible form of NOS (iNOS) is calcium-independent (Jacklet, 1997). Thus, any effect of imidacloprid and amitraz may be related to the production of iNOS at the gene expression level and not necessarily the activity level. It would be interesting to study the change in calcium flux in hemocytes exposed to pesticides and immune stimulators to connect the function of the electron transport chain to

the change in ROS production and the mechanism by which pesticides alter ROS concentrations. However, in phagocytes, NADPH oxidases and dual oxidases (DUOX) are the main sources of ROS (Al-Shehri, 2021). Since our approach includes the use of hemocytes, evaluating the activity or expression level of NADPH and DUOX would also be a good indicator for the change in ROS production.

Comparing the 3 species used, honeybees were the most affected by imidacloprid and amitraz in the production of NO and H₂O₂. Honeybees share the same environment with insects of different orders including their predators and pests. The Lepidopteran representative used; the cabbage moth (*M. brassicae*) MB-L2 cell line, was less affected than honeybee hemocytes in all compared parameters except for the decrease in protein production when they were exposed to pesticides and 10 µg/ml zymosan A. A concern arises as the greater wax moth and lesser wax moth are Lepidopteran pests of bee hives and may have an advantage over bees in areas where the pesticides are used. In addition, the fruit fly is a model for pesticide studies, and such variation in the immune response between honeybee and fruit fly hemocytes indicated that the true effect of pesticides on honeybees cannot be extrapolated from *Drosophila* models since they appear to be more resistant to imidacloprid and amitraz.

In addition, all three insect orders (Lepidoptera, Diptera, and Hymenoptera) represent pollinators of the same order, genus, and/or species (Devoto et al., 2011; J. Guo et al., 2022; Hung et al., 2018; Karremans et al., 2015; Stökl et al., 2010). Thus, imidacloprid and amitraz can also act on non-target insects and affect a wide range of pollinators including honeybees.

Regarding phagocytosis in honeybees, we observed that zymosan A induced the decrease of phagocytic hemocytes after exposure to imidacloprid and amitraz mixtures correlated with an increase in viability. However, the effect of pesticide mixtures was not pronounced when hemocytes were not exposed to zymosan A. Moreover, zymosan at 1 µg/ml resulted in a stronger negative correlation between phagocytosis and hemocyte viability. This infers that zymosan A redirects hemocyte response from phagocytosis when imidacloprid and amitraz are present as stressors. It could be that zymosan A induces a cytoprotective response that prioritizes attenuating cellular damage over eliciting phagocytosis as an immune response preventing over-extension of hemocytes. Linking the production of hydrogen peroxide and nitric oxide to viability and phagocytosis, it was clear that reduced ROS and RNS production was associated

with higher viability as well. A complex connection that links cellular responses and humoral immune responses is observed.

Nitric oxide produced via iNOS was connected to phagocytosis in mouse macrophages where increased nitric oxide was accompanied by increased phagocytosis and phagocytic index (Tümer et al., 2007) and it was also found to upregulate phagocytosis in other cell types like microglia in mice (Maksoud et al., 2019). Looking at the results obtained from our studies on honeybees, we observed that conditions that demonstrated decreased phagocytosis displayed a relative decrease in nitric oxide production. However, the decrease in nitric oxide concentration was not always accompanied by decreased phagocytosis. Henceforth, we can deduce that nitric oxide is not a limiting factor in phagocytosis in honeybee hemocytes but the association could explain why honeybee granulocytes produce nitric oxide at the beginning of the immune response as demonstrated by Negri et al., (2013).

Zymosan A had different effects on hemocytes depending if its concentration was at 1 or 10 µg/ml. At 1 µg/ml, the decrease of NO production was more intense in pesticide-treated hemocytes compared to hemocytes treated with the same pesticide concentrations but not with zymosan. Conversely, NO production was partially mitigated when hemocytes were treated with pesticides and 10 µg/ml zymosan A. Correlation between phagocytosis and viability was the strongest in treatments that included 1 µg/ml zymosan while 10 µg/ml zymosan demonstrated a weaker correlation. Thus, the lower concentration of zymosan had more of an impact on cellular immune responses such as phagocytosis. This could be due to a possible negative feedback loop caused by an excessive zymosan concentration of 10 µg/ml.

The immune genes of the Toll pathway including *toll*, *spaetzle*, and *myD88* were highly affected by pesticides, especially in mixtures, regardless of the presence of zymosan but it appears to partially mitigate the effect of imidacloprid and amitraz. Thus, there is a competition between pesticides and zymosan on acting on the Toll pathway.

However, looking at *relish* and *eater*, zymosan A appears to result in regulation of their expression after being dysregulated by pesticides. Zymosan A has a stronger regulatory effect on *relish* and *eater* than the Toll pathway. This leads us to conclude that the immune modulation is directed towards the Toll pathway in the presence of zymosan. Since phagocytosis is not an established outcome of Toll pathway signaling, redirection towards this pathway will lead to the

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observed reduced phagocytosis. Since the effect of reduced phagocytosis is prominent in pesticide mixtures, zymosan's effect will be mainly directed towards the Toll pathway in these conditions as a case of induced competition between zymosan and the applied pesticides.

Overall, imidacloprid and amitraz illustrate synergism when affecting honeybee hemocytes. Hemocyte viability is increased when exposed to both pesticides with a correlation with reduced phagocytosis and reduced ROS and RNS production. The relationship is established when hemocytes are exposed to zymosan. However, with 1 µg/ml zymosan, the effect is stronger than its higher concentration. Indeed, 1 µg/ml zymosan showed NO and H₂O₂ production even further reduced than when it is absent. While exposure to 10 µg/ml zymosan mitigates the effect of pesticides on NO production.

It is true that reduced oxidative molecules may have contributed to increased cellular viability and less oxidative damage, however, the production of such molecules is important for defense against pathogens. Our approach mimics immune stimulation by fungal PAMPs to illustrate the underlying effects of pesticide exposure but does not mimic the associated damage from pathogen infection. In fact, "healthier" hemocytes could pose a preferred target for infection. Not to mention that zymosan was the inducer that established a correlation for reduced phagocytosis. The correlation was detected with the higher concentration. zymosan at 10 µg/ml was able to mitigate the effect on NO production. Thus, the immune stimulation may be beneficial or deleterious to honeybees depending on the context and the concentration.

The innate immune system of invertebrates and specifically honeybees require evaluation at complex level of interaction and in different conditions of co-exposure. The inability to truly comprehend the main driving factor behind CCD lies in the complexity of the insect immune system and the variability of responses to different stressors.

Conclusion and Perspectives

6. Conclusion and Perspectives

Imidacloprid and amitraz have a context specific effect on the honeybee immune system. Imidacloprid and amitraz decrease the production of nitric oxide and hydrogen peroxide by honeybee hemocytes at the time of exposure. Zymosan A increased the effect of pesticides whether alone or in combination on nitric oxide production at 1 $\mu\text{g/ml}$ of zymosan but at 10 $\mu\text{g/ml}$ of zymosan, the negative effect of pesticides on honeybee hemocytes was attenuated implying that a stronger immune-stimulation results is masking the effect of pesticides on NO production. Compared to MB-L2 cells and Schneider-2 cells, the negative impact on NO production was the more severe on honeybee hemocytes. In fact, Schneider-2 cells may have an internal regulatory mechanism that renders them less affected by imidacloprid and amitraz since NO production did not change as drastically as honeybee or MB-L2 cells. The latter is true not just at different time points but the production rate from 15 minutes until 2 hours post-exposure as well. Hydrogen peroxide production decreases by honeybee hemocytes when exposed to imidacloprid and amitraz either without zymosan or with 1 $\mu\text{g/ml}$ zymosan but no significant change was observed with the higher concentration of zymosan, a pattern similar to that of NO production. Imidacloprid and amitraz decrease hydrogen peroxide production in Scheider-2 cells but only when zymosan is present. Overall, imidacloprid and amitraz appear to affect the production of RNS and ROS in honeybee hemocytes to a greater extent compared to the cell lines of the cabbage moth (MB-L2) and the fruit fly (Scheider-2). This implies that imidacloprid and amitraz affect pollinators and pests of different orders to different extents. Also, honeybee hives could be at a disadvantage in sites exposed to amitraz and imidacloprid and are more immunologically compromised. In addition, the total protein content in all cells of studies species was not affected compared to their respective controls except when MB-L2 were exposed to the combinations of pesticides at 10 $\mu\text{g/ml}$ zymosan. Hence, the effect of pesticides on cellular products is not associated with protein production. At least, any alteration in protein content is too minor to be detected by Bradford's assay.

Imidacloprid and amitraz reduce the phagocytic rate in honeybee hemocytes but only when the immune system is stimulated by zymosan regardless of the used concentrations used.

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Phagocytosis was not affected when hemocytes were not treated with zymosan A. However, when treated with zymosan, imidacloprid and amitraz showed a phagocytic decrease in a dose-dependent manner. The pesticide combinations have the strongest effect on decreasing phagocytosis. Phagocytosis is negatively correlated with hemocyte viability as detected by flow cytometry. The negative correlation was only present when cells were immune activated by zymosan suggesting the presence of a trade-off between the phagocytic rate and cellular viability. Specifically, reduced phagocytosis is correlated with a cyto-protective response that increases cellular viability even compared to the control which could also be related to reduced oxidative response. Thus, imidacloprid and amitraz co-exposure induces the protective response prominently present when hemocytes are immune-activated. Hemocytes also appear to prevent the cytotoxic effect of pesticides maybe by a mechanism that alters the cell membrane. As phagocytosis is associated with membrane activity it could be that the protective response would result in reducing phagocytosis. The increased viability of honeybee hemocytes when exposed to pesticide mixtures was also observed in the dye-exclusion method. The negative correlation between phagocytosis and viability suggested that hemocytes exposed to pesticides are unable to lean on phagocytosis for fending off pathogens making the organism more susceptible to infection.

On the molecular level, either in single exposure, mixtures, with zymosan A exposure or not, imidacloprid and amitraz decreased the expression of the *spaetzle*, *toll* and *myD88* genes of the Toll pathway. The expression of these 2 genes appear to be strongly correlated and since they are at the first level of pathogen recognition this could result in decrease of immune responses results from the Toll pathway like AMPs production. However, with zymosan exposure, the effect of pesticides is less severe but still significant.

The expression of *myD88* increases when cells are exposed to amitraz without immune challenge and with 10 µg/ml imidacloprid. Zymosan A exposure appears to attenuated to some degree the effect of pesticides on the variation of the gene expression. Thus, amitraz exposure could affect cellular processes that depend on *myD88*.

The expression of *relish* with pesticides without zymosan is variable but with a pronounced decrease in the pesticide mixtures that had pesticide mixtures of either a higher

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concentration of imidacloprid or amitraz. However, when challenged with zymosan the variation between treatments dissipated. Zymosan A appears to regulate the expression of *relish* to a homeostatic level.

Eater, as a crucial element in phagocytosis did not show any association with the phagocytosis reading from fluorescent beads. In fact, eater expression increased with pesticide combination contrasting to phagocytosis decrease with the same combinations. Thus, the action of pesticide in hindering phagocytosis in the context of immune stimulation is not necessarily related to eater expression. Instead, the results imply that phagocytosis is reduced by a different mechanism that could be related to membrane activity.

Zymosan exposure redirectes the immune activity towards the Toll pathway when hemocytes are exposed to both imidacloprid and amitraz propability as a regulatory function. This could also explain the reduced phagocytosis where it is implicated in other immune pathways instead of the Toll pathway.

Vitellogenin levels are not affected by any of the treatments of pesticides in single exposures or in combinations with the exception of the 10I-10A combination without zymosan where the gene expression increased. Thus, imidacloprid and amitraz may act an endocrine disruption in very specific conditions. This could lead to drastic effects on honeybee reproduction and development altering the normal consistency of the hive and brood.

With our finding we can state that imidacloprid and amitraz hinder the immune competence of honeybee hemocytes at the level of immune response and pathogen recognition mainly in the Toll pathway. Furthermore, pesticide combination reduce phagocytosis which is a crucial process in innate immunity. The synergism/antagonism between amitraz and imidacloprid is observed in our results.

More research is needed at the level of antioxidant enzymes like superoxide dismutase, catalase, glutathione-S- transferase and CYP450 and their activity in addition the expression of associated gene products that could better explain the observations in the production of RNS and ROS. Genes designating NADPH, iNOS, DUOX are a good

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indicator to understand the underlying immune modulatory mechanisms at play. It could also give a better understanding of the immune response and intrinsic interaction with pesticides and immune stimulators giving a detailed immunologic map comprising the mode of action. Calcium flux is also a good parameter to study when evaluating the effect of pesticide on the production of ROS and humoral responses. This could be done by measuring or observing the different distribution of calcium (bound and unbound) in hemocytes using specific dyes like Fluo-4 and Fura red. The trade-off between phagocytosis and the cyto-protective response is also an interesting aspect to study mainly on components on cell membrane action and components of phagocytic action in immune cells.

All further experimentation should be explored with different immune stimulators in addition to zymosan like LPS and PGN to observe the type of variation in the immune responses in the presence of different pesticides.

Amitraz and imidacloprid hinder the immune response in honeybees and be a contributing factor in CCD. Evaluation of the co-presence of different factors must be taken to consideration in future studies when conducting a risk assessment on pesticide usage as the underlying effects may not be apparent directly or in a simple context. The order of exposure to pesticides or immune activation may as well affect the response of honeybee hemocytes. Immune activation prior to pesticide exposure may regulate the hemocytes so not to be drastically affected by pesticides since our results have demonstrated the effect of zymosan in that manner in given conditions regarding concentrations and parameter studied.

On the other hand, immune activation may lead to a hyperinflammatory state that may render honeybees less fit for survival. This should be evaluated with infections that trigger the same immune pathways or different immune pathways. More in depth research is needed to fully understand the occurrence of CCD in different conditions and in a comprehensive approach.

Furthermore, the main limitation encountered when working with honeybee hemocytes is that they have to be freshly extracted and there is no commercially available cell line for immune related studies in honeybees. Larvae produced the largest volume of extractable

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hemolymph and they are only seasonally available and they depend on queen performance. This demands the production of a cell line corresponding to honeybee hemocytes to facilitate toxicologic studies with less conditional variability.

This work may lay a strong ground in assessing complex interactions in addition to shedding the light on the presence of yet to be deciphered mechanism of the immune system and cellular signaling.

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Annex

Annex 1. Statistical table of cellular products assays. N represents the sample size. Zym annotation represents the zymosan concentration group including all pesticide treatments.

Test	Cells	Variable	Exposure time	N	Statistical test	Post-Hoc	Confidence interval
Bradford	Honeybee	0 Zym	3 hrs	6	ANOVA	Tukey (HSD)	95%
		1 Zym			ANOVA	Tukey (HSD)	
		10 Zym			ANOVA	Tukey (HSD)	
	Schneider-2	0 Zym		9	Kruskal-Wallis	Dunn	
		1 Zym			Kruskal-Wallis	Dunn	
		10 Zym			ANOVA	Tukey (HSD)	
	MB-L2	0 Zym		6	Kruskal-Wallis	Dunn	
		1 Zym			ANOVA	Tukey (HSD)	
		10 Zym			Kruskal-Wallis	Dunn	
Viability (Trypan Blue)	Honeybee	0 Zym	18 hrs	3	ANOVA	Tukey (HSD)	95%
		1 Zym			Kruskal-Wallis	Dunn	
		10 Zym			Kruskal-Wallis	Dunn	
NO	Honeybee	0 Zym	15 min	6	ANOVA	Tukey (HSD)	95-99%
		1 Zym			Kruskal-Wallis	Dunn	
		10 Zym			Kruskal-Wallis	Dunn	
	Schneider-2	0 Zym		6	ANOVA	Tukey (HSD)	
		1 Zym			ANOVA	Tukey (HSD)	
		10 Zym			ANOVA	Tukey (HSD)	
	MB-L2	0 Zym		6	Kruskal-Wallis	Dunn	
		1 Zym			Kruskal-Wallis	Dunn	
		10 Zym			ANOVA	Tukey (HSD)	
NO	Honeybee	0 Zym	120 min	6	ANOVA	Tukey (HSD)	95-99%
		1 Zym			Kruskal-Wallis	Dunn	
		10 Zym			ANOVA	Tukey (HSD)	
	Schneider-2	0 Zym		6	ANOVA	Tukey (HSD)	
		1 Zym			ANOVA	Tukey (HSD)	
		10 Zym			ANOVA	Tukey (HSD)	
	MB-L2	0 Zym		6	ANOVA	Tukey (HSD)	
		1 Zym			Kruskal-Wallis	Dunn	
		10 Zym			ANOVA	Tukey (HSD)	
NO	Honeybee	0 Zym	Ratio 120/15	6	ANOVA	Tukey (HSD)	95-99%
		1 Zym			ANOVA	Tukey (HSD)	
		10 Zym			Kruskal-Wallis	Dunn	
	Schneider-2	0 Zym		6	Kruskal-Wallis	Dunn	
		1 Zym			ANOVA	Tukey (HSD)	
		10 Zym			Kruskal-Wallis	Dunn	
	MB-L2	0 Zym		6	ANOVA	Tukey (HSD)	
		1 Zym			ANOVA	Tukey (HSD)	

Annex

		10 Zym			ANOVA	Tukey (HSD)	
H ₂ O ₂	Honeybee	0 Zym	3 hrs	5	ANOVA	Tukey (HSD)	95-99%
		1 Zym			ANOVA	Tukey (HSD)	
		10 Zym			Kruskal-Wallis	Dunn	
		0 Zym			Kruskal-Wallis	Dunn	
	Schneider-2	1 Zym		5	Kruskal-Wallis	Dunn	
		10 Zym			Kruskal-Wallis	Dunn	
		0 Zym			ANOVA	Tukey (HSD)	
	MB-L2	1 Zym		5	Kruskal-Wallis	Dunn	
		10 Zym			Kruskal-Wallis	Dunn	

Annex

Annex 2. Statistical table of differences of variances test for flow cytometry analysis of phagocytosis and propidium iodide-mediated viability.

Test	Cells	Variable	Exposure time	N	Statistical test	Post-Hoc	Confidence interval
Phagocytosis	Honeybee	0 Zym	18 hrs	3	ANOVA	Tukey (HSD)	95%
		1 Zym			ANOVA	Tukey (HSD)	
		10 Zym			Kruskal-Wallis	Dunn	
Propidium iodide (viability)		0 Zym			ANOVA	Tukey (HSD)	95%
		1 Zym			ANOVA	Tukey (HSD)	
		10 Zym			Kruskal-Wallis	Dunn	

Annex

Annex 3. Difference of variances for gene expression analysis in honeybee cells after 18 hours of exposures to treatments.

Gene	Cells	Variable	Exposure time	N	Statistical test	Post-Hoc	Confidence interval
<i>spatzle</i>	Honeybee	All data set for respective gene	18 hrs	3	Two-way ANOVA	Dunnet's test	95%
<i>toll</i>							
<i>myD88</i>							
<i>relish</i>							
<i>eater</i>							
<i>vg</i>							

Abstract

Résumé

Les abeilles sont confrontées à la menace mondiale du syndrome d'effondrement des colonies (CCD), entraînant des décès de colonies et une diminution de leur nombre, affectant leur contribution environnementale et agronomique dans la pollinisation des plantes et des cultures commerciales, en plus de la production de miel. L'exposition aux pesticides peut être l'une des principales causes conduisant au CCD en affaiblissant le système immunitaire des abeilles et en altérant leurs réponses immunitaires. Les maladies de la nosérose causées par *Nosema* spp. peuvent avoir une contribution significative au CCD lorsque les abeilles sont exposées à différents pesticides simultanément. Dans cette étude, plusieurs facteurs de risque sont évalués, y compris les néonicotinoïdes les plus utilisés dans le monde, l'imidaclopride et l'amitraz qui est le pesticide utilisé directement en contact avec les abeilles pour traiter l'infection par les acariens. L'effet de ces pesticides est évalué au niveau de la stimulation immunitaire par zymosan A pour imiter l'infection par *Nosema*. L'effet des pesticides sur les produits cellulaires antimicrobiens, les réponses cellulaires et l'expression de gènes connexes est démontré.

Abstract

Honeybees are facing the global threat of colony collapse disorder (CCD) leading colony deaths and decline in their numbers affecting their environmental and agronomic contribution in pollination of plants and commercial crops in addition to honey production. Pesticide exposure may be of the main causes leading to CCD by weakening the immune system of honeybees and impairing their immune responses. Nosemosis diseases caused by *Nosema* spp. may have a significant contribution to CCD when bees are exposed to different pesticides simultaneously. Multiple risk factors are assessed in this study including the most used neonicotinoids worldwide, imidacloprid and amitraz which is the pesticide used directly in contact with honeybees to treat mite infection. The effect of these pesticides is evaluated at the level of immune stimulation by zymosan A to mimic *Nosema* infection. The effect of pesticides on antimicrobial cells products, cellular responses and related genes' expression are demonstrated.