

THÈSE

Pour l'obtention du Grade de

DOCTEUR DE L'UNIVERSITÉ DE POITIERS
(Faculté des Sciences Fondamentales et Appliquées)
(Diplôme National - Arrêté du 25 mai 2016)

École Doctorale 649 :
« *Rosalind Franklin* » - Énergie – Environnement - Biosanté

Secteur de Recherche :
Biologie des organismes

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Évaluer les effets différés des pesticides sur l'abeille mellifère : du gène à la colonie

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Soutenue le 07/05/2024 devant la Commission d'Examen

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Cette thèse a été soutenue par l'OFB dans le cadre de l'appel Ecophyto II+, lancé par le ministère français en charge de l'écologie, de l'agriculture, de la santé et de la recherche et également par la Fondation Lune de Miel.



RESUME

Cette thèse s'inscrit dans le contexte actuel de déclin des pollinisateurs et de l'utilisation intensive des pesticides. Il est donc essentiel, voire capital, de comprendre les conséquences de nos pratiques agricoles sur la biodiversité.

L'abeille mellifère (*Apis mellifera*) joue un rôle crucial en tant que pollinisateur vital pour les cultures et la biodiversité. Cependant, ces populations subissent des pertes importantes dues à divers facteurs. Dans le cadre de mes recherches, j'ai examiné les effets de sept facteurs de stress sur la biologie de l'abeille, appartenant à quatre catégories distinctes : (i) un élément inerte, l'acétone, (ii) une bactérie, *Paenibacillus larvae*, (iii) deux insecticides, le thiaméthoxame et la lambda-cyhalothrine, et (iv) trois fongicides, le boscalid, le tébuconazole et le prothioconazole.

La démarche choisie pour cette étude consiste à suivre et contrôler le cycle de vie complet des abeilles dans la ruche, en utilisant la technique d'élevage larvaire *in vitro* couplée à un système RFID. Cette approche originale m'a permis d'explorer avec précision les effets sublétaux dus à des expositions précoces, répétées et différées, ainsi qu'à des interactions avec des stress biotiques et abiotiques.

En adoptant une approche transversale, les effets sublétaux de ces facteurs ont pu être caractérisés sur la physiologie, le développement et le comportement au niveau individuel, tout en intégrant des simulations de dynamique des populations pour une vision globale de leur impact sur les colonies d'abeilles. Les résultats obtenus fournissent des réponses cruciales aux défis posés par les effets sublétaux des pesticides lors d'expositions précoces dans la vie des abeilles. Cette approche m'a permis une meilleure compréhension des effets différés des pesticides sur le cycle de vie des abeilles domestiques, tant au niveau individuel qu'au niveau de la colonie.

Mots clefs : *Apis Mellifera* ; Elevage *in vitro* ; RFID ; Activités de vol ; Expression de gènes ; Ecotoxicologie ; Pesticides ; Interactions Pathogènes – Pesticides

SUMMARY

This thesis is written in the current context of pollinator decline and intensive pesticide use. It is therefore essential, even vital, to understand the consequences of our agricultural practices on biodiversity.

The honeybee (*Apis mellifera*) plays a decisive role as a vital pollinator for crops and biodiversity. However, its populations are currently losing ground due to a variety of factors. As part of my research, I examined the effects of seven stressors on bee biology, belonging to four distinct categories: (i) an inert element, acetone, (ii) a bacterium, *Paenibacillus larvae*, (iii) two insecticides, thiamethoxam and lambda-cyhalothrin, and (iv) three fungicides, boscalid, tebuconazole and prothioconazole.

The approach chosen for this study is to monitor and control the entire life cycle of bees in the hive, using the *in vitro* larval rearing technique coupled with an RFID system. This original approach enables me to accurately explore the sublethal effects of early, repeated and delayed exposure, as well as interactions with biotic and abiotic stresses.

By adopting a cross-disciplinary approach, it has been possible to characterise the sublethal effects of these factors on physiology, development and behavior at the individual level, while integrating population dynamics simulations for a global view of their impact on honeybee colonies. The results obtained provide crucial answers to the challenges posed by the sublethal effects of pesticides due to early exposure in the life of bees. My approach has led me to a better understanding of the delayed effects of pesticides on the life cycle of honeybees, at both individual and colony levels.

Keywords : *Apis Mellifera* ; *In vitro* rearing ; RFID ; Flight activities ; Gene expression ; Ecotoxicology ; Pesticides ; Pathogen – Pesticide Interactions

REMERCIEMENTS

Certains résument une thèse par trois années d'un travail laborieux rempli de catastrophes, mais c'est oublier que dans un voyage ce qui compte ce n'est pas la destination, mais le chemin parcouru. Cette quête de la connaissance et du savoir est une aventure personnelle et professionnelle jalonnée de défis et de rencontres inoubliables.

Je remercie les membres du jury de me faire l'honneur d'évaluer mes travaux de thèse, notamment les rapporteurs **Mickaël Henry et Mathieu Lihoreau**, ainsi que les examinatrices **Colette Bertrand et Emmanuelle Porcher**.

Pour m'avoir permis de réaliser cette étude exceptionnelle et d'ainsi développer mes compétences, je remercie mes trois encadrants **Freddie-Jeanne, Pierrick et Fabrice** pour la confiance que vous m'avez accordée en me confiant le projet que vous avez imaginé et de m'avoir permis de travailler sur le modèle que j'aime tant. Merci **Fabrice** pour tes conseils précieux concernant les analyses RFID et les simulations. Sans toi, ces études n'auraient pas pris de telles proportions.

Trois personnes, trois localisations géographiques et deux sites d'expérimentation demandent une certaine organisation logistique et une flexibilité de dernière minute afin de pouvoir échanger avec vous tous. Vos expertises m'ont permis de me sensibiliser et de définir le chercheur que je souhaite devenir. Vous m'avez également offert l'opportunité de créer de nombreuses expérimentations, de présenter mes travaux en congrès scientifiques. Merci.

Je souhaite également remercier **Marie-Pierre Chauzat et Jean-Christophe Sandoz** pour leur implication dans le comité scientifique de thèse ainsi que pour leurs conseils et leurs avis éclairés.

Je remercie tous **les membres de l'équipe APIS de l'INRAE du Magneraud** et plus particulièrement **Carole et Daniel**. Votre aide était indispensable au cours des expérimentations. Vous êtes passés maîtres dans l'art du collage de tag RFID et l'élevage de larves d'abeilles. Merci **Stéphane** pour ton expertise et tes connaissances sans limites dans la gestion des ruches du Magneraud. Merci **Colombe et Fabien** pour votre infinie gentillesse. Merci **Thierry** pour tes anecdotes ornithologiques et palynologiques. Merci **Claude** pour tes petits coups de main administratifs afin de

pouvoir dormir « directement sur les ruches » et assurer les commandes de dernière minute.

Merci à vous les Post-doc et les compagnons de projet. **Maxime**, tes 1000 projets de vulgarisation me laissent sans voix ; merci **Elisa** pour ton abnégation et ta passion pour la recherche et surtout, merci pour ta patience. Sans vous, la vie au Magneraud aurait été clairement moins sympa ! Vos coups de main lors des sessions de collage de tags et les échanges autour des expérimentations étaient pour moi des moments d'échanges scientifiques inoubliables.

Merci **Manon** pour ton aide et ton soutien lors de ma première année de thèse, tu es la reine des petits trous dans les tubes et découpages de coton ! Merci **Barberine** de m'avoir fait confiance, (PS : ta petite plante va toujours très bien). En tous cas, ensemble nous avons appris la réalité des lenteurs administratives.

Merci à **toute l'équipe EES** pour la bienveillance dont vous avez fait preuve à mon égard, alors que je n'étais là qu'à « mi-temps ». Particulièrement, merci **Nicolas B., Hélène, Justine, Christine, Fred, Nicolas D., Romain, Christelle, Thierry, Joanne, Julia, Isabelle M., Jean, Maryline, Carine, Tiffany, Isabelle G., Alexandra**.

Je remercie tous mes anciens encadrants de stage : **Laurence, Ludovic, Anne et Dirk**. C'est grâce à vous et à toutes les expériences que nous avons menées ensemble que j'ai pu élargir et préciser ma sensibilité scientifique.

Une pensée aux membres de **l'équipe Abeille et Environnement d'Avignon** et toutes les personnes que j'ai croisées en congrès de **l'IUSSI et EURBEE**. C'est grâce à vous que je suis et reste piqué pour les abeilles.

Une grosse pensée à tous **mes compagnons d'aventure / doctorants**. Ensemble nous avons traversé monts et marées (au sens littéral avec les déménagements et les problèmes « d'humidité » des laboratoires et du bureau). Merci **Cécilia**, malgré ton air de pince-sans-rire à la Minerva McGonagall, tes conseils ont toujours été des petits *lumos* pour guider les voyageurs perdus. Nos petits cafés–marchés étaient toujours source de longues et passionnantes discussions. Tes « Rhooo » de dépit dans mon dos, lors de nos blagues pot-potache avec Elie me manquent un peu ! Merci **Elie** ou Monsieur le Cartographe entrepreneur qui a toujours su me guider dans les méandres de tes blagues *légèrement* douteuses. Nos (trop nombreux) cafés étaient toujours (ou

presque) des moments intenses « d'émulsion scientifique ». Ta vision de la recherche et nos discussions écologies / statistiques valaient de l'or. Merci **Baptiste** pour les blagues (parfois trop) recherchées et toutes les anecdotes décalées dont tu avais le secret et dont tu nous abreuvais lors des pauses du déjeuner. Ta bienveillance et ta culture scientifique (et pas que) ont toujours été une grande source d'inspiration pour moi. Merci **MOMO** (ou **Maud** pour les intimes) d'être la « petite crevette » du laboratoire et d'avoir supporté mes blagues et calembours au quotidien. Ton arrivée et ton côté bulldozer ont été un vrai plus pour le labo. Nos pauses canapé ont toujours été des moments de franche rigolade. Encore un grand merci à toute l'équipe des **thésards & Co**, et notamment ceux qui aiment les petits monstres avec trop de pattes : **Said, Louis, Thomas B., Charlotte, Cybèle, Axel, Camille, Sarah** ; ceux bercés par la chlorophylle : **Benoit, Mathias et Lisa**, et ceux navigant en eau trouble : **Amélie, Louis, Gaëlle et Eloïse** (l'OVNI) vous êtes géniaux ! N'oubliez pas que vous êtes la « force vive du labo » et que « l'émulsion scientifique » se fait sans pression !

Merci à **mes nombreux coloc, ex-coloc ou semi-coloc**, d'avoir toujours été là au quotidien. Le fait de pouvoir aborder d'autres sujets que les sciences est toujours enrichissant et permet d'ouvrir ses horizons. Merci **Flo**, ton engagement me laisse toujours admiratif. Promis, maintenant je me remets à cuisiner ; merci **Emilie**, tu es la plus jeune, pourtant j'ai l'impression que tu as eu 1000 vies ; merci **Agathe** pour ton côté chaotique ; merci **Bapti** pour tes (trop) nombreux traquenards ; merci **Emeline** pour ton accueil et toutes les personnes que tu m'as fait rencontrer ; merci **Baptiste** d'avoir supporté ma présence en coup de vent ; merci mes presque coloc, **WAgathe, WAllice et Val** pour tous ces brunch-jeux de société du dimanche. Évidemment, merci **Geraldine** (la tortue) pour ta présence discrète mais essentielle à côté de mon bureau en télétravail.

Merci à toutes mes belles rencontres de Poitiers : **Clément, Jean, Aurore, Cerise (Arnaud), Antho, Romain, Apolline et Jeanne**.

Gros big up à l'asso du Petit Plateau, notamment **Arthur, Alex et Clément** qui ont toujours été de bon conseil pour me permettre de garder un fidèle destrier opérationnel et pouvoir aller au travail.

Évidemment, je n'oublie pas les Lyonnais. Merci **Adrien**, je suis nostalgique de nos moments « révisons » de master et je suis toujours aussi admiratif de ta facilité et ta

passion à vulgariser un sujet ; merci **Brice**, tu es mon frère de parcours de l'EPHE, je n'oublie pas nos moments trottinette endiablés à Lyon ; merci **Lucille** pour les petits moments café-dessin, tu m'impressionnes par ton courage, tu es la prochaine que nous appellerons « doc » ; merci **Audrey** pour ton aide lors de la thèse et les « Yatta » d'encouragement ; merci **Mallaury** de nous régaler de tes anecdotes abracadabrantes sur les Lego ; merci **Amélie**, heureusement que tu es là pour garder cette fine équipe en un seul morceau !

En rédigeant ces remerciements, je me rends compte que ces derniers temps je me suis éloigné de mes racines et de mes vieux copains : **Arthur** et **Charles** vous êtes les prem's, même si on n'arrive jamais à se voir ; merci **Val**, promis je viens te voir dans le grand Nord ; merci **Martin**, grâce à toi je continue de toucher du bois ; merci **Alex** pour ton soutien sans compter au fil de ces années ; merci **Melou** pour ta bienveillance, les services au « salon » me semblent si lointains ; merci **Violette** de supporter le squatteur ; merci **Damien, Sibylle, Yann, Salomé** pour votre gentillesse. Promis, je serai là pour aller voir Michel la prochaine fois car, bientôt, je vais le faire mon tour de France et d'Europe pour venir vous voir !

Merci **Véronique** pour le temps que tu m'as accordé et les (très) nombreux échanges de fichiers et les corrections orthographiques.

Je termine en remerciant **mes parents et ma famille** de m'avoir toujours soutenu dans mes études et mes choix, et aussi pour mes escapades aux quatre coins de la France. Nous avons souvent été loin géographiquement, mais vous êtes là, bien présents. Et c'est pour cela qu'à chaque fois que je rentre à Guérande, j'ai l'impression de rentrer au « Nid ». Promis, je reviens vite vous voir. Merci **Basile** d'avoir littéralement TOUJOURS été présent à mes côtés, tu m'as toujours montré l'exemple et appris à prendre la vie avec légèreté. Promis, je me remets intensément aux sessions escalade et je vais retourner sur les voies noires ! Je ne te remercierai jamais assez **Sidonie** pour ton aide constante et nos appels quotidiens. Tu es vraiment un des piliers indéfectibles sur lesquels j'ai toujours pu m'appuyer. C'est quand même grâce à tes qualités d'oratrice et tes conseils que j'ai pu être finaliste régional pour MT180 ! Promis, je parlerai plus lentement ! Merci **Benoît** d'avoir engagé mon premier pas dans la recherche. Une pensée à ma famille d'Orange : **Michel et Martine**, « ma maman du sud », qui ont été si présents lors de mes arrivées du jour au lendemain. Merci à **mes**

grands-parents et à mes cousins Desclos le Peley, Boisguerin / Sauveroché & co. C'est là que l'on se rend compte de l'importance de la famille. Promis, je serai là aux prochaines cousinades.

Comme une abeille en présence de néonicotinoïde, j'ai la mémoire qui flanche et j'ai forcément oublié quelqu'un. Je m'en excuse. Ces dernières années, mon esprit a été intensément sollicité. Ces quelques mots me semblent trop pâles en regard de toute la gratitude que je ressens pour vous qui me montrez que c'est ensemble que l'on avance. Pour rester dans le champ lexical de l'aventure et comme le disait un troubadour des temps modernes : « Quand tu as le désert à traverser, il n'y a rien à faire, sauf avancer ». Votre soutien et nos rencontres sont pour moi de véritables petites oasis de fraîcheur croisées sur le chemin.

PS : Une dernière pensée ira **aux abeilles**. J'ai été un tortionnaire pour environ 10.000 d'entre vous au cours de ces années, l'équivalent d'un essaim. J'espère que vous considérez que ce sacrifice n'aura pas été vain et que le travail accompli est à la hauteur.

Table des matières

Résumé.....	6
Summary	7
Remerciements.....	10
Introduction générale.....	20
Les généralités sur <i>Apis mellifera</i>	20
L'importance de l'abeille mellifère	20
La biologie de l'abeille mellifère.....	21
Les multiples facteurs de stress	24
L'exposition des abeilles mellifères aux pesticides	27
Les voies d'exposition des abeilles	27
Les matrices contaminées.....	29
Les effets sublétaux sur les abeilles	32
Les effets physiologiques	33
Les effets cognitifs.....	35
Les effets comportementaux	36
Les pesticides	38
Les généralités	38
Les éléments inertes	39
Les fongicides	41
Les insecticides	46
Généralités sur les tests réglementaires	50
Problématique et objectifs de la thèse	53
Chapitre 1 - De la naissance, à la mort : Stratégie de surveillance individuelle et globale	56
Avant-propos	56
Les objectifs	57
Le résumé des résultats	58
Publication	59
Chapitre 2 - De l'individu à la colonie : évaluation des effets d'un fongicide sur l'activité et impact sur la colonie	82
Avant-propos	82
Les objectifs	82
Le choix des doses d'exposition	84
Le résumé des résultats	84
Publication	85

Chapitre 3 - de l'individu à la molécule : Caractérisation de l'impact physiologique d'un fongicide.....	110
Avant-propos.....	110
Les objectifs	110
Le résumé des résultats	112
Publication.....	112
Chapitre 4 - De la larve à l'abeille : l'effet des pesticides en interaction avec un pathogène	134
Avant-propos.....	134
Les effets sublétaux du pyriproxyfène	134
Le choix des doses d'exposition.....	135
Le résumé des résultats	136
Publication.....	136
Chapitre 5 - De la molécule à la colonie : évaluation de l'interaction entre insecticides et fongicides	162
Avant-propos.....	162
Les objectifs	162
Le choix des doses d'exposition.....	164
Le résumé des résultats	164
Publication.....	165
Discussions et perspectives	194
Discussion générale	194
Mise en place d'une stratégie de surveillance globale des abeilles mellifères	194
Évaluer les effets d'un fongicide sur l'abeille mellifère	196
Évaluer les interactions entre insecticides, fongicides et pathogènes sur l'abeille mellifère.....	199
Perspectives.....	205
Consolider les tests en laboratoire	205
Améliorer les tests terrain.....	206
Élargir la notion de risque.....	207
Références	214
Annexes du chapitre 1	256
Annexes du chapitre 2	268
Annexes du chapitre 3	278
Annexes du chapitre 4	288
Annexes du chapitre 5	304

INTRODUCTION GENERALE

Les généralités sur *Apis mellifera*

L'importance de l'abeille mellifère

Implication de l'abeille mellifère dans la pollinisation

La pollinisation entomophile joue un rôle d'une importance capitale dans les écosystèmes terrestres. Environ 90 % de l'ensemble des plantes à fleurs dépendent de ces pollinisateurs pour assurer leur survie (Southwick & Southwick, 1992). Ces insectes sont également vitaux pour la stabilité de la production agricole, car 75 % de nos espèces cultivées tirent des bénéfices de la pollinisation, représentant ainsi un service mondial évalué à 153 milliards de dollars pour la production alimentaire (Gallai et al., 2009 ; Klein, 2003). En Europe, on estime que 84 % des 264 plantes cultivées bénéficient de la pollinisation animale (Williams, 1994).

Les abeilles mellifères, *Apis mellifera*, assurent une partie de la pollinisation des plantes sauvages et cultivées et apportent ainsi une contribution significative aux services écosystémiques et à la biodiversité florale (Klein et al., 2007). Elles constituent la principale espèce responsable de la pollinisation par les abeilles dans le monde. Au Royaume-Uni, elles réalisent 34 % des demandes de services de pollinisation (Breeze et al., 2011 ; Carreck & Williams, 1998). Aux États-Unis, elles réalisent un rôle indispensable dans la stabilité de la production agricole et de la sécurité alimentaire, contribuant à hauteur de 17 milliards de dollars à l'amélioration de la qualité et de la quantité des cultures grâce à leurs services de pollinisation (Calderone, 2012).

Économie autour de l'abeille mellifère

Apis mellifera est considérée comme une espèce semi-domestiquée. Dans la majorité des cas, les apiculteurs ne contrôlent pas entièrement la reproduction des reines et ces dernières fécondent naturellement hors des ruches (Pernal, 2021). Cependant, l'apiculture moderne a introduit des pratiques de reproduction contrôlée, telles que l'insémination artificielle des reines pour sélectionner des caractéristiques spécifiques dans les colonies (Gillard & Oldroyd, 2020). En 2022, le nombre d'apiculteurs déclaré est de 62 784 en France (Avelin, 2024). Outre le service de pollinisation apporté par les abeilles mellifères, les ressources issues de la ruche ont des intérêts économiques.

Le miel est la ressource apicole la plus commercialisée pour un prix de vente moyen de 11 dollars par kg qui est relativement stable (Wilson-Rich et al., 2014). En France, la production de miel était estimée à 31 387 tonnes en 2022 (Avelin, 2024). Les autres produits de la ruche comme la cire, le pollen, la propolis, le venin, sont régulièrement utilisés dans des domaines très diversifiés allant de l'art à l'industrie cosmétique, pharmaceutique ou alimentaire (Wachkoo et al., 2024).

La biologie de l'abeille mellifère

Un insecte qui vit en société

Une colonie d'abeilles est une société à part entière, comprenant plusieurs milliers d'individus, estimés entre 20 000 et 30 000 (Goût, 2008). Les abeilles mellifères sont considérées comme des insectes sociaux en raison de leur mode de vie caractérisé par une coopération au sein du nid, notamment par la répartition des tâches liées à la protection, à la reproduction, au recrutement des ressources, à l'élevage des individus immatures (Michener, 1974). Ce nid est un environnement clos où les individus collaborent pour maintenir des conditions internes optimales en termes de température et d'hygrométrie, indépendamment des conditions extérieures (Seeley, 1989). Chez l'abeille mellifère, comme chez de nombreuses espèces sociales, différentes castes composent une colonie.

Les castes sexuelles au sein de la colonie

Le terme « caste » est fréquemment utilisé pour décrire des groupes d'individus spécialisés dans une fonction spécifique. Dans une colonie d'abeilles mellifères, on observe un système de détermination sexuelle haplodiploïde et ainsi trois castes distinctes :

- Les mâles, également appelés « faux bourdons », proviennent d'ovules non fécondés. Leur principale fonction est la fécondation des reines vierges.
- La reine provient d'un gamète fécondé et est dotée d'organes reproducteurs pleinement fonctionnels. Elle est responsable du renouvellement et de la cohésion de la colonie.
- Les ouvrières proviennent de gamètes fécondés, mais ne sont pas développées sexuellement. Elles sont quand même capables de pondre des œufs non fécondés qui se développent toujours en faux bourdons.

Au sein d'une colonie, les ouvrières n'ont pas la même parenté car la reine stocke le sperme des différents mâles qui l'ont fécondée. Elle utilisera tout au long de sa vie le sperme stocké dans sa spermathèque pour féconder ses œufs. Une reine peut pondre de 1500 à 2000 œufs par jour à certaines périodes de l'année, qui seront pour la majorité fécondés et donneront naissance à des ouvrières (Le Conte, 2002).

Le cycle de développement larvaire des ouvrières

La reine dépose un œuf fécondé (0,5 x 1,5 mm) de couleur blanc nacré dans une alvéole dont la taille est spécifiquement adaptée au bon développement de l'ouvrière. L'œuf est fixé grâce à une sécrétion semblable à de la colle et se dissout au bout de trois jours (Fig. 1). Ainsi, la petite larve devient active et est nourrie jusqu'au sixième jour par les abeilles participant au soin du couvain (Winston, 1987). Les larves reçoivent une alimentation spéciale, produite par les ouvrières, composée d'une combinaison de sécrétions issues des glandes hypopharyngiennes (riches en protéines) et des glandes mandibulaires (principalement des lipides). La composition de cette nourriture influe sur le développement ultérieur de l'ouvrière (Yadav et al., 2017). Au dixième ou onzième jour, la larve s'allonge dans la cellule avec la tête tournée vers l'extérieur et les alvéoles sont operculées par les abeilles ouvrières (Fig. 1). La larve entame alors le stade nymphal qui dure environ une dizaine de jours. Pendant ce temps, la nymphe subit plusieurs mues successives. Ses pièces buccales, sa tête, son thorax, son abdomen, ses yeux, ses antennes et tous les tissus internes (i.e. les muscles et les précurseurs de l'aiguillon) se développent et subissent des changements massifs. Vers le vingtième jour, la jeune abeille ouvrière utilise ses mandibules pour perforer l'opercule de sa cellule. Une fois émergée, l'ouvrière déploie ses ailes et ses antennes, laissant sécher les poils de son corps. Une fois parvenue à son développement final, elle commence à effectuer diverses tâches spécifiques au sein de la colonie, que nous appellerons « castes comportementales » (Le Conte, 2002 ; Winston, 1987).

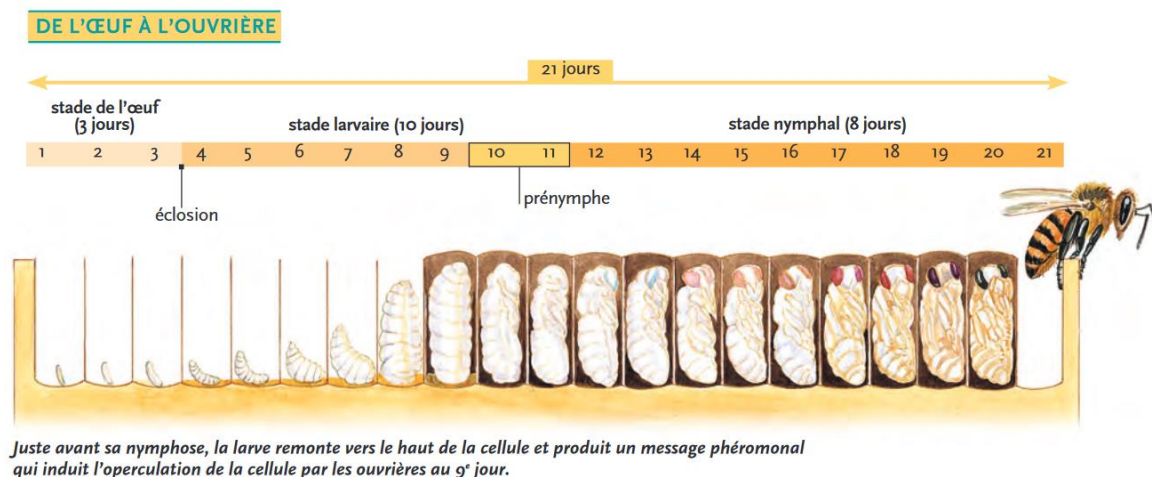


Figure 1 : Cycle de développement de l'abeille : de l'œuf à l'ouvrière (Le Conte, 2002).

Les castes comportementales des ouvrières

Contrairement à d'autres insectes sociaux, toutes les ouvrières chez *Apis mellifera* présentent une morphologie identique (Winston, 1987). La répartition des tâches au sein de la colonie est principalement liée à l'âge des ouvrières, bien que la génétique joue également un rôle majeur dans cette division du travail (Robinson et al., 1992). Ce changement graduel de comportement au fil du temps est désigné sous le terme de « polythéisme temporel » ou « polythéisme d'âge » (Wilson-Rich et al., 2014) (Fig. 2).

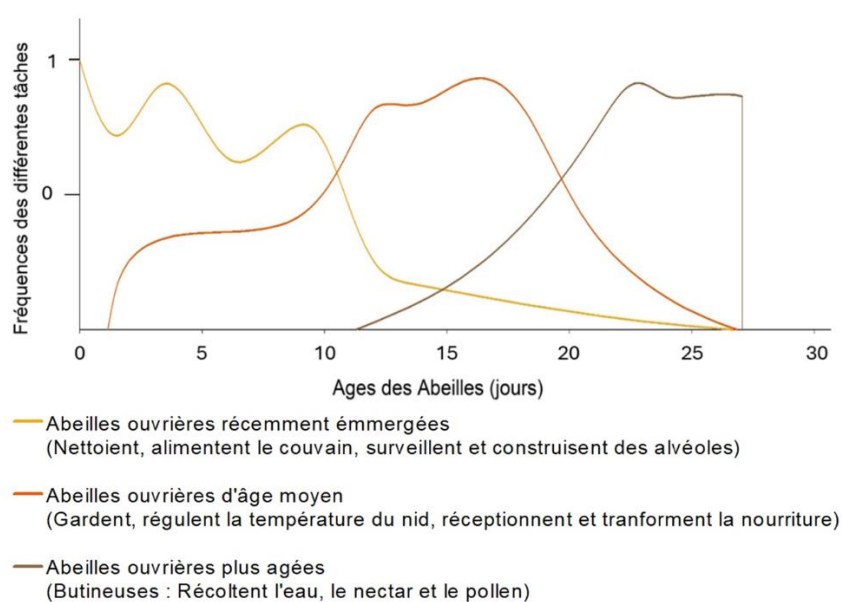


Figure 2 : Polythéisme d'âge chez l'abeilles (inspiré de Le Conte, 2002)

- Les abeilles ouvrières récemment émergées s'occupent des tâches à l'intérieur du nid, telles que le nettoyage, l'alimentation du couvain, la surveillance et la construction des alvéoles. Certaines d'entre elles prennent également soin de la reine.
- Les abeilles ouvrières d'âge moyen (environ deux semaines) s'engagent dans diverses activités à l'entrée de la colonie. Elles jouent alors le rôle de gardiennes, repoussant les abeilles étrangères et les prédateurs et régulant la température du nid. Elles réceptionnent et transforment également la nourriture pour produire du miel à partir du nectar ; du pain d'abeille à partir du pollen ; de la propolis à partir de la résine ou encore elles sécrètent de la cire à partir de leurs glandes.
- Les butineuses, les ouvrières les plus âgées (environ trois semaines), sortent de la colonie pour récolter des ressources. Leur rôle consiste à subvenir aux besoins de la colonie en récoltant de l'eau, du nectar ou du pollen.

Le polythéisme temporel, qui guide la transition de l'ouvrière du rôle de nourrice à celui de butineuse, s'avère extrêmement adaptable. Cette transformation est particulièrement influencée par l'expression de certaines hormones spécifiques, telles que la vitellogénine et l'hormone-juvénile, et peut être modifiée par divers facteurs internes et externes à la ruche (Page et al., 2006). Parmi ces facteurs, on peut noter la dynamique de la colonie, comprenant les ressources disponibles ; la taille de la population ; la production du couvain et la santé globale de la colonie (Amdam et al., 2009 ; Guzmán-Novoa et al., 1994). Il existe également un impact significatif de facteurs environnementaux tels que la saison, la disponibilité des ressources florales, la température et les conditions météorologiques (Amdam & Seehuus, 2006).

Les multiples facteurs de stress

Perte de colonies

En dépit de l'augmentation mondiale du nombre de colonies d'abeilles gérées au cours des dernières décennies (+ 85,0 % ; 1961 à 2017 ; Phiri et al., 2022), l'état de santé des populations d'abeilles est source d'inquiétude notamment pour les apiculteurs et les scientifiques (vanEngelsdorp & Meixner, 2010). Cela découle de l'apparition d'une mortalité anormale et récurrente des colonies dans les années 2000, appelée « colony

collapse disorder » (Amdam & Seehuus, 2006 ; Evans & Chen, 2021). En 2006, les États-Unis ont observé des mortalités importantes dans les cheptels d'abeilles mellifères lors de la période hivernale. Actuellement, des plans de surveillance sont en place dans de nombreuses régions du monde afin d'étudier les pertes de colonies : en Europe (Chlebo & Čápek, 2016 ; Gray et al., 2023 ; Kagiali et al., 2023) ; en Amérique du Nord et du Sud (Medina-Flores et al. 2023 ; Requier et al. 2023) ; en Asie (Tang et al., 2020, 2023) ; en Afrique (Pirk et al., 2014) et en Océanie (Brown et al., 2018 ; Kviesis et al., 2020). Ainsi, ces plans ont souligné d'importantes pertes de colonies d'abeilles à l'échelle mondiale (Gray et al., 2023). Jusqu'à présent, il n'a pas été possible de déterminer de cause commune à cette surmortalité mondiale. L'hypothèse sous-jacente au déclin des abeilles implique de multiples facteurs, agissant séparément ou en combinaison.

Les facteurs biotiques

L'introduction de l'abeille mellifère dans de nouveaux environnements d'une part, et les échanges commerciaux internationaux d'autre part, exposent ces abeilles à une diversité de pathogènes, parasites et prédateurs (K. M. Smith et al., 2013). Ils entraînent des conséquences néfastes sur les individus et peuvent causer un affaiblissement ou même la mort de la colonie (Hristov et al., 2020). De plus, durant ces dernières décennies, les effets non négligeables du frelon asiatique *Vespa velutina*, de l'ectoparasite *Varroa destructor* ou de la bactérie *Paenibacillus larvae* sur les populations d'abeilles mellifères ont été reportés (Brødsgaard et al., 1998 ; Goulson et al., 2015). Les conséquences de ces différents pathogènes et parasites peuvent être concomitantes, comme l'impact de *Varroa destructor* qui augmente l'intensité de l'infection du *Deformed wing* (Martin et al., 2012) (Fig. 3).

La loque américaine

La loque américaine est une maladie qui touche spécifiquement le couvain par la bactérie Gram-positive *Paenibacillus larvae* (*P. larvae*), en forme de bâtonnet et formant des spores (Genersch, 2010b). Les spores de *P. larvae* se propagent facilement lors de l'alimentation des larves par les nourrices et elles infectent les larves d'abeilles âgées de deux à trois jours. Sa colonisation est rapide et dix spores peuvent tuer une larve (Lindström, 2008). Les spores germent dans l'intestin de la larve et prolifèrent massivement pendant plusieurs jours. À un stade ultérieur, *P. larvae* va

pénétrer dans les cellules épidermiques, produisant une septicémie et provoquant finalement la mort. Dans le dernier cas, les cadavres sont digérés par des cellules bactériennes végétatives et transformés en écailles séchées contenant des millions de spores de *P. larvae* (Brødsgaard et al., 1998 ; Cornman et al., 2013 ; Hansen & Brødsgaard, 1999). Les symptômes cliniques les plus évidents dans les colonies s'appuient sur l'observation de cellules operculées ou non dispersées de façon irrégulière sur les cadres, d'opercules sombres enfoncés et souvent perforés dégageant une odeur fétide et de résidus de colle brune des larves mortes formant un fil filandreux au fond des cellules (de Graaf et al., 2006). Les effets synergiques entre pesticides et pathogènes doivent être pris en compte pour leur fort impact sur la santé des individus. En effet, la modification physiologique des larves lors de l'exposition aux pesticides pourrait faciliter la transmission et la virulence de pathogènes, affaiblissant le système immunitaire de l'abeille (Alaux et al., 2010). Par exemple, des effets synergiques ont déjà été démontrés, notamment chez des abeilles exposées au boscalid, puis infectées par *Nosema ceranae* (Herrera López et al., 2016). Néanmoins, ces interactions entre *P. larvae* et les pesticides restent pour le moment peu étudiées chez l'abeille. En revanche, il a été démontré que *P. larvae* n'avait pas d'effet synergique avec le thiaméthoxame pour ce qui concerne la mortalité et la cognition des abeilles après une exposition larvaire (Papach et al., 2017). Cependant, des doses sublétales de diméthoate ou de clothianidine et *P. larvae* entraînent une mortalité significativement plus élevée que la somme attendue des facteurs individuels et induisent une réduction drastique du nombre de cellules hématocytaires (López et al., 2017).

Les facteurs abiotiques

Les effets du changement climatique sur les pollinisateurs sont en partie connus. Il est prévu que les événements climatiques extrêmes tels que les tempêtes, les canicules ou les sécheresses soient plus fréquents (IPCC, 2018). Cela va induire une divergence entre la phénologie des pollinisateurs et celle des plantes que ces derniers pollinisent (Flores et al., 2019). La hausse de la température aura un effet direct sur la biologie des abeilles mellifères, en affectant négativement leur thermorégulation et perturbant ainsi la croissance et la survie des colonies (Cunningham et al., 2022). Enfin, il est certain que le changement climatique agit sur la répartition des espèces exotiques

envahissantes, comme le frelon asiatique *Vespa velutina* qui affecte les populations des pollinisateurs (Cornelissen et al., 2019).

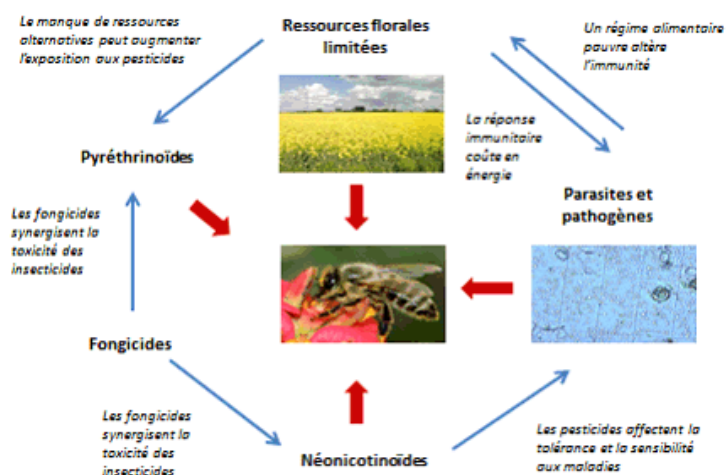


Figure 3 : Le déclin des abeilles est dû à de multiples stress (Goulson et al. 2015)

Des changements à plus large échelle affectent les capacités immunitaires de l'abeille mellifère et son aptitude à résister aux déclin. Plusieurs facteurs entraînent une diminution de la diversité et de la densité florale. On peut citer : l'urbanisation ; l'intensification de l'agriculture ; l'augmentation de la superficie des monocultures et la perte de l'habitat naturel (Theodorou et al., 2020). Les paysages d'agriculture intensive soumettent les abeilles à des périodes de pénurie à certaines périodes de l'année, y compris en été (Requier et al., 2015). Ces diminutions de la qualité nutritive des ressources et les pénuries de ressources vont avoir des effets négatifs sur les capacités et l'état de santé global des colonies (Alaux et al., 2017 ; Barascou et al., 2021).

L'exposition des abeilles mellifères aux pesticides

Les voies d'exposition des abeilles

Du fait des pratiques apicoles et agricoles, les abeilles mellifères sont soumises à diverses formes d'exposition aux pesticides qu'il faut différencier. D'une part, il y a les expositions volontaires : en tant qu'espèce en partie gérée par l'homme, les abeilles mellifères subissent un certain nombre de pratiques apicoles incluant la mise en place de traitements sanitaires destinés à lutter contre les parasites (Higes et al., 1999) ;

(Fig. 4). Ainsi, contre le *Varroa destructor*, les apiculteurs français emploient des acaricides tels que l'Apivar, basé sur la molécule chimique amitraz (Faugère & Dussy, 2021). D'autre part, les expositions accidentelles provenant en grande partie des pratiques agricoles intensives ont démontré que l'utilisation massive de pesticides dans l'agriculture moderne contribue significativement aux pertes d'abeilles (Gill et al., 2012 ; Goulson et al., 2015 ; Johnson et al., 2012 ; Sanchez-Bayo & Goka, 2014 ; Steinhauer et al., 2018).

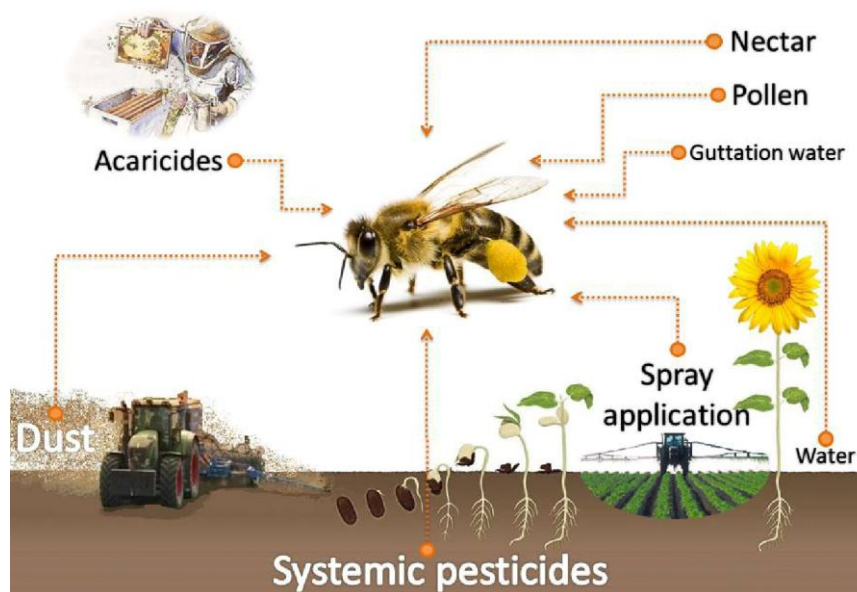


Figure 4 : Les différentes voies d'exposition des abeilles aux pesticides (T. Kiljanek, 2016)

Dans le paysage agricole, les abeilles sont exposées aux pesticides par de multiples voies. Cette exposition diffère en fonction des méthodes d'application, des systèmes de culture, des propriétés physico-chimiques des pesticides et de l'écologie des abeilles (EFSA, 2023). Deux types d'exposition sont caractérisés à la suite du traitement systémique des semences et de l'application des pesticides systémiques sur les cultures. La première est l'exposition par contact et par inhalation qui se produit au moment de l'application ou via la dérive de poussières associée à la plantation de semences traitées aux pesticides (EFSA, 2012). La seconde voie d'exposition passe par le biais de l'alimentation, lorsque les abeilles consomment du pollen et du nectar contaminés. C'est la principale voie d'exposition des abeilles ouvrières (Krupke et al., 2012). Ainsi, ces molécules présentes dans le régime alimentaire contaminent les aliments transformés pour abeilles (i.e le pain de pollen, la gelée ouvrière) (Böhme et al., 2019). La consommation de liquides non nectarifères tels que l'eau des flaques

contaminées est également une voie d'exposition des abeilles (Gradish et al., 2016) (Fig. 4). Des études récentes montrent que les pesticides peuvent se retrouver dans toutes les matrices des ruches (i.e. cire, miel...). Ainsi, au cours de sa vie, une abeille sera exposée à un cocktail de pesticides par de multiples voies, augmenté par les effets potentiellement synergiques de ces pesticides (Chauzat et al., 2006 ; Tosi et al., 2022 ; Willow et al., 2019).

Les matrices contaminées

Les ressources

En collectant des ressources contaminées dans l'environnement (i.e. pollen, nectar, eau de guttation...), les abeilles ouvrières introduisent de nombreux résidus de pesticides au sein de la ruche. Les abeilles sont notamment très attirées par le nectar et le pollen de grandes cultures et de vergers, tels que le colza, le tournesol, les haricots, les pommes, les abricots... (USDA, 2017). Même si l'exposition des abeilles provient majoritairement des nectars et des pollens de plantes cultivées, ceux de la flore sauvage peuvent aussi être contaminés (Végh et al., 2023). La concentration et les combinaisons de pesticides retrouvées dans le pollen varient également en fonction de l'intensité agricole du site, de l'année d'étude et de la période de floraison des cultures (Böhme et al., 2018 ; Chauzat et al., 2006 ; Friedle et al., 2021). Ainsi, dans des paysages de grande culture européens, une centaine de résidus de substances actives différentes a été détectée dans le pollen et le nectar collectés par les abeilles (Cappellari et al., 2024). En France, sur 36 pesticides recherchés dans les pollens collectés, 19 ont été retrouvés et seulement 12 % des échantillons ne contenaient aucune des molécules recherchées (Chauzat et al., 2006). Les résidus contenus dans le pollen et le nectar peuvent être transformés et/ou métabolisés par les abeilles à l'intérieur et à l'extérieur de la ruche. Ces processus complexes ne sont actuellement pas bien compris. En outre, ces résidus peuvent contaminer d'autres matrices au sein de la ruche (Fig. 5).

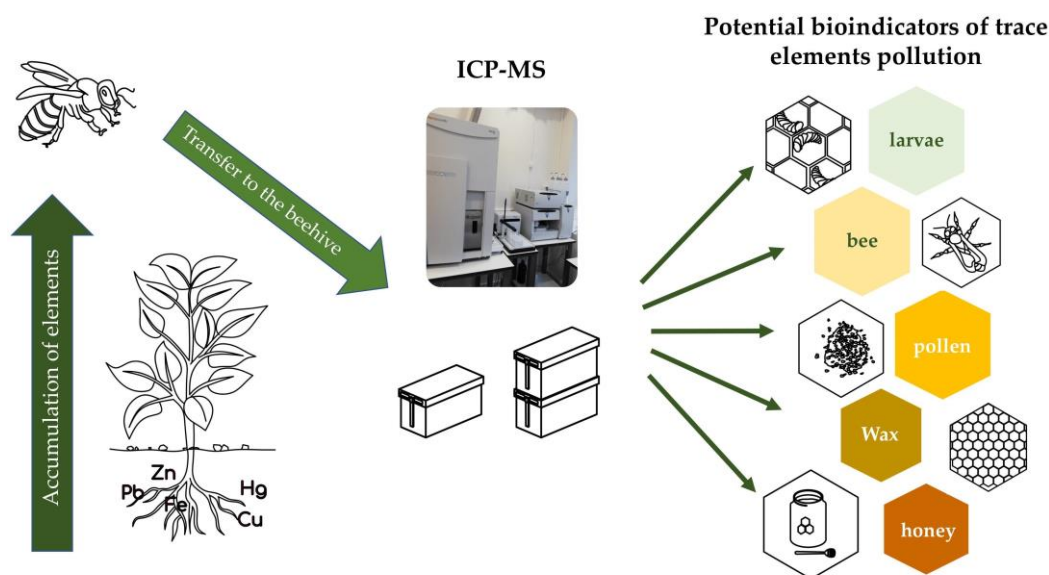


Figure 5 : Contamination des matrices de la ruche (modifié de Zafeiraki et al., 2022)

La cire

La cire d'abeille est un mélange complexe de composés principalement très lipophiles. Elle est utilisée par les abeilles dans la ruche pour construire les rayons de miel. Ainsi, la cire conserve les pesticides avec lesquels la ruche a été en contact (Daniele et al., 2018). C'est grâce aux propriétés physicochimiques particulières de la cire que les résidus de pesticides peuvent s'y accumuler (Herrera López et al., 2016). En France, 61 % des échantillons de cire récoltés contenaient des résidus de pesticides (Daniele et al., 2018). Étant généralement recyclée dans de nouvelles ruches, la cire d'abeille peut être, à long terme, une source de contamination dans les ruchers des apiculteurs (Martel et al., 2007). Cette contamination peut également être transférée à d'autres produits apicoles (i.e. gelée royale et gelée des ouvrières) avec lesquels elle entre en contact et induire un risque pour les abeilles, notamment lors du stade de développement larvaire (Raimets et al., 2022 ; Végh et al., 2023). Plusieurs études récentes utilisant des cires contaminées relèvent des mortalités sur le couvain et des effets sublétaux à long terme chez les reines (Alkassab et al., 2020 ; Milone & Tarpy, 2021). Pour le moment, la toxicité induite par les résidus dans les cires n'est pas testée lors de la mise sur le marché d'un nouveau pesticide. Néanmoins, un modèle expérimental *in vitro* a récemment été élaboré pour tester le risque des résidus de pesticides dans la cire d'abeille pour les larves d'ouvrières (Kast & Kilchenmann, 2022).

La propolis

La propolis est un produit résineux récolté sur les bourgeons mélangés à la cire et aux sécrétions salivaires des abeilles. Elle contient également d'autres éléments chimiques comme des acides organiques, des flavonoïdes, des oligo-éléments, de nombreuses vitamines et du parabène. Elle est utilisée par les abeilles comme substance antimicrobienne ainsi que pour réparer les fissures et renforcer les parois de la ruche (Naik et al., 2021). Aucune donnée n'est disponible à ce jour sur l'état de contamination de la propolis par les pesticides en France. En revanche plusieurs études ont signalé l'absence ou de faibles concentrations de résidus de pesticides dans de la propolis mesurée en Jordanie (Naik et al., 2021). Ces résidus détectés peuvent être liés aux acaricides utilisés pour traiter les ruches et à d'autres pesticides provenant du paysage agricole (Naik et al., 2021 ; Wang et al., 2020). Ainsi, les abeilles peuvent être exposées de façon chronique aux résidus de pesticides présents dans la propolis puisque ce produit est utilisé comme matériau de construction dans la ruche (Tomšič et al., 2020 ; Wang et al., 2020). À notre connaissance, aucune étude n'a spécifiquement observé les effets de la contamination de la propolis sur la santé des abeilles.

Le miel

Le miel est une substance naturelle sucrée produite par les abeilles mellifères à partir du nectar des fleurs, de sécrétions des parties vivantes des plantes et de l'excrétion d'insectes suceurs de plantes sur les parties vivantes des plantes (Kadri et al., 2017). C'est un mélange complexe d'hydrate de carbone, de protéines, d'acides aminés, d'enzymes, de vitamines, de lipides, de minéraux, d'acides phénoliques, de substances chimiques volatiles et de flavonoïdes (Avni et al., 2014). En France, 95,7 % des échantillons de miel récolté contenaient des résidus de pesticides (Lambert et al., 2013). Cette forte présence de résidus de pesticides dans le miel suggère un risque pour la santé humaine en cas de consommation (El-Nahhal, 2020). Les abeilles s'en servent également pour leur alimentation.

Le pain d'abeilles

Le pain d'abeilles est un mélange de pollen, de miel et d'enzymes digestives, fabriqué par les abeilles mellifères et naturellement fermenté dans la ruche. Il est composé d'une large gamme de nutriments : protéines, acides aminés, acides gras, hydrates de

carbone, sels minéraux, polyphénols et flavonoïdes qui dépendent de la source botanique du pollen d'abeille (Urcan et al., 2017). En France, une étude a montré que 77 % des échantillons de pain d'abeilles étaient contaminés au moins par un des pesticides ciblés. Cette matrice est également la plus contaminée en termes de nombres de composés retrouvés par échantillon (Daniele et al., 2018).

La gelée royale et la gelée des ouvrières

La gelée royale est produite par les glandes hypopharyngiennes et mandibulaires tandis que la gelée ouvrière reçoit en plus des grains de pollen provenant du jabot de la nourrice (Rembold & Dietz, 1966). Les principaux composants de la gelée des ouvrières sont les glucides, les lipides et les protéines (Ramadan & Al-Ghamdi, 2012). L'ajout du pollen couvre notamment les apports en protéines, en glucides et en vitamines pour le bon développement des larves. Ces pollens sont la principale source de contamination de la gelée ouvrière (Böhme et al., 2019 ; Rembold & Dietz, 1966).

Les effets sublétaux sur les abeilles

Un effet subléthal est un effet qui n'entraîne pas la mort, mais provoque des modifications aux niveaux biologique, physiologique, démographique ou comportemental chez un individu ou une population (EFSA, 2024). L'abeille mellifère est une espèce sociale et la survie de la colonie dépend de l'interaction et des efforts communs des individus. Ainsi, les effets sublétaux ont des impacts importants et subtils à différentes échelles (Belzunces et al., 2012).

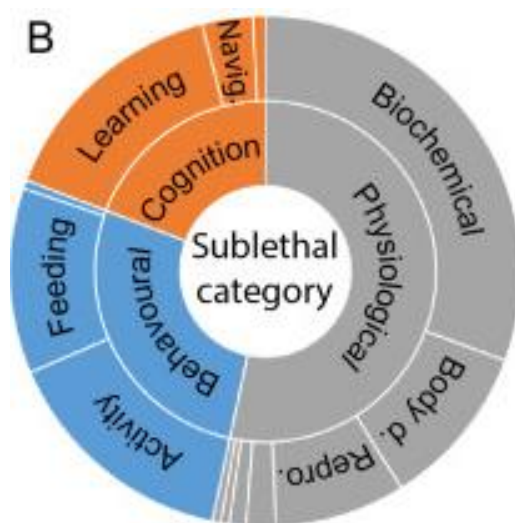


Figure 6 : Classification des effets sublétaux sur les abeilles (Tosi et al. 2022)

Les études sur les effets sublétaux et combinés sur les abeilles sont un domaine relativement nouveau. Les premières études publiées ont été réalisées dans les années 70, mais 75 % des travaux datent de moins d'une dizaine d'années (Tosi et al., 2022 ; Fig 6). Bien que diverses méthodes de détection des effets sublétaux aient été mises au point, elles ne sont prises en compte que de manière marginale dans les évaluations des risques (EFSA, 2023). Cela est notamment dû à une absence de normalisation et de classification de ces effets sublétaux. C'est pourquoi nous nous appuyons sur les travaux de Tosi et al. 2022 pour les décrire. Les effets sublétaux peuvent s'exercer à différents niveaux. Tout d'abord, par un impact sur la physiologie des individus, puis sur la cognition, et enfin sur le comportement. Les effets sublétaux observés sur le plan individuel entraînent des conséquences létales sur le plan social (Henry et al., 2012).

Les effets physiologiques

Les effets sublétaux sont étudiés sur la physiologie des abeilles, et également sur le rôle, le fonctionnement et l'organisation mécanique, physique et biochimique des individus et de leurs composants (organes, tissus, cellules et organites cellulaires). Cette catégorie représente plus de la moitié des études menées sur les effets sublétaux des pesticides (Tosi et al., 2022). Elles jouent un rôle extrêmement important dans la compréhension des impacts non spécifiques et mécanistiques des pesticides. Néanmoins, la majorité des données produites par la recherche universitaire ne sont pas prises en compte lors de la validation ou la réévaluation de la mise sur le marché des pesticides (EFSA, 2023). Le premier niveau d'études est la détection des modifications de l'activité biochimique de l'organisme face à un stress. Il est par exemple possible d'observer chez les abeilles les effets des pesticides en étudiant l'expression de certains gènes, la production de certaines protéines ou encore l'activité de certaines enzymes. Il est aussi possible de regrouper ces analyses en fonction du rôle fonctionnel de certains organes ciblés par ces stress chez l'abeille mellifère.

La neurophysiologie

Les paramètres neurophysiologiques sont impactés par les pesticides, notamment en entraînant des altérations du système nerveux avec, par exemple, la nécrose ou l'apoptose des neurones. Cela est très observé lors de tests avec certaines classes insecticides, comme les pyréthréinoïdes ou les néonicotinoïdes, qui ciblent directement

le système nerveux des insectes (Cabirol & Haase, 2019). L'activité métabolique ou neuronale des abeilles mellifères est également observable et analysable (Belzunces et al., 2012).

Le système immunitaire

Certains pesticides agissent sur la quantité ou la densité des hémocytes, la réponse d'encapsulation et l'activité ou la production de peptides antimicrobiens. Ces altérations du système immunitaire et de celui de la détoxification rendent plus sensibles les abeilles faisant face à des stress induits par les pathogènes ou parasites (Christen et al., 2016). Les pesticides étant des molécules chimiques, ils perturbent de façon récurrente les voies métaboliques associées à la désintoxication. Par exemple, le glyphosate entraîne une régulation à la hausse de l'enzyme glutathion SS-transférase ou, au contraire, baisse l'activité enzymatique de la superoxyde dismutase (Chen et al., 2023).

L'activité musculaire

Certains pesticides modifient l'activité musculaire des abeilles ainsi que le rythme respiratoire, la respiration mitochondriale et la contraction musculaire. L'imidaclopride en particulier agit sur la taille des glandes hypopharyngiennes et le rythme respiratoire des abeilles (Hatjina et al., 2013). Un effet significatif du boscalid sur l'activité des mitochondries a également été démontré. Par exemple, une exposition au fongicide Pristine® diminue la fréquence de battement des ailes et altère les paramètres de vol des abeilles (Desjardins et al., 2021 ; Glass et al., 2021 ; L.-H. Liao et al., 2019).

La thermorégulation

La capacité des abeilles à réguler, dissiper et produire de l'énergie est également perturbée par les pesticides. Par exemple, le thiaméthoxame bloque la thermorégulation des individus et les empêche de maintenir une température stable face à une augmentation ou une diminution de la température de l'environnement et cela à court et moyen termes (Tosi et al., 2016).

La reproduction

La capacité de reproduction des reines et des faux bourdons est affectée par une exposition à certains pesticides. Ces effets sublétaux sur ces individus sont alarmants, car seule la reine remplit les fonctions reproductives de l'ensemble de la colonie.

Notamment, il a été démontré une diminution du succès reproducteur et de la quantité de sperme dans la spermathèque des reines exposées au boscalid (Pineaux et al., 2023). Peu d'études se sont intéressées aux effets sublétaux sur les faux bourdons, néanmoins certains pesticides présents dans les cires altèrent la viabilité du spermatozoïde (Li & Rangel, 2018).

Le microbiote

Le microbiote est affecté négativement par les pesticides. Le coumaphos, le tau-fluvalinate et le chlorothalonil modifient la composition des communautés bactériennes du microbiote intestinal (Kakumanu et al., 2016). Cependant, les interactions complexes entre l'environnement, la communauté microbienne et l'hôte ne sont toujours pas claires (Engel et al., 2012). On constate néanmoins que la capacité des abeilles à métaboliser efficacement les acides phénoliques des pollens et les constituants toxiques du mannose du nectar est diminuée (Filannino et al., 2016 ; Zheng et al., 2016).

Développement de l'individu

Le développement des individus, des organes, des cellules, peut être altéré par de nombreux stress. La mise en place et la standardisation des élevages des larves *in vitro* permettent un suivi tout au long du développement des abeilles lors du stade immature (Aupinel et al., 2005). Elles permettent d'observer les étapes clés du développement larvaire (i.e. nymphose, émergence ; Czoppelt 1990 ; Dai et al. 2018 ; Yang et al. 2020), ainsi que les problèmes de développement sur les abeilles adultes. Par exemple, un mélange de six pesticides utilisés aux États-Unis réduit le poids corporel des abeilles exposées (Yao et al. 2018). La morphologie des abeilles peut être modifiée par les pesticides : le fénoxycarbe réduit la taille des acini des glandes hypopharyngiennes tandis que le boscalid et le pyraclostrobin diminuent la taille du thorax (Fisher et al. 2022 ; Glass et al. 2021).

Les effets cognitifs

L'apprentissage et la mémoire

Des méta-analyses globalisent les effets négatifs importants des pesticides sur l'apprentissage et la mémoire (Siviter et al., 2018). Précisément, des insecticides altèrent l'apprentissage olfactif et visuel (Decourtye et al., 2005 ; Delkash-Roudsari et

al., 2020, 2022). L'exposition sublétales aux pesticides affecte la mémoire et la perception des couleurs (Mayack et al., 2021), alors que les abeilles doivent apprendre la forme, la couleur et l'odeur des fleurs pour réussir à butiner (Leonard et al., 2011 ; Leonard & Masek, 2014). L'altération de cet apprentissage par les pesticides perturbe l'état de santé des colonies et réduit potentiellement leur implication dans la pollinisation (Wu et al. 2022).

La communication

Les abeilles étant une espèce sociale, différents moyens de communication sont utilisés dans la colonie que les pesticides affectent. Les ouvrières communiquant entre elles à l'aide de danses pour indiquer où se situent les ressources, leurs comportements sont perturbés par l'imidaclopride ou le clothianidine (Grüter & Farina 2009 ; Tison et al. 2020 ; Zhang et al. 2020). D'autre part, la communication entre les différents membres de la colonie passe également par l'échange de phéromones potentiellement affectées par le chlorothalonile et chlorpyrifos (Walsh et al. 2020 ; Wu et al. 2023)

L'orientation et la navigation

La capacité des abeilles à reconnaître les caractéristiques du paysage afin de s'orienter et de naviguer est altérée par certains pesticides (Decourtye et al., 2010). Cet effet sublétales a notamment été repéré pour les insecticides neurotoxiques tels que les néonicotinoïdes, ce qui a permis la mise en place de tests spécifiques du vol de retour à la ruche (Henry et al., 2012 ; OCDE, 2021). Par ailleurs, les abeilles exposées au glyphosate présentent des vols de retour perturbés, mettent plus de temps pour retrouver leur colonie et effectuent moins de vols directs vers leurs ruches en comparaison des abeilles non exposées (Balbuena et al., 2015).

Les effets comportementaux

Les expériences menées sur le comportement des abeilles représentent un peu plus d'un quart des études portant sur l'étude des effets sublétales (Tosi et al., 2022).

Les activités

Au sein de la colonie, les différentes castes vont réaliser diverses tâches au cours de leurs vies. Certains pesticides modifient ce répertoire comportemental (Tosi & Nieh, 2017). Chez les reines, ils vont notamment diminuer le succès des vols nuptiaux ou le

taux de ponte des œufs (Pineaux et al. 2023 ; Walsh et al. 2020). Chez les mâles, des néonicotinoïdes ont impacté le premier vol, ainsi que le comportement de dérive dans une autre ruche (Straub et al., 2021). Grâce au polythéisme temporel, les ouvrières possèdent un large répertoire de comportements qui peuvent néanmoins être altérés par un grand nombre de pesticides. En laboratoire, il a été constaté que les insecticides pyréthroïdes affectent la locomotion de vol et le toilettage des abeilles (Oliver et al., 2015). Dans les dernières décennies, nombre d'études ont observé les effets des pesticides sur les capacités et les activités de vol et de butinage. Cela a été facilité par l'émergence de techniques telles que la pose de puces d'identification par radiofréquence (RFID) ou de codes-barres bidimensionnels qui sont suffisamment légers et petits pour être utilisés sur les abeilles (Bagnis et al., 2010 ; Nunes-Silva et al., 2019 ; Prado et al., 2020). Ainsi ont pu être constatées une réduction de la durée de vie globale des abeilles dans les ruches ainsi qu'une diminution de leur intensité de vol et de leur activité de butinage induite par le sulfoxaflor ou le thiaméthoxame (Barascou et al. 2022 ; Prado et al. 2019).

La coordination des mouvements

Certains pesticides impactent les fonctions motrices des abeilles. Les insecticides pyréthroïdes par exemple affectent négativement la locomotion de vol et le toilettage des abeilles (Oliver et al., 2015). Le flupyradifurone quant à lui provoque des handicaps moteurs (Hesselbach & Scheiner, 2019). D'autres pesticides en mélanges agissent sur la coordination avec l'émergence de schémas stéréotypés, des mouvements erratiques ou irréguliers, ou encore l'incapacité à se tenir debout (Tosi & Nieh, 2017).

L'alimentation

La présence de pesticides dans l'alimentation impacte la consommation et l'absorption de nourriture. Par exemple, la consommation de pollen est augmentée dans les ruches exposées au boscalid ou au thiaclopride (Fisher et al., 2021). Les abeilles accordent une préférence au sirop contaminé au glyphosate, ce qui peut générer un risque plus élevé d'exposition à ce pesticide lors du butinage dans les champs traités (Liao et al., 2019).

Les pesticides

Les généralités

Les pesticides sont des substances utilisées afin de lutter contre des organismes considérés comme nuisibles. C'est un terme générique qui rassemble les insecticides, les fongicides, les herbicides et les parasitocides. C'est après la Seconde Guerre mondiale que l'utilisation de produits chimiques et d'insecticides organiques synthétiques apparaît, le premier étant le DDT (Ware, 1999). De nos jours, il existe de nombreuses familles de pesticides chimiques : les carbamates, les pyrethroïdes synthétiques, les organochlorines, les organochlorés, les organo-phosphorés et les organonitrogénés, les néonicotinoïdes... Les pesticides occupent une place particulière parmi les substances chimiques, car ils sont utilisés dans l'environnement dans le but d'éliminer ou d'endommager certains organismes. Idéalement, l'action nocive des pesticides se doit d'être hautement spécifique pour les organismes cibles et inoffensive pour ceux non ciblés. Mais la plupart des produits utilisés en tant que pesticides ne sont pas complètement sélectifs. Cette absence d'action sélective constitue un risque à la fois pour l'homme, pour la faune, la flore, les sols et les cours d'eaux. Par exemple, dans le contexte de la santé humaine, les insecticides organochlorés et organophosphorés seraient des facteurs de risque pour le cancer de la prostate (Pardo et al., 2020). Les pesticides ont également des effets délétères sur la richesse taxonomique régionale des invertébrés vivant dans les cours d'eau, en Europe et en Australie (Beketov et al., 2013). Néanmoins, en France, les conditions d'utilisation des pesticides durant la floraison ont fait l'objet d'un récent arrêté en date du 20/11/2021, visant à améliorer la protection des abeilles et des autres insectes pollinisateurs. Le texte réglementaire encadre notamment les dispositions de mise sur le marché et les conditions d'application de ces pesticides durant la floraison. Il concerne, entre autres, les grandes cultures oléoprotéagineuses attractives : colza, tournesol, féverole, pois-chiche, lin et lupin (Légifrance - JORF n° 0271 du 21/11/2021).

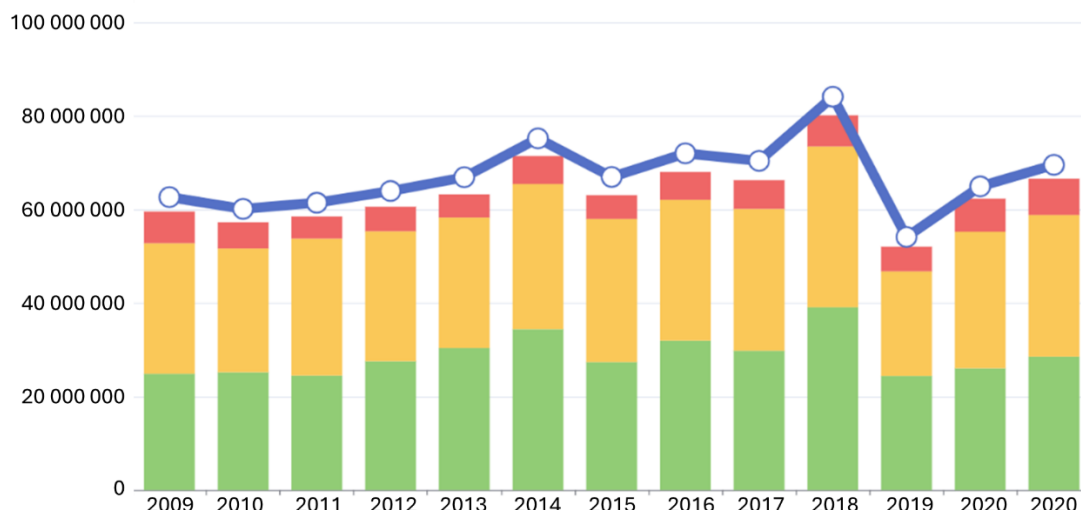


Figure 7 : Quantités totales de pesticides vendues en France de 2009 à 2021 (kg)

Vert : fongicides ; Orange : herbicides ; Rouge : insecticides

(Ministère de la transition écologique et de la cohésion des territoires, 2021)

En France, l'agriculture est, de loin, le premier utilisateur de pesticides, puisqu'elle emploie 90 % des utilisations totales (annexes Sénat, 2023). Depuis 10 ans, les quantités de pesticides vendus à l'échelle de la France sont globalement stables (Fig. 7). Néanmoins, la part de pesticides utilisable en agriculture biologique est de plus en plus importante (Ministère de la transition écologique et de la cohésion des territoires, 2021). Les pesticides utilisés pour l'agriculture sont sous la forme de formulations commerciales et sont généralement des mélanges de composants multiples. Chaque formulation comprend à la fois la substance active, des molécules volontairement toxiques pour les ravageurs et en second des coformulants afin d'améliorer les caractéristiques physiques des substances actives (US EPA, 2013).

Les éléments inertes

Ces coformulants, sont appelés « ingrédients inertes » car ils ne sont pas destinés à avoir une action antiparasitaire directe. On peut classer ces éléments inertes en trois grandes catégories en fonction de leurs rôles (Hazen, 2000) :

- Les surfactants qui facilitent la pénétration de l'ingrédient actif dans les feuilles.
- Les solvants qui contribuent à dissoudre l'ingrédient actif.
- Les émulsifiants qui assurent le maintien d'une bonne homogénéité des formulations commerciales.

L'utilisation globale des ingrédients inertes est assez opaque et, en Europe, seul leur nombre est connu sans qu'aucune donnée ne soit disponible concernant les quantités utilisées, par exemple 70 en France, 294 au Royaume-Uni (ANSES 2023, Straw et al. 2022). Toutefois, les formulations commerciales contiennent des ingrédients actifs appliqués avec des ingrédients inertes. Par conséquent, on suppose que leurs quantités sont proportionnelles, voire probablement supérieures à celles des ingrédients actifs (Straw et al. 2022).

La nature des composants inertes présents dans les solutions commerciales reste souvent confidentielle, ce qui entrave considérablement la capacité des chercheurs à comprendre leur effet sur la biodiversité et l'environnement (Regulation, 2009). Bien que désignés comme inertes, les produits étudiés ont montré que certains des additifs utilisés par les principaux fabricants de pesticides agricoles provoquent des effets délétères sur la santé et l'environnement (Cox & Sorgan, 2006 ; Pezzoli & Cereda, 2013). Contrairement aux principes actifs qui subissent une série d'essais de toxicité sur les abeilles afin d'être en conformité avec les organismes de réglementation, l'utilisation des ingrédients inertes n'exige aucun essai toxicologique. La connaissance spécifique des effets des ingrédients inertes sur les abeilles reste donc limitée, avec seulement 19 études publiées entre 1973 et 2021 (Straw et al., 2022). Certains solvants se sont avérés toxiques lors d'une application par voie topique ou administrés par voie orale (Goodwin & McBrydie, 2000). En plus des effets directs sur la mortalité, des études démontrent les effets sublétaux de ces produits, révélant des conséquences néfastes sur le comportement des abeilles mellifères. Par exemple, l'ingestion d'organosilicones et d'adjuvants tensioactifs perturbe le test du réflexe d'extension du proboscis (Ciarlo et al., 2012). De plus, la toxicité synergique des molécules actives avec les éléments inertes est reconnue, notamment pour certains herbicides (Bradberry et al., 2004 ; Eddleston et al., 2012). D'autres composés organiques volatils tels que le xylène, le toluène et l'acétone ont également été détectés dans l'environnement, soulevant des interrogations sur leurs interactions (Wang et al., 1995).

L'acétone est un ingrédient inerte couramment utilisé comme témoin dans le domaine de la recherche en écotoxicologie, car il est considéré comme ayant une faible toxicité directe sur les organismes (Dai et al., 2019 ; Kablau et al., 2023 ; Rembold et al., 1974). L'OCDE recommande empiriquement que les tests sur les abeilles incorporent

moins de 1 à 5 % d'ingrédients inertes dans le volume total de la solution d'exposition (OCDE, 1998b, 1998a, 2017a). De nombreuses études scientifiques n'évaluent pas les effets de l'acétone, car elles n'incluent pas de témoins non exposés dans leurs expériences (Ii & Rangel, 2018 ; Rinkevich et al., 2015). Cela entraîne des biais potentiels, car l'ingestion d'acétone peut provoquer des effets sublétaux et des altérations morphologiques et histochimiques. Par exemple, chez l'abeille, l'acétone n'altère pas les cellules cérébrales, mais affecte le neuropile du lobe antennaire et des cellules digestives (Oliveira et al., 2014 ; Sigg et al., 1997). De plus, l'acétone modifie le microbiote intestinal des abeilles adultes et inhibe la croissance glandulaire pendant l'exposition des larves (Quinn et al., 2018 ; Salles & Cruz-Landim, 2004).

Les fongicides

Les maladies fongiques menaçant une grande variété de cultures vivrières, l'utilisation de fongicides est considérée comme essentielle à la sécurité alimentaire mondiale (Strange & Scott, 2005). Les fongicides sont utilisés pour lutter contre les champignons pathogènes des cultures. Ils représentent 40 % du marché mondial des pesticides et leur utilisation devrait continuer à augmenter à l'avenir (Oliver & Hewitt, 2014). Cependant, le bénéfice de l'utilisation de ces produits est de plus en plus sujet à caution. De récentes méta-analyses montrent que l'usage global des fongicides contribue fortement à l'écotoxicité des eaux douces, à l'écotoxicité terrestre, à la toxicité humaine et à la sélection de pathogènes multi-résistants (Bastos et al., 2021 ; Fisher et al., 2018 ; Tsalidis, 2022).

Les fongicides ciblent théoriquement des mécanismes moléculaires spécifiques des champignons et, chez les abeilles, leur DL50 est généralement élevée par voie orale et par contact (Rondeau & Raine, 2022). Ils sont considérés par la réglementation comme plus sûrs pour les abeilles et peuvent même être pulvérisés lorsque les cultures sont en fleurs et attractives pour les pollinisateurs (Gierer et al., 2019). Ainsi, des résidus de fongicides sont fréquemment retrouvés dans les matrices de la ruche : miel, cire d'abeille, pollen, gelée d'ouvrière ou de reine... (Böhme et al. 2018 ; Friedle et al. 2021). Néanmoins, ces ingrédients actifs peu toxiques présentent des risques chroniques pour les abeilles en raison de leur forte présence dans l'environnement (Krupke et al. 2012 ; Tsvetkov et al. 2017). Des études récentes rapportent des impacts à la fois létaux et sublétaux à la suite de l'exposition à certains fongicides et

de probables synergies notamment avec des insecticides (Belsky et al. 2022 ; Cullen et al. 2019).

Le *Fungicide Resistance Action Committee* (FRAC) a référencé les modes d'action des fongicides et en dénombre actuellement quatorze différents. Ils sont classés selon les dix grands types de cibles physiologiques. Je me limiterai ici à la description des modes d'action qui concernent seulement deux types de cibles physiologiques : les inhibiteurs de la respiration et les inhibiteurs de la biosynthèse des stéroïdes.

Le boscalid

Le premier pesticide qui nous intéresse est le boscalid qui appartient à la famille des Carboxamides (Fig. 8). C'est un inhibiteur de la succinate déshydrogénase (SDHI) dont les premières formulations ont été commercialisées dans les années 60. La succinate déshydrogénase est essentielle à la chaîne respiratoire mitochondriale des organismes fongiques (Stammler et al., 2008). Parmi les différents fongicides utilisés en production agricole conventionnelle, le boscalid, commercialisé pour la première fois par BASF en 2002, est largement utilisé en raison de son large spectre d'action et de son efficacité contre les maladies fongiques (Li et al., 2021).

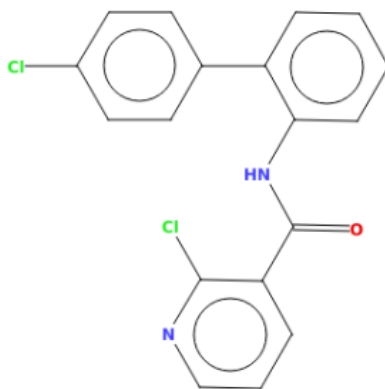


Figure 8 : Molécule du boscalid (Cambridge Crystallographic Data Centre diagrams)

En France, cette molécule active se retrouve seule ou en mélange dans 47 produits commerciaux couverts par la réglementation française (ANSES, 2023). Plusieurs des formulations commerciales de boscalid sont couramment appliquées aux cultures de pois, de haricots et de colza après l'émergence de la première fleur (BASF France SAS, 2022). Les abeilles sont donc exposées au boscalid par le biais de son utilisation dans les cultures, mais il semble également persister dans l'environnement puisqu'il a été retrouvé dans des fleurs sauvages et dans des champs, même certaines années

où il n'avait pas été appliqué (Daniele et al., 2018 ; Zioga et al., 2023). De plus, le boscalid est l'un des fongicides les plus fréquemment détectés dans les différentes matrices de la ruche (Böhme et al., 2018 ; Chauzat et al., 2006 ; Friedle et al., 2021).

Ces dernières années, des études ont révélé que l'exposition au boscalid peut avoir divers effets sur la physiologie des abeilles, touchant toutes les castes. L'exposition des larves à une formulation commerciale du boscalid a induit une réduction de la taille du thorax des abeilles adultes (Glass et al., 2021). De plus, cette exposition est associée à une diminution de la longévité des adultes en laboratoire et sur le terrain, bien que cela ne soit pas observable par les tests écotoxicologiques classiques (Fisher et al., 2022 ; Simon-Delso et al., 2018). Chez *Apis cerana*, le boscalid augmente les transcriptions de gènes associés à la réponse immunitaire et du métabolisme, il provoque des lésions de l'intestin moyen et un déséquilibre du microbiote intestinal des butineuses (Dong et al., 2023 ; Huang et al., 2023). La cognition et les capacités des abeilles sont altérées par une formulation commerciale contenant du boscalid (le Pristine®), ce qui diminue les performances d'apprentissage et la fréquence des battements d'ailes (DesJardins et al., 2021 ; Liao et al., 2019). De plus, ce produit modifie le comportement des abeilles, en induisant une transition plus précoce vers la recherche de nourriture chez les ouvrières (Fisher et al., 2021). Chez les reines, le boscalid diminue l'expression du gène de la vitellogénine, le nombre de spermatozoïdes stockés dans les spermathèques et augmente la mortalité des reines pendant et peu après la période des vols nuptiaux (Pineaux et al., 2023). De plus, l'exposition des reines au boscalid a des conséquences néfastes sur les colonies en ce qui concerne la production de couvain, l'infection par *Varroa destructor* et le stockage de pollen, mais est sans effet sur le stockage de nectar ou la taille de la population (Pineaux et al., 2023). Il est également intéressant de se concentrer sur les formulations commerciales du boscalid, car certaines produisent des effets négatifs sur la mémoire et cet effet n'était pas constaté chez les abeilles nourries avec les molécules brutes (DesJardins et al., 2023).

Les triazoles

Dans un second temps, je me suis intéressé aux pesticides appartenant à la famille chimique des triazoles, en particulier le prothioconazole qui appartient au groupe des triazolinethiones et le tébuconazole qui agissent contre les champignons en perturbant la membrane cellulaire fongique. Ils vont notamment inhiber la biosynthèse de

l'ergostérol, précurseur de la vitamine D2, qui est un composant de la membrane (Wang et al., 2020) (Fig. 9). Le tébuconazole et le prothioconazole ont été respectivement introduits sur le marché en 1986 et 2002 par Bayer AG, (Lewis et al., 2016). Ils ont rapidement gagné en popularité en raison de leur large spectre d'action contre de nombreuses maladies fongiques et sont destinés aux grandes cultures (i.e. avoine, blé, épeautre, cameline, colza...).

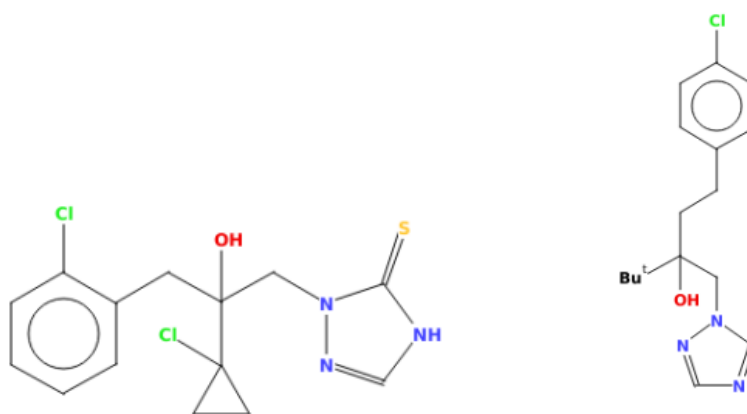


Figure 9 : Molécules du prothioconazole (gauche) et du tébuconazole (droite)
(Cambridge Crystallographic Data Centre diagrams)

Ces deux molécules sont les fongicides les plus appliqués sur les cultures dans certaines régions d'Allemagne centrale (Tauchnitz et al., 2020). En France, les ventes de tébuconazole en 2018 sont de 833 tonnes et ont doublé depuis 2011 (ANSES, 2023). La progression de l'utilisation du tébuconazole serait la conséquence de son arrivée dans le domaine des produits phytosanitaires génériques (Brigon & Lenoble, 2020). Comme le boscalid, ces fongicides triazoles sont considérés comme sûrs pour les abeilles du point de vue de la réglementation et peuvent être appliqués lorsque les cultures sont en fleurs et attractives pour les pollinisateurs (ANSES, 2023). De plus, les fongicides triazoles sont également des composés plutôt lipophiles et modérément persistants dans l'environnement (Tomlin, 1997). À cause de la résistance à l'hydrolyse et à la photolyse, ils possèdent des temps de demi-vie allant de quelques semaines à plusieurs mois (Lewis et al., 2016). Il est courant de les retrouver avec leurs métabolites dans des eaux de surface ou dans les sols ainsi que dans les matrices des colonies d'abeilles (Raimets et al. 2022 ; Tauchnitz et al. 2020). La présence de ces fongicides dans l'environnement soulève des questionnements quant à leur effet sur la santé humaine et la biodiversité. Ces produits auraient notamment

des conséquences sur la faune aquatique comme les zebrafishs et les daphnies (Sancho et al., 2016 ; Tian et al., 2019), ou le microbiote intestinal des insectes (Cang et al., 2023 ; Willow et al., 2019 ; Wood et al., 2020).

Très peu de données sont disponibles sur les effets sublétaux du prothioconazole sur les abeilles et sa LD50 pour les adultes est de 71 µg/bee (Böhme et al., 2018).. Il serait sans effet sur la survie et le développement des larves (Wood et al., 2020). Chez les abeilles adultes, l'exposition à cette molécule n'a pas influé sur la survie, le gène du cytochrome p450, le développement des glandes hypopharyngiennes ou la consommation d'aliments (Wood et al., 2020). Pour le moment, cette molécule ne montre aucun effet marqué d'interactions avec d'autres pesticides et n'a aucun effet additif avec le thiaméthoxame (insecticide) sur les adultes ou le couvain (Wood et al., 2020). Le seul effet d'interaction noté est une faible synergie lors de son association avec le flupyradifurone (insecticide) sur le système immunitaire des abeilles (Haas et al., 2021).

Les connaissances sur les effets du tébuconazole sur les abeilles sont plus larges. Des résultats démontrent que le tébuconazole affecte la physiologie des abeilles. Notamment, il diminue la capacité antioxydante totale et modifie le profil des acides gras du cerveau (Mackei et al., 2023). Il va également accélérer le métabolisme, la détoxification et réduire l'activité neurale (Cang et al., 2023). Son impact direct a également été reporté sur l'action d'enzyme impliquée dans le transport des ions et la régulation du pH (Soydan et al., 2017). Lors des tests en laboratoire et en tunnel, ce produit n'a pas induit de mortalité directe ni diminué l'intensité de recherche de nourriture (Schmuck et al., 2003). Cependant, chez les reines, le tébuconazole inhibe des enzymes de la spermathèque, augmente leurs poids et diminue l'acceptation des cellules royales (Raimets et al., 2022 ; Soydan et al., 2017). Le tébuconazole entre en interaction avec plusieurs insecticides ce qui entraîne des effets sublétaux plus importants. Son association avec le tetra-chlorantraniliprole présente une toxicité synergique aiguë sur l'expression des gènes associés à la réponse immunitaire, au stress oxydatif et au métabolisme de détoxification chez l'abeille mellifère (Cang et al., 2023). Cet affaiblissement du système immunitaire a été également reporté lors de sa coexposition avec le thiaméthoxame ce qui réduit significativement la survie des larves infectées par *M. plutonius* (Wood et al., 2020). Son association avec un cocktail de pesticides augmente la masse des larves et perturbe le développement des glandes

hypopharyngiennes en réduisant le diamètre des acinis (Böhme et al., 2017). Au laboratoire, son association avec le thiaclopirde entraîne un effet synergique et augmente la mortalité, mais cet effet n'a pas été observé lors des tests en tunnel lorsque les abeilles étaient directement pulvérisées (Schmuck et al., 2003). Également, son association avec le chlorpyrifos et la cyperméthrine induit un effet répulsif sur les cultures traitées (Stejskalová et al., 2021). Aucune étude n'a encore exploré l'effet de ce produit sur la cognition des abeilles mais, chez le bourdon, des effets négatifs sur l'apprentissage ont été reportés ainsi que la diminution du nombre d'ouvrières et d'alvéoles de couvain (Botías et al., 2015).

Les insecticides

Les insecticides sont utilisés dans la lutte contre les insectes ou d'autres invertébrés (acariens, myriapodes), nuisibles à l'homme, aux cultures, aux denrées alimentaires. Ils représentent une solution efficace contre les pucerons, les aleurodes, les tordeuses, les cochenilles... L'utilisation des insecticides représente 9 % des pesticides vendus. En revanche, leur utilisation entre 2009-2011 et 2018-2020 a augmenté de 105 % (Sedillot & Courtier, 2022 ; Fig. 6). La spécificité et la toxicité des insecticides sont déterminées par leur mode d'action biochimique qui affecte les insectes au niveau cellulaire ou physiologique. La spécificité va dépendre du type de cible physiologique, alors que la toxicité va être déterminée par la dose nécessaire pour provoquer la mort d'un organisme.

Le *Resistance Action Committee* (IRAC) a référencé les modes d'action des insecticides et en dénombre actuellement 36 différents. Ils sont classés selon les six grands types de cibles physiologiques (Fig. 10) :

- Les molécules ciblant le système nerveux et les muscles.
- Celles ciblant la croissance et le développement.
- Celles visant la respiration.
- Celles visant les voies digestives.
- Celles visant à supprimer les protéines.
- Celles dont le mode d'action est non spécifique ou encore inconnu à ce jour.

Je me limiterai à la description de deux produits examinés au cours de cette étude et qui agissent sur les deux premiers types de cibles physiologiques.

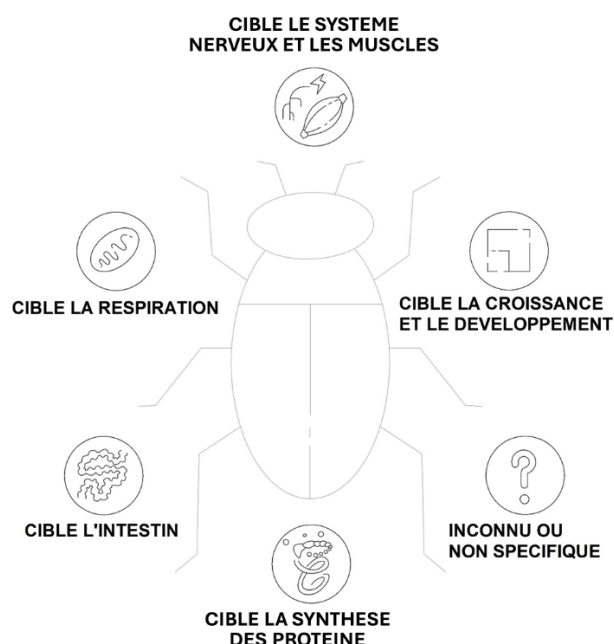


Figure 10 : Mode d'action des Fongicides (Inspiré de IRAC 2024)

La lambda-cyhalothrine

La lambda-cyhalothrine est un pyréthrianoïde synthétique qui agit sur les protéines des canaux sodium voltage-dépendants, que l'on trouve dans les membranes des cellules nerveuses des insectes (Ray & Forshaw, 2000) (Fig. 11). La liaison des insecticides à ces canaux va perturber la transmission de l'influx nerveux et ce processus entraînera une paralysie puis, à terme, la mort de l'insecte. Cette molécule a été utilisée pour la première fois en Amérique centrale en 1985 (Lewis et al. 2016 ; PPDB Pesticide Index 2024).

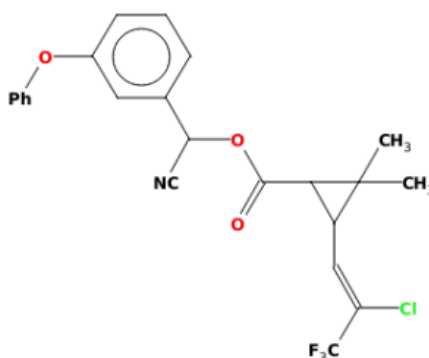


Figure 11 : Molécules de la lambda-cyhalothrine (Cambridge Crystallographic Data Centre diagrams)

En France, la lambda-cyhalothrine représente environ 1 % (29 tonnes) en masse des ventes de substances actives utilisées pour la formulation d'insecticides (Brigon & Lenoble, 2020). On dénombre 29 produits commerciaux contenant cette molécule active et qui sont autorisés (ANSES, 2023). Son application en France peut être effectuée pendant la période de floraison, mais en dehors de la présence des abeilles dans les champs, c'est-à-dire dans les deux heures qui précèdent le coucher du soleil ou dans les trois heures qui suivent le coucher du soleil. Cette molécule agit par contact et ingestion et présente une forte toxicité pour les mammifères, les poissons, les invertébrés aquatiques et les pollinisateurs. Elle est modérément toxique pour les vers de terre (PPDB Pesticide Index 2024). Lors de la dernière évaluation de ce produit par l'EFSA, il a été conclu que son utilisation sur le terrain entraînait un faible risque pour les abeilles, mais que des informations complémentaires seraient nécessaires, notamment au niveau des risques dans les vergers (EFSA, 2014).

L'exposition sublétales à la lambda-cyhalothrine va déclencher plusieurs réponses physiologiques chez l'abeille, avec notamment une activation des voies de désintoxication par le biais du cytochrome P450 (Piechowicz et al. 2021). La mémoire est affectée par l'inhibition de l'expression de gènes spécifiques dans le cerveau des abeilles (Liao et al., 2018). Au niveau cellulaire, cette molécule endommage les organes tels que l'intestin, la glande hypopharyngienne et le cerveau (Arthidoro de Castro et al., 2020). La lambda-cyhalothrine agit aussi sur la cognition et la mémoire des abeilles adultes, en diminuant la réponse au test d'extension du proboscis et sur le nombre d'individus capables de s'orienter et de rentrer à la ruche (Liao et al., 2018). Lors de tests en tunnel, cette molécule a réduit le nombre d'abeilles butinant pendant environ une demi-journée après la pulvérisation, ainsi que le nombre de butineuses se posant sur les plantes, sans toutefois augmenter la mortalité (Lewis et al., 1990). Cependant, les effets répulsifs de la lambda-cyhalothrine sont remis en cause (Wegorek et al., 2015). Des effets synergiques sont observés entre la lambda-cyhalothrine et le prochloraz (fongicide), augmentant la toxicité par inhibition de l'expression du cytochrome P450 et de la carboxylestérase, qui sont impliqués dans la dégradation de l'insecticide (Johnson et al., 2006). La lambda-cyhalothrine entre en synergie avec le thiaméthoxame et ils vont diminuer l'activité et l'expression des glutathion S-transférases impliqués dans la détoxification et entraîner une mortalité plus élevée (Wang et al., 2020). Plus largement, les pyréthrénoïdes, dont fait partie la

lambda-cyhalothrine, entrent en synergie avec les fongicides inhibiteurs de la biosynthèse de l'ergostérol et vont augmenter la mortalité après le traitement. (Pilling et al., 1995 ; Pilling & Jepson, 1993)

Le pyriproxyfène

Le pyriproxyfène appartient à la famille des pyridines et est un inhibiteur de croissance (Fig. 12). Cette molécule est un homologue des hormones juvéniles et va notamment bloquer le développement de la larve, l'empêchant de se métamorphoser en imago, ainsi qu'entraîner la mort de l'individu. Cette molécule a été homologuée pour la première fois en 1995 par l'entreprise japonaise Sumitomo Chemical. (Lewis et al. 2016 ; PPDB Pesticide Index 2024). Elle est très utilisée pour lutter contre les moustiques qui transmettent le virus zika ou la malaria dans les pays tropicaux (Darriet et al., 2007 ; Ishaaya & Horowitz, 1995).

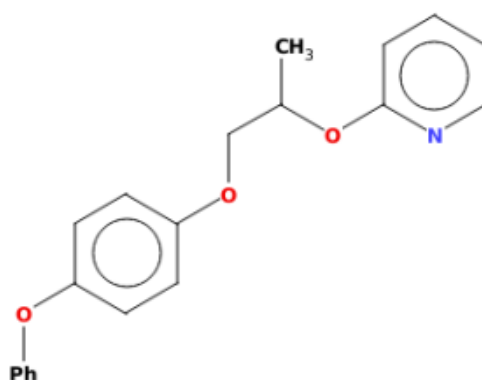


Figure 12 : Molécule du pyriproxyfène (Cambridge Crystallographic Data Centre diagrams)

En France, cinq produits commerciaux contiennent cette molécule active et sont autorisés à l'utilisation (ANSES, 2023). Son application peut être effectuée sur les cultures fruitières et maraichères pendant la période de floraison, mais en dehors de la présence des abeilles dans les champs (i.e. tomate, concombre, courgette, poivron, aubergine...) (SUMIMOTO, 2023). Peu d'informations sont disponibles quant à son utilisation en agriculture. Le pyriproxyfène a une forte toxicité pour les mammifères, les poissons, les invertébrés aquatiques (PPDB Pesticide, 2024). Plusieurs études soupçonnent cette molécule d'entraîner des cas de microcéphalie chez les nourrissons. Lors de la dernière évaluation de ce produit par l'EFSA, il a été conclu que son utilisation sur le terrain entraînerait un faible risque pour les abeilles, mais il est nécessaire d'apporter des informations complémentaires au niveau des risques dans les vergers (EFSA 2019).

Le pyriproxifène entraîne des effets délétères sur le développement des larves ainsi que sur l'adhésion des jeunes adultes aux tâches de la colonie (Chen et al., 2016 ; Devillers & Devillers, 2020 ; Fourrier et al., 2015). En raison de son homologie avec l'hormone de croissance, cette molécule impacte la physiologie des individus, altérant la synthèse du vitellogénine, de la prophenoloxidasase et de l'estérase-6 (Bitondi et al., 1998 ; Pinto et al., 2000 ; Zufelato et al., 2000). Ces modifications des voies métaboliques perturbent le cycle de développement des larves, induisant une initiation précoce de la nymphose qui se confond avec le début de la pigmentation et un retard dans la différenciation des muscles du vol (Bitondi et al., 1998 ; Fernandez et al., 2012). De plus, l'exposition des abeilles adultes au pyriproxifène provoque des altérations du tissu cérébral et de la capacité de vol, et augmente la mortalité (Litsey et al., 2021 ; Naiara Gomes et al., 2020). La reine peut également subir les effets de cette molécule, ce qui entraîne une diminution du taux d'acceptation des cellules reines, affectant ainsi l'expression des protéines ovariennes de la reine et la ponte d'œufs avec des taux d'éclosion accrus (Chen et al., 2016 ; Fine et al., 2023). Les expositions à l'échelle de la colonie augmentent la mortalité au stade nymphal et ont des effets néfastes sur l'operculation du couvain, l'émergence des adultes, et diminuent la production de la gelée royale (Chen et al., 2016). Des effets synergiques ont déjà été rapportés avec le méthoxyfénozide, entraînant une régulation à la baisse de l'expression de l'hormone juvénile (Litsey & Fine, 2024). Cela perturbe notamment la cognition des abeilles traitées qui se sont révélées moins sensibles à la phéromone de la reine (Litsey et al., 2021).

Généralités sur les tests réglementaires

Mandatée par la Commission européenne, l'EFSA a publié la première évaluation des risques liés aux pesticides pour les abeilles domestiques, les bourdons et les abeilles solitaires en 2013. En 2023, une révision de ce document d'orientation a été effectuée en décrivant une approche par étapes pour l'estimation de l'exposition dans différents scénarios et plusieurs niveaux. Des recommandations sont également suggérées pour les études de niveau supérieur, les risques liés aux métabolites et les pesticides en mélanges. Les nouvelles directives de l'EFSA recommandent d'évaluer les différentes voies d'exposition aux pesticides et d'analyser leurs effets à différents stades de la vie

pendant lesquels sont exposées les abeilles (adultes et larves) et sur différentes durées (aiguës et chroniques). Ainsi, quatre scénarios de risques ont été définis :

- Contact aigu,
- Contact aigu par l'alimentation,
- Chronique par l'alimentation,
- Larves par l'alimentation.

Ces scénarios d'exposition se basent sur des protocoles déjà reconnus par l'OCDE (OCDE, 1998a, 1998b, 2013a, 2017a) et, dans l'hypothèse où des effets des pesticides sont observés lors de ces tests, des expérimentations plus approfondies doivent être réalisées. Le nouveau guide recommande d'approfondir les connaissances des pesticides et notamment sur les deux aspects que sont les effets sublétaux et l'augmentation des effets toxiques due à une exposition à long terme à de faibles doses de pesticides. Par ailleurs, l'EFSA a défini des objectifs de protection spécifiques (SGP : *Specific Protections Goals*) de 10 % comme réduction maximale acceptable de la taille des colonies à la suite d'une exposition à un pesticide (EFSA 2023). Plus globalement, la protection des autres pollinisateurs et abeilles *non-Apis* reste limitée par le manque de connaissances, ce qui ne permet pas de fixer un seuil de protection.

PROBLEMATIQUE ET OBJECTIFS DE LA THESE

Ce projet s'inscrit dans un contexte global de perte de biodiversité où un quart des espèces animales et végétales est menacé d'extinction (Diaz et al., 2019). Les insectes volants, y compris les pollinisateurs et plus particulièrement les abeilles mellifères, ne sont pas épargnés (Hallmann et al., 2017). Actuellement, l'hypothèse sous-jacente au déclin des abeilles implique des facteurs multiples, agissant séparément ou de manière combinée, tels que la perte d'habitat et de ressources, les bioagresseurs (virus, bactéries, parasites) et l'exposition aux pesticides (Goulson et al., 2015). Afin de protéger les pollinisateurs, notamment les abeilles mellifères (*Apis mellifera*), divers tests de toxicité ont été développés pour évaluer les effets des pesticides. Le renforcement de ce cadre réglementaire vise à réduire encore l'exposition des abeilles aux produits phytopharmaceutiques (ANSES, 2018). Pourtant, ces produits étant toujours utilisés, ils sont présents dans l'environnement et sont donc retrouvés de manière quasi permanente dans toutes les matrices des ruches en France et dans le reste du monde (Böhme et al., 2018 ; Chauzat et al., 2006). Mis à part dans certains cas spécifiques, les effets sublétaux sur le comportement et les interactions entre différents facteurs biotique et abiotique ne sont pas pris en compte dans les tests.

Cette thèse s'inscrit dans une démarche originale visant à contrôler les élevages larvaires *in vitro* et à suivre le cycle de vie complet de l'abeille mellifère dans les ruches avec un système RFID. Cette approche permet d'explorer avec précision les effets sublétaux induits par les expositions précoces, répétées et en interaction des stress biotique et abiotique. De plus, une approche transversale a permis de caractériser leurs effets sublétaux sur la physiologie, le développement et le comportement à l'échelle individuelle, tout en intégrant des simulations de dynamiques des populations pour une vision globale de l'impact des stress sur les colonies d'abeilles. Ce travail propose également une ouverture sur une perspective plus large, liée aux évaluations écotoxicologiques visant à limiter les effets néfastes des pratiques agricoles sur les écosystèmes et sur la résilience des organismes vivants, notamment dans le contexte crucial de la pollinisation assurée par l'abeille mellifère et sur son rôle essentiel dans la production agricole et la sécurité alimentaire.

Le premier chapitre se focalise sur la mise en œuvre et l'évaluation d'une stratégie pour contrôler et suivre le cycle de vie de l'abeille mellifère. J'examine notamment les effets des conditions d'élevage larvaire *in vitro* sur l'activité des abeilles au sein des colonies, en utilisant la système RFID, et également les effets de l'exposition à un élément inerte couramment utilisé lors des tests écotoxicologiques.

Les deuxième et troisième chapitres visent à caractériser les effets précoces, répétés et différés d'un fongicide. Cette partie concerne les effets sublétaux du fongicide boscalid à des concentrations réalistes de terrain. Le deuxième chapitre montre les effets précoces répétés et différés d'une exposition au boscalid sur les altérations du développement et du comportement des abeilles et leurs effets sur les dynamiques des colonies. Le troisième chapitre analyse les effets précoces et différés du boscalid sur le développement et la physiologie des abeilles.

Les quatrième et cinquième chapitres visent à étudier les effets d'interactions précoces et différés entre des stress biotique et abiotique. Cette partie concerne les effets sublétaux d'un large panel de stress biotique et abiotique sur l'abeille. Le quatrième chapitre examine les effets d'interaction entre différents stress biotique et abiotique sur le développement des abeilles. Enfin, le dernier chapitre analyse les effets d'interactions précoces entre divers stress abiotiques sur le développement et le comportement des abeilles et leurs effets sur les dynamiques des colonies.

Dans le contexte actuel de déclin des pollinisateurs et d'utilisation intensive des pesticides, il est nécessaire, sinon capital, de connaître les conséquences des pratiques agricoles sur la biodiversité. L'abeille mellifère, à travers son utilisation pour la production de miel, est un organisme sentinelle qui contribue plus largement à protéger l'ensemble des pollinisateurs de l'exposition aux pesticides. Ces travaux de recherche contribuent ainsi à répondre aux enjeux liés aux effets sublétaux des pesticides et aux expositions à tout âge de l'organisme. Ces approches permettront non seulement de mieux connaître le cycle de vie des abeilles mellifères, mais également d'évaluer les effets d'un pesticide lors d'expositions précoces des individus. Enfin, ces travaux soulignent la nécessité d'évaluer les interactions lors de co-expositions entre des facteurs biotique et abiotique sur les abeilles mellifères.

CHAPITRE 1 - DE LA NAISSANCE, A LA MORT : STRATEGIE DE SURVEILLANCE INDIVIDUELLE ET GLOBALE

Avant-propos

Dans le contexte d'une meilleure prise en compte de l'impact des pesticides sur les abeilles et les pollinisateurs, il est essentiel de mettre en place de nouvelles procédures qui permettent d'évaluer les effets sublétaux des pesticides. Actuellement, les tests d'exposition des pesticides se font seulement à des stades spécifiques (i.e. larve, adulte) et les conséquences à long terme des pesticides sur les individus ne sont pas examinées (OCDE, 2013b, 2017a, 2017a). L'OCDE recommande empiriquement que les tests sur les abeilles incorporent moins de 1 à 5 % d'ingrédients inertes dans le volume total de la solution d'exposition (OCDE, 1998b, 1998a, 2017a). L'acétone est un ingrédient inerte couramment utilisé comme témoin et il est considéré comme ayant une faible toxicité directe sur les organismes (Dai et al., 2019 ; Kablau et al., 2023 ; Rembold et al., 1974). De nombreuses études scientifiques n'évaluent pas les effets de l'acétone, car elles n'incluent pas de témoins non exposés dans leurs expériences (Ii & Rangel, 2018 ; Rinkevich et al., 2015). Cela entraîne des biais potentiels, car l'ingestion d'acétone peut entraîner des effets sublétaux avec, entre autres, l'altération morphologique et histologique (Oliveira et al., 2014 ; Sigg et al., 1997).

L'élevage larvaire des abeilles est une technique couramment utilisée, elle permet un contrôle précis et un suivi rigoureux des facteurs de développement (OECD, 1998a). Les abeilles élevées au stade larvaire *in vitro* sont privées d'interactions sociales, et cela ne peut se faire sans compromis sur la physiologie et les activités des abeilles adultes (Mortensen et al., 2019 ; Mortensen & Ellis, 2023 ; Schilcher et al., 2022). Les abeilles élevées au stade larvaire *in vitro* vont adhérer aux différentes tâches de la colonie, mais plusieurs traits comportementaux sont affectés négativement par l'élevage *in vitro* (Mortensen & Ellis, 2018 ; Schilcher et al., 2022). Je propose donc ici une approche couplant la technique d'élevage *in vitro*, qui permet un suivi précis de l'exposition et du développement lors de la phase larvaire, et la technique RFID qui permet de suivre l'activité et des traits d'histoire de vie des abeilles adultes au sein de la colonie (Aupinel et al., 2005 ; Bagnis et al., 2010). Il est donc essentiel de comparer

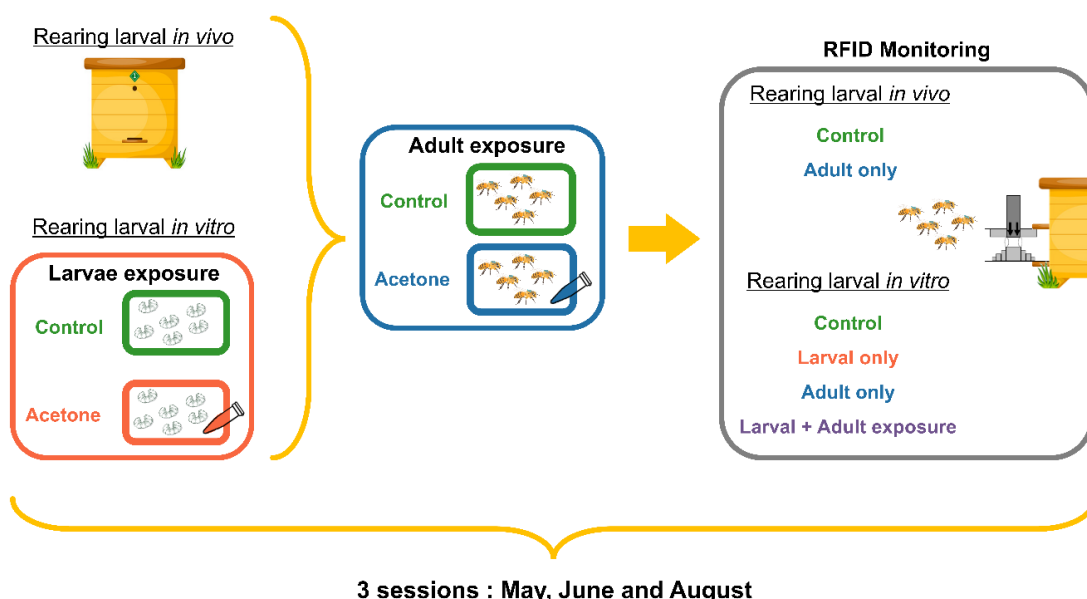
les traits d'histoire de vie avec des abeilles ayant été élevées *in vivo* au sein de la colonie (Prado et al., 2020 ; Requier & Rebaudo, 2020). De plus, lors des procédures expérimentales, des produits inertes sont utilisés pour permettre l'incorporation de molécules actives dans l'alimentation (telles que l'acétone). L'ingestion d'acétone pourrait perturber le développement au stade larvaire et au stade adulte, modifiant ainsi leurs comportements au sein des ruches.

Les objectifs

L'objectif de cette étude est de mesurer les conséquences de l'élevage larvaire au laboratoire et de l'exposition à un élément inerte sur les traits d'histoire de vie des larves et des abeilles adultes. Pour répondre à cet objectif, j'ai combiné l'utilisation de la méthode d'élevage larvaire *in vitro* et l'utilisation de la technique RFID, et j'ai exposé les individus à de l'acétone lors de la phase larvaire ou à l'émergence.

Sur la base des éléments développés précédemment, trois hypothèses émergent :

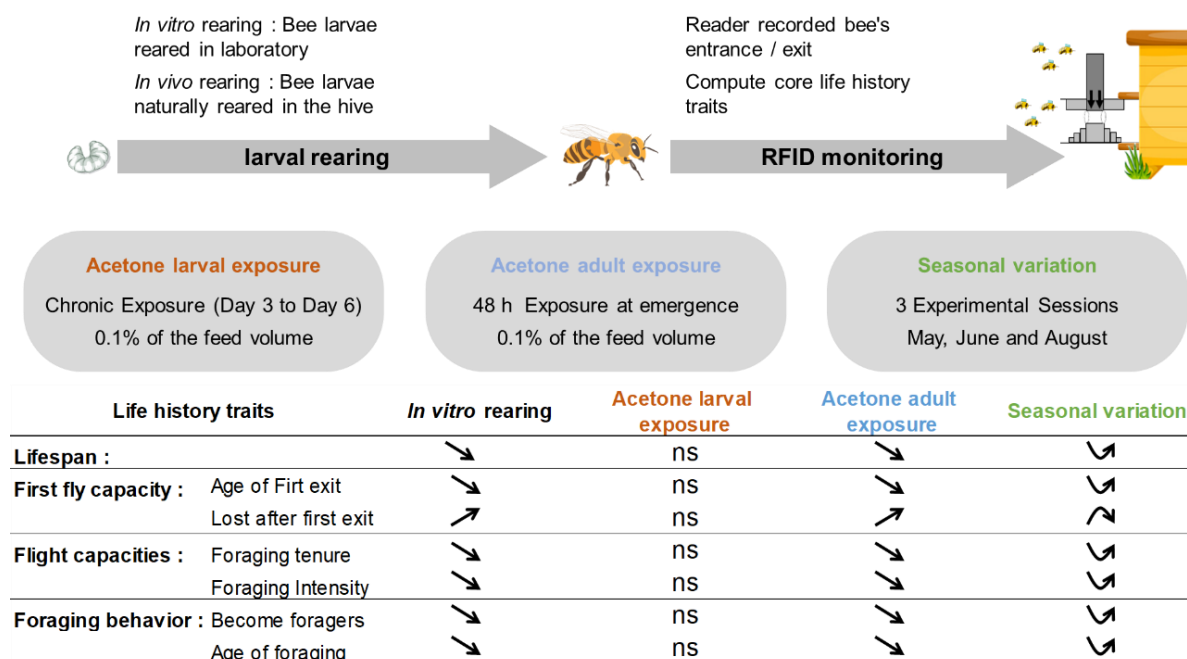
- Au vu des différences physiologiques, les abeilles élevées *in vitro* lors de leur phase larvaire vont avoir une longévité et des performances de vol réduites par rapport à leurs homologues élevées *in vivo* dans des ruches.
- Une exposition précoce à l'acétone va altérer les comportements des abeilles au sein des ruches.
- L'exposition récurrente, aux stades larvaire et adulte, va avoir des effets plus significatifs.



L'acétone a été incorporée à 0,1 % dans l'alimentation des larves et des abeilles. Les abeilles ont été exposées à l'acétone pendant leur stade larvaire et/ou adulte (abeille élevée *in vitro*) ou adulte (abeille élevée *in vitro* et *in vivo*), et j'ai comparé leur cycle de vie avec celui des individus qui n'ont pas été exposés à l'acétone (Fig. S1). J'ai mesuré les principales caractéristiques du cycle de vie de toutes les abeilles : la durée de vie, l'âge à la première sortie et l'âge au début du butinage, ainsi que la durée du butinage et l'intensité du butinage des abeilles adultes. De plus, j'ai cherché à savoir si l'effet potentiel de l'acétone pouvait dépendre de la saison en répétant l'expérience sur trois sessions (en mai, juin et août).

Le résumé des résultats

La méthode d'élevage des larves exerce une influence profonde sur divers aspects vitaux du cycle de vie des abeilles. Les abeilles qui ont été élevées *in vitro* ont une durée de vie nettement plus courte que les abeilles élevées *in vivo*. En ce qui concerne l'activité du premier vol, les abeilles élevées *in vitro* commencent leurs premiers vols plus tôt que les abeilles élevées *in vivo* et une proportion plus importante d'entre elles ne reviendra pas de ce premier vol. De plus, l'activité totale des vols et des comportements de butinage des abeilles élevées *in vitro* est affectée négativement. Ces résultats suggèrent que la méthode d'élevage des larves joue un rôle essentiel dans la mise en place des traits d'histoire de vie des abeilles, en particulier en ce qui concerne leurs schémas de vol et leurs comportements de recherche de nourriture.



L'exposition des larves à l'acétone n'a pas influencé les paramètres étudiés. Cependant, l'exposition des abeilles adultes à l'acétone a eu un impact significatif sur divers paramètres de leur cycle de vie. J'ai mis en évidence que la sensibilité des abeilles à l'exposition à l'acétone est variable selon les différentes sessions expérimentales sur leurs activités de butinage. Lors des sessions de mai et juin, l'exposition des adultes à l'acétone s'est traduite par une durée de vie plus courte et une diminution significative de l'âge au premier vol. En outre, la proportion d'abeilles perdues après le premier vol a augmenté lorsqu'elles ont été exposées à l'acétone à l'âge adulte, uniquement lors des sessions de juin et août. La capacité totale de vol et les activités de butinage ont été réduites chez les abeilles adultes exposées à l'acétone au cours des sessions de mai et juin. La double exposition à l'acétone lors des stades larvaire et adulte a conduit à une durée de vie plus courte, uniquement lors de la session de juin, et à un premier vol plus précoce pour les abeilles adultes.

Publication

Cette étude a été acceptée dans *Environmental Toxicology and Chemistry*.

Les figures et tables supplémentaires se trouvent dans l'annexe 1.

Experimental ecotoxicology procedures interfere with honeybee life history

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Running title : *In vitro* rearing and acetone effects on honeybees

ABSTRACT

Apis mellifera was used as a model species for ecotoxicological testing. In this study, I've tested the effects of acetone (0.1 % in feed), a solvent commonly used to dissolve pesticides, on bees exposed at different developmental stages (larval and/or adult). Moreover, I've explored the potential effect of *in vitro* larval rearing, a commonly used technique for accurately monitoring worker exposure at the larval stage, by combining acetone exposure and treatment conditions (*in vitro* larval rearing versus *in vivo* larval rearing). I then analyzed the life-history traits of the experimental bees using RFID (radio frequency identification) technology over three sessions (May, June and August) to assess the potential seasonal dependence of the solvent effects. My results highlight the substantial influence of *in vitro* larval rearing on the life cycle of bees, with a 47.7 % decrease in LSP, a decrease of 0.9 days in the age at first exit and an increase of 57.3 % in the loss rate at first exit, and a decrease of 40.6 % in foraging tenure. I haven't observed any effect of exposure to acetone at the larval stage on the capacities of bees reared *in vitro*. Conversely, acetone exposure at the adult stage reduced the bee lifespan by 21.8-60 %, decreased the age at first exit by 1.12-4.34 days and reduced the foraging tenure by 30-37.7 %. Interestingly, I've found a significant effect of season on acetone exposure, suggesting that interference with the life history traits of honeybees is dependent on season. These findings suggest improved integration of long-term monitoring for assessing sublethal responses in bees following exposure to chemicals during both the larval and adult stages.

KEYWORDS

Apis mellifera ; *in vitro* larval rearing; acetone ; RFID ; flight activities ; life history traits.

INTRODUCTION

Bees play a critical role in global food production by promoting the pollination of many plants, on which many crops depend directly (Garibaldi et al. 2014 ; Ollerton et al. 2011). Indeed, 90 % of all flowering plants and 75 % of our main crops require animal pollination (Klein et al., 2007 ; Tepedino, 1979). However, pollinators are facing increasing threats, particularly exposure to pesticides used to protect crops, and major concerns about their impact on bees have been raised (Goulson et al., 2015 ; Sanchez-Bayo & Goka, 2014).

Pesticides are generally composed of active substances that are toxic to pests, as well as inert substances designed to improve their effectiveness (US EPA, 2013). Although classified as inert ingredients, these additives are used by the main manufacturers of agricultural pesticides, and some have been shown to provoke harmful effects on human beings' health and on the environment (Cox & Sargan 2006 ; Pezzoli & Cereda 2013). The global use of inert ingredients is very unclear and, in Europe, no data are available on the quantities used, only on their number (e.g., 70 in France, 294 in the UK ; ANSES 2023 ; Straw et al. 2022). Almost all active ingredients are applied with inert ingredients, and it is assumed that their quantities are proportional or probably greater than those of the active ingredients (Straw et al. 2022). The identities of inert ingredients contained in commercial solutions are confidential, which makes it challenging to understand how they affect biodiversity and the environment (Regulation, 2009). Nevertheless, volatile organic compounds such as xylene, toluene and acetone have been found in the environment (Wang et al., 1995).

These inert ingredients are also widely used in regulatory ecotoxicological tests of the active ingredients (e.g., dissolving, emulsifying, and decanting). The Organization for Economic Co-operation and Development (OECD) empirically recommends that bee tests should incorporate less than 1 to 5 % of inert ingredients in the overall volume of the exposure solution (OCDE, 1998a, 1998b, 2017a). Overall, 19 studies between 1973 and 2021 directly tested the effects of inert ingredients on bees (Straw et al., 2022). Thus, some inert ingredients (e.g., Citowett®, Pulse®, Boost®) have been shown to be toxic when administered topically or orally (Goodwin & McBrydie, 2000). In addition, nonlethal effects of specific inert ingredients were observed. These compounds can modify the behavior of *Apis mellifera* ; for example, the proboscis

extension reflex (PER) is impaired by the ingestion of inert ingredients, such as organosilicons and tensioactifs (Ciarlo et al., 2012). Many active ingredients are not miscible in water, so protocols must use an inert ingredient, such as a solvent, to incorporate them into test solutions. Acetone is an inert ingredient that is commonly used as a control and is considered to have low direct toxicity (Dai et al. 2019 ; Kablau et al. 2023 ; Rembold et al. 1974). Many scientific studies do not evaluate the effects of acetone because they do not include unexposed controls in their experiments (li & Rangel, 2018 ; Rinkevich et al., 2015). This can lead to potential biases, as the ingestion of acetone can have consequences on organisms and modify the intestinal microbiota (Quinn et al., 2018) of adult bees, and acetone inhibits glandular growth during larval exposure (Salles & Cruz-Landim, 2004). It is therefore relevant to better assess the consequences of exposure to acetone and its effects on the behavior and life-history traits of bees.

In addition to the common use of acetone in ecotoxicological tests involving bees, the *in vitro* larval rearing method is commonly used for the assessment of environmental risks associated with bees, particularly pesticides (Aupinel et al., 2007 ; Fourrier et al., 2015). Ingestion of acetone could be one of the causes of developmental disturbance in the larval stage, which results in an increase in mass (Rembold et al. 1974). However, only a few studies have examined how the rearing method affects the behavior of adult bees, and this is important for better assessing the long-term consequences of the rearing method on the life of bees.

Few studies follow bees individually throughout their lives (Brodschneider et al., 2009 ; Fisher et al., 2021 ; Mortensen & Ellis, 2023 ; Schilcher et al., 2022). Most of these studies have focused on a single stage of bee life, such as larval, adult or forager stages (Fine et al., 2017 ; Kralj & Fuchs, 2006 ; Scofield & Mattila, 2015). *In vitro* larval rearing allows monitoring of the development of larvae in the laboratory until they emerge as young adult bees and analyses of the physiology of larvae and adults in the context of biotic or abiotic stresses (Aupinel et al., 2007 ; Schmehl et al., 2016). The use of radio frequency identification (RFID) devices provides automatic recording of the in-and-out activity of adult bees to estimate flight activity in bees (Nunes-Silva et al., 2019 ; Requier et al., 2020 ; Streit et al., 2003). For instance, this system is applied to assess the impact of sublethal doses of pesticides on the flight activity of bees (Bagnis et al., 2010 ; Henry et al., 2012 ; Prado et al., 2020).

Unfortunately, the environment used for larval rearing differs between *in vivo* and *in vitro* conditions and can affect the morphology, physiology and behavior of bees at the adult stage. For instance, bees reared larval *in vitro* (RL_{vitro}) can have smaller body sizes and smaller lateral calyces than bees reared larval *in vivo* (RL_{vivo}) (Steijven et al., 2017). The flight behavior and performance of suckling and foraging tasks are reduced in RL_{vitro} bees compared with RL_{vivo} bees (Brodschneider et al., 2009 ; Schilcher et al., 2022). This is because larvae are deprived of social contact in *in vitro* larval rearing methods, either through chemical interactions with pheromones or through the maintenance of nurse bees (Schmehl et al., 2016). Moreover, the use of artificial food in *in vitro* larval rearing methods could lead to a nutritional stress, affecting the physiology and morphology of adult bees (Mortensen et al. 2019). Thus, combining the *in vitro* larval rearing method with RFID monitoring could help researchers exploring whether the larval development environment can affect the flight activity of adult bees.

The aim of this study was to test whether acetone, an inert ingredient commonly used in bee ecotoxicological tests to solubilize pesticides, could affect larval and adult bee's life history. Therefore, I've combined the use of the *in vitro* larval rearing method to monitor the survival of larvae and the use of the RFID technique to monitor the life history of adult bees. I've exposed bees to acetone during their larval and/or adult stage (RL_{vitro}) or adult stage (RL_{vitro} and RL_{vivo}) and have compared their life histories to those of bees (RL_{vitro} and RL_{vivo}, respectively) that were not exposed to acetone (Fig. S1). We measured the core life-history traits of all the bees, including the lifespan (LSP), age at first exit (AFE), and age at onset of foraging (AOF), and the foraging tenure (FT) and foraging intensity (FI) of the adult bees (Prado et al., 2020 ; Requier et al., 2020). Moreover, I've tested whether the potential effect of acetone could be dependent on the season by replicating the experiment over three sessions (in May, June and August).

MATERIALS AND METHODS

All experiments were conducted in the entomological experimental unit of INRAE, Le Magneraud (46° 8' 59,104"N, 0° 41' 28,609"W), France, between April and August 2021. The apiary is in an intensive cereal farming system with a high proportion of wheat, sunflowers, oil seed rape and maize (Odoux et al., 2014 ; Requier et al., 2015). I've used *A. mellifera mellifera* × *caucasica* colonies for the experiment, treated with

Varroa destructor before winter, but not during the spring or summer of the present study.

IN VITRO LARVAL REARING AND EXPOSITION TO ACETONE

One-day-old larval workers were collected from three honeybee colonies and reared *in vitro* until emergence following the method described by Aupinel et al. (Aupinel et al., 2007 ; Medrzycki et al., 2013 ; Papach et al., 2017) The first-cycle larvae were obtained by enclosing the queen with a frame of built-up brood to an isolated Italian cage for 30 hours. Then, the queen was released, and the freshly laid frame was left in the hive for one more day to obtain a first cycle larvae (L1) for grafting. All graft procedures were performed in the laboratory at room temperature (approximately 20-24°C). The same experimenters performed all transplants. The larvae (L1) were transferred to disinfected plastic grafting dishes. The L1 cups were maintained in an incubator at 34.5 ± 5 °C from day 1 to day 8 with a relative humidity (RH) of 95 ± 5 % (K₂SO₄ solution). Cups were filled with a mixture of royal jelly, aqueous sugar and yeast on days 1, 3, 4, 5 and 6. Each larvae was unexposed (control) or chronically exposed to 0.1 % acetone (Larvae) for 4 days in their diet (day 3 to day 6). At the first grafting session, on day 8, the plates containing the pupae were capped with a layer of wax. Pupal plates were placed vertically in crystal polypropylene emergence boxes (11 × 15 × 12 cm) with a 5 mm piece of Be-Boost® (PseudoQueen) attached to a piece of wax (approximately 5 × 5 cm). The emergence boxes were transferred to a pupae incubator at 34.5 ± 5 °C with 80 ± 5 % RH (saturated NaCl solution). Prior to emergence (day 15), the emergence boxes were placed at 34.5 ± 5 °C and 50 ± 5 % RH with 50 % agarose-sugar solution *ad libitum*. The second and third grafting sessions were performed with the same protocol to obtain adult bees. The first part of the protocol was like the first grafting session until days 7 and 8. On days 7 and 8, the larvae were transferred to a new clean plate when they had finished feeding, and the plates containing the pupae were transferred to a pupae incubator. Prior to emergence (day 15), the pupae plates were placed in a crystal polypropylene emergence box equipped in the same way as those used for the first grafting (equipped in the same format as in session 1). Individuals who died before the start of feeding on day 2 were removed from the analysis. The percentages of larvae that died before ingesting the first feed were 4.3 %, 2.8 % and 3.2 % in May, June and August, respectively. For these individuals reared *in vitro*, we monitored daily mortality and the days of

emergence. Only in May was the mortality of the capped plates from day 7 to day 20 subsequently assessed according to the stage of development. Overall, 984 individuals were reared in May, 998 individuals in June and 994 individuals in August.

ADULT BEE EXPOSITION TO ACETONE AND RELEASE

At emergence (days 17 to 20), the RL_{vitro} bees were marked with an RFID transponder (see below the RFID Monitoring section). The RL_{vitro} bees were directly introduced to a single host hive after RFID was marked. The RL_{vitro} bees were placed in small wire frames, which were sealed with candy (8 hours after the first tagging). The frames were then placed directly in a bait comb host hive equipped with RFID readers. On day 18, some of the marked bees were kept in polypropylene boxes equipped in the same way as the emergence boxes under laboratory conditions for 48 hours at $34.5 \pm 5^\circ\text{C}$ and $50 \pm 5\%$ RH. The plants were then exposed to 0.1 % acetone for 48 hours in a sugar solution (50 % w/v sucrose). After exposure, the RL_{vitro} bees were introduced into the hives with a small wire frame. Thus, we used four modalities : (i) RL_{vitro} bees that were never exposed to acetone, called control (C) ; (ii) RL_{vitro} bees that were exposed to acetone only during the larval phase, called larvae exposed (L) ; (iii) RL_{vitro} bees that were exposed to acetone only during the adult phase, called adult exposed (A) ; and (iv) RL_{vitro} bees that were exposed to acetone during the larval and adult phases, called larvae and adult exposed (L+A) (Fig. S1). Overall, 43 C RL_{vitro}, 52 L RL_{vitro}, 83 A RL_{vitro} and 77 L+A RL_{vitro} bees were released in May. In June, 103 C RL_{vitro}, 98 L RL_{vitro}, 99 A RL_{vitro} and 92 L+A RL_{vitro} bees were released. In August, 100 C RL_{vitro}, 104 L RL_{vitro}, 98 A RL_{vitro} and 101 L+A RL_{vitro} bees were released in August.

On the other hand, RL_{vivo} bees that were less than 12 hours old were collected from the hives that provided the larvae for *in vitro* rearing. RL_{vivo} bees were marked with an RFID transponder and subjected to the same treatment as the RL_{vitro} bees at emergence : direct release or exposure for 48 hours and release (see above). There were two modalities : RL_{vivo} bees that were never exposed to acetone, control (C), and RL_{vivo} bees that were exposed to acetone during the adult phase, adult exposed (A) (Fig S1). Finally, 104 C RL_{vivo} and 104 A RL_{vivo} bees were released in May, 104 C RL_{vivo} and 90 A RL_{vivo} bees were released in June, and 102 C RL_{vivo} and 90 A RL_{vivo} bees were released in August.

RFID MONITORING

The adult bees were immobilized on their thoraxes using a piston cage to glue a mic3®-TAG 16 kbit RFID transponder (HF-RFID 13.56 MHz, Microsensys mic3 Technology). The transponders were glued to the thorax using biocompatible dental cement (TempoSIL2). The tags were $1.6 \times 1.9 \times 0.5 \text{ mm}^3$ in size and weighed approximately 3 mg (Streit et al., 2003). The impact of the weight of the tag may be marginal or null, given that it represents 3 % of bees' weight without considering its capacity to transport nectar or pollen (Marden, 1987 ; Winston, 1991). The operation and ID of the tags were checked and saved before they were attached to the bees with a portable USB holder (ID®PENmini 7.0, Microsystems).

All tagged bees from the three donor colonies were released into a single host colony equipped with an RFID system. The 10-frame Dadan hive equipped with the RFID system contained five brood frames, three honey + pollen reserve frames and two empty wax frames. The queen of the host colony was born in 2020. Two sets of four RFID readers (iID2000, 2k6 HEAD ; Microsensys) were placed at the entrance of the hive (with a total of eight readers). The two lines of readers allowed us to distinguish the direction of the activity, i.e., an entrance or an exit (Requier et al., 2020). Each well passed through a tunnel $80 \times 8 \text{ mm}$ high. The reader recorded the identity, date, and time at each passage of a tagged bee. The data were collected every 7 days.

LIFE HISTORY TRAITS

We measured the core life history traits of 604 RL_{vitro} bees and 1050 RL_{vivo} bees based on the RFID data after cleaning the raw dataset following Requier et al. (2020)'s method. The lifespan (LSP) was measured in days up to the last recorded RFID hit for each bee. The other traits were computed after removing bee trips shorter than 2 seconds and trips longer than 180 minutes (Prado et al., 2020). The age at first exit (AFE) of the bees was defined as the number of days from the first recorded RFID hit (the first flight). We then calculated the foraging tenure (FT) as the time span from the AFE to the LSP. We also analyzed the proportion of bees lost after their first exit from the hive (L-AFE) (Coulon et al., 2020). We measured foraging intensity (FI) as the total number of flights performed by bees (see also Prado et al., 2020). Finally, we computed the age at onset of foraging (AOF) of the bees in days using the *aof* R package (Requier and Rebaudo 2020) for 338 RL_{vitro} bees and 410 RL_{vivo} bees.

STATISTICAL ANALYSES

All the statistical analyses were performed using R software version 4.2.2 (R Core Team 2022). The significance level for the statistical tests was set at 5 % for the probability of rejecting the true null hypothesis. Graphs were generated with the *ggplot2* R package (Wickham et al. 2023). The compact letters of all pairwise comparisons were generated with the *multcomp* R package (Piepho, 2004).

Effects on bee larvae : I've generated a Cox proportional hazards model using the *coxme* package (Therneau, 2022) to assess whether larval survival in hazard ratios (HR) was affected by acetone exposure (control (C), larvae exposed (L)), session (May, June, and August) and the hive of origin of the larvae (R3, R4, and R6). I've considered the rearing plate as a random factor in the model (standard deviation = 0.250 ; variance = 0.062). The impact of technicians transferring larvae to D7-D8 (June and August session only) had a significant effect on larval survival and was considered in a specific model ($\chi^2 = 74.7523$; $df = 3$; $p < 0.001$; Table S1). I've used a linear mixed model (LMM) to assess the emergence day of RL_{vitro} bees using the *lme4* R package (Bates et al., 2023). The explanatory variables considered for this model are like those of the survival model (see above). I've considered the rearing plate as a random factor in the model (standard deviation = 0.010 ; variance = 0.10 ; Table S2). For both models, we performed post hoc (Tukey) pairwise comparisons with the *emmeans* R package (Lenth et al. 2023) to identify differences between sessions, acetone exposure and the impact of the hive of origin.

Effects of the experimental procedure on adult bees : We generated different models to test the effects of the larval rearing protocol (i.e., *in vitro* and *in vivo*), the origin of the larvae (i.e., R3, R4 and R6), acetone exposure (i.e., control (C), bees exposed as larvae (L), bees exposed as adults (A) and bees exposed as larvae + adults (L+A)) and their interactions with the sessions (i.e., May, June and August) on bee life history traits (see above). Survival analysis was performed using a Weibull hazard model with the *survival* package (Therneau et al., 2023) to test the effects of these explanatory variables on LSP. The AFEs of bees were compared with generalized linear mixed models (GLMMs) using the *glmmTMB* R package (Brooks, Bolker, and Kristensen 2023). L-AFEs (binary response variables) were compared with binomial GLMMs from the *ade4* R package (Dray and Dufour 2007). For bee flight capacity, FT and FI were analyzed with a negative binomial 1 GLMM (I've included in the formula "ziformula~0"). For foraging activities, the proportion of forager bees (binary response variables) was

analyzed with binomial GLMM. Finally, AOF was analyzed with gamma GLMM. I've carried out pairwise comparisons to analyze the effects of the different explanatory variables. I've calculated the value of the Akaike information criterion (AIC) to compare the log-likelihood of GLMM error distributions (AFE and OAF : Gamma and Gaussian ; FT and FI : Poisson, zero-inflated, negative binomial 1 and negative binomial 2) and selected the lowest AIC (Sakamoto et al., 1986). The residuals were checked, and analysis of variance was performed using the *DHARMA* R package (Florian Hartig 2022) and *car* (Fox et al., 2023).

RESULTS

EFFECT OF ACETONE ON BEE LARVAE

The survival of *in vitro*-reared larvae was affected by the session of the experiment ($\chi^2 = 40.306$; $df = 2$; $p < 0.001$; Fig. 1 ; Table S1). In May, larval mortality was significantly greater than that in June and August (May : 1.863 ± 0.194 HR (hazard ratio), June : 0.925 ± 0.100 HR and August : 0.587 ± 0.065 HR ; $p < 0.01$, Table S1). Acetone exposure had no impact on larval survival ($\chi^2 = 1.23$; $df = 1$; $p < 0.267$, Fig. 1 ; Table S1). The hives used to graft the larvae had no effect on larval survival ($\chi^2 = 5.422$; $df = 2$; $p = 0.066$, Table S1).

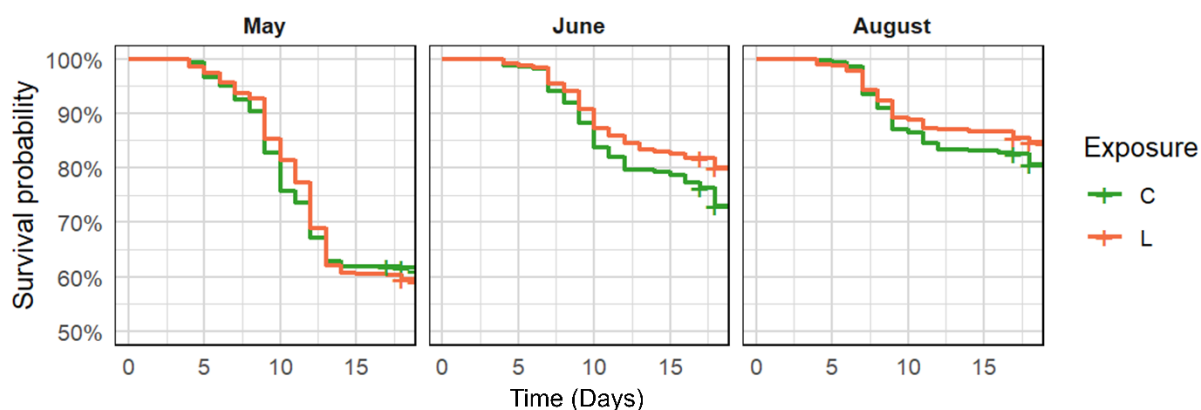


Figure 1 : *Effect of acetone on bee larvae survival.* Larvae were not exposed : control (C), Larvae were chronically exposed to 0.1 % acetone in their diet on days 3, 4 and 5 (L) for each session (May, June and August).

Experiment session was impacted on the emergence day of the RL_{vitro} bees (LMM, $\chi^2 = 27.878$; $df = 2$; $p < 0.001$; Table S2). The average bee emergence days for the May, June and August sessions were 19.13 ± 0.030 days, 19.18 ± 0.027 days and

18.98 ± 0.027 days, respectively. In the August session, the bees emerged earlier than those in the May and June sessions ($p < 0.001$; Table S2). Chronic exposure of larvae to acetone had no effect on bee emergence day (LMM, C : 19.07 ± 0.023 and L : 19.12 ± 0.023 ; $\chi^2 = 2.833$; $df = 1$; $p < 0.092$; Table S2). The hives of origin of the larvae impacted the days of emergence of the bees (LMM, $\chi^2 = 35.564$; $df = 2$; $p < 0.001$; Table S2). The days of emergence of the R6 bees were later than those of the R3 and R4 hives (R3 : 18.98 ± 0.023 days, R4 : 19.09 ± 0.028 days and R6 : 19.22 ± 0.028 days ; $p < 0,001$; Table S2).

EFFECTS OF EXPERIMENTAL PROCEDURES ON ADULT BEES

Impact of larval rearing conditions on adult bees

Larval rearing type had a significant effect on all GLMMs : LSP, AFE, L-AFE, FT, FI, foragers, and AOF ($\chi^2 > 5.4$; $df = 1$; $p < 0.020$; Table 1). The survival model indicated that the adult RL_{vitro} bees had significantly shorter lifespans than did the adult RL_{vivo} bees (11.0 ± 0.294 days for the RL_{vitro} bees, 21.2 ± 0.901 days for the RL_{vivo} bees ; $p < 0.001$; Fig. 2 ; Table S3). In terms of the first flight activity, the RL_{vitro} bees exhibited a reduced AFE and a significant increase in L-AFE compared to the RL_{vivo} bees (AFE : 5.66 ± 0.102 days for the RL_{vitro} bees, 6.58 ± 0.201 days for the RL_{vivo} bees ; $p < 0.001$; L-AFE : 68.6 ± 1.52 % for the RL_{vitro} bees, 30.4 ± 2.44 % for the RL_{vivo} bees ; $p < 0.001$; Fig. 3 ; Table S4 and S5).

Table 1: *Type II analysis of variance for the different generalized mixed effect models.* For all GLM, the chi-square tests of Wald's likelihood were calculated with their associated p values (*: p values <0.05; **: p values <0.01; ***: p values <0.001).

	Effects variable	Session		Exposure		Session : Exposure		Rearing		Hive	
		2		3		6		1		2	
		χ^2	p-value	χ^2	p-value	χ^2	p-value	χ^2	p-value	χ^2	p-value
Models	LSP	126,6	***	55,6	***	89,3	***	165,1	***	2,6	
	AFE	57,4	***	83,4	***	122,0	***	20,4	***	0,7	
	L-AFE	80,0	***	2,0		23,4	***	153,0	***	12,0	**
	FT	37,8	***	23,5	***	18,2	**	26,0	***	0,4	
	FI	51,8	***	6,8		12,3		47,9	***	0,1	
	Foragers	60,9	***	13,1	**	8,4		27,5	***	4,4	
	AOF	57,7	***	16,3	**	3,7		5,4	*	12,4	**

Regarding total flight activity, the RL_{vitro} bees had a significantly lower FT and FI than did the RL_{vivo} bees (FT : 13.5 ± 0.571 days for the RL_{vitro} bees, 18.5 ± 0.976 days for

the RL_{vivo} bees ; $p < 0.001$; FI : 18.0 ± 0.962 trips for the RL_{vitro} bees, 30.3 ± 1.852 days for the RL_{vivo} bees ; $p < 0.001$; Fig. 4 ; Table S6 and S7). For foraging activities, the RL_{vitro} bees exhibited significantly fewer foragers and earlier AOFs compared to the RL_{vivo} bees (foragers : 54.4 ± 3.03 % for the RL_{vitro} bees, 77.9 ± 2.93 % for the RL_{vivo} bees ; $p < 0.001$; AOF : 18.7 ± 0.565 days for the RL_{vitro} bees, 20.9 ± 0.825 days for the RL_{vivo} bees ; $p < 0.05$; Fig. S2 and Fig. S3 ; Table S8 and S9).

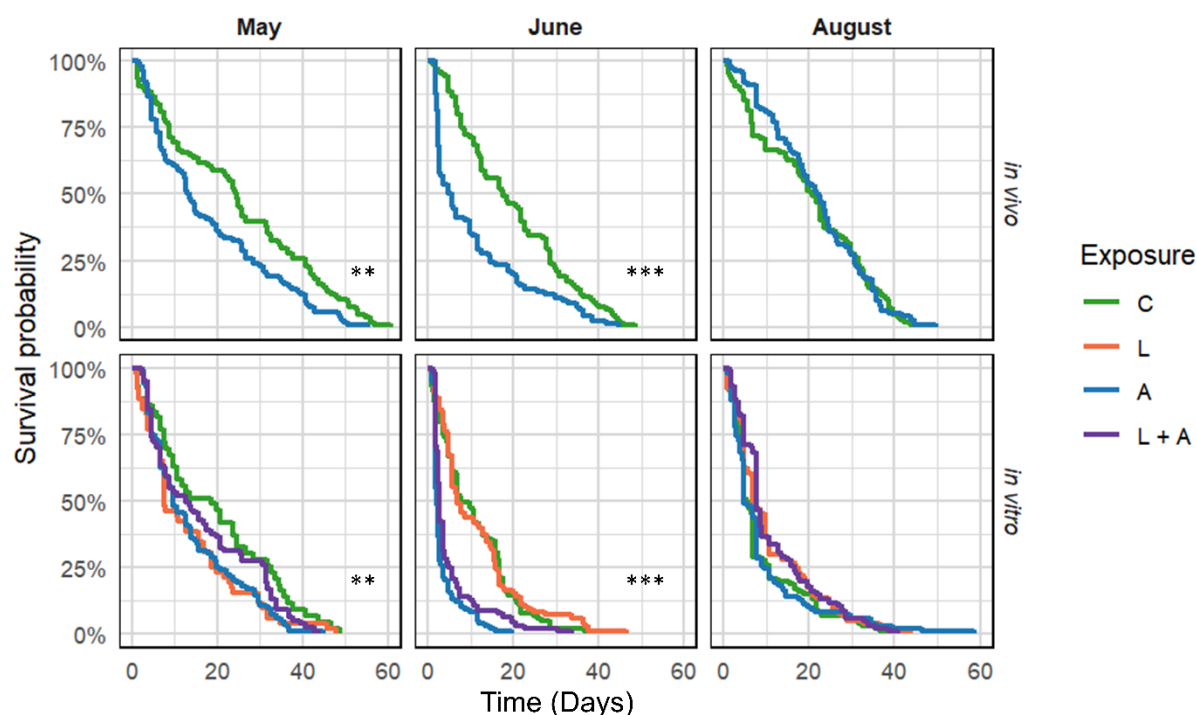


Figure 2 : Effect of acetone on the survival of adult bees. Adult bees were reared following the larval rearing protocol (i.e., *in vitro* and *in vivo*) for each experimental session (May, June and August). The bees were in the unexposed control group (C) or were exposed to acetone at the larval (L) stage, at the adult (A) stage or at both the larval and adult (L+A) stages. **: p values < 0.01 ; *** : p values < 0.001 ; if acetone exposures affect LSP for each session.

Effects of seasonal variation on adult bees

The session of the experiment had an impact on all GLMMs : LSP, AFE, L-AFE, FT, FI, foraging, and AOF ($\chi^2 > 37.8$; $df = 2$; $p < 0.001$; Table 1). The average LSP differed significantly among adult bees in May, June, and August (May : 20.6 ± 0.928 days, June : 11.0 ± 0.421 days, and August : 15.8 ± 0.529 days ; $p < 0.001$; Fig. 2 ; Table S3). The AFE of the bees was significantly greater in the May session than in the June and August sessions (7.57 ± 0.239 , 5.36 ± 0.161 , and 5.78 ± 0.150 , respectively ;

$p < 0.001$; Fig. 3A ; Table S4). The L-AFE of the bees was significantly greater in the June session than in the May and August sessions (May : 39.3 ± 2.74 %, June : 68.6 ± 0.015 %, and August : 42.3 ± 2.39 % ; $p < 0.001$; Fig. 3B ; Table S5). The FT of the bees was significantly lower in the June session than in the May and August sessions (May : 18.7 ± 0.983 days, June : 12.6 ± 0.909 days, and August : 16.4 ± 0.753 days ; $p < 0.004$; Fig. 4A ; Table S6). The FI of the bees was significantly greater in the August session than in the May and August sessions (May : 22.0 ± 0.151 trips, June : 19.4 ± 1.57 trips, and August : 29.7 ± 1.55 trips ; $p < 0.01$; Fig. 4B ; Table S7). The proportion of foragers was significantly lower in the June session than in the May and August sessions (May : 70.5 ± 3.59 %, June : 47.3 ± 4.86 %, and August : 80.1 ± 2.55 % ; $p < 0.001$; Fig. S2 ; Table S8). The AOF of the bees was significantly earlier in the August session than in the other sessions (May : 22.5 ± 0.858 days, June : 20.5 ± 1.245 days, and August : 17.1 ± 0.494 days ; $p < 0.05$, Fig. S3 ; Table S9).

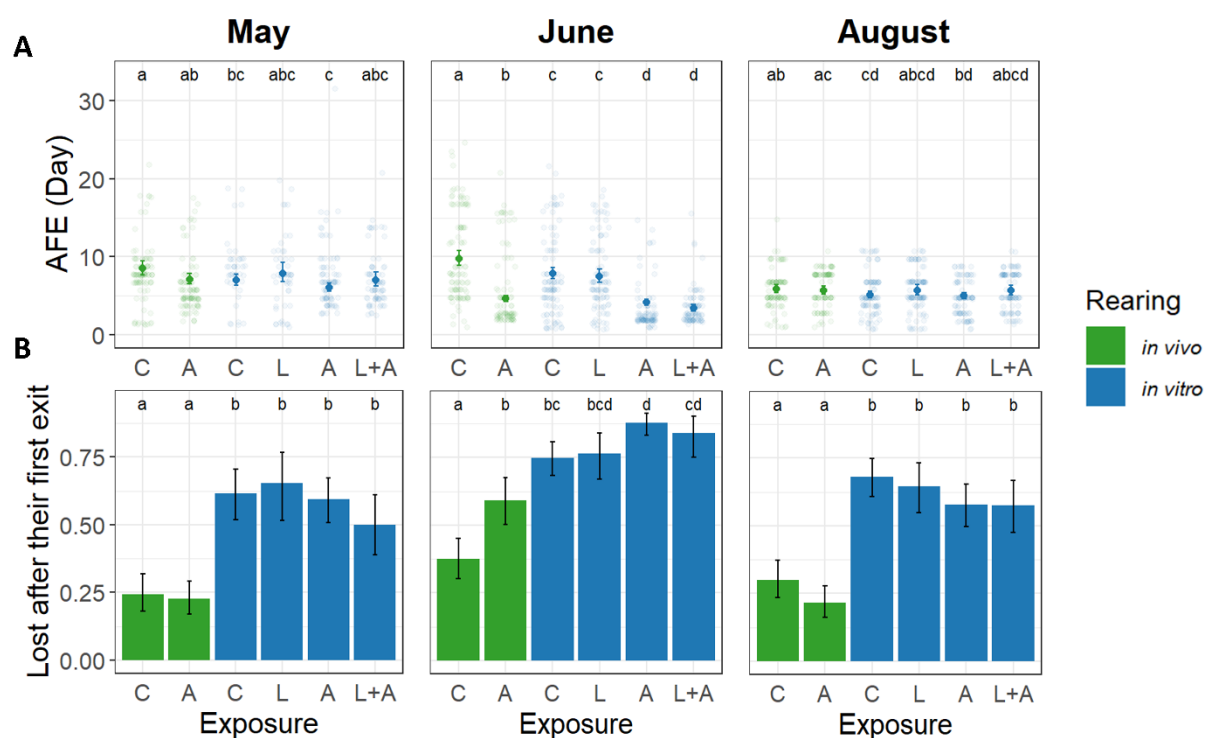


Figure 3 : Effect of acetone on the first flight activity of adult bees. (A) AFE in days. (B) L-AFE in proportion. The bees were in the unexposed control group (C) or were exposed to acetone at the larval stage (L), at the adult stage (A) or at both the larval and adult (L+A) stages for each experimental session (May, June and August). The colours show that the adult bees followed the larval rearing protocol (*in vitro* and *in vivo*). c

Effects of exposure on adult bees

Acetone exposure had significant effects on the GLMM LSP, AFE, FT, foraging, and AOF ($\chi^2 > 13.1$; $df = 3$; $p < 0.004$; Table 1). Additionally, an interaction effect between experimental session and exposure was reported for GLMM LSP, AFE, L-AFE, and FT ($\chi^2 > 18.2$; $df = 6$; $p < 0.006$; Table 1). Larval acetone exposure had no discernible effect on the parameters examined in this study ; the RL_{vitro} bees and those exposed as larvae exhibited similar LSP, AFE, L-AFE, FT, foraging, and AOF to the control RL_{vitro} bees across all the experimental sessions ($p > 0.05$; Table S3, S4, S5, S6, S7, S8, and S9). The life cycle of the bees exposed to acetone in the adult stage was affected ; the bees exposed to the adults had a shorter LSP than did the control bees, but only during the May and June sessions (in May, C : 21.72 ± 1.477 HR, and in bees A : 17.05 ± 1.022 HR ; in June, C : 15.53 ± 0.882 days, and in bees A : 6.90 ± 0.418 days ; $p < 0.007$; Fig. 2 ; Table S3). Bees exposed as adults exhibited a significant decrease in AFE compared to control bees during the May and June sessions (in May, C : 7.68 ± 0.359 days, and A : 6.56 ± 0.273 days ; in June, C : 8.69 ± 0.344 days, and A : 4.35 ± 0.227 days ; $p < 0.001$; Fig. 3A ; Table S4). Bees exposed as adults showed a significant increase in L-AFE compared to the control bees in the June and August sessions (in June, C : 57.2 ± 3.71 %, and A : 76.4 ± 3.21 % ; in August, C : 49.0 ± 3.82 %, and A : 38.0 ± 3.70 % ; $p < 0.05$; Fig. 3B ; Table S5). Bees exposed as adults significantly reduced FT compared to control bees during the May and June sessions (in May, C : 20.83 ± 1.293 days, and A : 14.56 ± 0.951 days ; in August, C : 13.49 ± 1.012 days, and A : 8.41 ± 1.009 days ; $p < 0.001$; Fig. 4A ; Table S6). Bees exposed as adults had significantly fewer foragers compared to control bees (C : 67.0 ± 3.22 %, and A : 53.8 ± 3.74 % ; $p < 0.05$; Fig. S2 ; Table S8). Bees exposed to acetone as larvae and adults experienced an impact on their life cycle compared to control bees. During the June session, the adult RL_{vitro} bees that were exposed as larvae + adults had a shorter LSP than did the RL_{vitro} bees, but their LSP was equal to the RL_{vitro} bees that were exposed as adults (C RL_{vitro} bees : 11.19 ± 0.689 HR, A RL_{vitro} bees : 4.97 ± 0.336 HR, and L+A RL_{vitro} bees : 5.69 ± 0.448 HR ; $p < 0.001$ and $p < 0.2132$, respectively ; Fig. 2 ; Table S3). The RL_{vitro} bees that were exposed as larvae + adults showed a significant decrease in AFE compared to the control RL_{vitro} bees and the RL_{vitro} bees that were exposed as adults during the May session (C RL_{vitro} bees : 7.85

± 0.345 days, A RL_{vitro} bees : 4.13 ± 0.171 days, and L+A RL_{vitro} bees : 3.42 ± 0.206 days ; $p < 0.01$; Fig. 3A ; Table S4).

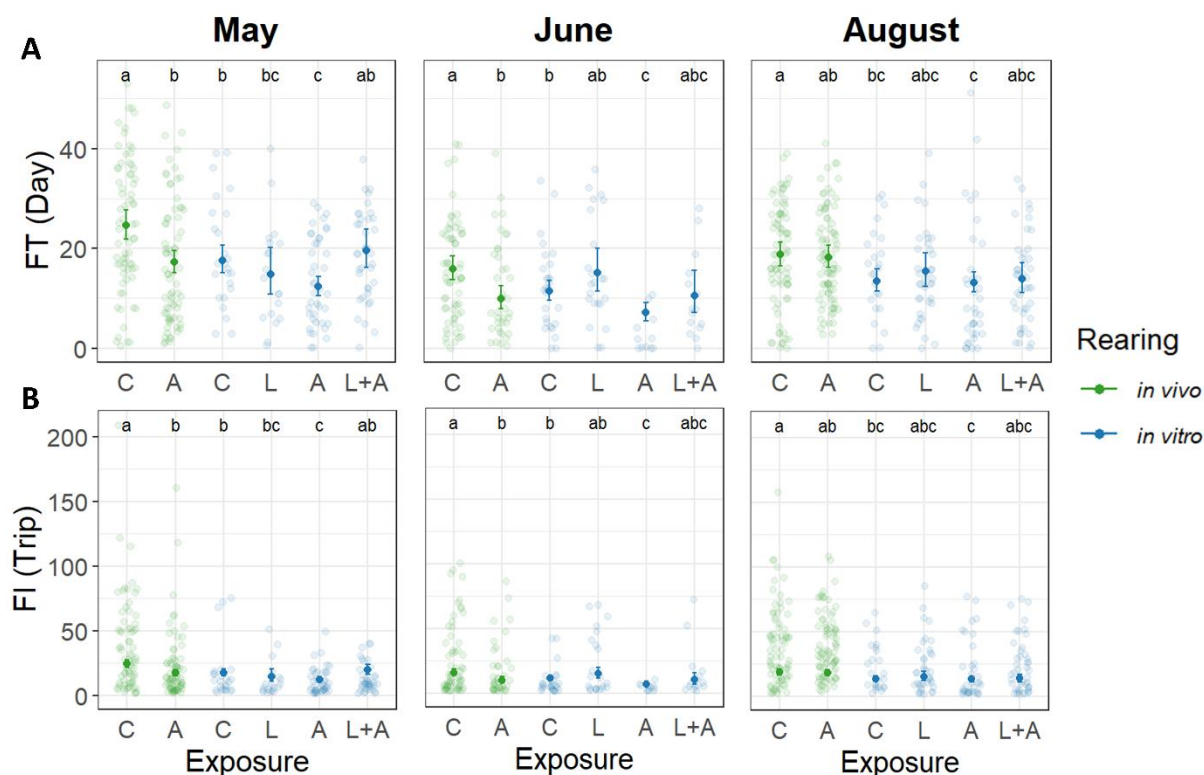


Figure 4 : Effect of acetone on the total flight capacity of adult bees. (A) FT in days. (B) FI in total number of trips. The bees were in the unexposed control group (C) or were exposed to acetone at the larval stage (L), at the adult stage (A) or at both the larval and adult (L+A) stages for each experimental session (May, June and August). The colours show that the adult bees followed the larval rearing protocol (*in vitro* and *in vivo*). Transparent dots represent the raw data. Larger dots represent model estimates $\pm$ confidence intervals of the models (95 %). Significant differences according to Tukey's pairwise comparisons are indicated with letters for each session.

Effect of hive origin on adult bees

The hives of origin had a significant effect on the GLMM L-AFE and AOF of adult bees ($\chi^2 = 12.0$; $df = 2$; $p < 0.002$; $\chi^2 = 12.4$; $df = 2$; $p < 0.002$; Table 1). Bees from hive R4 exhibited a significantly greater L-AFE than did bees from hives R3 and R6 (R3 : 44.7 ± 2.47 %, R4 : 55.9 ± 2.56 %, and R6 : 47.7 ± 2.63 % ; $p = 0.042$; Table S4). Bees from hive R4 had a significantly earlier AOF than did bees from hives R3 and R6 (R3 : 18.5 ± 0.574 days, R4 : 21.2 ± 0.774 days, and R6 : 19.8 ± 0.774 days ; $p < 0.021$; Table S9).

DISCUSSION

EFFECT OF ACETONE ON BEE LARVAE

During *in vitro* larval rearing, survival rates varied between experimental sessions, with higher mortality occurring in May. Regarding development time and bee emergence day, bees from the August session emerged earlier than those from the May and June sessions. We fed bee larvae a regular diet containing 0.1 % acetone, and we did not observe any effect of this chronic exposure on larval survival or development time with respect to bee emergence day. The hive of origin influenced survival, with larvae from R6 having a higher mortality rate and bees having longer development times and emerging later than bees from the two other hives.

The higher mortality in the May session is associated with a protocol that was under development. Vertical capping plates are thought to significantly reduce the deformation rate of newly emerged adults but, in our experience, increase mortality and do not allow us to monitor mortality daily (Kim et al., 2021). During the June and August sessions, we applied the modification proposed by Schmel et al. to transfer the larvae at D8, as has been done in other publications on larval rearing (Mortensen and Ellis 2023 ; Mortensen et al. 2019 ; Schmehl et al. 2016). This greatly reduced larval mortality during our experimental sessions, but we were slightly above the OECD recommendation of less than 15 % in negative controls (OECD, 2013b). In terms of larval life history traits, acetone had no negative effect on survival or developmental delay with respect to the date of emergence. This observation is consistent with numerous *in vitro* larval rearing studies that used dual controls : unexposed and exposed to acetone (Dai et al. 2019 ; Kablau et al. 2023 ; Rembold et al. 1974). It cannot be ignored that a low dose of acetone could inhibit the development of certain pathogens and bacteria present in the larval gut (Ola-Fadunsin and Ademola 2013). In the long term, this disruption could have an impact on immunity, as symbionts and intestinal bacteria play essential roles in the digestion and assimilation of nutrients, including pollen (Daisley et al., 2020). It has also been shown that some larvae gain weight because of this exposure (Rembold et al. 1974). However, the effects of this acetone on other larval parameters remain unknown.

EFFECTS OF EXPERIMENTAL PROCEDURE ON ADULT BEES

Impact of larval rearing conditions on adult bees

The method of rearing larvae has a profound influence on various vital aspects of the life cycle of bees. RL_{vitro} bees may experience nutritional deficits during their development, potentially resulting in underdeveloped adult bees having notably shorter lifespans than RL_{vivo} bees, which may experience nutritional deficits during their development, potentially resulting in underdeveloped bees. In addition, the microbiota of RL_{vitro} and RL_{vivo} bees also differ, but such differences are subsequently reversed by individual transfer after emergence (Kowallik & Mikheyev, 2021). In terms of their first flight activity, RL_{vitro} bees may experience nutritional deficits during their development, potentially resulting in underdeveloped RL_{vitro} bees that initiate their first flight earlier and have a higher proportion of bees that are lost after the first flight. Additionally, they show a reduction in total flight activity and foraging behaviors, including fewer foragers and an earlier onset of foraging. These results suggest that the larval rearing method plays a crucial role in shaping bee life history traits, particularly in terms of their flight patterns and foraging behaviors.

Morphologically, RL_{vitro} bees can suffer nutritional deficits during development, potentially resulting in underdeveloped bees with marked differences (Scofield and Mattila 2015). This leads to variations in forewing length and width, dry body weight and hypopharyngeal gland size (Mortensen et al. 2019). However, in line with the literature, the average flight times of the RL_{vitro} and RL_{vivo} bees were generally similar (Brodschneider et al. 2009). Nevertheless, it is essential to note that *in vitro* rearing may deprive bees of essential behavioral and social learning experiences that occur in colonies and could lead to differences in their suckling and foraging behavior (Scofield and Mattila 2015). More specifically, a decrease in the foraging capacity and activity of RL_{vitro} bees has already been noted (Schilcher et al., 2022).

An interesting juvenile rearing environment hasn't significantly altered the behavioral repertoire of adult bees (Mortensen et al. 2019). RL_{vitro} bees continue to perform tasks such as assisting the queen, collecting pollen, performing dances, and engaging in defense against parasites (Mortensen et al. 2019). Thus, rearing *in vitro* is a well-established method that allows for precise control and rigorous monitoring of developmental factors, including the targeted application of pesticides or pathogens. However, it is imperative to acknowledge the substantial differences between RL_{vitro}

bees and those naturally developing in hives. These distinctions underscore the critical importance of considering these variations when conducting ecotoxicological tests under properly controlled environments.

Effects of seasonal variation on adult bees

These results highlight the impact of season on the life history characteristics of bees. The lifespan of adult bees varied significantly between the experimental sessions. In particular, the age at the first flight significantly differed from the age on the first flight, being older during May than in June and August. Similarly, June was characterized by a greater proportion of bees lost after their first flight and reduced overall foraging tenure. Furthermore, the proportion of foragers was significantly greater in the May and August sessions than in the June session. Additionally, the intensity of foraging is greater, and the age at which foraging begins is earlier in August than in other months.

The observed variations were consistent with natural fluctuations in bee behavior throughout the season, a phenomenon previously described (Fukuda & Sekiguchi 1966). Life history traits are known to be influenced by environmental factors such as climate effects, seasonality, landscape structure and weather conditions (Danner et al., 2016 ; Goulson et al., 2015 ; Henry et al., 2014 ; Steffan-Dewenter & Kuhn, 2003 ; Vanbergen, 2014). During our study period, rainfall levels were highly variable at 75 mm, 141 mm and 32 mm in the May, June and August sessions, respectively (Infoclimat, 2021). These climatic differences could have a significant impact on bee activity, as their first flight activity is essential for bees. Conversely, temperatures that are too high or too low lead to a reduction in foraging activity (Abou-Shaara et al., 2017).

For example, approximately 20 °C is the optimum temperature for foraging activity by bees (Tan et al., 2012). The landscape in relation to the season and availability of flowers has a major influence on the behavior of bee colonies (Danner et al., 2016 ; Free & Winder, 1983 ; Klein et al., 2007 ; Steffan-Dewenter & Kuhn, 2003). The diversity of the local landscape structure can influence bee pollen-based diets, as well as periods of resource depletion. In agricultural landscapes, it has been reported that colony dynamics are largely influenced by the phenology of the main massive-flowering crops foraged by bees, which at our experimental site were oilseed rape and sunflower (Odoux et al., 2014). Thus, agricultural landscapes in late spring colonies

suffer a period of food shortage between the two massive flowerings, which would modify foraging behavior (i.e., between late May and early July ; Requier et al., 2015). These results highlight the importance of considering colony dynamics, seasonal variations, climatic conditions and the availability of floral resources when analyzing bee life history traits and colony management.

Effects of exposure on adult bees

Exposure to acetone had a significant impact on various life-history parameters of bees. However, the exposure of larvae to acetone did not influence the studied parameters. These results highlight the sensitivity of bees to acetone exposure and its consequences for their life cycle and foraging activities, with variations between the different experimental sessions. Adult exposure to acetone resulted in a shorter lifespan and a significant reduction in age at first flight (May and June sessions). In addition, the proportion of bees lost after the first flight increased when they were exposed to acetone as adults (June and August). Total flight capacity and foraging activities (fewer foragers) were reduced in adult bees exposed to acetone (during the May and June sessions). Exposure to acetone during the larval and adult stages led to a shorter lifespan for adult bees (June session) and earlier first flight (May session). The age at first exit could be attributed to the inhibition of glandular growth (Salles and Cruz-Landim 2004) and the development of the hypopharyngeal gland (Sigg et al. 1997). In addition, similar concentrations of acetone altered morphological and histochemical digestive cells but also had no impact on the brain cells of Africanized bees (Oliveira et al., 2014). However, in *Apis mellifera*, the neuropil of the antennal lobe was impacted by acetone (Sigg et al 1997).

These results show that acetone, an inert ingredient commonly used in pesticide validation tests and in commercial pesticide formulations, can cause sublethal effects. Thus, scientists' preoccupation regarding the potential toxicity risks of inert ingredients in pesticide formulations is justified (Mullin, 2015 ; Straw et al., 2022). Regulatory agencies (i.e., the European Food Safety Authority – EFSA – in Europe or the Environment Protection Agency – EPA – in the US) evaluate the risk assessment of the active ingredient before approval. Thus, potential effects of commercial formulations are probably underestimated (Fine et al., 2017 ; Straw & Brown, 2021).

Effect of hive origin on adult bees

The bees used in this experiment came from three different colonies whose queens were genetically related. However, significant variations were observed in several aspects of their life cycle, including the duration of larval development, the proportion of bees lost after the first flight and the age at which foraging begins.

Indeed, genetically related colonies can show notable variations in key aspects of the bee life cycle (George et al., 2020 ; Junca et al., 2019 ; Page et al., 1995), including behavioral differences such as aggressive colony behavior in response to stress (Provost et al., 2003). Genetic diversity has also been identified as a major explanatory factor for variations in foraging behavior (Dreller et al., 1995). To clarify the extent to which our results have a genetic basis, future work could compare the behavior of queens inseminated by a monodrone or use genomic sequencing to determine the paternal lineage of each bee (Junca et al., 2019). Additionally, certain genetic characteristics could increase the susceptibility of some individuals to health pressures (Kralj & Fuchs, 2006). The rearing hives showed no visible symptoms, but I do not know about nonsymptomatic infections such as viral infections or parasites such as *Varroa destructor*, which of them affect the capabilities of the bee (Penn et al., 2022). It is important to consider the diversity of genetic patterns when studying different stresses because it is essential for colony-level functioning (Mattila & Seeley, 2007).

EXPERIMENTAL RECOMMENDATION

Notably, larval rearing conditions have a significant impact on the life characteristics of adult bees. Consequently, it is important to recognize the differences between RL_{vitro} and RL_{vivo} bees, particularly in the context of ecotoxicological or behavioral studies. This would make it more efficient to transfer the effects observed in the laboratory to field conditions. In the future, it would be wise to explore the mechanisms and consequences underlying the morphological variations observed in RL_{vitro} bees and their influence on flight and foraging behavior in adult bees. When studying the effects of biotic or abiotic stresses on bee life characteristics, it is essential to consider seasonal variations and genetic diversity as potential synergistic effects. Chronic exposure to acetone during the larval phase had no significant effect on the survival, developmental duration or day of emergence of bee larvae. Acetone did not affect life-history traits, despite variations between the different sessions. Some future studies

could examine other physiological or molecular parameters that might be influenced by larval exposure to acetone to better understand its effects on bee development. Finally, exposure to acetone in the adult stage had a significant impact on the different life-history traits of the bee. These results underline the fact that experiments using only acetone controls without unexposed controls need to be conducted. Exposure to acetone can influence the life history of bees. For future research, it would be relevant to examine other physiological parameters, such as the development of mandibular glands in adult bees. We highlight the necessity of having a control solvent, if any, to better evaluate the risk of active ingredients.

CHAPITRE 2 - DE L'INDIVIDU A LA COLONIE : EVALUATION DES EFFETS D'UN FONGICIDE SUR L'ACTIVITE ET IMPACT SUR LA COLONIE

Avant-propos

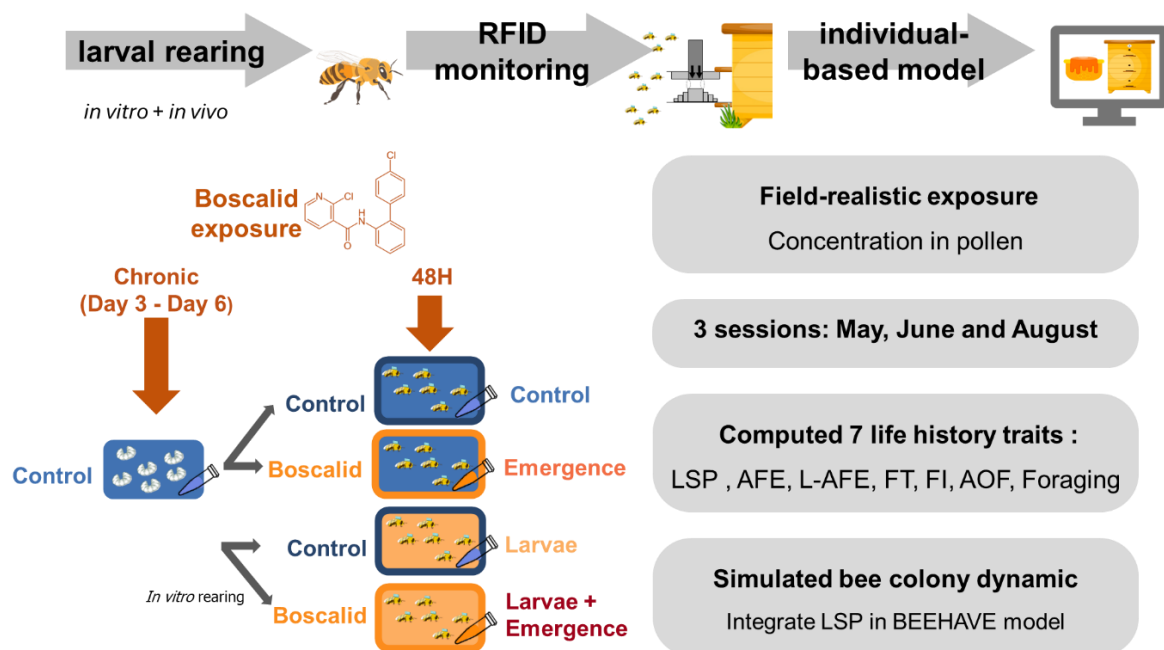
Dans le contexte de l'utilisation intensive du boscalid dans le paysage agricole, il est essentiel de prendre en compte les effets sublétaux de ce pesticide sur la vie globale des individus. Comme nous venons de le voir en détail dans l'introduction, le boscalid induit de nombreux effets sublétaux sur les abeilles. Ces effets ne sont pas observables par les tests classiques d'écotoxicologie (Simon-Delso et al., 2017). Il va notamment perturber le développement et l'activité des individus au sein des ruches (Fisher et al., 2021 ; Glass et al., 2021). L'action du boscalid va également réduire la taille des colonies et augmenter le pollen stocké au sein des colonies (Fisher et al., 2021 ; Pineaux et al., 2023). Néanmoins, les concentrations utilisées dans la majorité de ces études sont très fortes par rapport à celles retrouvées au sein des ruches en Europe (voir ci-dessous). Les larves et les jeunes adultes sont particulièrement exposés au boscalid via le pollen présent dans leur alimentation (Chauzat et al., 2006). Il apparaît ainsi primordial d'améliorer nos connaissances sur les réponses des abeilles face à ce stress, notamment sur leurs activités au sein de la colonie.

Les objectifs

L'objectif de ce chapitre est d'évaluer les effets sublétaux du boscalid lors d'expositions précoces sur l'activité des abeilles et de simuler cet effet à l'échelle de la colonie. Pour répondre à cet objectif, j'ai combiné l'utilisation de la méthode d'élevage larvaire in vitro avec la technique RFID pour contrôler le cycle de vie des abeilles adultes, comme présenté dans le chapitre 1.

Sur la base des éléments développés précédemment, trois hypothèses émergent :

- Sous l'action non-spécifique du boscalid, des larves ou des jeunes abeilles adultes exposées réduisent leurs activités à long terme au sein de la colonie avec un effet d'interaction lors d'expositions récurrentes.
- L'effet du boscalid sur le cycle de vie des abeilles sera variable au cours du temps, du fait de la dynamique de la colonie et de l'environnement.
- La transposition de l'effet du boscalid sur la longévité des individus dans une simulation de développement de colonie va entraîner une diminution de la taille de population des ruches et peut-être aussi sur les ressources.



J'ai mesuré les principales caractéristiques du cycle de vie des abeilles, notamment le développement larvaire, la durée de vie, l'âge de la première sortie, la mort après la première sortie, l'âge de début de butinage, la durée et l'intensité de butinage des abeilles mellifères adultes (Prado et al., 2020 ; Requier et al., 2020). Ces expérimentations ont été répétées au cours de trois sessions, afin de pouvoir observer

si l'effet du boscalid est modifié en fonction de la saison. Pour évaluer l'effet du boscalid sur la colonie d'abeilles mellifères, j'ai intégré les changements de la durée de vie des abeilles dans un modèle informatique simulant le développement d'une colonie d'abeilles mellifères (BEEHAVE).

Le choix des doses d'exposition

Les abeilles ont été exposées au boscalid pendant le stade larvaire et/ou adulte (élevage larvaire in vitro) ou adulte (élevage larvaire in vivo et in vitro). J'ai exposé des larves et des adultes sur la base de la contamination en boscalid présente dans le pollen qui nourrit ces individus, puis évalué la contamination du pollen par le boscalid sur la base des données disponibles dans la littérature. J'ai choisi d'utiliser la quantité maximale détectée en Allemagne, car le paysage agricole est relativement similaire à celui de la France. Ainsi, la concentration utilisée est de 1496,4 ng/g de pollen, qui est aussi la dose retrouvée dans les pollens des champs au moment de la pulvérisation (BASF 2016 ; Böhme et al. 2018). Sur la base de la consommation de pollen par les larves et les jeunes abeilles adultes, j'ai ainsi estimé qu'elles ingéreraient respectivement environ 3,05 ng et 15.6 ng de boscalid (Babendreier et al., 2004 ; Böhme et al., 2019 ; Daniele et al., 2018). J'ai adopté une approche réaliste et parcimonieuse de l'exposition des abeilles au boscalid, car elles peuvent également être exposées via d'autres matrices. Par exemple, des résidus sont retrouvés dans la cire des ruches et le nectar des plantes (BASF, 2016 ; Daniele et al., 2018).

Le résumé des résultats

Mes résultats ont montré des effets significatifs de l'exposition au boscalid sur le cycle de vie des abeilles lors du stade larvaire et adulte. À la suite de l'exposition chronique au boscalid, les larves ont émergé avec un retard de 0.15 jour. Les effets du boscalid sur les activités des abeilles ont été variables en fonction de la saison. Lors des sessions de juin et août, l'exposition larvaire et des adultes a causé une diminution de la longévité de 28 %-32.04 %. Lors des sessions de juin et août, l'exposition larvaire et des adultes a induit une diminution de la longévité de 28 % à 32.04 %. En juin, l'exposition larvaire et adulte au boscalid a provoqué une première sortie plus précoce de 0.94 à 1.49 jour. En août, l'effet de l'exposition larvaire du boscalid sur les abeilles était lié à une augmentation du taux de 1.39-1.52 directement après la première sortie.

La simulation de l'effet relatif du boscalid lors de période de pré-croissance (du jour 76 au jour 122) a réduit la taille de la population des colonies simulées de 18,5 % et leur production de miel de 13,0 % au cours de cette période d'exposition. Ces affaiblissements impactent plus fortement les colonies les plus faibles et peuvent être observés jusqu'à deux mois après la fin de l'impact simulé. Il est donc démontré que le boscalid a des effets à long terme sur le cycle de vie des abeilles et qu'il altère le développement et les ressources des colonies. Ainsi, les tests écotoxicologiques conventionnels, qui se concentrent uniquement sur le cycle larvaire ou adulte, ne sont pas efficaces pour caractériser les effets sublétaux des pesticides.

Publication

Cette étude a pour ambition d'être soumise dans Science of The Total Environment

Les figures supplémentaires se trouvent dans l'annexe 2.

Realistic exposure to boscalid induces shorter-lived honeybees and disturbs colony dynamics

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Running title : Boscalid impairs honeybee behaviour

ABSTRACT

Although levels of many pesticides in hives are relatively low compared with laboratory-defined toxicity levels, recent meta-analyses indicate a potential underestimation of the effects of fungicides on honey bees (*Apis mellifera*), particularly under field conditions. Our objectives were to evaluate the effects of early and recurrent exposure to boscalid at field-realist on the life history traits and also to evaluate the consequences on colony population size and honey production. We explored the potential effect boscalid at field-realist exposure on the life history traits of bees on the life history traits of bees exposed at different developmental stages (larval and/or adult). We then analysed the life history traits over three seasons (May, June and August) under field condition. We exposed bees during the larval stage following in vitro protocol, and then tagged the bees individually with RFID (radio frequency identification) chips in order to follow them throughout their lives under natural conditions. Our results show significant effects of boscalid exposure on larvae and adult bees. Exposure of larvae to boscalid delays emergence of young bees. Boscalid effect on the life-history traits of adult bees fluctuate between the season and could lead to significant lifespan decrease (29.9 %) when exposed at either larvae or adult stage. These decreases can be explained by a disruption in early activity, inducing early foragers by 1.49 days for the first flight, and the probability to come back after the first fly is divided by 1.4. We found that simulated colonies were more sensitive to boscalid when exposed to realistic field conditions in spring before the colony growth period. In addition, the effect of boscalid in spring reduced population size by 18.5 % and honey production by 13.0 %. The results of this study show overall negative impact of boscalid on honeybees. Our results will contribute to a more accurate assessment of the risks to bees at individual and colony level associated with the use of fungicides in the environment.

KEYWORDS

Apis mellifera ; in vitro rearing ; Larvae exposure ; simulation ; RFID ; SDHI

HIGHLIGHTS

We investigated boscalid at field-realist exposure on honeybee's life history traits and on simulated colony dynamics.

Boscalid delays the development of honeybees reared in vitro

Exposure of larvae and adults to boscalid induces shorter-lived honeybees and disrupts the first flight activities

Boscalid exposure affects differently honeybee's life history traits during the season.

Simulations of the relative effect of boscalid reveal a vulnerability before the growth period of colonies and decreases population size and honey production.

INTRODUCTION

The global decline in insect populations, including pollinators and bees, raises many concerns (Hallmann et al., 2017 ; Potts, Roberts, et al., 2010 ; Sánchez-Bayo & Wyckhuys, 2019). Insect pollination plays an essential role in the successful reproduction of flowering plants and is vital to the functioning of terrestrial ecosystems, providing vital ecosystem services that are essential to human production (Garibaldi et al., 2014 ; Ollerton et al., 2011). In Europe, 84 % of the 264 crops grown benefit from insect pollination (Williams 1994), and this service is estimated to represent 9.5 % (153 billion dollars) to the world's food production worldwide (Gallai et al., 2009).

This alarming decline of bees is assumed to be caused by a combination of multiple factors (e.g. lack of flower resources, pesticides, diseases and pests ; (Goulson et al., 2015 ; Kuchling et al., 2018). The intensive use of pesticides in modern agriculture is emerging as a significant cause of the decline of bees (Johnson et al., 2010 ; Sanchez-Bayo & Goka, 2014). Scientists, politicians and the public are alarmed by the impact of pesticides on the environment and wish to limit their effects (Carson, 1962 ; Mrema et al., 2013). Pesticides can have both lethal or sublethal impacts, such as altered behavior, and development of individuals and reducing colony dynamics. (Chmiel et al., 2020 ; Di Noi et al., 2021 ; Henry et al., 2012).

Bees can be directly exposed to pesticides when foraging on recently treated crops (Ciarlo et al., 2012). In addition, spray drift from adjacent fields, contamination of food sources such as nectar and pollen, and the presence of pesticide residues in hive components contribute to this widespread exposure (Drummond et al., 2018 ; Sanchez-Bayo & Goka, 2014). Therefore, bees could be continuously exposed to pesticides with potentially synergistic effects on their life history traits (Chauzat et al., 2006 ; Schmuck et al., 2003 ; Tosi et al., 2022). Although the concentrations of many pesticides in beehives are relatively low compared with the toxicity levels defined by laboratories, recent meta-analyses indicate a potential underestimation of the effects of fungicides on bees, particularly in field conditions (Calatayud-Vernich et al., 2018 ; Di Noi et al., 2021).

Fungicides are a crop protection product designed to eliminate or limit the development of parasitic fungi and are not designed to target bees and other pollinating insects, so knowledge of their potential risks is limited. However, previous studies

suggest that fungicides can affect bee's behavior (navigation, learning), and their larval development (Liao et al., 2019). Among the various fungicides used in conventional agricultural production, boscalid is widely used due to its broad spectrum of action and effectiveness against fungal diseases (Li et al., 2021). In France, this active molecule is found alone or in mixtures in 44 commercial products covered by French regulation (ANSES 2023). Commercial formulations of boscalid (e.g. ASTRIAL DG, PICTOR PRO®, FILAN® SC...) are notably applied to crops such as peas, beans and oilseed rape after the first flower has appeared (BASF France SAS 2022). Bees are thus exposed to this molecule through its use in crops (Friedle et al., 2021). This product raises questions about the risk to bees and is frequently detected in hive matrices such as honey, beeswax, pollen and worker jelly. (Böhme et al., 2018 ; Friedle et al., 2021).

Considering the effects of pesticides at different stages in bees' life seems critical to ensure a comprehensive assessment of the impact of these products on colony health. Boscalid is a commonly used fungicide, member of the Carboxamide family, acts by inhibiting the activity of succinate dehydrogenase, an enzyme essential to the mitochondrial respiratory chain of fungal organisms (Stammeler et al., 2008). Although registration ecotoxicology tests demonstrated low acute toxicity to bees, several studies have begun to report effects of boscalid that are not observable by conventional ecotoxicology tests, chronic exposure in the laboratory and in the field has been associated with a reduction in the longevity of worker bees (Aubee & Lieu, 2010 ; Fisher et al., 2022 ; Simon-Delso et al., 2018). Other sublethal effects induced by boscalid have also been observed on bees (i.e altered physiology, morphology, learning, behavior; (DesJardins et al., 2021 ; Fisher et al., 2022 ; Glass et al., 2021 ; Liao et al., 2019 ; Tadei et al., 2019). Nevertheless, the harmful consequences of exposure to boscalid during the larval or adult phase on the life history of bees are still unclear.

In this study we experimented whether field-realistic exposure of boscalid could affect individual life history of bees at larval and adult stages, and if this potential impact on individuals could disturb colony dynamics and survival. We combined the use of the in vitro larval rearing method to monitor larval survival with the RFID technique to monitor the life cycle of adult bees. Bees were exposed to boscalid at field-realistic exposure during their larval and/or adult stage (in vitro larval rearing) or adult stage (in vivo and in vitro larval rearing). We compared their respective life cycles with those of

control bees (in vitro and in vivo larval rearing, respectively). We measured the main life cycle traits of all bees, including lifespan (LSP), age of first emergence (AFE), age of onset of foraging (AOF), foraging duration (FT), and foraging intensity (FI) of adult honeybees (Prado et al., 2020 ; Requier et al., 2020). We then explored by simulation if boscalid induced impact on bee individuals could disturb colony dynamics and survival with the use of an individual-based model. Results of this study will contribute to a more accurate assessment of the risks associated with the use of this fungicide in the field and will help to minimize any negative effects on pollinators.

MATERIALS AND METHODS

All experiments were conducted in the entomological experimental unit of INRAE, Le Magneraud (46° 8' 59,104"N, 0° 41' 28,609"W), France, between April and August 2021.

In vitro larval rearing

One-day-old larvae worker bees were collected from three honeybee colonies (*A. mellifera mellifera* × *caucasica*) and reared larval in vitro until emergence following the method described in Aupinel et al. 2007 (Aupinel et al., 2007 ; Medrzycki et al., 2013 ; Papach et al., 2017) The experiments involving exposure to boscalid were carried out at the same time as those whose results are described in the publication (Desclos le Peley, in publication). Further details of the larval rearing protocol are provided in more detail in that publication and can be found in the appendix (Appendix 1). Each larva was exposed to 0.1 % of acetone (Control) or boscalid included in 0.1 % acetone for 4 days in their diet (day 3 to day 6). Three graphing sessions were carried out in May, June and August. The rate of larvae that died before ingesting the first feed was 4.3 %, 2.8 % and 3.2 % respectively for the May, June and August sessions. For these in vitro reared individuals, we monitored daily mortality and the days of emergence. Only in May, the mortality of capped plates from D7 to D20 was assessed subsequently according to the stage of development. Overall, 946 individuals were grafted and reared in May, 971 individuals in June and 960 individuals in August.

Adult bee rearing

At emergence (days 18) in vitro larval reared bees were marked with an RFID transponder (see below). Bees were kept in polypropylene boxes (11 × 15 × 12 cm) with a 5 mm piece of Be-Boost® (PseudoQueen) attached to a piece of wax (approximately 5 × 5 cm) under laboratory conditions for 48 h at $34.5 \pm 5^\circ\text{C}$ and $50 \pm 5\%$ RH. They were then exposed to 0.1 % acetone or boscalid included in sugar solution (see below). After exposure the bees were in vitro larval reared are placed in small wire frames, which were sealed with candy. The frames were then placed directly in the bait comb host hive equipped with the RFID readers (see below the RFID Monitoring section).

On the other side, in vivo larval reared bees less than 12 hours old were collected from the hives that provided the larvae for the in vitro rearing. In vivo larval reared bees were marked with an RFID transponder and administered the same treatment as the bees reared larval in vitro at emergence : exposure for 48 h to acetone or boscalid and release.

Boscalid exposure

Based on data available in the literature, we assessed field-realistic exposure dose by boscalid. The concentration of boscalid in pollen brought back to the hive by bees varies widely throughout the world. For example, in California, the maximum dose detected is 6060 ng/g (Fisher et al., 2021) or in Canada it is 2200 ng/g (Tsvetkov et al., 2017). In our study We decided to use the quantity detected in Germany it is 1496.4 ng because the agricultural landscape is relatively like that which we have in France (Böhme et al., 2018). Considering that a bee larva consumes around 0.002 g of pollen over a period of four days (Babendreier et al., 2004), we estimated that the larvae would ingest around 3.05 ng of boscalid, while the bees would absorb around 15.6 ng of pollen over an exposure period of 48 hours after emergence. Boscalid stock solutions (99 % purity, Cluzeau Info Labo, France) were prepared using acetone and incorporated into the diet of larvae and young adult bees. The pesticide stock solutions were prepared at the beginning of the beekeeping season and stored at -20°C , only being thawed for later use. The larvae were exposed chronically for 4 days, from day 3 to day 6. They were either fed a larval diet containing 0.1 % acetone (C) or exposed to 3.05 ng boscalid (B_L) via larval diets B and C containing a boscalid concentration of 19.5 ng/g (see Appendix 1B). The young adult bees were exposed for 48 hours in their

diet, the food was available ad libitum. They received either sugar solution (50 % w/v sucrose) containing either 0.1 % acetone only (C and intoxicated larvae groups) or exposed to 15.6 ng of boscalid (intoxicated emergence and intoxicated larvae + emergence), the concentration of boscalid was 0.25 ng/μl in the sugar solution.

Thus, we had four modalities of exposure : (i) bees exposed to acetone (C ; bees reared larval in vitro + in vivo) ; (ii) bees exposed to boscalid during the larval phase (B_L ; bees reared larval in vitro) ; (iii) bees exposed to boscalid during the adult phase (B_A ; bees reared larval in vitro + in vivo) ; and (iv) bees exposed to boscalid during the larval and adult phase (B_{L+A} ; bees reared larval in vitro).

RFID monitoring

Adult bees were immobilised using a piston cage to attach mic3®-TAG 16 kbit RFID transponders (HF-RFID 13.56 MHz, microsensus mic3 technology) to their thorax. The transponders were attached using biocompatible dental cement (TempoSIL2). The dimensions of the tags were 1.6 x 1.9 x 0.5 mm³, with a weight of approximately 3 mg (Streit et al., 2003). Given its weight relative to the bees (representing 3 % of their total weight) and considering its ability to carry nectar or pollen (Marden, 1987 ; Winston, 1991) the impact of the tag's weight was regarded as marginal or negligible.

Overall, 289 bees, reared larval in vitro, were introduced in RFID monitored hive in May, 354 in June and 405 in August. Finally, 198 bees, reared larval in vivo, were introduced in RFID monitored hive May, 175 in June and 204 in August.

The operational status and unique identifiers of the tags were checked and recorded before installation, using a portable USB device (ID®PENmini 7.0, microsensus). Two sets of four RFID readers (iID2000, 2k6 HEAD ; Microsensus) were positioned at the hive entrance, for a total of eight readers. These two lines of readers facilitated differentiation of the direction of activity, distinguishing between entry and exit movements (Requier et al., 2020). Each reader was designed to pass through an 80 x 8 mm tunnel. As a tagged bee passed through, the reader recorded the data, including identity, date and time. Data was collected at seven-day intervals.

Life history traits

Statistical analyses were performed using R software version 4.2.2 (R Core Team 2022). The significance level for the statistical tests was set at 5 % for the probability of rejecting the true null hypothesis. Graphs were generated with the ggplot2 R package (Wickham et al. 2023). The compact letters of all pairwise comparisons were generated with the multcomp R package (Piepho, 2004).

Boscalid on bee larvae : cox proportional hazards model was developed using the coxme R-package (Therneau 2022). This model considered the different sessions (May, June and August) ; exposure (C and B_L) and the hive of origin of the larvae (R3, R4 and R6). The rearing plates were considered as a random factor in the model, with a standard deviation of 0.485 and a variance of 0.235. A specific model was performed to consider the significant effect of technicians transferring larvae in D7-D8 (June and August sessions only ; Table S1). Linear mixed model (LMM) was used to assess the day of emergence of bees reared in vitro, using lme4 R-package (Bates et al., 2023). The explanatory variables considered for this model were like those of the survival model previously described. Rearing plates were included as a random factor, with a standard deviation of 0.010 and a variance of 0.10 (Table S2).

Boscalid on adult bees : we assessed the main life history characteristics of 1048 bees reared in vitro and 577 bees reared in vivo based on RFID data, following the data cleaning method of Requier et al. (2020) and statistical were carried out using the same method as Desclos le Peley et al. (2024). This analysis aimed to compare the influence of exposure (C, B_L, B_A and B_{L+A}), as well as their interactions with the sessions (May, June, and August), the larval rearing protocol (in vitro and in vivo), the hive of origin of the larvae (R3, R4, and R6).

Lifespan (LSP) was measured in days to the last recorded RFID response for each bee. Other characteristics were calculated after removing bee activity less than 2 seconds and greater than 180 minutes. The LSP of bees analysis were performed using a Weibull hazard model with the survival package (Therneau et al., 2023). The age at first exit (AFE) of the bees was defined in days until the first recorded RFID contact (first flight) and were analysed with generalized linear mixed models (GLMMs) using the glmmTMB R package (Brooks et al., 2023). We then calculated foraging duration as the time elapsed between AFE and the point of exit from the hive (FT). We also analysed the proportion of bees lost after their first exit from the hive (L-AFE ;

binary response variables) with binomial GLMMs from the *ade4* R package (Coulon et al., 2020 ; Dray & Dufour, 2007). We then calculated foraging tenure (FT) as the time elapsed between AFE and the point of exit from the hive and foraging intensity (FI) was measured as the total number of flights made by the bees (see also Prado et al., 2020). The FT and FI's bees were performed with a negative binomial 1 GLMM (we included in the formula "ziformula~0"). Finally, we calculated the age at foraging initiation (AOF) of the bees, defined in days using the R *aof* package (Requier et al., 2020) and analyzed with gamma GLMM for 319 larvae reared in vitro and 364 larvae reared in vivo.

Simulation of honeybee colony dynamics

In order to test whether the observed effects of boscalid on individual bees could affect the colony dynamic and survival, we simulated the effect of shorter-lived bees into a model of bee colony dynamic, the BEEHAVE model (Becher et al., 2014). A total of 700 simulations were computed with $n=600$ simulations for assessing the boscalid-induced effect of shorter-lived bees on colony dynamics, and $n=100$ simulations for sensitivity analysis on colony survival. We first calibrated the model with Becher et al. (2014) initial colony settings associated with a random parameterization of four parameters to improve stochasticity and realism of the model, as follows: we randomly attributed a maximal egg-laying capacity of the queen between 500 and 1,600 eggs per day, an initial adult population size ranging from 7,000 to 10,000 bees, a larval mortality rate ranging from 0.009 to 0.011, and an infestation with 3 to 6 Varroa destructor mites from which the prevalence virus-infected mites range from 0 to 100 % on the first day of simulation as default value. To consider beekeeping management conditions, we also enabled the ad-hoc options such as the Varroa treatment and the honey harvests. We considered the gradient of maximal egg-laying capacity of the queen as the demographic strength of the colony, by converting the value from 0.4 (500 eggs per day) to 1 (1,600 eggs per day).

We incorporated the observed effect of boscalid into the model through modulations of the forager mortality rate for inducing shorter-lived bees. To this end, we gradually increased the forager mortality rate from the default value of 0.00001 to 0.0000156, representing an increase coefficient of $\times 1.56$ according to the maximum boscalid effect in our results. Thus, each simulation involved a relative effect of

boscalid according to our experimental observations, linearly increasing from no-effect (0 % of the effect) to full impact (100 % of the effect). We included these impacts in our simulations by increment of 46 days (i.e. the average duration of flowering crops Requier et al., 2015) for different time intervals during the colony dynamic, i.e. before the growth period of the population size (day 76 to day 122, interval 1), at the beginning of the growth period of the population size (day 122 to day 168, interval 2), and at the end of the growth period of the population size (day 168 to day 214, interval 3). We ran six scenarios of exposure for different time intervals (interval 1, interval 2, interval 3) and by combining the different time intervals (intervals 1+2, intervals 2+3, intervals 1+2+3). We computed 100 simulations per scenario with a gradual increase of boscalid impact. Moreover, we computed 100 other simulations with an extended gradient of forager mortality rate from the default value of 0.00001 to 0.00006 (i.e. increase coefficient of $\times 6$) in order to run a sensitivity analysis on colony survival. The objective was to analyze the risk of colony mortality due to the maximum boscalid effect observed in our results.

We registered the adult population size (i.e., the number of adult worker bees in the colony) and the quantity of honey reserves (quantity of honey in the brood chamber and in the super chambers together, as it is mentioned in the model) for the last day of the exposition period for each simulation. Moreover, we registered the simulation endpoint deemed insufficient for colony survival using two following thresholds according to Becher et al. (2014) : (i) simulations that reach population size smaller than 4,000 adult bees during the winter, and (ii) simulations that reach population size smaller than 2,500 adult bees from spring to autumn.

We analyzed the effects of the boscalid impact (i.e. simulating shorter-lived bees) on the population size and honey reserves (response variables) using linear models (LMs) and including the level of boscalid impact (for 0 to 100 %), the demographic strength of the colony (from 0.4 to 1) as explanatory variables. We also tested the interaction between the level of boscalid impact and the demographic strength of the colony. We analyzed the direct time effect of simulating shorter-lived bees on changes in population size, as well as delayed effects with +1 month, +2 months and +3 months after the end of the simulated impact. Finally, we performed the sensitivity analysis (i.e. risk of colony mortality) by fitting a standard logistic function with the use of a generalized linear model (GLM) with a binomial error distribution.

RESULTS

We found a significant effect of sessions of the experiment in all GLMM : larvae mortality, emergence, LSP, AFE, L-AFE, FT, FI, foraging, and AOF ($\chi^2 > 28.85$; df = 2 ; $p < 0.001$; Table 1). Additionally, we found a significant effect of boscalid exposure and an interaction effect with experiment sessions on GLMM : emergence, LSP, AFE, L-AFE ($\chi^2 > 11.54$; df = 1 or 3 ; $p < 0.009$; Table 1). In the other GLMM : larvae mortality, FT, FI, forager and AOF we did not measure significant effect of boscalid ($\chi^2 > 6.30$ df = 3 ; $p < 0.098$; Table 1). We reported a significant effect of the hives from which the individuals were taken in GLMM : larvae mortality, emergence, LSP, AFE, L-AFE and AOF ($\chi^2 = 6.12$; df = 2 ; $p < 0.047$, Table 1). Moreover, we found a significant effect of the larval rearing protocol on the life history traits of adult bees, particularly in the GLMM : LSP, L-AFE, FI ($\chi^2 = 60.07$; df = 1 ; $p < 0.001$, Table 1).

Table 1: Type II analysis of variance for the different generalized mixed effect models. For all GLMM, the chisquare tests of Wald's likelihood are calculated with their associated p-values (• : p-values < 0.1 ; * : p-values < 0.05 ; ** : p-values < 0.01 ; *** : p-values < 0.001).

Variables	Session		Exposure		Session : Exposure		Rearing		Hive		Data analyse
	χ^2	p-value	χ^2	p-value	χ^2	p-value	χ^2	p-value	χ^2	p-value	
Larvae Mortality	65.06	<0.001	0.02	0.88874					6.12	0.04698	Fig.1A; Tab.S1
Emergence	28.85	<0.001	18.67	<0.001					36.61	<0.001	Fig.1B; Tab.S2
LSP	365.37	<0.001	19.17	<0.001	26.24	<0.001	60.07	<0.001	9.24	0.001	Fig.2; Tab.S3
AFE	211.43	<0.001	11.54	0.009	43.74	<0.001	1.70	0.192	8.79	0.012	Fig.3A; Tab.S4
L-AFE	202.76	<0.001	12.38	<0.001	25.32	<0.001	97.16	<0.001	5.92	0.052	Fig.3B; Tab.S5
FT	41.75	<0.001	7.17	0.067	5.38	0.496	0.64	0.425	0.40	0.818	Tab.S6
FI	53.50	<0.001	3.77	0.288	10.03	0.123	28.06	<0.001	0.40	0.815	Tab.S7
Forager	60.01	<0.001	6.30	0.098	2.04	0.916	2.45	0.118	1.30	0.523	Tab.S8
AOF	40.62	<0.001	1.66	0.645	2.37	0.882	2.27	0.132	21.11	<0.001	Tab.S9

Boscalid delays the development of bee larvae

We did not find any effect of the boscalid exposure on larval survival ($\chi^2 = 0.02$; df = 1 ; $p > 0.89$, Figure 1). The survival of in vitro reared larvae bees was affected by the session of the experiment ($\chi^2 = 65.06$; df = 2 ; $p < 0.001$; Table 1). In May, larval mortality was significantly higher than in June and August (May : 1.863 ± 0.194 HR (Hazard Ratio), June : 0.925 ± 0.100 HR and August : 0.587 ± 0.065 HR ; Tukey test > 0.701 ; $p < 0.003$).

Chronic exposure of larvae to boscalid had effect on LMM bee emergence day ($\chi^2 = 18.674$; df = 1 ; $p < 0.001$; Table 1). Larvae exposed to boscalid will have a delayed

day of emergence compared to bees exposed to the control (C : 19.12 ± 0.023 and B_L : 19.27 ± 0.023 ; Tuckey test = -0.142 ; $p < 0.001$; Fig.1). Also, we found an effect of the experiment session on the emergence day of in vitro bees ($\chi^2 = 28.85$; $df = 2$; $p < 0.001$, Table 1)

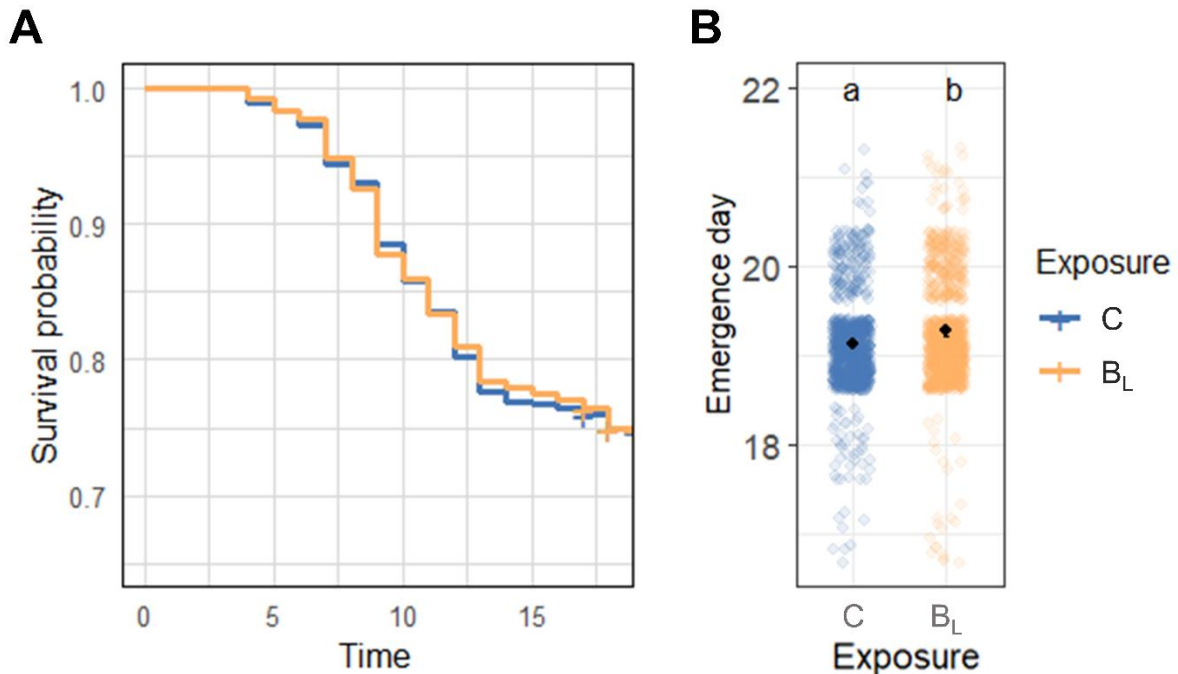


Figure 1. Impact of boscalid on larvae development. (A) on larvae survival, (B) on emergence in days. The colours show that the larvae were chronically exposed to Acetone (C) or boscalid (B_L) in their diet on days 3, 4 and 5. Transparent dots represent raw data. Larger dots or bar represent model estimates $\pm$ confidence level (95 %). Significant differences of Tuckey pairwise comparisons indicated with letters for each session.

Boscalid impact on the life span and first activity of bees

We found a significant effect of experiment sessions on LSP ($\chi^2 > 365.37$; $df = 2$; $p < 0.001$; Table 1). Bee survival was significantly different between the different sessions (May : 19.01 ± 0.87 Days, June : 6.026 ± 0.026 Days and August : 13.49 ± 0.52 Days ; Tukey test > 0.34 ; $p < 0.001$). Additionally, we found a significant effect of boscalid exposure ($\chi^2 > 19.17$; $df = 3$; $p < 0.001$; Table 1) and an interaction effect with experiment sessions on LSP ($\chi^2 > 26.24$; $df = 6$; $p < 0.001$; Table 1). In bees reared in vivo exposed to B_A had a shorter LSP than bees exposed to C, but only during the June sessions (C : 9.99 ± 0.66 days ; B_A : 7.10 ± 0.51 days ; Tukey test = 0.40 ; $p < 0.001$; Fig. 2). In bees reared in vitro exposed to B_L, B_{L+A} had a shorter LSP than

bees exposed to C, but only during the June and August sessions (in June, C : 6.70 ± 0.47 days, B_L : 4.27 ± 0.38 HR, B_A : 4.77 ± 0.33 days and B_{L+A} : 4.44 ± 0.41 days ; in August, C : 13.61 ± 0.90 days, B_L : 9.43 ± 0.79 days, B_A : 12.55 ± 0.82 days and B_{L+A} : 179.25 ± 0.77 days ; Tukey test > 0.34 ; $p < 0.001$; Figure 2). Bees reared in vitro exposed to B_A had shorter LSP than bees exposed to C (B_A : 4.77 ± 10.33 days ; Tukey test = 0.34 ; $p < 0.001$; Figure 2). Bees reared in vitro exposed to B_{L+A} had a shorter LSP than bees exposed to B_A bees but only in August (Tukey test = 0.30 ; $p < 0.001$, Figure 2).

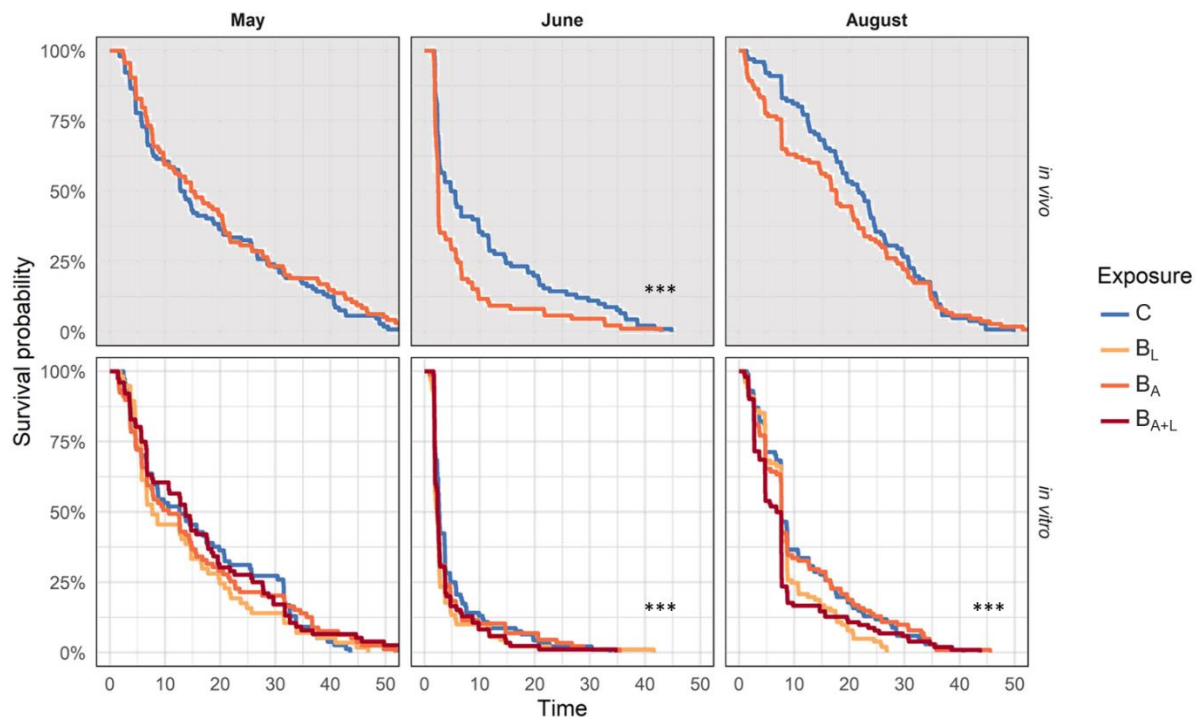


Figure 2. Impact of boscalid on the survival of adult bees. Adult bees were following larvae rearing protocol (i.e. in vitro and in vivo) for each experiment session (May, June and August). The bees were exposed to control (C), bees exposed to boscalid at larvae (L), bees exposed to boscalid at adults (A) and bees exposed to boscalid to larvae + adults (L+A). *** p -values < 0.001, if boscalid affect LSP for each session.

We found a significant effect of sessions of the experiment in GLMM : AFE, L-AFE, ($\chi^2 > 37.8$; $df = 2$; $p < 0.001$; Table 1). In addition, we observed a significant effect of boscalid exposure ($\chi^2 > 19.17$; $df = 3$; $p < 0.001$; Table 1) and an interaction effect with experimental sessions ($\chi^2 > 26.24$; $df = 6$; $p < 0.001$; Table 1). We found that bees reared in vivo exposed to B_A, a significant decrease in AFE compared to bees exposed to C during the June sessions (C : 4.61 ± 0.19 days and B_A : 3.64 ± 0.16

days ; Tukey test = -0.06 ; $p < 0.001$; Fig. 3A). We reported that bees reared in vitro exposed to B_L , B_A , B_{L+A} a significant decrease in AFE compared to bees exposed to C during the June sessions ($C : 4.44 \pm 0.19$ days $B_L : 2.95 \pm 0.17$ days, $B_A : 3.55 \pm 0.15$ days and $B_{L+A} : 3.28 \pm 0.19$ days ; Tukey test > 1.274 ; $p < 0.007$; Fig. 3A). We showed that bees reared in vitro exposed B_L , B_{L+A} showed a significant increase in L-AFE compared to bees exposed to C in August sessions ($C : 0.51 \pm 0.03$, $B_L : 0.74 \pm 0.04$, $B_A : 0.58 \pm 0.04$ and $B_{L+A} : 0.78 \pm 0.04$; Tukey test > 0.28 ; $p < 0.001$; Fig. 3B).

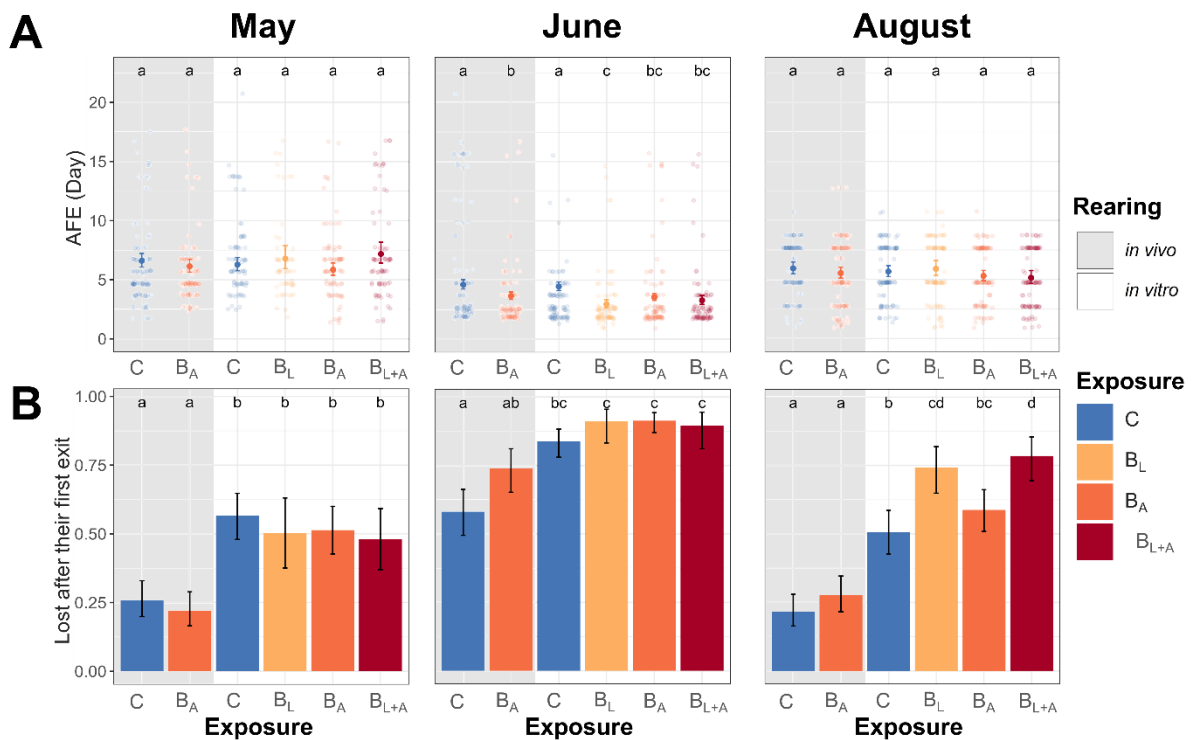


Figure 3. Impact of boscalid on first flight activity of adult bees. (A) AFE in days (B) L-AFE in proportion. The bees were exposed control (C), bees exposed to larvae (L), bees exposed to adults (A) and bees exposed to larvae + adults (L+A) for each experiment session (May, June and August). The colors show that the adult bees followed the larval rearing protocol (in vitro and in vivo). Transparent dots represent raw data. Larger dots or bars represent model estimates $\pm$ Confidence level (95 %). Significant differences of Tuckey pairwise comparisons indicated with letters for each session.

Simulated effect of shorter-lived bees on colony dynamics

Sensitivity analysis showed that simulating shorter-lived bees (i.e. the relative effect of boscalid) reduced colony survival (GLM with binomial error distribution : $Z = -5.097$, $p < 0.001$) and that the observed maximum effect of boscalid on individual bees (i.e. an increase coefficient of $\times 1.56$) led to a predicted risk of colony mortality of 2 % (95 % CI : 0-10%, Figure S1). On the other hand, we observed a single colony collapse in our set of 600 simulations (Figure S2). However, we found disturbances in the simulated colony dynamics.

We found that simulating shorter-lived bees (i.e. the relative effect of boscalid) before the growth period of the population size (day 76 to day 122, interval 1) reduced the population size of the simulated colonies by 18.5 % ($t = -3.052$, $P = 0.003$, Figure 1A) and their honey production by 13.0 % ($t = -2.864$, $p = 0.005$, Figure 1B) during this exposure period. We also found significant interactions between the level of boscalid impact and the demographic strength of the colony, showing that the demographically weaker colonies were more affected by this boscalid impact on both population size ($t = 2.080$, $p = 0.040$) and honey production ($t = 2.026$, $p = 0.046$). We found that these impacts of boscalid on colony dynamics can be observed up to two months after the end of the simulated impact, without interaction with the colony demographic strength, on population size (+1month : $t = -2.133$, $p = 0.036$; +2months : $t = -2.121$, $p = 0.037$, Figure 1A) and honey production (+1 month : $t = -2.737$, $p = 0.007$; +2 months : $t = -0.587$, $p = 0.011$, Figure 1B). Three months after the end of the simulated impact, we observed no significant effect of the previously simulated shorter-lived bees on population size ($t = -0.230$, $p = 0.818$) and honey production ($t = 1.999$, $p = 0.048$).

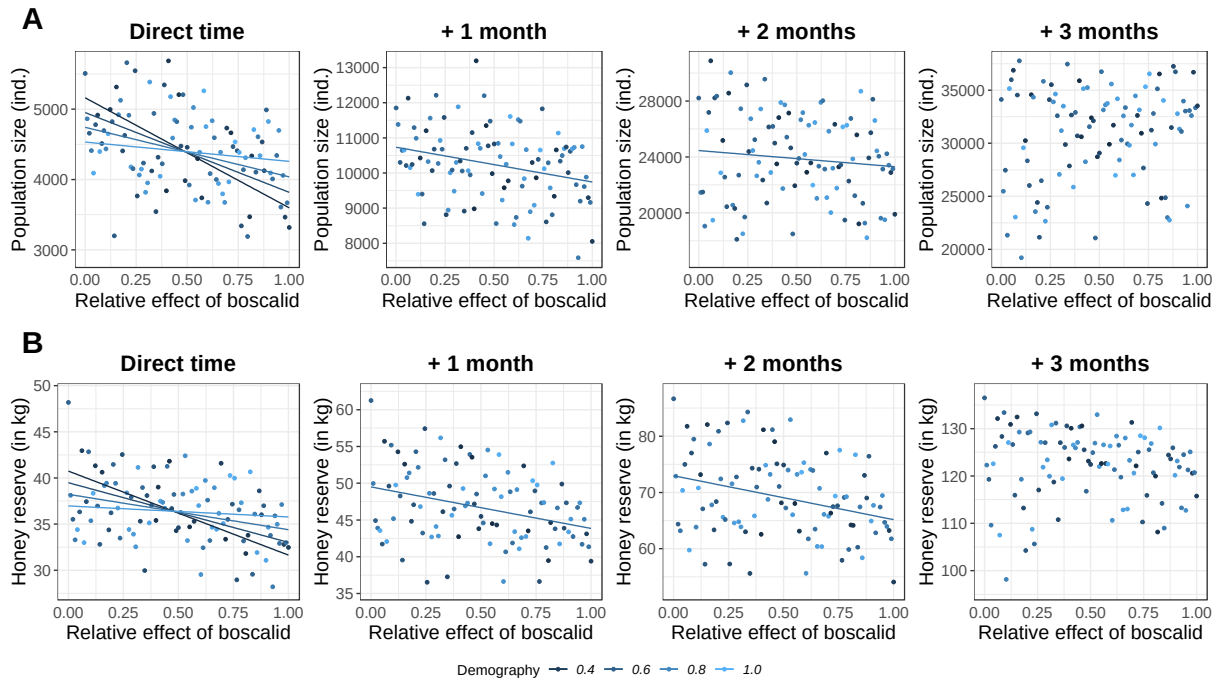


Figure 4. Simulated effect of shorter-lived bees (the relative effect of boscalid) from day 76 to day 122 (interval 1) on colony dynamics. (A) Changes in population size were analyzed along a gradient of relative effect of boscalid (from 0 to 1, no-effect and full impact, respectively) simulating shorter-lived bees from day 76 to day 122 (i.e. before the growth period of the population size, interval 1). We analyzed the direct time effect of simulating shorter-lived bees on changes in population size, as well as delayed effects with +1 month, +2 months and +3 months after the end of the simulated impact. (B) Changes in honey reserves were also analysed along a gradient of relative effect of boscalid simulating shorter-lived bees from day 76 to day 122 (i.e. interval 1). We analyzed the direct time effect of simulating shorter-lived bees on changes in honey reserves, as well as delayed effects with +1 month, +2 months and +3 months after the end of the simulated impact.

We found no significant effects of the relative effect of boscalid on changes in population size and honey reserves when simulating shorter-lived bees at the beginning of the growth period of the population size (day 122 to day 168, interval 2, Figure S3), at the end of the growth period of the population size (day 168 to day 214, interval 3, Figure S4), and in the scenario combining intervals 1+2 (Figure S5). However, we found significant decreases in the population size and honey reserve related with the relative effect of boscalid simulated during the intervals 1+2 (Figure S6) and the intervals 1+2+3 (Figure S7). Interestingly, we found significant interactions

between the level of boscalid impact and the demographic strength of the colony, showing that the demographically weaker colonies were generally more affected by this boscalid impact (Figure S6, Figure S7). Moreover, we found that these impacts of boscalid on colony dynamics can be observed up to three months after the end of the simulated impact (Figure S6, Figure S7).

DISCUSSION

Scenario of exposure

The main aim of our study was to assess the impact of exposure to boscalid, at field-realistic exposure, on various aspects of the bee life cycle. We adopted a parsimonious approach, to realize larval and adult exposure, based on boscalid contamination present in the pollen that feeds these individuals (Böhme et al., 2018). As ingestion of contaminated food, particularly pollen from pesticide-treated plants, has been identified as one of the main routes of larval exposure (Babendreier et al., 2004 ; Böhme et al., 2019). In our research, individuals were exposed to concentrations of 3.05 ng/larva or 15.6 ng/bee. Limited information is available on the routes of contamination, accumulation or degradation of boscalid in various bee matrices and their impact on the larvae and adult bees (Wueppenhorst et al., 2022). Nevertheless, various exposure pathways contribute to the accumulation of pesticides throughout the have not considered the fact that bee larvae come into direct contact with wax, which may contain pesticide residues from a variety of sources (Friedle et al., 2021 ; Xiao et al., 2022). In addition, we did not estimate direct exposures through contact or ingestion in the field, notably those linked to contaminated nectar. boscalid seems to persist in the environment and can be found in wildflowers around agricultural fields (Zioga et al., 2023). Although the presence of boscalid metabolites has been measured in bee samples from various hives in France, the deleterious effects of these metabolites on bees are still unclear (Jabot et al., 2015, 2016). Consequently, it is likely that we have use field-realistic hight exposure doses of pesticides to which individuals may be exposed in their environment, by considering only pollen ingestion way.

We integrated mortality rate for inducing shorter-lived bees in our simulations for different time intervals during the colony dynamic, starting before the growth period of the population size (day 76) and finished at the end of the growth period of the

population size (day 214) by increment of 46 days (i.e. the average duration of flowering crops ; Requier et al. 2015). We have also combined the time sea in order to propose more pessimistic scenarios (Figure S1). The phenology of bees depends on the floral resources made available to them during the season. (Requier et al., 2015 ; Wood et al., 2020). We can identify our intervals based on the seasonal variation in pollen loads collected by bees (Bilisik et al., 2008). Thus, our intervals correspond to pollen from (interval 1) Rosaceae families (interval 2) Brasicaceae families and (interval 3) *Helianthus annuus* by foragers. Boscalid is authorized for use on plants during the flowering period of our intervals (apple, pear, quince, apricot, rapeseed, sunflower), (ANSES, 2023). This is confirmed by the frequent detection of boscalid in pollen collected by bees during this period (Böhme et al., 2018 ; Daniele et al., 2018 ; Friedle et al., 2021 ; Tosi et al., 2022). Our scenario proves to be protective for bees, as it includes general exposure to boscalid, given its irregular use in the landscape during flowering periods. Furthermore, boscalid appears to persist in the environment, as evidenced by its presence in wildflowers on the edge of fields (Froger et al. 2023). Furthermore, its detection in nectar or pollen is not necessarily linked to application during the year (Zioga et al. 2023).

Boscalid effects on individuals

Our results show significant effects of boscalid exposure on larvae and adult bees. Larval exposure to boscalid caused a delay in the emergence of adult bees, disrupting the normal life cycle. In adult bees, we observed a significant reduction in longevity, particularly during the June and August sessions when both larval and adult bees were exposed. For larvae, no significant effects were observed following larval exposure, which is consistent with other studies (Simon-Delso et al., 2018). Conventional ecotoxicological tests, which focus only on the larval cycle, are not effective for monitoring sublethal effects. Here we show that boscalid can have long-term effects on the bee's life cycle. These individuals exposed during their larval phase are consistent with field observations indicating a reduction in the longevity of worker bees (Fisher et al., 2022), as well as earlier initiation of foraging activity (DesJardins et al., 2021 ; Fisher et al., 2021 ; Liao et al., 2019). These behavioral changes may result from larval exposure, causing notable alterations in bee flight behavior, reduced wing beat frequency, changes in thorax mass, and late alterations in the expression of

certain developmental genes (Glass et al. 2021 ; Tadei et al. 2019, Desclos le Peley et al. in press). In addition, the health of exposed adult bees may be affected by changes in gut morphology and the fungal and bacterial communities of the gut and cuticular microbiota (Dong et al., 2023 ; Reiß et al., 2023).

Seasonal effect of boscalid

The different experimental sessions had significant effects on several aspects of the bees' life cycle. All variables : larval mortality, emergence, lifespan, first flight activity, and the day of delayed first flight activity were impacted by sessions. These results highlight the importance of seasonal conditions on bee development, independent of boscalid exposure. In addition, boscalid exposure also showed significant effects on emergence, lifespan, first flight activity, and day of first delayed flight activity, highlighting the complex interaction between environmental factors and exposures in affecting bee's life history traits.

The behavioral variations of the bees during the sessions are consistent with an experiment conducted in parallel and with seasonal fluctuations (Desclos le Peley, In press ; Decourtye et al., 2003). Seasonal variations also influence the availability of resources, modifying the availability and composition of the pollen and nectar available to bees (Henry et al., 2014 ; Requier et al., 2015). Studies have clearly shown that variations in the nutritional quality of food resources can have repercussions on the health and lifespan of bees, acting in synergy with the negative effects of pesticides (Barascou et al., 2021 ; Barraud et al., 2022 ; Henry et al., 2014b). More over, during the summer season, the health status of colonies varies and deteriorates, with an increase in the prevalence of diseases such as varroa mites and the deformed wing virus (Chen et al., 2021 ; Pfeiffer & Crowder, 2022 ; Tentcheva et al., 2004). It has also been shown that colony health also plays a major role in the sensitivity of bees to pesticides/ boscalid, due to synergistic effects and an overall reduction in immune and detoxification capacities (Johnson et al., 2013 ; Pineaux et al., 2023).

Boscalid disturbs colony dynamics

We found no significant effects of the relative effect of boscalid on changes in population size and honey reserves at the beginning of the growth period (interval 2) and at the end of the growth period (interval 3). Significant decreases in population size and honey reserves were observed in the scenario with to the relative effect of boscalid at before the growth period (interval 1, interval 1+2 and 1+2+3). Thus, colonies could be most sensitive to boscalid at field-realistic exposure in spring, before the growth period. This corresponds to the period when foragers recruit pollen from plants in the Rosaceae family (Bilisik et al., 2008). Boscalid is used in arboriculture (i.e. cherry, apple, pear, quince, apricot ; ANSES 2023), It is therefore plausible that the application of boscalid during flowering in arboriculture plays an important role in weakening colonies in orchards.

We found that simulating the relative effect of boscalid at before the growth period (interval 1) reduced the population size of simulated colonies by 18.5% and their honey production by 13.0 %. We also found that demographically weaker colonies were more affected by the impact of boscalid, which can be observed up to two months after the end of the simulated impact. Thus, we show boscalid at field-realistic exposure could have a direct role in colony weakening and could reduce income for beekeepers. This decrease in population size did not result in the collapse of the colony, but the population decrease is above the critical value of the specific protection objectives set by EFSA (SPG : 10 %), which is extremely alarming. These results are confirmed by several field studies showing a decrease in populations at the hive site and an increase in pollen storage (Fisher et al., 2021). They are also supported by the observation that boscalid is one of the pesticides found in colonies when bees are disturbed or die in apiaries (Daniele et al., 2018). this reduction in populations size could have a detrimental effect on their ability to cope with other biotic or abiotic stresses, as well as on their reproduction, competitiveness and longevity, particularly during the winter season (Free & Racey, 1968 ; Harbo, 1983 ; Locke & Fries, 2011 ; Winston, 1987). Furthermore, this reduction in honey production could lead to a lack of resources during dearth periods and in winter, which would compromise colony survival (Free & Racey, 1968 ; Requier et al., 2015).

CONCLUSION

Our results show that boscalid at field-realistic exposure on larvae and adults has sublethal effects on the life history trait of bees in the hive in three sessions. In vitro rearing, used to expose bees to boscalid during the larval stage, revealed an increase in development time to emergence. Monitoring adult bees using RFID technology has shown that boscalid has a variable effect during the season. Boscalid at field-realistic exposure on Larval and adult decrease in their lifespan due to an earlier age of first flight or failure to return from this flight. Boscalid, under realistic field conditions, decreases the lifespan of larvae and adults due to an earlier age of first flight or lack of return from this flight. Simulation of the relative effect of boscalid on shorter-lived bees to disrupt colony dynamics simulated. In particular, before the growth period exposure (the spring) showed a simulated colony sensibility and decreased population size in greater than the GSP set by EFSA. Also honey production was reduced during simulations indicating that boscalid could induce a decrease in honey harvest for beekeepers.

ACKNOWLEDGMENTS

The authors would like to thank Maxime Pineaux, Elisa Gomes, Manon Desaiivres, Colombe Chevallereau, Thierry Tamic and Fabien Dequine for their contribution to the implementation of the experiment and to the logistics.

FUNDING

Research reported in this publication was supported by OFB as part of the Ecophyto II+ call on “Phytopharmaceuticals products: from exposure to impacts on human health and ecosystems” launched by the French Ministry in charge of Ecology, Agriculture, Health and Research for the project EXPLORA granted to F.-J. Richard (2021–2024).

AUTHORS' CONTRIBUTIONS

Victor Desclos le Peley : conceptualization ; data curation ; methodology ; formal analysis ; investigations ; visualization ; writing – original draft ; Writing – review & editing.

Fabrice Requier : conceptualization ; methodology ; supervision ; formal analysis ; investigations ; visualization ; writing – original draft ; Writing – review & editing.

Stéphane Gâteau : ressources ; writing – review & editing.

Carole Moreau-Vauzelle : Ressources ; Writing – review & editing.

Daniel Raboteau : resources ; Writing – review & editing.

Pierrick Aupinel : conceptualization ; funding acquisition ; supervision ; writing – review & editing.

Freddie-Jeanne Richard : conceptualization ; funding acquisition ; investigation ; supervision ; methodology ; project administration ; Writing – review & editing.

CHAPITRE 3 - DE L'INDIVIDU A LA MOLECULE :

CARACTERISATION DE L'IMPACT PHYSIOLOGIQUE D'UN FONGICIDE

Avant-propos

Comme nous l'avons vu dans le chapitre précédent, le boscalid induit des effets sublétaux sur les abeilles, notamment en affectant leur développement et leur activité à l'échelle individuelle, avec des répercussions sur la colonie (Fisher et al., 2022 ; Simon-Delso et al., 2017). Ces modifications des traits d'histoire de vie découlent d'altérations physiologiques et métaboliques induites par le boscalid, qu'il est crucial d'identifier. Chez les reines, le boscalid perturbe les voies métaboliques associées au développement et aux réserves, en modifiant l'expression des gènes du cytochrome P450 pour la vitellogénine ou l'hexamérine (Jabot et al. 2016 ; Pineaux et al. 2023). En combinaison avec la pyraclostrobine, il perturbe les voies associées à la détoxification chez les ouvrières adultes, en régulant à la hausse plusieurs gènes du cytochrome p450 (Mao et al., 2017). Cependant, l'impact du boscalid sur le métabolisme des larves est actuellement mal compris. En outre, le boscalid est utilisé dans l'agriculture par le biais de formulations commerciales composées d'un cocktail de molécules inertes en plus de la molécule active (ANSES, 2023). Certaines formulations commerciales se sont révélées plus nocives pour les abeilles que les formulations brutes de molécules actives en raison de la synergie avec des éléments inertes (Chen et al. 2023 ; Straw & Brown 2021). Il est ainsi important d'évaluer les effets potentiels d'interactions de ces formulations, comme le Pictor Pro® (composé à 50 % de boscalid), par rapport à la molécule brute seule.

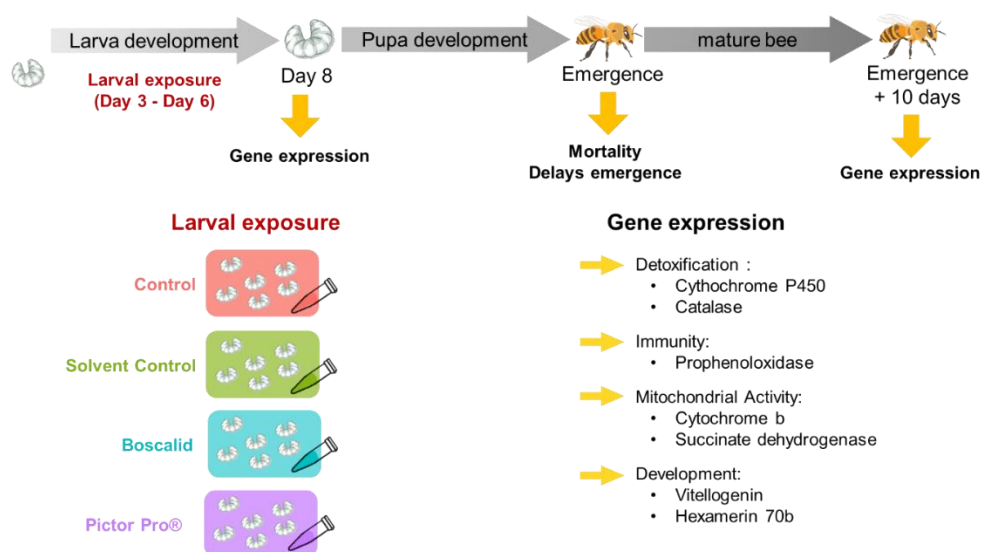
Les objectifs

L'objectif de ce chapitre vise à mieux comprendre les effets des différentes formulations du boscalid sur la physiologie et le développement de l'abeille. Afin de répondre à cet objectif, les effets du boscalid ont été mesurés sur les paramètres de développement et sur la mortalité des larves ; l'expression des gènes juste après exposition des larves et pendant le stade adulte.

Sur la base des éléments développés précédemment, deux hypothèses émergent :

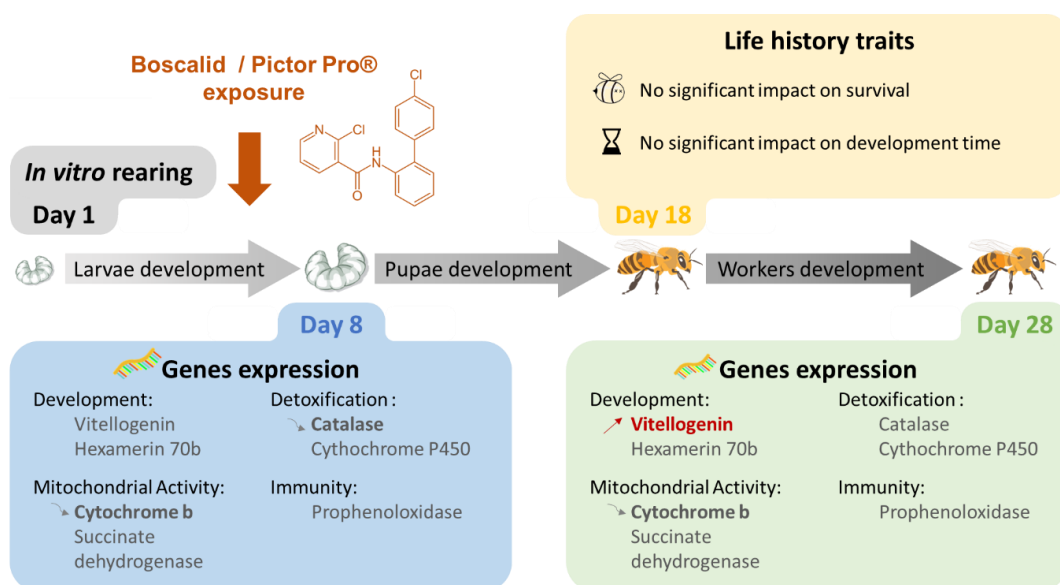
- L'exposition des larves au boscalid pourrait induire une perturbation du métabolisme qui sera mesurable juste après exposition, mais également à plus long terme.
- La formulation commerciale de boscalid, par sa composition complexe, pourrait affecter différemment la réponse physiologique des individus.

Les niveaux d'expression des gènes ont également été mesurés à la fois quelques jours après l'exposition chronique des larves et à la fois sur des individus adultes âgés de 10 jours après l'émergence. Les gènes cibles étaient liés aux voies métaboliques de l'immunité (prophénoloxidase), de la détoxification (catalase et cythochrome P450), du développement et de réserve (hexamerine 70b et vitellogénine) et de l'activité mitochondriale (cythochrome b et succinate déshydrogénase).



La dose d'exposition de boscalid utilisée est similaire à celles utilisées dans les expérimentations du chapitre 2. Nous avons procédé à une exposition chronique des larves au boscalid en utilisant une formulation pure (3,05 ng/larve de boscalid + 0,1 % d'acétone), ainsi qu'une formulation commerciale (6,1 ng/larve avec Pictor Pro®, 50 % de boscalid). Nous avons inclus des groupes de larves contrôle sans exposition et un contrôle avec solvant (0,1 % d'acétone).

Le résumé des résultats



Nos résultats ne montrent pas d'effets significatifs de l'exposition chronique au boscalid sur la survie et l'émergence des individus. Cependant, les larves exposées au boscalid présentaient des niveaux d'expression significativement plus faibles pour certains gènes associés à la détoxification et à l'activité mitochondriale (catalase et cytochrome b) par rapport aux larves exposées au contrôle. Cependant, cette différence n'était pas significative en regard de celle exposée au contrôle solvant. Les analyses ont révélé des effets tardifs sur la physiologie des abeilles adultes, notamment sur les gènes liés aux mitochondries et au développement. Les abeilles adultes présentaient des niveaux d'expression significativement plus faibles du gène du cytochrome b par rapport aux abeilles exposées au contrôle, mais cette différence n'était pas significative en regard de celles exposées au contrôle solvant. Quant au gène de la vitellogénine, il était significativement sur-exprimé chez les abeilles exposées par rapport aux abeilles contrôles et cette différence était seulement une tendance par rapport aux abeilles exposées au contrôle solvant. Pour finir, les effets observés avec la molécule brute de boscalid n'ont pas été observés avec le Boscalid en formulation commerciale Pictor Pro®.

Publication

Cette étude a été soumise dans la revue *Apidologie* (25/09/2023).

Les annexes supplémentaires se trouvent dans l'annexe 3.

IMPACT OF EXPOSURE OF LARVAE TO BOSCALID AT FIELD CONCENTRATION ON GENE EXPRESSION IN HONEY BEES

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Running title : Impact of larval exposure to Boscalid

ABSTRACT

Apis mellifera can be confronted with the fungicide Boscalid in agricultural landscape. In this study, we assessed the impacts of ingesting Boscalid and Pictor Pro® (a commercial formulation of Boscalid) at environmentally relevant doses during the larval development phase. After chronic exposure, we recorded survival, development time and gene expression in larvae and in ten-day-old workers. The targeted genes are associated with immune, detoxification, development, and mitochondrial activity pathways. We did not observe a significant impact of Boscalid in larval survival and emergence. Genes linked to cytochrome b, succinate dehydrogenase and catalase were also under-expressed in larvae and adult workers. Late variation was observed in ten days adult bees' overexpression of the vitellogenin gene, which is linked to development. The effects observed with Boscalid molecule was not observed with the commercially formulated product, Pictor Pro®.

KEYWORDS

Apis mellifera, *In vitro*, Fungicide, SDHI, field concentration, Vitellogenin

INTRODUCTION

The process of pollination, facilitated by insects, plays an essential role in the successful reproduction of flowering plants and is vital to the functioning of terrestrial ecosystems, providing vital ecosystem services that are essential to human production (Garibaldi et al., 2014 ; Ollerton et al., 2011). Their role in plant reproduction is crucial, contributing to the production of fruit, vegetables and seeds in many species 75 % of our crop species, and 90 % of all flowering plants (Tepedino 1979) require pollinators to reproduce (Klein et al. 2007 ; Tepedino 1979). Overall, pollinators provide a global service worth \$153 billion to food production (Gallai et al., 2009).

Insect populations, including honeybees, are declining worldwide and poses significant concerns and prompts extensive inquiries into the contributing factors (Hallmann et al., 2017). This decline is the result of multiple factors acting separately or in combination (Goulson et al., 2015). Habitat modification and loss (Kuchling et al., 2018), parasites and diseases (Fünfhaus et al., 2018) and pesticides (Sanchez-Bayo & Goka, 2014) are major factors contributing to the decline of bees. Many chemicals can have harmful effects on bees, including immediate mortality at high doses and sublethal effects such as alterations in development, behavior and overall capacity at lower doses (Henry et al., 2012).

Pesticides, including insecticides, fungicides and herbicides, are extensively utilized in agriculture to safeguard crops against pests and diseases (Mitchell et al., 2017). The total number of 454 approved active substances in the EU in May 2023, which further translates into a vast number of commercial formulations incorporating one or more active substances with adjuvants (EU Pesticides Database - Active substances 05/2023). Bees can encounter pesticides through various pathways, such as direct exposure while foraging on treated crops, spray drift, contamination of food sources, and the presence of pesticides in hive components (Sanchez-Bayo & Goka, 2014). Although the concentrations of many pesticides found in beehives are relatively low compared to the oral and contact LD50, recent meta-analyses suggest that the effects of fungicides on bees are inadequately studied and potentially underestimated, particularly in real-world conditions (Di Noi et al. 2021). For bee larvae, one primary pathway of pesticide exposure is through the consumption of pollen contaminated with pesticides from treated plants. Studies have observed a correlation between pesticide

quantity and the presence of pollen grains in worker jelly, indicating the risk of larval exposure to pesticides (Böhme et al., 2019 ; Drummond et al., 2018). These findings emphasize the necessity for further investigation into the impacts of pesticide exposure on bees, including a comprehensive understanding of sublethal effects on larval development and health status.

Fungicides are not specifically designed to target bees and other pollinating insects, so knowledge of their potential risks is limited. However, previous studies suggest that fungicides can affect navigation, learning, and larval development in bees (Liao et al., 2019). Among the various fungicides used in conventional agricultural production, Boscalid is widely used due to its broad spectrum of action and effectiveness against fungal diseases (Li et al. 2021). In France, this active molecule is found alone or in mixtures in 47 commercial products covered by French regulation (ANSES 2023). Pictor Pro® is a commonly used commercial formulation of Boscalid, applied to crops such as peas, beans, and oilseed rape after the first flower emerges (BASF France SAS 2022).

Notably Boscalid, a commonly used, is frequently detected in hive matrices such as honey, beeswax, pollen, worker jelly (Böhme et al., 2018 ; Friedle et al., 2021). Boscalid belongs to the Carboxamide family and acts by inhibiting the activity of succinate dehydrogenase, an enzyme essential for the mitochondrial respiratory chain in fungal organisms (Stammler et al., 2008). In addition, recent studies have shown that exposure to Boscalid can have harmful effects on bees and is associated with a reduction in the longevity of worker bees (Fisher et al. 2022 ; Simon-Delso et al. 2018). Exposure can also lead to sublethal effects with a decrease in the capacities and behaviour changes of individuals : such as lower pollen consumption, learning reduction, earlier foraging, and a decrease in the frequency of wing beats (DesJardins et al., 2021 ; Fisher et al., 2021 ; Fisher et al., 2022 ; Liao et al., 2019). Although the effects of several pesticides (chlorpyrifos, imidacloprid, amitraz, fluvalinate, coumaphos, myclobutanil, chlorothalonil) on gene expression have been observed in larvae, no study has specifically examined the effects of Boscalid (Gregorc et al., 2012 ; Shi et al., 2020 ; Tesovnik et al., 2017).

Boscalid has been shown to modify feeding behaviour by disrupting the nutritional intake necessary for the overall health and survival of the colony, particularly

during the winter (Fisher et al., 2021). In the *Apis Cerana*, the genes involved in detoxification and immunity were also strongly up-regulated following treatment with a cocktail of pesticides containing boscalid (Dong et al., 2023). In addition, exposure to boscalid led to physiological changes in queens, notably in the expression of the vitellogenin gene, and among many other consequences had negative effect on colonies in terms of brood production (Pineaux et al., 2023).

This study aims to better understand the effects of a fungicide, the boscalid, on honeybees' gene expression. We realized chronic exposed larvae to boscalid at field concentration. We used a parsimonious approach by only considering the exposure of the larvae to an equivalent amount of boscalid that they could ingest through contaminated pollen in the environment. The effects of boscalid on gene expression were measured few days after chronic exposure of the larvae. We also evaluate the effect of gene expression in adult workers was measured 10 days after emergence. The target genes were related to metabolic pathways of immunity, detoxification, development, and mitochondrial activity. The results of this study will contribute to a better assessment of the risks associated with the use of this fungicide in the field and help to minimize adverse effects on pollinators.

MATERIALS AND METHODS

Experiments were conducted in the entomological experimental unit of INRA, Le Magneraud (46° 8' 59,104"N, 0° 41' 28,609"W), France between April and August 2021. In this study we recorded larval and pupal mortality and adult emergence rates.

We compared all variables between the untreated larvae (Control), treated with 0.1 % acetone (Solvent Control), and treated with pesticides. The two treatments were as follows : exposure to 3.05 ng pure same dose of active molecule to a commercial solution : Pictor Pro® (contains 50 % boscalid).

larval rearing

One-day-old larvae were collected from 3 colonies of *A. mellifera* and reared in vitro until emergence. For artificial rearing, we followed the method adapted by (Aupinel et al., 2005 ; Papach et al., 2017).

Comb with first instar larvae was obtained by enclosing the queen with a frame of built-up brood to an isolated Italian cage for 30 h. Then, the queen was released and

the freshly laid frame was left in the hive for one more day to obtain first cycle larvae (L1) for grafting.

All grafts were performed in the laboratory at room temperature. The transplants were performed by the same experimenters. A first larval rearing was carried out, until the eighth day of rearing in the laboratory. The larvae (L1) were transferred into disinfected plastic grafting dishes. The cups with L1 were maintained in an incubator at 34.5 ± 5 °C from day 1 to day 8 with a relative humidity (RH) of $95 \pm 5\%$ (K₂SO₄ solution). Cups were filled with a mixture of royal jelly, aqueous sugar and yeast extract with or without pesticide treatment (see below for details) on days 1, 3, 4, 5 and 6. Larvae were then collected on day 8 for molecular biology analyses.

A second round of grafting was performed with the same protocol to obtain adult workers. The first part of the protocol was like the first grafting round. Then the larvae were transferred to a new clean plate when they had finished feeding (days 7 and 8). The plates containing the pupae were transferred to a sealed incubator at 34.5 ± 5 °C, with $80 \pm 5\%$ RH (saturated NaCl solution). Prior to emergence (day 15), pupal plates were placed in crystal polypropylene emergence boxes (11 × 15 × 12 cm) at 34.5 ± 5 °C and $50 \pm 5\%$ RH with 50 % agarose-sugar solution ad libitum. A 5 mm piece of Be-Boost® (PseudoQueen) was attached to a piece of wax (approximately 5 × 5 cm) in each emergence box. After emergence, the bees were transferred to a new polypropylene box, equipped in the same way as the emergence boxes, with the addition of pollen powder. They were maintained under laboratory conditions for 10 days at 34.5 ± 5 °C and $50 \pm 5\%$ RH before being collected for molecular biology analysis.

For the first series, larvae were collected at day 8 and for the second series worker bees were collected at 10 days of age. The individuals were collected alive and immediately stored at -80 °C until gene expression analyses (the experimental design is graphically summarized in Appendix 1).

Pesticide exposure

Firstly, we estimated the contamination of the natural environment by boscalid based on data available in the literature. The concentration of boscalid found in pollen brought back by bees to the hives varies widely around the world. For example, the maximum dose found in California is 6060 ng/g (Fisher et al., 2021), in Canada is

2200 ng/g (Tsvetkov et al., 2017) and at 1496.4 ng/g in Germany (Böhme et al., 2018). In our study, we use the quantity of 1496.4 ng/g, and considering that a bee larva feeds on around 0.002 g of pollen in four days (Babendreier et al., 2004), we estimated that under natural conditions, the larvae would consume 3.05 ng of boscalid.

Stock solutions of boscalid (99 % purity, Cluzeau Info Labo, France) were prepared in acetone and added to the larval diet. Pictor Pro® is a commercial solution and is composed of 50 % boscalid. Thus, the concentration of boscalid and commercial solution were respectively 19.5 ng/g and 38.9 ng/g in diet B and C (Appendix 1). The stock solutions of the pesticides were made at the beginning of the beekeeping season and stored at -20 °C, they were only thawed when used. Each larva was chronically exposed to water (Control), 0.1 % of acetone only (Solvent Control), or 3.05 ng of boscalid solution or 6.1 ng of Pictor Pro® for 4 days in their diet (day 3 to day 6).

Laboratory analysis

We measured the expression of seven genes (Appendix 1) at both larvae and adult stage (10-day-old workers). The analyses were carried out at the laboratory Ecology and Biology of Interactions (Vienne-France) in 2021, on frozen individuals. The individuals analysed come from two hives used for grafting. In larvae and bees, gene expression was performed in 13-16 individuals per modality.

Extraction of total RNA : each larva was homogenized in 900 µl of QIAzol (Qiagen) with 3 beads of 2 mm diameter beads, and a FastPrep-24 (MP) (1 time 30 s at 6.5 m/s). For each bee, the abdomen was homogenized in 900 µl of QIAzol (Qiagen) with 3 beads of 2 mm diameter beads, and a FastPrep-24 (MP) (2 times 30 s at 6.5 m/s with 15 min in ice for adult bees). The homogenate was incubated for 5 min at room temperature. Total RNA was isolated from each larva using a RNeasy Plus Universal Mini kit (QIAGEN). The optional DNA digestion step by adding 50 µl of DNase. The final volume of the suspension was 50 µl. RNA was quantified using a NanoDrop 1000 Spectrophotometer (Thermofisher).

Quantification of gene expression : the reverse transcription was performed with SuperScript® First-Strand cDNA Synthesis Reaction Kit (Invitrogen) using 500 ng RNA in 20 µl reaction volumes containing random hexamer primer. The RNase H step was performed to digest the RNA residue, by adding 1 µl of RNase H and incubating at

37 °C for 20 min, according to the producer's protocol. The expression levels of 2 reference genes and 5 target genes were quantified by qPCR.

The epMOTION automated system (Eppendorf) was used to prepare the qPCR plates. For the RT-qPCR assays, LighCycler 480 SYBERGREEN I Master (Roche) qPCR was used. Each reaction of 10 µl consisted of 1.5 µl sample cDNA, 0.5 µM forward and 0.5 µM reverse primers (Integrated DNA Technologies) and 7.5 µl master mix, using the LighCycler 480 (Roche) detection system. Amplification was performed with the following program : 10 min at 95 °C and 35 cycles : denaturation cycles of 10 s at 95 °C, hybridation cycles of 10 s at 60 °C and elongation cycle of 20s at 72 °C. At the end of this program, a melting curve is generated by measuring the fluorescence over a range of 65 °C to 97 °C to verify the presence of the desired amplicon. All reactions were performed in triplicate and the variation between the triples was less than 1 ct. Controls, containing water, were included in each assay. The average cycle threshold values of our different target genes were normalized to the geometric mean of the reference genes The ribosomal protein S5 and β -actin, which were found to have stable expression levels. The Δ Ct value of each group was subtracted from the Δ Ct value of the Solvent Control to obtain $\Delta\Delta$ Ct. For gene expression profiling were collected from 2 different colonies, 14–16 larvae of each modality were collected live and immediately frozen on day 8 days and 16 bees were frozen 10 days after emergence for blind analysis.

Statistical analyses

Statistical analysis was computed with statistical software R 4.0.3 (R Core Team 2020), add to package survival 3.3-1 (Therneau et al., 2023), emmeans 1.8.5 (Lenth et al., 2023), Lme4 1.1-32 (Bates et al., 2023).

The survival of individuals was analysed with a Cox proportional hazard model (Therneau, 2021). We checked the proportionality of the hazard ratios using a score test in the "*cox.zph*" function of the *survival* package. Selection of the best-fitted model was based on analyses of variance and the Akaike information criterion (AIC ; Guthery et al., 2003). The modality, the experimenter transferring the larvae at D8, and the hive of origin of the larvae were considered fixed effect variables in the model.

The variation in emergence days of bees was analysed using a linear random effects model built in the *lme4* package. Model selection was performed by analysis of

variance and AIC (Guthery et al., 2003). Variations in emergence days between rearing plates were accounted in a random effect. The exposure modalities and the hive of origin of the larvae were also included in the model as fixed effect variables. Pairwise comparisons were performed using R package *emmeans* (Tukey's HSD) to identify differences between treatment modalities and the impact of the hive of origin of the larvae.

We determined whether the effects of our different modalities altered gene expression using linear models included in the *r* software. For each of the genes, we also considered the effects of the hive of origin of the individuals reared *in vitro*. To identify differences between treatment modalities and the impact of the hive of origin of the larvae, pairwise comparisons were performed using the R *emmeans* package (Tukey's HSD).

RESULTS

Larva survival

Larval survival during *in vitro* rearing was not significantly impacted by the different treatments added in their diet. Larvae in both control and solvent control modalities had the same chance of survival (Tuckey test = -0.301 ; $p = 0.480$). There was no significant difference in survival rates between larvae exposed to boscalid or Pictor Pro® and those exposed to solvent control (Tuckey test < 0.24 ; $p > 0.668$).

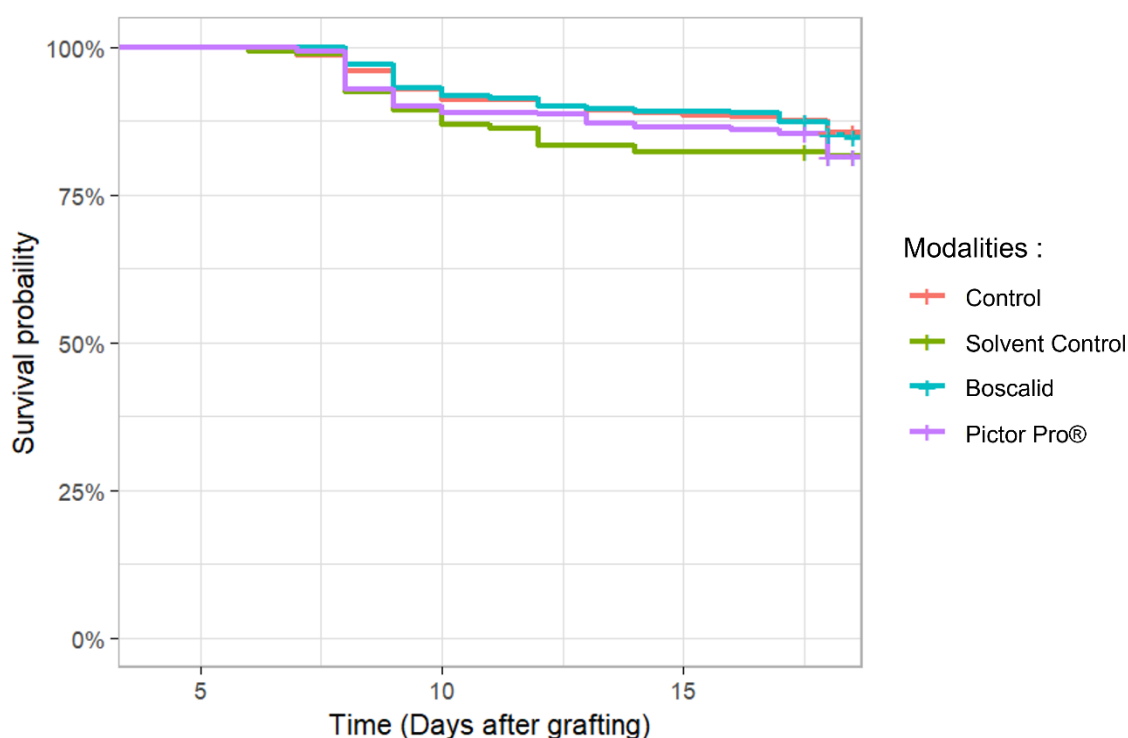


Figure 1 : Larvae survival when unexposed (Control), exposed to Solvent (Solvent Control), to boscalid and to Pictor Pro® - Larval survival did not differ significantly between the experimental groups (Log-Rank test ; $\chi^2 = 3.2$; $p = 0.4$)

Larvae survival was not significantly different between the hives R3 and R6 (Tuckey test = -0.01 ; $p = 0.997$) but mortality was significantly higher mortality for the hive R4 (Tuckey test > -0.54 ; $p = 0.013$). In addition, larvae manipulation at 8 days (D8) was realized by two experimenters and we observed a significant difference of larvae survival between these two groups (Tuckey test = 0.920 ; P-value < 0.005 ; Appendix 2).

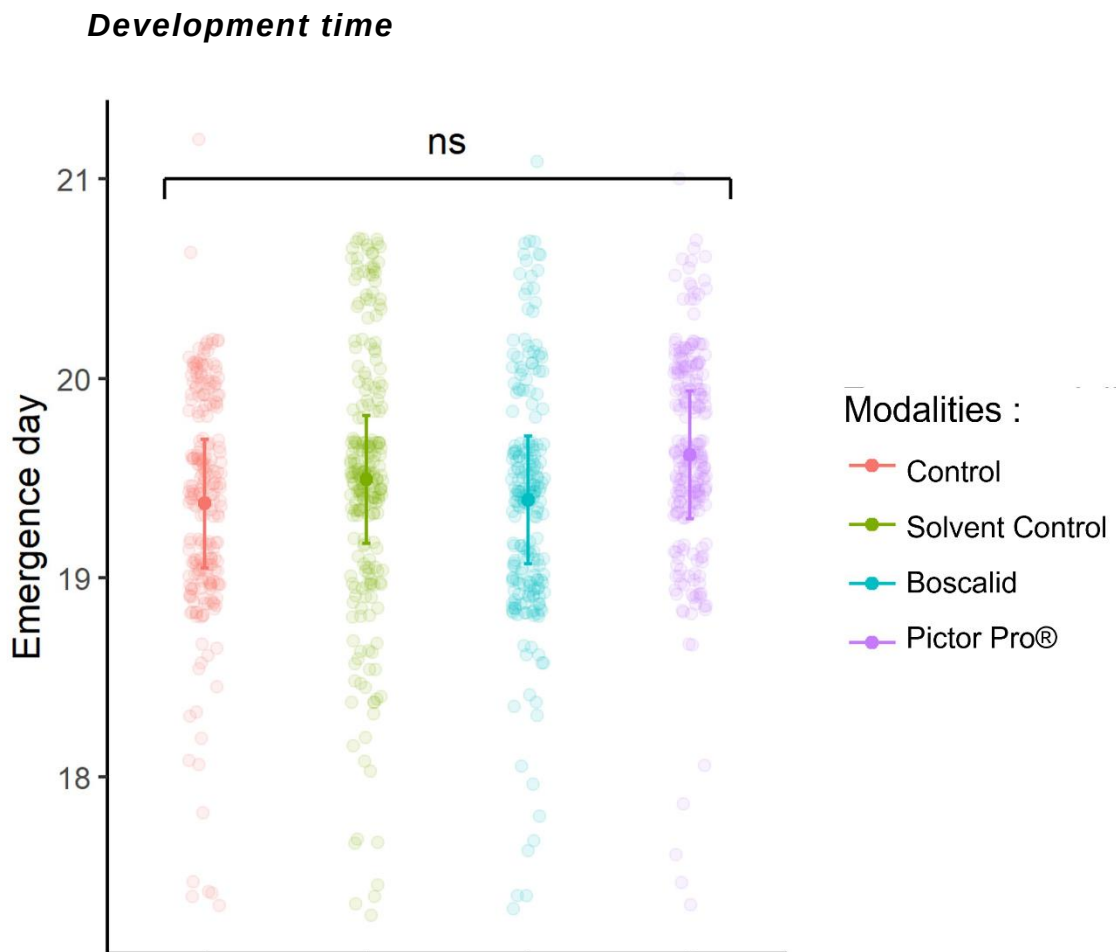


Figure 2 : Days of workers' emergence unexposed (Control), exposed to Solvent (Solvent Control), to Boscalid and to Pictor Pro® (from left to right) - Larger points represent mean $\pm$ SE and transparent points represent raw data. Pairwise comparison (Tukey's HSD) : ns when $p > 0.05$

Larvae development duration until emergence was not affected by the hive used for grafting (Tuckey test < 0.302 ; $p > 0.2653$; Fig 2B) neither by boscalid or Pictor Pro® exposure and was an average of 19.2 days (Tuckey test $p < 0.24$; $p > 0.6770$; Figure 2). Also, comparisons between the hive of origin of the larvae had no effect on the time it took our larvae to emerge (Appendix 2).

Larvae's gene expression

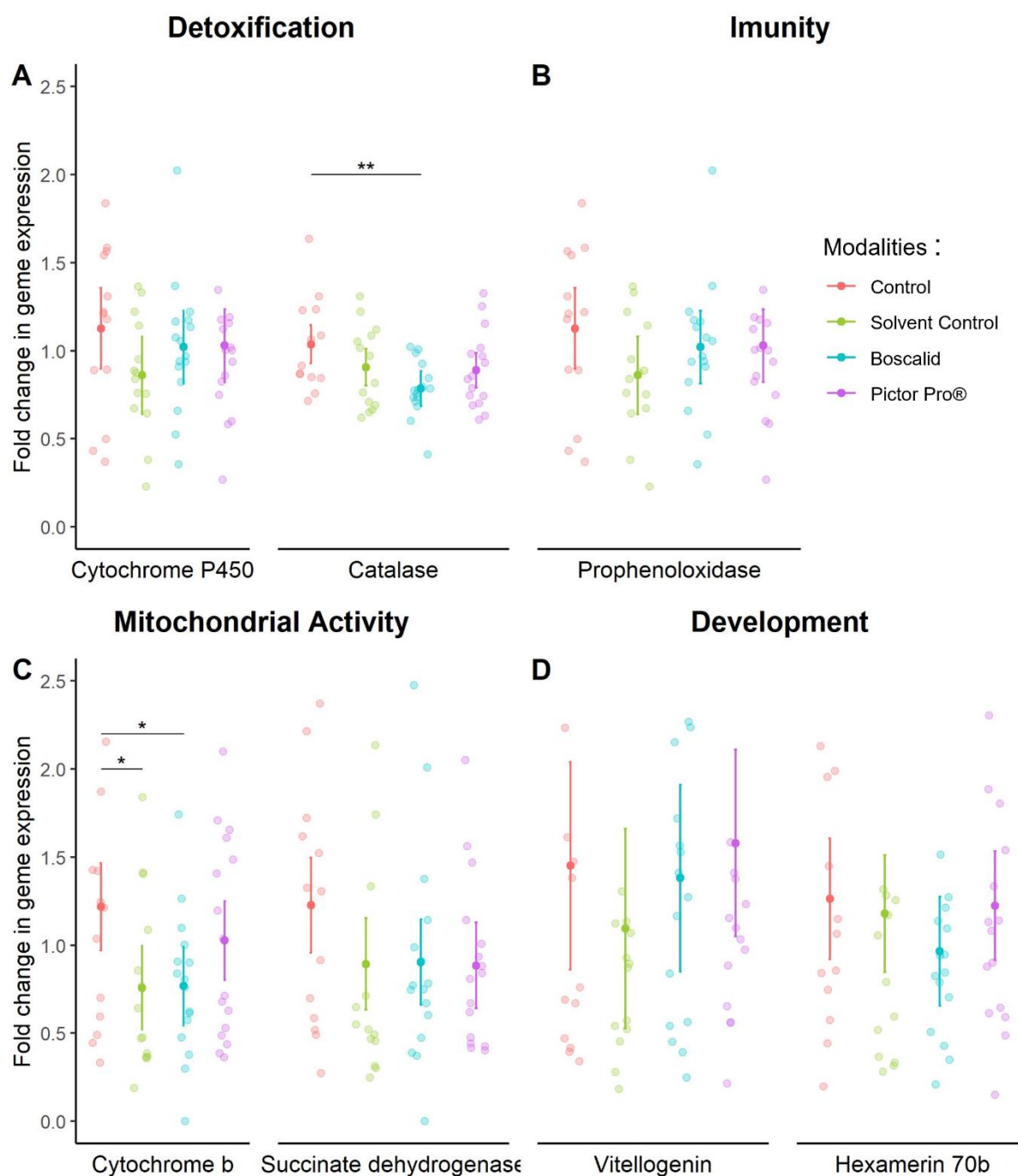


Figure 3 : relative expression of seven genes : cytochrome P450, catalase, prophenoloxidase, cytochrome b, succinate dehydrogenase, vitellogenin and hexamerin, in treated larvae with Control, Solvent Control, Boscalid and Pictor Pro® (from left to right) - Genes are grouped according to their function ; A : Detoxification ; B : Immunity ; C : Mitochondrial Activity ; D : Development. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ (Livak, 2001) - Larger points represent mean $\pm$ SE

*and transparent points represent raw data - Pairwise comparison (Tukey's HSD) : * when $p < 0.05$; ** when $p < 0.01$ (Appendix 3)*

Larvae exposed to solvent control had significantly lower expression levels of cytochrome b, associated with mitochondrial activity, compared with control larvae (Tuckey test = -0.25 ; $p = 0.006$; fig.3C). Larvae exposed to boscalid had significantly lower expression levels of catalase (detoxification) and cytochrome b (mitochondrial activity), compared solvent control larvae (respectively Tuckey test = -0.25 Fig.3A ; $p = 0.006$ and Tuckey test = -0.45 ; $p = 0.042$; Fig.3C). The gene expression of larvae exposed to Pictor Pro® were not significantly different for all genes compared larvae exposed to control solvents (Tuckey test < 0.147 , $p > 0.195$). For genes prophenoloxidase, succinate dehydrogenase and hexamerin, no significant differences were observed between treatments (Tuckey test < 0.147 , $p > 0.195$; Figure 3 ; Appendix 3).

The hive of origin from which the larvae had been collected influenced the expression levels of several genes. Larvae from the hive R3 had significantly higher expression levels of cytochrome 450 (Tuckey test = -8.36 ; $p < 0.001$) and lower expression levels for catalase (Tuckey test = -0.191 ; $p < 0.001$), for prophenoloxidase (Tuckey test = -0.248 ; $p = 0.025$), cytochrome b (Tuckey test = -0.63 ; $p < 0.001$), for succinate dehydrogenase (Tuckey test = -0.792 ; $p < 0.001$), for vitellogenin (Tuckey test = -1,395 ; $p < 0.001$), for hexamerin (Tuckey test = -1,015 ; $p < 0.001$), compared to larvae from hive R4 (Appendix 3).

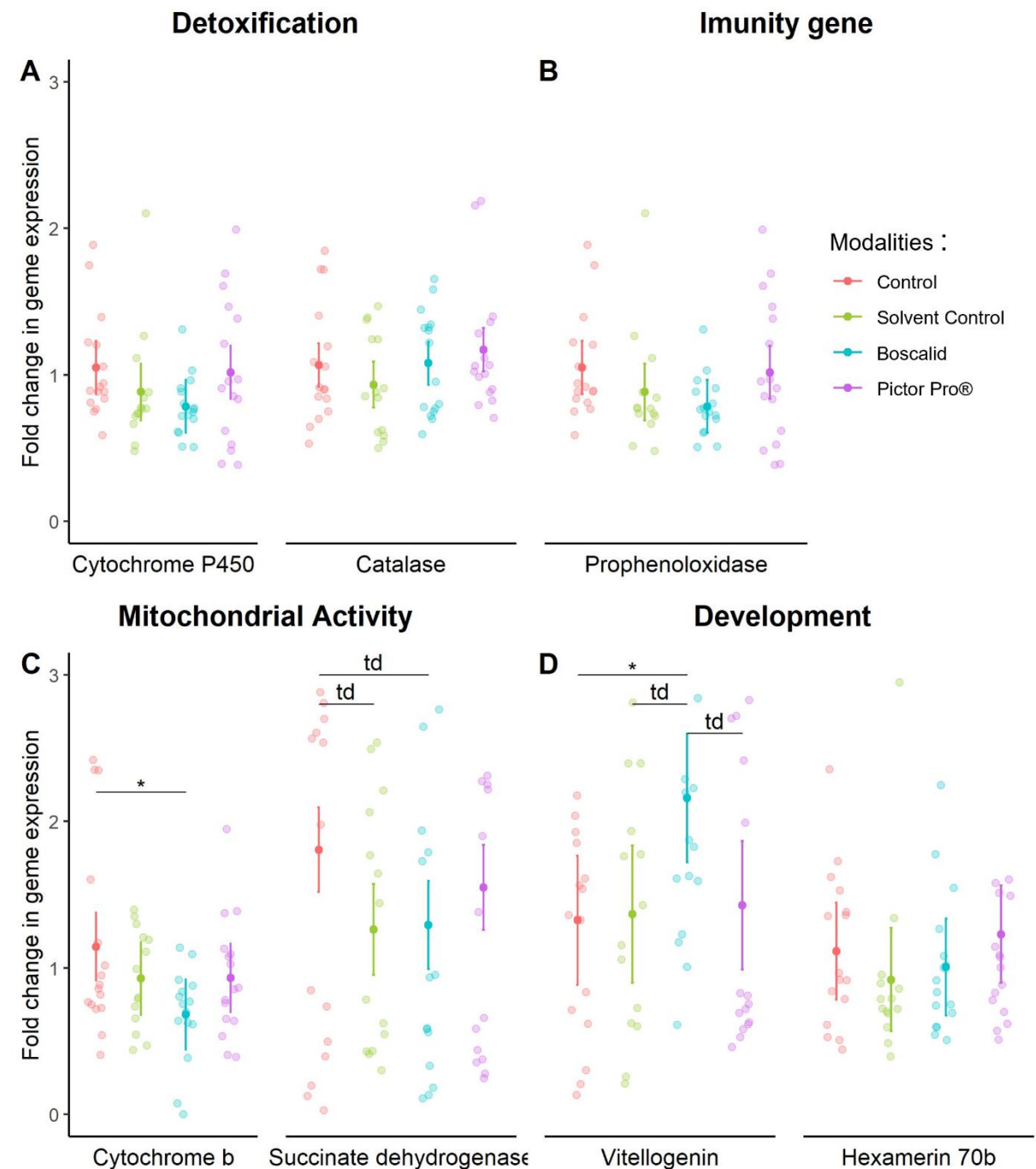


Figure 4: Relative expression of seven genes : cytochrome P450, catalase, prophenoloxidase, cytochrome b, succinate dehydrogenase, vitellogenin and hexamerin, in treated worker bees of 10 days with Control, Solvent Control, boscalid and Pictor Pro® (from left to right) - Genes are grouped according to their function ; A : Detoxification ; B : Immunity ; C : Mitochondrial Activity ; D : Development - Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ (Livak, 2001) - Larger points

*represent mean $\pm$ SE and transparent points represent raw data - Pairwise comparison (Tukey's HSD) : td when $P < 0.1$; * when $p < 0.05$ (Appendix 4)*

Ten-day-old workers exposed to control solvent showed significantly lower expression levels of succinate dehydrogenase, associated with mitochondrial activity, than workers exposed to control (Tuckey test = -544; $p = 0.599$; fig.3C). The activity of succinate dehydrogenase was altered in ten-day-old workers after exposure to Boscalid (fig.4C). Workers exposed to Boscalid tended to under-express the succinate dehydrogenase gene (Tuckey test = -0.514 ; $p = 0.075$) and significantly under-expressed the cytochrome b gene (Tuckey test = -0.463 ; $p = 0.036$) compared with workers exposed to Solvent Control. In addition, workers exposed to boscalid had higher levels of vitellogenin gene expression, associated with development, than workers in the solvent control group (Tuckey test = 0.835 ; $p = 0.045$). The gene expression of workers exposed to Pictor Pro® were not significantly different for all genes compared with larvae exposed to control solvent (Tuckey test < 0.214 ; $p > 0.564$). For the other five genes, associated with detoxification (cytochrome P450, catalase), immunity (prophenoloxidase) and development (hexamerin), no significant differences were observed between bees from the different modalities (Tuckey test < 0.238 ; $p > 0.133$; Fig 4).

We have a strong effect of the grafting hive. Workers from the hive R3 had significantly lower gene expression levels for succinate dehydrogenase and vitellogenin (respectively Tuckey test = -1.879 ; $p < 0.001$ and Tuckey test = -1.000 ; $p < 0.001$), higher expression levels for catalase and prophenoloxidase (respectively Tuckey test = 0.495 ; $p < 0.001$ and Tuckey test = 0.246 ; $p = 0.010$) compared workers from hive R4 (Appendix 4). There was no significant difference in the expression of cytochrome P450, cytochrome b and hexamerin genes between workers from hives R3 and R4 (Tuckey test ≤ 0.538 ; $p \geq 0.343$).

DISCUSSION

Choice of intoxication dose

The aim of our study was to examine the effects of exposure to boscalid at does present in the environment, and the consequences of the gene expression of bees in the larval and adult stages. Previous study estimated that the LD50 values on Day 8 and Day 15 were respectively 86,786 $\mu\text{g/larva}$ and 78,782 $\mu\text{g/larva}$ (Simon-Delso et

al., 2017), Here, larval individuals were chronically exposed to a sublethal dose at a concentration of 3.05 ng/larvae. Our approach was parsimonious, particularly regarding larval exposure, which was assessed based on boscalid contamination in the pollen that feeds the larvae (Böhme et al., 2018). Limited information is available on the pathway of contamination, accumulation, or degradation of boscalid in different bee matrices and their effects on larval physiology (Wueppenhorst et al., 2022). Boscalid metabolites have been quantified in bee samples collected from different hives in France, and their deleterious effects on beehives are unclear (Jabot et al., 2016). However, ingestion of contaminated food and pollen from pesticide-treated plants is one of the main pathways of larval exposure (Babendreier et al., 2004 ; Böhme et al., 2018). Additionally, bee larvae come into direct contact with wax, which can contain pesticide residues from various sources such as antiparasitic treatments used in hives, pesticide applications on crops, or environmental contamination (Friedle et al., 2021 ; Xiao et al., 2022). These different exposure routes contribute to the accumulation of pesticides in bee larvae, which can result in lethal or sublethal consequences. We have probably underestimated the doses of pesticides to which bee larvae may be exposed in the field by considering only one route and a short time of exposure.

Impact on worker's larvae

As expected, we did not measure a significant impact on larval survival following exposure to Boscalid. Some studies, ranging from 10 to 10,000 times higher than the dose we used, did not observe in larval mortality and effect in the development time of the bees until emergence (Simon-Delso et al., 2018). However, other studies have shown an effect of Boscalid on larval mass during the prenympchal stage (Glass et al., 2021).

We measure the consequences of this exposure on gene expression after exposure to understand the impact of this molecule on bee physiology. Our results show the expression of specific genes was significantly impacted by the different larval treatments with boscalid. We show under-regulation of the cytochrome b transcription gene. This gene is associated with cellular respiration because it is responsible for transmembrane electron transfer, by which redox energy is converted into a proton

motive force (Esposti et al., 1993). However, the commercial product Pictor® had no effect on gene expression.

Delayed impact on adult workers

We assess the long-term effects by examining the gene expression of worker bees aged 10 days, who were exposed during the larval phase, the worker bees showed slight modifications in gene expression. We report a clear effect of boscalid on downregulation of the cytochrome b gene and a less clear effect on the succinate dehydrogenase gene, this gene is associated with mitochondrial activity. A previous study, conducted on *Apis cerana*, showed that mixture of fungicides containing boscalid increased significantly up-regulated gene transcripts associated with immune response and coding for enzymes involved in oxidative phosphorylation and metabolism (Dong et al., 2023). The decrease in transcripts related to cellular respiration notably of cytochrome b could lead to a reduction in the flying capacity of bees (Chance & Sacktor, 1958). It has been demonstrated that high doses of Boscalid can reduce thorax size (Glass et al., 2021), potentially affecting the efficiency of foraging by lowering wingbeat frequency (Liao et al., 2019). In addition, when tested under field conditions, it reduced the survival rate of worker bees. (Fisher et al. 2022).

We report an upregulation of the vitellogenin gene associated with development. A joint study conducted on queens with the same doses of pesticides had a significant impact on vitellogenin gene expression in queens (Pineaux et al., 2023). Long-term upregulation of the vitellogenin gene could induce earlier foraging (Fisher et al., 2021). These findings highlight the potential effects of Boscalid exposure on long-term bee capacity. Young bees exposed during the larval phase showed change in motion behaviour (Tadei et al., 2019).

Individual sensitivity on pesticide

In our study, we observed different sensitivities to boscalid in workers from different hives. The laying queens used were sisters, but the workers could be genetically different, presenting variations that can influence the bees' sensitivity to pesticides. For example, some colonies may possess natural pesticide resistance genes, making them less likely to be affected by certain substances (Rinkevich et al., 2015).

Commercial solutions and pesticides synergy

Literature reviews reveal a significant lack of information on the sublethal and combined toxicity of pesticides to bees, particularly in regulatory toxicological bioassays (Barascou et al., 2021 ; Tosi et al., 2022). This is particularly considering the substantial amount of different pesticide residues found in all hive matrices (Friedle et al. 2021 ; Sanchez-Bayo et Goka 2014 ; Tosi et al. 2018). Our study also focused on one commercial formulation, Pictor Pro®, and we did not observe specific effects of this formulation at sublethal doses of 6.1 ng/larvae). However, there is some evidence in the literature of adverse effects caused by adjuvant ingredients present in these formulations, with reports of increased nuptial flight mortality after exposure of queen bees (Pineaux et al., 2023). This highlights the need to assess the risk of inactive ingredients and commercial pesticide formulations to bees. (Wernecke et al., 2022). Other commercial products, particularly Pristin®, a mixture of boscalid and Pyraclostrobin, have had effects on the bees' individual capacities, on the size of their thorax and their microbiota, their pollen consumption behaviour and the number of individuals in the colonies (Fisher et al., 2021 ; Fisher et al., 2022 ; Glass et al., 2021).

Limits of laboratory tests

Laboratory sublethality studies can be useful to address compound-specific concerns, it is necessary to complement this with field and semi-field trials which may observe different effects (Thompson & Maus, 2007). Laboratory studies can underestimate the true effects of fungicides by not accurately reflecting the complex interactions and real-world conditions that bees face in their natural environment (Henry et al., 2012). The sensitivity of bees to Boscalid could vary according to different factors, such as the caste and age of the bee, the general health of the colony and the

interaction between other pesticides and pathogens, as has already been shown with other pesticides (Coulon et al., 2020 ; Wood et al., 2020). This has also been demonstrated with many pesticides in interaction (David et al., 2016 ; Wood et al., 2020). It is therefore important to conduct in-depth studies to assess the specific effects of Boscalid on different bee species, and to consider the complex interactions between this pesticide and other environmental stressors to which bees are exposed. Despite its usefulness in crop protection, it is important to better understand the potential effects of Boscalid on non-target organisms, particularly bees, which play a crucial role in crop pollination and the preservation of biodiversity. Recent studies have highlighted the need to assess the risks associated with Boscalid use and to adopt more sustainable agricultural practices to minimize impacts on bees and other pollinator populations (Perrot et al., 2022 ; Wintermantel et al., 2019).

CONCLUSION

Conclusion, the aim of this study was to assess the effects of exposure to boscalid at field concentration on the survival, development time and gene expression of larvae. The results obtained indicate that exposure to boscalid had no significant effect on the survival and emergence of the larvae compared with the control groups. We found effects on gene expression suggesting that boscalid impact on certain physiological processes in honeybee larvae. Larvae exposed to boscalid showed significantly lower levels of the expression of certain genes associated with detoxification and mitochondrial activity (catalase and cytochrome b). In addition, the analyses revealed late impacts on adult bees exposed during the larval phase, with effects on genes linked to mitochondria and development (cytochrome b, succinate dehydrogenase, vitellogenin). However, the effects observed with the crude boscalid molecule were not observed with the product in the commercial formulation Pictor Pro®. Differences in gene expression between larvae and bees from different hives, indicating the influence of the hive of origin of the expression levels of the genes studied. This highlights the importance of taking individual and environmental variations into account when interpreting the results. Overall, this study contributes to our understanding of the potential effects of boscalid exposure on larvae. The results suggest that Boscalid can modulate the gene expression of certain enzymes and mitochondrial activities. Although the functional consequences have not been studied

here, this physiologically characterizes the decreases in bee capacity observed in the literature.

ACKNOWLEDGMENT

The authors would like to thank Fabrice Requier, Maxime Pineaux, Elisa Gomes, Manon Desaivres, Colombe Chevallereau, Thierry Tamic and Fabien Dequine for their contribution to the implementation of the experiment and to the logistics.

FUNDING

Research reported in this publication was supported by OFB as part of the Ecophyto II+ call on “Phytopharmaceuticals products : from exposure to impacts on human health and ecosystems” launched by the French Ministry in charge of Ecology, Agriculture, Health and Research for the project EXPLORA granted to F.-J. Richard (2021–2023).

CHAPITRE 4 - DE LA LARVE A L'ABEILLE : L'EFFET DES PESTICIDES EN INTERACTION AVEC UN PATHOGENE

Avant-propos

Les effets sublétaux du pyriproxifène

Au sein des ruches, les larves d'abeilles peuvent être soumises à de nombreux stress, notamment aux pesticides de différentes classes ou des pathogènes (Goulson et al., 2015). La relation entre les différents facteurs biotiques et abiotiques et le stress qu'ils induisent sur les abeilles est encore mal connue, mais des interactions inquiétantes entre les pathogènes et les pesticides ont déjà été notifiées (Aufauvre et al., 2012 ; Wood et al., 2020). Trois facteurs de stress sont particulièrement préoccupants par leurs effets délétères sur les abeilles et par leurs prévalences dans l'environnement :

- Le fongicide boscalid, censé inhiber spécifiquement la respiration mitochondriale des champignons, diminue la longévité des abeilles (Chauzat et al., 2006 ; Simon-Delso et al., 2017 ; Desclos le Peley, *in prep*).
- L'insecticide pyriproxifène, homologue des hormones juvéniles, il bloque le développement des insectes. Il est fortement toxique pour les larves d'abeilles (PPDB Pesticide 2024).
- La bactérie Gram-positive *Paenibacillus larvae* qui est responsable de la maladie de la loque américaine. Elle infecte spécifiquement les larves et provoque des septicémies, conduisant finalement à la mort (Brødsgaard et al., 1998).

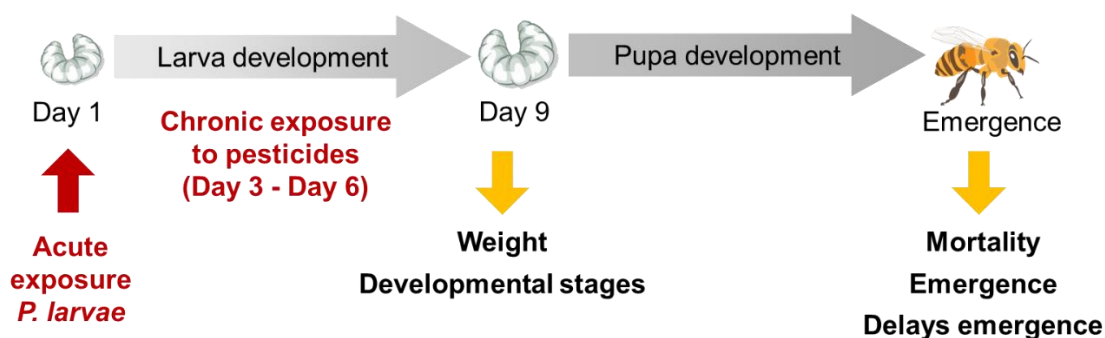
Ces facteurs de stress ont déjà été signalés comme étant synergiques avec d'autres facteurs (e.g. *P. Larvae*-clothianidine ou boscalid-pyraclostrobin ou pyriproxifène-méthoxyfénozide ; (Litsey & Fine, 2024 ; López et al., 2017 ; Wade et al., 2019).

L'objectif de ce chapitre vise à comprendre l'interaction entre le boscalid, le pyriproxifène et *P. larvae* et leurs effets sur le développement larvaire de l'abeille. Les larves élevées *in vitro* ont été exposées de manière chronique pour la dose de pyriproxifène et de boscalid et par contact à *P. larvae*. Nous avons mesuré les principales caractéristiques du cycle de développement des larves, la mortalité

cumulative, le taux d'émergence et le processus de développement (poids et nymphose).

Sur la base des éléments développés précédemment, deux hypothèses émergent :

- Les expositions simples des larves à des doses non mortelles de pyriproxyfène et de *P. larvae* peuvent entraîner des effets sublétaux sur les paramètres de développement des larves.
- Les coexpositions des larves aux combinaisons de boscalid et de pyriproxyfène et *P. larvae* vont davantage perturber le développement de que les expositions simples.



Exposures

	1	2	3	4	5	6	7	8	9
Solvent Control	X								
Control		X							
Boscalid			X			X	X		X
Pyriproxyfen				X		X		X	X
<i>Paenibacillus larvae</i>					X		X	X	X

Le choix des doses d'exposition

Les concentrations sans effets observés (NOEC) ont été déterminées pour les trois expositions simples : pyriproxyfène, boscalid et *P. Larvae* sur des larves provenant de quatre colonies d'un rucher intérieur. Des gammes de cinq concentrations ont été testées pour chaque traitement et ont été diluées dans l'alimentation des larves. Les concentrations retenues correspondent aux doses les plus élevées sans différences

statistiques significatives pour la mortalité cumulée à J22 entre les traitements et les contrôles. Ainsi, pour tester les coexpositions, les larves ont été exposées à 80 ppm de boscalid et/ou 0.08 ppm de pyriproxifène et/ou 2-3 spores de *P. larvae*.

Le résumé des résultats

L'exposition simple des larves à 80 ppm de pyriproxifène entraîne une mortalité plus élevée que les contrôles et diminue le taux de nymphose et l'émergence des individus. Les bi-expositions boscalid + *P. larvae* et pyriproxifène + *P. larvae* ainsi que la tri-exposition boscalid + pyriproxifène + *P. larvae*, ont un impact sur la mortalité, le taux de nymphose et le taux d'émergence. Mais ces combinaisons ne montrent pas de différences significatives avec les simples expositions. Ces résultats pourraient suggérer une interaction entre le pathogène *P. larvae* et le boscalid, mais sa nature n'a pas été clairement établie.

Publication

Cette étude a pour ambition d'être soumise dans *Journal of apicultural research*

Les tables se trouvent dans l'annexe 4.

Pesticides and parasite cocktail : effects of tri-exposure at larvae stage on honeybees life history traits

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Running title : Effect of Pathogen and pesticides coexposure on honeybees

ABSTRACT

In natural conditions, honeybees are exposed to multiple stressors. In agricultural landscapes they can be exposed at the same time to insecticides, fungicides and attacked by pathogens. Here we exposed *Apis mellifera* larvae reared *in vitro* to sublethal doses of insecticide (pyriproxyfen) and/or a fungicide (SDHI boscalid) and/or to a pathogen (American foulbrood). Our results show a significant effect of co-exposure with the fungicide boscalid, the insecticide pyriproxyfen and the bacterium *Paenibacillus larvae* on survival, pupation rate and emergence of bee larvae. No synergistic was observed but our results suggest an interaction between the pathogen *P. larvae* and the two pesticides. Here we evaluated exposure impact at the larvae stage, but further studies should investigate the potential consequence in the long term on the subsequent emerging individual life history traits. Decrease of larvae survival and weaknesses of exposed individuals could contribute to decrease colony efficiency, mainly its adaptation to face extra stressors (biotic or abiotic).

KEYWORDS

pyriproxyfen, boscalid, *Paenibacillus larvae*, mortality, emergence, pre-pupae weight, pupation

INTRODUCTION

Among pollinators communities, bees (22,000 species listed) are recognized for their effectiveness (Goulson et al., 2015). Pollinators worldwide have been decline, since the 2000s, particularly in the northern hemisphere (mainly Europe and USA) (Currie et al., 2010 ; Neumann & Carreck, 2010 ; vanEngelsdorp et al., 2009). Until now, it has not yet been possible to find a common cause for this global excess mortality. Currently, the underlying hypothesis for pollinators decline involves multiple factors, acting separately or in combination, are pesticides, pathogens, habitat fragmentation, climate change and exotic species (Vanbergen & Initiative, 2013).

Wild and domestic bees are exposed to various insecticides such as neonicotinoids, insects growth regulators (IGRs), but also to fungicides, herbicides (Mitchell et al., 2017). Thus, through foragers all colony members are exposed directly or indirectly to environment contaminants. Even if they are usually applied in crops at sub-lethal doses for bees many studies revealed that others side-effects such as alteration of motor skills, learning abilities, growth rates of colonies (Decourtye et al., 2010 ; Devillers & Devillers, 2020 ; Fourrier et al., 2015 ; Gill et al., 2012 ; Papach et al., 2017 ; Scott-Dupree et al., 2009 ; Urlacher et al., 2016). Insect growth regulators (IGRs) such as tebufenozide, diflubenzuron and pyriproxyfen influence moulting process of target species, all of which are widely used in the agricultural world to control insect pest (Fourrier et al., 2015 ; Goulson et al., 2015 ; Henry et al., 2012 ; Tasei, 2001). IGRs have different action mode : tebufenozide influences moulting as acting, as ecdysone, through nuclear receptor, diflubenzuron are an inhibitors of chitin synthesis and pyriproxyfen mimics juvenile hormone action by deteriorating the cuticular larvae development of target species (Tasei, 2001). pyriproxyfen has been used for over 25 years on farms and residues of this pesticide are still present in bee products (0.0049 ppm in beeswax, for example) Mullin et al., 2010 ; Tasei, 2001). This pesticide is not considered to have high toxic level at field concentrations to bees (Costa et al., 2014 ; Sullivan, 2000 ; Tasei, 2001). However, numerous studies showed that exposure to this molecule has consequences on larval development (deformities) with notably an acceleration of larval development and a delay in muscular differentiation in flight (Bitondi et al., 1998 ; Fernandez et al., 2012) and on the social interactions inside the hive by reducing social tasks and the rejection of young

contaminated workers (Chen et al., 2016 ; Fourrier et al., 2015 ; Ko et al., 2017 ; Pinto et al., 2000).

Fungicides are applied on fruits and vegetables crops during flowering period. They are used to control fungal crop pathogens and are one of the most common agrochemicals encountered by honeybees in the environment (Mullin et al., 2010 ; Ostiguy et al., 2019). Fungicides residues are frequently found in hive matrices : honey, wax, nectar, pollen, or queen jelly... (Böhme et al., 2018 ; Daniele et al., 2018 ; Friedle et al., 2021). Although their concentration is relatively low compared to the LD50 (Ostiguy et al., 2019), the synergistic, chronic, and long-term effects are not yet fully understood and consequences on workers longevity and behaviour, are probably underestimated. Carboxamide such as boscalid inhibit cellular respiration of fungi by blocking ubiquinone binding sites in the mitochondrial complex involved in the Krebs cycle (Aubee & Lieu, 2010 ; Avenot et al., 2008). Boscalid residues found in beekeeping matrices were at non-lethal doses for domestic bees (Medrzycki et al., 2013 ; Simon-Delso et al., 2017). In addition, workers exposed to boscalid show shorter longevity, decrease pollen consumption, earlier foraging, and decrease wing beat frequency with overall negative impact on colony population size and winter survival (Fisher et al., 2021 ; Glass et al., 2021 ; Liao et al., 2019 ; Simon-Delso et al., 2017).

Wild and domestic bees are prone to many diseases or pathogens : bacteria (e.g. *Melissococcus plutonius* responsible of European foulbrood), fungi (e.g. *Nosema ceranae*, *N. apis*), viruses (e.g. *deformed wing virus* (DWV) or black queen cell virus (BQCV), or parasite (e.g. *Varroa destructor*) (Boncristiani et al., 2021 ; Genersch, 2010a; Higes et al., 2006 ; Manley et al., 2015 ; Neumann & Carreck, 2010 ; Vanbergen & Initiative, 2013). American foulbrood is a significant disease due to the bacterium *Paenibacillus larvae* (Genersch, 2010a). Spores' forms of *P. larvae* are easily spread during larvae feeding by nurses, and it can only infect honeybee larvae at early stage (within 53h after hatching), its colonization is rapid, and 10 spores can kill one larva (Lindström et al., 2008). Shortly after infection, individuals turn brown and their bodies become a hard scale of material deposited on the side of cells (López et al., 2017 ; Matheson & Reid, 1992).

Under natural conditions, not only foragers but all colony members were exposed to multiple stress factors. Only a few studies explored the impact of stress combinations between products used in agriculture and natural stress environments such as pathogens. Coexistence of pesticides was proved worldwide and can increase damage to pollinators (Mitchell et al., 2017 ; Woodcock et al., 2017). Co-exposures can result in different consequences : potentiation, synergy and additive effect. Among all co-exposure studies, most have focused on combinations of pesticides-pathogens on adult pollinators (Alaux et al., 2010 ; Aufauvre et al., 2012 ; Prisco et al., 2013 ; Vidau et al., 2011). Interactions between substances have synergistic and negative effects on bees, even at sublethal doses (Alaux et al., 2010 ; Aufauvre et al., 2012 ; Doublet et al., 2015 ; Pettis et al., 2012 ; Prisco et al., 2013 ; Retschnig et al., 2014 ; Vidau et al., 2011). For example, the association of an insecticide chlorpyrifos and a fungicide Pristine® (commercial form of boscalid) compromised the immunity of developing queens (DeGrandi-Hoffman et al., 2013). Based on mortality rates an interactive effect between tau-fluvalinate (acaricide) and fungicides was observed (Johnson et al., 2013). Honeybees exposed to clothianidin neonicotinoid have reduced immune defenses and are more susceptible to virus infection (Prisco et al., 2013). Indeed, when larvae were exposed to sublethal doses of dimethoate or clothianidin in combination with *P. larvae*, it leads to a significant decrease in mortality and hemocyte count (López et al., 2017). Finally, tri-exposures including two pesticides and a pathogen showed stronger synergistic effects than a single product with higher decrease in larval survival (Thiamethoxam, Propiconazole and *M. plutonius* ; Wood et al., 2020).

To improve our knowledge of pollinators' decline and co-exposition, our study aims to compare impacts of simple exposure and co-exposure (bi-exposure and tri-exposure) to a fungicide : boscalid and an IGRs / pyriproxyfen and a pathogen *P. larvae* (the infectious agent of American foulbrood) on development. Pyriproxyfen and boscalid can be applied to the same type of crops that are pollinated by bees, such as floral crops, strawberry, cherry, tomato, cucumber, or pepper crops (Free, 1971 ; Mullin et al., 2010). At first, we exposed honeybees (*Apis mellifera*) to determine the chronic doses of pyriproxyfen and boscalid and the acute dose of *P. larvae*. The results are used to determine the doses with low effect on mortality and emergence at day 18 post-hatching. Then, we used the low doses determined in the first step to compare

the different exposures effects on cumulative mortality and emergence rate of honeybee larvae, as well as on development process (weight and pupation).

MATERIALS AND METHODS

All experiments were conducted in the experimental entomological unit of INRA in le Magneraud, France, in Spring 2017. In this study, we recorded larval and pupal mortality and adult emergence rates, and the weight of pupae.

Larval rearing

Honeybees' larvae were raised *in vitro* until the adult stage, according to the procedure of Aupinel et al., 2005, 2007 ; Medrzycki et al., 2013) and following OECD guidelines (2013).

To obtain eggs, we confine the queen on a frame surrounded by a cage on the hive for 30 hours. It pushes the queen to lay eggs on this frame. This device allows the workers passage through the grids. Then, the queen is removed from the cage but the frame with eggs is maintained in the cage for another 2 days. After, frames containing larvae (L1) were brought in the laboratory for grafting. It consists in extracting larvae from cells and dropping them off one by one on sterilized artificial cells. These artificial cells were set up in a 48 wells cellular culture plate on aseptic dental cotton (moistened with 15 % glycerol in a Milton solution). To get closer to natural conditions, larvae were kept in an airtight chamber at $34.5\pm 5^{\circ}\text{C}$, from days 1 to 8 with a 90 ± 5 % relative humidity (RH) (K₂SO₄ solution), and after with 80 ± 5 % RH (saturated NaCl solution) for the pupal stage (from D9 to D15). For emergence stage plates containing pupal were placed at 50 ± 5 % RH.

The larvae were fed with 3 diet compositions (A, B and C) - all containing 50 % of royal jelly and 50 % of aqueous sugar (fructose and glucose in equal proportions) and yeast extract solution - successively from day 1 to 6, excepted on day 2. Mixture A was composed with 24 % of sugars and 2 % of yeast extract ; mixture B with 30 % of sugars and 3 % of yeast extract and mixture C with 36 % of sugars and 4 % of yeast extract. The diet quantities increased according to the stage of development, the total amount corresponding to 160 μL per larvae from D1 to D6 (Figure 1). On day 15, nymphs were placed on plastic box containing Candi, 50 % sugar solution, pollen and water ad libitum for emerging adults.

Toxicological measurement

Solutions' volumes containing the pesticides were equivalent to 1 % of the final diet volume and all of stock solutions were realized for each grafting and stocked at - 5°C.

Pyriproxyfen, boscalid and *P. larvae* exposure

A semi-chronical exposure has been done for pesticides (pyriproxyfen and boscalid). The total amount of pesticides was allocated from day 3 to 6 in equal proportions. Both pesticides were purchased from Cluzeau Info Labo, France (compounds purity at 99 %) and were diluted in acetone. American Foulbrood is a disease affecting honeybee larvae and caused by the bacteria *P. larvae*, where the infection is induced by spores (Genersch 2010). An acute exposure has been done for *P. larvae* spores on day 1. The *P. larvae* of genotype ERIC I was collected and isolated from an infected colony in INRAE. Different genotypes of *P. larvae* differ into virulence on the larval level (Rauch et al., 2009). The strain was purified and stored at 5°C in water. The same biological sample was used for all exposures in this study.

Ecotoxicology measurement of pyriproxyfen, boscalid and *P. larvae*

The non-effect dose was determined for the 3 types of exposure (pyriproxyfen, boscalid and *P. larvae*) in March. To achieve this, we monitored the impact of 5 dose ranges diluted in larvae diet (Table 1-A). In parallel, for each exposure, untreated larvae were used as control. Every day, mortality was recorded until adult emergence. In total, the experiment was done with 48 larvae per treatment. In total, 1 008 larvae were hatched for the dose determination of pyriproxyfen, boscalid and *P. larvae*. Doses used in the second study ("exposure and co-exposure measurements"), correspond to the highest doses without significant statistical differences for cumulative mortality at D22 between treatment and controls (Table 1-B).

Exposure and Co-exposure measurements

Co-exposure was carried out with larvae from an ordinary hive. Nine exposure modalities were used. (i) Two control modalities, Control : untreated larvae : Control Solvent : larvae fed with 1% acetone. (ii) Three single exposures, pyriproxyfen, boscalid, and *P. larvae*. Three co-exposures : pyriproxyfen and boscalid, pyriproxyfen and *P. larvae*, boscalid and *P. larvae*. (iii) finally, a tri-exposure pyriproxyfen and

boscalid and *P. larvae*. The doses of pyriproxyfen, boscalid and *P. larvae* were determined in the first "ecotoxicological measurement" experiment, based on the last no-effect dose, so the larvae were exposed to 80 ppm boscalid and/or 0.08 ppm pyriproxyfen and/or 2-3 spore of *P. larvae*. A total of 1717 larvae were grafted and pooled over two sessions (Table 2).

The impacts of exposure and co-exposure were assessed based on several parameters recorded each working day from grafting to day 18. We reported larval mortality at all developmental stages, pre-pupae were weighed at day 9 (in their artificial combs - to avoid developmental disruption), the number of pupae (at the pupal stage, from day 8 to day 13). The time between the first and the last pupa was also calculated and subsequently referred to as "pupation time". Finally, the emergence rate was measured on day 22.

Statistical analysis

Statistical analysis was computed with statistical software R 3.3.1, add to package survival 3.3-1, coxme 2.2-16, emmeans 1.7.5, SJPlot, Lme4.

Dose determination used for exposures

Generalized linear mixed effect model (GLMM) logistic regression were computed to compare cumulative mortality of larvae at D22 of different tested doses to controls. A binomial distribution with logit link function was used. Each type of exposure (pyriproxyfen, boscalid, and *P. larvae*) was analysed separately.

Exposition and Co-exposition measurements

The survival of individuals was analyzed with a Cox hazard regression analysis with a random effect. The Schoenfeld residuals of our data were not time dependent thus an AIC selection of the model was performed. The hive of origin of the larvae and type of exposure were considered in the model as a fixed effect variable. Interacting between rearing plates for each session was again included as a random factor (standard deviation : $7.621.10^{-03}$; variance : $5.809.10^{-05}$), affected larval survival (Table 3). Survivorship of all treatments was then converted to hazard ratios (instantaneous risk of death compared to the model average) for statistical analysis.

Prepupae weights were compared with a GLMM. The hive of origin of the larvae and type of exposure were considered in the model as a fixed effect variable. Variations

in prepupae weight between rearing plates were considered in a nested random effect. In the analysis of the change in pre-pupal weight after feeding, there was a large variance that is accounted for in the random effects : the effect between sessions (variance : 8.39) and the nested random effect of the breeding plates (variance : 39.78). Pupation rate, at D13, survival rate at J7 and emergence rate were analyzed with a binomial generalized linear model (GLM binomial). Model selection was performed by analysis of variance. In all our models, estimates of the exposure modalities were tested with a Sidak test between the Solvent Control and the other exposure modalities and pairwise comparison with the Tukey test.

RESULTS

Ecotoxicology measurement of pyriproxyfen, boscalid and *P. larvae*

During larval stage from day 3 to day 9 (D₃₋₉), cumulative mortality in controls group was 3.6%, which is below the standard acceptance threshold (< 15 %) for *in vitro* rearing conditions (OECD, 2013). The average cumulative mortality of control for pyriproxyfen test was 32.83 %, for boscalid test was 18.75 %, and for *P. larvae* test was 33.3 %. Larvae mortality was significantly higher when larvae were exposed to 40ppm, 160 and 320 ppm of boscalid (Table 1-B). Larvae mortality was significantly affected when exposed to 0.16 ppm to 0.64 ppm doses of pyriproxyfen (Table 1-B). Mortality comparison for larvae exposed showed that the minimal quantity of spores with no significant effect should be lower than 10 spores per larvae (Table 1-B). Doses were therefore chosen at 2-3 spores per larvae for *P. larvae* exposure, 0.08 ppm for pyriproxyfen, and 80 ppm for boscalid exposure.

Effect of co-exposure on larval survival

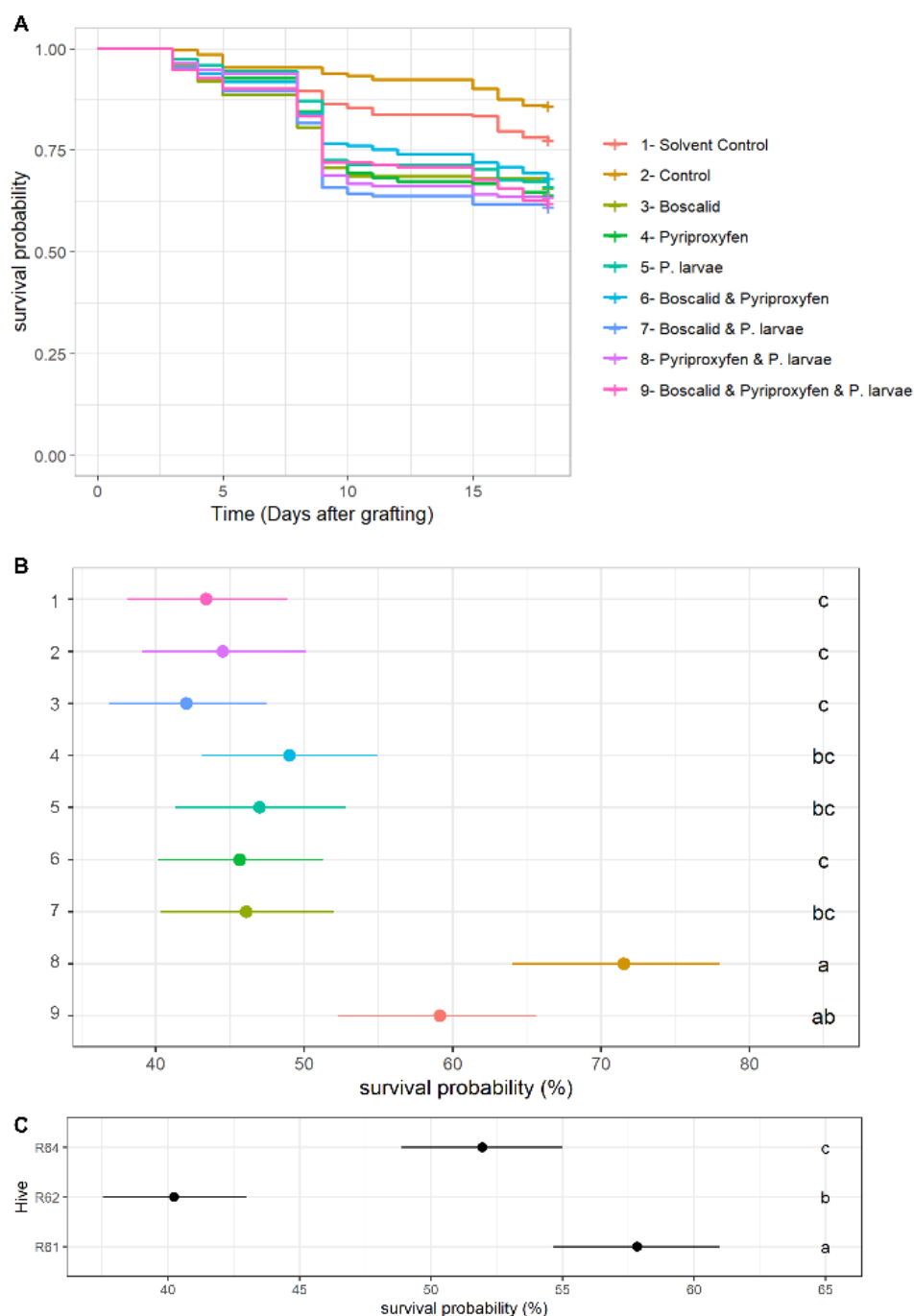


Figure 1. Survival probability on larval honeybees. **A** : Survival curves of larval worker honeybees raised *in vitro* exposed during development to boscalid, pyriproxyfen and *P. larvae* alone or in combination and two control treatments. **B** : Statistical comparison between the different treatments (estimates of the Cox regression model and confidence level 95 %). **C** : Comparison of survival between larvae depending on their hive of origin (Tukey test; $p < 0.05$). Different letters indicate a significant difference.

Mortality of larvae was significantly caused by both pesticides and *P. larvae* ($\chi^2 = 44.958$; $df = 8$; $p < 0.001$; Table 3-A). *In vitro* rearing of larvae in both control with water and control with solvent had no effect on mortality (12.5 % and 18 % respectively at day 18 ; Sidak test = - 0.551 ; $p = 0.18$) but all other modalities were significantly different from control (Tuckey test ≤ 1.078 , $p > 0.0002$; Table 2-D). When compared to the control with solvent, larvae exposed only to pyriproxyfen showed a significant higher mortality at D18 (Sidak test = 0.544 ; $p < 0.004$ Table 3-C) or a tendency when exposed to boscalid or *P. larvae* (32.4 % and 29.6 % respectively Sidak test ≤ 0.052 ; $p \geq 0.06$ Table 3-C). Larvae exposed to combination of treatment were also significantly impacted (Sidak test ≥ 0.591 ; $p \leq 0.01$ Table 3-C) but to not with the mix of boscalid and pyriproxyfen (Sidak test = 0.410 ; $p = 0.276$). Comparison effect between single or combination of stressor revealed no additional or cumulative effect on survival probability (Tukey test ≤ 0.280 ; $p \geq 0.795$). We also observed a significant effect of colony of origin on mortality (Cox proportional hazard mixed model : $\chi^2 < 49.441$; $df=2$; $p < 0.001$; Table 3-A) due to differences between hive R62 and the other two (Tukey test ≤ -0.474 ; $p < 0.001$; Table 3-B)

Comparison of cumulative larval mortality at day 7 showed no significant effect between experimental session (GLM binomial ; $\chi^2 = 0.013$ $df = 1$; $p = 0.909$) neither between hive of origin ($\chi^2 = 4.778$; $df = 2$; $p = 0.0917$). Finally, larvae mortality was not significantly affected by the addition of single or combination of stressor ($\chi^2 = 11.427$; $df = 8$; $p = 0.178$). In all these groups, cumulative mortality from day 4 to day 7 did not exceed 13 %.

Effect of co-exposure on pre-pupae weight

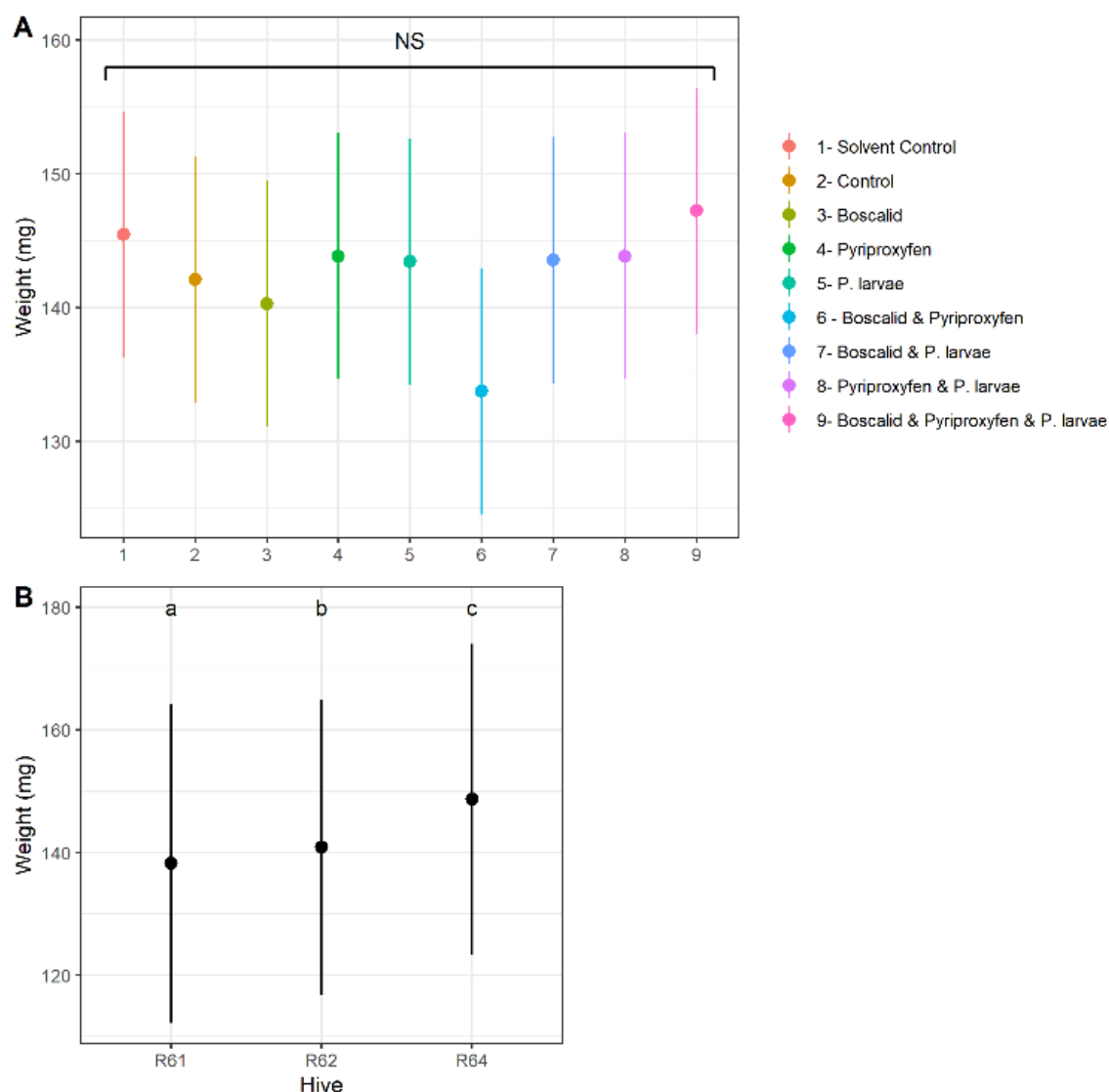


Figure 2 : weight of pre-pupae honeybees. **A :** weight of pre-pupae worker honeybees raised *in vitro* exposed during development to boscalid, pyriproxyfen and *P. larvae* alone or in combination and a control treatment. **B :** comparison of pre-pupae weight depending on their hive of origin (Tukey test; $p < 0.05$). Different letters indicate a significant difference ; NS : not significant.

To evaluate the impact of pesticides and pathogens on honeybee larvae growth, we weighted pre-pupae of the different treatments. No significant effect of boscalid, pyriproxyfen and *P. larvae* alone or in combination were measured (Tukey test ≤ 13.49 ; $p \geq 0.1384$; Table 4). However, the hive of origin had a significant impact on pre-pupal weight (Tukey test ≥ 2.66 ; $p \leq 0.003$).

Effect of co-exposure on pupation rate

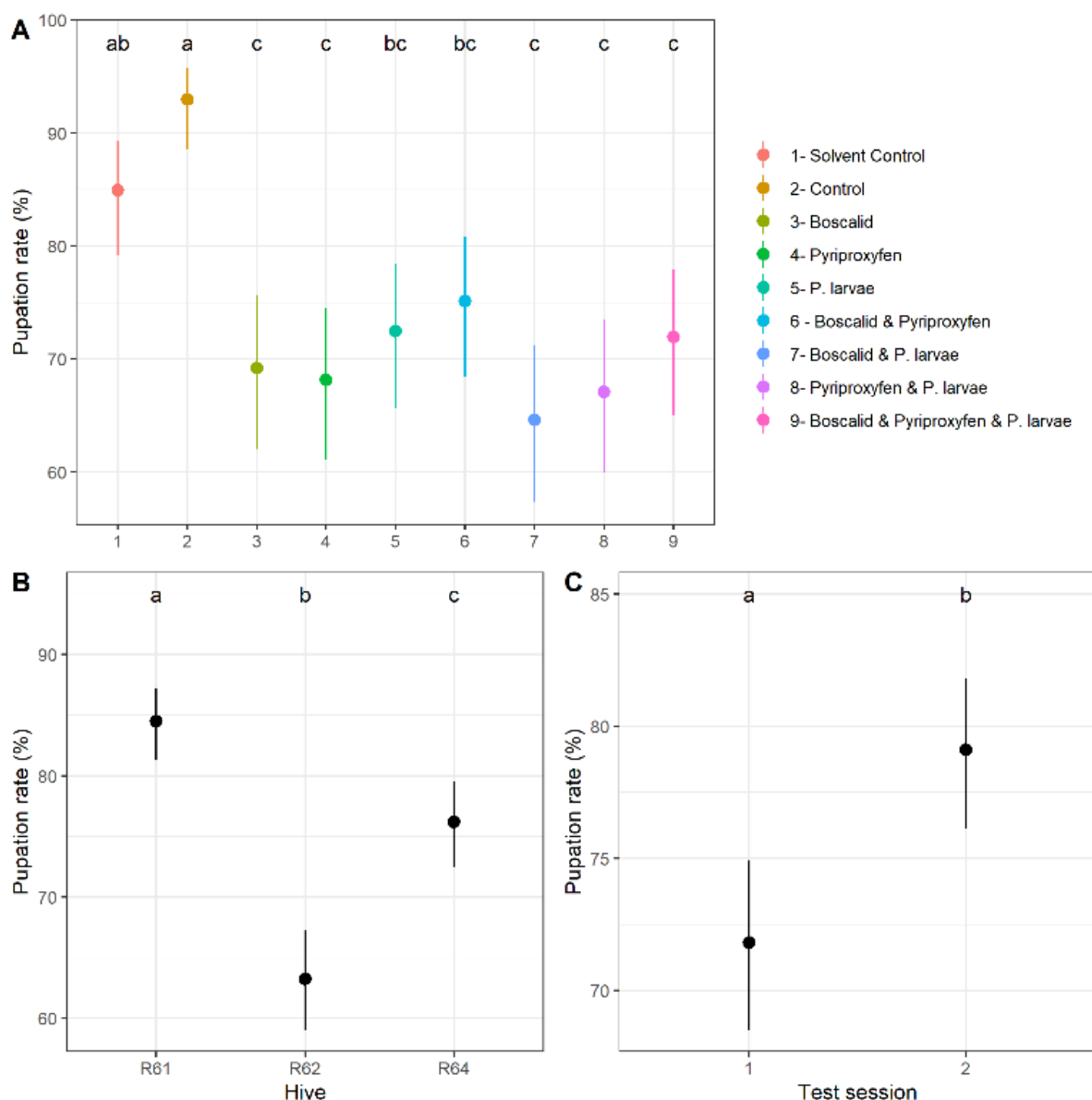


Figure 3 : pupation rate in vitro rearing honeybees larvae. **A** : pupation rate of larvae raised in vitro exposed during development to boscalid, pyriproxyfen and *P. larvae* alone or in combination and a control treatment. **B** : comparison of pupation rate depending on their hive of origin and **C** : test session (Fig 3C), (GLM binomial model, Tuckey test, ($P < 0.05$)).

Pupation rate of in vitro larvae rearing was significantly affected by the different treatments (Fig 3A). More specifically, pupation rate decreased when larvae were exposed to solvent alone (Sidak test = -0.847 ; $p = 0.09$; Table 5-D). Pupation rate of all larvae exposed to boscalid, pyriproxyfen alone or in combination with and *P. larvae* were significantly more impacted (comparison with control : Tukey test ≥ 1.471 ;

$p < 0.0001$; comparison with solvent control Sidak ≥ -0.763 ; $p \leq 0.02$; Table 5-D) but not for the larvae co-exposed to boscalid and pyriproxyfen (Sidak test = 0.625 ; $p = 0.12$). Finally, we had no significant effect between larvae exposed to boscalid, pyriproxyfen and *P. larvae* alone or in combination (Tukey test ≤ 0.504 ; $p \geq 0.404$; Table 5-E).

All exposures were repeated at two different times, with twenty days in between. Our results showed a significant impact of pupation rate between the two sessions of experiment ($\chi^2 = 12.099$; $df = 1$; $p < 0.05$; Fig 3-C ; Table 5-A) with higher pupation in the second session (Tukey test = -0.396 ; $p < 0.05$; Table 5-B). The hive of origin also impact significantly rates of pupation ($\chi^2 = 68.838$; $df = 2$; $p < 0.005$; Fig 3-B ; Table 5-A) with significant differences in all pairwise comparison (Tukey test ≥ -0.533 ; $p < 0.001$; fig 3-B ; Figure 3C, Table 5-C).

Impact of co-exposure on emergence rate

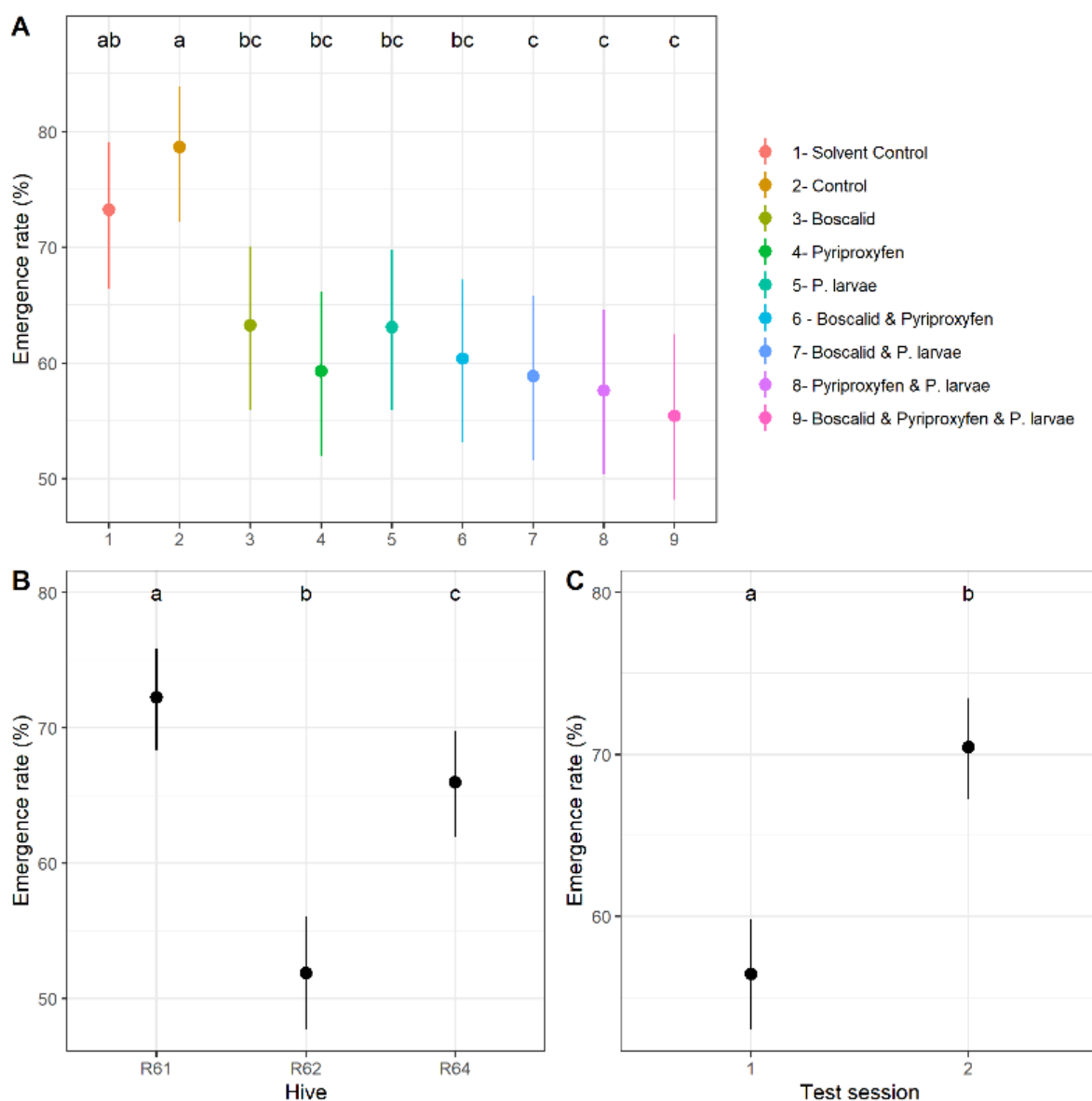


Figure 4 : emergence rate of *in vitro* rearing honeybees larvae. **A :** emergence rate of larvae raised *in vitro* exposed during development to boscalid, pyriproxyfen and *P. larvae* alone or in combination and a control treatment. **B :** comparison of emergence rate depending on their hive of origin and **C :** test sessions, (GLM binomial model, Tuckey test, ($P < 0.05$)).

Emergence rate of *in vitro* larvae rearing was significantly affected by the different treatments ($\chi^2 = 39.511$; $p < 0.05$; Fig 4-A; Fig 4-A; Table 6-A). More specifically, the emergence rate did not decrease when larvae were exposed to solvent alone (Sidak = 0.30, $p = 0.86$; Table 6-D). Emergence rate of larvae exposed to pyriproxyfen alone or combination with boscalid and *P. larvae* were significantly

impacted (comparison with solvent control Sidak test = 0.630 ; $P = 0.03$; Table 6-D). Emergence rate of larvae exposed to *P. larvae* and boscalid alone or combination to boscalid and pyriproxyfen were no impacted (Sidak test > 0.584 ; $p > 0.07$; Table 6-D). Finally, we had no significant difference between larvae exposed to boscalid, pyriproxyfen and *P. larvae* alone or in combination (Tukey test = -0.325 ; $p \geq 0.853$; Table 6-E).

All exposures were repeated at two different times, with twenty days in between. Our results showed a significant impact of emergence rate between the two sessions of experiment ($\chi^2 = 34.855$; $df = 1$; $p < 0.05$; Fig 4-C; Table 6-A), with higher emergence in the second session (Tukey test = -0.396 ; $p < 0.05$; Table 6-B). The hive of origin also impact significantly rates of emergence ($\chi^2 = 51.317$; $df = 2$; $p < 0.005$; Fig 6-B ; Table 5-A) with significant differences in all pairwise comparison (Tuckey test ≥ 0.30 ; $p < 0.005$; fig 4-B; Table 6-C).

DISCUSSION

Ecotoxicology measurement of pyriproxyfen, boscalid and P. larvae

We determined lower doses effect, in the diet, at 0.08 ppm for pyriproxyfen, 80ppm for boscalid and 2-3spores per larvae for the pathogen *P. larvae*. Doses determined in our study were similar than in previous studies. Indeed, Yang et al. (2010) showed that an exposure at 1ppm of pyriproxyfen in larval food induced 49.7 % larval death. For boscalid the lethal dose was 75.19 μ g/larvae and the no effect dose at 40 μ g/larvae (equivalent to 257.6 ppm ; Simon-Delso et al., 2017). Finally, for *P. larvae*, exposure to about 9 spores per larva at two days old induces 50 % death (Brødsgaard et al., 1998).

Exposure measurements

Effects of single exposures : analyses showed that different exposures impacted mortality at different stage of development. Boscalid exposed bees had an increasing of mortality during larvae and pupal stage, pyriproxyfen exposed bees. Moreover, single exposure to pyriproxyfen and exposure of *P. larvae* with acetone impacted rate emergence of bees. Duration of pupation are also impacted by single exposure to pyriproxyfen and boscalid. pyriproxyfen were shorter, while boscalid accelerated it. No single exposure except *P. larvae* with acetone exposure had an

effect on pre-pupae weight. No studies have been investigated effects of fungicide or IGRs on prepupae weight, indeed, our study showed no significant effects on prepupae weight after a larvae exposure to pyriproxyfen or boscalid or *P. larvae*.

Boscalid at 80 ppm had a significant impact on the rate of larvae reaching pupation at D13. Our result is consistent with previous study (Simon-Delso et al., 2017). Several studies reported negative consequences of fungicides on bee brood development. They hypothesis that carboxamide pesticides, such as boscalid, may interfere with hormonally regulated development (Atkins & Kellum, 1986 ; Aubee & Lieu, 2010 ; Frazier et al., 2008 ; Ladurner et al., 2005). Fungicides (Captan, Rovral or Ziram) can disturb larvae, prepupae development to adults and even lead to amorphogenic effects (Mussen, Lopez, et al. 2004). In addition, boscalid exposure reduces worker life span and colony population size (Fisher et al., 2021). The increased toxicity under field conditions is at least partly due to the activation of nutritional responses in the colonies that are not considered by laboratory tests. (Fisher, Cogley, et al., 2021 ; Fisher, DeGrandi-Hoffman, et al., 2021 ; Fisher et al., 2022).

Field realistic concentration of Pristine (2.3ppm) and a concentration two order of magnitude higher (230 ppm) reduces thorax mass but not the workers' body mass (Glass et al., 2021). The highest dose also reduced maximal flight performance and metabolic rates (Glass et al., 2021). The authors concluded that Pristine reduces forager longevity by negatively impacting digestive or nutritional processes.

Field studies provide essential informations and are representative of real conditions with various environmental changes throughout the period of colony activities. Although field tests are most complex to set up and require higher investment (equipment, monitoring...), they are essential to properly evaluate pesticides effects on non-target species.

Pyriproxyfen : our study showed the effect of an IGRs pesticides on mortality, rate emergence and duration of pupation. Larva exposure to 0.08 ppm pyriproxyfen resulted in higher mortality with notably a lower pupation rate at D13 and a lower emergence rate at D22 than the solvent control. This insecticide is considered to have low acute toxicity to adults by the oral and contact routes (Devillers & Devillers, 2020). IGRs impact insect development at larvae phase by inhibiting metamorphism and disturb molting processes in honeybees (Chen et al., 2016 ; Pinto et al., 2000 ; E. Yang

et al., 2010). In addition, other studies showed that pyriproxyfen induced shorter pupation and morphological disturbances and increased melanisation of the pupal cuticle via topical application or also delayed differentiation of the flight musculature (Bitondi et al., 1998 ; Fernandez et al., 2012 ; Zufelato et al., 2000). Negative effects have been observed under field conditions on larval hatching, adult bees, royal jelly production, and queen cell acceptance particularly at concentrations of 10 and 100 ppm (Chen et al., 2016). At the metabolic level, pyriproxyfen has been shown to inhibit the production of vitellogenin (a protein related to the immune system and juvenile hormone) in young workers. Pyriproxyfen treatment of larvae can also have negative consequences at the adult stage. Adult workers have different odors which are essential for acceptance by nest members and exhibited fewer social behaviors (Fourrier et al., 2015).

P. larvae : larvae exposed to 2-3 *P. larvae* spores had a lower pupation rate at D13 compared to larvae receiving solvent control, but no impact on larvae mortality or emergence rate. Different genotypes of *P. larvae* induce variations in virulence at the larval stage (Rauch et al., 2009). In the current experiment we used the same genotype and biological sample for all experiments. The use of acetone could contribute to increase the spread of the bacterium in the larval digestive system, and thus contribute to increase the virulence of *P. larvae*. However, this is still partly representative due to the composition with adjuvants of the commercial formulations (Fine et al., 2017 ; Wernecke et al., 2022). In addition to the direct virulence of the bacterium, *P. larvae* could cause oxidative stress in infected larvae and constitute an additional cytotoxic factor (María de la Paz Moline, 2022).

Multiple exposure effects

Studies on the toxicity of mixtures between different classes of pesticides at relevant concentrations and their interactions in bees and on pollinators are scarce in view of the potential combinations available (Johnson et al., 2013). Indeed, detecting potential statistical effect leads to increase the number of individuals per modality (Baguley, 2004 ; Lieber, 1990). Moreover, using different laying queens to include genetic diversity may reveal sensitivities to these different stresses thus more variation between replicats (Büchler et al., 2014 ; Mattila & Seeley, 2007). Larvae co-exposed to boscalid-pyriproxyfen were not significantly more affected in pupation and emergence rates compared to larvae exposed to just one of these two pesticides alone.

A synergistic effect between pesticides in honeybees was observed in a previous study testing fungicides (Aliouane et al., 2009 ; Johnson et al., 2013 ; Pilling & Jepson, 1993 ; Thompson & Wilkins, 2003). These effects might be explained by the fact that fungicides can inhibit mixed-function oxidase enzymes in bees, thus preventing the metabolism and detoxification of other pesticides (Pilling & Jepson, 1993).

Exposures to combination to boscalid, *P. larvae* clearly impacted survival, pupation rate and emergence rate and no significant differences between larvae exposed to just one of these stressors. Thus, with the variables measured we observed no synergistic, additive, subtractive effects of the interactions between these xenobiotic and pathological stressors. The developmental stage of the individuals conducts to different sensibility of the organisms. For example, the exposure to *Nosema ceranae* fipronil combination induced a synergistic effect compared to the sum of the effects observed in honeybees exposed to each stressor alone with the most significant impacts when stressors we applied at the emergence of honeybees (Aufauvre et al., 2012).

Previous studies tested the interactions between factors and adult bees showed a negative synergistic effect of pesticide / pathogen or pesticide / pesticide on bee survival (Alaux et al., 2010 ; Aufauvre et al., 2012 ; Coulon et al., 2018, 2020 ; Genersch, 2010b). Indeed, the physiological modification of larvae after exposure to pesticides could facilitate the transmission of *P. larvae*, by reducing the activity of an enzyme used by the workers to combat the transmission of the pathogen (Alaux, Ducloz, et al., 2010). For example, an interaction between sublethal doses of dimethoate or clothianidin with *P. larvae* has been shown to result in significantly higher mortality than the expected sum of the individual factors and in parallel with a drastic reduction in total and differential haemocyte cell counts (Hernandez Lopez et al., 2017).

Co-exposure to parasite (*Nosema*) and pesticides (fipronil, or thiamethoxam or boscalid) affects the gut microbiota composition by a decrease in the abundance of alphaproteobacteria and an increase in the abundance of gammaproteobacteria (Paris et al., 2020). Giving the essential contribution of microbial community of the digestion and metabolism of the *A. mellifera* diet (Zheng et al., 2016) and overall host fitness (Romero et al., 2019), modifying the regulation of competition between different strains could thus disturbing the overall functional role of the microbiota. In addition, fungi

represent a fundamental key insect nutrition, by biotransforming complex compounds that insects are unable to assimilate but the fungi convert into simpler molecules such as more labile sugars and free amino acid (Abril, 2011 ; Calheiros et al., 2019 ; Khadempour et al., 2020). Thus, by altering gut microbiota composition and targeting fungi, boscalid contributes to the weakness of individuals.

Exposures to boscalid and pyriproxyfen combination had a similar effect on mortality, emergence rate and duration of pupation than exposure to a pesticide alone. We also observed an additive effects of pesticides combination on the reduction of the prepupae weight and emergence probability. The prepupae weight is an index of viability for many *Lepidoptera* and is directly correlated with higher capacity (as reproduction) after emergence (Rodriguez et al., 2001). Previous studies also showed a synergic effect of several pesticides in honeybees (Aliouane et al., 2009 ; Johnson et al., 2013 ; Pilling & Jepson, 1993 ; Thompson, 2012). fungicide can inhibit mixed function oxidase enzymes in honeybees, thus preventing the metabolism and detoxication of pesticides (Pilling & Jepson, 1993), and in our study induce a lower weight.

Even though we used doses with lower single effects, we showed, at D23, that cumulative mortality was increased from 10 % to 15 % in single exposures to pesticides, and twice when larvae were biexposed to pesticides/pathogens. Moreover, the emergence rate can be reduced to 20 % when larvae were biexposed to pesticides and pathogens.

Colony and « genetics » effects

Here we have strong evidence of differences in insecticide resistance between colonies at the larval stage and on pathogen resistance in honeybees. Our results revealed differences between larvae from different colonies in larval mortality, pre-pupal weight, pupation rate and emergence rate to the different exposure. Previous studies have shown that diet, such as pollen availability, during larval development can affect their physiology and lifespan (Human et al., 2013 ; Mattila & Seeley, 2007). Furthermore, the addition of a toxic compound can also alter larval survival (Czoppelt, 1990 ; Doublet et al., 2015 ; Fourrier et al., 2015 ; Papach et al., 2017) using three colonies but the authors did not provide information regarding the effect of colonies. Genetic diversity may explain the differences observed between colonies (Breed et al.,

1988). Colony genetics can affect productivity, fitness, survival (and insecticide resistance in worker bees (Büchler et al., 2014 ; Laurino et al., 2013 ; Mattila & Seeley, 2007 ; Meixner et al., 2014 ; Rinkevich et al., 2017). Indeed, in our study, all queens came from the same genetic lines and mated naturally. Therefore, the main variability in our experiment is the queen mating history since the larvae were reared *in vitro*. Genetic and environmental factors (e.g. seasonality) can affect the weight of queen eggs (Taber et al., 1963 ; Winston, 1987). In addition, we used colonies that were visually symptomless and healthy, but it has been shown that the level of pathogens (e.g. viruses, fungi, etc.) varies from one hive to another.

Other significant parameters

In perspective, many new studies recently published have brought new insights into a better understanding of the interactions between bees, boscalid, Pyriproxifene and *P. larvae* but also the environment. In particular, the effects of pesticides are increasingly observed at sublethal doses and at different levels. (i) At the metabolic level, (ii) At the level of the microbiota. (iii) At the individual level, (iv) In the field, there is co-exposure to different active substances or adjuvants, which are most frequently found in beekeeping matrices (Böhme et al., 2019 ; Friedle et al., 2021 ; Herrera López et al., 2016). A few studies have already shown the importance of this co-exposure in larval development (Johnson et al., 2013 ; Zhu et al., 2014).

CONCLUSION

Our results show a significant effect of exposure and co-exposure of the common fungicide boscalid, the insecticide pyriproxifen and the bacterium *P. larvae* on survival, pupation rate and emergence of honeybee larvae *in vitro* rearing. Besides the lack of a strong synergistic effect, our results suggest an interaction between the pathogen *P. larvae* and pesticides. Even at low doses and single exposures pyriproxifen (0.08 ppm/larvae), boscalid (80 ppm/larvae) and *P. larvae* (2-3. Bacteria/larvae) tend to impact on the survival of individuals, and the pupation rate of individuals, which demonstrates the importance of larval toxicity tests and long-term monitoring of individuals. Our study contributes to better knowledge of dose effect of xenobiotic stresses on pollinators and the environment. It is important to assess the needs of farmers to protect crops from pests while maintaining the health of pollinators to provide an ecosystem service.

AUTORS CONTRIBUTION

AV : conceptualization ; methodology ; investigation ; writing – original draft.

VDP : data curation ; formal analysis ; investigation ; visualization ; writing – review & editing.

DF : resources ; editing.

SG : resources; editing.

PA : conceptualization ; supervision ; writing – review & editing.

FJR : conceptualization ; funding acquisition ; investigation ; supervision ; methodology ; project administration ; writing – review & editing.

FUNDING

This project was financially supported by the European Commission through the program Erasmus Mundus “Master Course - International Master in Applied Ecology” (EMMC-IMAE) (FPA 2023 – 0224/ 532524-1-FR- 2012-1-ERA MUNDUS-EMMC) – coordinator F-J Richard, Université de Poitiers.

CONFLIT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CHAPITRE 5 - DE LA MOLECULE A LA COLONIE : EVALUATION DE L'INTERACTION ENTRE INSECTICIDES ET FONGICIDES

Avant-propos

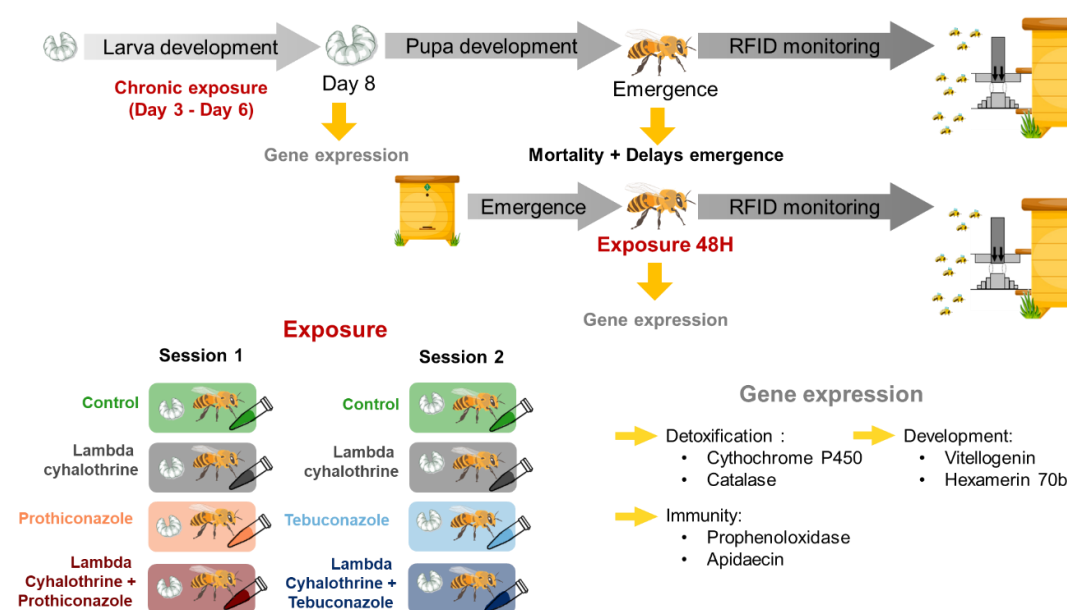
Au cours de leur vie, les abeilles sont continuellement exposées à différentes combinaisons de pesticides (Barascou et al., 2021). L'ingestion d'aliments contaminés est notamment considérée comme le risque le plus élevé pour les butineuses, les abeilles d'hiver et les larves (Böhme et al., 2019). Cette situation met en évidence une préoccupation croissante concernant les mélanges de pesticides susceptibles d'induire des effets toxiques synergiques chez les abeilles (Almasri et al. 2020 ; Cang et al. 2023 ; Wang et al., 2020). La toxicité interactive entre les fongicides à base de triazole et les pyréthrinoïdes est également très préoccupante. La lambda-cyhalothrine, le tébuconazole et le prothioconazole sont souvent détectées de façon concomitante dans des échantillons de nectar et de pollen en raison de leurs forte utilisation (David et al. 2016 ; Kaila et al. 2022 ; Yao et al. 2018). La synergie des fongicides triazoles avec les pyréthrinoïdes est attribuée à leur capacité à interférer avec la détoxification oxydative en inhibant le cytochrome P450 (Johnson et al., 2012). Néanmoins, peu d'informations sont disponibles sur les modifications physiologiques, comportementales et sur les colonies à des faibles doses de ces pesticides.

Les objectifs

L'objectif de ce chapitre est de caractériser l'interaction entre la lambda-cyhalothrine et les triazoles (prothioconazole et le tébuconazole) sur la physiologie et l'activité des abeilles, tout en intégrant des simulations de dynamiques des populations pour généraliser cet effet sur la colonie. Pour répondre à cet objectif nous avons caractérisé l'état physiologique des individus en mesurant l'expression de gènes spécifiques après l'exposition sur les larves et les jeunes abeilles adultes. Nous avons utilisé la technique RFID pour observer le cycle de vie des abeilles adultes. Pour contrôler précisément l'exposition des larves, cette technique est combinée à la méthode d'élevage *in vitro* des abeilles (chapitre 1).

Sur la base des éléments développés précédemment, quatre hypothèses émergent :

- L'exposition simple aux triazoles n'entraînera pas d'effets sublétaux sur les individus.
- L'exposition simple à la lambda-cyhalothrine n'aura pas d'effet sur les traits d'histoire de vie des individus, mais induira une légère altération physiologique
- La toxicité interactive entre la lambda-cyhalothrine et les triazoles entraînera des effets sublétaux sur les traits d'histoire de vie et des effets physiologiques plus forts que ceux induits par la lambda-cyhalothrine seule.
- Le report de ces effets synergiques sur les abeilles dans des simulations des colonies induira des effets néfastes sur la dynamique de de la colonie.



Nous avons analysé les niveaux d'expressions des gènes après l'exposition chronique des larves (7 jours) et des jeune abeilles adultes (48h) après l'émergence. Les gènes cibles étaient liés aux voies métaboliques de l'immunité (prophenoloxidase et apidaecine), de la détoxification (catalase et cythochrome P450), du développement et de réserve (hexamerine 70b et vitellogénine) et de l'activité mitochondriale (cythochrome b et succinate déshydrogénase). Nous avons mesuré les principales caractéristiques du cycle de vie des abeilles, notamment le développement larvaire, la durée de vie, l'âge de la première sortie, la mort après la première sortie, l'âge du début de la recherche de nourriture, la durée de la recherche de nourriture, et l'intensité de la recherche de nourriture des abeilles adultes (Prado et al., 2020 ; Requier et al., 2020). L'effet d'interaction entre la lambda-cyhalothrine et le prothioconazole ou le

tébuconazole est simulé à plusieurs intervalles de temps sur les colonies d'abeilles mellifères. Pour cela, les changements de la durée de vie des abeilles ont été intégrés dans un modèle informatique simulant le développement d'une colonie d'abeilles mellifères (BEEHAVE).

Le choix des doses d'exposition

Le prothioconazole et tébuconazole font partie des fongicides les plus utilisés en France, mais les informations sur leur présence dans les matrices des ruches font défaut (ANSES, 2023). Cependant, les abeilles sont bien exposées à ces produits car ils ont été détectés dans des pollens, le nectar et le pain d'abeilles (Roszko et al., 2016 ; Zioga et al., 2023). Pour ces produits, nous avons donc décidé d'utiliser une dose qui n'affecte pas la survie des larves et des abeilles (Wood et al., 2020). Cette dose provient d'une étude qui avait examiné les concentrations maximales présentes dans les pollens en Pologne (Roszko et al., 2016). Ainsi nous avons décidé d'exposer les larves et abeilles à 50 ng/individu de prothioconazole et de tébuconazole afin de pouvoir comparer leurs sensibilités. La dose de lambda cyhalothrine utilisée est de 6 ng/individu et correspond à 1/40 de la DL50 pour les adultes (241 ng/abeille ; (Decourtye et al., 2005) et à 1/140 de la DL50 pour les larves (830 ng/larve) (Waseem et Thakur 2023). À ce niveau d'exposition, seuls des effets cytotoxiques ont été observés sur les cellules de l'intestin moyen, de l'hypopharynx, des glandes et du cerveau des ouvrières mellifères, sans que cela affecte la cognition ni la survie des abeilles (Arthidoro de Castro et al., 2020 ; Decourtye et al., 2005).

Le résumé des résultats

La lambda-cyhalothrine a réduit la survie des larves en laboratoire et l'intensité de butinage de 55,92 % de ces individus lors du stade adulte dans la ruche, mais seulement pendant une session. Les larves coexposées *in vitro* à la lambda-cyhalothrine et au prothioconazole ou au prothioconazole surexpriment des gènes liés au développement (hexamérine ou vitellogénine), mais nous n'avons pas mesuré d'impact différé sur leurs traits d'histoire de vie au sein de la colonie. Cependant, la coexposition à la lambda-cyhalothrine et au prothioconazole des abeilles adultes réduit leurs durées de vie et leur intensité de butinage de respectivement 29,00 % et 36,35 %. Cette réduction globale des capacités pourrait être liée à la surexpression du gène de réponse immunitaire (apidecine). La coexposition à la lambda-cyhalothrine et au

tébuconazole des abeilles adultes agit en interaction et diminue de 61,00 % la longévité des abeilles adultes. Cela est directement associé à une date de première sortie plus précoce de 1,47 jour et à une augmentation de la mort directement après de 28,55 %.

La simulation de l'effet de la coexposition à la lambda-cyhalothrine et au tébuconazole (longévité réduite des abeilles) a conduit à la mortalité de 27 % des colonies et la plupart de ces effondrements se sont produits lors de la période de reprise de croissance. La simulation de l'effet de la coexposition sur toute la période de croissance (le scénario extrême) a réduit la population des colonies simulées de 34,4 % et leur production de miel de 53,8 % pendant cette période d'exposition. De plus nous constatons dans les simulations que (i) taille de la population et (ii) la production en miel sont impactés jusqu'à respectivement 2 et 3 mois après la fin de l'impact simulé. De plus des tendances très similaires ont été observées pour les autres scénarios d'exposition.

Ce travail offre une nouvelle perspective en fournissant des résultats solides sur les effets synergiques des pesticides dans l'affaiblissement des colonies d'abeilles mellifères.

Publication

Des simulations sont encore envisagées pour cette étude. Nous souhaitons approfondir l'analyse des risques induits par la coexposition lambda-cyhalothrine et au prothioconazole sur les colonies. Cette coexposition a induit une diminution de la longévité moins importante que l'autre combinaison, mais elle a aussi induit une réduction de l'intensité de butinage. Il sera pertinent de comparer les risques induits par les deux coexpositions, afin de globaliser les effets des interactions entre la lambda-cyhalothrine et les triazoles.

Les figures supplémentaires se trouvent dans l'annexe 5.

Co-exposures of lambda-cyhalothrin and triazole fungicides have sub-lethal effects on honeybees and increase the risk of colony mortality

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Running title : Co-exposure of pesticides have sub-lethal effects on honeybees

ABSTRACTS

Honeybees (*Apis mellifera*) are continually exposed to different combinations of pesticides throughout their life. The triazole fungicides and pyrethroids are of high concern because of their potential and interactive toxicity and the fact that they are often detected in nectar and pollen samples due to their intensive use. We monitored activity in hives using RFID systems of co-exposed bees during the adult stage and during the larval stage using *in vitro* rearing. The bees were exposed to sublethal doses of lambda-cyhalothrin and tebuconazole and/or prothioconazole. Lambda-cyhalothrin reduced the survival of larvae in the laboratory and the foraging intensity of 55.92 % of these individuals during the adult stage in the hive, but only during one session. Larvae co-exposed *in vitro* to lambda-cyhalothrin and tebuconazole or prothioconazole overexpressed development-related genes (hexamerin or vitellogenin), but we did not measure any delayed impact of their activity within the colony. Coexposure of adult bees to lambda-cyhalothrin and prothioconazole reduced their lifespan and foraging intensity by 29.00 % and 36.35 % respectively. This global decrease in abilities could be associated with the overexpression of the immune response gene (apidecin). Adult bees co-exposed to lambda-cyhalothrin and tebuconazole interacted to reduce the longevity of adult bees by 61.00 %. This is directly associated with the disruption of early flight activity. The repercussion of this effect at colony level by simulation models (short-lived bees) resulted in a 27 % mortality of colonies, a 34.4 % reduction in the population of simulated colonies and a 53.8 % reduction in their honey production during this exposure period. This work offers a new perspective by providing solid results on the effects of pesticides in the weakening of bee colonies at different levels of physiological disruption, and capacity at the individual and colony level.

KEYWORDS

Apis mellifera, *in vitro* rearing, RFID, co-exposition, gene expression, individual-based model.

INTRODUCTION

Apis mellifera, plays an essential role as a pollinator, contributing significantly to the reproduction of flowering plants, and supporting crop pollination services and beekeeping activities (Garibaldi et al., 2014 ; Klein et al., 2007 ; Quigley et al., 2019). However, colony losses of honeybees are reported worldwide (Castilhos et al., 2019 ; Neumann & Carreck, 2010 ; Potts et al., 2010 ; Requier et al., 2023), as well as a decline in bee diversity that, together, lead to concerns about the sustainability of beekeeping and pollination services (Potts et al., 2016). Several factors have been identified as being associated with bee decline and colony losses, e.g. habitat loss, parasite infestation, presence of pathogens, nutritional deficiencies (Goulson et al., 2015 ; Johnson et al., 2012). However, the intensive use of pesticides in modern agriculture has been recognized as a major risk involved in bee decline and honeybee colony losses (Gill et al., 2012 ; Goulson et al., 2015 ; Johnson et al., 2012 ; Sanchez-Bayo & Goka, 2014 ; Steinhauer et al., 2018).

Honeybees are subject to multiple routes of exposure to pesticides in agricultural fields (Krupke et al., 2012 ; Long & Krupke, 2016). This exposure can occur through direct foliar spraying or through contact with residues on plants, resulting in acute exposures of bee foragers (Thompson et al., 2014 ; Tosi et al., 2018). In addition, spray drift from adjacent fields, contamination of food sources such as nectar and pollen, and the presence of residues in hive components contribute to widespread exposure. (Böhme et al., 2019 ; Drummond et al., 2018 ; Sanchez-Bayo & Goka, 2014). This accumulation of chemicals in pollen and nectar can contaminate broods, queens and honeycomb (Böhme et al., 2018 ; Flores et al., 2021 ; Nougadère et al., 2012). Ingestion of contaminated food is a major risk for foragers, winter bees and larvae. (Böhme et al., 2019 ; Daniele et al., 2018 ; Zhu et al., 2014).

During their lifespan, bees may be continuously exposed to cocktails of pesticides (Barascou et al., 2021 ; Chauzat et al., 2006 ; Tosi et al., 2022). The origin of this cocktail exposure derives from the use of pesticides, which can be applied simultaneously or sequentially in agricultural systems (Belden & Brain, 2018). This situation highlights a serious and expanding concern about pesticide mixtures susceptible to inducing synergistic toxic effects in bees (Almasri et al., 2020 ; Cang et al., 2023 ; Wang et al., 2020).

Coexposure toxicity between triazole-based fungicides and pyrethroids is a growing concern. These substances are often detected together in nectar and pollen samples due to their frequent application (David et al., 2016 ; Kaila et al., 2022 ; Yao et al., 2018 ; Zioga et al., 2023). As a pyrethroid, lambda-cyhalothrin targets the voltage-dependent sodium channel present in the bees' nervous system, including flight muscles and the brain, directly impacting their survival and memory (Liao et al., 2018 ; Soderlund et al., 2002). Triazole fungicides, such as tebuconazole and prothioconazole, are among the most widely used fungicides for the control of fungal diseases in agricultural crops and can have a cytotoxic effect and influence microbial communities in the gut of insects (Cang et al., 2023 ; Mackei et al., 2023 ; Raimets et al., 2022). The synergy of triazole fungicides with pyrethroid is attributed to their ability to interfere oxidative detoxification by inhibiting cytochrome P450. (Johnson et al., 2012 ; Mullin et al., 2010 ; Pilling et al., 1995 ; Pilling & Jepson, 1993). However, little information is available on the physiological, behavioral and hive effects of lower doses on bees.

In this study, we investigated the potential effects of co-exposures between lambda-cyhalothrin and prothioconazole, and between lambda-cyhalothrin and tebuconazole on the physiology and life history of individual bees. Bees were exposed or co-exposed to low doses of pesticides during their larval stage (*in vitro* larval rearing) or adult stage (*in vivo* larval rearing). We assessed physiology by examining the expression of seven targeted genes following exposure of larvae and adult bees. Larval mortality and emergence were measured on larvae reared *in vitro* in the laboratory. The life history of adult bees was monitored using the RFID technique (*in vitro* and *in vivo* larval rearing). We computed seven essential characteristics of each bee's life history, including lifespan (LSP) (Prado et al., 2020 ; Requier et al., 2020 ; Desclos le Peley 2024). Subsequently, we explored, through simulation, the impact of co-exposures on individual bees and their potential effect on colony dynamics and survival using a mechanistic modeling approach. The results of this study will contribute to a better comprehensive assessment of the risks associated with co-exposures, to minimize negative effects on pollinators.

MATERIAL AND METHODS

The experiments were conducted at the INRAE Entomology Experimental Unit, Le Magneraud (46°8'59.104"N, 0°41'28.609"W), France, between may and august 2022. Molecular analyses were carried out at the laboratory Ecology and Biology of Interactions (Vienne-France). The apiary is in intensive farmland habitat with a high proportion of wheat, sunflower, roil seed rape and maize (Odoux et al., 2014 ; Requier et al., 2015). For the experiment we used *A. mellifera mellifera* × *caucasica* colonies. We treated *Varroa destructor* before winter but not during the spring or summer of the present study.

In vitro larval rearing

We collected one-day-old larvae from 2 and 3 colonies of *A. mellifera* and reared them *in vitro* until emergence. For artificial rearing, we followed the method adapted by (Aupinel et al., 2005 ; Medrzycki et al., 2013 ; Papach et al., 2017). By isolating the queen with a frame of built-up brood in an Italian cage for 30 hours, we obtained comb with first instar larvae. To obtain first-cycle (L1) larvae for grafting, the freshly laid frame was left in the hive for a further day after queen release. Two grafts were carried out by the same experimenters in the laboratory at room temperature. The first larval rearing was carried out, until the eighth day of rearing in the laboratory. The larvae (L1) were transferred into disinfected plastic grafting dishes. The cups with L1 were maintained in an incubator at 34.5 ± 5 °C from day 1 to day 8 with a relative humidity (RH) of $95 \pm 5\%$ (K₂SO₄ solution). Cups were filled with a mixture of royal jelly, aqueous sugar and yeast extract with or without pesticide treatment (see below for details) on days 1, 3, 4, 5 and 6. Each larva was chronically exposed to water (Control) or 0.1 % of acetone (Larvae exposure) for 4 days in their diet (day 3 to day 6). On days 7 and 8 the larvae were transferred to a new clean plate when they had finished feeding and the plates containing the pupae were transferred to a pupae incubator. In the same time larvae were collected and frozen for molecular biology analyses (see below). Prior to emergence (day 15), pupae plates are placed in crystal polypropylene emergence boxes (11 × 15 × 12 cm) with a 5 mm piece of Be-Boost® (PseudoQueen) attached to a piece of wax (approximately 5 × 5 cm). and placed at 34.5 ± 5 °C and 50 ± 5 % RH with 50 % agarose-sugar solution ad libitum. Individuals that died before the start of feeding on day 2 were removed from the analysis. We

monitored daily mortality and the days of emergence. Overall, 1007 individuals were reread in the first session and 1145 individuals in the second session.

RFID monitoring

We used RFID tags (mic3®-TAG 16 kbit, HF-RFID 13.56 MHz microsensys mic3 technology) to monitor bee activity. The tags, weighing around 3 mg and measuring $1.6 \times 1.9 \times 0.5 \text{ mm}^3$ (Streit et al., 2003). Tag presents a weight (3 % of the bee's total weight) that could have a marginal impact, (Marden, 1987 ; Winston, 1991). Before attaching the tags to the bees, we checked that they were working properly and recorded the tag ID using a portable USB device (ID®PENmini 7.0, microsensys). At the emergence of bees reared *in vitro* (between days 17 and 20), the bees were immobilized in a piston cage and tags were fixed to their thorax using biocompatible dental cement (TempoSIL2). Once marked, the bees were introduced into the hives using small wire frames, sealed with candy and then positioned in the RFID hive.

The hive considered for the monitoring was equipped with RFID readers (iID2000, 2k6 HEAD ; Microsensys) used to record the identity, date, and time at each passage of a tagged bee passed through a tunnel $80 \times 8 \text{ mm}$ high. We can distinguish the direction of the bees' activity, i.e. entry or exit, with the help of two lines of readers (Requier et al., 2020). Lines are constituted of four RFID readers (with a total of eight readers at the hive entrance) and recorded data are collected every 7 days.

Adult bees' exposure

Frames from the same hive used for *in vitro* grafting are recovered 17 days after grafting, when the brood is closed. The frame is placed in the incubator at $34.5 \pm 5^\circ\text{C}$ and $50\% \pm 5\%$ relative humidity. Emergent bees less than 12 hours old were marked with an RFID transponder (see above). Bees were maintained in small wire frames under laboratory conditions for 48 h at $34.5 \pm 5^\circ\text{C}$ and $50\% \pm 5\%$ relative humidity. The small wire frame was equipped with water and a 5 mm piece of Be-Boost® (PseudoQueen) was attached to a piece of wax (approximately $5 \times 5 \text{ cm}$). The sides of the wire frame were covered with perforated plastic which was removable. Bees were then exposed for 48 h via a sugar solution (50 % w/v sucrose) *ad libitum* and water. At the end of exposure some bees were collected and frozen for molecular

biology analyses (see above). After exposure, the small wire frames are sealed with candy and placed in the RFID hive comb.

Pesticide exposure and modalities analysed

Stock solutions of lambda-cyhalothrin (94.8 % purity, Cluzeau Info Labo, France), Tebuconazole (98.6 % purity, Cluzeau Info Labo, France) and Prothioconazole (98.6 % purity Cluzeau Info Labo, France) were prepared in acetone and added to the larval or young bee diet. The stock solutions of the pesticides were prepared at the time of use. In first session, larvae were chronically exposed to either 0.1 % of acetone (C_L), or 0.1 % of acetone and 6 ng of lambda-cyhalothrin (L_L), or 0.1 % of acetone and 50 ng prothioconazole (P_L), or coexposure 0.1 % of acetone and 6 ng of lambda-cyhalothrin + 50 ng prothioconazole (L+P_L), during 4 days in their diet (day 3 to day 6). Thus, the concentration of lambda-cyhalothrin and prothioconazole was respectively 39.6 ng/g and 296,64 in feed B and C Bees were exposed to 0.1 % of acetone (C_A), to 0.1 % of acetone and 6 ng of lambda-cyhalothrin (L_A), to 0.1 % of acetone and 50 ng prothioconazole (P_A), to 0.1 % of acetone and 6 ng of lambda-cyhalothrin + 50 ng prothioconazole (L+P_A). Thus, the concentration of lambda-cyhalothrin and prothioconazole was respectively 0.1 ng/μl and 0.84 ng/μl in a sugar solution (50 % w/v sucrose) and used to feed the young bees for 48 h.

In second session, larvae were chronically exposed to 0.1 % of acetone (C_L), to 0.1 % of acetone and 6 ng of lambda-cyhalothrin (L_L), to 0.1% of acetone and 50 ng tebuconazole (T_L), to 0.1 % of acetone and 6 ng of lambda-cyhalothrin + 50 ng tebuconazole (L+P_L), during 4 days in their diet (day 3 to day 6). Thus, the concentration of lambda-cyhalothrin and tebuconazole was respectively 39.6 ng/g and 296,64 in feed B and C Bees were exposed to 0.1 % of acetone (C_A), to 0.1 % of acetone and 6 ng of lambda-cyhalothrin (L_A), to 0.1 % of acetone and 50 ng tebuconazole (T_A), to 0.1 % of acetone and 6 ng of lambda-cyhalothrin + 50 ng tebuconazole (L+T_A). Thus, the concentration of lambda-cyhalothrin and tebuconazole was respectively 0.1 ng/μl and 0.84 ng/μl in a sugar solution (50 % w/v sucrose) and used to feed to the young bees for 48 h.

Extraction of total RNA and quantification of gene expression

Each larva was homogenized in 900 µl of QIAzol (Qiagen) with 3 beads of 2 mm diameter using a FastPrep-24 (MP) for 30 seconds at 6.5 m/s. For each adult bee, the abdomen was homogenized in 900 µl of QIAzol (Qiagen) with 3 beads of 2 mm diameter, using a FastPrep-24 (MP) for 2 cycles of 30 seconds at 6.5 m/s, separated by 15 minutes on ice. The homogenate was then incubated for 5 minutes at room temperature. Total RNA was extracted from each sample using the RNeasy Plus Universal Mini kit (QIAGEN). The DNA digestion step was performed by adding 50 µl of DNase. The final volume of the suspension was adjusted to 50 µl and the RNA was quantified using a NanoDrop 1000 spectrophotometer (Thermofisher).

The reverse transcription process utilized the SuperScript® First-Strand cDNA Synthesis Reaction Kit (Invitrogen) with 500 ng of RNA in 20 µl reaction volumes, incorporating random hexamer primers. Following cDNA synthesis, the RNase H step was implemented to digest RNA residues, involving the addition of 1 µl of RNase H and incubation at 37 °C for 20 min, following the manufacturer's protocol. Quantification of expression levels for 2 reference genes and 5 target genes was carried out using qPCR, encompassing both larvae and adult bees (TabS1). The RT-qPCR assays employed the LighCycler 480 SYBERGREEN I Master (Roche) qPCR. Each 10 µl reaction contained 1.5 µl of sample cDNA, 0.5 µM forward and 0.5 µM reverse primers (Integrated DNA Technologies), and 7.5 µl master mix, utilizing the LighCycler 480 (Roche) detection system. The amplification program comprised an initial denaturation step of 10 min at 95 °C, followed by 35 cycles : denaturation cycles of 10 s at 95 °C, hybridization cycles of 10 s at 60 °C, and elongation cycles of 20 s at 72 °C. Subsequently, a melting curve was generated by measuring fluorescence over a range of 65 °C to 97 °C to confirm the presence of the desired amplicon. All reactions were conducted in triplicate, ensuring a variation of less than 1 ct between the replicates. Water controls were included in each assay. For each session (1 and 2) and each exposure stage (L and A), the influence of the exposure modality (C, L, P, and L+P or L, T, and L+T).

Life history traits

To examine the life history characteristics of 927 bees, in the first session, and 866 bees in the second session, we used RFID data. The raw data was cleaned using the method described by Requier et al (2020). We computed 7 life history traits on the individuals : (i) The lifespan (LSP) was estimated in days up to the last recorded RFID hit for each bee. The other traits were computed after removing bee trips shorter than 2 seconds and trips longer than 180 minutes (Prado et al., 2020). (ii) The age of first exit (AFE) of the bees was defined as the number of days from the first recorded RFID tag (the first flight) and the days of release. (iii) The proportion of bees lost after their first exit from the hive (L-AFE) was estimated when the AFE was the equivalent of the LSP (Coulon et al., 2020). (iv) the foraging tenure (FT) as the time between AFE and PSL. (v) the foraging intensity (FI) as the total number of flights performed by bees (see also Prado et al., 2020). (vi) the age at onset of foraging (AOF) was the ontogenetic changes in the series of univariate temporal activity budgets in days using the *aof* R package (Requier and Rebaudo 2020), (vii) the foragers were the proportion of bees for which AOF could be determined.

Simulation of honeybee colony dynamics

To test whether the observed effects of pesticide co-exposure on individual bees could affect the colony dynamic and survival, we simulated the effect of shorter-lived bees into a model of bee colony dynamic, the BEEHAVE model (Becher et al., 2014). A total of 700 simulations were computed with $n = 600$ simulations for assessing the coexposure-induced effect of shorter-lived bees on colony dynamics, and $n = 100$ simulations for sensitivity analysis on colony survival. We first calibrated the model with Becher et al. (2014) initial colony settings associated with a random parameterization of four parameters to improve stochasticity and realism of the model, as follows : we randomly attributed a maximal egg-laying capacity of the queen between 500 and 1,600 eggs per day, an initial adult population size ranging from 7,000 to 10,000 bees, a larval mortality rate ranging from 0.009 to 0.011, and an infestation with 3 to 6 *Varroa destructor* mites from which the prevalence virus-infected mites range from 0 to 100 % on the first day of simulation as default value. To consider beekeeping management conditions, we also enabled the ad-hoc options such as the *Varroa* treatment and the honey harvests. We considered the gradient of maximal egg-laying capacity of the

queen as the demographic strength of the colony, by converting the value from 0.4 (500 eggs per day) to 1 (1,600 eggs per day).

We incorporated the observed effect of pesticide co-exposure into the model through modulations of the forager mortality rate for inducing shorter-lived bees. For that, we gradually increased the forager mortality rate from the default value of 0.00001 to 0.0000263, representing an increase coefficient of $\times 2.63$ according to the maximum pesticide co-exposure effect in our results. Thus, each simulation involved a relative effect of pesticide co-exposure according to our experimental observations, linearly increasing from no-effect (forager mortality rate of 0.00001, i.e. the default value) to full impact (forager mortality rate of 0.0000263). We included these impacts in our simulations by increment of 46 days (i.e. the average duration of flowering crops, Requier et al. 2015) for different time intervals (called seasons) during the colony dynamic, i.e. before the growth period of the population size (day 76 to day 122, season 1), at the beginning of the growth period of the population size (day 122 to day 168, season 2), and at the end of the growth period of the population size (day 168 to day 214, season 3). We ran six scenarios of exposure for different seasons (season 1, season 2, season 3) and by combining the different seasons (seasons 1+2, seasons 2+3, seasons 1+2+3). We computed 100 simulations per scenario with a gradual increase of pesticide co-exposure impact. Moreover, we computed 100 other simulations with an extended gradient of forager mortality rate from the default value of 0.00001 to 0.00006 (i.e. increase coefficient of $\times 6$) to run a sensitivity analysis on colony survival. The objective was there to analyze the risk of colony mortality due to the maximum pesticide co-exposure effect observed in our results.

We registered the adult population size (i.e., the number of adult worker bees in the colony) and the quantity of honey reserves (quantity of honey in the brood chamber and in the super chambers together, as it is mentioned in the model) for the last day of the exposition period for each simulation. Moreover, we registered the simulation endpoint deemed insufficient for colony survival using two following thresholds according to Becher et al. (2014), (i) simulations that reach population size smaller than 4,000 adult bees during the winter, and (ii) simulations that reach population size smaller than 2,500 adult bees from spring to autumn.

Statistical analyses

Statistical analyses were carried out with the R software version 4.2.2 (R Core Team, 2020). Analysis of variance and residuals of all GLMM were performed using *DHARMA R-package* (Florian Hartig, 2022) and *car R-package* (Fox et al., 2023). Statistical significance was set at 5 % for all tests. Model estimates were compared with posthoc pairwise comparisons using the *emmeans R-package* (Tukey's HSD ; Lenth et al. 2023). The results of the values shown in the visualizations represent the estimates and 95 % confidence intervals of the models, which have been back-transformed for visualization on the raw data using the *ggplot R-package* (Wickham et al., 2023). We have exploited the *multcomp R-package* to succinctly condense all pairwise comparisons of estimated marginal means (Piepho, 2004).

Assessing the impact of pesticide co-exposure on Gene expression : The average cycle threshold values for the target genes (apidaecin, catalase, cytochrome p450, hexamerin, prophenoloxidase and vitellogenin). Analysis of relative gene expression was performed using the $2^{-\Delta\Delta Ct}$ method were normalized to the geometric mean of reference genes, (ribosomal protein 32 and β -actin). Statistical analysis was performed using the ΔCt value, which was obtained by subtracting the Ct value of the internal reference gene from the Ct value of the experimental group. For each session (1 and 2) and each exposure stage (L and A), the influence of the exposure modality (C, L, P, and L+P or L, T, and L+T) on the $\Delta\Delta Ct$ of each genes was compared using the non-parametric Kruskal-Wallis method, followed by the Wilcoxon test for multiple comparisons, with *rstatix R-package* (Kassambara, 2023).

Measuring the impact of pesticide co-exposure on bee larvae : to analyze larval survival, Cox proportional hazards model was developed using the *coxme R-package* (Therneau 2022). For each Session (1 and 2) models considered exposure (C_L , L_L , P_L and $L+P_L$ or C_L , L_L , T_L and $L+T_L$) and the hive of origin of the larvae (R3, R4 and R6). The rearing plates were considered as a random factor in the model. In addition, a Linear mixed model (LMM) was used to assess the day of emergence of bees reared in vitro, using *lme4 R-package* (Bates et al. 2023). The explanatory variables considered for this model were like those of the survival model previously described.

Evaluation of the impact of pesticide co-exposure on adult bees : the statistical analysis process was like that described in Desclos le Peley et al. (2024). To monitor the bees' life-history traits, for each session (1 and 2) the models considered, the hive of origin of the larvae (R3, R4 and R6), the exposure (C, L, P and L+P or C, L, T and L+T) in interaction with the exposure stage (larval _(L) or adult _(A)). After eliminating bee movements lasting less than 2 seconds and those lasting more than 180 minutes, we calculated several parameters. The LSP were conducted using a Weibull hazard model from the *Survival R-package* (Therneau et al., 2023). The AFE were modeled using Generalized Linear Mixed Models (GLMM) gamma with the *glmmTMB R-package* (Brooks et al., 2023). The L-AFE were performed using binomial GLMM using the *ade4 R package* (Dray & Dufour, 2007). The FT were conducted with a negative binomial 2 GLMM. The FI were analyzed with a negative binomial 2 GLMM (Prado et al., 2020). The proportion of foraging bees using a binomial GLMM and analyzed AOF using a gamma GLMM of 278 bees from the first session and 140 bees from the second session.

Assessing the impact of pesticide co-exposure on colony dynamics and survival : we analyzed the effects of the pesticide co-exposure impact (i.e. simulating shorter-lived bees) on the population size and honey reserves (response variables) using linear models (LMs) and including the level of pesticide co-exposure impact, the demographic strength of the colony as explanatory variables. We also tested the interaction between the level of pesticide co-exposure impact and the demographic strength of the colony. We analyzed the direct time effect of simulating shorter-lived bees on changes in population size, as well as delayed effects with +1 month, +2 months and +3 months after the end of the simulated impact. Finally, we performed the sensitivity analysis (i.e. risk of colony mortality) by fitting a standard logistic function with the use of a generalized linear model (GLM) with a binomial error distribution.

RESULTS

Impact on gene expression after co-exposure

Larve gene expression was altered by chronic coexposure. Hexamerin gene was significantly overexpressed in L+P_L compared to C_L and L_L but showed no significant difference in expression to P_L (Wilcoxon paired test, respectively $p < 0.042$, $p = 0.648$). Expression of the vitellogenin gene was not significantly altered in L_L, T_L and L + T_L compared with C_L (Wilcoxon paired test ; $p > 0.23$). However, this gene was overexpressed in L+T_L compared to L_L and T_L (Wilcoxon paired test ; $p < 0.026$). There was no significant difference between the expression levels of the prophenoloxidase, catalase, cytochrome p450 and apidaecin genes at different larval exposures (Wilcoxon paired test ; $p > 0.097$).

Adult bee's gene expression of apidaecin was significantly overexpressed after exposed from the L+P_A compared to the C_A, L_A and P_A (Wilcoxon paired test ; $p < 0.05$). There was no significant difference in the expression of the other targeted genes catalase, hexamerin, cytochrome p450, vitellogenin and prophenoloxidase by adult bees exposures (Wilcoxon paired test ; $p > 0.208$).

Effect of pesticides on larvae development

Larval survival was affected by survival in session 1 ($\chi^2 = 11.87$; $df = 3$; $p = 0.008$, Fig.1, Tab.S1) but not in session 2 ($\chi^2 = 6.94$; $df = 3$; $p = 0.07$, Fig.1, Tab.S1). survival rates were lower in L_L larvae than C_L larvae during session 1, (C_L : 0.34 ± 0.08 HR ; L_L : 0.91 ± 0.21 HR ; Tukey test = 0.38 ; $p < 0.02$, Tab.S1). Survival rates of P_L and L+P_L larvae were not significantly different than C_L or L_L larvae (P_L : 0.82 ± 0.20 HR and L_L : 0.42 ± 0.11 HR ; Tukey test > 0.701 ; $p < 0.003$; Tab.S1). Moreover, the day of emergence of the bees was not altered by the exposure modalities over the two sessions ($\chi^2 < 5.30$; $df = 3$; $p > 0.15$; Tab.S2).

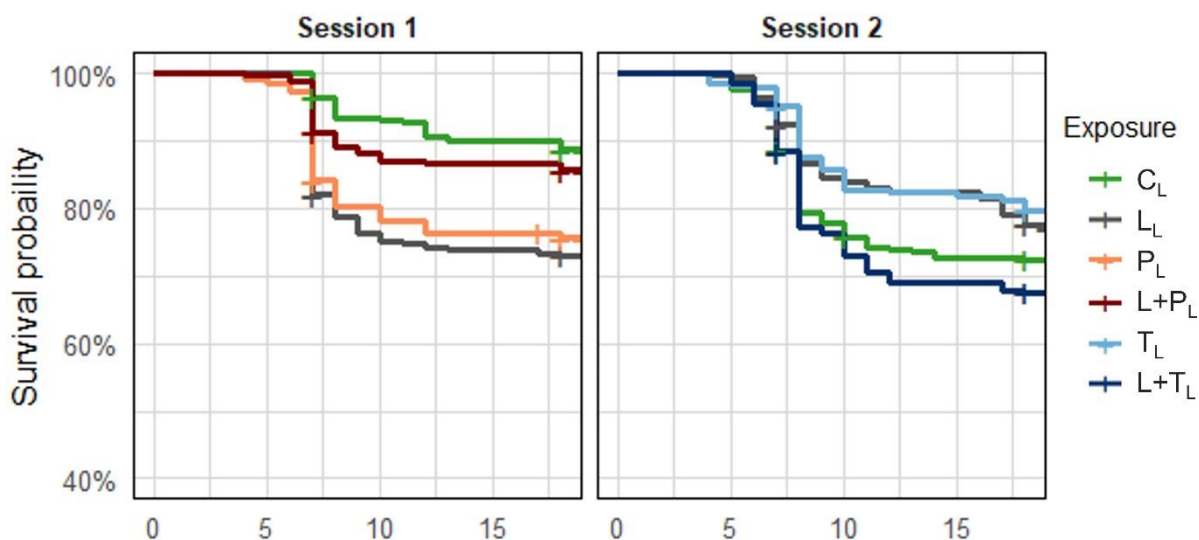


Figure 1. Impact of coexposure pesticides on larvae survival. The colors show that the larvae were chronically exposed during their diet to acetone (C_L), lambda-cyhalothrin (L_L), prothioconazole (P_L), lambda-cyhalothrin + prothioconazole ($L+P_L$) in session 1 ; or tebuconazole (T_L) and lambda-cyhalothrin + tebuconazole ($L+T_L$) in session 2.

Survival rate and day of emergence were affected by hive of origin of the larvae in both sessions (Survival : $\chi^2 > 17.61$; $df = 1$ or 2 ; $p < 0.001$; emergence : $\chi^2 > 14.08$; $df = 1$ or 2 ; $p < 0.001$; Tab.S1-S2).

Impact of co-exposure on life history trait of adult bees

LSP, AFE and L-AFE were affected by stages of exposure stage in both sessions ($\chi^2 > 13.52$; $df = 1$; $p < 0.001$; Tab.1), FI only in session 1 ($\chi^2 > 13.52$; $df = 1$; $p < 0.001$; Tab.1) and AOF just in session 2 ($\chi^2 > 28.33$; $df = 1$; $p = 0.001$; Tab.1). Also, LSP were impacted by pesticide exposure modality on the two sessions ($\chi^2 > 11.63$; $df = 3$; $p < 0.009$ Tab.1), FT and FI only in session 1 ($\chi^2 > 8.06$; $df = 3$; $p < 0.045$; Tab.1), and L-AFE just in session 2 ($\chi^2 > 8.86$; $df = 3$; $p < 0.031$; Tab.1). LSP, AFE and L-AFE were influenced by an interaction between exposure stages and exposure modalities in session 2 ($\chi^2 < 13.36$; $df = 3$; $p < 0.004$; Tab.1).

Hive of origin of the larvae had an incidence on LSP, AFE and L-AFE in both sessions ($\chi^2 > 6.66$; $df = 2$ or 3 ; $p < 0.036$; Tab.1). This parameter has an influenced FT, FI and AOF only in session 1 ($\chi^2 > 20.79$; $df = 2$ or 3 ; $p < 0.001$; Tab.1).

Table 1. Type II analysis of variance for the different generalized mixed effect models. For all GLM, the chi-squared tests of Wald's likelihood were calculated with their associated p-values.

		Stade		Exposure		Hive		Stade : Exposure	
		χ^2	p-value	χ^2	p-value	χ^2	p-value	χ^2	p-value
Session 1	LSP	38,66	<0,001	9,12	0,028	63,53	<0,001	2,23	0,527
	AFE	104,05	<0,001	2,64	0,450	23,11	<0,001	4,36	0,220
	L-AFE	146,43	<0,001	1,10	0,777	14,30	<0,001	0,73	0,866
	FT	0,36	0,550	8,06	0,045	29,98	<0,001	1,21	0,751
	FI	13,52	<0,001	16,02	0,001	25,23	<0,001	2,60	0,458
	Foragers	4,50	0,034	0,66	0,882	4,50	0,105	3,00	0,391
	AOF	1,80	0,180	5,83	0,120	20,79	<0,001	1,54	0,672
Session 2	LSP	20,34	<0,001	15,84	0,001	6,66	0,036	38,19	<0,001
	AFE	298,75	<0,001	1,80	0,615	10,70	0,005	10,70	0,005
	L-AFE	10,56	0,001	8,86	0,031	7,74	0,021	16,13	0,001
	FT	0,34	0,557	2,29	0,514	0,87	0,649	2,92	0,404
	FI	0,06	0,812	6,01	0,111	2,80	0,247	3,04	0,386
	Foragers	3,36	0,067	1,93	0,588	3,18	0,204	5,31	0,150
	AOF	28,33	<0,001	2,07	0,557	0,35	0,838	0,43	0,934

Pesticides coexposure decrease lifespan

Exposure stage influence on LSP GLMMs in both sessions ($\chi^2 > 20.34$; df = 1 ; $p < 0.001$; Tab.1). In addition, we found a significant effect of our pesticide exposure modality on GLMMs LSP ($\chi^2 > 9.12$; df = 3 ; $p = 0.028$; Tab.1). We also report an interaction between exposure stage and with the different exposure modalities in the LSP GLMM only during Session 2 ($\chi^2 = 38.19$; df = 3 ; $p < 0.001$; Fig.2A, Tab.1).

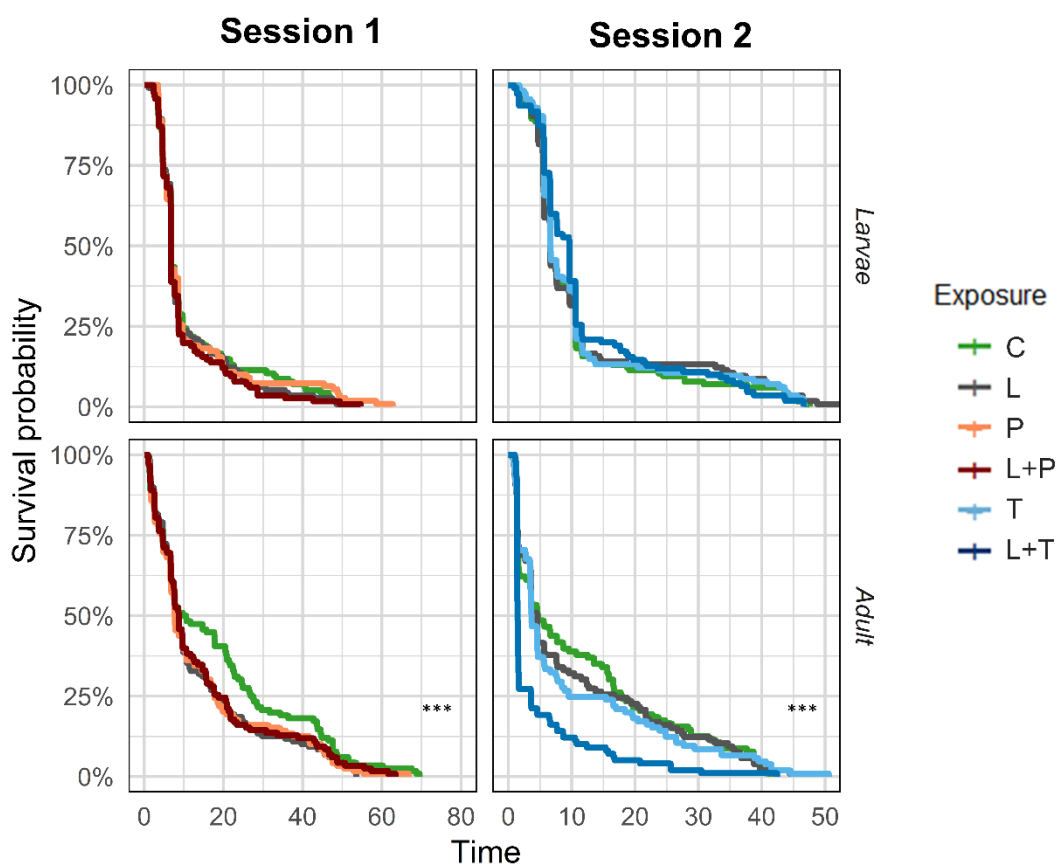


Figure 2. Effect of co-exposure to pesticides on the survival of adult bees. Adult bees were exposed to pesticides during two sessions (Session 1 and Session 2) either at the larvae (Chronic) or at adult stage (48h). The bees were exposed to Control (C), lambda-cyhalothrin (L), prothioconazole (P), lambda-cyhalothrin + prothioconazole (L+P), tebuconazole (T) and lambda-cyhalothrin + tebuconazole (L+T). *** p -values < 0.001 ; if pesticides affect LSP for each session.

We showed that bees exposed during the larval stage (C_L , L_L , P_L , $L+P_L$, T_L and $L+P_L$) did not have a shortened lifespan ($t < 1.1$; $p > 0.794$; Fig.2). We found that simple expositions of bees during the adult stage (L_A , P_A and T_A). had similar lifespan than Bees C_A ($t < 1.18$; $p > 0.0138$; Fig.2). On the other hand, bees coexposed during adult stage to $L+P_A$ had a shorter LSP by 28.3 % than the bees C_A ($t = 1.34$; $p < 0.047$; Fig.2), but not than bees L_A and P_A ($t < 1.34$; $p < 0.99$; Fig.2). We showed that co-exposure did not result in any significant difference in the LSP of the Bees L_L , T_L and $L+P_L$ ($t > 0.951$; $p > 0.980$; Fig.2). We found that bees coexposed on adult stage to $L+T_A$ had a shorter LSP by 62.00 % than the bees C_A . We found that bees coexposed during adult stage to $L+T_A$ had a shorter LSP by 28.3 % than the bees C_A ($t = 2.631$; p

< 0.001 ; Fig.2 On the other hand bees co-exposure showed a strong synergistic effect compared to single exposures on longevity, increasing significantly the effect of lambda-cyhalothrin and tebuconazole by 2.44 and 2.23 respectively ($t > 2.23$; $p > 0.001$; Fig.2).

Co-exposure to pesticides reduces the first activities of bees

In Session 2, the stage of exposure had an influence on AFE and L-AFE GLMMs ($\chi^2 > 10.56$; $df = 1$; $p < 0.001$; Tab.1). Pesticide exposure modality has a significant effect on GLMM L-AFE ($\chi^2 > 8.86$; $df = 3$; $p = 0.031$; Tab.1). We also report an interaction between exposure stage and exposure modalities in the AFE and L-AFE GLMMs ($\chi^2 = 10.70$; $df = 3$; $p < 0.005$; Tab.1).

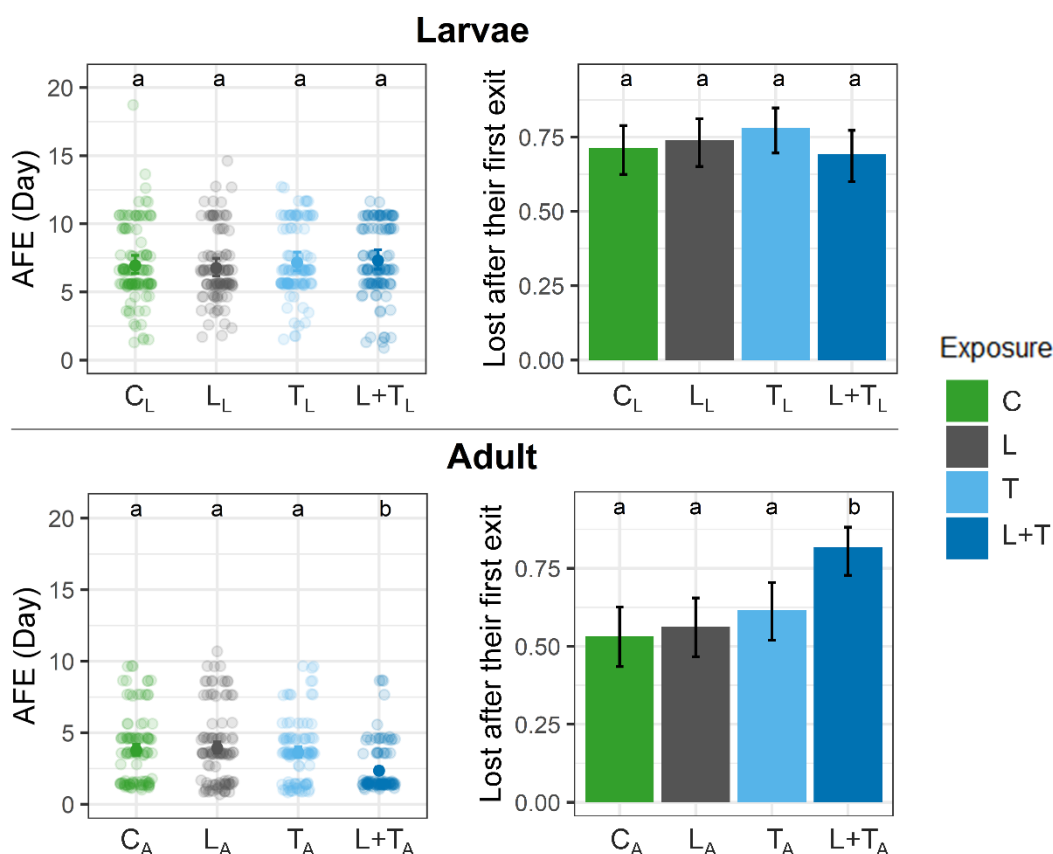


Figure 3. Effect of co-exposure to pesticides on AFE and L-AFE of adult bees on session 2. Adult bees were exposed on the larvae stage (Chronic) or at adult stage (48h). The colors show that the bees were exposed to Control (C), lambda-cyhalothrin (L), tebuconazole (T) and lambda-cyhalothrin + tebuconazole (L+T). Transparent dots represent the raw data. Larger dots or bars represent model estimates $\pm$ Confidence

intervals of the models (95%). Significant differences according to Tukey's pairwise comparisons are indicated with letters for each session.

We observed that bees exposed at the larval stage (C_L , L_L , T_L and $L+T_L$) had similar AFE and L-AFE (AFE : $t < 0.01$; $p > 0.674$; L-AFE : $t < 1.59$; $p > 0.438$; Fig.3). We also found that simple expositions during the adult stage (L_A , P_A and T_A). had a similar AFE and L-AFE (AFE : $t < 1.18$; $p > 0.138$; L-AFE : $t < 0.71$; $p > 0.612$; FigA .2). We observed that bees coexposed during adult stage to $L+T_A$ had a earlier AFE by 1.47 days and greater L-AFE of 28.55 % than bees C_A ($t > 0.254$; $p < 0.001$; Fig.3). On the other hand, we showed that coexposure of bees had a strong synergistic effect compared to single exposures on AFE, significantly increasing the effect of lambda-cyhalothrin and tebuconazole by 1.67 and 1.54 respectively ($t > 0.254$; $p < 0.001$; Fig.3). This co-exposure also increased the effects of lambda-cyhalothrin by 1.45 and tebuconazole by 1.34 on the L-AFE of bees exposed to the adult stage. ($t < 0.35$; $p < 0.0106$; Fig.3).

Co-exposure to pesticides reduces bee flight performance.

In session 1, we found a significant effect of the stage of exposure on FI GLMMs (Larvae : 22.11 ± 2.46 trips, Adult : 35.22 ± 2.05 trips ; $\chi^2 > 13.52$; $df = 1$; $p > 0,002$; Tab.1). In addition, we found a significant effect of our pesticide exposure modality on FI and FT GLMMs ($\chi^2 > 8.06$; $df = 3$; $p < 0.045$; Tab.1).

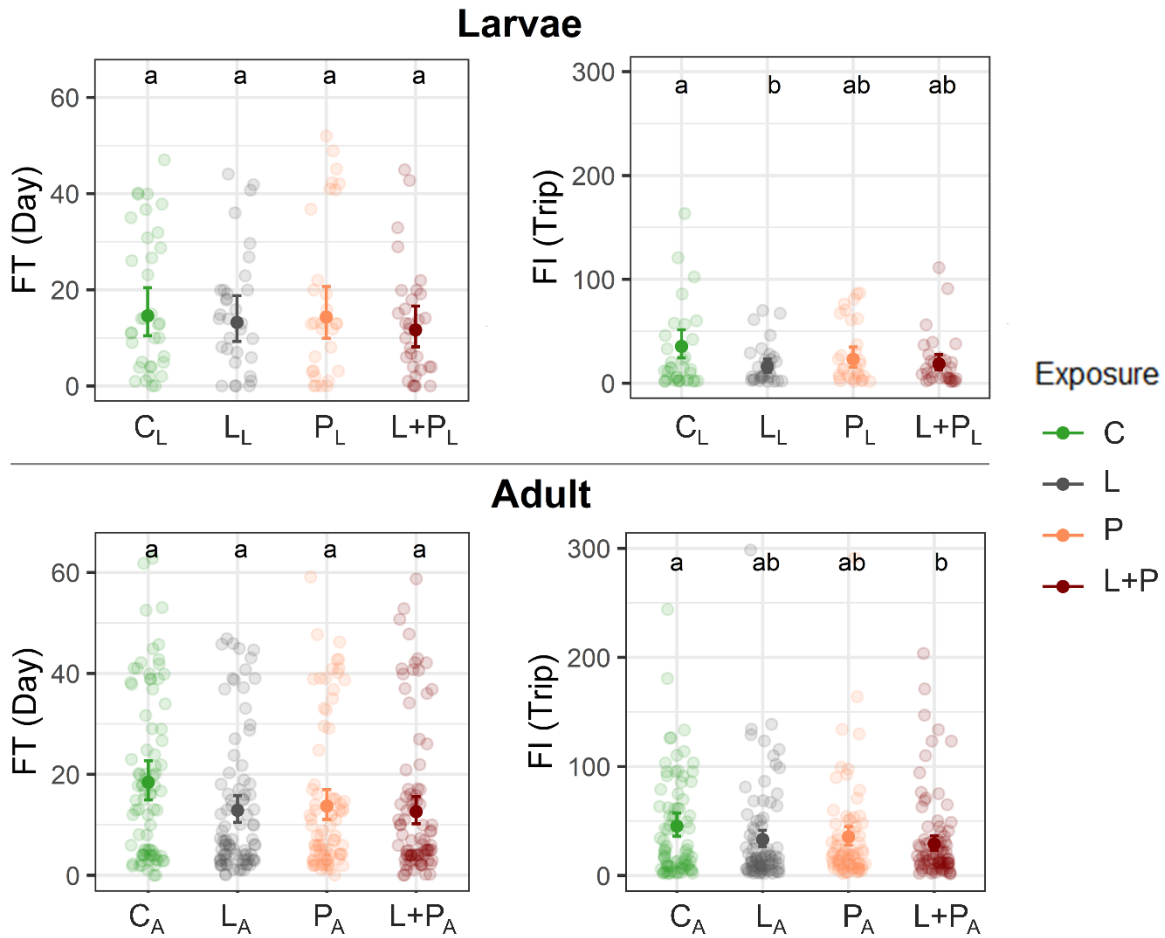


Figure 4. Effect of co-exposure to pesticides on FT and FI of adult bees in session 1. Adult bees were exposed to pesticides on the larval stage (Chronic) or at adult stage (48h). The colors show that the bees were exposed to Control (C), lambda-cyhalothrin (L), prothioconazole (P), lambda-cyhalothrin + prothioconazole (L+P). Transparent dots represent the raw data. Larger dots or bar represent model estimates $\pm$ Confidence intervals of the models (95 %). Significant differences according to Tukey's pairwise comparisons are indicated with letters for each session.

We showed that bees exposed at the larval stage (C_L , L_L , P_L and $L+P_L$) and adult stage (C_A , L_A , P_A and $L+P_A$) had similar FT (Larval stage : $t > 1.26$; $p > 0.782$; adult stage : $t < 1.46$; $p > 0.060$; Fig.4). We observed that bees exposed during the larval stage L_L had significantly lower FI by 44.49% than bees C_L ($t = 2.25$; $p = 0.012$; Fig. 4). We, found bees that coexposed during adult stage ($L+P_A$) had significantly lower FI by 36.33% than bees C_A ($t = 1.57$; $p = 0.03$; Fig. 3). We notified those bees exposed during adult stage (L_A , P_A and $L+P_A$) had similar FI ($t < 1.22$; $p > 0.62$; Fig. 3)

Impact of pesticide co-exposure on colony dynamics and survival

Sensitivity analysis showed that simulating shorter-lived bees (i.e. the relative effect of pesticide co-exposure) reduced colony survival (GLM with binomial error distribution : $Z = -5.397$, $p < 0.001$) and that the observed maximum effect of pesticide co-exposure on individual bees (i.e. an increase coefficient of $\times 2.63$) led to a predicted risk of colony mortality of 27% (95% CI : 16-42%, Figure 5, Figure S1). We observed that most of the colony collapses occurred during the first season (day 76 to day 122) both in Sensitivity analysis simulations (Figure S1) and experiment simulations (Figure S2).

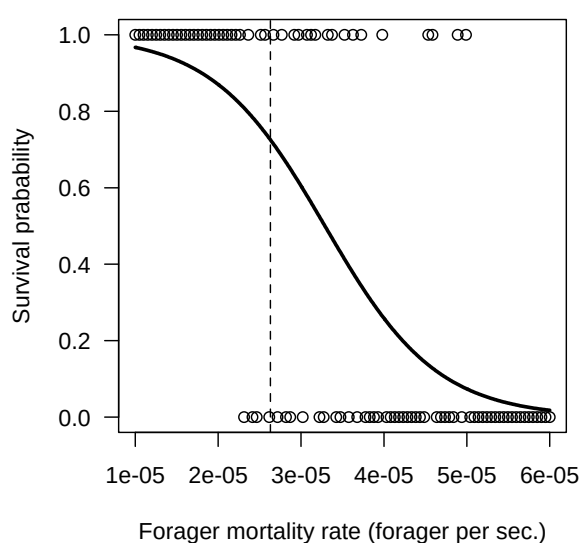


Figure 5. Simulated effect of shorter-lived bees on honeybee colony survival based on the sensitivity analysis ($n = 100$ simulations). The full line represents the binomial GLM prediction. The vertical dotted line represents the observed maximum effect of pesticide co-exposition on individual bees (i.e. an increase coefficient of $\times 2.63$).

In addition to the effect on colony survival, we found that simulating shorter-lived bees (i.e. the relative effect of pesticide co-exposure) reduced the population size of the simulated colonies and/or their honey production in all the six seasonal scenarios of exposure, in direct time and/or with delayed effects with +1 month, +2 months and +3 months after the end of the simulated impact (Figure 6, Figures S3-S7). The most alarming scenario (i.e. exposure during seasons 1+2+3) showed that simulating shorter-lived bees reduced the population size of the simulated colonies by 34.4% ($t = 3.792$, $p < 0.001$, Figure 6A) and their honey production by 53.8 % ($t = -4.445$;

$p < 0.001$, Figure 6B) during this exposure period. We found no significant interactions between the level of pesticide co-exposure impact and the demographic strength of the colony, showing that this pesticide co-exposure impact on population size and honey production is independent on the demographic strength of the colony. We found that these impacts of pesticide co-exposure on colony dynamics can be observed up to two months after the end of the simulated impact on population size (+1 month : $t = -3.463$, $p < 0.001$; +2 months : $t = -2.466$, $p = 0.016$; +3 months : $t = -1.634$, $p = 0.106$, Figure 6A), and up to three months after the end of the simulated impact on honey production (+1 month : $t = -5.065$, $p < 0.001$; +2 months : $t = -4.455$, $p < 0.001$; +3 months : $t = -4.504$, $p < 0.001$, Figure 6B). Very similar patterns were observed for the other seasonal scenarios of exposure (see Figures S3-S7).

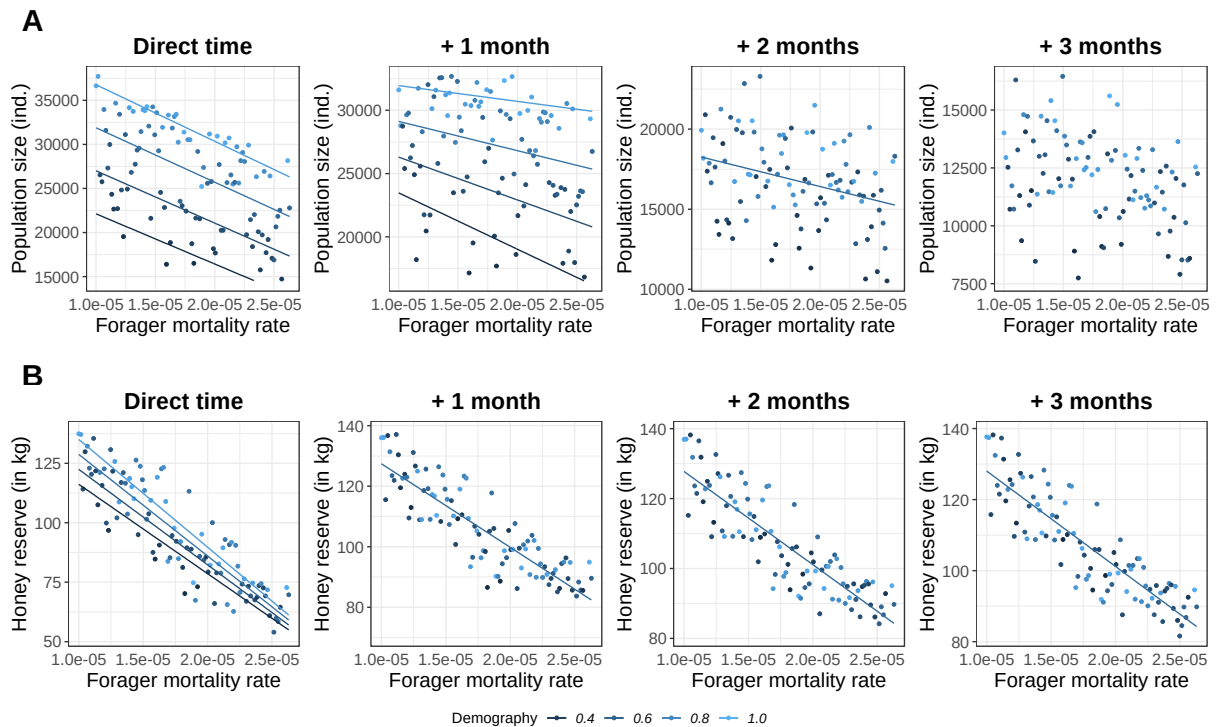


Figure 6. Simulated effect of shorter-lived bees (the relative effect of pesticide co-exposure) from day 76 to day 214 (seasons 1+2+3) on colony dynamics. (A) Changes in population size were analyzed along a gradient of relative effect of pesticide co-exposure simulating shorter-lived bees from day 76 to day 214 (i.e. seasons 1+2+3). We analyzed the direct time effect of simulating shorter-lived bees on changes in population size, as well as delayed effects with +1 month, +2 months and +3 months after the end of the simulated impact. (B) Changes in honey reserves were also analyzed along a gradient of relative effect of pesticide co-exposure simulating shorter-

lived bees from day 76 to day 214 (i.e. seasons 1+2+3). We've analysed the direct time effect of simulating shorter-lived bees on changes in honey reserves, as well as delayed effects with +1 month, +2 months and +3 months after the end of the simulated impact.

DISCUSSION

During their lifespan, honeybees may be continuously exposed to cocktails of pesticides (Barascou et al., 2021 ; Chauzat et al., 2006). Nevertheless, the effects of different categories of pesticides on bees are still poorly understood. (Schuhmann et al., 2022). Ecotoxicological toxicity tests are for the most part performed under laboratory cage conditions, which can bias the results by not considering social interactions within the colony, or external environmental influences (OCDE, 1998a, 1998b, 2013, 2017). Moreover, these tests are generally short and do not necessarily demonstrate long-term effects on individuals (Simon-Delso et al., 2017). In this study, we've adopted a realistic field approach to assess the effects of an insecticide in combination with fungicides on larvae and adult bees. We observed gene expression, survival and life-history traits on an individual scale, and based on these differences, simulated colony dynamics and survival.

The physiology and life-history traits of bees exposed during the larval or adult stage were not altered by exposure to tebuconazole and prothioconazole alone. These molecules are among the most widely used fungicides in France and in the world, but information about their presence in hive matrices is low (ANSES, 2023). However, bees are well exposed to these products, as they have been detected in pollen, nectar and bee bread (Roszko et al., 2016 ; Zioga et al., 2023). For these products, we've decided to use low doses that do not affect larval, pupal and bee survival (Wood et al, 2020). This dose comes from a study that looked at the maximum dose present in pollen in Poland (Roszko et al. 2016). We therefore decided to expose larvae and bees to 50 ng/individual of prothioconazole and tebuconazole to compare their sensitivity. Especially, only higher doses of tebuconazole than used here disrupted genes associated with immune response, oxidative stress, and detoxification. (Cang et al.,

2023). Its direct impact has also been reported on the action of enzymes involved in ion transport and pH regulation. (Soydan et al., 2017).

Lambda-cyhalothrin reduced larval survival in the laboratory and the FI by 55.92 % of these individuals to adult stage in the hive, but only during one session. The effects of different pesticides on bee activity have already been reported and would be caused by different colony dynamics and resource availability (Desclos le Peley , in prep ; Drummond et al. 2018 ; Requier et al. 2015). A wide range of doses of lambda-cyhalothrin are found in pollen collected by bees, some with potentially deleterious effects on individuals. (Böhme et al., 2018 ; Liao et al., 2018 ; Sanchez-Bayo & Goka, 2014 ; Tong et al., 2018 ; Xiao et al., 2022). High doses of lambda-cyhalothrin have disrupted genes associated with detoxification and the nervous system (Cang et al., 2023 ; Liao et al., 2018 ; Piechowicz et al., 2021). This disruption to the nervous system will have a direct impact on cognition, memory, social interaction and the orientation of bees (Decourtye et al., 2005 ; Ingram et al., 2015 ; Liao et al., 2018). The dose we decided to use, 6 ng/individual, corresponds to 1/40 of the LD₅₀ for adults. (241 ng/bee ; Decourtye et al., 2005) and 1/140 of the LD₅₀ for larvae (830 ng/larvae ; Waseem et Thakur 2023). At this range of concentrations, cytotoxic effects were observed on cells of the midgut, hypopharynx, glands and brain of honeybee workers (Arthidoro de Castro et al., 2020).

Lambda-cyhalothrin and prothioconazole are synergistic and reduce the LSP and FI of exposed adult bees by 29.00 % and 36.35 % respectively. This overall reduction in the capacity of exposed adult bees could be linked to the overexpression of the immune response gene (apidecin). In vitro larvae exposed to lambda-cyhalothrin and prothioconazole overexpressed development-related genes (hexamerin), but we did not measure any delayed impact on their life-history traits within the colony. These results confirm the synergistic effects of prothioconazole with insecticides on the bees' immune system, which had already been observed with flupyradifurone (Neonicotinoid). (Haas et al., 2021). Lambda-cyhalothrin and tebuconazole synergized and decreased LSP by 61.00 % of exposed adult bees. This reduced longevity on individual is directly associated to 1.47 day reduction of AFE and increase L-AFE by 28.55 %. Co-exposed in vitro larvae overexpressed development-related genes (vitellogenin). Tebuconazole interacted with several insecticides, resulting in

greater sublethal effects. In combination with tetrachlorantraniliprole produced acute synergistic toxicity on the expression of genes associated with immune response, oxidative stress and detoxification metabolism in honeybees (Cang et al., 2023). Combined with thiaclopirde or thiamethoxam and the pathogen *M. plutonius* synergistically increases bee and larval mortality in the laboratory. (Schmuck et al., 2003 ; Wood et al., 2020). This fungicide in cocktail increases the mass of larvae and disturbs the development of hypopharyngeal glands (Böhme et al, 2017). Synergies between certain triazoles and neurotoxic insecticides (e.g. pyrethroids and Neonicotinoids) are increasingly recognized, but this had not been noted on pesticides of this study and at such low doses (Mackei et al., 2023 ; Pilling et al., 1995 ; Schmuck et al., 2003 ; Wood et al., 2020). Some regulatory agencies regulate concomitant use, but this will not prevent these molecules from being present at the same time across the landscape. (ANSES, 2018).

Simulation of the risk of co-exposure to lambda-cyhalothrin and tebuconazole (reducing the lifespan of bees) led to the mortality of 27% of colonies, and most of these collapses occurred during the re-growth period. The early collapse of colonies suggests a heightened sensitivity of bee colonies at this season. This has already been observed in simulations of the risk effect induced by fungicide boscalid, but the product exclusively reduced honey production and population size (Desclos Le Peley, *in prep*). Simulation of the risk of co-exposure to lambda-cyhalothrin and tebuconazole over the entire growth period (the extreme scenario) reduced the population of simulated colonies by 34.4 % and their honey production by 53.8% during this exposure period. In addition, simulated and co-exposed colonies saw their population size and honey production significantly decrease over 2 and 3 months respectively. In addition, very similar trends were observed for the other exposure period scenarios. The colony losses and population declines are well above the 10% GSP set by EFSA (EFSA et al. 2023). Thus, bee colonies coexposed to lambda-cyhalothrin and tebuconazole during the growing period between March and June could be impacted by a higher mortality rate or a reduction in population size and honey reserves. In addition, the reduction in honey production would diminish one of the major sources of income for beekeepers (Wilson-Rich et al., 2014). It can also lead to a lack of available resources during periods of summer or winter dearth, which could compromise the survival of colonies (Free & Racey, 1968 ; Requier et al., 2015). Finally, declining populations within

colonies could have a disruptive effect on their ability to respond to other biotic or abiotic stresses, as well as on their reproduction, competitiveness and survival during the winter season (Free & Racey, 1968 ; Lee & Winston, 1987 ; Locke & Fries, 2011).

This period of exposure coincides with bees foraging for plants in the Acer genus, the Rosaceae family, the Brassicaceae family Salix spp Papaver spp, and Helianthus annuus (Bilisik et al. 2008). These two products are used in orchards and field crops (i.e. rapeseed, hazelnut, walnut, plum, cherry, cabbage, peach, apricot, etc. ; ANSES 2023). It is therefore plausible that the use of lambda-chalothrin, prothioconazole and tebuconazole may be found together in fields and exposed to honeybees and other pollinators.

Differences in all adult bees' life history traits in the hive and during rearing in the laboratory were observed between bees from different hives. The experiments were carried out on *A.mellifera mellifera* × *caucasica* hybrids. The common parentage of laying queens was established, but without any specific control of the genetics of every individual. The genetic diversity of populations can influence their ability to adapt to biotic or abiotic stresses (Laurino et al., 2013 ; Mattila & Seeley, 2007 ; Rinkevich et al., 2015). This may explain the significant differences observed in our results.

CONCLUSION

Our results have shown that co-exposures between these insecticides and fungicides altered the expression of certain genes, especially those implicated in development or the immune response in larvae or adults. These co-exposures will induce short-lived bees, and impact early flight activity or overall flight capacity, according to these combinations. Finally, this work offers a new perspective by providing solid results on the interactive effects of the pesticides lambda-cyhalothrin and triazole on bee longevity and flight performance. This transposition on colony simulation induced colony death or weakening of population size and honey production weakening and colony death of honeybee colonies. These results support the hypothesis that coexposure lambda-cyhalothrin and prothioconazole would induce deleterious effects on colonies in realistic field scenarios.

ACKNOWLEDGMENTS

The authors would like to thank, Carole Moreau-Vauzelle, Daniel Raboteau, Stéphane Grateau, Elisa Gomes Tiffany Laverre, Barberine Grospiron, Colombe Chevallereau, Thierry Tamic and Fabien Dequine for their contribution to the implementation of the experiment and to the logistics.

FUNDING

Research reported in this publication was supported by OFB as part of the Ecophyto II+ call on « Phytopharmaceuticals products : from exposure to impacts on human health and ecosystems » launched by the French Ministry in charge of Ecology, Agriculture, Health and Research for the project EXPLORA granted to F.-J. Richard (2021–2024).

AUTHORS' CONTRIBUTIONS

Victor Desclos Le Peley : conceptualization ; data curation ; methodology ; formal analysis ; investigation ; visualization ; writing – original draft.

Fabrice Requier : conceptualization ; supervision ; investigation ; methodology ; formal analysis ; investigation ; visualization ; methodology ; writing – original draft.

Pierrick Aupinel : conceptualization ; funding acquisition ; supervision ; writing – review & editing.

Freddie-Jeanne Richard : conceptualization ; funding acquisition ; investigation ; supervision ; methodology ; project administration ; writing – review & editing.

DISCUSSIONS ET PERSPECTIVES

Discussion générale

Pour mieux comprendre les enjeux de ces travaux, il est important de les recontextualiser. L'utilisation de pesticides est souvent jugée nécessaire par l'agriculture conventionnelle, car l'agriculture biologique ne peut répondre seule aux besoins actuels de la population. Toutefois, son usage excessif est préoccupant. En effet, la contamination des milieux (eau, sol, air) par les pesticides est un problème majeur qui inquiète la population soucieuse de l'usage de certaines substances et de leurs conséquences sur la santé, l'environnement et la biodiversité. L'ANSES émet régulièrement de nouvelles recommandations visant à renforcer le cadre réglementaire au niveau de l'usage des pesticides et à réduire encore l'exposition des abeilles aux produits phytopharmaceutiques (ANSES, 2018). La mise en œuvre d'une stratégie de surveillance des abeilles mellifères tout au long de leur vie et d'un contrôle précis du développement larvaire est une approche pertinente pour évaluer les effets des facteurs biotique et abiotique. Cette approche permet de combiner les analyses physiologiques et comportementales à l'échelle individuelle, avec les simulations de dynamiques des populations pour une vision globale sur les colonies d'abeilles. Ainsi, nous avons pu évaluer les effets d'un fongicide lors d'expositions précoces et récurrentes, et les interactions entre insecticides, fongicides et pathogènes lors du développement des abeilles.

Mise en place d'une stratégie de surveillance globale des abeilles mellifères

L'élaboration de cette méthodologie présente plusieurs défis importants.

Premièrement, l'élevage *in vitro* des abeilles au stade larvaire modifie significativement toutes les activités au sein de la colonie lorsque les abeilles sont devenues adultes. Les abeilles subissent des déficits nutritionnels lors de leur développement qui réduisent leurs capacités (Schilcher et al., 2022). Cela peut également provenir de la divergence de microbiote des abeilles élevées *in vitro*, mais ces différences sont ensuite inversées par le transfert individuel après l'émergence, grâce à leurs interactions sociales lors de leur réintroduction dans la ruche (Kowallik & Mikheyev, 2021). L'élevage larvaire *in vitro* influence la morphologie des abeilles adultes (Scofield & Mattila, 2015), entraînant des variations dans la longueur et la largeur des ailes

antérieures, du poids corporel sec et de la taille des glandes hypopharyngiennes (Mortensen et al., 2019). Ce protocole en laboratoire prive les larves d'interactions avec les nourrices et affecte négativement les apprentissages comportementaux et sociaux qui interviennent dans la colonie (Scofield & Mattila, 2015). Cependant, les abeilles élevées au stade larvaire *in vitro* adhèrent aux différentes tâches de la colonie et s'occupent de la reine, de la collecte de pollen, participent aux danses et à la défense contre les parasites (Mortensen & Ellis, 2018). Néanmoins, plusieurs traits comportementaux sont affectés négativement par l'élevage *in vitro*, par exemple la capacité de butinage des abeilles (Schilcher et al., 2022). Nos résultats montrent un âge de première sortie plus tardif, une diminution des capacités de vol et de butinage ainsi qu'une réduction globale de la longévité des individus élevés *in vitro*. Les tests en laboratoires, du fait de leur réalisation en espace très contrôlé, vont impliquer de nombreux compromis chez les espèces modèles et vont modifier différents traits biologiques (Avarguès-Weber & Mota, 2016 ; Kimball & Levin, 1985 ; Van Oystaeyen et al., 2021). Ainsi, la mise en place des protocoles d'élevage larvaire *in vitro* pourrait modifier les capacités des abeilles à long terme face à des stress biotique ou biotique.

Afin de limiter les effets indésirables des produits inertes lors des tests écotoxicologiques, nous recommandons à la suite de nos expérimentations l'utilisation d'une concentration de 0.1 % d'acétone pour solubiliser les molécules actives et intoxiquer les larves via leur alimentation. À cette concentration, nous n'avons pas observé d'effet sur le développement ou le comportement des individus devenus adultes au sein de la colonie. En revanche, nous avons observé une modification à long terme des traits d'histoire de vie des jeunes adultes ayant suivi le protocole d'exposition avec l'acétone à 0.1 % et ces effets varient également au cours de la saison (Danner et al., 2016 ; Fisher et al., 2022). L'utilisation des ingrédients inertes est recommandée de façon empirique entre 1-5 % dans le volume total de la solution d'exposition par les protocoles OCDE (OCDE, 1998a, 1998b, 2017a). Néanmoins, en fonction des paramètres chez les abeilles, ces concentrations de solvant induisent des effets sublétaux (Desclos le Peley et al. 2024 ; Quinn et al 2018 ; Salles Cruz-Landim 2004). Il est ainsi nécessaire de caractériser les compromis que les protocoles écotoxicologiques vont impliquer sur la biologie des organismes modèles.

Évaluer les effets d'un fongicide sur l'abeille mellifère

Nous avons évalué les effets d'un fongicide, le boscalid, lors d'expositions précoces sur différents traits d'histoire de vie de l'abeille mellifère. Nos résultats mettent en évidence des effets sublétaux significatifs à différentes échelles biologiques, observés à une dose de boscalid présent dans l'environnement. La dose de 1496,4 ng/g correspond à la dose la plus élevée de boscalid à laquelle sont exposés les individus par les pollens dans un paysage agricole européen (Böhme et al. 2018, Böhme et al. 2019). Cette dose varie considérablement dans le reste du monde, par exemple, en Californie, la dose maximale détectée est de 6060 ng/g (DeGrandi-Hoffman et al 2017 ; Fisher et al 2022) ou au Canada de 2200 ng/g (Tsvetkov et al., 2017). Elle est également plus faible que celle retrouvée directement dans le pollen des plantes juste après épandage (2050 – 6890 ng/g) lors des tests complémentaires de mise sur le marché (BASF, 2016). Au vu de ces informations, nous avons utilisé des doses élevées, mais réalistes, de pesticides auxquelles les individus peuvent être exposés dans leur environnement, en ne considérant que l'exposition par ingestion du pollen. Néanmoins, d'autres voies d'exposition sont envisageables : le contact direct des larves avec des cires contaminées ou l'ingestion de nectar contaminé (Friedle et al 2021 ; Xiao et al 2022). De plus, les doses que nous avons employées étaient inférieures ou égales à toutes celles présentées dans la littérature pour étudier les effets sublétaux.

Nous montrons que l'exposition larvaire au boscalid modifie la physiologie des abeilles à long terme, notamment l'expression du gène de la vitellogénine. Une étude menée en parallèle révèle, à une concentration similaire, une perturbation des gènes du développement et de la physiologie des reines, mais ce gène était sous exprimé (Pineaux et al., 2023). Ces différences peuvent s'expliquer par le fait que la vitellogénine influe sur de multiples aspects de l'histoire de vie de l'abeille mellifère, et notamment elle est impliquée dans l'ovogenèse chez les reines (Corona et al., 2007). Chez les abeilles domestiques, les ouvrières ne sont pas sexuellement développées, et la vitellogénine influence le comportement, la résistance au stress, et serait impliquée dans l'organisation sociale de la colonie (Amdam et al., 2012). Nous n'observons pas d'augmentation de la mortalité des larves à la suite de cette exposition, mais elle va retarder légèrement l'émergence des individus au laboratoire. Dans les expérimentations de coexposition des larves, nous montrons également qu'à

0.08 ppm, le boscalid seul va diminuer le taux de larves atteignant la nymphose à J13, et ce résultat est cohérent avec une étude précédente (Simon-Delso et al., 2017). Dans le même domaine, plusieurs études ont rapporté des conséquences négatives des fongicides sur le développement du couvain des abeilles. Des études ont démontré que le décalage de la date d'émergence des abeilles va modifier leurs activités, induire une adhésion plus tardive au rôle de nourricière et, à long terme, impacter la dynamique de la colonie (Becher et al., 2014 ; Russell et al., 2013 ; Traynor et al., 2017). Des études menées aux Etats-Unis ont montré que le boscalid à une concentration terrain (deux fois plus élevée que celles utilisées lors des expérimentations de cette thèse), modifie la morphologie en diminuant la taille du thorax ; le métabolisme, en diminuant la respiration mitochondriale des individus et la cognition en diminuant le réflexe du PER (Campbell et al., 2016 ; DesJardins et al., 2021 ; Fisher et al., 2021 ; Glass et al., 2021).

Ces observations ont été confirmées dans nos études : l'exposition larvaire et des jeunes adultes diminue la longévité, l'âge de première sortie et une très forte proportion d'abeilles ne revient pas après leur première sortie. La diminution globale de la longévité des abeilles au sein des ruches peut provenir des effets induits par le boscalid sur la physiologie, ainsi que nous l'avons cité précédemment. Les effets délétères du boscalid sur les premières activités de vol peuvent provenir de la diminution des capacités cognitives et d'orientation et du fait que les abeilles exposées se soient perdues lors de leur trajet de retour (DesJardins et al., 2021 ; Henry et al., 2012 ; Liao et al., 2019). Il est également possible que les individus exposés aient pu être exclus par leurs partenaires dans le cadre de l'immunité sociale de la colonie (Baracchi et al., 2012 ; Coulon et al., 2020) ou qu'ils se soient retirés de manière altruiste de la ruche (Rueppell et al., 2016). Ces phénomènes sont généralement induits par la présence de stress biotique sur les colonies. Enfin, il est possible que ces abeilles soient simplement mortes au sein de la colonie

Nous montrons également que la simulation du risque relatif au boscalid (abeilles avec une longévité réduite) est néfaste sur les colonies lors de l'application de ce stress pendant la période pré-développement. Ainsi, à la fin de la période de stress nous observons une diminution de 18 % de la taille de la population et également des ressources en miel. De plus, nous avons constaté que ces effets délétères du boscalid sur la dynamique de la colonie peuvent être observés jusqu'à deux mois après la fin

de l'impact simulé. Il est intéressant de noter que les colonies démographiquement plus fortes sont généralement plus affectées par cet impact du boscalid. Cette diminution de population est au-dessus de la valeur critique des objectifs de protection spécifiques fixés par l'EFSA (SPG : 10 %). Ces résultats sont confirmés par plusieurs études de terrain qui montrent une diminution des populations au sein des ruches et une augmentation du stockage du pollen directement sur le terrain (Fisher et al., 2021 ; Fisher et al., 2022).

D'autres simulations du risque induit par le boscalid à différentes périodes du développement de la colonie nous montrent une sensibilité accrue lors de la phase de pré-développement. Cette période correspond à la reprise d'activité, et donc il est envisageable que l'utilisation du boscalid à des conditions de terrain au printemps ait un effet négatif sur la croissance des colonies. Pour aller plus loin, la phénologie des abeilles varie en fonction des ressources florales qui sont à sa disposition au cours de la saison (Requier et al., 2015 ; Wood et al., 2020). Ainsi, cette période va correspondre à la période de recrutement du pollen des plantes appartenant au genre *Acer*, à la famille des Rosacées, par les butineuses (Bilisik et al., 2008). Le boscalid est utilisé en arboriculture (i.e. cerisier, pommier, poirier, cognassier, abricotier ; ANSES, 2023). Il est alors envisageable que l'application du boscalid en arboriculture lors de la floraison joue un rôle significatif sur l'affaiblissement des colonies dans les vergers.

Nous n'observons pas d'effondrement des colonies lors de nos simulations, mais cette diminution des populations au sein des colonies pourrait avoir un effet néfaste sur leur capacité à faire face à d'autres stress biotique ou abiotique, ainsi que sur leur reproduction, leur compétitivité et leur longévité notamment pendant la saison hivernale (Free & Racey, 1968 ; Harbo, 1983 ; Lee & Winston, 1987 ; Locke & Fries, 2011 ; Smith et al., 2017). De plus cette diminution du stock de miel peut conduire à un manque de ressources pendant les périodes de disette et en hiver, ce qui compromettrait la survie des colonies (Free & Racey, 1968 ; Requier et al., 2015). De plus la production et la commercialisation du miel étant l'une des sources principales de revenus des apiculteurs, son affaiblissement mettrait en péril de petites installations apicoles et à plus large échelle affaiblir l'industrie autour de l'abeille (Carreck & Williams, 1998).

Pour conclure, nos résultats et d'autres études mettent en évidence l'incapacité des tests écotoxicologiques traditionnels en laboratoire à détecter les effets du boscalid sur les abeilles (Simon-Delso et al., 2017). Bien que peu d'informations soient disponibles les concernant, d'autres pollinisateurs sont également exposés et affectés par le boscalid (i.e. *Bombus sp.* et *Osmia lignaria* ; Benner et al., 2023 ; Kopit et al., 2022 ; Kuivila et al., 2021). Ainsi, le boscalid, à des doses réalistes de terrain, contribue à l'affaiblissement des capacités individuelles des abeilles et pourrait induire un risque sur les colonies. À plus large échelle, le boscalid pourrait impacter les populations des pollinisateurs et compromettre le service de pollinisation essentiel à la survie de nombreuses cultures et espèces végétales sauvages (Biesmeijer et al., 2006 ; Calderone, 2012). De fait, le boscalid pourrait entraîner des conséquences sur la sécurité alimentaire et sur la santé des écosystèmes (Catarino et al., 2019 ; Gallai et al., 2009). Il est donc essentiel d'élaborer des tests écotoxicologiques spécifiques et des stratégies de gestion des pesticides plus durables, qui préservent la santé des pollinisateurs et la biodiversité dans son ensemble.

Évaluer les interactions entre insecticides, fongicides et pathogènes sur l'abeille mellifère.

Les abeilles mellifères sont continuellement exposées à des cocktails de pesticides au cours de leur vie (Chauzat et al., 2006 ; Daniele et al., 2018). Néanmoins, les connaissances relatives aux effets entre combinaisons de pesticides sont encore lacunaires sur les abeilles (Barascou et al., 2021). Nous avons évalué les effets d'un insecticide, la lambda-cyhalothrine, en interaction avec les fongicides tébuconazole ou prothioconazole sur les abeilles après une exposition adulte ou larvaire. La dose de lambda-cyhalothrine à laquelle nous avons exposé les larves et les adultes est de 6 ng/individu et correspond à 1/140 de la DL50 des larves (830 ng/larve ; Waseem & Thakur, 2023) et à 1/40 de la DL50 des adultes (241 ng/abeille ; Decourtye et al., 2005). Cette dose a été sélectionnée car la littérature ne rapporte aucun effet sur la cognition, l'orientation et la mémoire (Decourtye et al., 2005 ; Liao et al., 2018). Des effets cytotoxiques sur les cellules de l'intestin moyen, de l'hypopharynx, des glandes et du cerveau des ouvrières mellifères ont été observées à une dose de 3.72 ng/abeille (Arthidoro de Castro et al., 2020). Cette molécule à des doses équivalentes a une action peu claire sur la mobilité des abeilles (Ingram et al., 2015). La lambda-cyhalothrine a déjà été trouvée à une concentration maximum de 2206 ng/g dans les

pollens rapportés dans les ruches (Xiao et al., 2022). En cas d'ingestion de ces pollens contaminés par les jeunes abeilles, ces dernières seraient exposées à des doses plus élevées que celles que nous avons administrées à nos ouvrières. Pour les fongicides, le choix a été fait d'exposer les larves et les abeilles à 50 ng/individu de prothioconazole et de tébuconazole. Nous nous sommes basés sur cette dose qui provient d'une étude portant sur la dose maximale présente dans le pollen en Pologne et en Europe (Roszko et al., 2016). Cette dose a déjà été utilisée avec le prothioconazole et ne montre aucun effet sur la survie, le développement des glandes hypopharyngiennes et la consommation (Wood et al., 2020).

Nous observons dans notre étude, que les effets d'une exposition simple au tébuconazole et au prothioconazole n'affecte pas le développement des larves lors des élevages *in vitro*. De même, nous n'avons observé aucune altération de l'expression des gènes et des traits d'histoire de vie des individus adultes. Ces résultats concordent avec la littérature qui observe, seulement lors d'exposition des abeilles à de fortes doses de tébuconazole, une altération des gènes associés à la réponse immunitaire et au stress oxydatif de la détoxification (Cang et al., 2023).

Nos résultats indiquent que l'exposition simple à la lambda-cyhalothrine des jeunes adultes n'a pas altéré leur physiologie et leur cycle de vie au sein de la ruche. Cependant, cette exposition a réduit la survie des larves en laboratoire ainsi que leurs activités au sein des ruches, mais seulement pendant une session. Nous avons notamment observé une diminution de l'intensité de vol de ces individus au stade adulte dans la ruche. Nous n'avons pas identifié les voies métaboliques qui pourraient expliquer cette diminution des compétences de vol. Néanmoins, la lambda-cyhalothrine des doses élevées perturbe les gènes associés à la détoxification et au système nerveux (Cang et al., 2023 ; Liao et al., 2018 ; Piechowicz et al., 2021). Le fait d'observer des effets de cette molécule sur l'activité des abeilles seulement lors de sessions spécifiques a déjà été rapporté, les hypothèses sous-jacentes à cette interaction seraient liées à différentes dynamiques de colonies et à la disponibilité des ressources, à l'état sanitaire de la ruche et à la météo (Desclos le Peley et al, *in prep* ; Drummond 2022). Nous constatons une interaction lors d'une coexposition de la lambda-cyhalothrine et le prothioconazole sur les abeilles. Cette coexposition diminue la longévité et l'intensité de vol des abeilles au sein des ruches. Ces diminutions globales des capacités des abeilles adultes exposées pourraient être liées (i) à la

surexpression du gène de réponse immunitaire (apidecine). Cette surexpression du système immunitaire pourrait être directement liée à une interaction des molécules utilisées ici. La littérature reporte plutôt une sous-expression des capacités immunitaires lors de coexposition avec les triazoles (Bernabò et al., 2016 ; Cang et al., 2023). (ii) Une seconde hypothèse serait l'interaction concomitante avec un pathogène que nous n'avons pas mesuré ici. En effet, les triazoles sont connus pour perturber les membranes des cellules et peuvent entrer en interaction avec les effets cytotoxiques de la lambda-cyhalothrine sur les cellules de l'intestin, ce qui pourrait faciliter une infection par un pathogène (Arthidoro de Castro et al., 2020). Une telle interaction a été observée entre le pathogène *M. plutonius* et une coexposition avec un triazole et un pesticide neurotoxique sur les abeilles (Wood et al., 2020).

Nos expériences montrent une interaction lors d'une coexposition entre la lambda-cyhalothrine et le tébuconazole sur l'abeilles. La lambda-cyhalothrine et le tébuconazole ont agi en interaction et diminué la longévité des abeilles adultes exposées. Cette réduction de la longévité individuelle est directement associée à une réduction de l'âge de première sortie et à une augmentation très forte de la proportion d'abeilles qui ne sont jamais revenues dans la colonie après cette première sortie. Déjà citées ci-dessus pour le boscalid, plusieurs hypothèses sont sous-jacentes à cette sortie sans retour : (i) soit les abeilles exposées se sont perdues, (ii) soit les abeilles sont mortes dans ruche, (iii) soit les abeilles ont été exclues par les partenaires, (iv) soit les abeilles se sont retirées de manière altruiste de la ruche. Le tébuconazole interagit avec plusieurs insecticides et diminue la taille des glandes hypopharyngiennes (Böhme et al., 2017). Par ailleurs, il va avoir des effets sur la cognition, l'orientation et la mémoire en augmentant l'action des insecticides neurotoxiques, bien que cela n'ait pas été noté sur les pesticides de cette étude et à des doses aussi faibles (Mackei et al., 2023 ; Pilling et al., 1995 ; Schmuck et al., 2003 ; Wood et al., 2020)

Nous observons que les larves coexposées à la lambda-cyhalothrine et au tébuconazole *in vitro* ont surexprimé les gènes liés au développement (vitellogénine). Quant aux larves coexposées *in vitro* à la lambda-cyhalothrine et au prothioconazole, elles ont surexprimé des gènes liés au développement (hexamérine). Néanmoins, une fois les individus devenus adultes, nous n'avons pas constaté de différences significatives sur leurs traits d'histoire de vie au sein de la colonie. Il est possible que

(i) les perturbations de ces voies métaboliques soient seulement observables mais n'induisent pas de conséquences à long terme (Barchuk et al., 2002). Cette hypothèse est soutenue par le fait que leur retour à la ruche coïncide avec le moment où leur consommation de pollen est la plus importante et ce dernier stimule spécifiquement l'expression de ces gènes (Barascou et al., 2021). (ii) Une seconde hypothèse provient du fait que l'élevage *in vitro* des larves n'est pas sans conséquences sur le développement des abeilles adultes. Il est possible que ces compromis induits sur la biologie des individus ne permettent pas d'observer les effets synergiques de ces pesticides sur les larves (Desclos le Peley et al., 2024). Cette hypothèse est en partie soutenue par les résultats synergiques obtenus sur les adultes à des concentrations similaires, mais il faut prendre ces comparaisons avec prudence car les temps d'exposition, les aliments d'intoxication et le cycle biologique des individus divergent énormément.

La simulation de l'effet de la coexposition à la lambda-cyhalothrine et au tébuconazole (réduisant la durée de vie des abeilles) a conduit à la mortalité de 27 % des colonies et la plupart de ces effondrements se sont produits lors de la période de reprise de croissance. Le phénomène d'effondrement des colonies lors du début de la saison laisse supposer une sensibilité accrue des colonies d'abeilles à cette période. Cela a déjà été observé lors des simulations de l'effet de risque induit par le boscalid, mais celui-ci réduisait exclusivement la production de miel et la taille des populations.

La simulation de l'effet de la coexposition sur toute la période de croissance (le scénario extrême) a réduit la population des colonies simulées de 34,4 % et leur production de miel de 53,8 % pendant cette période d'exposition. De plus des tendances très similaires ont été observées pour les autres scénarios d'exposition. Ces pertes de colonies et les diminutions des populations sont bien supérieures au SPG de 10 % fixé par l'EFSA (EFSA et al. 2023). Ainsi la coexposition des colonies à la lambda-cyhalothrine et au tébuconazole pourrait induire des réductions de taille de population et des réserves en miel, peu importe le moment de l'exposition des ruches. Cette période d'exposition est concomitante à la récolte des plantes appartenant au genre *Acer*, à la famille des Rosacées, à la famille des Brassicaceae *Salix spp* *Papaver spp*, et *Helianthus annuus* par les butineuses (Bilisik et al., 2008). Ces deux produits sont notamment utilisés en vergers et en grandes cultures (i.e. colza, noisetier, noyer, prunier, cerisier, chou, pêcher, abricotier... ANSES 2023). Ainsi il est envisageable que

l'utilisation conjointe du tébuconazole et du prothioconazole en grandes cultures de colza et en arboricultures, lors de la floraison, joue un rôle significatif sur l'affaiblissement des colonies. Lors des simulations, nous avons constaté que les impacts de la coexposition aux pesticides sur la dynamique de la colonie simulée sont observés sur la taille de la population jusqu'à deux mois après la fin de l'impact simulé, et jusqu'à trois mois sur la production de miel. Les généralisations et les hypothèses émises sur les effets de la diminution de la dynamique des colonies pour les apiculteurs et les écosystèmes sont similaires à ceux cités pour le boscalid dans la partie précédente.

En plus d'être continuellement exposées à des cocktails de pesticides, les abeilles mellifères sont également soumises à un stress abiotique, en particulier pendant leur phase larvaire. (Brødsgaard et al., 1998 ; Chauzat et al., 2006 ; Daniele et al., 2018). Nous avons choisi étudier l'interaction entre le boscalid, le pyriproxifène et *Paenibacillus larvae*. Ces facteurs sont respectivement un fongicide, un insecticide et une bactérie. Les abeilles mellifères sont continuellement exposées à des cocktails de pesticides au cours de leur vie, mais également à des pathogènes (Chauzat et al., 2006 ; María de la Paz Moline, 2022). L'exposition à ces facteurs seuls entraînent des conséquences très diverses qui interrogent quant aux potentielles interactions qu'ils pourraient entraîner sur la biologie de l'abeille. Les doses retenues pour tester ces interactions entre ces facteurs correspondent à celles les plus élevées n'entraînant pas d'effet de mortalité lors du stade larvaire. Ainsi, les larves ont été exposées à 80 ppm de boscalid et/ou 0.08 ppm de pyriproxifène et/ou 2-3 spores de *P. larvae*. Les effets significatifs d'une exposition simple au boscalid sur le développement des larves est abordé au début de la discussion.

Nous avons observé que l'exposition des larves à 80 ppm de pyriproxifène entraîne une mortalité plus élevée, avec notamment un taux de nymphose et un taux d'émergence inférieurs à ceux du contrôle par solvant. Cet insecticide est considéré comme ayant une faible toxicité aiguë pour les adultes par voie orale et par contact (Devillers & Devillers, 2020). Les insecticides inhibiteurs de croissance perturbent le métamorphisme en altérant les processus de mue, bloquant ainsi le développement lors de phase larvaire chez les insectes comme les abeilles (Chen et al., 2016 ; Pinto et al., 2000). De plus, le pyriproxifène par application topique induit une nymphose plus courte, perturbe la mélanisation de la cuticule nymphale et retarde la

différenciation des muscles associés au vol (Bitondi et al., 1998 ; Fernandez et al., 2012 ; Zufelato et al., 2000). En outre, chez les jeunes abeilles, il va induire des perturbations métaboliques en inhibant un gène lié au développement (vitellogénine ; Pinto et al., 2000). Nous avons montré que les larves exposées à 2-3 spores de *P. larvae* avaient un taux de nymphose plus faible que les larves recevant le solvant contrôle, mais aucun impact n'a été mesuré sur la mortalité ou le taux d'émergence des larves. L'utilisation d'acétone peut augmenter la virulence de *P. larvae* en perturbant les cellules de l'intestin et en provoquant un stress oxydatif, mais cela reste partiellement représentatif en raison de la présence d'éléments inertes dans les formulations commerciales (Fine et al., 2017 ; Wernecke et al., 2022). Les différents génotypes de *P. larvae* induisent des variations de virulence au stade larvaire (Rauch et al., 2009).

Nous observons un effet d'interaction pour la bi-exposition au boscalid et *P. larvae* sur les larves avec notamment un effet négatif sur la survie, le taux de nymphose et le taux d'émergence, alors que les expositions simples ne sont pas significatives. De plus, nous observons que les larves biexposées au pyriproxifène et *P. larvae* ainsi que la tri-exposition (boscalid pyriproxifène et *P. larvae*) a un effet délétère sur la survie, le taux de nymphose et le taux d'émergence, mais ce dernier n'est pas différent de l'exposition simple au pyriproxifène. Nous avons précédemment démontré que le boscalid perturbe l'expression des gènes. Nous pouvons donc émettre l'hypothèse que le boscalid va perturber la vitellogénine et faciliter l'action de *P. larvae* à produire une septicémie chez les larves (Wood et al., 2020). Également, il est possible que ce soit l'effet cytotoxique causé par la bactérie qui augmente l'effet délétère du boscalid (María de la Paz Moline, 2022). Il semblerait que la première hypothèse soit la plus pertinente car, chez les adultes, des pesticides affectent une enzyme liée au système de l'immunité en facilitant la transmission de *P. larvae*, en réduisant l'activité (Alaux et al., 2010). Des études montrent des effets synergiques négatifs pesticide / pathogène ou pesticide / pesticide sur la survie des abeilles (Alaux et al., 2010 ; Aufauvre et al., 2012 ; Coulon et al., 2018, 2020 ; Genersch, 2010a). Par exemple, il a été démontré un effet synergique entre le diméthoate ou le clothianidine et *P. larvae* sur la mortalité, associé à une réduction du nombre de cellules hématocytaires (Hernández et al., 2017).

Il est nécessaire de mieux prendre en compte les effets sublétaux causés par les interactions complexes entre différents pesticides et pathogènes sur les abeilles. Les

conséquences des interactions pathogènes/pesticides sur le système immunitaire de l'abeille sont relativement bien établies (Cremer et al., 2007). En revanche, les autres effets sublétaux (i.e. le comportement, la cognition, la reproduction) sont particulièrement peu étudiés (O'Neal et al., 2018 ; Papach et al., 2017). Pourtant, l'abeille a à sa disposition tout un répertoire de comportements afin de lutter contre les pathogènes. Il s'agit de « l'immunité sociale » (Cremer et al., 2007). Elle comprend par exemple le toilettage, l'utilisation de matériaux antimicrobiens pour la construction des nids (Christe et al., 2003 ; Simone et al., 2009), la fièvre sociale (Starks et al., 2000), l'hygiène des nids (Cremer et al., 2007), le transfert des réponses immunitaires (Traniello et al., 2002) et la prise de risque accrue par les individus infectés (Schmid-Hempel, 2005). Cependant, les effets directs des pesticides sur la cognition et la mémoire ou sur la thermorégulation, pourraient impacter directement le toilettage, l'hygiène du nid et la fièvre sociale (Decourtye et al., 2005 ; Tosi et al., 2016). Ainsi, l'exposition aux pesticides pourrait induire une sensibilité accrue aux pathogènes et ravageurs causant à long terme la disparition de la colonie.

Perspectives

Consolider les tests en laboratoire

L'amélioration des tests en laboratoire est essentielle pour une évaluation plus précise des effets des pesticides sur les abeilles mellifères. La littérature met en avant que l'élevage larvaire présente un compromis significatif sur les capacités des individus adultes, mettant en lumière la nécessité d'améliorer les méthodes de test en laboratoire (Brodschneider et al., 2009 ; Mortensen et al., 2019). Plusieurs avancées récentes pourraient limiter ces effets, telles que l'aménagement des élevages avec des plaques positionnées à la verticale ou la mesure de la masse des individus afin d'obtenir une meilleure reproductibilité des résultats et des analyses (Kim et al., 2021 ; Vance et al., 2009).

Les observations faites au cours de cette étude révèlent que de nombreux individus ne rentrent pas après leur première sortie. Pour mieux comprendre ces effets, il faudrait approfondir les compromis sur la cognition et l'orientation avec des tests de réflexes conditionnés (PER) et de vol de retour. De plus, une meilleure caractérisation de l'adhésion à la colonie et des individus exposés est nécessaire et il serait pertinent de mesurer les phéromones de communication échangées entre les individus (Slessor

et al., 2005). Il convient également d'évaluer si les effets des pesticides mesurés dans des environnements *in vitro* peuvent être transposés à des observations *in situ* afin d'améliorer la compréhension des risques encourus par les abeilles mellifères lors d'expositions (Fisher et al., 2021).

Concernant l'amélioration des tests en laboratoire classique, nous recommandons de prolonger la durée des tests, car ils ne sont pas adaptés pour observer les effets sublétaux des pesticides, notamment des fongicides. Nos résultats, ainsi que d'autres études, indiquent que le boscalid n'induit pas de mortalité, mais seulement une réduction de la longévité, un effet qui n'est pas détectable avec les tests classiques d'exposition chronique de 10 jours (OCDE, 2017a). De plus, la mise en place des suivis au laboratoire sur les abeilles adultes post-élevage *in vitro*, ainsi que des suivis des butineuses, permettraient une évaluation plus complète des effets des pesticides sur les différents stades de vie des abeilles ouvrières (Amdam, 2011 ; Barascou et al., 2021 ; Fisher et al., 2021).

Améliorer les tests terrain

Plusieurs mesures peuvent être prises pour apprécier les facteurs biotique et abiotique sur la longévité et les activités des abeilles sur le terrain. Tout d'abord, la mise en place du test de vol de retour a été particulièrement utile pour évaluer l'impact des pesticides neurotoxiques sur la cognition des abeilles. (OCDE, 2021). Il est crucial de consolider les connaissances sur les tests d'intoxication afin de mieux suivre l'activité des abeilles, en prenant en compte des variables telles que le solvant utilisé, sa concentration, le temps d'exposition et l'heure de relâcher. Il est nécessaire de standardiser et de développer les métriques mesurées sur les activités des individus telles que celles que nous avons utilisées dans nos analyses (LSP, AFE, FT...). Également, la période de pré-butinage est essentielle et nous avons montré notamment un fort effet des pesticides sur les premières activités de vol des abeilles (Coulon et al., 2020 ; Prado et al., 2020). Il paraît primordial de mieux caractériser les mécanismes impliqués dans la proportion d'abeilles qui ne reviennent pas dans la colonie après leur première sortie et notamment l'implication des relations sociales (i.e immunités sociales, exclusion par les partenaires, auto-exclusion ; (Coulon et al., 2020 ; Cremer et al., 2007). Plus largement, il est nécessaire de mieux identifier les mécanismes comportementaux susceptibles de réduire l'exposition aux pesticides au sein des colonies (i.e. l'évitement

de nectar et de pollen contaminés, le traitement du nectar en miel, la culture de champignons et d'autres microbes symbiotiques (Berenbaum & Johnson, 2015).

Pour le moment, dans nos simulations de développement de colonies, nous n'avons pris en compte que la diminution de la longévité des individus fasse à des stress. Il sera nécessaire de transposer les métriques individuelles des ouvrières (citées ci-dessus) afin d'affiner l'évaluation des stress à l'échelle de la colonie. Il paraît également primordial d'intégrer les effets des stress induits sur les autres castes, (i.e reine, faux bourdons, larves), car elles sont directement impliquées dans la reproduction et le maintien des dynamiques de la population. Plusieurs modèles pourraient servir de support à leurs implémentations, notamment celui utilisé ici : BEEHAVE, mais également le model Bee++, ou ceux proposés par Magal ou Chen (Becher et al., 2014 ; Betti et al., 2017 ; Chen et al., 2021 ; Magal et al., 2019).

Un moyen rapide d'intégrer les mesures de l'activité dans les procédures d'écotoxicologie classiques seraient de les inclure dans le test 75 de l'OCDE « Test du couvain dans des conditions de semi-champs » (OCDE, 2014). Des colonies d'abeilles saines sont placées dans des tunnels (des enceintes construites) peu de temps avant la floraison et l'application de molécules testées. Lors de ce test, le développement du couvain est observé en priorité. L'activité de vol des butineuses est également identifiée, mais sur de courtes périodes et les observations sont réalisées à l'œil sur les fleurs. L'implémentation de technologies de suivi automatique de l'activité des abeilles, telles que les systèmes RFID ou les lecteurs optiques, serait pertinente pour des mesures plus précises et exhaustives sur l'activité (Alaux et al. 2014 ; Bagnis et al. 2010). Il serait également intéressant de mesurer les effets de ces expositions en pleins champs sur les différentes castes d'ouvrières : les jeunes abeilles, les butineuses, en même temps que l'application des molécules lors de la floraison (Barascou et al., 2021 ; Prado et al., 2020). De plus, puisque le couvain est observé, il est possible de mesurer l'activité des ouvrières devenues adultes qui auront été exposées lors de la phase larvaire (Fisher et al., 2021).

Élargir la notion de risque

Pour étendre la compréhension des risques liés à l'utilisation des pesticides, plusieurs mesures nous semblent nécessaires. Les récentes recommandations de l'ANSES et de l'EFSA mettent en avant plusieurs étapes pour évaluer les effets des pesticides.

Cependant, les tests sur le terrain, ne sont réalisés que si des effets préoccupants sont constatés lors des tests de mortalité en laboratoire (EFSA. 2023). Néanmoins, de nombreuses études sur le terrain ont permis d'observer des effets sublétaux sur les abeilles mellifères, notamment induits par les fongicides et les herbicides, qui n'avaient pas été identifiés comme préoccupants lors des tests en laboratoire (Aliouane et al., 2009 ; Bernabò et al., 2016 ; Pineaux et al., 2023). Il est alors pertinent que les pesticides utilisés pendant les périodes de floraison, avec un risque d'exposition élevé pour les pollinisateurs, soient testés dans des conditions semi-champs (OCDE, 2014, 2021). Notamment, l'EFSA propose d'utiliser la PEQ (Quantité d'exposition prévue). Cette dose serait intéressante notamment pour limiter le nombre de tests (EFSA 2023)

Il est primordial d'approfondir nos connaissances sur les effets des pesticides en prenant en considération la diversité génétique des abeilles. Les expérimentations menées au cours de cette thèse ont été réalisées sur des hybrides d'*Apis mellifera mellifera* × *caucasica*. J'ai tenu compte de la parenté commune des reines pondeuses, mais sans contrôle spécifique de la génétique de chaque individu. La diversité génétique des populations peut influencer leurs capacités d'adaptation aux stress biotique ou abiotique (Laurino et al., 2013 ; Mattila & Seeley, 2007 ; Rinkevich et al., 2015). Cela peut expliquer les différences significatives observées dans nos résultats en réponse aux pesticides sur les paramètres associés à l'activité, au comportement et à la physiologie. Il est également important d'explorer les effets des pesticides sur d'autres castes de la colonie, telles que les mâles et les reines, dont dépendent la reproduction, la production d'individus et la cohésion de la colonie (Barascou et al., 2021 ; Betti et al., 2017 ; Kairo et al., 2016).

Elargir les recherches écotoxicologiques aux autres insectes pollinisateurs est d'une importance majeure, car ils sont souvent négligés dans l'évaluation des effets sublétaux des pesticides malgré leurs contributions significatives à la pollinisation (MacInnis & Forrest, 2019 ; Parker & Torchio, 1987). Les abeilles solitaires et sociales n'ont pas les mêmes cycles biologiques et peuvent donc être exposées par des voies différentes et à des niveaux différents aux pesticides (Woodcock et al., 2017). Certaines abeilles sauvages sont plus sensibles que d'autres aux pesticides (Azpiazu et al., 2021). Une méta-analyse montre que seulement 15 % des études sur les effets sublétaux sur les abeilles concernent les espèces d'abeilles sauvages (autres que *Apis mellifera* ; Tosi et al., 2022). Actuellement les bourdons sont les seules abeilles

sauvages ayant des protocoles d'élevage validés par l'OCDE pour évaluer la toxicité des pesticides (Test No. 246 ; Test No. 247 ; OCED, 2017a, 2017b). Il existe des protocoles expérimentaux sur d'autres espèces d'abeilles telles que les osmies et les mélipones, mais ils n'ont pas encore été validés par cette organisation (Azpiazu et al., 2021 ; Belsky et al., 2022 ; Biddinger et al., 2013 ; Hydrology et al., 2016). Ces protocoles permettent d'obtenir une vision plus large des pesticides sur les abeilles sauvages, mais celles-ci ne représentent qu'une fraction des quelque 1000 espèces présentes en France et possédant des cycles biologiques très diversifiés (Pouvreau, 2004). On peut alors se questionner sur l'effet des pesticides sur le reste des insectes pollinisateurs qui représentent une grande diversité d'espèces floricoles, estimées entre 20 000 et 25 000, appartenant aux ordres des Diptères, des Lépidoptères et des Coléoptères (Pouvreau, 2004). Pour tenir compte de cette diversité, d'autres méthodes de recherche doivent être développées pour limiter les effets délétères des pesticides.

Les programmes de sciences participatives offrent une opportunité précieuse pour étudier la biodiversité de manière globale et les effets des pesticides (Bimonte et al., 2021 ; Jacquet et al., 2022). Ces programmes impliquent chercheurs et membres de la société civile dans la collecte de données. Par exemple, le programme « Spipoll » a mis en évidence le déclin des populations de divers insectes pollinisateurs dans les zones urbaines, ainsi que la diminution de la diversité des espèces dans plusieurs taxons (Deguines et al., 2012, 2016). Ces programmes permettent également de suivre l'expansion des espèces invasives, comme *Megachile sculpturalis* (Le Féon et al., 2018). En utilisant les données recueillies, il serait possible de dresser des « listes rouges » des insectes pollinisateurs en danger, ce qui aiderait à cibler les espèces nécessitant une protection particulière et à mettre en place des protocoles de suivi spécifiques. Ces études contribueront à élaborer des stratégies de gestion plus efficaces pour protéger la biodiversité et maintenir la santé des écosystèmes agricoles qui occupent une grande partie du territoire français. De plus, elles sensibiliseront le grand public au déclin des pollinisateurs et à l'importance de leur conservation.

Par ailleurs, l'utilisation des pesticides varie en fonction des cultures, des pratiques mises en place par les agriculteurs, ainsi que des pathogènes et ravageurs présents dans l'environnement. Compte tenu du grand nombre de pesticides présents dans l'environnement, il est essentiel de tenir compte de leurs impacts concomitants sur les pollinisateurs et la biodiversité (Al Nagggar & Paxton, 2021 ; Cedergreen, 2014 ;

Hernández et al., 2017). En raison du nombre important de pesticides mis sur le marché et des nombreuses combinaisons possibles, il est nécessaire d'établir des priorités pour ceux présentant les plus hauts risques. Ainsi, il est crucial de déterminer l'usage temporel et spatial de ces pesticides dans le paysage. L'émergence récente d'analyses spatiales de leur utilisation permet d'avoir une vue d'ensemble quant à leur répartition en France et dans d'autres pays en Europe (Esposito et al., 2005 ; Habran et al., 2022 ; Martin et al., 2023). Cependant, les modélisations saisonnières de leur présence dans l'environnement demeurent limitées à des zones géographiques restreintes (Lonsdorf et al., 2024 ; Materu et al., 2021 ; Nicholson et al., 2019) et les informations sur leur utilisation temporelle sont limitées. Par conséquent, il est important de poursuivre ce travail afin de proposer des scénarios d'exposition les plus pertinents, permettant de mesurer l'impact réel des pesticides sur les populations d'abeilles au cours de la saison, en prenant en considération les aspects paysagers, les ressources disponibles et la dynamique des colonies. Il serait également possible de transposer ces scénarios d'exposition sur les autres pollinisateurs, car ils fournissent également un service écosystémique essentiel.

CONCLUSION

Au terme de ces trois années de doctorat et d'un manuscrit de plus ou moins 200 pages de textes, nous pouvons retenir de ce travail que les abeilles font face à de nombreuses sources de stress, qu'ils soient biotiques ou abiotiques. Au cours de mes recherches, j'ai étudié les conséquences de sept facteurs de stress sur la biologie de l'abeille, appartenant à quatre familles différentes :

- Un pathogène : la bactérie *Paenibacillus larvae*.
- Un élément inerte : l'acétone.
- Deux insecticides : le thiaméthoxame et la lambda-cyhalothrine.
- Trois fongicides : le boscalid, le tébuconazole, et le prothioconazole.

Ces différents facteurs présents seuls ou en combinaison induisent des effets sublétaux sur la biologie des abeilles que j'ai choisi d'étudier à l'aide d'une approche transversale répartie sur quatre niveaux différents :

- La molécule : en analysant l'expression des gènes
- La larve : en analysant son développement
- L'abeille : en caractérisant ses traits d'histoire de vie
- La colonie : simulant le développement des colonies

J'ai ainsi pu caractériser les altérations différées de ces stress sur la physiologie, le développement, le comportement des abeilles. De plus, la transposition de ces effets individuels a induit une perturbation des simulations des dynamiques des colonies.

Les résultats obtenus offrent ainsi des réponses essentielles aux défis posés par les effets sublétaux des pesticides, induits par des expositions aux stades précoces de la vie de l'abeille. Ainsi, cette approche permet de mieux appréhender les effets différés des pesticides sur le cycle de vie des abeilles mellifères au niveau individuel, mais également collectif sur l'ensemble de la colonie. Ma thèse s'inscrit dans le contexte actuel d'utilisation intensive de pesticides et de déclin des insectes volants. Il est essentiel, de prendre en considération les conséquences de nos pratiques agricoles sur la biodiversité.

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ANNEXES DU CHAPITRE 1

Annex : Experimental ecotoxicology procedures interfere with honeybee life history

Content

Figure S1	Graphical protocol for the experiment.
Figure S2	Effect of acetone on proportion of forager bees.
Figure S3	Effect of acetone on the age at onset of foraging bees.
Table S1	Type II analysis of variance for Cox proportional hazard of larval survival.
Table S2	Type II analysis of variance for Linear Mixed Model of emergence day.
Table S3	Pairwise comparison of the coefficients of the Weibull hazard model to bee LSP.
Table S4	Pairwise comparison of the coefficients of the gamma GLMM on bee AFE.
Table S5	Pairwise comparison of the coefficients of the binomial GLMM on bee L-AFE;
Table S6	Pairwise comparison of the coefficients of the negative binomial 1 GLMM on bee FT.
Table S7	Pairwise comparison of the coefficients of the negative binomial 1 GLMM on bee FI.
Table S8	Pairwise comparison of the coefficients of the binomial GLMM on bee reaching the AOF.
Table S9	Pairwise comparison of the coefficients of the gamma GLMM on bee AOF.

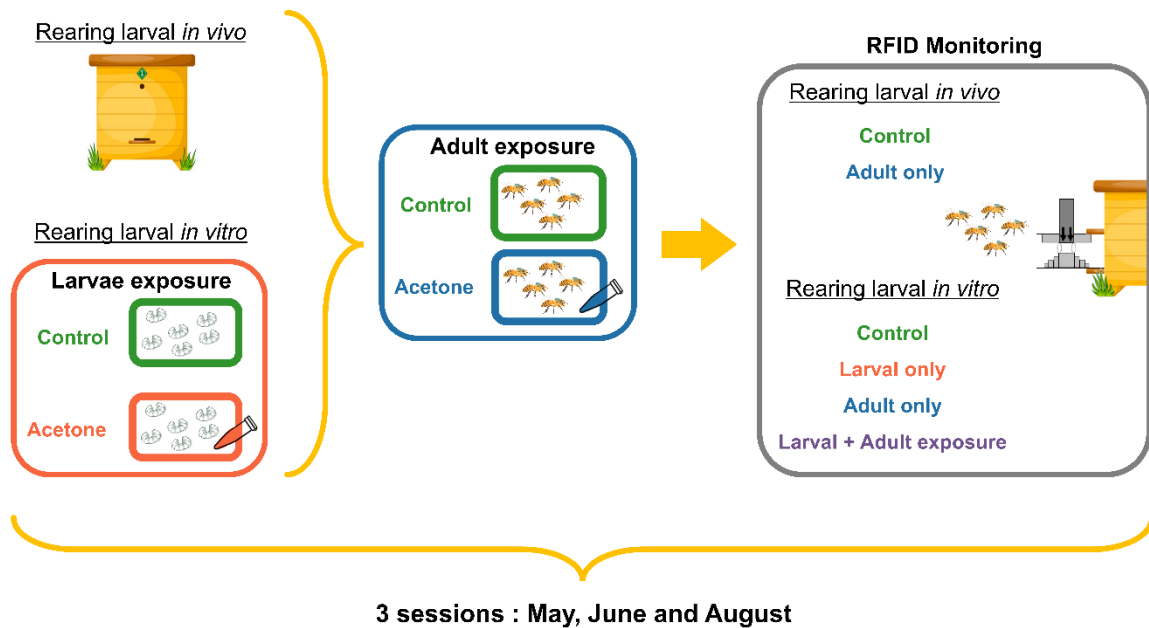


Figure S1. graphical protocol for the experiment. Larvae following two rearing protocols (i.e., in vitro and in vivo). We exposed bees to acetone during their larval stage and/or adult stage (bees reared in vitro) or only adult stage (bees reared in vivo), and we compared their life histories to those of bees that were not exposed to acetone. We conducted three experimental sessions (May, June and August).

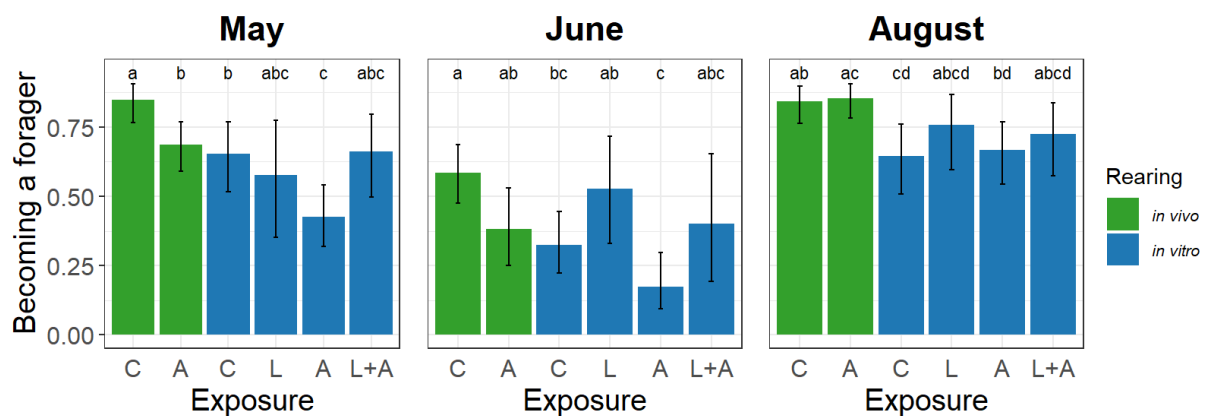


Figure S2. effect of acetone on the proportion of forager bees. The bees were in the unexposed control group (C) or were exposed to acetone at the larval stage (L), at the adult stage (A) or at both the larval and adult (L+A) stages for each experimental session (May, June and August). The colours show that the adult bees followed the larval rearing protocol (in vitro and in vivo). The bars represent the model estimates $\pm$ confidence intervals of the models (95 %). Significant differences in Tuckey pairwise comparisons are indicated with letters for each session.

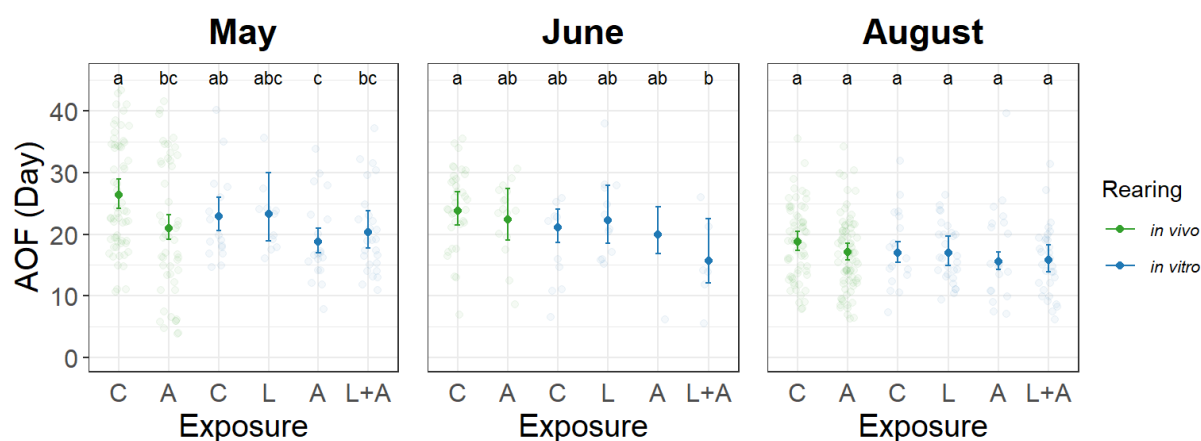


Figure S3. effect of acetone on the age at onset of foraging bees. The bees were in the unexposed control group (C) or were exposed to acetone at the larval stage (L), at the adult stage (A) or at both the larval and adult (L+A) stages for each experimental session (May, June and August). The colours show that the adult bees followed the larval rearing protocol (in vitro and in vivo). Transparent dots represent the raw data. Larger dots or bars represent model estimates $\pm$ confidence intervals of the models (95 %). Significant differences in Tuckey pairwise comparisons are indicated with letters for each session.

Table S1: type II analysis of variance for Cox proportional hazard of larval survival. The chi-square tests of Wald's likelihood are calculated with their associated p values. If $< 0,1^*$ if < 0.05 ; ** if < 0.01 and *** if < 0.001).

	Df	Chisq	Pr(>Chisq)
Session	2,000	40,306	0,000 ***
Exposure	1,000	1,233	0,267
Hive	2,000	5,422	0,066 .

Table S2: Type II analysis of variance for the linear mixed model of emergence day. The chi-square tests of Wald's likelihood are calculated with their associated p values. If $< 0,1^*$ if < 0.05 ; ** if < 0.01 and *** if < 0.001).

	Chisq	Df	Pr(>Chisq)
Exposure	2,833	1,000	0,092 .
Session	27,878	2,000	0,000 ***
Hive	35,564	2,000	0,000 ***

Table S3: Pairwise comparison of the coefficients of the Weibull hazard model to bee LSP. Tests were performed on a log scale (P value adjustment: Tukey method).

coefficient	contrast	ratio	SE	df	null	t.ratio	p.value
Rearing	in vivo / in vitro	1,926	0,095	1638,000	1,000	13,358	<0,001
Hive	R3 / R4	1,081	0,053	1638,000	1,000	1,579	0,255
	R3 / R6	1,021	0,051	1638,000	1,000	0,421	0,907
	R4 / R6	0,945	0,048	1638,000	1,000	-1,121	0,501
Session	May / June	1,882	0,106	1638,000	1,000	11,278	<0,001
	May / August	1,305	0,072	1638,000	1,000	4,798	<0,001
	June / August	0,693	0,035	1638,000	1,000	-7,265	<0,001
May : exposure	Cvivo / A vivo	1,274	0,115	1638,000	1,000	2,679	0,008
	Cvivo / C vitro	1,926	0,095	1638,000	1,000	13,358	<0,001
	Cvivo / L vitro	2,114	0,282	1638,000	1,000	5,621	<0,001
	Cvivo / A vitro	2,454	0,243	1638,000	1,000	9,084	<0,001
	Cvivo / LA vitro	1,695	0,199	1638,000	1,000	4,485	<0,001
	Avivo / C vitro	1,512	0,161	1638,000	1,000	3,870	<0,001
	Avivo / L vitro	1,659	0,217	1638,000	1,000	3,868	<0,001
	Avivo / A vitro	1,926	0,095	1638,000	1,000	13,358	<0,001
	Avivo / LA vitro	1,330	0,152	1638,000	1,000	2,501	0,013
	Cvitro / L vitro	1,098	0,148	1638,000	1,000	0,689	0,491
	Cvitro / A vitro	1,274	0,115	1638,000	1,000	2,679	0,008
	Cvitro / LA vitro	0,880	0,106	1638,000	1,000	-1,067	0,286
	Lvitro / A vitro	1,161	0,151	1638,000	1,000	1,150	0,250
	Lvitro / LA vitro	0,802	0,118	1638,000	1,000	-1,505	0,133
	Avitro / LA vitro	0,690	0,078	1638,000	1,000	-3,276	0,001
June : exposure	Cvivo / A vivo	2,251	0,186	1638,000	1,000	9,831	<0,001
	Cvivo / C vitro	1,926	0,095	1638,000	1,000	13,358	<0,001
	Cvivo / L vitro	1,755	0,181	1638,000	1,000	5,455	<0,001
	Cvivo / A vitro	4,335	0,431	1638,000	1,000	14,740	<0,001
	Cvivo / LA vitro	3,789	0,400	1638,000	1,000	12,607	<0,001
	Avivo / C vitro	0,856	0,079	1638,000	1,000	-1,686	0,092
	Avivo / L vitro	0,780	0,081	1638,000	1,000	-2,403	0,016
	Avivo / A vitro	1,926	0,095	1638,000	1,000	13,358	<0,001
	Avivo / LA vitro	1,684	0,178	1638,000	1,000	4,929	<0,001
	Cvitro / L vitro	0,911	0,093	1638,000	1,000	-0,906	0,365
	Cvitro / A vitro	2,251	0,186	1638,000	1,000	9,831	<0,001
	Cvitro / LA vitro	1,967	0,206	1638,000	1,000	6,453	<0,001
	Lvitro / A vitro	2,470	0,262	1638,000	1,000	8,521	<0,001
	Lvitro / LA vitro	2,159	0,256	1638,000	1,000	6,499	<0,001
	Avitro / LA vitro	0,874	0,095	1638,000	1,000	-1,245	0,213
August : exposure	Cvivo / A vivo	0,952	0,078	1638,000	1,000	-0,604	0,546
	Cvivo / C vitro	1,926	0,095	1638,000	1,000	13,358	<0,001
	Cvivo / L vitro	1,674	0,169	1638,000	1,000	5,108	<0,001
	Cvivo / A vitro	1,834	0,174	1638,000	1,000	6,375	<0,001
	Cvivo / LA vitro	1,619	0,165	1638,000	1,000	4,735	<0,001
	Avivo / C vitro	2,023	0,192	1638,000	1,000	7,419	<0,001
	Avivo / A vitro	1,926	0,095	1638,000	1,000	13,358	<0,001
	Avivo / LA vitro	1,700	0,174	1638,000	1,000	5,201	<0,001
	Cvitro / L vitro	0,869	0,089	1638,000	1,000	-1,380	0,168
	Cvitro / A vitro	0,952	0,078	1638,000	1,000	-0,604	0,546
	Cvitro / LA vitro	0,840	0,086	1638,000	1,000	-1,692	0,091
	Lvitro / A vitro	1,096	0,112	1638,000	1,000	0,894	0,371
	Lvitro / LA vitro	0,967	0,110	1638,000	1,000	-0,293	0,770
	Avitro / LA vitro	0,883	0,091	1638,000	1,000	-1,209	0,227

Table S4: Pairwise comparison of the coefficients of the gamma GLMM on bee AFE. Tests were performed on a the inverse scale (P-value adjustment: Tukey method).

coefficient	contrast	estimate	SE	df	t.ratio	p.value
Rearing	in vivo - in vitro	-0,025	0,005	1638,000	-4,516	<0,001
Hive	R3 - R4	0,004	0,005	1638,000	0,799	0,704
	R3 - R6	0,001	0,005	1638,000	0,255	0,965
	R4 - R6	-0,003	0,005	1638,000	-0,532	0,856
Session	May - June	-0,054	0,007	1638,000	-7,938	<0,001
	May - August	-0,042	0,006	1638,000	-7,022	<0,001
	June - August	0,012	0,007	1638,000	1,709	0,202
May : exposure	Cvivo - A vivo	-0,022	0,009	1638,000	-2,572	0,010
	Cvivo - C vitro	-0,025	0,005	1638,000	-4,516	<0,001
	Cvivo - L vitro	-0,010	0,012	1638,000	-0,811	0,417
	Cvivo - A vitro	-0,047	0,010	1638,000	-4,786	<0,001
	Cvivo - LA vitro	-0,025	0,011	1638,000	-2,216	0,027
	Avivo - C vitro	-0,002	0,011	1638,000	-0,214	0,830
	Avivo - L vitro	0,013	0,012	1638,000	1,033	0,302
	Avivo - A vitro	-0,025	0,005	1638,000	-4,516	<0,001
	Avivo - LA vitro	-0,002	0,011	1638,000	-0,215	0,830
	Cvitro - L vitro	0,015	0,013	1638,000	1,187	0,236
	Cvitro - A vitro	-0,022	0,009	1638,000	-2,572	0,010
	Cvitro - LA vitro	0,000	0,012	1638,000	-0,015	0,988
	Lvitro - A vitro	-0,037	0,013	1638,000	-2,968	0,003
	Lvitro - LA vitro	-0,015	0,014	1638,000	-1,084	0,279
	Avitro - LA vitro	0,022	0,012	1638,000	1,870	0,062
June : exposure	Cvivo - A vivo	-0,115	0,011	1638,000	-10,786	<0,001
	Cvivo - C vitro	-0,025	0,005	1638,000	-4,516	<0,001
	Cvivo - L vitro	-0,031	0,009	1638,000	-3,308	0,001
	Cvivo - A vitro	-0,139	0,012	1638,000	-11,854	<0,001
	Cvivo - LA vitro	-0,189	0,018	1638,000	-10,374	<0,001
	Avivo - C vitro	0,090	0,012	1638,000	7,425	<0,001
	Avivo - L vitro	0,084	0,013	1638,000	6,676	<0,001
	Avivo - A vitro	-0,025	0,005	1638,000	-4,516	<0,001
	Avivo - LA vitro	-0,075	0,020	1638,000	-3,700	<0,001
	Cvitro - L vitro	-0,006	0,010	1638,000	-0,621	0,535
	Cvitro - A vitro	-0,115	0,011	1638,000	-10,786	<0,001
	Cvitro - LA vitro	-0,165	0,018	1638,000	-8,943	<0,001
	Lvitro - A vitro	-0,109	0,013	1638,000	-8,575	<0,001
	Lvitro - LA vitro	-0,159	0,019	1638,000	-8,277	<0,001
	Avitro - LA vitro	-0,050	0,020	1638,000	-2,477	0,013
August : exposure	Cvivo - A vivo	-0,005	0,011	1638,000	-0,467	0,641
	Cvivo - C vitro	-0,025	0,005	1638,000	-4,516	<0,001
	Cvivo - L vitro	-0,004	0,013	1638,000	-0,328	0,743
	Cvivo - A vitro	-0,030	0,012	1638,000	-2,477	0,013
	Cvivo - LA vitro	-0,006	0,013	1638,000	-0,440	0,660
	Avivo - C vitro	-0,020	0,012	1638,000	-1,645	0,100
	Avivo - L vitro	0,001	0,013	1638,000	0,066	0,948
	Avivo - A vitro	-0,025	0,005	1638,000	-4,516	<0,001
	Avivo - LA vitro	-0,001	0,013	1638,000	-0,049	0,961
	Cvitro - L vitro	0,020	0,013	1638,000	1,610	0,108
	Cvitro - A vitro	-0,005	0,011	1638,000	-0,467	0,641
	Cvitro - LA vitro	0,019	0,013	1638,000	1,473	0,141
	Lvitro - A vitro	-0,025	0,013	1638,000	-1,975	0,049
	Lvitro - LA vitro	-0,001	0,014	1638,000	-0,104	0,917
	Avitro - LA vitro	0,024	0,013	1638,000	1,835	0,067

Table S5: Pairwise comparison of the coefficients of the gamma GLMM on bee L-AFE. Tests were performed on a log odds ratio scale (P-value adjustment: Tukey method).

coefficient	contrast	odds,ratio	SE	df	null	z,ratio	p,value
Rearing	in vivo / in vitro	0,200	0,027	Inf	1,000	-11,853	<0,001
Hive	R3 / R4	0,638	0,085	Inf	1,000	-3,360	0,002
	R3 / R6	0,888	0,118	Inf	1,000	-0,890	0,647
	R4 / R6	1,392	0,191	Inf	1,000	2,409	0,042
Session	May / June	0,330	0,051	Inf	1,000	-7,109	<0,001
	May / August	0,883	0,127	Inf	1,000	-0,865	0,662
	June / August	2,674	0,381	Inf	1,000	6,910	<0,001
May : exposure	Cvivo / A vivo	1,100	0,272	Inf	1,000	0,387	0,699
	Cvivo / C vitro	0,200	0,027	Inf	1,000	-11,853	<0,001
	Cvivo / L vitro	0,171	0,059	Inf	1,000	-5,094	<0,001
	Cvivo / A vitro	0,220	0,060	Inf	1,000	-5,602	<0,001
	Cvivo / LA vitro	0,320	0,096	Inf	1,000	-3,800	<0,001
	Avivo / C vitro	0,182	0,053	Inf	1,000	-5,811	<0,001
	Avivo / L vitro	0,155	0,053	Inf	1,000	-5,476	<0,001
	Avivo / A vitro	0,200	0,027	Inf	1,000	-11,853	<0,001
	Avivo / LA vitro	0,291	0,084	Inf	1,000	-4,255	<0,001
	Cvitro / L vitro	0,853	0,302	Inf	1,000	-0,448	0,654
	Cvitro / A vitro	1,100	0,272	Inf	1,000	0,387	0,699
	Cvitro / LA vitro	1,601	0,495	Inf	1,000	1,522	0,128
	Lvitro / A vitro	1,289	0,436	Inf	1,000	0,752	0,452
	Lvitro / LA vitro	1,876	0,695	Inf	1,000	1,699	0,089
	Avitro / LA vitro	1,455	0,420	Inf	1,000	1,298	0,194
June : exposure	Cvivo / A vivo	0,413	0,096	Inf	1,000	-3,792	<0,001
	Cvivo / C vitro	0,200	0,027	Inf	1,000	-11,853	<0,001
	Cvivo / L vitro	0,184	0,053	Inf	1,000	-5,853	<0,001
	Cvivo / A vitro	0,083	0,023	Inf	1,000	-8,990	<0,001
	Cvivo / LA vitro	0,114	0,037	Inf	1,000	-6,649	<0,001
	Avivo / C vitro	0,484	0,127	Inf	1,000	-2,773	0,006
	Avivo / L vitro	0,444	0,134	Inf	1,000	-2,699	0,007
	Avivo / A vitro	0,200	0,027	Inf	1,000	-11,853	<0,001
	Avivo / LA vitro	0,275	0,093	Inf	1,000	-3,832	<0,001
	Cvitro / L vitro	0,918	0,269	Inf	1,000	-0,291	0,771
	Cvitro / A vitro	0,413	0,096	Inf	1,000	-3,792	<0,001
	Cvitro / LA vitro	0,569	0,188	Inf	1,000	-1,711	0,087
	Lvitro / A vitro	0,450	0,140	Inf	1,000	-2,570	0,010
	Lvitro / LA vitro	0,620	0,230	Inf	1,000	-1,292	0,196
	Avitro / LA vitro	1,376	0,475	Inf	1,000	0,925	0,355
August :	Cvivo / A vivo	1,568	0,344	Inf	1,000	2,052	0,040
	Cvivo / C vitro	0,200	0,027	Inf	1,000	-11,853	<0,001
	Cvivo / L vitro	0,235	0,062	Inf	1,000	-5,459	<0,001
	Cvivo / A vitro	0,314	0,079	Inf	1,000	-4,600	<0,001
	Cvivo / LA vitro	0,317	0,083	Inf	1,000	-4,373	<0,001
	Avivo / C vitro	0,128	0,034	Inf	1,000	-7,809	<0,001
	Avivo / L vitro	0,150	0,041	Inf	1,000	-7,011	<0,001
	Avivo / A vitro	0,200	0,027	Inf	1,000	-11,853	<0,001
	Avivo / LA vitro	0,202	0,054	Inf	1,000	-5,963	<0,001
	Cvitro / L vitro	1,175	0,311	Inf	1,000	0,608	0,543
	Cvitro / A vitro	1,568	0,344	Inf	1,000	2,052	0,040
	Cvitro / LA vitro	1,586	0,416	Inf	1,000	1,759	0,079
	Lvitro / A vitro	1,335	0,353	Inf	1,000	1,091	0,275
	Lvitro / LA vitro	1,350	0,389	Inf	1,000	1,041	0,298
	Avitro / LA vitro	1,011	0,265	Inf	1,000	0,043	0,966

Table S6: Pairwise comparison of the coefficients of the negative binomial 1 GLMM on bee FT.

Tests were performed on a inverse scale (P-value adjustment: Tukey method).

coefficient	contrast	ratio	SE	df	null	t,ratio	p,value
Rearing	in vivo / in vitro	1,39	0,09	732,00	1,00	5,09	<0,001
Hive	R3 / R4	1,03	0,06	732,00	1,00	0,50	0,87
	R3 / R6	1,03	0,06	732,00	1,00	0,55	0,85
	R4 / R6	1,00	0,06	732,00	1,00	0,03	1,00
Session	May / June	1,49	0,13	732,00	1,00	4,56	<0,001
	May / August	1,14	0,08	732,00	1,00	1,94	0,13
	June / August	0,77	0,06	732,00	1,00	-3,20	<0,001
May : exposure	Cvivo / A vivo	1,431	0,123	732,000	1,000	4,182	<0,001
	Cvivo / C vitro	1,394	0,091	732,000	1,000	5,094	<0,001
	Cvivo / L vitro	1,662	0,278	732,000	1,000	3,038	0,003
	Cvivo / A vitro	1,996	0,208	732,000	1,000	6,622	<0,001
	Cvivo / LA vitro	1,254	0,144	732,000	1,000	1,971	0,049
	Avivo / C vitro	0,974	0,108	732,000	1,000	-0,236	0,814
	Avivo / L vitro	1,161	0,197	732,000	1,000	0,882	0,378
	Avivo / A vitro	1,394	0,091	732,000	1,000	5,094	<0,001
	Avivo / LA vitro	0,876	0,103	732,000	1,000	-1,127	0,260
	Cvitro / L vitro	1,192	0,208	732,000	1,000	1,006	0,315
	Cvitro / A vitro	1,431	0,123	732,000	1,000	4,182	<0,001
	Cvitro / LA vitro	0,899	0,113	732,000	1,000	-0,845	0,398
	Lvitro / A vitro	1,201	0,210	732,000	1,000	1,046	0,296
	Lvitro / LA vitro	0,754	0,139	732,000	1,000	-1,528	0,127
	Avitro / LA vitro	0,628	0,079	732,000	1,000	-3,699	<0,001
June : exposure	Cvivo / A vivo	1,603	0,221	732,000	1,000	3,422	0,001
	Cvivo / C vitro	1,394	0,091	732,000	1,000	5,094	<0,001
	Cvivo / L vitro	1,057	0,170	732,000	1,000	0,342	0,732
	Cvivo / A vitro	2,235	0,340	732,000	1,000	5,290	<0,001
	Cvivo / LA vitro	1,514	0,324	732,000	1,000	1,943	0,052
	Avivo / C vitro	0,870	0,133	732,000	1,000	-0,911	0,362
	Avivo / L vitro	0,659	0,123	732,000	1,000	-2,244	0,025
	Avivo / A vitro	1,394	0,091	732,000	1,000	5,094	<0,001
	Avivo / LA vitro	0,945	0,220	732,000	1,000	-0,245	0,807
	Cvitro / L vitro	0,758	0,127	732,000	1,000	-1,655	0,098
	Cvitro / A vitro	1,603	0,221	732,000	1,000	3,422	0,001
	Cvitro / LA vitro	1,086	0,238	732,000	1,000	0,377	0,706
	Lvitro / A vitro	2,116	0,405	732,000	1,000	3,913	<0,001
	Lvitro / LA vitro	1,433	0,353	732,000	1,000	1,462	0,144
	Avitro / LA vitro	0,677	0,161	732,000	1,000	-1,639	0,102
August : exposure	Cvivo / A vivo	1,028	0,089	732,000	1,000	0,323	0,747
	Cvivo / C vitro	1,394	0,091	732,000	1,000	5,094	<0,001
	Cvivo / L vitro	1,218	0,155	732,000	1,000	1,548	0,122
	Cvivo / A vitro	1,434	0,150	732,000	1,000	3,453	0,001
	Cvivo / LA vitro	1,356	0,169	732,000	1,000	2,441	0,015
	Avivo / C vitro	1,356	0,153	732,000	1,000	2,706	0,007
	Avivo / L vitro	1,184	0,149	732,000	1,000	1,341	0,180
	Avivo / A vitro	1,394	0,091	732,000	1,000	5,094	<0,001
	Avivo / LA vitro	1,318	0,163	732,000	1,000	2,238	0,026
	Cvitro / L vitro	0,873	0,121	732,000	1,000	-0,982	0,327
	Cvitro / A vitro	1,028	0,089	732,000	1,000	0,323	0,747
	Cvitro / LA vitro	0,972	0,132	732,000	1,000	-0,208	0,836
	Lvitro / A vitro	1,177	0,157	732,000	1,000	1,224	0,222
	Lvitro / LA vitro	1,113	0,171	732,000	1,000	0,700	0,485
	Avitro / LA vitro	0,945	0,124	732,000	1,000	-0,428	0,669

Table S7: Pairwise comparison of the coefficients of the negative binomial 1 GLMM on bee FI.

Tests were performed on a inverse scale (P-value adjustment: Tukey method).

coefficient	contrast	ratio	SE	df	null	t,ratio	p,value
Rearing	in vivo / in vitro	1,686	0,127	732,000	1,000	6,918	<0,001
Hive	R3 / R4	0,977	0,066	732,000	1,000	-0,346	0,936
	R3 / R6	0,994	0,066	732,000	1,000	-0,096	0,995
	R4 / R6	1,017	0,072	732,000	1,000	0,243	0,968
Session	May / June	1,131	0,115	732,000	1,000	1,217	0,443
	May / August	0,741	0,060	732,000	1,000	-3,673	0,001
	June / August	0,655	0,061	732,000	1,000	-4,579	<0,001
May : exposure	Cvivo / A vivo	1,375	0,144	732,000	1,000	3,032	0,003
	Cvivo / C vitro	1,686	0,127	732,000	1,000	6,918	<0,001
	Cvivo / L vitro	2,055	0,431	732,000	1,000	3,434	0,001
	Cvivo / A vitro	2,318	0,294	732,000	1,000	6,632	<0,001
	Cvivo / LA vitro	1,845	0,285	732,000	1,000	3,966	<0,001
	Avivo / C vitro	1,226	0,162	732,000	1,000	1,549	0,122
	Avivo / L vitro	1,495	0,315	732,000	1,000	1,907	0,057
	Avivo / A vitro	1,686	0,127	732,000	1,000	6,918	<0,001
	Avivo / LA vitro	1,342	0,209	732,000	1,000	1,892	0,059
	Cvitro / L vitro	1,219	0,264	732,000	1,000	0,913	0,362
	Cvitro / A vitro	1,375	0,144	732,000	1,000	3,032	0,003
	Cvitro / LA vitro	1,094	0,180	732,000	1,000	0,546	0,586
	Lvitro / A vitro	1,128	0,244	732,000	1,000	0,556	0,578
	Lvitro / LA vitro	0,898	0,214	732,000	1,000	-0,454	0,650
	Avitro / LA vitro	0,796	0,130	732,000	1,000	-1,393	0,164
June : exposure	Cvivo / A vivo	1,220	0,181	732,000	1,000	1,337	0,182
	Cvivo / C vitro	1,686	0,127	732,000	1,000	6,918	<0,001
	Cvivo / L vitro	1,096	0,208	732,000	1,000	0,482	0,630
	Cvivo / A vitro	2,056	0,347	732,000	1,000	4,276	<0,001
	Cvivo / LA vitro	1,295	0,303	732,000	1,000	1,108	0,268
	Avivo / C vitro	1,382	0,228	732,000	1,000	1,966	0,050
	Avivo / L vitro	0,899	0,187	732,000	1,000	-0,514	0,608
	Avivo / A vitro	1,686	0,127	732,000	1,000	6,918	<0,001
	Avivo / LA vitro	1,062	0,264	732,000	1,000	0,242	0,809
	Cvitro / L vitro	0,650	0,128	732,000	1,000	-2,182	0,029
	Cvitro / A vitro	1,220	0,181	732,000	1,000	1,337	0,182
	Cvitro / LA vitro	0,768	0,184	732,000	1,000	-1,099	0,272
	Lvitro / A vitro	1,876	0,407	732,000	1,000	2,904	0,004
	Lvitro / LA vitro	1,182	0,325	732,000	1,000	0,609	0,542
	Avitro / LA vitro	0,630	0,161	732,000	1,000	-1,806	0,071
August : exposure	Cvivo / A vivo	0,976	0,090	732,000	1,000	-0,264	0,792
	Cvivo / C vitro	1,686	0,127	732,000	1,000	6,918	<0,001
	Cvivo / L vitro	1,528	0,220	732,000	1,000	2,948	0,003
	Cvivo / A vitro	1,645	0,188	732,000	1,000	4,348	<0,001
	Cvivo / LA vitro	1,564	0,214	732,000	1,000	3,265	0,001
	Avivo / C vitro	1,728	0,215	732,000	1,000	4,399	<0,001
	Avivo / L vitro	1,566	0,223	732,000	1,000	3,149	0,002
	Avivo / A vitro	1,686	0,127	732,000	1,000	6,918	<0,001
	Avivo / LA vitro	1,603	0,217	732,000	1,000	3,479	0,001
	Cvitro / L vitro	0,907	0,142	732,000	1,000	-0,627	0,531
	Cvitro / A vitro	0,976	0,090	732,000	1,000	-0,264	0,792
	Cvitro / LA vitro	0,928	0,139	732,000	1,000	-0,500	0,617
	Lvitro / A vitro	1,076	0,163	732,000	1,000	0,486	0,627
	Lvitro / LA vitro	1,023	0,177	732,000	1,000	0,133	0,894
	Avitro / LA vitro	0,951	0,138	732,000	1,000	-0,349	0,727

Table S8: Pairwise comparison of the coefficients of the binomial GLMM on bee reaching the AOF. Tests were performed on a inverse scale (P-value adjustment: Tukey method).

coefficient	contrast	odds,ratio	SE	df	null	z,ratio	p,value
Rearing	in vivo / in vitro	2,952	0,613	Inf	1,000	5,210	<0,001
Hive	R3 / R4	1,161	0,244	Inf	1,000	0,711	0,757
	R3 / R6	1,516	0,305	Inf	1,000	2,073	0,095
	R4 / R6	1,306	0,278	Inf	1,000	1,253	0,422
Session	May / June	2,664	0,682	Inf	1,000	3,826	<0,001
	May / August	0,596	0,136	Inf	1,000	-2,267	0,061
	June / August	0,224	0,056	Inf	1,000	-6,028	<0,001
May : exposure	Cvivo / A vivo	2,537	0,832	Inf	1,000	2,841	0,005
	Cvivo / C vitro	2,952	0,613	Inf	1,000	5,210	<0,001
	Cvivo / L vitro	4,095	2,214	Inf	1,000	2,607	0,009
	Cvivo / A vitro	7,490	2,902	Inf	1,000	5,197	<0,001
	Cvivo / LA vitro	2,842	1,274	Inf	1,000	2,329	0,020
	Avivo / C vitro	1,163	0,452	Inf	1,000	0,389	0,697
	Avivo / L vitro	1,614	0,830	Inf	1,000	0,931	0,352
	Avivo / A vitro	2,952	0,613	Inf	1,000	5,210	<0,001
	Avivo / LA vitro	1,120	0,461	Inf	1,000	0,275	0,783
	Cvitro / L vitro	1,387	0,762	Inf	1,000	0,596	0,551
	Cvitro / A vitro	2,537	0,832	Inf	1,000	2,841	0,005
	Cvitro / LA vitro	0,963	0,442	Inf	1,000	-0,083	0,934
	Lvitro / A vitro	1,829	0,956	Inf	1,000	1,154	0,248
	Lvitro / LA vitro	0,694	0,406	Inf	1,000	-0,624	0,533
	Avitro / LA vitro	0,379	0,161	Inf	1,000	-2,290	0,022
June : exposure	Cvivo / A vivo	2,293	0,870	Inf	1,000	2,187	0,029
	Cvivo / C vitro	2,952	0,613	Inf	1,000	5,210	<0,001
	Cvivo / L vitro	1,260	0,599	Inf	1,000	0,486	0,627
	Cvivo / A vitro	6,769	3,017	Inf	1,000	4,292	<0,001
	Cvivo / LA vitro	2,109	1,213	Inf	1,000	1,297	0,195
	Avivo / C vitro	1,287	0,540	Inf	1,000	0,602	0,547
	Avivo / L vitro	0,549	0,287	Inf	1,000	-1,145	0,252
	Avivo / A vitro	2,952	0,613	Inf	1,000	5,210	<0,001
	Avivo / LA vitro	0,920	0,564	Inf	1,000	-0,136	0,891
	Cvitro / L vitro	0,427	0,212	Inf	1,000	-1,718	0,086
	Cvitro / A vitro	2,293	0,870	Inf	1,000	2,187	0,029
	Cvitro / LA vitro	0,714	0,423	Inf	1,000	-0,568	0,570
	Lvitro / A vitro	5,373	2,967	Inf	1,000	3,045	0,002
	Lvitro / LA vitro	1,674	1,131	Inf	1,000	0,762	0,446
	Avitro / LA vitro	0,312	0,199	Inf	1,000	-1,827	0,068
August : exposure	Cvivo / A vivo	0,909	0,312	Inf	1,000	-0,278	0,781
	Cvivo / C vitro	2,952	0,613	Inf	1,000	5,210	<0,001
	Cvivo / L vitro	1,718	0,797	Inf	1,000	1,166	0,244
	Cvivo / A vitro	2,683	1,052	Inf	1,000	2,517	0,012
	Cvivo / LA vitro	2,030	0,871	Inf	1,000	1,649	0,099
	Avivo / C vitro	3,247	1,331	Inf	1,000	2,874	0,004
	Avivo / L vitro	1,890	0,866	Inf	1,000	1,389	0,165
	Avivo / A vitro	2,952	0,613	Inf	1,000	5,210	<0,001
	Avivo / LA vitro	2,233	0,945	Inf	1,000	1,898	0,058
	Cvitro / L vitro	0,582	0,279	Inf	1,000	-1,131	0,258
	Cvitro / A vitro	0,909	0,312	Inf	1,000	-0,278	0,781
	Cvitro / LA vitro	0,688	0,306	Inf	1,000	-0,842	0,400
	Lvitro / A vitro	1,562	0,727	Inf	1,000	0,958	0,338
	Lvitro / LA vitro	1,182	0,607	Inf	1,000	0,325	0,745
	Avitro / LA vitro	0,757	0,326	Inf	1,000	-0,648	0,517

Table S9: Pairwise comparison of the coefficients of the binomial GLMM on bee reaching the AOF. Tests were performed on a log odds ratio scale (P-value adjustment: Tukey method).

coefficient	contrast	estimate	SE	df	t,ratio	p,value
Rearing	in vivo - in vitro	-0,006	0,002	483,000	-2,336	0,020
Hive	R3 - R4	0,007	0,002	483,000	3,526	0,001
	R3 - R6	0,004	0,002	483,000	1,752	0,187
	R4 - R6	-0,003	0,002	483,000	-1,685	0,212
Session	May - June	-0,004	0,003	483,000	-1,274	0,410
	May - August	-0,014	0,002	483,000	-5,930	<0,001
	June - August	-0,010	0,003	483,000	-2,858	0,012
May : Exposure	Cvivo - A vivo	-0,010	0,003	483,000	-3,510	0,001
	Cvivo - C vitro	-0,006	0,002	483,000	-2,336	0,020
	Cvivo - L vitro	-0,005	0,005	483,000	-0,977	0,329
	Cvivo - A vitro	-0,015	0,004	483,000	-4,318	<0,001
	Cvivo - LA vitro	-0,011	0,004	483,000	-2,783	0,006
	Avivo - C vitro	0,004	0,004	483,000	1,104	0,270
	Avivo - L vitro	0,005	0,005	483,000	0,858	0,391
	Avivo - A vitro	-0,006	0,002	483,000	-2,336	0,020
	Avivo - LA vitro	-0,001	0,004	483,000	-0,334	0,739
	Cvitro - L vitro	0,000	0,006	483,000	0,088	0,930
	Cvitro - A vitro	-0,010	0,003	483,000	-3,510	0,001
	Cvitro - LA vitro	-0,006	0,005	483,000	-1,246	0,213
	Lvitro - A vitro	-0,010	0,006	483,000	-1,797	0,073
	Lvitro - LA vitro	-0,006	0,006	483,000	-0,997	0,319
	Avitro - LA vitro	0,004	0,005	483,000	0,899	0,369
June : Exposure	Cvivo - A vivo	-0,003	0,005	483,000	-0,561	0,575
	Cvivo - C vitro	-0,006	0,002	483,000	-2,336	0,020
	Cvivo - L vitro	-0,003	0,005	483,000	-0,582	0,561
	Cvivo - A vitro	-0,008	0,005	483,000	-1,514	0,131
	Cvivo - LA vitro	-0,022	0,010	483,000	-2,168	0,031
	Avivo - C vitro	-0,003	0,005	483,000	-0,561	0,575
	Avivo - L vitro	0,000	0,006	483,000	-0,062	0,951
	Avivo - A vitro	-0,006	0,002	483,000	-2,336	0,020
	Avivo - LA vitro	-0,019	0,011	483,000	-1,807	0,071
	Cvitro - L vitro	0,003	0,006	483,000	0,453	0,651
	Cvitro - A vitro	-0,003	0,005	483,000	-0,561	0,575
	Cvitro - LA vitro	-0,016	0,010	483,000	-1,586	0,113
	Lvitro - A vitro	-0,005	0,007	483,000	-0,784	0,433
	Lvitro - LA vitro	-0,019	0,011	483,000	-1,731	0,084
	Avitro - LA vitro	-0,014	0,011	483,000	-1,254	0,211
August :	Cvivo - A vivo	-0,005	0,003	483,000	-1,705	0,089
	Cvivo - C vitro	-0,006	0,002	483,000	-2,336	0,020
	Cvivo - L vitro	-0,006	0,005	483,000	-1,201	0,230
	Cvivo - A vitro	-0,011	0,004	483,000	-2,756	0,006
	Cvivo - LA vitro	-0,010	0,005	483,000	-2,077	0,038
	Avivo - C vitro	0,000	0,004	483,000	-0,056	0,956
	Avivo - L vitro	0,000	0,005	483,000	-0,056	0,955
	Avivo - A vitro	-0,006	0,002	483,000	-2,336	0,020
	Avivo - LA vitro	-0,005	0,005	483,000	-0,964	0,336
	Cvitro - L vitro	0,000	0,005	483,000	-0,009	0,993
	Cvitro - A vitro	-0,005	0,003	483,000	-1,705	0,089
	Cvitro - LA vitro	-0,004	0,005	483,000	-0,858	0,392
	Lvitro - A vitro	-0,005	0,005	483,000	-1,045	0,296
	Lvitro - LA vitro	-0,004	0,006	483,000	-0,742	0,458
	Avitro - LA vitro	0,001	0,005	483,000	0,177	0,860

ANNEXES DU CHAPITRE 2

Annex : Realistic exposure to boscalid induces shorter-lived honey bees and disturbs colony dynamics

Content

- Figure S1** Sensitivity analysis of the simulated effect of shorter-lived bees on honeybee colony survival.
- Figure S2** Set of 600 simulations ran to analyse the effect of boscalid induced shorter-lived bees on the colony demographic growth (adult population size).
- Figure S3** Simulated effect of shorter-lived bees from day 122 to day 168 (interval 2) on colony dynamics.
- Figure S4** Simulated effect of shorter-lived bees from day 168 to day 214 (interval 3) on colony dynamics.
- Figure S5** Simulated effect of shorter-lived bees from day 76 to day 168 (intervals 1+2) on colony dynamics.
- Figure S6** Simulated effect of shorter-lived bees from day 122 to day 214 (i.e. intervals 2+3) on colony dynamics.

Figure S1. Sensitivity analysis of the simulated effect of shorter-lived bees on honeybee colony survival. We computed 100 simulations with an extended gradient of forager mortality rate from the default value of 0.00001 to 0.00006 (i.e. increase coefficient of $\times 6$) to run a sensitivity analysis. (A) Survival probability of simulated bee colonies during an 18-month period. The vertical dotted line represents the observed maximum effect of boscalid on individual bees (i.e. an increase coefficient of $\times 1.56$). (B) Demographic growth of simulated colonies (adult population size). X-axis represents the time series in day. Blue lines represent surviving colonies and red lines represent dead colonies with the vertical line indicating the time of colony death. The grey polygons show the period of stress simulated for the different time intervals during the colony dynamic. For this sensitivity analysis we considered the maximal duration of stress, corresponding to the combination of the three grey polygons starting before the growth period of the population size (day 76) at the end of the growth period of the population size (day 214).

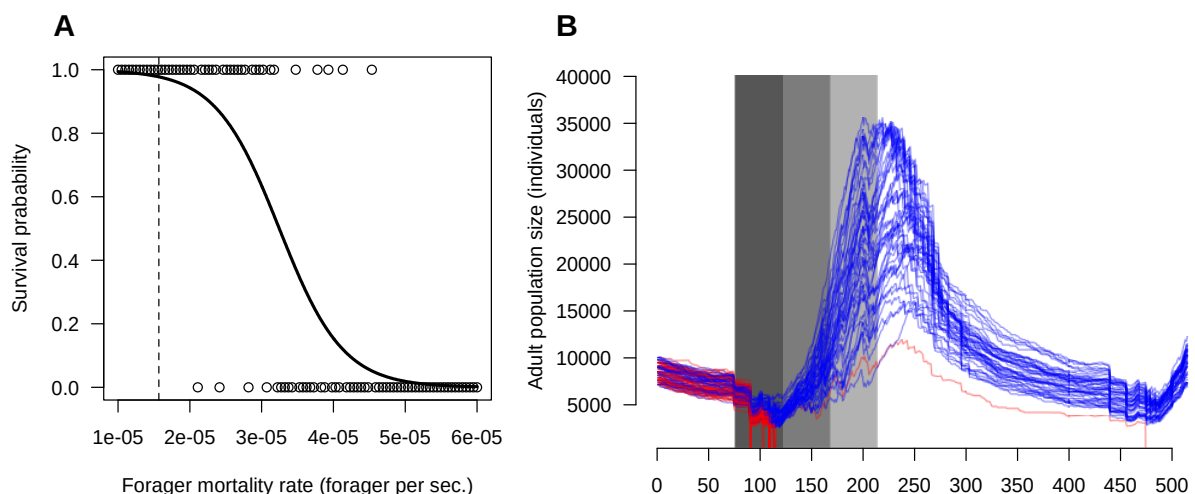


Figure S2. Set of 600 simulations ran to analyse the effect of boscalid induced shorter-lived bees on the colony demographic growth (adult population size). X-axis represents the time series in day. Blue lines represent surviving colonies and red lines represent dead colonies with the vertical line indicating the time of colony death (a single colony died in the set of simulations). The grey polygons show the period of stress simulated for the different time intervals during the colony dynamic, i.e. before the growth period of the population size (day 76 to day 122, interval 1 in dark grey), at the beginning of the growth period of the population size (day 122 to day 168, interval 2 in medium grey), and at the end of the growth period of the population size (day 168 to day 214, interval 3 in light grey). We ran six scenarios of exposure for different time intervals (interval 1, interval 2, interval 3) and by combining the different time intervals (intervals 1+2, intervals 2+3, intervals 1+2+3).

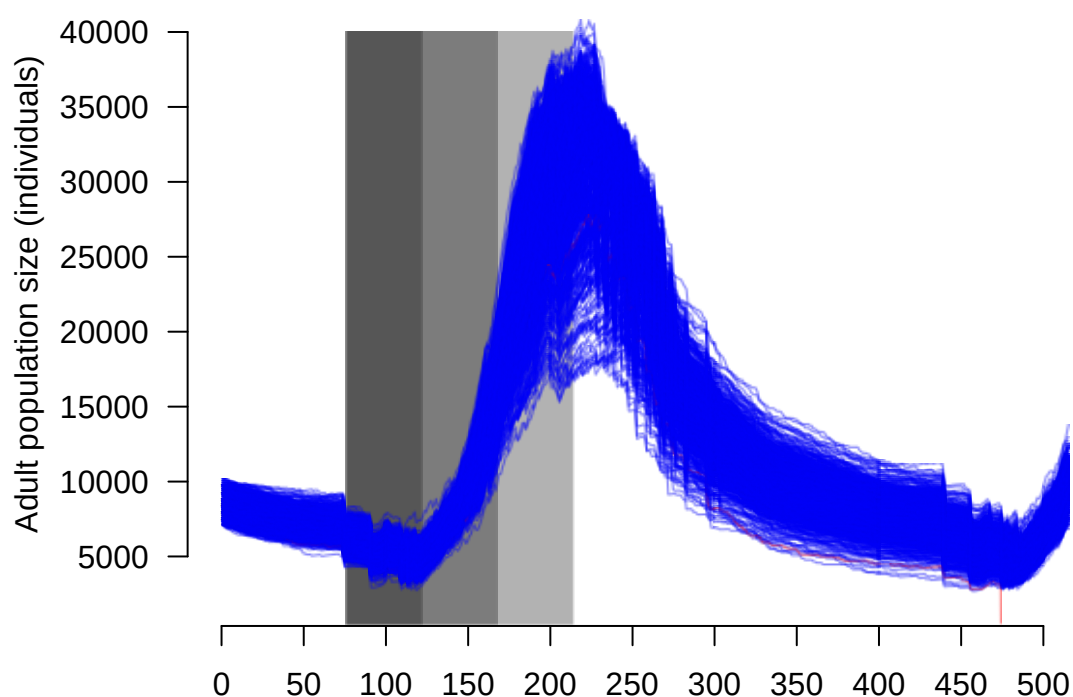


Figure S3. Simulated effect of shorter-lived bees from day 122 to day 168 (interval 2) on colony dynamics. (A) Changes in population size were analysed along a gradient of relative effect of boscalid (from 0 to 1, no-effect and full impact, respectively) simulating shorter-lived bees day 122 to day 168 (i.e. at the beginning of the growth period of the population size, interval 2). We analysed the direct time effect of simulating shorter-lived bees on changes in population size, as well as delayed effects with +1 month, +2 months and +3 months after the end of the simulated impact. (B) Changes in honey reserves were also analysed along a gradient of relative effect of boscalid simulating shorter-lived bees from day 122 to day 168 (i.e. interval 2). We analysed the direct time effect of simulating shorter-lived bees on changes in honey reserves, as well as delayed effects with +1 month, +2 months and +3 months after the end of the simulated impact. We found no significant effects of the relative effect of boscalid (i.e. simulating shorter-lived bees) on changes in population size and honey reserves, in direct time exposure and delayed times (+1 month, + 2 months, +3 months).

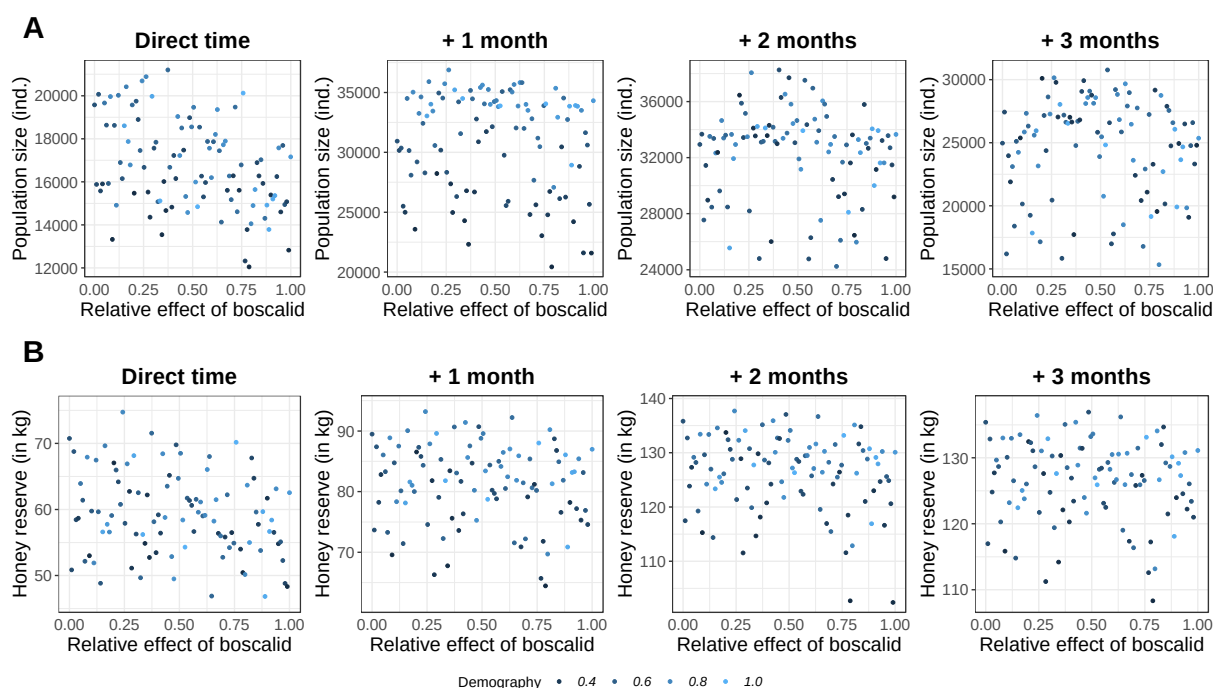


Figure S4. Simulated effect of shorter-lived bees from day 168 to day 214 (interval 3) on colony dynamics. (A) Changes in population size were analysed along a gradient of relative effect of boscalid (from 0 to 1, no-effect and full impact, respectively) simulating shorter-lived bees day from day 168 to day 214 (i.e., at the end of the growth period of the population size, interval 3). We analysed the direct time effect of simulating shorter-lived bees on changes in population size, as well as delayed effects with +1 month, +2 months and +3 months after the end of the simulated impact. (B) Changes in honey reserves were also analysed along a gradient of relative effect of boscalid simulating shorter-lived bees from day 168 to day 214 (i.e. interval 3). We analysed the direct time effect of simulating shorter-lived bees on changes in honey reserves, as well as delayed effects with +1 month, +2 months and +3 months after the end of the simulated impact. We found no significant effects of the relative effect of boscalid (i.e. simulating shorter-lived bees) on changes in population size and honey reserves, in direct time exposure and delayed times (+1 month, + 2 months, +3 months).

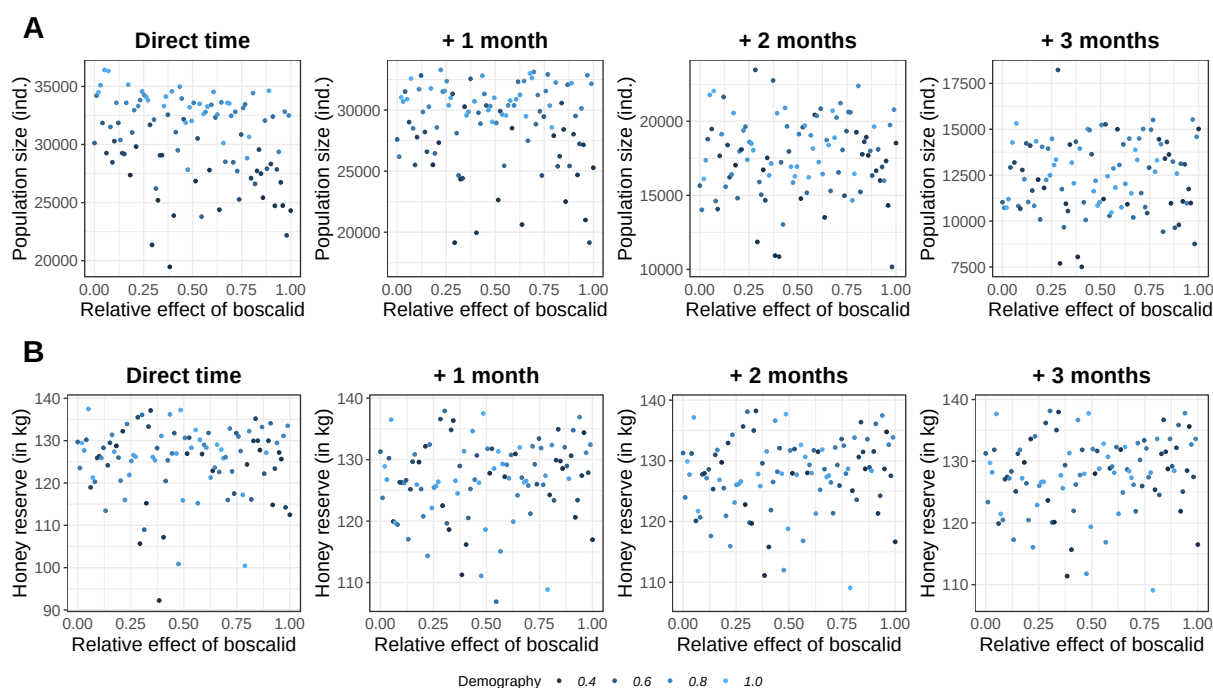


Figure S5. Simulated effect of shorter-lived bees from day 76 to day 168 (intervals 1+2) on colony dynamics. (A) Changes in population size were analysed along a gradient of relative effect of boscalid (from 0 to 1, no-effect and full impact, respectively) simulating shorter-lived bees day from day 76 to day 168 (i.e. intervals 1+2). We analysed the direct time effect of simulating shorter-lived bees on changes in population size, as well as delayed effects with +1 month, +2 months and +3 months after the end of the simulated impact. (B) Changes in honey reserves were also analysed along a gradient of relative effect of boscalid simulating shorter-lived bees from day 76 to day 168 (i.e. intervals 1+2). We analysed the direct time effect of simulating shorter-lived bees on changes in honey reserves, as well as delayed effects with +1 month, +2 months and +3 months after the end of the simulated impact. We found no significant effects of the relative effect of boscalid (i.e. simulating shorter-lived bees) on changes in population size and honey reserves, in direct time exposure and delayed times (+1 month, + 2 months, +3 months).

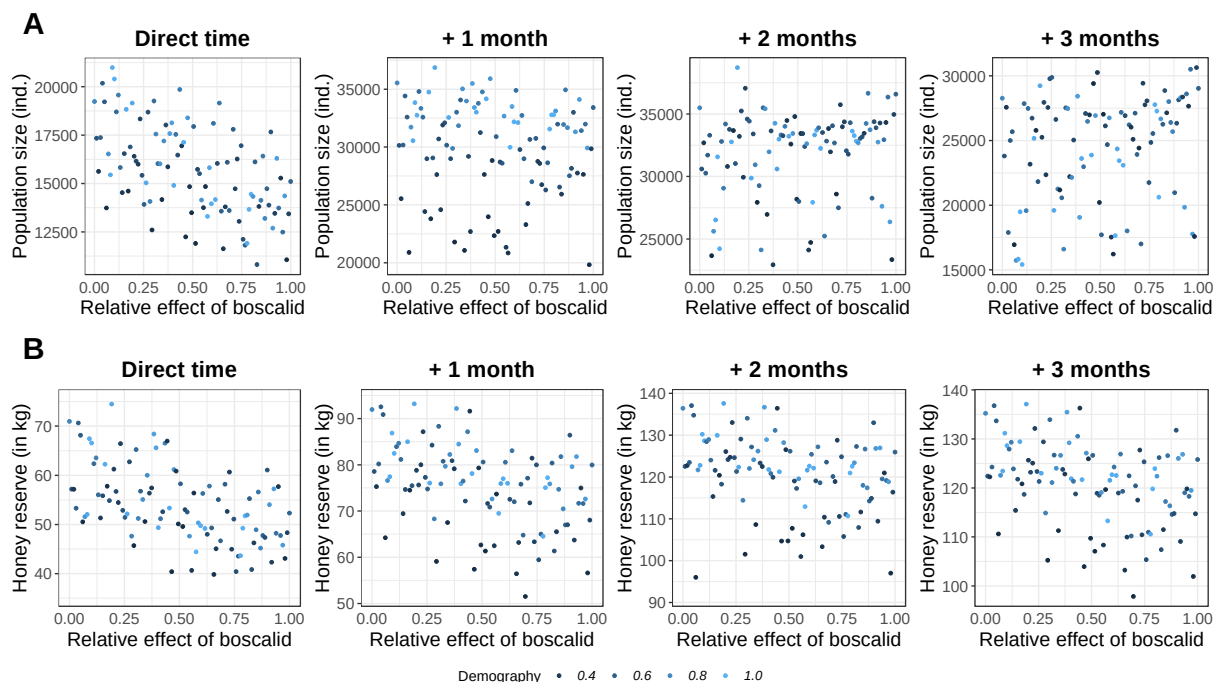


Figure S6. Simulated effect of shorter-lived bees from day 122 to day 214 (i.e. intervals 2+3) on colony dynamics. (A) Changes in population size were analysed along a gradient of relative effect of boscalid (from 0 to 1, no-effect and full impact, respectively) simulating shorter-lived bees day from day 122 to day 214 (i.e. intervals 2+3). We analysed the direct time effect of simulating shorter-lived bees on changes in population size, as well as delayed effects with +1 month, +2 months and +3 months after the end of the simulated impact. (B) Changes in honey reserves were also analysed along a gradient of relative effect of boscalid simulating shorter-lived bees from day 122 to day 214 (i.e. intervals 2+3). We analysed the direct time effect of simulating shorter-lived bees on changes in honey reserves, as well as delayed effects with +1 month, +2 months and +3 months after the end of the simulated impact. We found that simulating shorter-lived bees reduced the population size of the simulated colonies by 15.8% ($t = -4.729$, $p < 0.001$, **Figure S6A**) and their honey production by 14.1% ($t = -4.992$, $p < 0.001$, **Figure S6B**) during the exposure period of intervals 2+3. We also found significant interactions between the level of boscalid impact and the demographic strength of the colony, showing that the demographically stronger colonies were more affected by this boscalid impact on both population size ($t = 3.013$, $p = 0.003$) and honey production ($t = 3.882$, $p < 0.001$). The impacts of boscalid on population size can be observed up to one month after the end of the simulated impact ($t = -3.061$, $p = 0.003$, **Figure S6A**), with interaction with the colony demographic strength ($t = 2.529$, $p = 0.013$, **Figure S6A**). No significant effects of the relative effect of boscalid on changes in population size for + 2 months and +3 months have been found, while the impacts of boscalid on honey reserve can be observed up to three months after the end of the simulated impact (+1 month: $t = -3.710$, $p < 0.001$; +2 months : $t = -3.240$, $p = 0.002$; +3 months: $t = -3.269$, $p = 0.002$, **Figure S6B**), with interaction with the colony demographic strength (+1 month: $t = 2.959$, $p = 0.004$; +2 months: $t = 2.492$, $p = 0.014$; +3 months: $t = 2.506$, $p = 0.014$, **Figure S6B**).

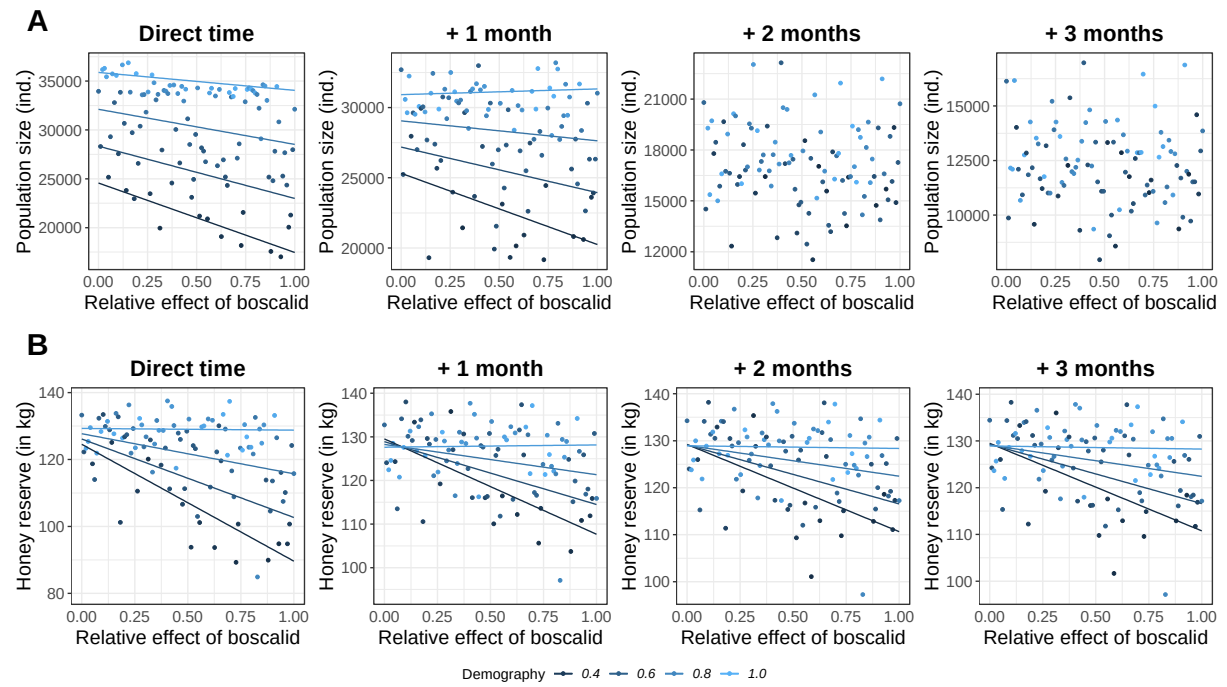
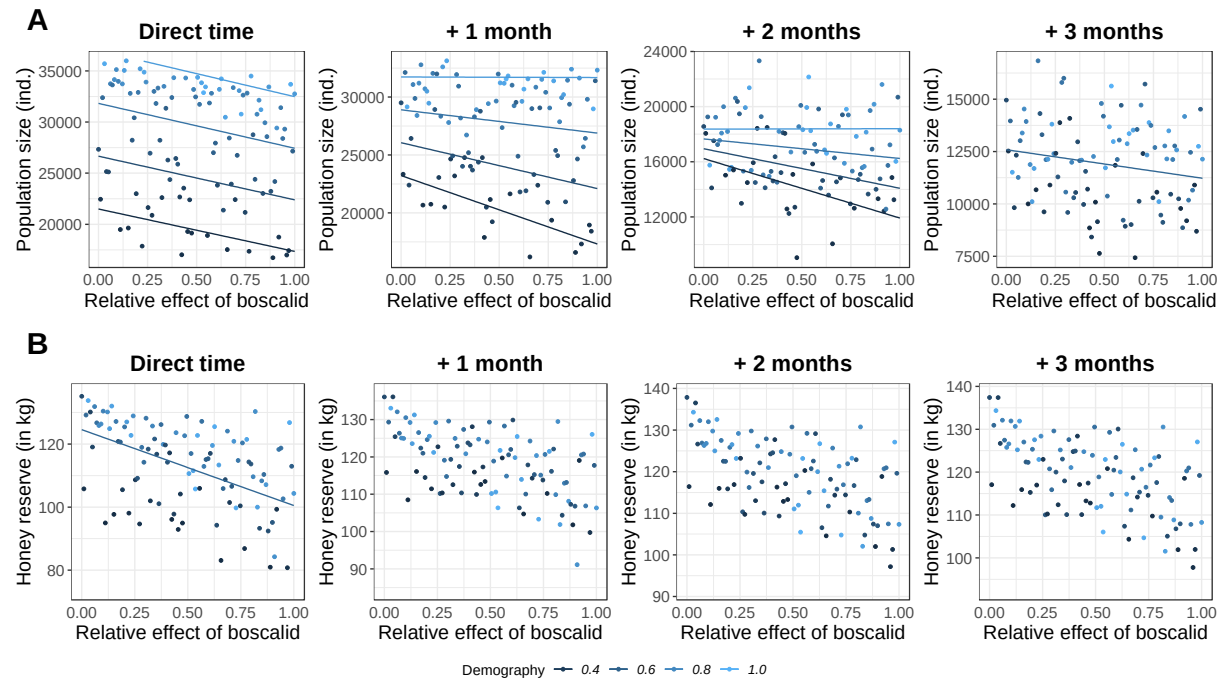


Figure S7. Simulated effect of shorter-lived bees from day 76 to day 214 (i.e. intervals 1+2+3) on colony dynamics. (A) Changes in population size were analyzed along a gradient of relative effect of boscalid (from 0 to 1, no-effect and full impact, respectively) simulating shorter-lived bees day from day 76 to day 214 (i.e. intervals 1+2+3). We analysed the direct time effect of simulating shorter-lived bees on changes in population size, as well as delayed effects with +1 month, +2 months and +3 months after the end of the simulated impact. (B) Changes in honey reserves were also analysed along a gradient of relative effect of boscalid simulating shorter-lived bees from day 76 to day 214 (i.e. intervals 1+2+3). We analysed the direct time effect of simulating shorter-lived bees on changes in honey reserves, as well as delayed effects with +1 month, +2 months and +3 months after the end of the simulated impact. I've found that simulating shorter-lived bees reduces the population size of the simulated colonies by 15.3% ($t = -1.849$, $p = 0.050$, **Figure S7A**) and their honey production by 19.3 % ($t = -2.434$, $p = 0.017$, **Figure S7B**) during the exposure period of intervals 1+2+3. Significant interactions between the level of boscalid impact and the demographic strength of the colony on population size (and not on honey production) have also been found, showing that the demographically stronger colonies were more affected by this boscalid impact on population size ($t = 2.0830$, $p = 0.042$, **Figure S7A**). The impacts of boscalid on population size can be observed up to three months after the end of the simulated impact have also been observed (+1 month: $t = -3.452$, $p < 0.001$; +2 months: $t = -2.611$, $p = 0.011$; +3 months : $t = -2.288$, $p = 0.024$, **Figure S7A**), with interaction with the colony demographic strength (+1 month: $t = 2.596$, $p = 0.011$; +2 months: $t = 1.985$, $p = 0.050$; no significant effect on +3 months, **Figure S7A**). No significant effects of the relative effect of boscalid on changes in honey reserves in delayed times have been found (+1 month, + 2 months, +3 months). (**Figure S7B**).



ANNEXES DU CHAPITRE 3

Annex : Impact of exposure of larvae to Boscalid at field concentrations on gene expression in honeybees

Content

Appendix 1A Experimental design.

Appendix 1B Diet composition.

Appendix 1C Primer sequences used for quantitative qPCR analysis.

Appendix 2A Analysis of individual survival.

Appendix 2B Analysis of emergence days.

Appendix 3A Analysis of modalities on larvae's gene expression.

Appendix 3B Analysis of hive of origin on larvae's gene expression.

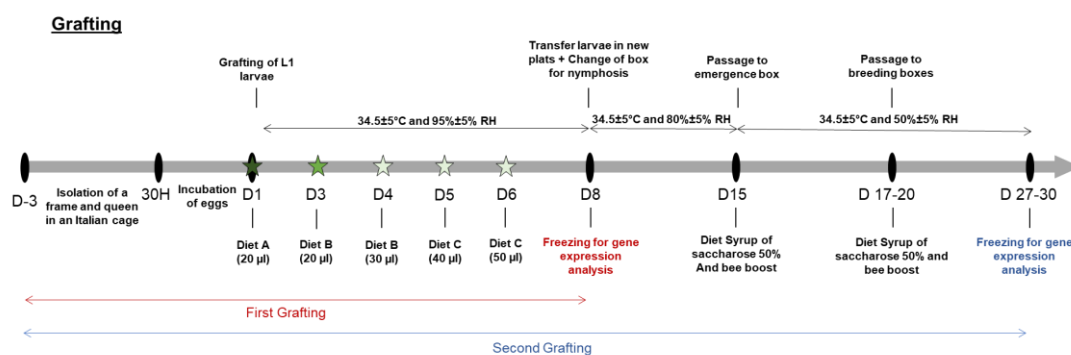
Appendix 4A Analysis of modalities on worker's gene expression.

Appendix 4B Analysis of hive of origin on worker's gene expression.

Appendix 1A - Experimental design: graphic representation of the experimental design of the artificial larval rearing carried out for the analyses. I've followed the method adapted by (Aupinel et al., 2005 ; Papach et al., 2017).

The individuals were exposed to the different modalities (Control, control Solvent, Bosclaid, Pictor®) chronically from day 3 to day 6 (Appendix 1B).

Two series were carried out : the first grafting, the individuals were collected and frozen at -80°C on D8 for gene expression analyses (Red). The second grafting, the individuals were followed individually until emergence and were then reared for 10 days in the laboratory before being frozen for gene expression analyses (Bleu).



Appendix 1B - Diet composition : composition of feed distributed to larvae during artificial rearing, for the different models (Control : C, Control Solvent : CS, Boscalid : B, and Pictor Pro® : P).

Diet composition	 A	 B	 C
Royal jelly (g)	50%	50%	50%
Sugar solution (g)	50%	50%	50%
Acetone (SC, B)	/	0.1%	0.1%
Boscalid (ng/g) (B)	/	19.5	19.5
Pictor Pro® (P)	/	38.9	38.9

Sugar solution	Sol A	Sol B	Sol C
Glucose %	12,0%	15,0%	18,0%
Fructose %	12,0%	15,0%	18,0%
Yeast extract %	2,0%	3,0%	4,0%
Osmosed water (g)	10	10	10

Appendix 1C - Primer sequences used for quantitative qPCR analysis:

Category	Gene description	Forward sequence (5'-3')	Reverse sequence (3'-5')	Reference
House	β-actin	TGCCAACACTGTC CTTTCTG	AGAATTGACCCAC CAATCCA	Lourenço et
House	Ribosomal protein	TGGATGTTCAACAGGGTTCATA	CTGGTGGTGGACGTATTGATAA	Evans et al.
Detoxification	Catalase	GTCTTGGCCCCAA CAATCTG	CATTCTCTAGGCCCA CCAAA	Evans et al.
Detoxification	Cytochrome 450	GTTCCGGGAAAATGACTAC	GGTCAAAATGGTGGTGAC	Mao et al.
Development	Vitellogenin	AGTTCCGACCGAC GACGA	TTCCCTCCCACGGA GTCC	Simone et al.
Development	Hexamerin 70b	AACTCGCTCAACTT TCCACAA	GGCTCACATAACTA ACCTCACC	Evans et al.
Mitochondrial	SDHA	AGCAGGATTACGTGCAGCTT	ACCCAAGGCAGCATTGATAC	Scharlaken
Mitochondrial	Cytochrome b	CAACTCATATTAAACCTGAATG	CCGATTACACCTCCTAATTTATT	wang et al.
Immunity	Prophenoloxidase	GTTTGGTCGACGG AAGAAAA	CCGTCGACTCGAAA TCGTAT	Gregorc et

Appendix 2B - Analysis of emergence days: Emergence days of bees was analyzed using a linear random effects model. Fixed effects were included in the model: modalities (Control: C, Control Solvent: CS, Boscalid: B and Pictor Pro: P) and grafted hives (R3, R4 and R6). A random effect was applied to the rearing plates in which the larvae were reared. Pairwise comparisons were performed with Tukey's HSD to identify differences between modalities and hives.

Linear random effects model

Model

Emergence_Days ~ Modality + Hive + (1 | ID_plate)

Random effects:

Groups	Name	Variance	Std,Dev,
ID_plate	(Intercept)	0,134	0,366
Residual		0,231	0,481

Fixed effects:

	Estimate	Std, Error	t value
(Intercept)	19,210	0,187	102,640
ModalityB	0,122	0,216	0,565
ModalityP	0,018	0,216	0,085
ModalityT	0,244	0,216	1,129
HiveR4	0,302	0,187	1,615
HiveR6	0,187	0,187	0,996

Correlation of Fixed Effects:

	(Intr)	MdltyB	MdltyP	MdltyT	HiveR4
ModalityB	-0,578				
ModalityP	-0,577	0,500			
ModalityT	-0,578	0,500	0,500		
HiveR4	-0,501	0,001	-0,001	0,000	
HiveR6	-0,500	0,000	0,000	0,000	0,500

Linear model fixed coefficients pairwise

Contrast	estimate	SE	df	t,ratio	p,value	p,value,signif
Modalities						
CS - B	-0,122	0,216	18,000	-0,565	0,941	ns
CS - P	-0,018	0,216	18,038	-0,085	1,000	ns
CS - C	-0,244	0,216	17,986	-1,129	0,677	ns
B - P	0,104	0,216	18,013	0,480	0,963	ns
B - C	-0,122	0,216	17,960	-0,564	0,941	ns
P - C	-0,225	0,216	17,999	-1,044	0,727	ns
Hives						
R3 - R4	-0,302	0,187	17,965	-1,615	0,265	ns
R3 - R6	-0,186	0,187	18,048	-0,996	0,588	ns
R4 - R6	0,115	0,187	17,986	0,617	0,813	ns

Appendix 3A – Analysis of modalities on larvae’s gene expression: Fixed effects were included in the model: modalities (Control: C, Control Solvent: CS, Boscalid: B and Pictor Pro: P) and grafted hives (R3 and R4). Pairwise comparisons were performed with Tukey's HSD to identify differences between modalities. Pairwise comparison (Tukey's HSD): * when $p < 0.05$; ** when $p < 0.01$.

contrast	estimate	SE	df	t,ratio	p,value	p,value,signif
cytochrome p450						
CS - B	0,106	0,154	54,000	0,688	0,901	ns
CS - P	0,097	0,154	54,000	0,631	0,922	ns
CS - T	0,266	0,159	54,000	1,673	0,348	ns
B - P	-0,009	0,146	54,000	-0,060	1,000	ns
B - C	0,160	0,151	54,000	1,059	0,716	ns
P - C	0,169	0,151	54,000	1,117	0,681	ns
catalase						
CS - B	0,251	0,073	54,000	3,442	0,006	**
CS - P	0,147	0,073	54,000	2,016	0,195	ns
CS - T	0,129	0,075	54,000	1,721	0,323	ns
B - P	-0,104	0,069	54,000	-1,507	0,441	ns
B - C	-0,122	0,071	54,000	-1,701	0,333	ns
P - C	-0,018	0,071	54,000	-0,246	0,995	ns
prophenoloxidase						
CS - B	0,106	0,154	54,000	0,688	0,901	ns
CS - P	0,097	0,154	54,000	0,631	0,922	ns
CS - T	0,266	0,159	54,000	1,673	0,348	ns
B - P	-0,009	0,146	54,000	-0,060	1,000	ns
B - C	0,160	0,151	54,000	1,059	0,716	ns
P - C	0,169	0,151	54,000	1,117	0,681	ns
cytochrome b						
CS - B	0,452	0,166	54,000	2,720	0,042	*
CS - P	0,194	0,166	54,000	1,166	0,650	ns
CS - T	0,461	0,171	54,000	2,693	0,045	*
B - P	-0,258	0,157	54,000	-1,642	0,364	ns
B - C	0,010	0,163	54,000	0,059	1,000	ns
P - C	0,268	0,163	54,000	1,645	0,363	ns
succinate dehydrogenase						
CS - B	0,324	0,181	54,000	1,789	0,290	ns
CS - P	0,343	0,181	54,000	1,894	0,243	ns
CS - T	0,334	0,187	54,000	1,790	0,289	ns
B - P	0,019	0,171	54,000	0,110	1,000	ns
B - C	0,010	0,177	54,000	0,058	1,000	ns
P - C	-0,009	0,177	54,000	-0,049	1,000	ns
vitellogenin						
CS - B	0,071	0,395	54,000	0,181	0,998	ns
CS - P	-0,127	0,395	54,000	-0,322	0,988	ns
CS - T	0,358	0,408	54,000	0,878	0,816	ns
B - P	-0,199	0,374	54,000	-0,531	0,951	ns
B - C	0,287	0,387	54,000	0,740	0,880	ns
P - C	0,485	0,387	54,000	1,254	0,596	ns
Hexamerin						
CS - B	0,299	0,231	54,000	1,298	0,568	ns
CS - P	0,038	0,231	54,000	0,166	0,998	ns
CS - T	0,084	0,238	54,000	0,353	0,985	ns
B - P	-0,261	0,218	54,000	-1,195	0,633	ns
B - C	-0,215	0,226	54,000	-0,953	0,777	ns
P - C	0,046	0,226	54,000	0,202	0,997	ns

Appendix 3B - Analysis of hive of origin on larvae's gene expression: fixed effects were included in the model. Modalities (Control : C, Control Solvent : CS, Boscalid : B and Pictor Pro : P) and grafted hives (R3 and R4). Pairwise comparisons were performed with Tukey's HSD to identify differences between hive. Pairwise comparison (Tukey's HSD) : * when $p < 0.05$; ** when $p < 0.01$ and *** when $p < 0.01$.

contrast	estimate	SE	df	t,ratio	p,value	p,value,signif
cytochrome p450						
R3 - R4	8,366	1,677	51,000	4,990	<0,001	***
catalase						
R3 - R4	-0,191	0,051	54,000	-3,746	<0,001	***
prophenoloxidase						
R3 - R4	-0,248	0,107	54,000	-2,309	0,025	**
cytochrome b						
R3 - R4	-0,632	0,116	54,000	-5,453	<0,001	***
succinate dehydrogenase						
R3 - R4	-0,792	0,126	54,000	-6,273	<0,001	***
vitellogenin						
R3 - R4	-1,396	0,276	54,000	-5,063	<0,001	***
Hexamerin						
R3 - R4	-1,015	0,161	54,000	-6,315	<0,001	***

Appendix 4A – Analysis of modalities on worker’s gene expression : fixed effects were included in the model: modalities (Control : C, Control Solvent : CS, Boscalid : B and Pictor Pro : P) and grafted hives (R3 and R4). Pairwise comparisons were performed with Tukey's HSD to identify differences between modalities. Pairwise comparison (Tukey's HSD) : td when $p < 0.01$;* when $p < 0.05$.

contrast	estimate	SE	df	t,ratio	p,value	p,value,signif
cytochrome p450						
CS - B	0,265	0,128	57,000	2,072	0,175	ns
CS - P	0,033	0,128	57,000	0,254	0,994	ns
CS - T	0,166	0,132	57,000	1,253	0,596	ns
B - P	-0,232	0,128	57,000	-1,818	0,276	ns
B - C	-0,099	0,132	57,000	-0,749	0,877	ns
P - C	0,133	0,132	57,000	1,007	0,746	ns
catalase						
CS - B	-0,012	0,104	57,000	-0,119	0,999	ns
CS - P	-0,105	0,104	57,000	-1,010	0,744	ns
CS - T	0,133	0,108	57,000	1,232	0,610	ns
B - P	-0,093	0,104	57,000	-0,891	0,809	ns
B - C	0,145	0,108	57,000	1,347	0,538	ns
P - C	0,238	0,108	57,000	2,208	0,133	ns
prophenoloxidase						
CS - B	0,265	0,128	57,000	2,072	0,175	ns
CS - P	0,033	0,128	57,000	0,254	0,994	ns
CS - T	0,166	0,132	57,000	1,253	0,596	ns
B - P	-0,232	0,128	57,000	-1,818	0,276	ns
B - C	-0,099	0,132	57,000	-0,749	0,877	ns
P - C	0,133	0,132	57,000	1,007	0,746	ns
cytochrome b						
CS - B	0,463	0,167	56,000	2,779	0,036	*
CS - P	0,214	0,164	56,000	1,304	0,564	ns
CS - T	0,219	0,170	56,000	1,291	0,573	ns
B - P	-0,249	0,167	56,000	-1,497	0,446	ns
B - C	-0,244	0,172	56,000	-1,417	0,494	ns
P - C	0,005	0,170	56,000	0,031	1,000	ns
succinate dehydrogenase						
CS - B	0,514	0,208	56,000	2,476	0,075	td
CS - P	0,257	0,204	56,000	1,259	0,593	ns
CS - T	0,544	0,212	56,000	2,572	0,060	td
B - P	-0,257	0,208	56,000	-1,238	0,606	ns
B - C	0,030	0,215	56,000	0,138	0,999	ns
P - C	0,287	0,212	56,000	1,356	0,532	ns
vitellogenin						
CS - B	-0,835	0,310	57,000	-2,691	0,045	*
CS - P	-0,103	0,310	57,000	-0,332	0,987	ns
CS - T	-0,041	0,321	57,000	-0,128	0,999	ns
B - P	0,732	0,310	57,000	2,359	0,097	td
B - C	0,794	0,321	57,000	2,471	0,075	td
P - C	0,062	0,321	57,000	0,192	0,997	ns
Hexamerin						
CS - B	0,108	0,234	57,000	0,464	0,967	ns
CS - P	-0,115	0,234	57,000	-0,493	0,960	ns
CS - T	0,196	0,242	57,000	0,809	0,850	ns
B - P	-0,224	0,234	57,000	-0,957	0,774	ns
B - C	0,087	0,242	57,000	0,360	0,984	ns
P - C	0,311	0,242	57,000	1,285	0,576	ns

Appendix 4B – Analysis of hive of origin on worker's gene expression : fixed effects were included in the model: modalities (Control : C, Control Solvent : CS, Boscalid : B and Pictor Pro : P) and grafted hives (R3 and R4). Pairwise comparisons were performed with Tukey's HSD to identify differences between hive. Pairwise comparison (Tukey's HSD): * when $p < 0.05$; ** when $p < 0.01$ and *** when $p < 0.01$.

contrast	estimate	SE	df	t,ratio	p,value	p,value,signif
cytochrome p450						
R3 - R4	0,539	0,563	57,000	0,957	0,343	ns
catalase						
R3 - R4	0,496	0,075	57,000	6,630	<0,001	***
prophenoloxidase						
R3 - R4	-0,246	0,092	57,000	-2,683	0,010	**
cytochrome b						
R3 - R4	0,014	0,119	56,000	0,114	0,910	ns
succinate dehydrogenase						
R3 - R4	-1,880	0,148	56,000	-12,693	<0,001	***
vitellogenin						
R3 - R4	-1,000	0,223	57,000	-4,487	<0,001	***
Hexamerin						
R3 - R4	0,069	0,168	57,000	0,411	0,683	ns

ANNEXES DU CHAPITRE 4

Annex : Pesticides and parasite cocktail : effects of tri-exposure at larvae stage on honeybees' life history traits

Content

Table 1 Dose range and GLMM statistical values for bee larvae used in the ecotoxicological test.

Table 2 Details of the sample size of the test sessions.

Table 3 Analysis of worker bee survival data to D18 when exposed or co-exposed in the larval stage.

Table 4 Analysis of *Apis mellifera* pre-pupal weight data in case of exposure or co-exposure at the larval stage.

Table 5 Analysis of pupation success data of worker bees in case of exposure or co-exposure at larval stage.

Table 6 Analysis of emergence success data of worker bees in case of exposure or co-exposure at larval stage.

Table 1. Dose range and GLMM statistical values for bee larvae used in the ecotoxicological test.

Table 1-A : Range dose used for ecotoxicology test for pyriproxyfen, boscalid and *P. larvae* exposure.

pyriproxyfen	boscalid	<i>P. larvae</i>
Controls	Controls	Controls
6.25 ng/larvae – 40 ppb	3 µg/larvae – 20 ppm	5 spores/larvae
12.5 ng/larvae – 80 ppb	6.25 µg/larvae – 40 ppm	10 spores/larvae
25 ng/larvae – 160 ppb	12.5 µg/larvae – 80 ppm	20 spores/larvae
50 ng/larvae – 320 ppb	25 µg/larvae – 160 ppm	40 spores/larvae
100 ng/larvae – 640 ppb	50 µg/larvae – 320 ppm	80 spores/larvae

Table 1-B : Statistical values of the GLMM to see effects of exposures on cumulative mortality rate at D23 (M) in larvae bees. n indicates the number of bees tested per treatment groups and P value refers to Wald test probabilities (z). 48 larvae were tested /doses/exposure.

	M (%)	Estimate	±S.E.	z	P
boscalid					
Intercept		-1.47	0.26	-5.61	0.000 ***
20 ppm	27.1	0.48	0.42	1.14	0.254
40 ppm	39.6	1.04	0.39	2.65	0.008 **
80 ppm	22.9	0.25	0.43	0.59	0.557
160 ppm	35.4	0.87	0.40	2.17	0.030 *
320 ppm	37.5	0.96	0.40	2.41	0.016 *
<i>P. larvae</i>					
Intercept		-0.69	0.22	-3.20	0.001 **
5 spores	45.83	0.53	0.36	1.46	0.146
10 sp/larvae	54.1	0.86	0.36	2.38	0.017 *
20 sp/larvae	41.7	0.36	0.36	0.98	0.327
40 sp/larvae	56.3	0.94	0.36	2.60	0.009 **
80 sp/larvae	77.1	1.91	0.41	4.70	0.000 ***
pyriproxyfen					
Intercept		-0.74	0.29	-2.50	0.012 *
40 ppb	20.68	-0.31	0.40	-0.78	0.434
80 ppb	33.8	0.10	0.38	0.26	0.799
160 ppb	70.9	1.32	0.37	3.53	0.000 ***
320 ppb	74.8	1.70	0.39	4.36	0.000 ***
640 ppb	96.8	3.97	0.76	5.23	0.000 ***

Table 2. Details of the sample size of the test sessions. Each exposure was replicated on 3 out of 2 hives of larval origin in each test session. The number of individuals corresponds to the number of individuals that were observed for the analyses of individual survival, pupation success and emergence success. The number of prenympths weighed corresponds to the number of pre-pupal that were weighed by each modality.

Test Session	Hive	Exposure Modalities	Number of individuals	Number of prenympths weighed
114	R61	1- Solvent Control	31	30
		2- Control	32	29
		3- Boscalid	29	22
		4- Pyriproxyfen	32	27
		5- P. larvae	32	27
		6- Boscalid & Pyriproxyfen	32	28
		7- Boscalid & P. larvae	30	25
		8- Pyriproxyfen & P. larvae	32	23
		9- Boscalid & Pyriproxyfen & P. larvae	32	25
	R62	1- Solvent Control	32	24
		2- Control	32	30
		3- Boscalid	28	20
		4- Pyriproxyfen	32	21
		5- P. larvae	32	12
		6- Boscalid & Pyriproxyfen	32	25
		7- Boscalid & P. larvae	32	11
		8- Pyriproxyfen & P. larvae	32	16
		9- Boscalid & Pyriproxyfen & P. larvae	32	15
	R64	1- Solvent Control	32	26
		2- Control	32	30
		3- Boscalid	31	18
		4- Pyriproxyfen	32	16
		5- P. larvae	32	20
		6- Boscalid & Pyriproxyfen	32	20
		7- Boscalid & P. larvae	32	20
		8- Pyriproxyfen & P. larvae	32	19
		9- Boscalid & Pyriproxyfen & P. larvae	32	22
135	R61	1- Solvent Control	32	29
		2- Control	32	31
		3- Boscalid	32	24
		4- Pyriproxyfen	32	26
		5- P. larvae	32	28
		6- Boscalid & Pyriproxyfen	32	27
		7- Boscalid & P. larvae	32	24
		8- Pyriproxyfen & P. larvae	32	30
		9- Boscalid & Pyriproxyfen & P. larvae	32	28
	R62	1- Solvent Control	32	24
		2- Control	32	29
		3- Boscalid	32	17
		4- Pyriproxyfen	32	21
		5- P. larvae	32	20
		6- Boscalid & Pyriproxyfen	32	14
		7- Boscalid & P. larvae	32	17
		8- Pyriproxyfen & P. larvae	32	17
		9- Boscalid & Pyriproxyfen & P. larvae	32	19
	R64	1- Solvent Control	32	29
		2- Control	32	29
		3- Boscalid	32	25
		4- Pyriproxyfen	32	21
		5- P. larvae	32	29
		6- Boscalid & Pyriproxyfen	32	30
		7- Boscalid & P. larvae	32	26
		8- Pyriproxyfen & P. larvae	32	23
		9- Boscalid & Pyriproxyfen & P. larvae	32	30

Table 3. Analysis of worker bee survival data to D18 when exposed or co-exposed in the larval stage. (SC : Solvent Control ; C : Control ; B : Boscalid ; P : Pyriproxyphen ; L : *P. Larvae* ; B & P : Boscalid & Pyriproxyphen ; B & L : Boscalid & *P. Larvae* ; P : Pyriproxyphen & *P. Larvae* ; B & P & L : Boscalid & Pyriproxyphen & *P. Larvae*).

Table 3-A : Cox regression analysis with a random effect

Fixed coefficients	coef	exp(coef)	se(coef)	z	p
C	-0,551	0,576	0,246	-2,240	0,025
B	0,527	1,694	0,198	2,660	0,008
P	0,544	1,723	0,194	2,800	0,005
L	0,491	1,634	0,197	2,500	0,013
B & P	0,410	1,507	0,199	2,060	0,040
B & L	0,690	1,994	0,192	3,600	<0,001
P & L	0,591	1,806	0,194	3,050	0,002
B & P & L	0,636	1,889	0,192	3,310	0,001
R62	0,712	2,039	0,107	6,640	<0,001
R64	0,238	1,269	0,115	2,070	0,039

Random effects group	Variable	Std Dev	Variance
session_test/ID plate	(Intercept)	0,008	5.808531e-05
session_test	(Intercept)	0,136	1.848822e-02

Analysis of Deviance Table (Type II tests)

	LR Chisq	Df	Pr(>Chisq)
Exposure	8,000	44.958	0,001
hive	2,000	49.441	0,001

Table 2-B : Tuckey pairwise comparison the grafted hives

Contrast	Estimate	SE	z,ratio	p,value
R61 - R62	-0,712	0,107	-6,642	<0,001
R61 - R64	-0,238	0,115	-2,067	0,097
R62 - R64	0,474	0,101	4,706	<0,001

Table 3-C: Sidak pairwise comparison of Solvent Control vs exposures modalities

Contrast	Estimate	SE	z,ratio	p,value
C - SC	-0,551	0,246	-2,244	0,182
B - SC	0,527	0,198	2,663	0,060
P - SC	0,544	0,194	2,797	0,041
L - SC	0,491	0,197	2,496	0,096
B & P - SC	0,410	0,199	2,057	0,277
B & L - SC	0,690	0,192	3,596	0,003
P & L - SC	0,591	0,194	3,047	0,018
B & P & L - SC	0,636	0,192	3,307	0,008

Table 3-D: Tuckey pairwise comparison of exposures modalities vs exposures modalities

Contrast		Estimate	SE	z,ratio	p.value
SC	- C	0,551	0,246	2,244	0,377
SC	- B	-0,527	0,198	-2,663	0,161
SC	- P	-0,544	0,194	-2,797	0,116
SC	- L	-0,491	0,197	-2,496	0,234
SC	- B & P	-0,410	0,199	-2,057	0,503
SC	- B & L	-0,690	0,192	-3,596	0,010
SC	- P & L	-0,591	0,194	-3,047	0,059
SC	- B & P & L	-0,636	0,192	-3,307	0,026
C	- B	-1,078	0,230	-4,684	<0,001
C	- P	-1,095	0,227	-4,820	<0,001
C	- L	-1,042	0,229	-4,548	<0,001
C	- B & P	-0,961	0,231	-4,155	<0,001
C	- B & L	-1,241	0,225	-5,516	<0,001
C	- P & L	-1,142	0,227	-5,037	<0,001
C	- B & P & L	-1,187	0,225	-5,267	<0,001
B	- P	-0,017	0,174	-0,097	1,000
B	- L	0,036	0,177	0,204	1,000
B	- B & P	0,117	0,180	0,651	0,999
B	- B & L	-0,163	0,172	-0,951	0,990
B	- P & L	-0,064	0,174	-0,368	1,000
B	- B & P & L	-0,109	0,172	-0,635	0,999
P	- L	0,053	0,173	0,305	1,000
P	- B & P	0,134	0,176	0,761	0,998
P	- B & L	-0,146	0,168	-0,874	0,994
P	- P & L	-0,047	0,170	-0,277	1,000
P	- B & P & L	-0,092	0,168	-0,550	1,000
L	- B & P	0,081	0,178	0,454	1,000
L	- B & L	-0,199	0,170	-1,171	0,963
L	- P & L	-0,100	0,172	-0,580	1,000
L	- B & P & L	-0,145	0,171	-0,851	0,995
B & P	- B & L	-0,280	0,173	-1,619	0,795
B & P	- P & L	-0,181	0,175	-1,032	0,983
B & P	- B & P & L	-0,226	0,174	-1,303	0,931
B & L	- P & L	0,099	0,167	0,595	1,000
B & L	- B & P & L	0,054	0,165	0,327	1,000
P & L	- B & P & L	-0,045	0,167	-0,271	1,000

Table 4. Analysis of *Apis mellifera* pre-pupal weight data in case of exposure or co-exposure at the larval stage. (SC : Solvent Control ; C : Control ; B : Boscalid ; P : Pyriproxyphen; L : P. Larvae ; B & P : Boscalid & Pyriproxyphen ; B & L : Boscalid & P. Larvae ; P: Pyriproxyphen & P. Larvae ; B & P & L : Boscalid & Pyriproxyphen & P. Larvae).

Table 4-A: Random-effects linear mixed effects model

Fixed effects	Estimate	Std, Error	t value
(Intercept)	141,119	3,895	36,231
C	-3,400	4,637	-0,733
B	-5,191	4,672	-1,111
P	-1,642	4,666	-0,352
L	-2,034	4,664	-0,436
B & P	-11,729	4,657	-2,519
B & L	-1,929	4,675	-0,413
P & L	-1,638	4,671	-0,351
B & P & L	1,761	4,661	0,378
R62	2,665	0,826	3,228
R64	10,461	0,778	13,443

Random effects group,	Name	Variance	Std,Dev,
IDplate:session_test	(Intercept)	39,760	6,306
session_test	(Intercept)	8,390	2,897
Residual		136,810	11,696

Analysis of Deviance Table (Type II tests)

	LR Chisq	Df	Pr(>Chisq)
Exposure	10.859	8,000	0.2098
hive	190.030	2,000	<2e-16

Table 4-B: Tuckey pairwise comparison the grafted hive of the transplanted larvae

Contrast		Estimate	SE	df	t,ratio	p,value
R61	- R62	-2,665	0,826	1232,253	-3,228	0,004
R61	- R64	-10,461	0,778	1231,918	-13,441	<0,001
R62	- R64	-7,797	0,847	1232,647	-9,205	<0,001

Table 4-C: Tuckey pairwise comparison of exposures modalities vs exposures modalities.

Contrast	Estimate	SE	df	t,ratio	p,value
SC - C	3,400	4,637	25,141	0,733	0,998
SC - B	5,191	4,672	25,898	1,111	0,967
SC - P	1,642	4,666	25,776	0,352	1,000
SC - L	2,034	4,664	25,736	0,436	1,000
SC - B & P	11,729	4,657	25,580	2,518	0,269
SC - B & L	1,929	4,675	25,974	0,413	1,000
SC - P & L	1,638	4,671	25,880	0,351	1,000
SC - B & P & L	-1,761	4,661	25,671	-0,378	1,000
C - B	1,791	4,663	25,713	0,384	1,000
C - P	-1,759	4,657	25,591	-0,378	1,000
C - L	-1,366	4,656	25,558	-0,293	1,000
C - B & P	8,329	4,649	25,399	1,792	0,687
C - B & L	-1,471	4,667	25,795	-0,315	1,000
C - P & L	-1,762	4,663	25,700	-0,378	1,000
C - B & P & L	-5,161	4,653	25,492	-1,109	0,967
B - P	-3,549	4,692	26,356	-0,756	0,997
B - L	-3,157	4,690	26,315	-0,673	0,999
B - B & P	6,538	4,683	26,158	1,396	0,889
B - B & L	-3,262	4,701	26,555	-0,694	0,998
B - P & L	-3,553	4,697	26,461	-0,757	0,997
B - B & P & L	-6,952	4,687	26,249	-1,483	0,853
P - L	0,392	4,685	26,194	0,084	1,000
P - B & P	10,087	4,678	26,036	2,156	0,460
P - B & L	0,288	4,696	26,436	0,061	1,000
P - P & L	-0,004	4,691	26,335	-0,001	1,000
P - B & P & L	-3,403	4,682	26,131	-0,727	0,998
L - B & P	9,695	4,676	25,990	2,073	0,511
L - B & L	-0,104	4,693	26,379	-0,022	1,000
L - P & L	-0,396	4,689	26,288	-0,084	1,000
L - B & P & L	-3,795	4,680	26,077	-0,811	0,995
B & P - B & L	-9,800	4,686	26,229	-2,091	0,500
B & P - P & L	-10,091	4,682	26,136	-2,155	0,461
B & P - B & P & L	-13,490	4,673	25,926	-2,887	0,138
B & L - P & L	-0,291	4,700	26,529	-0,062	1,000
B & L - B & P & L	-3,690	4,690	26,315	-0,787	0,996
P & L - B & P & L	-3,399	4,686	26,226	-0,725	0,998

Table 5. Analysis of pupation success data of worker bees in case of exposure or co-exposure at larval stage. (SC : Solvent Control ; C : Control ; B : Boscalid ; P : Pyriproxyphen ; L : P. Larvae ; B & P : Boscalid & Pyriproxyphen ; B & L : Boscalid & P. Larvae ; P : Pyriproxyphen & P. Larvae ; B & P & L : Boscalid & Pyriproxyphen & P. Larvae).

Table 5-A : Binomial generalised linear model

Fixed coefficients	Estimate	Std, Error	z value	Pr(> z)
(Intercept)	2,094	0,230	9,109	0,000
C	0,847	0,337	2,513	0,012
B	-0,920	0,258	-3,567	<0,001
P	-0,970	0,255	-3,805	<0,001
L	-0,763	0,258	-2,953	0,003
B & P	-0,625	0,261	-2,392	0,017
B & L	-1,130	0,253	-4,461	<0,001
P & L	-1,019	0,254	-4,011	<0,001
B & P & L	-0,790	0,258	-3,062	0,002
Hive R62	-1,153	0,144	-7,986	<0,001
Hive R64	-0,533	0,150	-3,558	<0,001
<u>session_test2</u>	0,396	0,114	3,465	0,001

Analysis of Deviance Table (Type II tests)

	LR Chisq	Df	Pr(>Chisq)
Exposure	79.325	8,000	6.683e-14
hive	68.838	2,000	1.127e-15
session_test	12.099	1,000	0.0005045

Table 5-B: Tuckey comparison of test session

Contrast	Estimate	SE	z,ratio	p,value
114 - 135	-0,396	0,114	-3,465	0,001

Table 5-C: Tuckey pairwise comparison the grafted hive

Contrast	Estimate	SE	z,ratio	p,value
R61 - R62	1,153	0,144	7,986	0,000
R61 - R64	0,533	0,150	3,558	0,001
R62 - R64	-0,621	0,132	-4,685	0,000

Table 5-D: Sidak pairwise comparison of Solvent Control vs exposures modalities

Contrast	Estimate	SE	z,ratio	p,value
C - SC	0,847	0,337	2,513	0,092
B - SC	-0,920	0,258	-3,567	0,003
P - SC	-0,970	0,255	-3,805	0,001
L - SC	-0,763	0,258	-2,953	0,025
B & P - SC	-0,625	0,261	-2,392	0,127
B & L - SC	-1,130	0,253	-4,461	<0,001
P & L - SC	-1,019	0,254	-4,011	<0,001
B & P & L - SC	-0,790	0,258	-3,062	0,017

Table 5-E: Tuckey pairwise comparison of exposures modalities vs exposures modalities

Contrast	Estimate	SE	z,ratio	p,value
SC - C	-0,847	0,337	-2,513	0,226
SC - B	0,920	0,258	3,567	0,011
SC - P	0,970	0,255	3,805	0,004
SC - L	0,763	0,258	2,953	0,077
SC - B & P	0,625	0,261	2,392	0,288
SC - B & L	1,130	0,253	4,461	<0,001
SC - P & L	1,019	0,254	4,011	0,002
SC - B & P & L	0,790	0,258	3,062	0,056
C - B	1,767	0,317	5,574	<0,001
C - P	1,817	0,314	5,777	<0,001
C - L	1,610	0,317	5,074	<0,001
C - B & P	1,472	0,320	4,604	<0,001
C - B & L	1,976	0,313	6,311	<0,001
C - P & L	1,866	0,314	5,945	<0,001
C - B & P & L	1,636	0,317	5,165	<0,001
B - P	0,050	0,227	0,218	1,000
B - L	-0,157	0,231	-0,681	0,999
B - B & P	-0,295	0,235	-1,259	0,943
B - B & L	0,209	0,225	0,930	0,991
B - P & L	0,099	0,226	0,438	1,000
B - B & P & L	-0,131	0,231	-0,566	1,000
P - L	-0,207	0,228	-0,908	0,993
P - B & P	-0,345	0,231	-1,492	0,860
P - B & L	0,160	0,222	0,722	0,999
P - P & L	0,050	0,223	0,223	1,000
P - B & P & L	-0,180	0,227	-0,793	0,997
L - B & P	-0,138	0,235	-0,587	1,000
L - B & L	0,367	0,226	1,624	0,792
L - P & L	0,256	0,227	1,130	0,970
L - B & P & L	0,027	0,231	0,116	1,000
B & P - B & L	0,505	0,229	2,201	0,405
B & P - P & L	0,394	0,230	1,712	0,739
B & P - B & P & L	0,165	0,234	0,702	0,999
B & L - P & L	-0,110	0,221	-0,500	1,000
B & L - B & P & L	-0,340	0,225	-1,510	0,851
P & L - B & P & L	-0,230	0,226	-1,015	0,985

Table 6. Analysis of emergence success data of worker bees in case of exposure or co-exposure at larval stage. (SC : Solvent Control ; C : Control ; B : Boscalid ; P : Pyriproxyphen ; L : P. Larvae ; B & P : Boscalid & Pyriproxyphen ; B & L : Boscalid & P. Larvae ; P : Pyriproxyphen & P. Larvae ; B & P & L : Boscalid & Pyriproxyphen & P. Larvae).

Table 5-A : Binomial generalised linear model

Fixed coefficients	Estimate	Std. Error	z value	Pr(> z)
(Intercept)	1,094	0,189	5,777	<0,001
C	0,298	0,242	1,230	0,219
B	-0,462	0,228	-2,029	0,042
P	-0,630	0,224	-2,817	<0,001
L	-0,469	0,225	-2,082	0,037
B & P	-0,585	0,224	-2,609	0,009
B & L	-0,648	0,224	-2,891	0,004
P & L	-0,698	0,223	-3,126	0,002
B & P & L	-0,787	0,223	-3,534	<0,001
Hive R62	-0,882	0,128	-6,899	<0,001
Hive R64	-0,295	0,130	-2,271	0,023
<u>session_test2</u>	0,609	0,104	5,861	<0,001

Analysis of Deviance Table (Type II tests)	LR Chisq	Df	Pr(>Chisq)
Exposure	39.511	8,000	<0,001
hive	51.317	2,000	<0,001
session_test	34.855	1,000	<0,001

Table 6-B : Tuckey pairwise comparison the grafted hive of the transplanted larvae

Contrast		Estimate	SE	z,ratio	p,value
R61	- R62	0,882	0,128	6,899	<0,001
R61	- R64	0,295	0,130	2,271	0,060
R62	- R64	-0,586	0,124	-4,728	<0,001

Table 6-C : Tuckey comparison of test session

Contrast		Estimate	SE	z,ratio	p,value
114	- 135	-0,609	0,104	-5,861	<0,001

Table 6-D : Sidak pairwise comparison of Solvent Control vs exposures modalities.

Contrast		Estimate	SE	z,ratio	p,value
C	- SC	0,297	0,242	1,230	0,861
B	- SC	-0,462	0,228	-2,029	0,293
P	- SC	-0,630	0,224	-2,817	0,038
L	- SC	-0,469	0,225	-2,082	0,262
B & P	- SC	-0,585	0,224	-2,609	0,070
B & L	- SC	-0,648	0,224	-2,891	0,030
P & L	- SC	-0,698	0,223	-3,126	0,014
B & P & L	- SC	-0,787	0,223	-3,534	0,003

Table 6-E : Tuckey pairwise comparison of exposures modalities vs exposures modalities.

Contrast	Estimate	SE	z,ratio	p,value
SC - C	-0,297	0,242	-1,230	0,950
SC - B	0,462	0,228	2,029	0,523
SC - P	0,630	0,224	2,817	0,110
SC - L	0,469	0,225	2,082	0,485
SC - B & P	0,585	0,224	2,609	0,183
SC - B & L	0,648	0,224	2,891	0,091
SC - P & L	0,698	0,223	3,126	0,046
SC - B & P & L	0,787	0,223	3,534	0,012
C - B	0,760	0,236	3,216	0,035
C - P	0,928	0,232	3,994	0,002
C - L	0,767	0,234	3,280	0,029
C - B & P	0,882	0,233	3,792	0,005
C - B & L	0,945	0,233	4,063	0,002
C - P & L	0,995	0,232	4,294	0,001
C - B & P & L	1,085	0,231	4,688	<0,001
B - P	0,168	0,217	0,773	0,998
B - L	0,007	0,219	0,031	1,000
B - B & P	0,122	0,218	0,563	1,000
B - B & L	0,186	0,218	0,853	0,995
B - P & L	0,236	0,217	1,087	0,976
B - B & P & L	0,325	0,216	1,503	0,855
P - L	-0,161	0,215	-0,751	0,998
P - B & P	-0,046	0,213	-0,213	1,000
P - B & L	0,018	0,213	0,083	1,000
P - P & L	0,068	0,212	0,319	1,000
P - B & P & L	0,157	0,212	0,741	0,998
L - B & P	0,116	0,215	0,537	1,000
L - B & L	0,179	0,215	0,831	0,996
L - P & L	0,229	0,214	1,068	0,979
L - B & P & L	0,318	0,214	1,489	0,861
B & P - B & L	0,063	0,214	0,296	1,000
B & P - P & L	0,113	0,213	0,532	1,000
B & P - B & P & L	0,203	0,212	0,954	0,990
B & L - P & L	0,050	0,213	0,235	1,000
B & L - B & P & L	0,139	0,212	0,656	0,999
P & L - B & P & L	0,089	0,211	0,423	1,000

ANNEXES DU CHAPITRE 5

Annex : Co-exposures of lambda-cyhalothrin and triazole fungicides have sub-lethal effects on honeybees and increase the risk of colony mortality

Content

- Figure S1** Demographic growth of simulated colonies (adult population size) for the sensitivity analysis of the simulated effect of shorter-lived bees on honey bee colony survival.
- Figure S2** Set of 600 simulations ran to analyse the effect of pesticide co-exposure induced shorter-lived bees on the colony demographic growth (adult population size).
- Figure S3** Simulated effect of shorter-lived bees from day 76 to day 122 (season 1) on colony dynamics.
- Figure S4** Simulated effect of shorter-lived bees from day 122 to day 168 (season 2) on colony dynamics.
- Figure S5** Simulated effect of shorter-lived bees from day 168 to day 214 (season 3) on colony dynamics.
- Figure S6** Simulated effect of shorter-lived bees from day 76 to day 168 (seasons 1+2) on colony dynamics.
- Figure S7** Simulated effect of shorter-lived bees from day 122 to day 214 (i.e. seasons 2+3) on colony dynamics.

Figure S1. Demographic growth of simulated colonies (adult population size) for the sensitivity analysis of the simulated effect of shorter-lived bees on honeybee colony survival. We computed 100 simulations with an extended gradient of forager mortality rate from the default value of 0.00001 to 0.00006 (i.e. increase coefficient of $\times 6$). X-axis represents the time series in day. Blue lines represent surviving colonies and red lines represent dead colonies with the vertical line indicating the time of colony death. The grey polygons show the period of stress simulated for the different seasons during the colony dynamic. For this sensitivity analysis we considered the maximal duration of stress, corresponding to the combination of the three grey polygons starting before the growth period of the population size (day 76) at the end of the growth period of the population size (day 214).

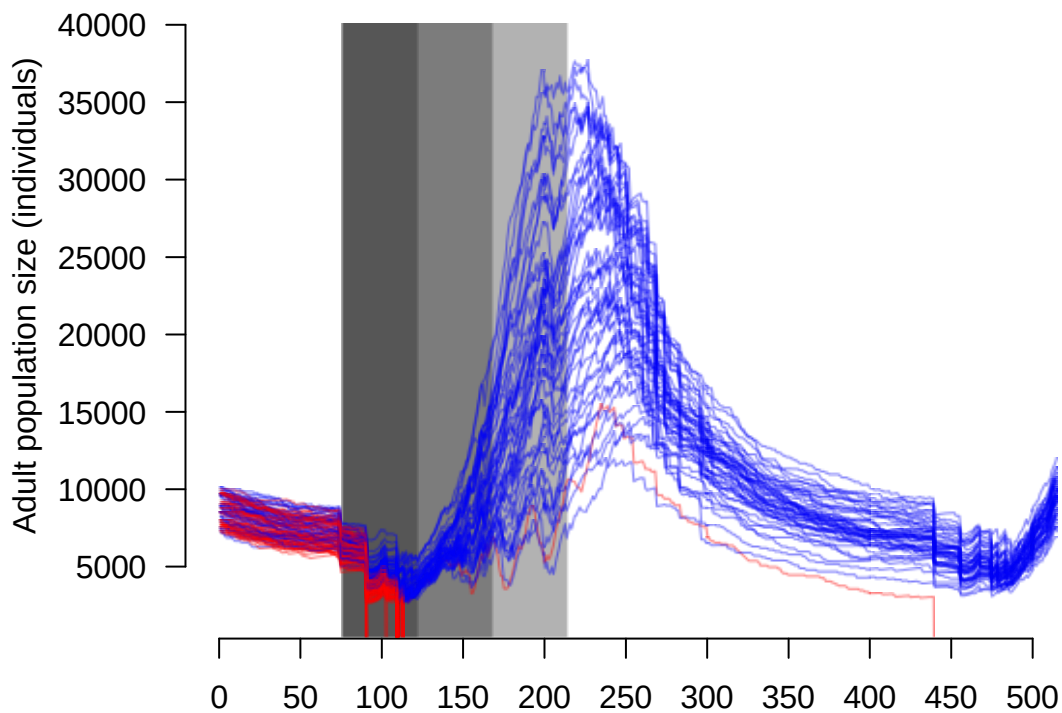


Figure S2. Set of 600 simulations ran to analyse the effect of pesticide co-exposure induced shorter-lived bees on the colony demographic growth (adult population size). X-axis represents the time series in day. Blue lines represent surviving colonies and red lines represent dead colonies with the vertical line indicating the time of colony death (a single colony died in the set of simulations). The grey polygons show the period of stress simulated for the different seasons during the colony dynamic, i.e. before the growth period of the population size (day 76 to day 122, season 1 in dark grey), at the beginning of the growth period of the population size (day 122 to day 168, season 2 in medium grey), and at the end of the growth period of the population size (day 168 to day 214, season 3 in light grey). We ran six scenarios of exposure for different seasons (season 1, season 2, season 3) and by combining the different seasons (seasons 1+2, seasons 2+3, seasons 1+2+3).

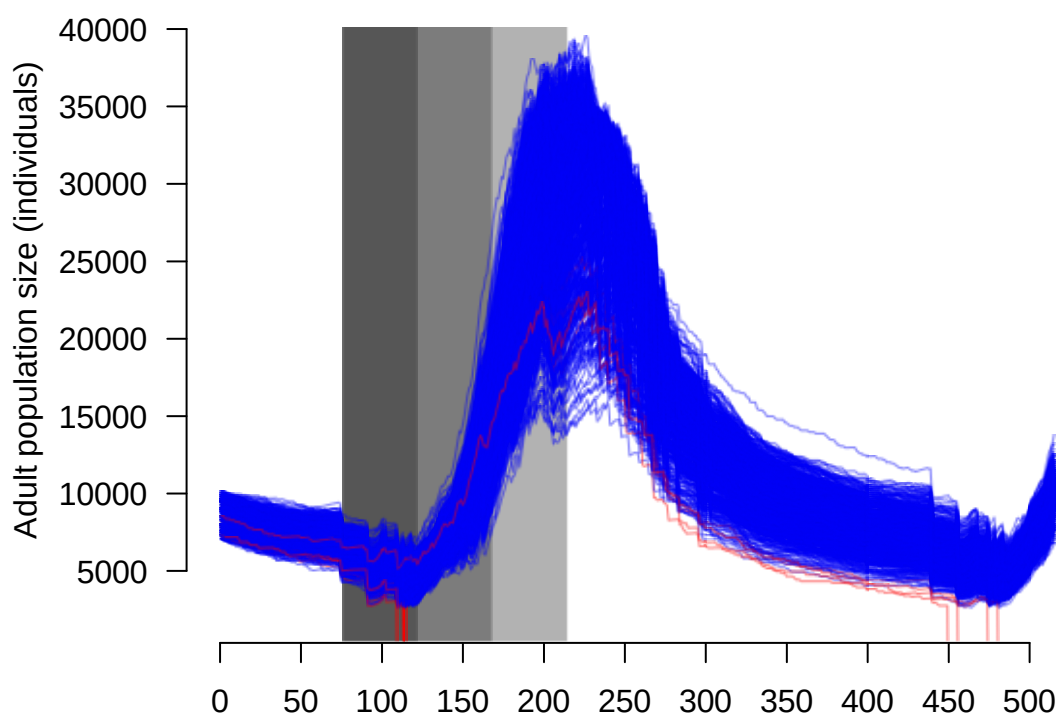


Figure S3. Simulated effect of shorter-lived bees from day 76 to day 122 (season 1) on colony dynamics. (A) Changes in population size were analysed along a gradient of relative effect of pesticide co-exposure simulating shorter-lived bees from day 76 to day 122 (i.e. before the growth period of the population size, season 1). We analysed the direct time effect of simulating shorter-lived bees on changes in population size, as well as delayed effects with +1 month, +2 months and +3 months after the end of the simulated impact. (B) Changes in honey reserves were also analysed along a gradient of relative effect of pesticide co-exposure simulating shorter-lived bees from day 76 to day 122 (i.e. season 1). We analysed the direct time effect of simulating shorter-lived bees on changes in honey reserves, as well as delayed effects with +1 month, +2 months and +3 months after the end of the simulated impact. Lines represent significant effects of the relative effect of pesticide co-exposure (LM predictions) and the colors represent the demographic strength of the colony.

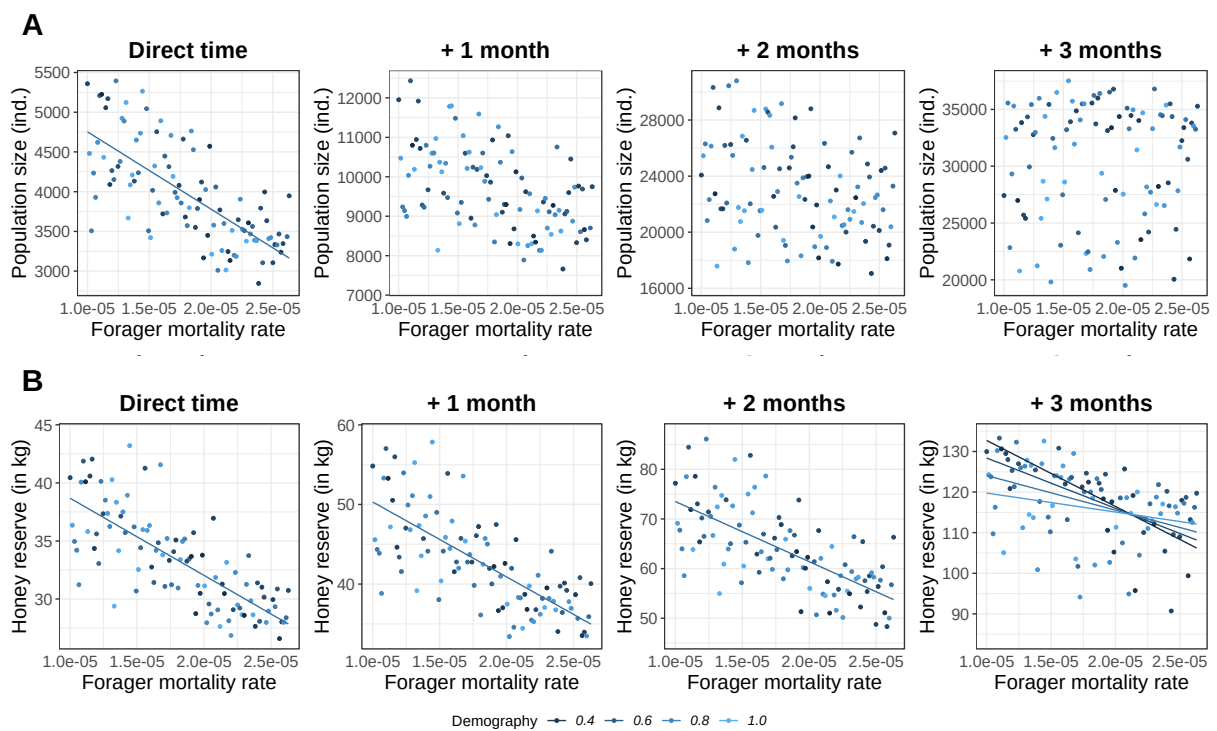


Figure S4. Simulated effect of shorter-lived bees from day 122 to day 168 (season 2) on colony dynamics. (A) Changes in population size were analysed along a gradient of relative effect of pesticide co-exposure simulating shorter-lived bees from day 122 to day 168 (i.e. at the beginning of the growth period of the population size, season 2). We analysed the direct time effect of simulating shorter-lived bees on changes in population size, as well as delayed effects with +1 month, +2 months and +3 months after the end of the simulated impact. (B) Changes in honey reserves were also analysed along a gradient of relative effect of pesticide co-exposure simulating shorter-lived bees from day 122 to day 168 (i.e. season 2). We analysed the direct time effect of simulating shorter-lived bees on changes in honey reserves, as well as delayed effects with +1 month, +2 months and +3 months after the end of the simulated impact. Lines represent significant effects of the relative effect of pesticide co-exposure (LM predictions) and the colors represent the demographic strength of the colony.

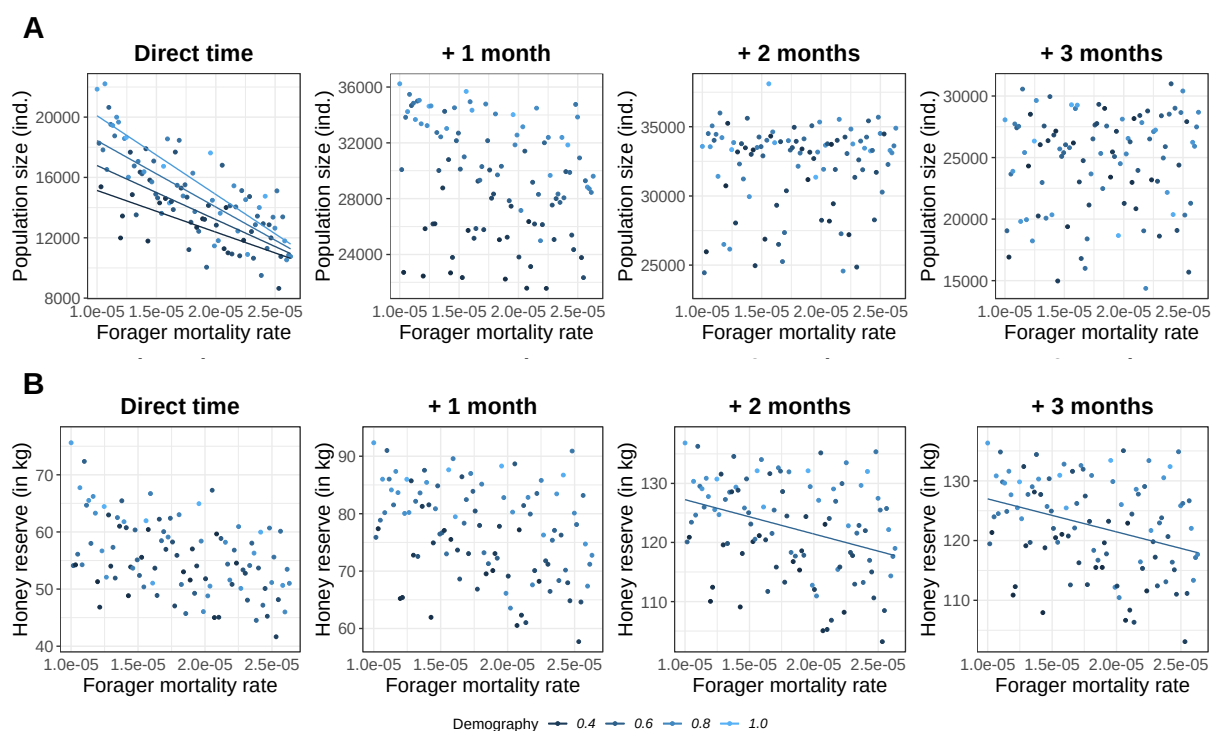


Figure S5. Simulated effect of shorter-lived bees from day 168 to day 214 (season 3) on colony dynamics. (A) Changes in population size were analysed along a gradient of relative effect of pesticide co-exposure simulating shorter-lived bees from day 168 to day 214 (i.e., at the end of the growth period of the population size, season 3). We analysed the direct time effect of simulating shorter-lived bees on changes in population size, as well as delayed effects with +1 month, +2 months and +3 months after the end of the simulated impact. (B) Changes in honey reserves were also analysed along a gradient of relative effect of pesticide co-exposure simulating shorter-lived bees from day 168 to day 214 (i.e. season 3). We analysed the direct time effect of simulating shorter-lived bees on changes in honey reserves, as well as delayed effects with +1 month, +2 months and +3 months after the end of the simulated impact. Lines represent significant effects of the relative effect of pesticide co-exposure (LM predictions) and the colors represent the demographic strength of the colony.

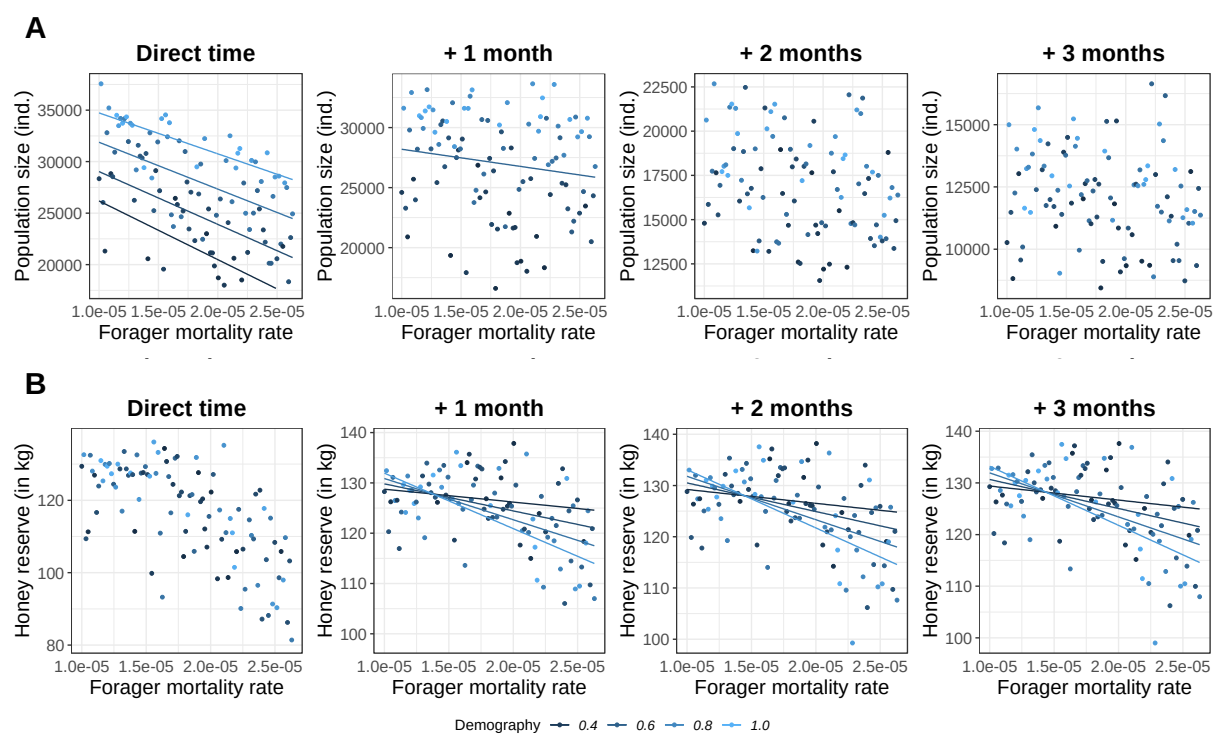


Figure S6. Simulated effect of shorter-lived bees from day 76 to day 168 (seasons 1+2) on colony dynamics. (A) Changes in population size were analysed along a gradient of relative effect of pesticide co-exposure simulating shorter-lived bees from day 76 to day 168 (i.e. seasons 1+2). We analysed the direct time effect of simulating shorter-lived bees on changes in population size, as well as delayed effects with +1 month, +2 months and +3 months after the end of the simulated impact. (B) Changes in honey reserves were also analysed along a gradient of relative effect of pesticide co-exposure simulating shorter-lived bees from day 76 to day 168 (i.e. seasons 1+2). We analysed the direct time effect of simulating shorter-lived bees on changes in honey reserves, as well as delayed effects with +1 month, +2 months and +3 months after the end of the simulated impact. Lines represent significant effects of the relative effect of pesticide co-exposure (LM predictions) and the colours represent the demographic strength of the colony.

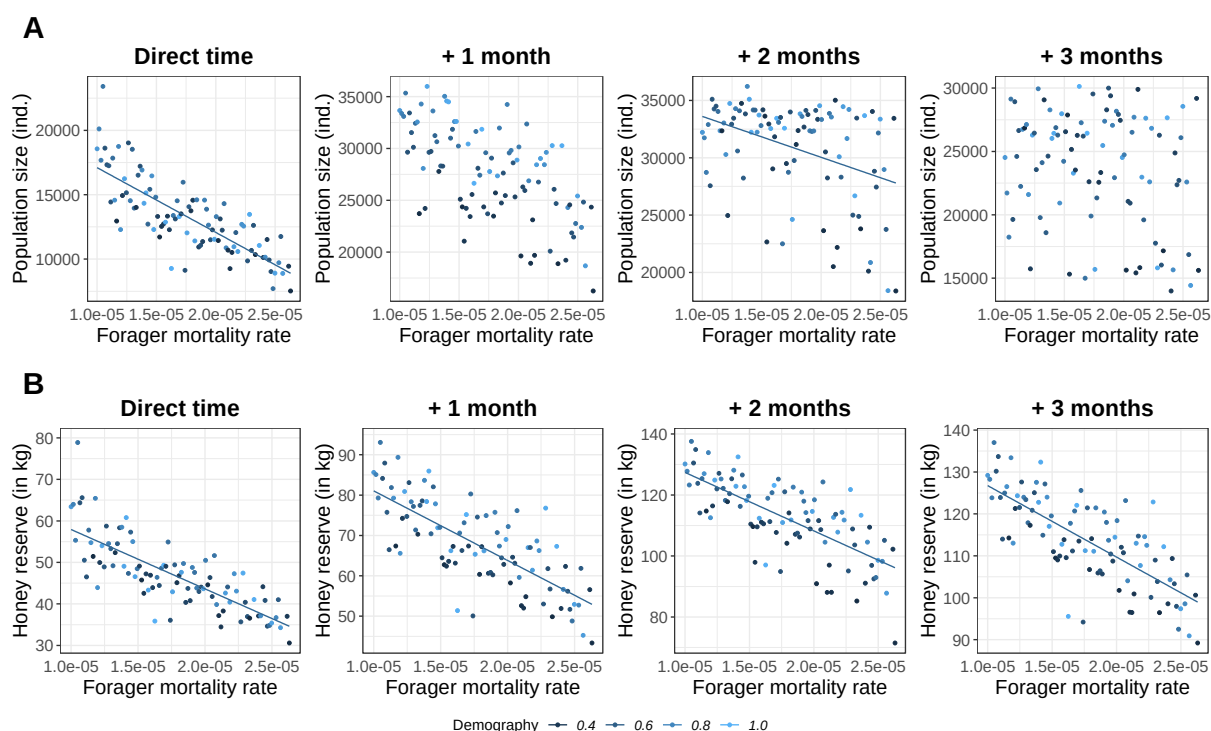
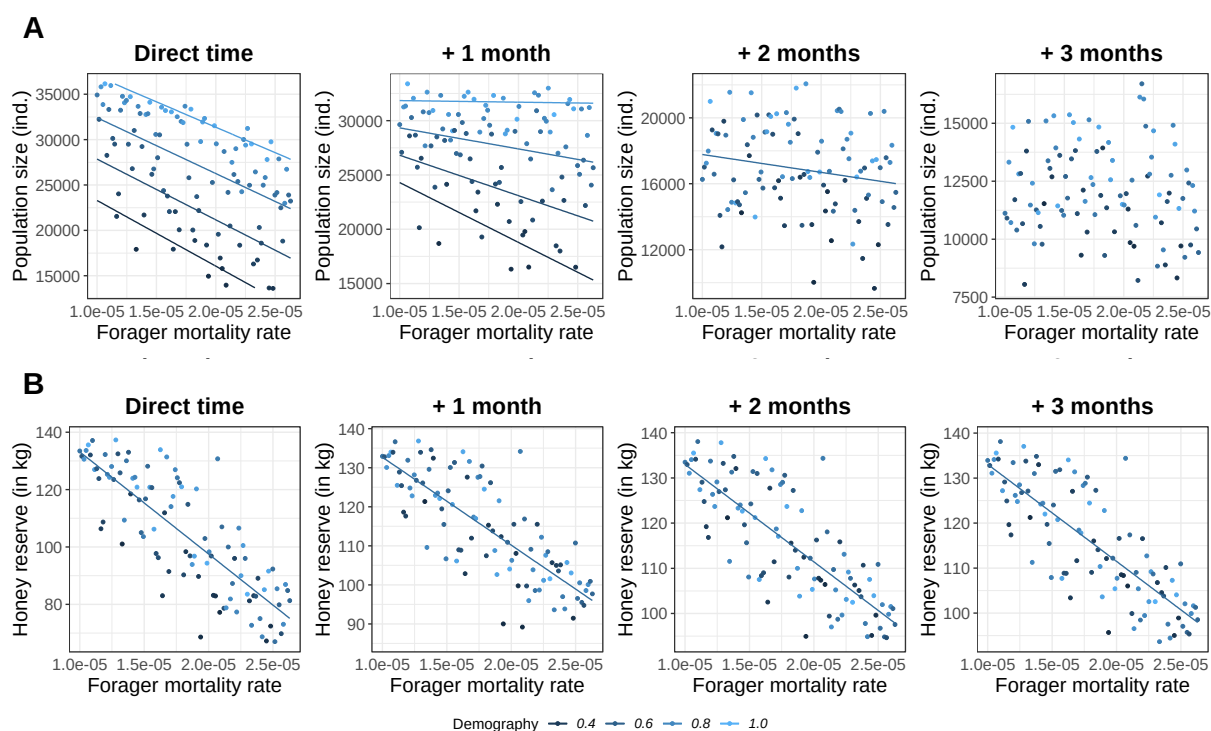


Figure S7. Simulated effect of shorter-lived bees from day 122 to day 214 (i.e. seasons 2+3) on colony dynamics. (A) Changes in population size were analysed along a gradient of relative effect of pesticide co-exposure simulating shorter-lived bees from day 122 to day 214 (i.e. seasons 2+3). We analysed the direct time effect of simulating shorter-lived bees on changes in population size, as well as delayed effects with +1 month, +2 months and +3 months after the end of the simulated impact. (B) Changes in honey reserves were also analysed along a gradient of relative effect of pesticide co-exposure simulating shorter-lived bees from day 122 to day 214 (i.e. seasons 2+3). We analysed the direct time effect of simulating shorter-lived bees on changes in honey reserves, as well as delayed effects with +1 month, +2 months and +3 months after the end of the simulated impact. Lines represent significant effects of the relative effect of pesticide co-exposure (LM predictions) and the colors represent the demographic strength of the colony.



SUMMARY

This thesis is written in the current context of pollinator decline and intensive pesticide use. It is therefore essential, even vital, to understand the consequences of our agricultural practices on biodiversity.

The honeybee (*Apis mellifera*) plays a decisive role as a vital pollinator for crops and biodiversity. However, its populations are currently losing ground due to a variety of factors. As part of our research, we have examined the effects of seven stressors on bee biology, belonging to four distinct categories: (i) an inert element, acetone, (ii) a bacterium, *Paenibacillus larvae*, (iii) two insecticides, thiamethoxam and lambda-cyhalothrin, and (iv) three fungicides, boscalid, tebuconazole and prothioconazole.

This study is part of an approach designed to supervise and monitor the entire life cycle of bees in the hive, using the *in vitro* larval rearing method coupled with an RFID system. This original approach enables us to accurately explore the sublethal effects of early, repeated and delayed exposure, as well as interactions with biotic and abiotic stresses.

By adopting a cross-disciplinary approach, the sublethal effects of these factors could be characterized on physiology, development and behavior at the individual level, while integrating them with population dynamics simulations for a global view of their impact on honeybee colonies. The obtained results provide crucial answers to the challenges posed by the sublethal effects of pesticides due to early exposure in the life of bees. Our approach has led to a better understanding of the delayed effects of pesticides on the life cycle of honeybees, at both individual and colony levels.

Keywords: *Apis Mellifera* ; *In vitro* rearing ; RFID ; Flight activities ; Gene expression ; Ecotoxicology ; Pesticides ; Pathogen – Pesticide Interactions

RESUME

Cette thèse s'inscrit dans le contexte actuel de déclin des pollinisateurs et de l'utilisation intensive des pesticides. Il est donc essentiel, voire capital, de comprendre les conséquences de nos pratiques agricoles sur la biodiversité.

L'abeille mellifère (*Apis mellifera*) joue un rôle crucial en tant que pollinisateur vital pour les cultures et la biodiversité. Cependant, ces populations subissent des pertes importantes dues à divers facteurs. Dans le cadre de nos recherches, nous avons examiné les effets de sept facteurs de stress sur la biologie de l'abeille, appartenant à quatre catégories distinctes : (i) un élément inerte, l'acétone, (ii) une bactérie, *Paenibacillus larvae*, (iii) deux insecticides, le thiaméthoxame et la lambda-cyhalothrine, et (iv) trois fongicides, le boscalid, le tébuconazole et le prothioconazole.

Cette étude s'inscrit dans une démarche visant à contrôler et suivre le cycle de vie complet des abeilles dans la ruche, en utilisant la technique d'élevage larvaire *in vitro* couplée à un système RFID. Cette approche originale nous permet d'explorer avec précision les effets sublétaux dus à des expositions précoces, répétées et différées, ainsi qu'à des interactions avec des stress biotiques et abiotiques.

En adoptant une approche transversale, les effets sublétaux de ces facteurs ont pu être caractérisés sur la physiologie, le développement et le comportement au niveau individuel, tout en intégrant des simulations de dynamique des populations pour une vision globale de leur impact sur les colonies d'abeilles. Les résultats obtenus fournissent des réponses cruciales aux défis posés par les effets sublétaux des pesticides dus à des expositions précoces dans la vie des abeilles. Notre approche a permis une meilleure compréhension des effets différés des pesticides sur le cycle de vie des abeilles domestiques, tant au niveau individuel qu'au niveau de la colonie.

Mots clefs : *Apis Mellifera* ; Elevage *in vitro* ; RFID ; Activités de vol ; Expression de gènes ; Ecotoxicologie ; Pesticides ; Interactions Pathogènes – Pesticides