THÈSE DE DOCTORAT D'AVIGNON UNIVERSITÉ

École Doctorale 536 Agrosciences et Sciences

Laboratoire de Toxicologie Environnementale
UR 406 Abeilles & Environnement, INRAE

Présentée par
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Toxicologie des mélanges de pesticides chez des abeilles exposées à un agent pathogène

Action combinée de l'agent pathogène *Nosema ceranae*, de l'insecticide imidaclopride, du fongicide difénoconazole et de l'herbicide glyphosate

Soutenue publiquement le 16 Décembre 2020
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Résumé

Les données scientifiques actuelles suggèrent un déclin de la diversité et de l'abondance des insectes, y compris les abeilles domestiques *Apis mellifera*. Ces dernières sont confrontées à de fortes pertes de colonies dans plusieurs régions du monde telles que l'ouest de l'Europe et les États-Unis. De nombreuses études suggèrent que l'origine du déclin des colonies d'abeilles est multicausale et identifient les pesticides et les agents pathogènes comme étant les principaux contributeurs à ce déclin. La co-exposition des abeilles à de multiples pesticides et l'infection par plusieurs pathogènes constituent un phénomène courant. Cependant, les recherches sur les effets des mélanges de pesticides n'ont pas fait l'objet d'un intense développement. Ainsi, les travaux conduits dans le cadre de cette thèse ont été focalisés sur la détermination de la toxicité des mélanges de pesticides, appliqués à des niveaux d'exposition environnementaux, en présence d'un agent pathogène. Le choix s'est porté sur l'étude des interactions entre un insecticide néonicotinoïde, l'imidaclopride, un fongicide azole, le difénoconazole, et un herbicide, le glyphosate, en présence de l'agent pathogène *Nosema ceranae*. Les résultats des différentes études effectuées durant cette thèse, révèlent la complexité des études sur les mélanges de pesticides. Ces travaux nous ont permis de constater que les effets d'un mélange de pesticides peuvent fortement varier en fonction des concentrations des pesticides constituant le mélange. L'augmentation du nombre de substances et du niveau d'exposition, n'induit pas nécessairement une augmentation de la toxicité du mélange. De plus, les effets du mélange peuvent varier en fonction de la séquence d'exposition aux pesticides et de l'état sanitaire des abeilles. Les mélanges de pesticides affectent l'état physiologique des abeilles suite à une réponse systémique liée à des perturbations de mécanisme généraux tels que le stress oxydant. Cependant, ces trois pesticides, seuls et en mélanges n'ont aucun effet sur l'installation du microbiote intestinal à des niveaux d'exposition environnementaux.

Mots-clés : *Apis mellifera*, mélanges de pesticides, *Nosema ceranae*, effet cocktail, microbiote intestinal

Abstract

Current scientific findings suggest a decline in the diversity and abundance of insects, including the honey bee *Apis mellifera*. The latter are facing high colony losses in several regions of the world such as Western Europe and the United States. Numerous studies suggest that the origin of bee colony decline is multi-causal and identify pesticides and pathogens as the main contributors to this decline. Co-exposure of honey bees to multiple pesticides and infection by multiple pathogens are common phenomena. However, research on the effects of pesticide mixtures has not been extensively developed. Thus, the thesis work has focused on determining the toxicity of pesticide mixtures, applied at environmental exposure levels, in the presence of pathogens. The choice was made to study the interactions between a neonicotinoid insecticide, imidacloprid, an azole fungicide, difenoconazole, and a herbicide, glyphosate, in the presence of the pathogen *Nosema ceranae*. The results of the different studies, carried out during this thesis, reveal the complexity of the studies on pesticide mixtures. The work allowed us to notice that the effects of a pesticide mixture can vary according to the concentrations of the pesticides constituting the mixture. The increase of the number of substances and the level of exposure does not necessarily induce an increase of the toxicity of the mixture. Furthermore, the effects of the mixture may vary depending on the sequence of exposure to the different pesticides and the health status of the honey bees. Pesticide mixtures affect the physiological state of individuals as a result of a systemic response related to disturbances of general mechanisms such as oxidative stress. However, these three pesticides, alone and in mixtures, have no effect on the installation of the intestinal microbiota at environmental exposure levels.

Keywords: *Apis mellifera*, pesticides mixtures, *Nosema ceranae*, cocktail effect, intestinal microbiota

Remerciements

Je tiens tout d'abord à remercier mes superviseurs de thèse, Dr. Luc Belzunces et Dr. Jean-Luc Brunet, d'avoir accepté de m'accueillir au sein de leur laboratoire pour effectuer ma thèse. Je vous remercie de votre confiance, vos conseils et votre encouragement tout le long de ces trois années. Merci pour toutes ces discussions qui m'ont apporté autant sur le plan scientifique que sur le plan humain et surtout merci pour votre bonne humeur.

Je remercie Dr. Angélique Vétillard et Dr. Catherine Armengaud pour avoir accepté d'être les rapporteurs de ces travaux de thèse, ainsi que Pr. Fréderic Delbac et Dr. Yves Le Conte pour avoir accepté de participer au jury en tant qu'examinateurs.

Un grand merci à l'ensemble des membres de l'unité *Abeilles & Environnement*. J'avais de la chance de vous côtoyer et de discuter avec beaucoup d'entre vous sur vos projets de recherche et sur beaucoup d'autres sujets.

Merci Marianne, pour m'avoir initié à l'histologie, pour ton aide et tes encouragements.

Déborah, un grand merci à toi et ta famille. Tu étais toujours prête à m'aider, à me conseiller, à me donner des coups de main dans mes manips. Tu as un grand cœur !

Daiana, merci pour cette collaboration extraordinaire et pour les beaux moments qu'on a partagés ensemble. Ta présence, tes encouragements, tes plaisanteries et tes conseils m'ont énormément aidé à démarrer ma thèse dans de bonnes conditions. C'est toujours un plaisir de parler et de travailler avec toi.

Merci à toi Clémentine pour ta bonne humeur, ta disponibilité et tes encouragements. Heureusement que tu étais là surtout durant la rédaction de ma thèse.

Un énorme merci pour toutes les personnes avec lesquelles j'ai partagé de beaux moments de détente. Merci à Mary, Jacques, Sébastien, Léna, Amélie, Mathilde, Coline, Alix, Maria, Fella, Rima, Sara, Fatina et Maya.

Maryam, ma voisine, j'ai eu la chance de t'avoir à côté de moi. Heureusement que tu étais là dans tous les moments difficiles surtout durant les longs mois de confinement.

Je ne peux évidemment pas poursuivre sans remercier la famille Tchamitchian. Je remercie Sylvie, Marc et Tigrane pour tous ce que vous m'avez apporté durant ces trois années. C'est grâce à vous que j'ai senti avoir également une famille en France. Merci Marc pour tes conseils en statistiques et tes encouragements. Sylvie, merci d'avoir été auprès moi dès les premiers jours de mon arrivée en France. Je te remercie pour ton aide précieuse dans mes manips et de tous les weekends qu'on a passés ensemble au labo. Je tenais à te remercier pour ton affection et ton soutien. Je sais très bien que je peux toujours venir frapper à ta porte en cas de problèmes.

Jamal, Juliana, Sanaa, Diaa et Walid un grand merci pour vous.

Maman et Papa cette thèse vous est dédiée. Merci d'avoir cru en moi et de m'avoir encouragée dans cette voie. C'est grâce à vous que j'ai pu arriver jusqu'ici. Vous êtes tous les deux ma source de force et je sais que vous êtes fiers de moi. Merci Papa de m'avoir transmis ta passion pour l'apiculture et la recherche. Je vous aime.

Samah, mes derniers mots sont pour toi. Merci pour ton soutien, ta patience et ta compréhension. Je sais que tu as fait beaucoup de concessions durant ces trois dernières années. J'espère qu'au bout de quelques mois, nous serons de nouveau ensemble pour la vie. Je t'aime.

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Liste des publications

Article publié

- Almasri, H., D. A. Tavares, M. Pioz, D. Sené, S. Tchamitchian, M. Cousin, J.-L. Brunet, and L. P. Belzunces. 2020. Mixtures of an insecticide, a fungicide and a herbicide induce high toxicities and systemic physiological disturbances in winter *Apis mellifera* honey bees. Ecotoxicol. Environ. Saf. 203:111013. https://doi.org/10.1016/j.ecoenv.2020.111013.

Articles soumis

- Almasri, H., D. A. Tavares, M. Diogon, M. Pioz, M. Alamil, D. Sené, S. Tchamitchian, M. Cousin, J.-L. Brunet, and L. P. Belzunces. (2020). Physiological effects of the interaction between *Nosema ceranae* and sequential and overlapping exposure to glyphosate and difenoconazole in the honey bee *Apis mellifera*. Soumis à *Ecotoxicology & Environmental Safety*.

Articles en préparation

† Co-premier auteur

- Pal, P.†, Almasri, H.†, L. Paris, M. Diogon, M. Pioz, M. Cousin, D. Sené, S. Tchamitchian, D. A. Tavares, F. Delbac, N. Blot, J.-L. Brunet, and L. P. Belzunces. Toxicity of the pesticides imidacloprid, difenoconazole and glyphosate alone and in binary and ternary mixtures to winter honey bees: effects on survival and antioxidative defenses. (article en préparation présenté dans cette thèse).

- Almasri, H., J. Liberti, M. Pioz, J.-L. Brunet, P. Engel and L. P. Belzunces. Mild chronic exposure to pesticides alters physiological markers of honey bee health without perturbing the core gut microbiota. (article en préparation présenté dans cette thèse).

- Almasri, H., D. A. Tavares, S. Tchamitchian, M. Pélissier, D. Sené, M. Cousin, J.-L. Brunet, and L. P. Belzunces. Effects of residual concentrations of an insecticide and a herbicide on the susceptibility of honey bees Apis mellifera to a single fungicidal spray application. (article en préparation présenté dans cette thèse).

- Tavares, D. A., H. Almasri, M. Pioz, D. Sené, S. Tchamitchian, M. Cousin, J.-L. Brunet, and L. P. Belzunces. Effects of an insecticide, a herbicide and a fungicide and their mixtures on the survival and physiology on summer honey bees *Apis mellifera*. (article en préparation).

- Tavares, D. A., H. Almasri, M. Pioz, D. Sené, S. Tchamitchian, M. Cousin, J.-L. Brunet, and L. P. Belzunces. Evaluation of the combined effects of *Nosema ceranae* with oral exposure to imidacloprid and topical exposure to boscalid on the survival, physiology and immunity genes. (article en préparation).

Liste des communications à des congrès

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- Almasri, H., P. Pal, D. A. Tavares, S. Tchamitchian, D. Sené, J.-L. Brunet, and L. P. Belzunces. (2018). Survival and physiological impacts of pesticides combinations in the honeybee (*Apis mellifera*). EurBee 8 Congress, Ghent, Belgium, September 2018. (Poster).

- Tavares, D. A., H. Almasri, S. Tchamitchian, D. Sené, J.-L. Brunet, and L. P. Belzunces. (2018). Impact of imidacloprid, difenoconazole and glyphosate alone or in mixtures on honey bee. EurBee 8 Congress, Ghent, Belgium, September 2018. (Poster).

- Almasri, H., P. Pal, D. A. Tavares, D. Sené, M. Cousin, S. Tchamitchian, J.-L. Brunet, and L. P. Belzunces. (2018). Alteration of survival and oxidative balance induced by subchronic exposure of overwintered honeybees to insecticide, fungicide and herbicide combinations. 11e journées des doctorants du département SPE de l'INRA, Rennes, France, Mai 2019. (Communication orale)

- Almasri, H., P. Pal, D. A. Tavares, D. Sené, M. Cousin, S. Tchamitchian, J.-L. Brunet, and L. P. Belzunces. (2018). Alteration of survival and oxidative balance induced by subchronic exposure of overwintered honeybees to insecticide, fungicide and herbicide combinations. 30ème de l'Union Internationale pour l'Étude des Insectes Sociaux – Section Française, Avignon, France, Août 2019. (Communication orale)

- Almasri, H., P. Pal, D. A. Tavares, D. Sené, M. Cousin, S. Tchamitchian, J.-L. Brunet, and L. P. Belzunces. (2018). Alteration of survival and oxidative balance induced by subchronic exposure of overwintered honeybees to insecticide, fungicide and herbicide combinations. The 46th Apimondia International Apicultural Congress, Montréal, Canada, September 2019. (Communication orale)

I. Introduction

A. Les abeilles

A.1. Origine des abeilles

Les abeilles appartiennent à l'ordre des hyménoptères et à la superfamille des Apoideae. Cette superfamille contient, en plus des abeilles, les guêpes dites apoïdes (Branstetter et al., 2017; Debevec et al., 2012). Parmi les Apoideae, les abeilles constituent le clade des Anthophila. Ce clade renferme notamment la famille des Apidae qui contient plus de 5700 espèces (Branstetter et al., 2017) (**Fig. 1**). La majorité des abeilles sont solitaires. D'autres espèces comme l'abeille domestique *Apis mellifera* de la sous-famille des Apinae sont sociales.

Les plus anciens fossiles d'abeilles sont ceux de l'espèce *Melittosphex burmensis*. Ils ont été découvertes à Myanmar et font remonter la date d'apparition des abeilles à 100 millions d'années (Poinar and Danforth, 2006). L'apparition des abeilles et leurs adaptations morphologiques et comportementales à la collecte et au transport de pollen ont contribué à la propagation et la diversification rapide des plantes angiospermes (Grant, 1994; Vamosi and Vamosi, 2010). Il existe plus de 16000 espèces d'abeilles qui dépendent exclusivement des angiospermes pour la collecte de produits tels que le nectar et le pollen, et qui contribuent ainsi à la pollinisation des plantes (Danforth et al., 2006). La majorité des espèces d'abeilles, à l'exception de l'abeille européenne *A. mellifera*, l'abeille asiatique *Apis ceranae* et quelques espèces de bourdons, ne sont pas domestiquées. L'abeille européenne *A. mellifera* présente un fort intérêt économique et l'exploitation de ses produits tels que la cire, par l'être humain, date de plus que 9000 ans (Roffet-Salque et al., 2015).

Près de 90% des plantes à fleurs dépendent des invertébrés et des vertébrés pour la pollinisation (Ollerton et al., 2011). La production de fruits et de légumes de 87 cultures agricoles dépend de la pollinisation animale ce qui correspond à 35% du volume de la production agricole mondiale (Klein et al., 2007). Les abeilles domestiques sont considérées comme étant les pollinisateurs des monocultures les plus rentables économiquement (McGregor, 1976) et la production de plusieurs cultures diminue de plus de 90% pendant leur absence (Southwick and Southwick Jr, 1992). Les autres espèces d'abeilles ont aussi un rôle écologique très important en pollinisant des espèces particulières de plantes à fleurs. En outre, d'autres espèces comme les bourdons sont plus efficaces que les abeilles domestiques dans la pollinisation de certaines cultures, comme les tomates, grâce aux vibrations que les bourdons émettent durant la pollinisation. Cette pollinisation est connue sous le nom de pollinisation vibratile (Banda and Paxton, 1990) (**Fig. 2**).

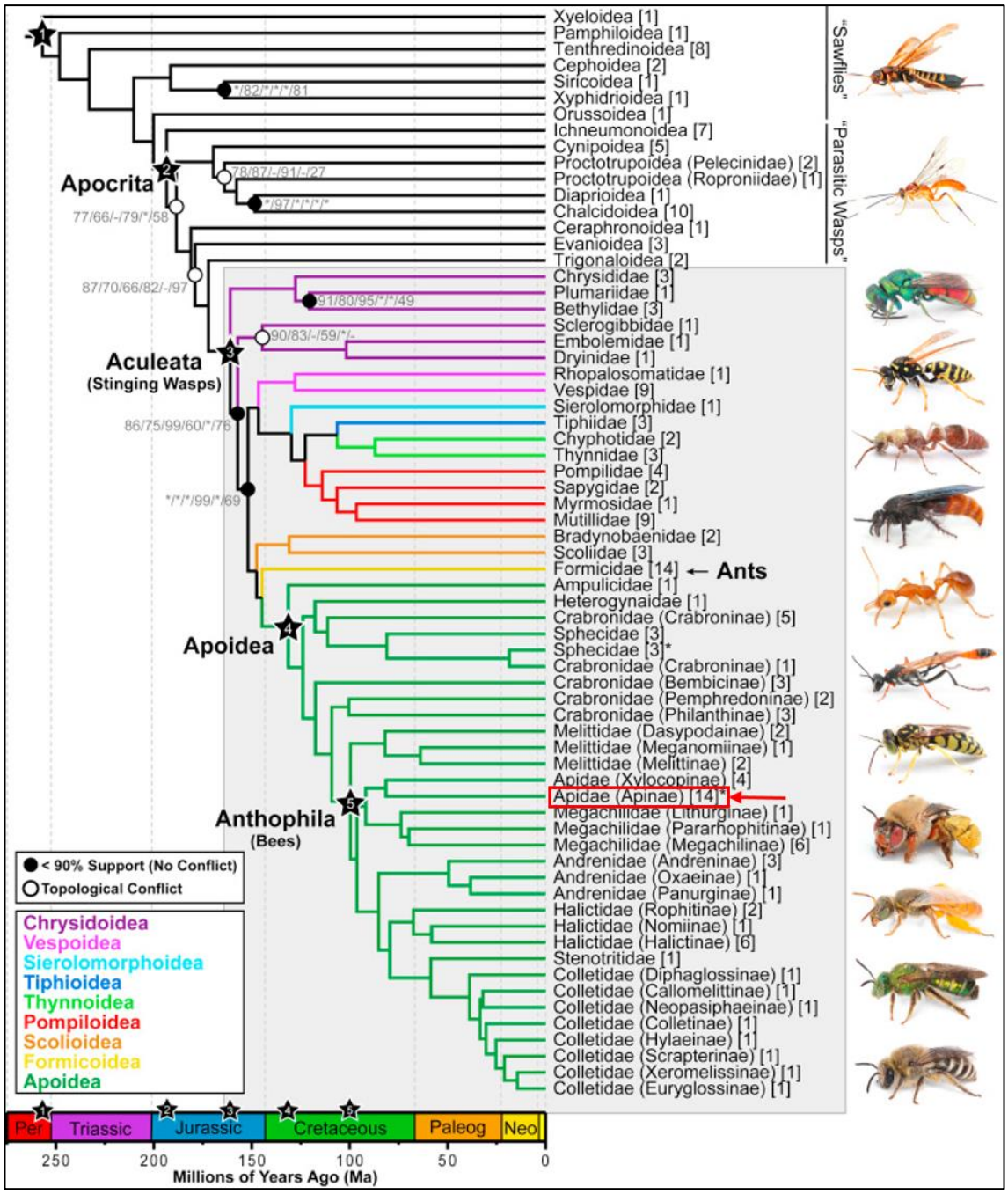

Figure 1 : Arbre phylogénétique de l'ordre des hyménoptères

Cet arbre phylogénétique est fondé sur l'analyse de plus de 800 loci dans 187 échantillons. Les points blancs correspondent aux nœuds où il existe des différences entres les analyses, et les points noirs correspondent aux nœuds retrouvés dans les différentes analyses mais avec moins de 90% de robustesse dans au moins une analyse. La famille des Apidae, à laquelle l'abeille domestique appartient, est encadrée en rouge. D'après Branstetter et al (2017).

A.2. Importance de l'abeille domestique

En plus de son rôle écologique et agronomique lié à sa grande capacité polinisatrice, l'abeille domestique a aussi un rôle économique du fait qu'elle constitue une source de revenus pour les apiculteurs dont le nombre dépasse 600000 en Europe (European Commission, 2016). Les produits de la ruche tels que le miel, le pollen, la cire, la gelée royale, la propolis et le venin d'abeille sont largement utilisés pour leurs valeurs nutritionnelles et médicales très élevées (Denisow and Denisow-Pietrzyk, 2016; El-Seedi et al., 2020; García, 2018; Jagua-Gualdron et al., 2020; Viuda-Martos et al., 2008).

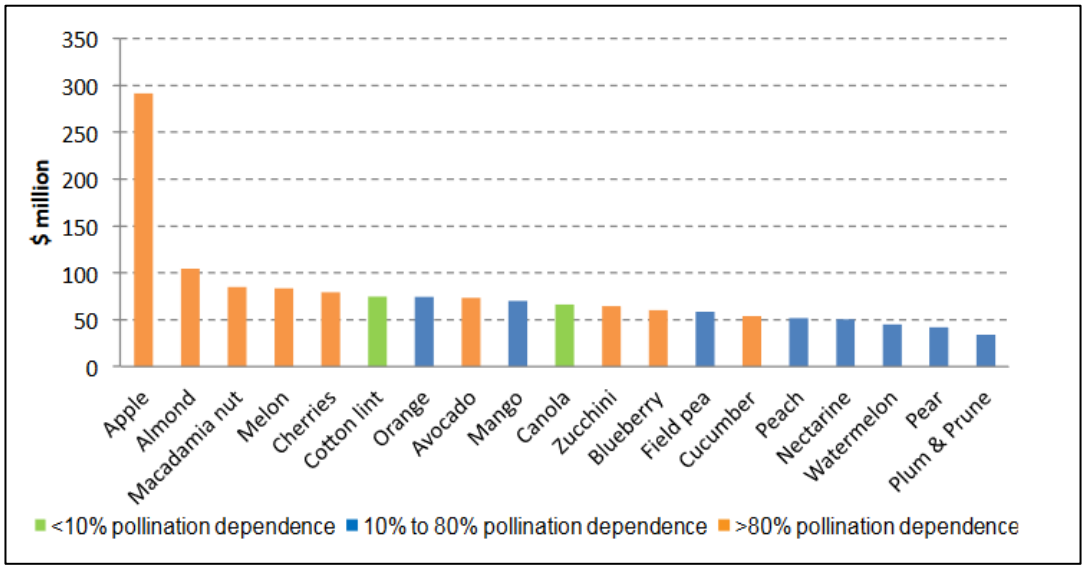

Figure 2 : Valeurs brutes des productions dépendantes de la pollinisation des abeilles dans 20 cultures en Australie

La valeur brute des productions dépendantes de la pollinisation des abeilles est le produit de la valeur de la production annuelle de la culture et le pourcentage de culture dépendant de la pollinisation des abeilles. Cette figure montre que la production de pommes, d'amandes, d'avocats et de plusieurs autres cultures est très fortement dépendante de la pollinisation par les abeilles. D'après Hafi et al (2012).

A.3. Vie sociale et organisation de la colonie de l'abeille domestique

A.3.1. Un super-organisme

La colonie d'abeilles est souvent considérée comme un super-organisme par analogie avec des organismes supérieurs formés de plusieurs cellules individuelles. La colonie est composée de plusieurs dizaines de milliers d'individus groupés en trois castes différentes : la reine, les ouvrières et les faux-bourdons. Comme dans chaque organisme complexe, chaque individu a une tâche bien particulière et la vie de ce super-organisme est dépendante de sa présence à l'intérieur

de la colonie. L'individu passe par quatre stades de développement (œuf, larve, pupe et adulte) quelle que soit sa caste. Cependant la période de développement du couvain (œufs, larves et pupes) et la composition de l'alimentation avec laquelle il se nourrit varie considérablement selon la caste.

Les deux castes reproductrices sont la reine et les faux-bourdons. La reine peut vivre plusieurs années et pond jusqu'à 2000 œufs par jour quand les conditions sont favorables au développement de la colonie. Les faux-bourdons sont produits dès le printemps jusqu'à à la fin d'été et leur nombre atteint quelques centaines d'individus par colonie. Ils assurent la fécondation des reines et vivent un mois au sein de la ruche avant qu'ils soient chassés ou tués par les ouvrières (Winston, 1987). Les ouvrières constituent la caste non reproductrice. Ce sont les individus les plus nombreux dans la colonie et ont des organes reproducteurs atrophiés grâce à l'action de la phéromone royale (Hoover et al., 2003). Ainsi, les ouvrières accomplissent toutes les tâches de la ruche hormis la reproduction.

A.3.2. Polyéthisme d'âge

Les ouvrières de l'abeille domestique sont caractérisées par une division de travail (des tâches de travail différentes) en fonction de leurs âges, ce qui est connu sous le nom de polyéthisme d'âge. Au contraire des autres insectes sociaux comme les fourmis, les ouvrières d'abeilles ayant des tâches distinctes dans la colonie sont différentes d'un point de vue physiologique et non pas morphologique (Moritz and Fuchs, 1998). Ces différences physiologiques sont particulièrement visibles au niveau du métabolisme, de la structure et l'expression des gènes du cerveau et surtout au niveau de la production et l'expression de l'hormone juvénile (HJIII) et de la glycolipoprotéine vitellogénine (Vg) (Robinson, 2002). Ainsi, chez les jeunes abeilles, le taux de Vg est élevé et celui de l'HJIII est faible. Ces taux s'inversent chez les butineuses, avec une inhibition de la synthèse de la Vg par l'HJIII. De plus, les nourrices présentent des taux de HJIII plus faibles que les butineuses.

Après la désoperculation de l'alvéole, l'abeille adulte sort de la cellule au sein de laquelle elle s'était développée et est considérée comme émergente. Elle s'occupe notamment durant les 20 premiers jours du nettoyage des cellules, de l'alimentation du couvain et de l'operculation des cellules de couvain, du soin des reines, de l'alimentation des adultes, de la construction et de l'entretien des rayons ainsi que de la réception du nectar rapporté par les butineuses (Calderone, 1998; Clément et al., 2013). Au-delà de ces 20 jours, les abeilles s'attellent à défendre la ruche et à récolter le pollen, le nectar, l'eau et la propolis dans un rayon allant jusqu'à 12 kilomètres de la ruche (Beekman and Ratnieks, 2000) (**Fig. 3**).

Le polyéthisme d'âge n'est pas un système très rigide. En effet, afin de maintenir la cohésion de la colonie, les abeilles présentent une flexibilité leur permettant d'avancer, de retarder ou de revenir à un stade particulier. Cela est contrôlé par les besoins de la colonie comme la perte des

butineuses ou le confinement pendant des situations climatiques défavorables (Huang and Robinson, 1996; Schmickl and Crailsheim, 2002; Schulz et al., 1998). Par exemple, une transition précoce du stade nourrice au stade butineuse, du fait d'un besoin élevé en butineuses (par exemple après une perte de butineuses liée à une exposition de ces dernières à un traitement insecticide), induit une augmentation du taux de HJIII chez les nourrices devenues nouvellement butineuses. De même, une injection de HJIII à des nourrices induira chez ces dernières un comportement de butineuses. À l'inverse, la transition du stade butineuse au stade nourrice chez des butineuses âgées (par exemple quand il y a un déficit en nourrices dans la colonie) induira une baisse du taux de HJIII. Ainsi, il est difficile de savoir si c'est le taux de HJIII qui induit le comportement différentiel nourrice/butineuse ou si c'est la fonction qui induit des changements dans le taux de HJIII (Robinson et al., 1989; Robinson et al., 1988; Robinson et al., 1992). De plus, certains pathogènes, comme *Nosema ceranae*, et des pesticides, comme le fenoxycarb et le thymol, entrainent des changements de l'expression de gènes et des taux de Vg chez les abeilles (Antunez et al., 2013; Boncristiani et al., 2012; Charpentier et al., 2014; Wegener et al., 2016). Cela entraine un butinage précoce et une durée de vie raccourcie par comparaison avec les abeilles en conditions normales (Goblirsch et al., 2013).

A.3.3. Abeilles d'hiver et abeilles d'été

Au contraire des autres hyménoptères, comme les guêpes et les bourdons, les abeilles domestiques passent l'hiver en colonie. Cet hivernage impose des adaptations physiologiques particulières chez les ouvrières. Durant la période hivernale, la ponte et le butinage sont suspendus, et les ouvrières restent à l'intérieur de la ruche en formant une grappe dont le centre a une température proche de 35°C. Ces abeilles sont appelées « abeilles d'hiver » ; elles sont nées aux dernières pontes en automne et restent vivantes durant les quatre à six mois qui suivent (Free and Spencer-Booth, 1959). Cependant, les abeilles qui naissent durant la belle saison ont une durée de vie beaucoup plus courte, ne dépassant pas les six semaines, et diffèrent physiologiquement des abeilles d'hiver. Les abeilles d'hiver ont des glandes hypopharyngiennes hypertrophiées, un tissu adipeux plus développé et une accumulation plus élevée de Vg dans leur hémolymphe en comparaison avec les abeilles d'été (Amdam and Omholt, 2002; Winston, 1987). Ces différences physiologiques semblent contribuer aux différences de sensibilité aux pesticides et aux infections virales entre les abeilles d'hiver et les abeilles d'été (Meled et al., 1998; Steinmann et al., 2015)

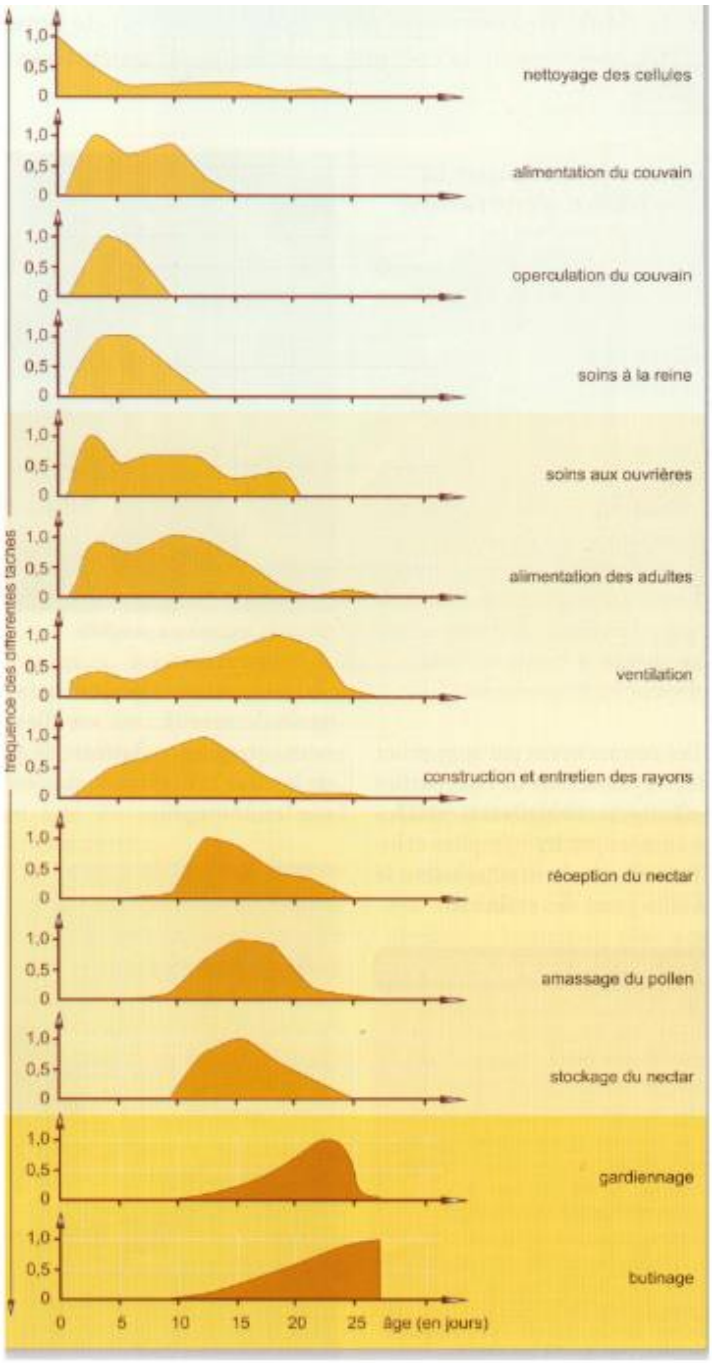

Figure 3 : Le polyéthisme d'âge des abeilles

Suite à son émergence, l'abeille accomplit des tâches différentes en fonction de son âge. Les premières tâches après l'émergence de l'abeille s'effectuent à l'intérieur de la ruche puis elle effectue à la fin de sa vie des tâches plus risquées de gardiennage et de butinage à l'extérieur de la ruche. La fréquence des tâches est représentée sur une échelle de 0 à 1 sur l'axe des ordonnées. D'après Clément et al (2013).

A.4. Physiologie de l'abeille

A.4.1. Système de détoxication

En récoltant le nectar, le pollen et la propolis, l'abeille s'expose à une large gamme de composés phytochimiques, tels que les flavonoïdes (Gheldof et al., 2002; Viuda-Martos et al., 2008), et à des xénobiotiques tels que les pesticides. Ces derniers sont ainsi fréquemment détectés en co-occurrence dans les matrices de la ruche en association avec les acaricides utilisés dans la lutte contre des acariens ectoparasites comme *Varroa* (*Varroa destructor*) (Chauzat et al., 2011; Pareja et al., 2019; Silvina et al., 2017). L'abeille dispose d'un système de détoxication qui permet de métaboliser les substances phytochimiques et les xénobiotiques. Ce système comporte des enzymes de phase I, qui sont des enzymes de fonctionnalisation et des enzymes de phase II, qui sont des enzymes de conjugaison. La structure de la molécule toxique est altérée et rendue incapable d'agir sur son site cible lipophile grâce aux enzymes de la phase I telles que les mono-oxygénases à cytochrome P450 (CYP450) et les carboxylestérases (CaEs). Les abeilles utilisent notamment les CYP450 pour la détoxication des flavonoïdes dans le miel et le pollen (Mao et al., 2009). De plus, ces enzymes sont actives dans la détoxication de nombreuses familles de pesticides telles que les pyréthrinoïdes, les organophosphorés (OP), les phényles pyrazoles et les néonicotinoïdes (Iwasa et al., 2004; Johnson et al., 2013), et sont impliquées dans le métabolisme d'hormones (Claudianos et al., 2006). Les carboxylestérases sont impliquées dans le métabolisme des lipides et des xénobiotiques par l'hydrolyse des liaisons amide et ester (Badiou-Beneteau et al., 2012; Ross et al., 2010). Elles interviennent surtout dans le métabolisme des pyréthrinoïdes, et diminuent l'effet neural des OP et des carbamates en piégeant ces deux derniers au niveau du site actif (Carvalho et al., 2013; Johnson et al., 2006; Ross et al., 2010).

Les enzymes de phase II comme la glutathion-S-transférase (GST) contribuent à la détoxication par la conjugaison aux xénobiotiques électrophiles. Les conjugués obtenus sont ainsi plus solubles. La GST participe aussi à la séquestration des xénobiotiques comme dans le cas des pyréthrinoïdes (Kostaropoulos et al., 2001).

Les transporteurs ABC, appelés aussi transporteurs ATP Binding Cassettes, participent aussi à la détoxication. Ils utilisent de l'énergie, en hydrolysant l'ATP, pour empêcher les molécules toxiques de rentrer dans les cellules ou pour capturer les molécules ayant déjà pénétré dans la cellule et les rejeter à l'extérieur. Ces transporteurs sont mal connus chez les insectes (Berenbaum and Johnson, 2015), pourtant, ils sont impliqués dans la protection des abeilles contre plusieurs pesticides tels que le fluvalinate, le coumaphos, l'acétamipride, le thiaclopride et l'imidaclopride (Guseman et al., 2016; Hawthorne and Dively, 2011).

Comparé à d'autres insectes, le génome de l'abeille présente un déficit des gènes codant les enzymes de détoxication des phases I et II. Il existe chez l'abeille 46 et 24 gènes codant pour les CYP450 et les CaEs, respectivement, contre 85 et 35 gènes chez la drosophile. Les gènes codant pour des transférases comme la GST sont aussi réduits presque de quatre fois chez l'abeille par comparaison avec la drosophile (Berenbaum and Johnson, 2015). Ce déficit de gènes de

détoxication semble être une conséquence évolutive de l'eusocialité très développée de l'abeille domestique (Berenbaum and Johnson, 2015; Claudianos et al., 2006). En effet, les abeilles évitent quelques nectars ou pollens riches en toxines et en pesticides et elles mélangent le pollen et le nectar de plusieurs sources ensemble diluant ainsi les concentrations des substances toxiques. De plus, la fermentation du pollen en pain d'abeilles, principalement par les champignons et les levures, et la transformation du miel en nectar peuvent aussi aider dans la dégradation des xénobiotiques (Hurst et al., 2014; Jaffe et al., 2019).

A.4.2. Immunité des abeilles

La réponse immunitaire de l'abeille est assurée grâce à une réponse cellulaire et/ou humorale. Les hémocytes assurent la réponse cellulaire qui se manifeste par la phagocytose des petites cibles microbiennes ainsi que la nodulation et l'encapsulation des bactéries et des parasites de plus grande taille (Collison et al., 2016). La production d'hémocytes est souvent couplée à la mélanisation qui est catalysée par la phénoloxydase (POx) dans le but de cicatriser les blessures et bloquer l'absorption de nutriments par les parasites (Kanost and Gorman, 2008).

La réponse humorale est assurée par la sécrétion de peptides antimicrobiens (PAM) suite aux infections virales, bactériennes, fongiques et microsporidiennes (Bull et al., 2012; Li et al., 2017a; Wu et al., 2018). La sécrétion des PAM est régulée par l'expression des gènes de l'immunité sous le contrôle des voies Toll et Imd (Brutscher et al., 2015).

Chez l'abeille, le nombre de gènes impliqués dans l'immunité représente environ un tiers du nombre gènes impliqués chez la drosophile (Evans et al., 2006). Ce déficit de gènes pourrait être une conséquence évolutive de l'eusocialité comme dans le cas des gènes de détoxication. Les abeilles possèdent une immunité comportementale coopérative contre les parasites et les pathogènes. Cette immunité apparait à travers : (i) l'auto et l'allo-épouillage, (ii) l'augmentation de la température pour tuer les bactéries et les prédateurs, (iii) la récolte des résines de plantes ayant des propriétés antimicrobiennes pour la fabrication de propolis, (vi) l'élimination du couvain malade ou mort à l'extérieur de la ruche, (v) la sécrétion de la glucose oxydase (GOx) par les glandes hypopharyngiennes. Cette enzyme est sécrétée dans le nectar, au cours de sa maturation en miel, et dans la gelée royale. Elle produit l'acide gluconique et le peroxyde d'hydrogène H_2O_2, stérilisant ainsi la nourriture du couvain et des reines ainsi que le miel (Bucekova et al., 2014). La GOx joue aussi un rôle dans la détoxication d'alcaloïdes toxiques présents dans le nectar permettant ainsi aux abeilles de bénéficier des nectars riches en alcaloïdes tels que la nicotine (Liu et al., 2005; Musser et al., 2002) (**Fig. 4**).

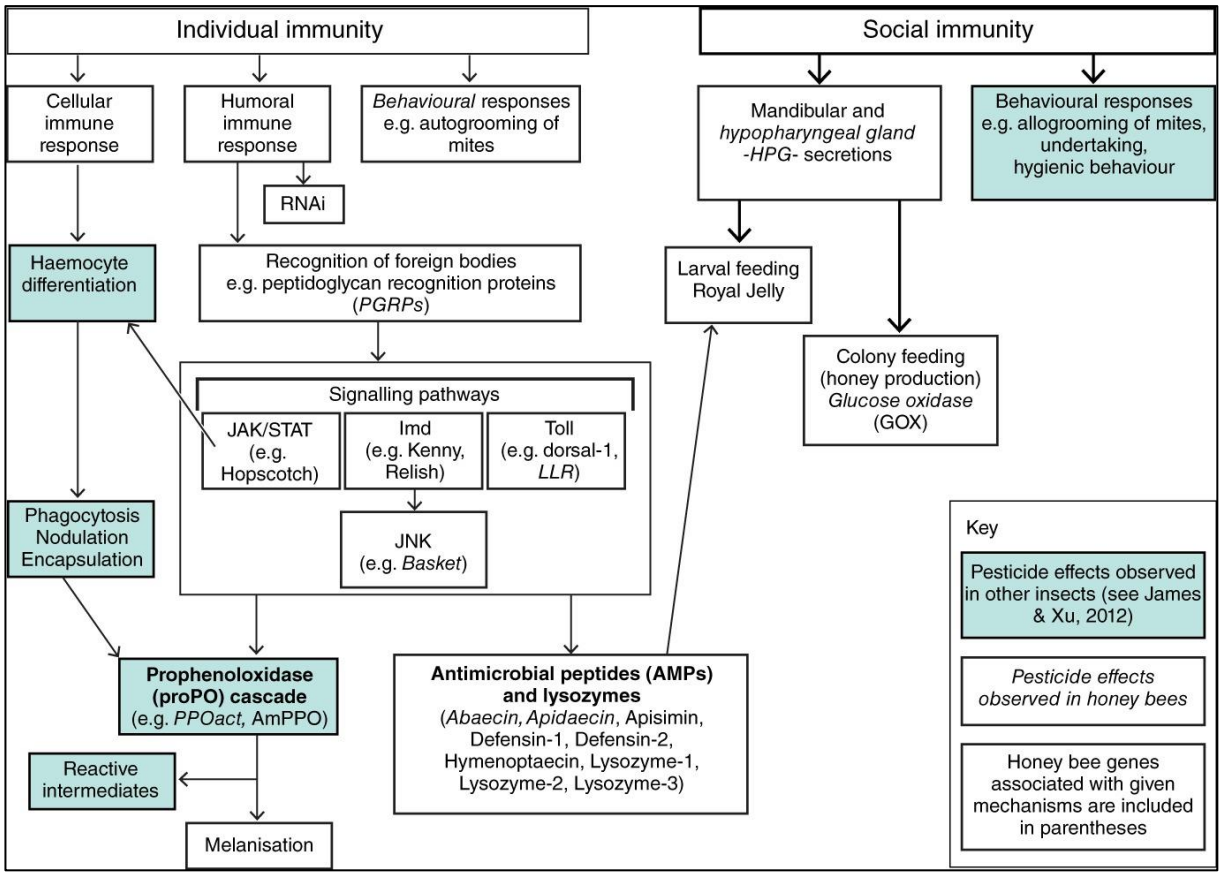

Figure 4 : Représentation schématique de la réponse immunitaire des abeilles

Le système immunitaire des abeilles est divisé en immunité individuelle et sociale. Les rectangles en blanc et bleu clair correspondent, respectivement aux composants qui sont affectés par les pesticides chez les abeilles et chez d'autres des organismes. AmPPO, *Apis mellifera* prophenoloxidase gene; Imd, immune deficiency; JAK/STAT, Janus kinase/signal transduction and transcription; JNK, c-Jun N-terminal kinases; LLR, leucine-rich repeats; PPOact, prophenoloxidase-activating enzyme; RNAi, ribonucleic acid interference. D'après Collison et al (2016).

A.4.3. Le stress oxydant

Sous les conditions physiologiques normales, il existe chez les organismes vivants un équilibre entre les pro-oxydants et les antioxydants. Cet équilibre est connu sous le nom d'équilibre oxydatif. Une augmentation dans la production des pro-oxydants due à des facteurs endogènes ou exogènes, ou une diminution de l'activité ou de la présence des antioxydants conduisent à un déséquilibre oxydatif et à un stress oxydant.

Les pro-oxydants sont principalement les radicaux libres de l'oxygène et les peroxydes connus sous le nom d'espèces réactives de l'oxygène ROS (Reactive Oxygene Species). De plus, il existe des espèces réactives azotées RNS (Reactive Nitrogen Species) qui jouent aussi un rôle dans le stress oxydant. La métabolisation des substances chimiques, xénobiotiques ou naturelles, et de certains dérivés de l'oxygène, entraine la formation de radicaux libres (ROS et RNS) tels que le

radical hydroxyle $HO^{\bullet}$, l'anion superoxyde $O_2^{\bullet-}$ et les monoxyde et dioxyde d'azote $NO^{\bullet}$ et $^{\bullet}NO_2$, principalement. Ces radicaux possèdent un électron célibataire sur leur couche externe, ce qui leur confèrent une grande instabilité et réactivité chimique, et tendent à échanger leurs électrons avec d'autres molécules formant ainsi de nouveaux radicaux libres ou de nouveaux composés chimiques (Barouki, 2006). Les peroxydes R-O-O-R', tels que le peroxyde d'hydrogène (H_2O_2), et les radicaux peroxyles $R\text{-}O\text{-}O^{\bullet}$ font aussi partie des ROS. Le peroxynitrite est aussi un peroxyde. Il est formé par la réaction rapide entre l'ion superoxyde et le monoxyde d'azote (NO) et conduit à la formation d'autres espèces de RNS comme le $^{\bullet}NO_2$ (Szabó et al., 2007).

Les ROS jouent un rôle physiologique considérable, particulièrement dans la signalisation cellulaire et l'équilibre entre la croissance, la sénescence cellulaire et l'apoptose (Finkel, 2003). Ils sont produits dans les conditions physiologiques normales, principalement lors du transfert d'électrons pendant la respiration mitochondriale. Les nicotinamide adénine dinucléotide phosphate oxydases (NADPH oxydases ou NOX/DUOX) sont aussi responsables de la production de ROS lors des infections (Ha et al., 2005; Nathan and Cunningham-Bussel, 2013). Ces NOX/DUOX sont des complexes enzymatiques localisés dans la membrane des cellules épithéliales intestinales et génèrent de l'anion superoxyde suite à la réaction de l'O_2 avec le NADPH. La détoxication des xénobiotiques peut être aussi une source de production de ROS. En effet, les CYP450 utilisent l'oxygène pour oxyder leurs substrats (Choi, 2002) et peuvent ainsi former, après plusieurs étapes séquentielles, de l'$O_2^{\bullet-}$ ou du H_2O_2 (Veith and Moorthy, 2018). La myéloperoxydase catalyse l'oxydation de l'ion chlorure par le H_2O_2, formant ainsi l'acide hypochloreux HOCl. Cet acide est extrêmement pro-oxydant et contribue grandement à l'explosion oxydative des cellules immunitaires ayant pour but de neutraliser les bactéries (Parker and Winterbourn, 2013). Le fer ferreux (Fe^{2+}), présent dans l'organisme, est aussi susceptible de produire des radicaux libres par la réaction de Fenton. Cette réaction transforme le H_2O_2 en $HO^{\bullet}$ suivant la réaction : $Fe^{2+}_{(aq)} + H_2O_2 \rightarrow Fe^{3+}_{(aq)} + OH^-_{(aq)} + HO\bullet$ (Kehrer, 2000; Kohen and Nyska, 2002). Cette réaction pourra aussi avoir lieu en présence du cation cuivreux (Cu^+). Les ROS peuvent aussi être générés par une source exogène telle que l'exposition aux irradiations ionisantes ou aux rayonnements ultraviolets, aux polluants de l'air tels que les fumées de toutes origines et aux contaminants anthropogéniques (Kohen and Nyska, 2002). L'exposition aux pesticides et aux métaux entrainent aussi une surproduction des ROS et un déséquilibre oxydatif chez les animaux, y compris les abeilles (Al Naggar et al., 2020; Gregore et al., 2018; Jumarie et al., 2017; Nwani et al., 2010).

L'organisme arrive en général à garder les concentrations de ROS en-dessous du seuil de toxicité grâce à un système d'antioxydants enzymatiques et non enzymatiques. Parmi les antioxydants non enzymatiques, il est possible de citer les vitamines C et E, le glutathion, les polyphénols et les caroténoïdes. D'autre part le système antioxydant enzymatique est constitué d'enzymes antioxydantes primaires et secondaires (Corona and Robinson, 2006). Les enzymes antioxydantes primaires agissent directement sur les ROS. Ces enzymes sont :

1- Les superoxydes dismutases (SOD) qui transforment le radical superoxyde en oxygène et en peroxyde d'hydrogène selon la réaction :

$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$.

Les SOD existent sous deux formes dans les cellules eucaryotes. Ces deux formes diffèrent dans leur localisation dans la cellule et la structure de leur site active. MnSOD est présente dans l'espace mitochondriale interne et Cu/ZnSOD est présente dans le cytoplasme (Corona and Robinson, 2006; Vaziri et al., 2003).

2- La Catalase (CAT) qui est présente au niveau du cytosol. Elle est formée de quatre chaines polypeptidiques comportant chacune une molécule d'hème. Elle permet la dismutation du peroxyde d'hydrogène en dioxygène et en eau selon la réaction : $2H_2O_2 \rightarrow 2H_2O + O_2$ (Corona and Robinson, 2006).

3- La glutathion peroxydase (GPOx) qui agit sur le peroxyde d'hydrogène et les autres hydroperoxydes organiques en catalysant leurs réductions en utilisant les électrons du glutathion (GSH), selon la réaction :

$2GSH + H_2O_2 \rightarrow GS–SG + 2H_2O$ (Michiels et al., 1994).

Les enzymes antioxydantes secondaires agissent indirectement sur les ROS. Parmi ces enzymes on note :

1- La glutathion réductase (GR) qui régénère le glutathion réduit (GSH) à travers le glutathion oxydé en présence du nicotinamide adénine dinucléotide phosphate réduit (NADPH) comme donneur d'électrons. La réaction est :

$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$.

2- La glucose-*6*-phosphate déshydrogénase (G6PDH) qui catalyse la première étape de la voie des pentoses phosphates. Cette voie est l'une des quatre voies principales du métabolisme énergétique. Elle permet ainsi la production de ribose-5-phosphate et d'érythrose-4-phosphate, utilisés respectivement pour la synthèse des nucléotides et d'acides aminés aromatiques. En plus, elle génère du NADPH qui est utilisé pour réduire le GSH sous l'influence de la GR (Ge et al., 2020). La réaction est :

Glucose-*6*-phosphate + $NADP^+ \rightarrow$ 6-phosphogluconolactone + NADPH.

3- La glutathion-S-transférase (GST), qui est une enzyme de détoxication de phase II, joue aussi un rôle dans la lutte contre le stress oxydant puisqu'elle a une grande affinité pour les lipides peroxydés et elle les transforme en des dérivés hydroxyles moins toxiques (Singh et al., 2001).

Le stress oxydant entraine des dommages sur différents composants cellulaires y compris les lipides, les protéines et les acides nucléiques :

1- Sur les lipides insaturés, la réaction qui se produit due au stress oxydant est connue sous le nom de peroxydation lipidique. Cette peroxydation a lieu en trois phases. La première phase est la phase d'initiation déclenchée par un ROS ; il y'a un réarrangement moléculaire qui induit la formation d'un acide gras modifié. Cet acide gras réagit, durant la deuxième phase de propagation, avec un acide gras se trouvant dans son voisinage, entrainant ainsi sa modification. D'où une simple initiation d'un acide gras insaturé dans une membrane cellulaire peut conduire à la peroxydation de tous les acides gras insaturés de la membrane. La phase finale a lieu suite à l'interaction de deux radicaux ensemble ou d'un radical avec un antioxydant (Kohen and Nyska, 2002) (**Fig. 5**).

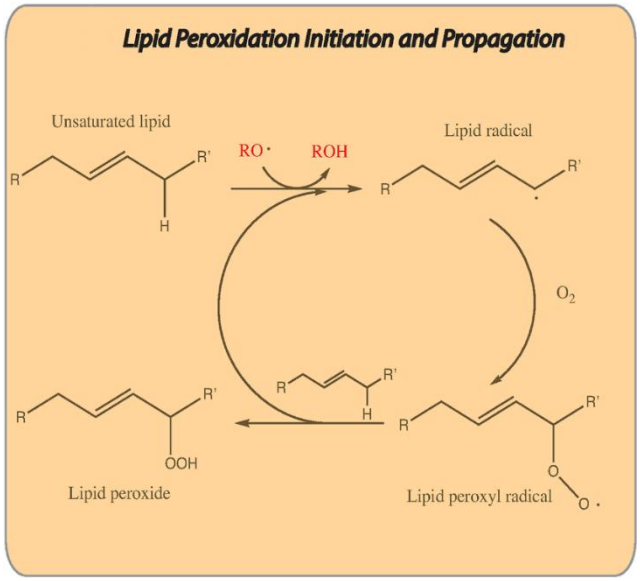

Figure 5 : Mécanismes de peroxydation lipidique

La peroxydation lipidique a lieu en trois phases : durant la phase I, les lipides insaturés sont oxydés par un ROS (RO•). Durant la phase II, l'acide gras oxydé réagit avec un autre acide gras se trouvant dans son voisinage, entrainant ainsi son oxydation. Durant la phase III, la propagation s'arrête par l'interaction de deux radicaux ensemble ou d'un radical avec un antioxydant. D'après Kohen and Nyska (2002).

2- Sur les protéines, le stress oxydant induit leur peroxydation, des changements de leurs structures tertiaires, des dégradations, des fragmentations et des modifications de certains acides aminés. Parmi les conséquences du stress oxydant sur les protéines, il est possible de noter une perte de l'activité enzymatique et des altérations des fonctions cellulaires comme la production d'énergie. Ainsi ces protéines seront plus sensibles à la dégradation protéolytique (Kohen and Nyska, 2002).

3- Sur les acides nucléiques, les ROS, surtout le HO•, entrainent plusieurs dommages tels que la modification des bases azotés comme l'adénine et la guanine, des cassures simple ou du double brin de l'ADN et des altérations des systèmes de réparation de l'ADN (Kohen and Nyska, 2002).

A.4.4. Le microbiote intestinal

Les communautés microbiennes existent pratiquement sur tous les sites du corps de tous les êtres vivants. Toutefois, les communautés microbiennes intestinales ont une importance particulière du fait de leurs effets directs et divers sur leur hôte (Rooks and Garrett, 2016; Trompette et al., 2014). Le progrès rapide dans les méthodes de cultures bactériennes et du séquençage ont permis de découvrir une communauté microbienne intestinale particulière et relativement stable chez les abeilles domestiques du monde entier (Kwong et al., 2017b). Cette communauté est formée de plusieurs phylotypes bactériens, qui sont des clusters de souches de bactéries partageant plus de 97% de séquences identiques dans leur ARN ribosomique 16S (ARNr 16 S)) (Corby-Harris et al., 2014; Martinson et al., 2011). Neufs phylotypes représentent à eux seuls 95 à 99% de la flore bactérienne intestinale de toutes les ouvrières d'abeille domestique, parmi lesquels cinq constituent le microbiote fondamental, appelé aussi microbiote « core ». Ces phylotypes sont : *Snodgrassella alvi*, *Gilliamella apicola*, *Lactobacillus* Firm-4, *Lactobacillus* Firm-5 et *Bifidobacterium asteroids*. Il existe d'autres phylotypes qui sont moins persistants tels que *Frischella perrara*, *Bartonella apis*, *Parasaccharibacter apium* et Alpha 2.1 (Kwong and Moran, 2016). D'autres bactéries beaucoup moins abondantes peuvent être aussi présentes dans les intestins telles que *Serratia marcescens*, *Lactobacillus kunkeii*, *Apibacter* et *Enterobacteriaceae* (**Tableau 1**).

Les différentes espèces bactériennes ne sont pas distribuées d'une manière uniforme tout le long de l'intestin de l'abeille. Le jabot, utilisé pour le stockage et le transport de nectar, renferme un nombre limité de bactéries transitoires. Ces bactéries, telles que *Lactobacillus kunkeei*, *Parasaccharibacter apium* et des bactéries membres des Enterobacteriaceae, sont en même temps présentes dans le nectar et les matrices de la ruche. L'intestin moyen, qui est le site de digestion et d'absorption de nutriments, n'est pas considéré comme un substrat stable pour la colonisation bactérienne. Il contient en conséquence un nombre limité de bactéries. Au niveau du pylore, qui constitue une jonction entre l'intestin moyen, les tubes de Malpighi et l'intestin postérieur, *F. perrara* est abondante et est responsable de la production de la mélanine dans cette région (Emery et al., 2017). L'intestin postérieur contient plus de 99% des bactéries intestinales. Il est divisé en deux parties : La première partie est l'iléon dans lequel *S. alvi* forme une couche sur la paroi intestinale au-dessus de laquelle il y'a une autre couche de *G. apicola*. La deuxième partie est le rectum où les matières fécales sont stockées en attente de la défécation. Le rectum peut aussi servir pour la réabsorption de l'eau et des sels. Cette partie contient principalement les *Lactobacillus* Firm-4 et *Lactobacillus* Firm-5 (Kwong and Moran, 2016) (**Fig. 6**).

La composition du microbiote intestinal des ouvrières diffère entre le stade larvaire et le stade adulte. Les larves contiennent beaucoup moins de bactéries que les adultes, et ces bactéries sont plutôt des bactéries transitoires acquises à travers l'alimentation et sont complétement éliminées à la fin de la pupaison. Ainsi, les abeilles émergentes sont dépourvues du microbiote intestinal (McFrederick et al., 2014) et elles acquièrent les communautés bactériennes à travers les interactions sociales surtout durant les trois premiers jours après l'émergence. Le nombre de bactéries dans l'intestin croît pour se stabiliser à 10^8-10^9 cellules bactériennes vers le quatrième jour après l'émergence (Zheng et al., 2018).

Les différents phylotypes microbiens interagissent entre eux et avec l'hôte et assurent plusieurs fonctions fondamentales. Le microbiote intestinal agit sur le gain de poids de son hôte, il induit des changements dans la signalisation hormonale et l'expression des gènes et fait augmenter les taux de vitellogénine (Vg) (Zheng et al., 2017). Le microbiote peut être aussi associé à des changements comportementaux chez l'abeille, puisque les taux des amines biogènes, y compris l'octopamine, la dopamine et la sérotonine sont moins élevés chez les abeilles émergentes, dépourvues naturellement de leur microbiote, en comparaison avec les abeilles adultes, ayant un microbiote bien développé (Harris and Woodring, 1992). Des membres spécifiques de la communauté microbienne intestinale jouent un rôle dans la digestion par la dégradation des polymères non digérables par l'abeille tels que la cellulose, l'hémicellulose et la pectine qui sont présentes dans la couche externe du pollen (Engel et al., 2012). Le microbiote intestinal contribue aussi à défendre l'hôte contre les agents pathogènes. En effet, la présence du microbiote intestinal fait augmenter le taux des peptides antimicrobiens et de l'hymenoptaecin dans les cellules épithéliales, il forme aussi un biofilm protecteur qui constitue une barrière biologique contre les pathogènes (Kwong et al., 2017a). La colonisation avec *F. perrara* augmente encore l'expression de plusieurs gènes du système immunitaire au niveau du pylore, tels que les gènes liés au processus de mélanisation. Ainsi, *F. perrara* conduit à la formation d'une couche de mélanine au niveau du pylore, pouvant protéger contre l'infection par des agents pathogènes. (Emery et al., 2017). Le microbiote pourrait aussi augmenter la toxicité des pesticides. C'est le cas des larves de quelques lépidoptères dont le microbiote augmente la sensibilité vis-à-vis de la toxine de *Bacillus thuringiensis* (Broderick et al., 2009). D'autre part, le microbiote participe à la dégradation des pesticides, ce qui peut conduire à une augmentation ou à une diminution de la toxicité en fonction de la toxicité des métabolites (Almeida et al., 2017; Kikuchi et al., 2012).

Tableau 1 : Les bactéries présentes dans l'intestin des abeilles et dans la ruche

Ce tableau présente les principales bactéries présentes dans l'intestin des abeilles et dans les matrices de la ruche. D'après Zheng et al (2018).

Taxa	Phylum	Primary location
Ubiquitous gut-restricted taxa		
Lactobacillus sp. Firm-5 *L. apis* *L. helsingborgensis* *L. kimbladii* *L. kullabergensis* *L. melliventris*	Firmicutes	Hindgut (ileum, rectum)
Lactobacillus sp. Firm-4 *L. melliferL. mellis*	Firmicutes	Hindgut (rectum)
Bifidobacterium sp. *B. asteroides* *B. coryneforme* *B. indicum*	Actinobacteria	Hindgut (rectum)
Snodgrassella alvi	Proteobacteria	Hindgut (ileum wall)
Gilliamella apicola	Proteobacteria	Hindgut (ileum lumen)
Frischella perrara	Proteobacteria	Hindgut (pylorus, ileum)
Bartonella apis	Proteobacteria	Hindgut, variably present
Commensalibacter sp. "Alpha 2-1"	Proteobacteria	Hindgut, variably present
Other common taxa		
Apibacter adventoris	Bacteroidetes	Adult gut
Parasaccharibacter apium	Proteobacteria	Larval gut, adult crop, queen gut, hive
Lactobacillus kunkeeii	Firmicutes	Larval and adult gut, hive, nectar
Fructobacillus fructosus	Firmicutes	Larval and adult gut, hive
Saccharibacter spp.	Proteobacteria	Bee stomach, honey, pollen
Opportunistic pathogens		
Serratia marcescens	Proteobacteria	Adult gut
Hafnia alvei	Proteobacteria	Adult gut

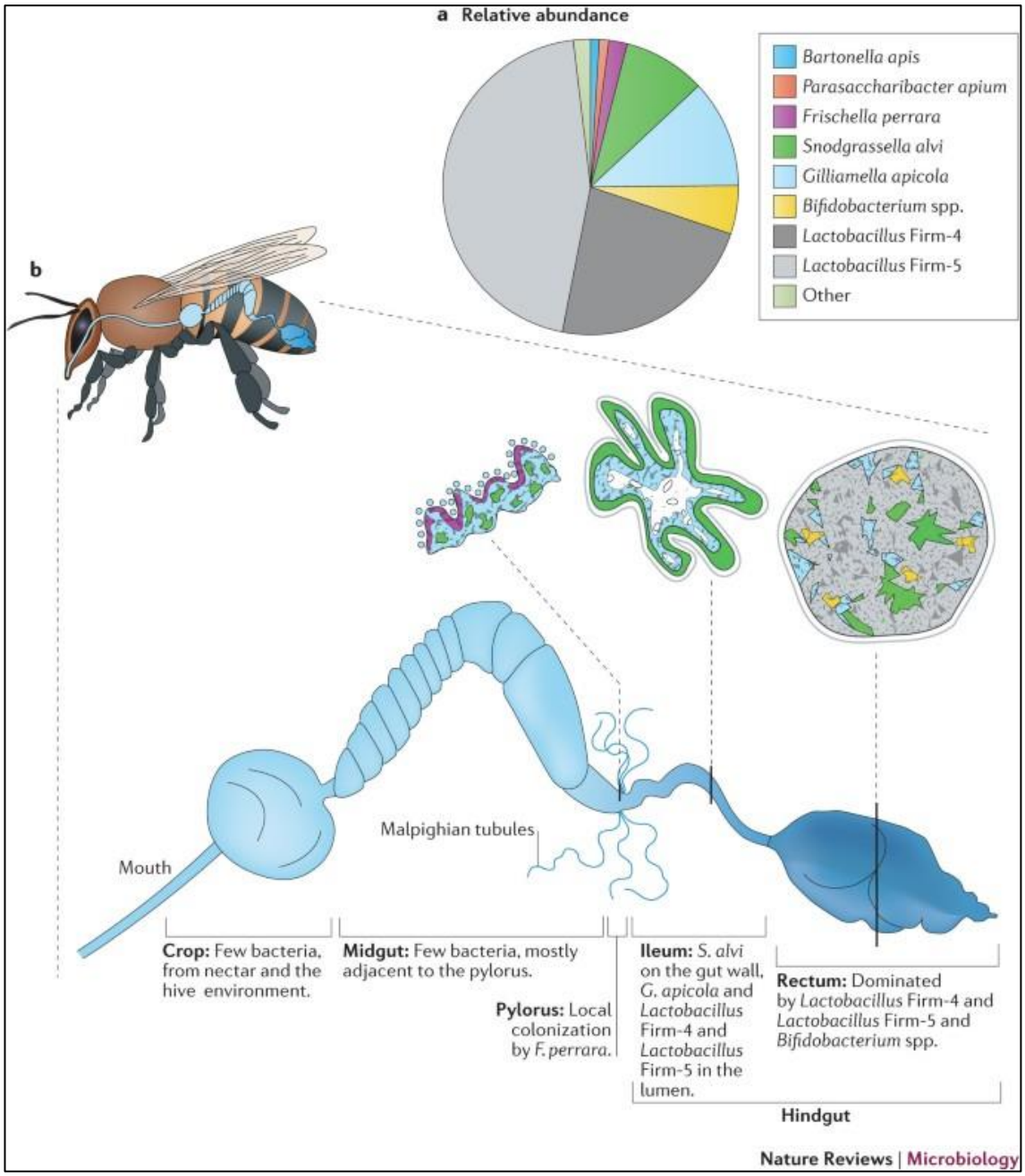

Figure 6 : Composition des communautés microbiennes et leurs localisations dans l'intestin d'une abeille adulte

La composition du microbiote de l'intestin d'abeille en se fondant sur des techniques de séquençage de l'ARN ribosomal 16S (a). La localisation des espèces bactériennes dans les différents compartiments de l'intestin de l'abeille, fondée sur des données obtenues à travers les techniques d''hybridation *in situ* en fluorescence (FISH) et des PCR quantitatives (qPCR). D'après Kwong et Moran (2016).

B. Perte de colonies et facteurs contributeurs

Les données scientifiques actuelles suggèrent un déclin de la diversité et de l'abondance des insectes dans plusieurs régions du monde. Par exemple, il a été noté une baisse de 75% des insectes volants en 27 ans dans les zones protégées de l'Allemagne (Hallmann et al., 2017). De plus, de nombreuses études ont montré un déclin de plusieurs espèces de papillons et d'abeilles sauvages y compris les bourdons (Conrad et al., 2006; Conrad et al., 2002; Goulson et al., 2008; Thomas et al., 2004). Le déclin du nombre d'insectes a certainement des effets néfastes sur l'écosystème du fait que les insectes assurent plusieurs fonctions telles que la pollinisation, l'herbivorie, la détritivorie, le cyclage des nutriments et une source de nutrition pour les niveaux trophiques supérieurs tels que les oiseaux et les amphibiens (Öckinger and Smith, 2007; Yang and Gratton, 2014). Les populations d'abeilles domestiques sont aussi confrontées à des mortalités élevées dans plusieurs régions du monde. Par exemple, à l'ouest de l'Europe et aux États-Unis, le nombre de colonies d'abeilles a diminué respectivement de 13% et de 32% entre 1980 et 2018 (Faostat, 2018) (**Fig. 7**). La période hivernale parait une période délicate pour la survie des colonies d'abeilles. Par exemple, en 2012-2013, les mortalités des colonies dans 11 sur 17 pays européens ont dépassé le seuil de mortalité hivernale acceptable de 10%, et étaient supérieures à 20% dans quelques pays tels que la Belgique, la Suède, l'Angleterre et la Finlande (Chauzat et al., 2016) (**Fig. 8**).

L'abeille est soumise à plusieurs facteurs de stress, y compris des facteurs biotiques et abiotiques. Cependant, il parait peu probable qu'un seul facteur soit responsable à lui seul des mortalités élevées. Celles-ci ont plutôt une origine multicausale avec des facteurs majeurs et d'autres facteurs de moindre importance et une hausse des mortalités quand plusieurs facteurs coexistent ensemble (Goulson et al., 2015; vanEngelsdorp and Meixner, 2010). Les différents facteurs biotiques et abiotiques contribuant au déclin du nombre de colonies sont traités en détails dans les paragraphes qui suivent (cf. B.1 et B. 2).

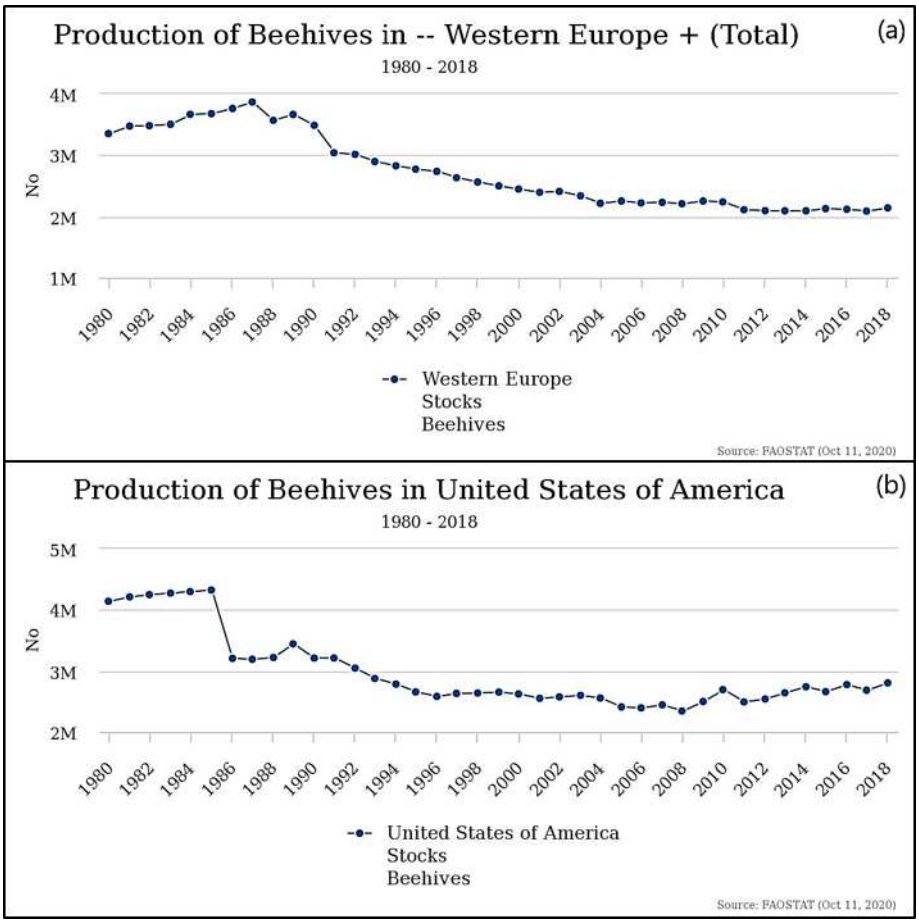

Figure 7 : Évolution du nombre de ruches entre 1980 et 2020 dans quelques régions du monde

Le nombre de colonies d'abeilles a diminué en Europe (a) et aux États-Unis (b) entre 1980 et 2018. Ces graphes sont obtenus à partir des statistiques de la FAO (Faostat. 2018).

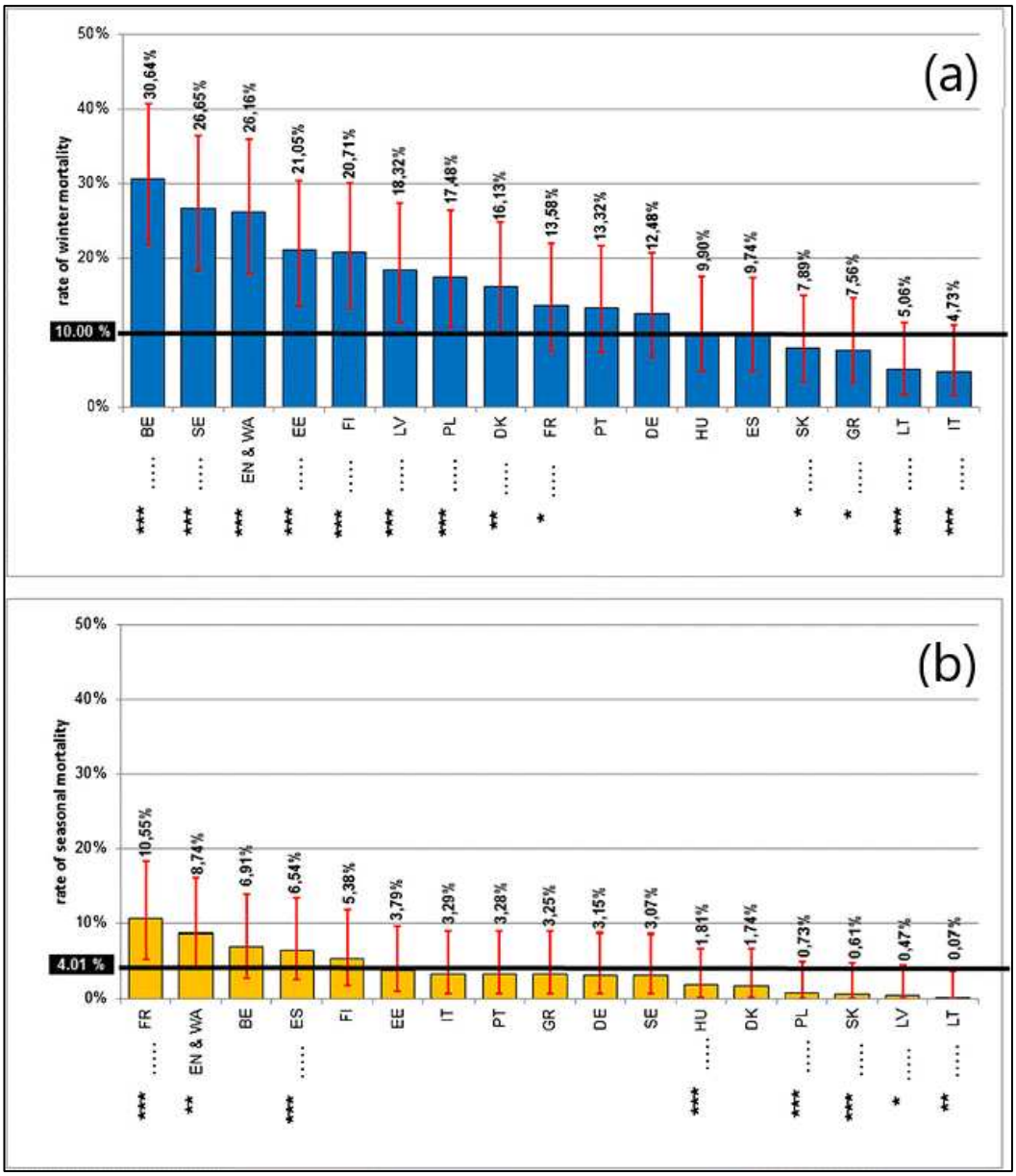

Figure 8 : Mortalités observées dans 17 pays européens

Ces graphes représentent les moyennes des mortalités des colonies dans 17 pays européens durant l'hiver 2012-2013 (a) et la saison apicole du printemps-été 2013 (b). La moyenne des mortalités hivernales de chaque pays est comparée au seuil de mortalité hivernale acceptable de 10%. La moyenne de mortalité durant la saison apicole de chaque pays est comparée à la moyenne des mortalités saisonnières des 17 pays qui ont participé à cette étude. BE Belgique, SE Suède, EN&WA Angleterre et Pays de Galles, EE Estonie, FI Finlande, LV Lettonie, PL Pologne, DK Danemark, FR France, PT Portugal, DE Allemagne, HU Hongrie, ES Espagne, SK Slovaquie, GR Grèce, LT Lituanie, IT Italie. Les différences statistiques sont indiquées par des astérisques (* : $p < 0.05$; ** : $p < 0.01$; *** : $p < 0.001$). Les barres d'erreurs représentent l'intervalle de confiance à 95%. D'après Chauzat et al. (2016).

B.1. Les facteurs biotiques impliqués dans les pertes de colonies d'abeilles

L'abeille domestique est exposée à une large variété de parasites, parasitoïdes, champignons, bactéries et virus. Plusieurs parasites, pathogènes et prédateurs ont émergé durant les dernières décennies suite aux échanges commerciaux internationaux et l'introduction de l'abeille domestique dans de nouvelles régions. Les exemples les plus connus sont le parasite *Varroa destructor*, la microsporidie *Nosema ceranae* et le frelon asiatique *Vespa velutina* (Arca et al., 2015; Goulson et al., 2015).

B.1.1. Les acariens

Il existe plusieurs espèces d'acariens qui parasitent l'abeille. *Acarapis woodi* provoque l'acariose. Il parasite les trachées respiratoires des abeilles et se nourrit de l'hémolymphe (Sammataro and Needham, 1996). *V. destructor* est un ectoparasite qui affecte le couvain et les adultes de l'abeille *A. mellifera* et se nourrit d'hémolymphe et du corps gras. Les effets néfastes du *Varroa* ne se limitent pas à un affaiblissement des abeilles et à une réduction de leur longévité. *Varroa* est aussi un vecteur de plusieurs virus comme le virus des ailes déformées (DWV), le virus de la paralysie aiguë de l'abeille (ABPV), le virus israélien de la paralysie aiguë (IAPV) et la paralysie du Cachemire (KBV). Différents traitements chimiques existent pour lutter contre *Varroa* ; les plus répandus sont l'amitraze, le coumaphos et le tau-fluvalinate (Jesus Gracia et al., 2017).

B.1.2. Les endoparasites

Nosema apis et *Nosema ceranae* sont des microsporidies qui infectent le tissu épithélial intestinal. Leurs rôles dans les pertes de colonies sont controversés et seront discutés en détails par la suite (cf. C.5).

Lotmaria passim et *Crithidia mellificae* sont deux espèces de trypanosomatides qui infectent les abeilles. Peu d'études existent sur ces deux espèces. Leurs interactions avec *N. ceranae* altèrent la transcription des gènes d'immunité chez les abeilles (Schwarz and Evans, 2013).

Ascophaera apis est un champignon ascomycète responsable de l'ascosphérose du couvain des abeilles, connue sous le nom de couvain plâtré (Gilliam et al., 1988). Le diagnostic clinique de cette maladie est simple, il s'appuie principalement sur la perception de momies de couvain au fond de la ruche et sur la planche d'envol.

B.1.3. Les prédateurs

Le petit coléoptère des ruches (*Aethina tumida*) et le frelon asiatique (*Vespa velutina*) sont parmi les prédateurs les plus néfastes pour l'abeille européenne (*A. mellifera*). *A. tumida* est un coléoptère qui se nourrit à l'état larvaire de miel, de pollen, des œufs et des larves d'abeilles. Il peut être aussi un vecteur de virus comme le DWV (Eyer et al., 2009). D'autre part, l'abeille européenne est complétement fragilisée par les frelons asiatiques qui furent introduits pour la première fois en Europe en 2004. Près de 70% du régime alimentaire des frelons est constitué des abeilles domestiques (Villemant et al., 2014). Ces dernières, suite à l'invasion par les frelons, diminuent leurs vols de butinage ou sont attaquées durant leur vol de retour à leurs ruches, ce qui affecte négativement la dynamique de la colonie et augmente le risque des pertes hivernales (Requier et al., 2019).

B.1.4. Les bactéries

Paenibacillus larvae et *Mellissococus plutonius* sont deux bactéries qui attaquent l'abeille et qui provoquent respectivement la loque américaine et la loque européenne. La loque américaine est beaucoup plus grave que la loque européenne. Elle est très contagieuse, induit de très fortes mortalités au sein du couvain et est difficile à contrôler causant ainsi la perte de la colonie (Genersch et al., 2010a). La loque européenne affecte aussi le couvain et, dans les cas les plus sévères, peut conduire à la perte de la colonie (Budge et al., 2010).

B.1.5. Les virus

La virologie de l'abeille était relativement simple avant le développement rapide des techniques de séquençage à haut débit. Les seuls virus connus étaient ceux qui induisaient des symptômes physiques (DWV, CWV, AmFV, AIV), comportementaux (CBPV, ABPV, SBPV), développementaux (SBV, BQCV) et démographiques (BVX, BVY) (Beaurepaire, 2020) (pour les acronymes et les symptômes, voir tableau 2). Le développement rapide des techniques de séquençage a permis la découverte d'une très large gamme de virus en plus des virus précités. Ces virus sont en majorité asymptomatiques et juste une petite proportion cause des maladies (**Tableau 2**).

La transmission des virus peut être horizontale (ingestion de nourriture contaminée ou trophallaxie) ou verticale (transmis par la reine à sa descendance). *V. destructor* sert aussi de vecteur pour la transmission de plusieurs virus (cf. B.1.1), ce qui augmente l'impact de l'infestation par cet ectoparasite.

Tableau 2 : Les virus d'abeille dont l'infection peut être symptomatique

Liste des virus qui peuvent causer des symptômes physiques ou physiologiques chez les abeilles. Le tropisme correspond aux organes dans lesquels les virus étaient trouvés. D'après Beaurepaire et al (2020).

Virus	Tropism	Symptoms
Deformed wing virus (DWV)	Whole body, including the queen ovaries, queen fat body, spermatheca, and drone seminal vesicles, tissues of wings, head, thorax, legs, hemolymph and gut	Crumpled or aborted wings, shortened abdomens, paralysis, severely shortened adult life span for emerging worker and drone bees, modified responsiveness to sucrose, impaired learning, impaired foraging behavior
Cloudy wing virus (CWV)	Tracheal tissue and thoracic muscles	Opaque wings, shortened lifespan of adult bees
A. mellifera filamentous virus (AmFV)	NA	Milky-white hemolymph
Apis iridovirus (AIV)	NA	Iridescence of most internal organs
Invertebrate iridescent virus Type 6	NA	Flightless clustering bees
Chronic bee paralysis virus (CBPV)	Nervous system, alimentary tract, mandibular and hypopharyngeal glands	Syndrome 1: trembling of the wings and bodies, bloated abdomen, inability to fly, crawling on the ground and upward on grass, gather in groups in the warmest areas of the nest, death within few days
		Syndrome 2 ('black robbers'): hairless (thus appearing smaller), darker, greasy in appearance, shiny, suffer nibbling attacks by the healthy bees, death within few days
Acute bee paralysis virus complex (ABPV)	Nervous system, cytoplasm of fat body cells, brain and hypopharyngeal glands	Trembling, inability to fly, gradual darkening and loss of hair from the thorax and abdomen, crawling on the ground and upward on grass, rapid death for highly infected bees
Slow bee paralysis virus (SBPV)	Nervous system	Paralysis of the two anterior legs a day or two before death
Sacbrood virus (SBV)	Hypopharyngeal glands of worker bees, cytoplasm of fat, muscle and tracheal-end cells of larvae	Pupation failure, 'sac' phenotype: swollen larvae filled with ecdysial fluid full of viral particles, precocious foraging, reduction of adult life span and metabolic activities, impaired foraging activity
Black queen cell virus (BQCV)	Gut tissue	Yellowish queen larvae with sac-appearance that resembles SBV and with time evolves to dark brown, infected pupae turn brown and die, dark brown to black colored walls in queen cells, significantly shortened life span in adult bees
Bee virus X (BVX)	NA	Shortened lifespan of adult bees
Bee virus Y (BVY)	NA	Shortened lifespan of adult bees

B.2. Les facteurs abiotiques

B.2.1. Le changement climatique, la limitation des ressources et les pratiques apicoles

Les effets du changement climatique sur les pollinisateurs sont très peu connus. Cependant, le changement climatique pourrait induire une divergence entre la phénologie des pollinisateurs et celle des plantes que ces derniers pollinisent (Willmer, 2012). En outre, il est prévu que des conditions climatiques extrêmes telles que la sécheresse, les canicules, les inondations et les tempêtes apparaissent plus fréquemment avec le changement climatique affectant ainsi la période de floraison et les réserves de miel et de pollen (Flores et al., 2019). Enfin, le changement climatique augmente les risques d'invasion par des espèces nuisibles comme le frelon asiatique *Vespa velutina* et le petit coléoptère des ruches *Aethina tumida* (Barbet-Massin et al., 2013; Cornelissen et al., 2019) et les ravageurs de culture, ce qui risque d'induire une plus grande utilisation des produits phytopharmaceutiques dommageable pour l'abeille.

Actuellement, la densité et la diversité florale régressent rapidement suite à l'urbanisation et le développement de l'agriculture intensive (Naug, 2009). Cette dernière est fortement fondée sur la monoculture, entrainant ainsi un appauvrissement de la qualité nutritive des ressources et une pénurie des ressources (Alaux et al., 2010b; Dolezal et al., 2019).

De par l'influence des facteurs abiotiques, les pratiques apicoles et l'expérience de l'apiculteur peuvent aussi avoir un effet sur les pertes des colonies. Les apiculteurs par exemple ont de plus en plus recours à la transhumance, souvent sur de longues distances comme le cas des États-Unis, pour assurer la pollinisation d'arbres fruitiers ou de grandes cultures. Cette pratique semble avoir des effets négatifs sur la colonie suite aux stress liés aux taux élevés de dioxyde de carbone, par manque de ventilation, et aux stress liés aux fortes variations de température en traversant de nombreuses régions d'altitudes et de latitudes très variées (Melicher et al., 2019). D'autre part les apiculteurs amateurs, qui possèdent moins de ruches et pourraient éventuellement avoir moins d'expérience que les apiculteurs professionnels, perdent presque le double de leurs colonies à la fin de la période hivernale, d'où l'importance de l'expérience de l'apiculteur et des pratiques apicoles dans la lutte contre les pertes de colonies (Jacques et al., 2017). Cependant, compte tenu des faibles pertes hivernales et des belles récoltes de miel enregistrées au printemps 2020 chez presque tous les apiculteurs, il est difficile de penser que la compétence des apicultures soit une cause majeure pouvant expliquer les pertes hivernales (L'Abeille de France, n°1080 Juin 2020).

B.2.2. Exposition aux pesticides et aux métaux lourds

La pollution environnementale est l'un des principaux facteurs contribuant au déclin d'insectes y compris les abeilles. Les pesticides sont les polluants qui présentent le plus de danger pour les abeilles du fait de leur toxicité intrinsèque attendue. Les abeilles sont exposées à ces produits en visitant des champs agricoles traités ou par ingestion de leur nourriture contaminée par les résidus

de pesticides. Les voies d'exposition aux pesticides, leurs différentes classes et leurs effets sont traités en détails dans les paragraphes qui suivent (cf. D).

Les métaux lourds constituent le deuxième groupe de polluants les plus dangereux pour les abeilles, après les pesticides (Feldhaar and Otti, 2020). Ils se trouvent dans le sol et peuvent être absorbés par les plantes et transmis aux abeilles par contamination du nectar ou du pollen qui seront récoltés par les abeilles pour leur alimentation. Les métaux lourds peuvent aussi se lier à d'autres composés et former des particules fines qui pollueront l'air et se déposeront sur les surfaces des plantes ou des insectes, seront absorbées par le système respiratoire trachéal de l'abeilles ou iront contaminer les eaux de surface (Negri et al., 2015; Pellecchia and Negri, 2018). Les effets des métaux lourds sur les insectes y compris les abeilles reçoivent beaucoup moins d'attention que ceux des pesticides, bien que les métaux lourds soient souvent détectés dans les matrices de la ruche (Conti and Botrè, 2001; Skorbiłowicz et al., 2018). Le peu d'études sur les métaux lourds montre qu'ils nuisent aux abeilles en augmentant le taux de mortalités et en diminuant leurs capacités d'apprentissage et de mémoire (Bromenshenk et al., 1991; Burden et al., 2019; Chicas-Mosier et al., 2017; Lambert et al., 2012).

C. *Nosema ceranae*

C.1. Classification

Nosema ceranae est un parasite de l'abeille qui fait partie des microsporidies. Ces dernières sont des parasites eucaryotes, intracellulaires, obligatoires qui infectent une large gamme d'hôtes, causant des maladies chez les êtres humains et les animaux. Ils se rattachent à une branche au sein du règne des Mycètes (Chen et al., 2009; Hibbett et al., 2007)**.** La première espèce décrite de microsporidies est *Nosema bombycis*, elle est responsable de la maladie de pébrine qui attaque le ver à soie *Bombyx mori* et cause des pertes importantes dans la production de soie.

Il existe près de 1500 espèces de microsporidies groupées en 200 genres. Cependant, il est possible qu'il y ait de nombreuses espèces non encore découvertes (Keeling and Fast, 2002; Vávra and Lukeš, 2013). Sur la base de la large gamme d'hôtes infectés par les microsporidies et de la spécificité que les microsporidies ont d'infecter une seule espèce ou plusieurs espèces proches, il a été estimé que le nombre d'espèces de microsporidies serait égal au nombre d'espèces animales.

Les microsporidies partagent plusieurs points en commun entre-elles, indépendamment des hôtes qu'elles infectent. Elles peuvent exister à l'extérieur de leurs hôtes uniquement sous formes de spores et ont un génome très compact. La mitochondrie est absente chez les microsporidies et est remplacée par un mitosome qui n'a pas la capacité de produire l'ATP, via la phosphorylation oxydative, ce qui rend ces organismes énergétiquement dépendants de leurs hôtes (Keeling, 2009).

C.2. Origine de *Nosema ceranae*

Nosema apis et *Nosema ceranae* sont les deux microsporidies qui attaquent l'abeille *A. mellifera* et sont responsables des Nosémoses type A et C, respectivement. *N. apis* a été isolé de *A. mellifera* en 1909. Cependant *N. ceranae* a été détectée pour la première fois dans *A. cerana* en 1996 (Fries et al., 1996) et dans *A. mellifera* en 2005 en Espagne et à Taiwan (Higes et al., 2006; Huang et al., 2007) et dans des échantillons en Finlande stockés depuis l'année 1998 (Paxton et al., 2007). C'est pour cette raison que les chercheurs considèrent que *N. ceranae* a fait un saut d'espèce depuis quelques décennies de *A. cerana* vers *A. mellifera*. Ce saut d'espèce serait lié à l'implantation des colonies de *A. mellifera* en Asie.

Les deux espèces de *Nosema* ont des effets différents sur l'abeille *A. mellifera* à l'échelle de l'individu et de la colonie. La nosémose de type A se caractérise par des abeilles tremblantes ayant des abdomens dilatés et par la présence de fèces sur les cadres et l'entrée de la ruche, souvent au printemps. Dans les cas sévères, cette maladie conduit à une réduction de la taille des colonies suite à la réduction de la quantité de couvain. Cependant, la nosémose de type C ne présente pas de symptômes spécifiques à l'échelle individuelle et son effet à l'échelle des colonies est controversé.

C.3. Le cycle de vie de *Nosema ceranae*

N. ceranae est transmis aux abeilles après ingestion de spores présentes dans l'eau et la nourriture contaminée et durant les échanges de nourriture entre les abeilles, connus sous le nom de trophallaxie. La microsporidie est encore transmise durant leurs activités de nettoyage et de manutention de la colonie (Martín-Hernández et al., 2018). Le pouvoir infectieux de *N. ceranae* est estimé à 149 spores par abeille, bien que la dose minimale de spores capable de produire une infection est de 1,28 spores par abeille (McGowan et al., 2016). Les spores de *N. ceranae* ont une double paroi entourant la membrane plasmique, formée d'une couche externe de glycoprotéines (exospore) et d'une couche interne de chitine (endospore). Cette double paroi leur assure aux spores une résistance contre la pression osmotique et les protège contre les conditions environnementales défavorables quand elles sont expulsées à l'extérieur de leur hôte. Elles ont en plus un sporoplasme formé d'un noyau double (diplocaryon), d'une vacuole dans sa partie postérieure, de couches de membranes dans la partie antérieure, appelées polaroplaste, et d'un tube polaire qui est attaché à la partie supérieure de la spore (**Fig. 9**). Après ingestion par l'hôte, les spores présentent une germination favorisée par la composition ionique et le pH à l'intérieur de l'intestin. En conséquence, le tube polaire est extrudé et le sporoplasme est transféré à l'intérieur des cellules épithéliales intestinales. Le parasite se développe à l'intérieur du cytoplasme et se multiplie par scission binaire sous forme de mérontes. Cette phase est suivie d'une phase de maturation durant laquelle les mérontes se différencient en sporontes puis en sporoblastes qui deviennent matures dès la formation de l'endospore. Les spores matures sont

ensuite libérées dans la lumière intestinale et sont expulsées à l'extérieur du corps. Les spores peuvent aussi infecter d'autres cellules épithéliales conduisant à une destruction extensive et même totale de l'épithélium intestinal (Fries et al., 1996; Martín-Hernández et al., 2018) (**Fig. 10**).

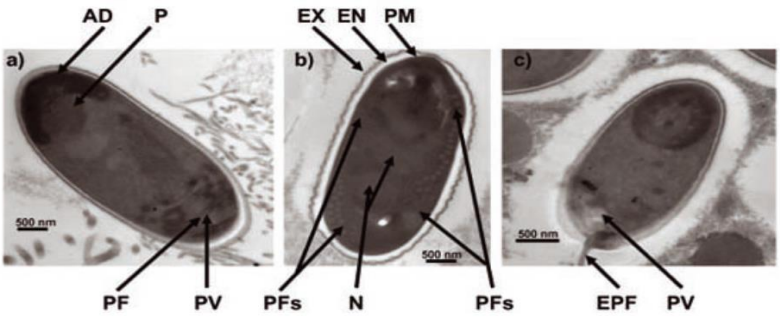

Figure 9 : Micrographies électroniques de trois coupes longitudinales de spores de *N. ceranae*

Les micrographies électroniques des coupes longitudinales de spores de *N. ceranae* montrent (a) le disque d'ancrage (AD), le polaroplaste (P), la vacuole postérieure (PV), le tube polaire (filament polaire) (PF); (b) l'endospore (EN), l'exospore (EX), la membrane plasmique (PM), le noyau (N), le filament polaire (PFs); et (c) le filament polaire extrudé (EPF). D'après Chen et al (2009).

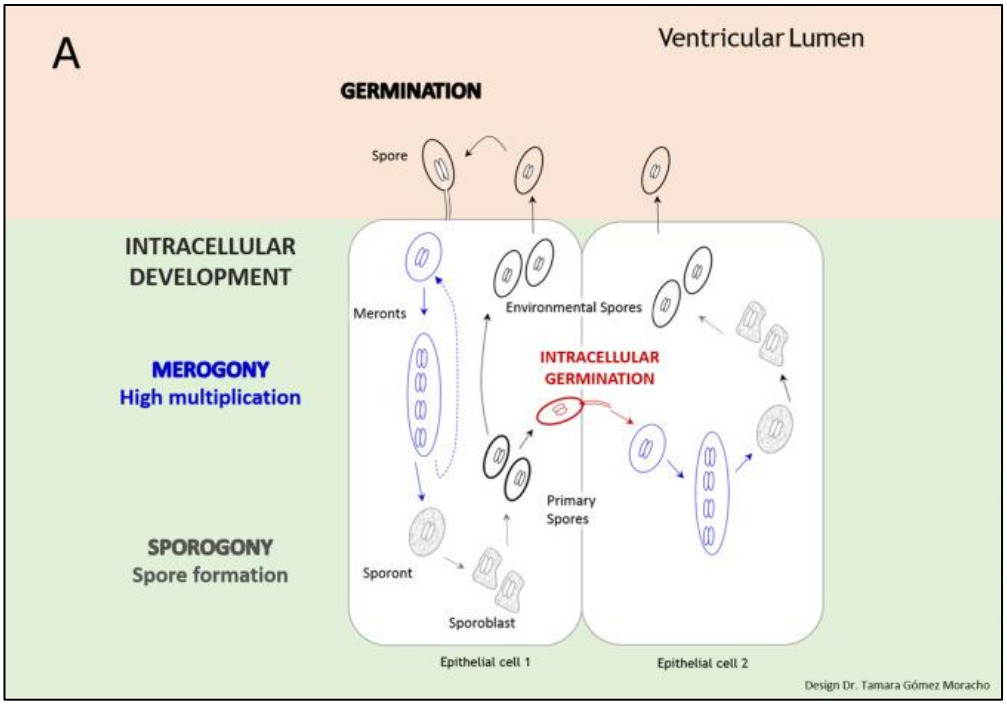

Figure 10 : Cycle de vie de *N. ceranae*

Les spores de *N. ceranae* arrivent dans l'intestin de l'abeille. Ils extrudent le filament polaire et injectent le sporoplasme à l'intérieur des cellules épithéliales intestinales. Le sporoplasme mature en méronte et se multiplie durant la phase de mérogonie par scission binaire. Cette phase est suivie de la sporogonie durant laquelle les sporontes se transforment en sporoblastes puis en spores matures. La première génération de spores sont des spores primaires qui réinfectent les mêmes cellules ou des cellules adjacentes. La deuxième génération de spores est constituée par des spores ayant une membrane plus épaisse qui seront expulsées dans l'environnement. D'après Martín-Hernández et al (2018).

C.4. Impact de *Nosema ceranae* sur les abeilles à l'échelle individuelle

De plus en plus d'études se concentrent sur les effets de l'infection des abeilles par *N. ceranae*. Les effets de *Nosema* ne sont pas seulement localisés au niveau du tissu épithélial intestinal mais ils présentent un caractère systémique et affectent plusieurs fonctions telles que le métabolisme, l'immunité et le comportement.

C.4.1. Effet de *Nosema ceranae* sur la mortalité

L'infection par *N. ceranae* réduit significativement la durée de vie des abeilles à l'échelle individuelle. Cependant la sévérité de l'infection expérimentale par les spores (de l'ordre de 100 000 spores) varie considérablement entre les études, avec des taux de mortalités ne dépassant pas les 30% dans certaines études et arrivant à 100% deux à trois semaines après l'infection dans d'autres études (Alaux et al., 2010a; Aufauvre et al., 2012; Aufauvre et al., 2014; Dussaubat et al., 2012a; Higes et al., 2007). Cette variabilité du taux de survie consécutive à l'infection par

Nosema entre les différentes études pourrait être attribuée à plusieurs facteurs tels que (i) l'utilisation de protocoles expérimentaux différents (abeilles en cagettes, en colonies sous tunnel ou en plein champ). (ii) La diversité du régime alimentaire utilisé pour nourrir les abeilles. (iii) L'utilisation de souches différentes d'abeilles et/ou de pathogènes et la vitalité des spores utilisées dans les études expérimentales. (iv) le statut toxico-physiologique des abeilles.

C.4.2. Perturbation métabolique

Des études ont rapporté des modifications dans le métabolisme des hydrates de carbone chez les abeilles suite à leur infection par *N. ceranae*. Les modifications sont détectées sous forme de perturbations de plusieurs gènes impliqués dans le métabolisme des hydrates de carbone. Suite à l'infection par *N. ceranae*, il y'a une augmentation de l'expression de trois gènes impliqués dans le transport du tréhalose (principale molécule de stockage glucidique chez les insectes) et le gène codant pour la α-glucosidase (enzyme qui hydrolyse le saccharose du nectar en glucose et en fructose dans les glandes hyopharyngiennes) (Dussaubat et al., 2012a). De plus, il y'a une diminution de l'expression d'un gène codant pour la tréhalase (enzyme qui hydrolyse le tréhalose et génère du glucose, qui sera après catabolisé par la glycolyse ou la voie des pentoses phosphates) (Aufauvre et al., 2014). Les abeilles infectées par *Nosema* ont aussi des faibles teneurs en acides aminés tels que la L-proline et en glucides tels que le fructose, le tréhalose, le sorbitol et le glycérol (Aliferis et al., 2012; Kurze et al., 2016). D'autre part, une augmentation de la consommation de saccharose chez les abeilles infectées a été signalée dans plusieurs études et une augmentation de mortalité a été observée quand l'alimentation des abeilles était restreinte à une quantité limitée de saccharose (Alaux et al., 2010a; Mayack and Naug, 2009; Vidau et al., 2011). L'augmentation des besoins en sucres et les perturbations de l'expression de gènes du métabolisme et du taux de sucre et d'acides aminés dans l'hémolymphe, peuvent être liées à la dépendance de *Nosema* à l'énergie produite par l'hôte et à l'utilisation de cette énergie pour sa croissance.

C.4.3. Effet de *Nosema ceranae* sur l'équilibre oxydatif

Plusieurs études ont montré que l'infection par *N. ceranae* induit une augmentation des capacités antioxydantes chez l'abeille. En effet, suite à l'infection, l'expression des gènes codant pour la CAT et la GPOx augmente dans l'intestin ainsi que l'activité de la GST dans l'intestin et le tissu adipeux (Dussaubat et al., 2012a; Vidau et al., 2011). Ces trois enzymes précitées font partie des enzymes antioxydantes, ayant comme but de lutter contre les ROS (cf. A.4.3). Ainsi, suite à l'activation de ces enzymes, le taux de ROS diminue et les dégâts induits par le stress oxydant sont minimisés. Cela est confirmé par la diminution des concentrations des peroxydes solubles (qui font partie des ROS) et des protéines et de lipides oxydés quelques jours après l'infection par *N. ceranae.* Le stress oxydant, qui se produit juste après l'infection par *Nosema*, pourrait être dû à l'intensification du métabolisme énergétique dans les cellules de l'abeille pour assurer les

besoins énergétiques de *Nosema*. Ainsi, la phosphorylation oxydative génère simultanément de l'ATP et des ROS conduisant à une activation du système antioxydant par l'abeille pour minimiser les effets négatifs des ROS. D'autre part, *Nosema* pourrait être capable de manipuler l'hôte en augmentant les capacités antioxydantes afin de se protéger contre les effets délétères des ROS (Paris et al., 2017).

C.4.4. Effet de *Nosema ceranae* sur le système immunitaire

L'infection de l'abeille par *N. ceranae* affecte la réponse immunitaire humorale mais pas la réponse cellulaire. En effet, le nombre total d'hémocytes et l'activité de la POx ne changent pas suite à l'infection par *Nosema* (Alaux et al., 2010a; Kairo et al., 2017a; Vázquez et al., 2020). Cependant, les perturbations de la réponse immunitaire humorale suite à l'infection par *N. ceranae* se traduisent par une diminution de l'expression de plusieurs gènes codant pour des PAM (Antunez et al., 2009; Aufauvre et al., 2014; Chaimanee et al., 2012) ainsi qu'une diminution de l'expression des PAM et des récepteurs de reconnaissance de motifs moléculaires (PRR, acronyme de Pattern Recognition Receptor) (Li et al., 2018). Ces derniers jouent aussi un rôle dans la réponse immunitaire humorale en assurant la reconnaissance d'un ensemble de molécules qui signent la présence de pathogènes.

C.4.5. Effet de *Nosema ceranae* sur le polyéthisme

L'infection par *N. ceranae* conduit à des altérations du polyéthisme avec, notamment, une activité de butinage précoce et une diminution de la survie des ouvrières. En effet, dans les conditions physiologiques normales, les jeunes abeilles s'occupent des tâches à l'intérieur de la ruche et présentent des taux élevés de Vitellogénine (Vg) et des taux faibles d'hormone juvénile (HJIII). Quand les abeilles se transforment en butineuses, les taux de Vg et de l'HJIII s'inversent. Lors de l'infection des jeunes abeilles par *N. ceranae*, l'expression du gène codant pour la Vg diminue, et le taux de HJIII augmente. Cela entraine une activité de butinage précoce et une réduction de la longévité de ces abeilles infectées de moins de neuf jours (Goblirsch et al., 2013). De plus, l'infection par *N. ceranae* provoque une augmentation du taux de l'oléate éthyle (OE). Cette phéromone est émise par les butineuses et inhibe la transition des jeunes abeilles en futur butineuses. Ainsi, le taux élevé d'OE chez les butineuses infectées par *N. ceranae*, est capable de perturber l'organisation de la colonie, ce qui peut retarder le démarrage de l'activité de butinage chez les jeunes abeilles non infectées (Dussaubat et al., 2010). D'autre part, les abeilles infectées présentent des difficultés à retourner à leur ruche ; elles mettent plus de temps pour y arriver ou n'y retournent jamais (Kralj and Fuchs, 2010).

C.5. Effets de *Nosema ceranae* à l'échelle de la colonie

Il est difficile de juger l'importance de l'effet de l'infection par *N. ceranae* sur les pertes de colonies. Des études conduites en Espagne, en Europe méditerranéenne et au Moyen-Orient ont mis en évidence un lien entre l'infection par *N. ceranae* et les pertes de colonies (Higes et al., 2009), tandis que d'autres études conduites dans le nord de l'Europe et des États-Unis n'ont pas mis en évidence ce lien (Forsgren and Fries, 2013). Cette différence d'effets entre les régions pourrait être liée à la sensibilité des spores de *N. ceranae* aux faibles températures et à l'incapacité de *N. ceranae* à envahir les régions ayant un climat plus froid.

L'infection par *Nosema* entraine des modifications du taux d'OE, de Vg et de l'HJIII chez les ouvrières. Ainsi, l'infection induit un butinage précoce, une longévité plus faible et des capacités d'orientation réduites chez les jeunes abeilles. De plus, l'augmentation de la production d'OE par les abeilles infectées entraine un retard de l'activité de butinage chez les jeunes abeilles non infectées (Dussaubat et al., 2010; Goblirsch et al., 2013). Toutes ces modifications entrainent des altérations au niveau de l'homéostasie de la colonie et pourraient être responsables de l'affaiblissement des ruches.

D'autre part, *N. ceranae* infecte aussi les castes reproductrices de la colonie par transmission horizontale. L'infection des reines par *N. ceranae* augmente les risques de suppression des reines et perturbe l'homéostasie de la colonie et la production de couvain et du miel. Cela est dû à l'altération du taux de vitellogénine (qui est un indicateur de la longévité et de la fertilité des reines) et des capacités antioxydantes des reines ainsi que la production de la phéromone mandibulaire (Queen mandibular pheromone, QMP). Cette phéromone est sécrétée par les reines et permet de maintenir la cohésion de la colonie, d'inhiber le développement des ovaires des ouvrières et de réguler la maturation de ces derniers (Alaux et al., 2011; Dussaubat et al., 2016).

D. Exposition aux pesticides

D.1. Consommation mondiale

Les pesticides sont des substances chimiques naturelles ou synthétiques, utilisées le plus souvent dans l'agriculture pour lutter contre les insectes ravageurs, les mauvaises herbes et les agents pathogènes qui conduisent ensemble à une perte annuelle de 31% de la production agricole à l'échelle mondiale (**Tableau 3**). Les mauvaises herbes provoquent les pertes les plus élevées suivies par les ravageurs et les agents pathogènes (34%, 18% et 16% de pertes respectivement) (Oerke, 2006). À l'heure actuelle, deux millions de tonnes de pesticides sont utilisés chaque année dans le monde, et il est prévu que ce nombre augmente dans les années qui suivent. Près de 50% des pesticides utilisés sont des herbicides, 29,5% sont des insecticides et 17,5% sont des fongicides (De et al., 2014). La Chine, les États-Unis et le Brésil sont les plus gros consommateurs actuels de pesticides (Faostat, 2018) (**Fig. 11**).

Tableau 3 : Estimation des pertes en agriculture provoquées par les stresseurs biotiques des plantes

Estimation des pertes provoquées par les mauvaises herbes, les ravageurs, les pathogènes et les virus sur six cultures principales dans 19 régions du monde entier. D'après Oerke (2006).

		Crop losses [%][1] due to									
	Attainable production	Weeds		Animal pests		Pathogens		Viruses		Total	
Crop	[M t]	Potential	Actual	Potential	Actual	Potential	Actual	Potential	Actual	Potential	Actual
Wheat	785·0	23·0 (18–29)	7·7 (3–13)	8·7 (7–10)	7·9 (5–10)	15·6 (12–20)	10·2 (5–14)	2·5 (2–3)	2·4 (2–4)	49·8 (44–54)	28·2 (14–40)
Rice	933·1	37·1 (34–47)	10·2 (6–16)	24·7 (13–26)	15·1 (7–18)	13·5 (10–15)	10·8 (7–16)	1·7 (1–2)	1·4 (1–3)	77·0 (64–80)	37·4 (22–51)
Maize	890·8	40·3 (37–44)	10·5 (5–19)	15·9 (12–19)	9·6 (6–19)	9·4 (8–13)	8·5 (4–14)	2·9 (2–6)	2·7 (2–6)	68·5 (58–75)	31·2 (18–58)
Potatoes	517·7	30·2 (29–33)	8·3 (4–14)	15·3 (14–20)	10·9 (7–13)	21·2 (20–23)	14·5 (7–24)	8·1 (7–10)	6·6 (5–9)	74·9 (73–80)	40·3 (24–59)
Soybeans	244·8	37·0 (35–40)	7·5 (5–16)	10·7 (4–16)	8·8 (3–16)	11·0 (7–16)	8·9 (3–16)	1·4 (0–2)	1·2 (0–2)	60·0 (49–69)	26·3 (11–49)
Cotton	78·5[2]	35·9 (35–39)	8·6 (3–13)	36·8 (35–41)	12·3 (5–22)	8·5 (7–10)	7·2 (5–13)	0·8 (0–2)	0·7 (0–2)	82·0 (76–85)	28·8 (12–48)

[1] Figures in parentheses indicate variation among 19 regions.
[2] Seedcotton.

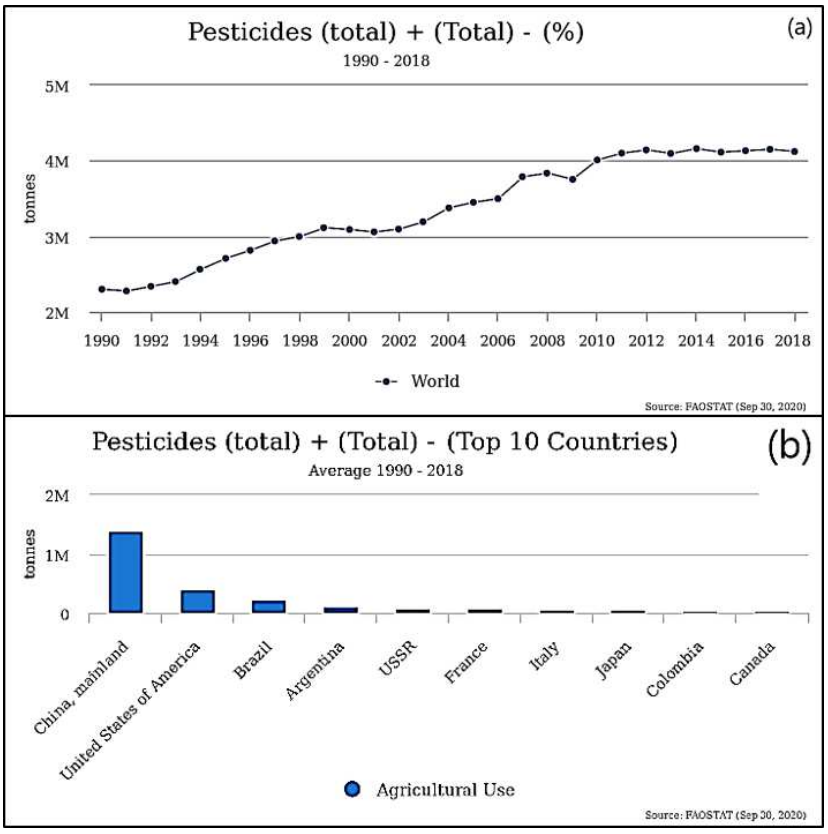

Figure 11 : Évolution de la quantité de pesticides utilisée en agriculture entre 1990 et 2018

La quantité de pesticides utilisée en agriculture a augmenté à l'échelle mondiale entre 1990 et 2018 (a). Les trois pays qui ont utilisé la plus grande quantité de pesticides en agriculture entre 1990 et 2018 sont la Chine, les États-Unis et le Brésil (b). Ces graphes sont obtenus à partir des statistiques de la FAO (Faostat. 2018).

D.2. Origine des pesticides

L'utilisation des pesticides remonte à plus de 4500 ans, quand les sumériens ont utilisé le soufre pour protéger leurs cultures. Pline, naturaliste romain du I^{er} siècle, préconisait l'utilisation de l'arsenic comme insecticide et de la soude et de l'huile d'olive pour le traitement protecteur des graines de légumes. Avant le 16ème siècle, les Chinois utilisaient des quantités modérées de composés arsenicaux comme insecticides, puis vers le 17ème siècle, ils utilisaient le premier insecticide naturel, la nicotine, sous forme d'extrait de feuilles de tabac. Au 18ème siècle, Hamberg proposait le chlorure mercurique comme protecteur du bois. Les extraits de pyrèthres et le savon étaient utilisés pour prévenir l'action des insectes et Forsyth a décrit l'utilisation combinée d'extrait de tabac, de soufre et de chaux pour combattre les insectes et les champignons. Puis au 19ème siècle, Prévost décrivait l'inhibition de la croissance des champignons par le sulfate de cuivre. L'essor véritable des pesticides fut lié au développement de la chimie organique de synthèse dès les années 1930. Ainsi, les propriétés insecticides du dichlorodiphényltrichloroéthane (DDT) furent découvertes en 1939 et ce dernier a dominé le marché des insecticides jusqu'aux années 1970 (EPA, 1975). La recherche sur les armes chimiques, notamment durant la deuxième guerre mondiale, a permis de développer d'autres pesticides comme les organophosphorés. La prise de conscience progressive des impacts environnementaux et sanitaires de ces pesticides a conduit à limiter leurs usages voire même à interdire l'utilisation de certains d'entre eux (Curtis and Lines, 2000). Cela a stimulé l'industrie phytopharmaceutique à trouver d'autres molécules ayant un mode d'action restreint sur des espèces cibles et efficaces à faible dose. Ainsi, le développement des insecticides pyréthrinoïdes a débuté dans les années 1970. Avec ces substances, les dosages sont passés de 500-2000 g/ha à quelques grammes par ha. Par exemple, avec la deltaméthrine, insecticide pyréthrinoïde, le dosage était de 7,5 g/ha (Elliott, 1989). Par la suite, dans les années 1990 ont été développées les familles des phényles pyrazoles, dont le premier représentant était le fipronil, et les néonicotinoïdes, dont le premier représentant était l'imidaclopride. Avec les substances de ces familles, les dosages se situaient aux alentours de 70 g/ha (Simon-Delso et al., 2015).

D.3. Exposition des abeilles aux pesticides

D.3.1. Exposition lors de l'activité de butinage

Afin de protéger les cultures, les agriculteurs ont souvent recours à la pulvérisation des pesticides en plein champ. Ainsi les pollinisateurs, y compris les abeilles butineuses, sont exposés par contact, de façon aiguë et directe (à des doses très élevées), aux gouttelettes de pesticides dans les champs traités ou aux alentours suite à la dérive des pesticides, phénomène pouvant être amplifié par le vent. L'exposition aiguë de contact aux pesticides, surtout aux insecticides, conduit à une mortalité élevée chez les abeilles exposées. Cette mortalité élevée est due à la toxicité élevée de ces substances, même à faible dose (Suchail et al., 2001) (**Fig. 12**).

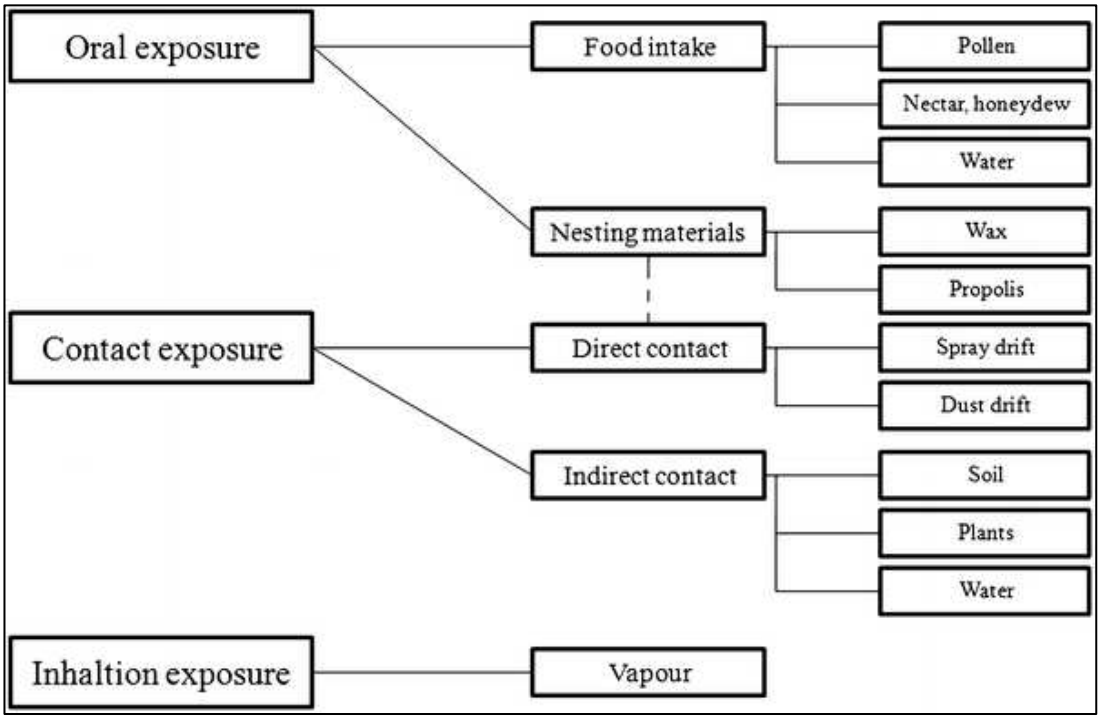

Figure 12 : Voies d'exposition des abeilles aux pesticides

Les abeilles sont exposées aux pesticides par de multiples voies d'exposition. Les butineuses peuvent être exposées par voie orale suite à la contamination du pollen, du nectar et de l'eau par les pesticides. Elles sont encore exposées par contact direct avec les produits pulvérisés en pleine floraison (cas des fongicides et des herbicides), ou aux dérives suite à la pulvérisation et aux poussières émises lors des semis de semences enrobées avec des pesticides. De plus, les butineuses sont exposées par contact indirect avec les plantes et l'eau contaminées. Les butineuses rapportent les produits contaminés à la ruche et c'est ainsi que les autres individus, tels que les castes reproductrices et les abeilles nourrices, seront exposés par voie orale à ces contaminants. D'après Alkassab et al (2017).

Afin de minimiser le danger pour l'environnement, l'usage des pesticides et, surtout, des insecticides, n'est pas autorisé en plein champ durant la floraison. Ainsi, certains pesticides systémiques, comme par exemple les insecticides néonicotinoïdes, ont plutôt été appliqués aux cultures par enrobage de semences. Lors de son développement, cette technique était considérée moins dommageable que la pulvérisation pour les organismes non cibles. Toutefois, elle pose aussi des risques élevés pour ces organismes à travers la formation de poussières toxiques générées par l'abrasion qui provoque la séparation des pesticides de la surface externe des semences lors du semis (Biocca et al., 2019; Girolami et al., 2012; Greatti et al., 2003). Ces poussières se déposent sur les plantes, aux alentours du champ cultivé, et sur les abeilles lors du butinage, les exposant ainsi par contact à ces pesticides.

Les pesticides systémiques ont une solubilité dans l'eau relativement élevée. Par conséquent, ils seront absorbés par la plante et transportés par le phloème ou le xylème aux parties distales non traitées suite à leur application sur le sol ou sur la semence par enrobage. Ces pesticides systémiques persistent à l'intérieur des plantes et contaminent sur de longues durées le nectar, le pollen, les exsudats et l'eau de guttation (Laurent and Rathahao, 2003). Ainsi, les abeilles seront

exposées oralement, et d'une façon chronique, à ces pesticides (Bonmatin et al., 2015; Krupke et al., 2012; Tapparo et al., 2011).

L'eau constitue une autre voie d'exposition orale aux pesticides. Elle peut être contaminée par les pesticides suite à leur lessivage du sol vers l'eau souterraine ou leur dérive vers les eaux de surface (Masiá et al., 2013).

D.3.2. Exposition aux résidus de pesticides

Les butineuses ayant été exposées oralement aux produits phytopharmaceutiques à travers les ressources contaminées (nectar, pollen, exsudat et eau) rapportent les substances actives à leurs colonies. Cela est confirmé par de nombreuses études qui montrent une forte contamination des matrices de la ruche par les résidus de pesticides. Ces résidus se retrouvent dans le miel, le pain d'abeilles, la cire, la propolis, et même les abeilles, des ruches de toutes les régions du monde (Bridi et al., 2018; Gonzalez-Martin et al., 2017; Kanga et al., 2019; Lambert et al., 2013).

En plus des pesticides issus des traitements agricoles, les apiculteurs se voient contraints d'utiliser des pesticides à l'intérieur de leurs ruches pour traiter des parasites tels que *Varroa destructor*. De nombreux acaricides organiques, naturels et synthétiques, sont utilisés et laissent également des résidus dans les matrices de la ruche (Chauzat and Faucon, 2007; Lopez et al., 2016).

Il existe de grandes différences dans l'abondance totale et relative des pesticides entre les nombreuses études effectuées. L'existence des pesticides ainsi que la fréquence de leurs détections et leurs concentrations dépendent de l'emplacement des ruches par rapport aux surfaces agricoles, des types de cultures et des variations saisonnières (Lambert et al., 2013; Villalba et al., 2020). Lambert et al (2013) ont collecté des échantillons de pollen, de miel et d'abeilles butineuses de 18 ruchers situés dans l'ouest de la France, pendant deux ans, dans le but d'identifier et de quantifier 80 pesticides utilisés pour le traitement des cultures et la lutte contre *Varroa*. Dans les abeilles butineuses, le miel et le pollen, 72,3%, 95,7% et 58,6% des échantillons, respectivement, étaient contaminés par au moins un pesticide. Les pesticides les plus fréquemment détectés dans les abeilles butineuses étaient le carbendazime (fongicide, dans 41,1% des échantillons), le triphénylphosphate (insecticide, 24,8%), le coumaphos (acaricide, 17,8%) et l'amitraze II (acaricide, 16,3%). Les pesticides les plus détectés dans le miel étaient le coumaphos (acaricide utilisé contre *Varroa destructor*, 78,0%), l'amitraze II (acaricide utilisé contre *Varroa destructor*, 68,8%), le carbendazim (fongicide, 64,5%), le phosmet (insecticide, 12,8%) et le cyproconazole (fongicide, 11,3%). Dans le pollen, les pesticides les plus détectés étaient le carbendazime (fongicide, 34,4%) et l'amitraze II (acaricide, 14,8%). Ainsi, dans cette étude, les acaricides et les fongicides étaient les pesticides les plus fréquemment retrouvés dans la matrice de la ruche.

La contamination des différentes matrices de la ruche par les pesticides persiste même en-dehors de la saison apicole. Ainsi, plus de 49% des échantillons de pain d'abeille, de miel et de cire sont contaminés par des pesticides au début du printemps (Ostiguy and Eitzer, 2014; Pohorecka et al., 2017). Ces études sur les résidus de pesticides dans les matrices apicoles soulèvent la question de l'implication des pesticides dans les pertes élevées de colonies à la sortie de l'hiver. En effet, les abeilles d'hiver, de par leur grande longévité et la durée de la saison hivernale, sont exposées aux pesticides pendant une longue période de temps, ce qui pourrait conduire à un affaiblissement global de la colonie et pourrait expliquer l'effondrement des colonies pendant l'hiver.

D.4. Les trois pesticides d'intérêt

Dans le cadre de cette thèse, les études ont été focalisées sur les effets de trois pesticides, l'imidaclopride, le glyphosate et le difénoconazole appartenant aux classes des insecticides, des herbicides et des fongicides, respectivement. Ces trois classes de pesticides regroupent à elles seules 98% des substances actives utilisées en phytoprotection. Le choix de ces substances sera détaillé dans la section « Objectifs de la thèse ».

D.4.1. L'imidaclopride

D.4.1.1. Mode d'action

L'imidaclopride a été le premier insecticide commercialisé appartenant à la famille des néonicotinoïdes. Il a été introduit sur le marché en 1994. Les insecticides de cette famille sont divisés en trois sous-familles chimiques : (i) Les N-nitroguanidines (imidaclopride, thiaméthoxame, clothianidine et dinotéfuran). (ii) Les nitrométhylènes (nitenpyrame). (iii) Les N-cyanoamidines (acétamipride et thiaclopride). Ce sont tous des agonistes des récepteurs postsynaptiques nicotiniques de l'acétylcholine (nAChR). Ils se lient à ces récepteurs chez l'insecte par des interactions de haute affinité et provoquent une hyperstimulation prolongée des neurones cholinergiques qui conduit à une paralysie fatale, même à de très faibles doses. En effet, les doses létales médianes orales ($DL_{50,}$ dose qui tue 50% des individus par ingestion) de l'imidaclopride et de la clothianidine sont de 4 et 5 ng par abeille, respectivement. Cette DL_{50} correspond approximativement à $1/10.000^{ème}$ de la DL_{50} du DDT (**Fig. 13**).

La toxicité des insecticides néonicotinoïdes est beaucoup plus élevée chez les insectes que chez les vertébrés. Cette différence de toxicité est due à une affinité plus élevée pour les nAChR des insectes que pour les nAChR des vertébrés (**Fig. 14**).

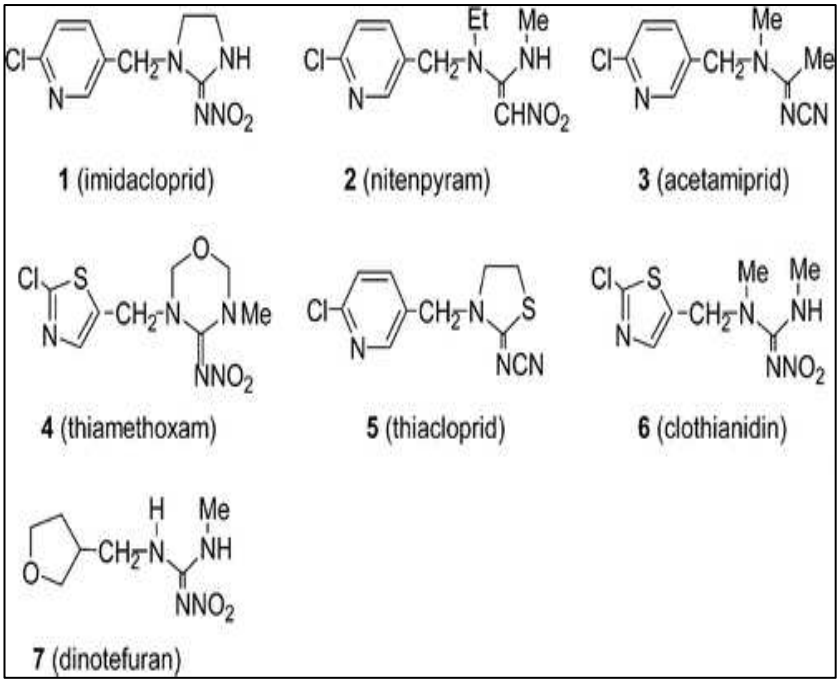

Figure 13 : Structures chimiques des néonicotinoïdes

La figure représente les structures chimiques des néonicotinoïdes commercialisés. D'après Kagabu et al (2011).

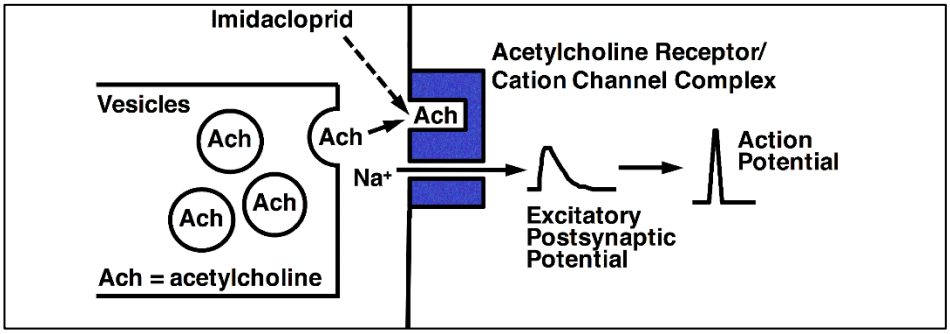

Figure 14 : Mode d'action de l'imidaclopride sur les récepteurs synaptiques

Sous les conditions physiologiques normales, l'acétylcholine (ACh) se fixe sur récepteurs postsynaptiques nicotiniques de l'acétylcholine (nAChR) après sa libération des vésicules présynaptiques. En se liant à ces récepteurs, elle provoque une modification de la perméabilité ionique membranaire et un passage des cations sodiums (Na^+) et potassium (K+) à travers la membrane plasmique du neurone postsynaptique. L'action de l'acétylcholine s'achève par l'action de l'acétylcholinestérase (AChE) qui hydrolyse l'ACh. La nicotine et les néonicotinoïdes imitent l'action de l'ACh en se fixant sur les nAChR et les activant d'une manière persistante car ils ne sont pas dégradés par l'AChE. Cela conduit à une hyperstimulation des synapses cholinergiques qui aboutit à une hyperexcitation, des convulsions, des paralysies et la mort de l'insecte. Figure modifiée d'après Bloomquist (2009).

D.4.1.2. Utilisation des néonicotinoïdes

Les néonicotinoïdes présentent des propriétés systémiques dans la plante ; ils sont relativement solubles dans l'eau et sont absorbés par la plante à travers leurs feuilles ou leurs racines, puis distribués dans tous ses tissus par les voies xylémique et phloémique (Goulson, 2013). Ils sont utilisés pour lutter contre une large gamme d'insectes nuisibles comprenant les pucerons, les aleurodes, les cicadelles et les taupins. Le large spectre d'action des néonicotinoïdes, leurs activités systémiques, leur faible toxicité pour les vertébrés et leur toxicité élevée pour les insectes, sont des facteurs qui ont contribué à l'expansion de leur utilisation dès l'année 1994. Parmi les différentes familles d'insecticides, la famille des néonicotinoïdes regroupe les substances les plus utilisées à l'échelle mondiale. En 2014, la part des néonicotinoïdes dans le marché international des pesticides était équivalente à 25%, dont 85% étaient constituées par l'imidaclopride, la clothianidine et le thiaméthoxame (Bass et al., 2015) (**Tableau 4**)

Tableau 4 : Évolution du marché mondial des néonicotinoïdes entre 2003 et 2009

L'évolution du marché mondial de sept néonicotinoïdes entre 2003 et 2009 (valeurs en millions de dollars américains) ainsi que le nombre de culture traitées par chaque néonicotinoïdes. D'après Simon-Delso et al (2015).

Product	Crop uses	Company	2003	2005	2007	2009
imidacloprid	140	Bayer CropScience	665	830	840	1091
thiamethoxam	115	Syngenta	215	359	455	627
clothianidin	40	Sumitomo//Bayer CS	<30	162	365	439
acetamiprid	60	Nippon Soda	60	95	130	276
thiacloprid	50	Bayer CropScience	<30	55	80	112
dinotefuran	35	Mitsui Chemicals	<30	40	60	79
nitenpyram	12	Sumitomo	45	<10	<10	8

D.4.1.3. Réglementation d'usage des néonicotinoïdes

Les réglementations concernant l'utilisation des insecticides en agriculture ont commencé à apparaitre en France dès l'année 1975 avec un arrêté interdisant l'utilisation des insecticides en pulvérisation durant toute la période de floraison et de production d'exsudats consécutifs aux attaques de pucerons. Les réglementations concernant l'utilisation des néonicotinoïdes en agriculture ont commencé en Europe dès l'année 2013 avec un moratoire qui a suspendu l'utilisation de l'imidaclopride, du thiaméthoxame et de la clothianidine en enrobage de semences (Règlement d'exécution (UE) No 485/2013) (Commission européenne, 2013). Ensuite, l'article L.123-19-1 du code de l'environnement a interdit en France, depuis le premier septembre 2018, l'utilisation des produits phytopharmaceutiques contenant une ou des substances actives de la famille de néonicotinoïdes. Toutes ces mesures d'interdiction d'usage des néonicotinoïdes ont été prises sur la base des résultats obtenus par les structures de recherche académiques qui ont

permis de démontrer le fort impact de cette famille d'insecticides sur les pollinisateurs, y compris les abeilles domestiques et sauvages (Bonmatin et al., 2015; Chagnon et al., 2015; Furlan and Kreutzweiser, 2015; Pisa et al., 2015).

D.4.1.4. Effets sublétaux des néonicotinoïdes sur les abeilles

D.4.1.4.1. Altérations physiologiques

Les études sur les effets des néonicotinoïdes à des doses sublétales ont montré un fort impact de ces produits sur la physiologie des abeilles. L'exposition des abeilles aux néonicotinoïdes affecte le système immunitaire. Une exposition orale de 24 heures au thiaclopride, à l'imidaclopride et à la clothianidine à des concentrations environnementales, conduit à une diminution du taux d'hémocytes dans l'hémolymphe et à une altération de la réaction d'encapsulation des agents exogènes et de l'activité antimicrobienne (Brandt et al., 2016). De plus, l'exposition des abeilles à la clothianidine durant leur stade larvaire affecte aussi l'immunité sociale des abeilles adultes qui se traduit par une réduction du comportement d'hygiène chez les colonies traitées (Tsvetkov et al., 2017).

Les néonicotinoïdes affectent le métabolisme des abeilles. Une exposition des colonies d'abeilles à l'imidaclopride, pendant 15 jours, à la concentration de 2 µg/L, dans la nourriture, affecte le métabolisme énergétique des larves d'abeilles. Cet insecticide affecte aussi le métabolisme cérébral des abeilles adultes par une altération de la cytochrome c oxydase au niveau des corps pédonculés (Armengaud et al., 2000; Decourtye et al., 2004a; Decourtye et al., 2004b; Derecka et al., 2013).

De nombreuses études ont montré des modifications de l'activité et/ou de l'expression de gènes codant des enzymes de détoxication (GST, CaEs, CYP450) (Alptekin et al., 2016; Badiou-Beneteau et al., 2012; Tesovnik et al., 2020b) et des enzymes impliquées dans la lutte contre le stress oxydant (CAT, GST) (Badiou-Beneteau et al., 2012). Ces résultats reflètent ainsi des perturbations des capacités de détoxication et l'induction d'un stress oxydant à suite d'une exposition des abeilles aux néonicotinoïdes (Gauthier et al., 2018; Gregore et al., 2018).

Les néonicotinoïdes induisent une cytotoxicité lors de leur diffusion dans les organes de l'abeille. Des altérations morphologiques et histochimiques ont été détectées dans le cerveau des abeilles adultes suite à une exposition chronique au thiaméthoxame à 1/10 et 1/100 de la concentration létale médiane orale (CL_{50}) (Oliveira et al., 2014). De plus, les néonicotinoïdes peuvent affecter d'autres organes. L'imidaclopride, par exemple, entraine une réduction de la taille des glandes hyopharyngiennes (Heylen et al., 2011; Škerl and Gregorc, 2010). Des phénomènes apoptotiques ont été détectées dans les intestins des larves d'abeilles exposées pendant quatre jours à l'imidaclopride à 400 mg/kg de nourriture, qui est un niveau d'exposition non environnemental (Gregorc and Ellis, 2011), et dans les intestins et les tubes de Malpighi des abeilles adultes exposées pendant huit jours au thiaméthoxame à 1/10 de la CL_{50} par jour (Catae et al., 2014). De

plus, de nombreuses études récentes mettent aussi en évidence la capacité des néonicotinoïdes à perturber le microbiote intestinal de l'abeille en altérant l'abondance totale et relative des communautés bactériennes (Liu et al., 2020; Raymann et al., 2018; Rouze et al., 2019).

D.4.1.4.2. Altérations comportementales et cognitives

Le bon fonctionnement de l'abeille à l'échelle individuelle et à l'échelle de la colonie dépend du bon fonctionnement du système nerveux. Ainsi, les néonicotinoïdes constituent un danger pour l'abeille en affectant le système nerveux. Ce danger est mis en évidence grâce aux nombreuses études qui montrent l'implication des néonicotinoïdes dans l'altération de l'apprentissage et de la mémoire des abeilles. L'apprentissage et la mémoire des abeilles sont affectés suite à des expositions aiguës et chroniques à l'imidaclopride à des niveaux sublétaux (Decourtye et al., 2003a; Guez et al., 2001). De plus, l'ingestion de l'imidaclopride à une concentration de 100 µg/kg dans la nourriture perturbe le retour des abeilles à la ruche (Bortolotti et al., 2003), ce qui met en danger le devenir de toute la colonie (Henry et al., 2012). D'autres néonicotinoïdes, tels que le thiaclopride, sont aussi capables d'altérer le comportement des abeilles. L'exposition orale des abeilles à des concentrations sublétales de thiaclopride dans la nourriture, pendant plusieurs semaines, conduit aussi à des perturbations des capacités des abeilles à butiner, à retourner à leur ruche et à communiquer avec les autres abeilles (Tison et al., 2016).

D.4.1.5. Effets des néonicotinoïdes sur la longévité des individus

Les néonicotinoïdes se caractérisent par une toxicité aiguë orale et de contact très élevées. En plus d'une toxicité aiguë très élevée, les néonicotinoïdes présentent une forte toxicité chronique à de très faibles doses. Cette toxicité a été particulièrement étudiée dans des expérimentations de laboratoire. L'imidaclopride peut induire un taux de mortalité de 50% après 8 jours d'exposition à une nourriture contaminée à des concentrations de 0.1, 1 et 10 µg/L (Suchail et al., 2001). Il est intéressant de noter que la dose cumulée ingérée après 8 jours d'exposition est de 60 à 6000 fois inférieure à la dose nécessaire pour produire les mêmes effets en exposition aiguë. En ce qui concerne le thiaclopride, une exposition de 13 jours à des concentrations de 600 et 2000 µg/L conduit à une augmentation significative de mortalités chez les abeilles traitées (Liu et al., 2020).

Néanmoins, des études en conditions plus naturelles, telles que les études en tunnel ou en plein champ avec des colonies nourries avec un sirop contenant des néonicotinoïdes, n'ont pas démontré d'effet létal significatif (Cutler and Scott-Dupree, 2007; Faucon et al., 2005; Schmuck et al., 2001). Cependant, les apiculteurs rapportent de nombreuses et importantes mortalités d'abeilles quand les colonies sont placées à proximité des cultures traitées à l'imidaclopride. Ainsi, les différences de résultats obtenus entre les études de laboratoire et de terrain pourraient s'expliquer par des différences dans les conditions expérimentales et l'état physiologique des abeilles testées. L'état physiologique pourrait jouer un rôle important car il a été montré que,

pour une même colonie d'abeilles, la DL_{50} peut varier de 5 à 500 ng/abeille (Suchail et al., 2001). Ainsi, selon l'état des colonies et leur statut toxicologique, les abeilles utilisées dans les tests et les études (éco)toxicologiques pourraient présenter, dans certain cas, une faible sensibilité à l'imidaclopride et, éventuellement, aux néonicotinoïdes. Cet aspect de la sensibilité des abeilles aux toxiques sera traité dans la Section II de cette thèse, Résultats et Discussion, Partie 3.

D.4.1.6. Effets des néonicotinoïdes sur la colonie

Plusieurs études ont été conduites afin de détecter les effets d'une exposition chronique aux néonicotinoïdes sur la colonie. Les résultats de ces études sont, dans certains cas, contradictoires et dépendants de la durée et du niveau d'exposition. Plusieurs études ont montré une absence d'effet sur le développement et la santé des colonies exposées à des cultures traitées par les néonicotinoïdes, telles que la culture de colza traitée par enrobage de semences à la clothianidine, au thiaméthoxame et à l'imidaclopride, et par pulvérisation à l'acétamipride et au thiaclopride (Cutler and Scott-Dupree, 2007; Cutler et al., 2014; Pohorecka et al., 2012). Cependant d'autres études ont montré de forts impacts sur la colonie suite à une exposition chronique aux néonicotinoïdes. Par exemple, l'exposition chronique des colonies d'abeilles aux pollens contaminés par le thiaméthoxame et la clothianidine à des doses environnementales, ont conduit à des impacts rapides sur les colonies tels que la réduction du nombre d'adultes, du couvain et de la quantité de miel et de pollen récoltés. Ces colonies ont pu hiverner normalement mais plus de 60% d'elles ont connu, à la sortie de l'hiver, des suppressions des reines et une réduction de la capacité d'essaimage (Sandrock et al., 2014).

D.4.2. Le glyphosate

D.4.2.1. Mode d'action

Le glyphosate ou *N*-phosphonométhyl glycine appartient à la famille des aminophosphonates. C'est un herbicide de post-émergence, non sélectif, à large spectre d'activité agissant sur toutes les plantes pérennes et annuelles. Il présente des propriétés systémiques et est absorbé par les feuilles et véhiculé vers toutes les autres parties de la plante. Le glyphosate est le seul herbicide qui agit sur l'enzyme 5-énolpyruvylshikimate-3-phosphate synthétase (EPSPS) en inhibant son activité. Cette enzyme est impliquée dans la sixième étape de la voie du shikimate qui est une voie métabolique aboutissant à la biosynthèse de plusieurs acides aminés aromatiques (phénylalanine, tyrosine et tryptophane). Ainsi, l'inhibition de l'EPSPS par le glyphosate conduit à la diminution de la concentration en acides aminés précités, une cessation de la croissance suivie d'une nécrose et de la mort cellulaire (Amrhein et al., 1980) (**Fig. 15**). Le glyphosate a été longtemps considéré comme très faiblement toxique pour les animaux du fait de l'absence de EPSPS chez ces organismes. La DL_{50} du glyphosate chez des organismes non cibles comme les abeilles, dépasse les 100 µg par abeille (National Center for Biotechnology Information). Cependant de nombreuses études récentes révèlent des effets secondaires sur les organismes non

cibles qui pourraient être liés à des modes d'action secondaires (Avigliano et al., 2014; Guilherme et al., 2010; Jasper et al., 2012; Seide et al., 2018).

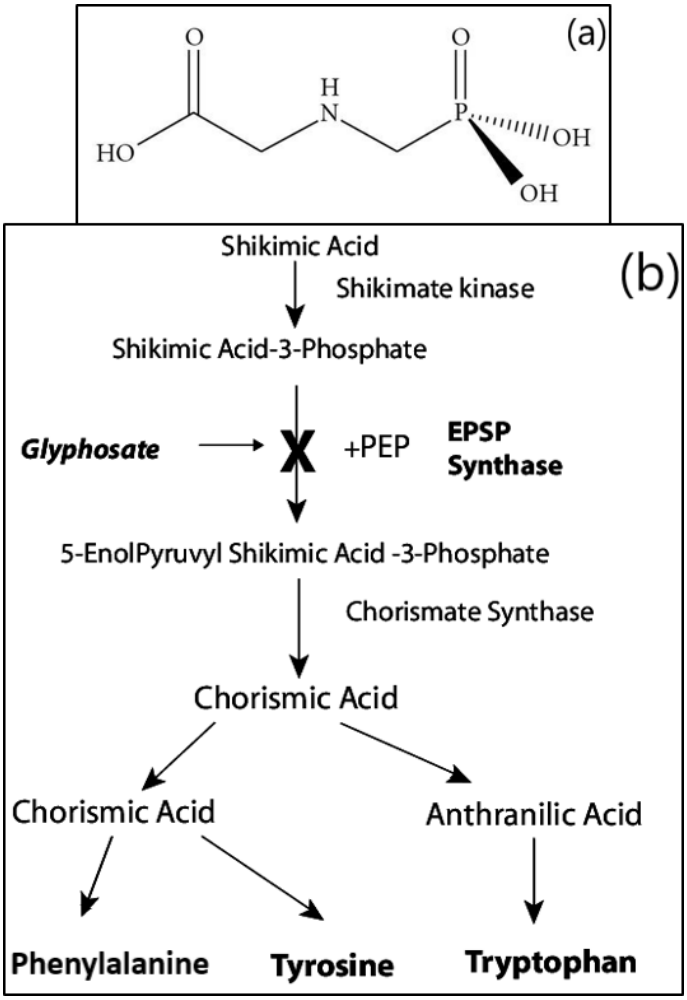

Figure 15 : Structure chimique et mode d'action du glyphosate

Le glyphosate est un herbicide organophosphoré non inhibiteur des cholinestérases (a). Le glyphosate inhibe l'enzyme 5-Enolpyruvylshikimate-3-phosphate synthase (EPSP Synthase) qui catalyse le transfert du groupement enolpyruvyl du phosphoenolpyruvate (PEP) au shikimate-3-phosphate. Cette inhibition bloque la synthèse de plusieurs molécules clés chez la plante telles que les acides aminés aromatiques (phénylalanine, tyrosine et tryptophane) (b). Figure 15-a : d'après Prasad et al (2009), figure 15-b : d'après Dill (2005).

D.4.2.2. Utilisation du glyphosate

Les propriétés herbicides du glyphosate furent découvertes en 1970 puis, en 1974, la première formulation à base du glyphosate fut commercialisée sous le nom de Roundup®. L'utilisation du glyphosate resta très limitée jusqu'à l'introduction des plantes génétiquement modifiées en 1996. Ainsi, l'utilisation agricole mondiale de ce pesticide a augmenté de 14.6 fois de 1995 à 2014 et, à l'heure actuelle, il continue d'être le pesticide le plus vendu au monde (Benbrook, 2016) (**Tableau 5**).

Tableau 5 : Évolution des quantités de glyphosate utilisées à l'échelle mondiale

L'évolution des quantités de glyphosate (en kg et en lb) utilisées à l'échelle mondiale dans l'agriculture et dans d'autres usages non agricoles. D'après Benbrook (2016).

	1994	1995	2000	2005	2010	2012	2014
Glyphosate use (1000 kg)	56,296	67,078	193,485	402,350	652,486	718,600	825,804
Agricultural	42,868	51,078	155,367	339,790	578,124	648,638	746,580
Non-agricultural	13,428	16,000	38,118	62,560	74,362	69,962	79,224
Glyphosate use (1000 lb)	124,112	147,882	426,561	887,030	1,438,485	1,584,242	1,820,585
Agricultural	94,508	112,608	342,525	749,108	1,274,546	1,430,002	1,645,927
Non-agricultural	29,604	35,274	84,036	137,922	163,940	154,240	174,658
Share agricultural (%)	76	76	80	84	89	90	90
Share non-agricultural (%)	24	24	20	16	11	10	10

Data in thousands of kilograms or pound of glyphosate active ingredient. See Additional file 1: Table S24 Table for details

D.4.2.3. Effets sublétaux du glyphosate chez les abeilles

D.4.2.3.1. Altérations physiologiques

Jusqu'au mois de novembre 2018, le glyphosate était l'herbicide qui a présenté le plus grand nombre d'études concernant les effets des herbicides sur les abeilles, avec 11 études sur les effets de la substance active et 4 études sur les effets de ses formulations (Cullen et al., 2019). Le glyphosate provoque un stress oxydant chez les abeilles au stade adulte et larvaire, par la réduction des taux de molécules antioxydantes, telles que le bêta-carotène, et par l'altération de l'expression de plusieurs gènes codant des enzymes impliquées dans la régulation de l'équilibre oxydatif chez des larves d'abeilles (Helmer et al., 2015; Vázquez et al., 2020).

Le développement des larves d'abeilles est altéré par une exposition au glyphosate dans la nourriture. Le glyphosate induit des taux élevés d'apoptose dans l'épithélium intestinal, les ovaires et les glandes salivaires des larves traitées (Gregorc and Ellis, 2011). De plus, il provoque un changement de l'expression de plusieurs gènes liés à la détoxication (gène codant les CYP450)

(Gregorc et al., 2012). D'autres effets plus prononcés peuvent aussi être induits chez les larves. Les larves exposées au glyphosate à des niveaux allant de 1,25 à 5.0 mg/L ont des mues retardées et des poids plus petits que les larves élevées en absence de glyphosate (Vazquez et al., 2018). Le glyphosate conduit aussi à des perturbations au niveau des communautés bactériennes intestinales chez les larves et chez les abeilles adultes (Blot et al., 2019; Dai et al., 2018; Motta et al., 2020; Motta et al., 2018).

D.4.2.3.2. Altérations comportementales et cognitives

Le glyphosate induit des effets cognitifs délétères chez l'abeille et affecte la navigation, l'apprentissage et la perception gustative des abeilles. Les abeilles exposées au glyphosate présentent des vols de retour altérés, mettent plus de temps pour retrouver leur colonie et effectuent moins de vols directs vers leurs ruches en comparaison avec les abeilles non traitées (Balbuena et al., 2015). Le glyphosate affecte l'apprentissage associatif chez les abeilles et réduit la mémoire à court terme (Herbert et al., 2014). De plus, les abeilles présentent une préférence pour le sirop contaminé à 10 µg/kg de glyphosate, ce qui peut engendrer un risque plus élevé d'exposition à ce pesticide lors du butinage dans les champs traités (Liao et al., 2017).

D.4.3. Le difénoconazole

D.4.3.1. Mode d'action

Le difénoconazole est un fongicide qui appartient à la famille des azoles (sous-famille des triazoles). Ce sont des fongicides inhibiteurs de la biosynthèse des stérols (ergosterol biosynthesis inhibitor (EBI)). Ce sont des inhibiteurs de la lanosterol 14α-déméthylase (CYP51) qui appartient aux isoenzymes des cytochromes P450 des mitochondries des cellules fongiques. Cette enzyme permet la transformation du lanostérol en ergostérol qui est un composant essentiel de la membrane cellulaire des champignons. L'inhibition de cette enzyme a lieu quand un des atomes d'azote du fongicide se lie à l'atome de fer de l'hème qui est situé sur le site actif de l'enzyme du cytochrome P450. Cette inhibition conduit à l'accumulation des précurseurs de la chaine de synthèse d'ergostérol comme le lanostérol et à l'épuisement de l'ergostérol, ce qui compromet l'intégrité de la membrane cellulaire et inhibe la croissance des champignons (Leroux, 2003).

Ainsi, les fongicides azoles sont actifs sur les enzymes dépendantes du cytochrome P450 qui sont présentes non seulement chez le champignon mais également chez les autres organismes. Ils peuvent donc inhiber les CYP450 qui sont des enzymes de détoxication des xénobiotiques (cf. A.4.1) chez les insectes et les vertébrés. L'inhibition des CYP450 conduit à l'accumulation des xénobiotiques à l'intérieur de l'organisme au lieu de leur dégradation en métabolites. Cela peut conduire à l'augmentation de la toxicité des xénobiotiques et à l'apparition d'effets additifs ou synergiques lorsque les azoles sont associés aux insecticides (Colin and Belzunces, 1992). Les

fongicides azoles peuvent aussi induire l'expression des gènes codants pour les CYP450 et augmentent ainsi la détoxication des xénobiotiques (D'Agostino et al., 2018; Johnston et al., 1996; Johnston et al., 1994; Rivière, 1983).

D.4.3.2. Utilisation des fongicides azoles

Les inhibiteurs de la 14α-déméthylation des stérols ont été introduits sur le marché il y'a une quarantaine d'années (Stensvold et al., 2012). Ils représentent près de 25% du marché mondial des fongicides. Les triazoles sont actifs contre un large spectre de maladies fongiques telles que la rouille, la moniliose et l'oïdium chez les végétaux et, particulièrement, les grandes cultures, et les arbres fruitiers.

Les triazoles sont des substances systémiques et ont un double effet, préventif et curatif. Leur toxicité aiguë est très faible pour les organismes non cibles comme les abeilles, avec une DL_{50} dépassant les 100 µg par abeille (National Center for Biotechnology Information). C'est pour cette raison que l'emploi des triazoles est autorisé en pleine floraison. Par exemple la formulation de difénoconazole (Score® 250 EC) est autorisée en pulvérisation sur les arbres fruitiers tels que les amandiers, les pêchers et les abricotiers en pleine floraison à la dose de 0.3 L de produit par hectare (équivalent à 75 g de difénoconazole par hectare) (**Fig. 16**).

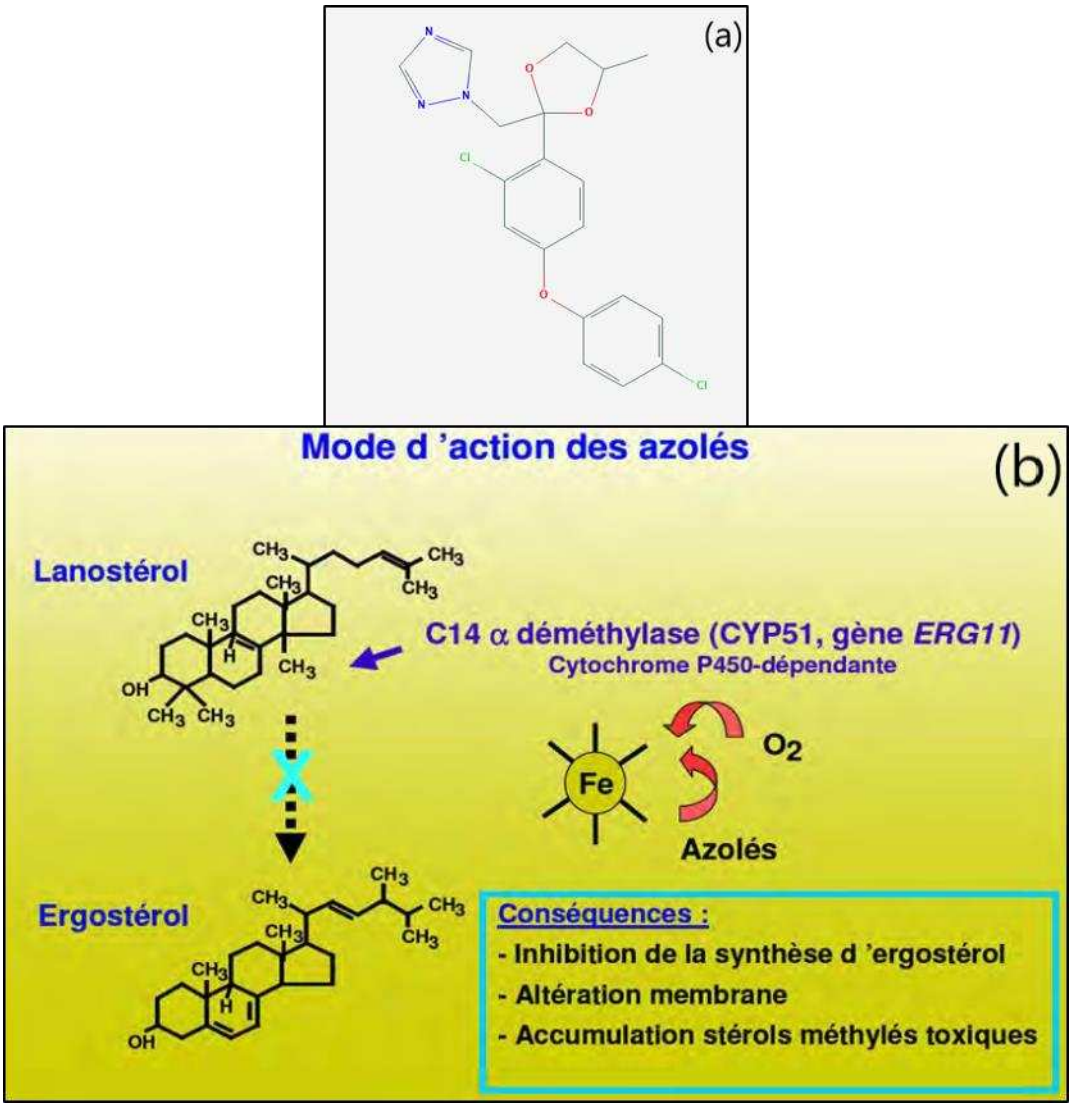

Figure 16 : Structure chimique du difénoconazole et mode d'action des fongicides azoles

Les fongicides azoles inhibent la synthèse d'ergostérol, en se liant à travers un de ces atomes d'azotes à l'atome de fer de l'enzyme lanosterol 14α-déméthylase (CYP51). Cette inhibition a comme conséquence une accumulation du lanosterol et des 14-méthylstérols dans la membrane et conduit à un arrêt de la synthèse d'ergostérol entrainant l'altération de la membrane plasmique et un changement de sa perméabilité. Figure 16-a : d'après National Center for Biotechnology Information (https://pubchem.ncbi.nlm.nih.gov/compound/Difenoconazole), figure 16-b : d'après la thèse de Sibrac-Pelayo, C (2013).

D.4.3.3. Effets des azoles sur les abeilles

Il existe très peu d'études sur les effets des fongicides azoles sur les abeilles par comparaison avec le nombre d'études sur les insecticides ou quelques herbicides. Les études sur les azoles ont été focalisées sur le potentiel de ces derniers à induire des interactions synergiques lorsqu'ils sont associés à des pesticides d'autres classes tels que les pyréthrinoïdes (Colin and Belzunces, 1992).

L'exposition des abeilles aux fongicides azoles peut avoir des effets directs sur la survie des abeilles. Cela est mis en évidence par les mortalités retardées qui apparaissent à la suite d'exposition orale des abeilles adultes au propiconazole (fongicide triazole) à des concentrations élevées de l'ordre de 65 µg de substance active par abeille (Ladurner et al., 2005). Cependant d'autres études ont montré des résultats contradictoires. Par exemple, le propiconazole n'a pas conduit à des effets létaux sur les larves à 2,25 µg/larve et sur les adultes suite à une exposition de contact à des doses comprises entre 0,011 et 1,260 µg/abeille. Les effets létaux sont apparus uniquement quand le propiconazole est associé à des insecticides tels que le diflubenzuron et le chlorantraniliprole (Wade et al., 2019). De même l'exposition orale des abeilles pendant 14 jours, à 0,084 mg/L de Domark®, ayant le tétraconazole (fongicide triazole) comme substance active, et par contact au même fongicide à 2500 mg/L, n'a aucun effet négatif sur la survie de l'abeille (Zhu et al., 2017a; Zhu et al., 2017b).

Les azoles conduisent aussi à des effets sublétaux tels qu'une répression de l'expression de plusieurs gènes d'immunité chez les prépupes exposées au prochloraz (fongicide imidazole) et une augmentation de l'expression de ces mêmes gènes chez les abeilles adultes (Cizelj et al., 2016). De même, l'exposition orale chronique au tétraconazole en formulation augmente significativement l'activité de la phénoloxydase chez les abeilles adultes, et diminue l'activité de la glutathion-S-transférase. L'altération de l'activité de ces deux enzymes reflète des altérations des systèmes immunitaire et de détoxication suite à l'exposition aux azoles (Zhu et al., 2017a). De plus, la contamination du pain d'abeilles par des fongicides, y compris des azoles tels que le propiconazole, réduit le nombre de champignons dans cette matrice, ce qui peut affecter la capacité de la colonie à fermenter le pollen en pain d'abeilles, donc à apporter de nouveaux nutriments, et augmente le risque d'infection par des pathogènes (Yoder et al., 2013).

E. Interactions des stresseurs

E.1. Interaction entre pesticides

Face au nombre élevé de ravageurs, de pathogènes et de mauvaises herbes, les agriculteurs utilisent une large gamme de pesticides simultanément ou consécutivement au fil de la saison agricole. Le mélange en cuve (tank mixture) est une technique qui consiste à utiliser au moins deux produits phytopharmaceutiques lors d'une même pulvérisation. Elle parait être une bonne stratégie agricole afin d'économiser du temps, de la main d'œuvre, du carburant et de causer moins de compaction du sol. Cette technique devient de plus en plus répandue dans plusieurs

pays tels que l'Australie, le Canada, les États-Unis et le Royaume-Uni (Tornisielo et al., 2013). Par exemple, au Royaume-Uni, plus de 50% des terres arables et des vergers sont traités en utilisant la technique de mélange en cuve avec un nombre de produits qui peut atteindre six produits par mélange (Fryday et al., 2011) (**Tableau 6**). Les abeilles peuvent donc subir une exposition simultanée à plusieurs substances lorsqu'elles sont présentes au moment du traitement de la culture. D'autre part, les abeilles au cours de leur activité de butinage peuvent être exposées successivement à plusieurs pesticides pulvérisés séparément à plusieurs reprises sur une même culture ou en visitant plusieurs cultures traitées par des produits différents. En conséquence, les abeilles seront exposées à ces mélanges soit en butinant, soit par l'ingestion d'une nourriture contaminée par les substances actives, par contamination du nectar et du pollen, que les abeilles butineuses auront rapportées à la ruche. Enfin, il ne faut pas oublier l'exposition directe des abeilles aux acaricides utilisés à l'intérieur des colonies pour traiter le *Varroa*.

La multi-exposition des abeilles à une large gamme de produits phytopharmaceutiques est confirmée par de nombreuses études qui ont permis de détecter la présence de plusieurs pesticides dans un même échantillon (Chauzat et al., 2009; Chauzat and Faucon, 2007; Chauzat et al., 2006; Kanga et al., 2019; Lambert et al., 2013; Mullin et al., 2010; Wiest et al., 2011). Par exemple, l'étude de Mullin et al. (Mullin et al., 2010) a permis de montrer la présence de 6,5 pesticides différents en moyenne par échantillon de cire, de pain d'abeilles, de pollen et d'abeilles, avec un maximum de 39 pesticides différents par échantillon. De plus, l'étude de Lambert et al. (Lambert et al., 2013) (cf. D.3.2) a permis de montrer que jusqu'à six, huit et sept pesticides différents pouvaient être détectés ensemble dans un même échantillon d'abeilles, de miel et de pollen, respectivement. Ainsi, la multi-exposition des abeilles aux pesticides est un phénomène très courant et de nombreuses études ont été conduites afin de comprendre les effets des interactions entre deux ou plusieurs pesticides. Plus de 55% des études d'interaction se sont intéressées aux interactions entre les insecticides pyréthrinoïdes/pyréthrines et les fongicides azoles (principalement des triazoles), et 35% portaient sur les interactions entre les néonicotinoïdes et les fongicides azoles. Plus de 61% des études d'interactions étaient fondées sur des co-expositions aiguës de contact aux pesticides d'intérêt. Cependant, uniquement 20 et 19% des études s'intéressaient à des expositions orales-chroniques et orales-aiguës aux pesticides d'intérêt, respectivement (Carnesecchi et al., 2019).

Les études menées sur les interactions montrent que ces dernières peuvent conduire à des phénomènes de synergie, d'additivité ou d'antagonisme. Le phénomène de synergisme apparait quand la toxicité du mélange est supérieure à la somme des toxicités des substances constituant le mélange. Le phénomène d'additivité apparait quand la toxicité du mélange est égale à la somme des toxicités des substances constituant le mélange. Enfin, le phénomène d'antagonisme apparait quand la toxicité du mélange est inférieure à la toxicité des substances constituant le mélange.

Tableau 6 : Les classes de pesticides utilisées en mélange pour le traitement des vergers au Royaume-Uni

Les classes de pesticides utilisées en mélange pour le traitement des vergers en 2008 au Royaume-Uni, avec le nombre de combinaisons possibles, les superficies traitées (ha) et le pourcentage de la superficie totale traitée par chaque type de mélange. Avec « PGR » : Régulateurs de croissance des plantes (plant growth regulator). D'après Fryday et al (2011).

Mélange	**N**	**Surface (ha)**	**% surface totale**
Fongicides	243	62758	46.02
Fongicide(s) + Insecticide(s)	384	33911	24.87
Fongicide(s) + PGR(s)	175	17224	12.63
Herbicides	54	13298	9.75
Fongicide(s) + Insecticide(s) + PGR(s)	181	6602	4.84
Acaricide(s) + Fungicide(s)	21	1370	1.00
Insecticides	13	614	0.45
Insecticide(s) + PGR(s)	13	254	0.19
PGRs	5	238	0.17
Acaricide(s) + Fongicide(s) + PGR(s)	6	92	0.07
Fongicide(s) + Herbicide(s)	2	7	0.01
Herbicide(s) + PGR(s)	2	4	0.003

De nombreuses études ont montré la capacité des fongicides azoles à accroitre la toxicité des insecticides tels que les pyréthrinoïdes chez les organismes aquatiques (Bjergager et al., 2011; Cedergreen et al., 2006; Kretschmann et al., 2015) et les abeilles (ChalvetMonfray et al., 1996; Colin and Belzunces, 1992; Meled et al., 1998; Pilling et al., 1995; Pilling and Jepson, 1993). Les fongicides azoles ont montré aussi une capacité à accroitre la toxicité des néonicotinoïdes (Han et al., 2019; Iwasa et al., 2004; Schmuck et al., 2003; Thompson et al., 2014a; Zhu et al., 2017b) et celle de substances appartenant à la classe des acaricides comme c'est le cas entre le fongicide fenpyroximate et l'acaricide coumaphos (Johnson et al., 2013). Les effets synergiques observés seraient dus à l'inhibition des CYP450 par les fongicides azoles (cf. D.4.3.1), ce qui induit une inhibition de la détoxication des pesticides et l'accumulation de ces derniers à des concentrations élevées à l'intérieur des abeilles (Pilling et al., 1995).

L'importance des CYP450 dans la détoxication des pesticides peut être illustrée par l'utilisation d'inhibiteurs métaboliques spécifiques des CYP450 tels que le butoxyde de piperonyl (PBO, piperonyl butoxide). L'action du PBO synergise la toxicité induite par les pesticides et cette propriété est mise à profit pour lutter contre les insectes résistants aux insecticides (Tozzi, 1999). De même, d'autres inhibiteurs peuvent être utilisés tels que les S,S,S-tributylphosphorotrithioate (DEF) et le diethylmaleate (DEM). Le DEF et le DEM sont utilisés en agriculture pour augmenter la toxicité des pesticides par leur action inhibitrice sur les estérases et les GST, respectivement (Iwasa et al., 2004). Cependant, la synergie n'est pas toujours d'origine métabolique, elle peut se produire par une interaction sur une cible commune comme par exemple, les neurones. Cela était constatée avec l'interaction du tau-fluvalinate avec le coumaphos. La Synergie se produit dans ce cas par une action excitatrice sur le même neurone en deux sites différents sur l'acétylcholinestérase par action bloquante du coumaphos et sur l'axone par l'action hyperpolarisante du tau-fluvalinate. De plus, une hypothermie est aussi détectée suite à une co-exposition à des doses environnementales de prochloraze et de difénoconazole. Ainsi, la synergie observée, lorsque ces deux pesticides sont associés aux insecticides pyréthrinoïdes, pourrait être due à une interaction des azoles et des pyréthrinoïdes sur des cibles communes, les ATPases (Vandame and Belzunces, 1998).

La prédiction des interactions entre des pesticides ayant le même mode d'action ou un mode d'action différent est très complexe. La complexité de prédiction est due à plusieurs facteurs tels que le niveau d'exposition, la séquence des traitements ou des expositions, et la saison.

(i) Effet du niveau de l'exposition : L'interaction entre les fongicides azoles et les pesticides peut ne pas toujours conduire à des effets synergiques. En effet, les azoles connus pour avoir un effet inhibiteur sur la CYP450 mais ils peuvent aussi induire l'expression des gènes codants pour cette enzyme. Par exemple, les fongicides azoles ont un effet synergique sur le tau-fluvalinate à des doses de 1 ou 10 nmol par abeille. Cependant à 0.01 nmol, ces mêmes fongicides ont un effet antagoniste sur le tau-fluvalinate qui pourrait être expliqué par une induction des gènes de la CYP450 par les azoles à faibles doses (Johnson et al., 2013; Willoughby et al., 2007).

(ii) Effet séquence d'exposition : L'effet d'une interaction entre deux pesticides peut changer suivant la séquence d'exposition de l'abeille à ces pesticides. C'est le cas, par exemple, de l'interaction entre le tau-fluvalinate et le fenpyroximate, un fongicide azole. Le traitement préalable au fenpyroximate augmente de huit fois la toxicité du tau-fluvalinate, tandis que le traitement préalable au tau-fluvalinate ne change pas la toxicité du fenpyroximate. Cette différence peut être expliquée par la capacité du fenpyroximate à inhiber les CYP450 responsables de la détoxication du tau-fluvalinate alors que ce dernier n'agit pas sur les CYP450, donc sur le métabolisme du fenpyroximate (Johnson et al., 2013).

(iii) Effet de la saison : la sensibilité aux pesticides peut varier d'une année à l'autre et même d'une saison à l'autre. Par exemple, la DL_{50} du tau-fluvalinate a diminué de 19,8 µg/abeille en 2009 à 9 µg/abeille en 2010. Cette légère variation de sensibilité au tau-fluvalinate pourrait être liée à la variation de la composition génétique de la colonie d'abeilles suite au changement annuel des reines ou à la variation de la représentation des différentes fratries dans la colonie. De plus, les abeilles d'été paraissent huit fois plus sensibles que les abeilles d'hiver à l'interaction synergique entre le prochloraze et la deltaméthrine (Meled et al., 1998). Cette différence pourrait être liée aux variations physiologiques qui existent entre les abeilles d'été et celles de l'hiver (cf. A.3.3).

E.2. Interactions entre les stresseurs biologiques

De nombreuses études ont révélé l'existence d'interactions entre les parasites et les agents pathogènes qui attaquent l'abeille domestique. Par exemple, l'infection par *N. ceranae* induit une action synergique sur la réplication du CBPV (chronic bee paralysis vrius) chez les abeilles d'hiver. De plus, *N. ceranae* est capable, à partir d'un certain niveau d'infection, d'accélérer la réplication de DWV (deformed wing virus) dans les phases précoces d'infection par ce virus (Toplak et al., 2013; Zheng et al., 2015). De même, la co-infection des abeilles avec *N. ceranae* et le BQCV conduit aussi à un effet synergique adverse sur la longévité des abeilles. En effet, après 11 jours d'infection, il était observé un taux de mortalité de 50% chez les abeilles co-infectées, tandis que les taux de mortalité observés chez les abeilles infectées seulement par *N. ceranae* ou BQCV (bee queen cell virus) étaient de 20% et 5%, respectivement (Doublet et al., 2014).

L'interaction synergique entre *V. destructor* et plusieurs virus qu'il transmet a été démontrée chez les abeilles. Ainsi, la charge virale chez les abeilles est corrélée aux taux d'infestation par *Varroa* dans la colonie. Les populations de *Varroa* augmentent exponentiellement dans les colonies infestées, ce qui conduit à une épidémie massive liée au DWV et/ou à l'ABPV (acute bee paralysis virus). Cette épidémie conduit ainsi à la perte de la colonie (Francis et al., 2013; Martin, 2001; Martin et al., 2012).

E.3. Interaction entre les agents pathogènes et les pesticides

Les abeilles peuvent être infectées par des pathogènes et être simultanément exposées à une large gamme de pesticides. Ainsi, de nombreuses études ont été conduites afin de détecter la présence d'interactions toxico-pathologiques chez les abeilles et de déterminer les mécanismes d'action de telles interactions (Harwood and Dolezal, 2020). Plusieurs études ont montré que les pesticides ont un effet additif ou synergique sur la réplication de virus et les mortalités induites par ces derniers. Par exemple, l'exposition à la clothianidine réduit les défenses immunitaires en diminuant l'expression de l'apidaecine qui fait partie des AMP, et augmente la réplication du DWV chez l'abeille (Di Prisco et al., 2013). Cependant, la co-exposition des abeilles au thiaméthoxame et au DWV n'a aucun effet notable sur la charge virale de ce virus mais elle conduit à un butinage précoce et diminue la survie des abeilles co-infectées par comparaison avec la survie des abeilles exposées à ces deux facteurs séparément (Coulon et al., 2020).

Les interactions entre les agents pathogènes et les pesticides ont aussi été démontrées avec la microsporidie *N. ceranae*. La toxicité induite par des insecticides tels que les néonicotinoïdes (thiaclopride et imidaclopride) et les phényles pyrazoles (fipronil) peut être augmentée lorsque les abeilles sont co-exposées aux pesticides et à l'agent pathogène (Alaux et al., 2010a; Aufauvre et al., 2012; Aufauvre et al., 2014; Retschnig et al., 2014; Vidau et al., 2011).

Les pesticides peuvent aussi sensibiliser les abeilles aux agents pathogènes. Ainsi, la probabilité d'infection par *N. ceranae* est augmentée lorsque les abeilles sont exposées aux fongicides (pyraclostrobine et chlorothalonil) via leur nourriture (Glavinic et al., 2019; Pettis et al., 2013). Toutefois, si les pesticides peuvent sensibiliser les abeilles aux agents pathogènes, les agents pathogènes peuvent aussi accroître la sensibilité des abeilles aux pesticides. Ainsi, comme cela a été démontré avec les insecticides phényles pyrazoles et l'agent pathogène *Nosema*, les stresseurs biologiques et chimiques xénobiotiques peuvent exercer, l'un vis-à-vis de l'autre, une action sensibilisante réciproque et peuvent agir en synergie quel que soit l'ordre dans lequel les individus sont exposés à ces deux types de stresseurs (Aufauvre et al., 2012).

Contexte de la thèse

Les travaux de cette thèse s'inscrivent dans le projet MIXTRESS (Pesticide MIXTURES and pathogen STRESSORS in the bee), qui est un projet scientifique de 4 ans (2016-2020) regroupant deux laboratoires, le *Laboratoire de Toxicologie Environnementale* (LTE) appartenant à l'unité *Abeille & Environnement*, INRAE, Avignon, et le *Laboratoire de Microorganismes Génome et Environnement* (LMGE) situé à Clermont-Ferrand. Ce projet est financé par l'Agence Nationale de la Recherche (ANR) (ANR-15-CE34-0004-01). L'objectif principal de ce projet est d'étudier la toxicité des mélanges de pesticides et d'évaluer les interactions entre les mélanges de pesticides et un agent pathogène émergeant, *Nosema ceranae* chez *A. mellifera*. Mon séjour à l'unité *Abeille & Environnement* a été financé par une bourse de l'Université Libanaise et par une bourse d'excellence entrante de la région PACA pour l'année 2017-2018.

De plus, dans le cadre de mon inscription dans le parcours de l'École Internationale de Recherche d'Agreenium (EIR-A), j'ai effectué un séjour de trois mois au Département de Microbiologie Fondamentale (DMF) de la Faculté de Biologie et de Médecine à l'Université de Lausanne, Suisse, sous la direction de Philipp Engel. Ce séjour était financé par la bourse Perdiguier de l'Université d'Avignon et par un financement de la part de la DARESE de l'INRAE.

Objectifs de la thèse

Les pesticides et les pathogènes semblent être les principaux contributeurs au constant déclin des populations d'abeilles dans plusieurs régions du monde (Goulson et al., 2015). Les abeilles domestiques *Apis mellifera* sont exposées à une large gamme de pesticides tout au long de leur vie. Les butineuses sont exposées aux pesticides, surtout les fongicides, lors de leurs vols de butinages. Elles entrent aussi en contact avec les pesticides présents sur les surfaces traitées et récoltent le nectar, le pollen, l'eau et les exsudats de plantes contaminés par les résidus de pesticides (Fisher et al., 2017; Piechowicz et al., 2018c). Les butineuses transportent la nourriture contaminée à la colonie et c'est ainsi que le couvain, les abeilles nourrices et les castes reproductrices sont exposés chroniquement à de nombreux pesticides (Blaga et al., 2020; Böhme et al., 2018; David et al., 2016; Skerl et al., 2009). Par conséquent, la multi-exposition des abeilles aux pesticides est un phénomène très courant, pour ne pas dire banal, et peut conduire à des interactions antagonistes, additives ou synergiques, mais seules les interactions additives et synergiques sont considérées pour leurs impacts environnementaux délétères. De telles interactions ont principalement été observées entre les pesticides (pyréthrinoïdes, néonicotinoïdes) et les fongicides azoles. Cependant, de par ces études, les recherches sur les effets des mélanges de pesticides n'ont pas connu un intense développement (Colin and Belzunces, 1992; Meled et al., 1998; Spurgeon et al., 2016; Thompson et al., 2014a). Il convient aussi de noter que la multi-exposition aux pesticides peut être simultanée, séquentielle ou chevauchante et très peu d'études ont exploré la variation des effets en fonction de la séquence d'exposition aux différents pesticides (Johnson et al., 2013).

D'autre part, les abeilles sont exposées à de nombreux stresseurs biotiques tels que des prédateurs, des ectoparasites et des agents pathogènes. Ces trois derniers ont des impacts sévères sur les colonies d'abeilles et peuvent, en présence des pesticides, conduire à des interactions toxico-pathologiques. De telles interactions ont été observées entre des pesticides de plusieurs classes et la microsporidie *Nosema* (Abou-Shaara, 2018; Alaux et al., 2010a; Glavinic et al., 2019; Pettis et al., 2013; Retschnig et al., 2014; Tesovnik et al., 2020a).

Ainsi, le projet de thèse avait pour objectif premier de déterminer la toxicité des mélanges de pesticides, appliqués à des niveaux d'exposition environnementaux, soit entre eux soit en association avec des agents pathogènes. Le deuxième objectif était d'étudier les impacts des associations de stresseurs sur de grandes fonctions physiologiques dans le but de déterminer si les effets délétères induits par les mélanges de pesticides dépendaient de mécanismes d'action communs entre les différents stresseurs ou spécifiques à chacun des pesticides et des pathogènes.

Compte tenu du nombre de pesticides et des niveaux de doses auxquels les abeilles peuvent être exposées et, par conséquent, du nombre considérable de combinaisons possibles, le choix de molécules pour nos études s'est orienté vers trois pesticides : un insecticide néonicotinoïde (l'imidaclopride), un fongicide azole (le difénoconazole) et un herbicide (le glyphosate). Le choix s'est porté sur ces trois pesticides car ils constituent une large part du marché de pesticides à l'échelle mondiale et sont fréquemment détectés dans toutes les matrices de la ruche. En effet, l'imidaclopride et son métabolite l'acide 6-chloronicotinique étaient, entre 2002 et 2005, les pesticides les plus abondants dans les matrices des ruches en France, avec une concentration moyenne d'imidaclopride de 0,7 µg/kg dans le miel et de 0,9 µg/kg dans le pollen (Chauzat et al., 2011). Plusieurs autres études ont montré que l'imidaclopride pouvait être aussi détecté à des concentrations allant de 0,14 à 0,275 µg/kg dans le miel, de 3 à 5,09 µg/kg dans la cire et de 1,35 µg/kg dans le pollen (Lambert et al., 2013; Lopez et al., 2016; Nguyen et al., 2009). De plus, l'imidaclopride présente une toxicité très variable qui pourrait modifier la toxicité induite par les interactions entre ce toxique et d'autres stresseurs environnementaux (Suchail et al., 2001). Le glyphosate est aussi très fréquemment détecté dans les ruches à des concentrations allant de 17 à 342 µg/kg dans le miel et de 50 à 58.4 µg/kg dans le pain d'abeilles (Berg et al., 2018; El Agrebi et al., 2020; Pareja et al., 2019; Rubio et al., 2015). Enfin, le difénoconazole est détecté dans les échantillons de miel à une concentration de 0.6 µg/kg, de pain d'abeilles à une concentration de 270 µg/kg, de cire à une concentration de 1 µg/kg et de pollen à des concentrations allant de 43 à 171.4 µg/kg (Kubik et al., 2000; Lopez et al., 2016; Pettis et al., 2013). En outre, les fongicides azoles, dont fait partie le difenoconazole, sont aussi autorisés pour le traitement des cultures en pleine floraison et augmentent ainsi le risque d'exposition aiguë des butineuses à ces pesticides. Ce sont aussi des substances qui présentent des effets très variables en fonction du type de séquence d'association avec d'autres stresseurs.

Au niveau des agents pathogènes, nous nous sommes focalisés sur *Nosema ceranae* car ce pathogène présente une distribution géographique mondiale. De plus, *N. ceranae* est connu pour interagir avec des pesticides de plusieurs familles et de nombreuses données expérimentales ont

été obtenues sur ce pathogène, pouvant aider à mieux comprendre la modulation de sa virulence en présence des pesticides (Martín-Hernández et al., 2018).

Ainsi le travail de la thèse a consisté à étudier la toxicité des mélanges binaires et ternaires d'imidaclopride, de difénoconazole et de glyphosate, associés ou non à *N. ceranae.* Autour de la problématique des co-expositions aux différents stresseurs biotiques et abiotiques, différents questionnements liés aux problématiques du déclin des populations d'abeilles étaient sous-jacents :

(i) En partant du fait que ces trois pesticides coexistent dans le miel et le pollen durant toute la période hivernale, il était légitime de se demander si une longue période d'exposition des individus à une nourriture contaminée pouvait rendre compte des pertes hivernales importantes de colonies d'abeilles. Pour répondre à cette question, il a été étudié la toxicité induite par une exposition des abeilles d'hiver aux trois pesticides, seuls et en mélanges binaires et ternaires. De plus, si ces pesticides étaient capables d'induire une toxicité, seuls ou en mélanges, il était important de savoir si leur nocivité s'exerçait préférentiellement par une action locale ou par une action systémique.

(ii) Si les mélanges de pesticides induisent une toxicité chez les abeilles d'hiver, il est important de savoir si leur action s'exerce par des mécanismes spécifiques des substances, ou des familles de substances, ou par des mécanismes communs pouvant avoir des impacts sur les grandes fonctions physiologiques. La réponse à cette question a été approchée en exposant les abeilles d'hiver aux trois pesticides d'intérêts, seuls ou en mélanges, et en observant, dans un premier temps, les effets sur le stress oxydant.

(iii) Au sein de la ruche, les abeilles peuvent être exposées à une nourriture contaminée à partir de leur naissance. Cependant, dès leur première sortie de la ruche (vers l'âge de 8-12 jours), les abeilles peuvent être aussi exposées à des résidus de pesticides localisés sur les surfaces végétales ou dans l'eau, ou lors de pulvérisations de produits phytopharmaceutiques. Ces expositions, lors des pulvérisations, se produisent essentiellement avec les herbicides et les fongicides dont l'usage est autorisé en pleine floraison. Ainsi, il est important de savoir si le statut toxicologique de l'abeille a une influence sur les effets d'une exposition aiguë à d'autres pesticides. Pour essayer de mimer, autant que faire se peut, cette situation d'exposition environnementale, il a été étudié les effets d'expositions chroniques orales des abeilles naissantes à l'imidaclopride et au glyphosate, seuls ou en mélanges binaires, suivie d'une exposition aiguë par pulvérisation d'une préparation phytopharmaceutique à base du fongicide difénoconazole. Les impacts physiologiques de ces expositions ont été recherchés en considérant la modulation de marqueurs métaboliques.

(iv) Dans la réalité environnementale, les abeilles peuvent être exposées à plusieurs pesticides de façon concomitante, séquentielle ou chevauchante, en présence ou non d'agents pathogènes. Ainsi, il devenait intéressant de savoir si les effets d'une exposition à des stresseurs, sur une même durée et à des niveaux d'exposition identiques, est modulée par la séquence d'exposition des abeilles à ces stresseurs. Il a donc été étudié les impacts d'expositions chevauchantes et

consécutives au glyphosate et au difénoconazole sur les abeilles, tout en considérant l'effet aggravant ou modérateur d'une infection par *Nosema ceranae* sur les impacts de ces expositions.

(v) Les abeilles présentent un microbiote intestinal qui est impliqué dans plusieurs fonctions physiologiques chez l'abeille. Sachant que les pesticides peuvent altérer la composition ou l'installation du microbiote, la question qui se posait, en termes de co-exposition aux pesticides, était de savoir si les pesticides en mélanges présentaient une potentialité plus élevée pour modifier le microbiote que les pesticides seuls. Ainsi, nous avons conduit une étude portant non pas sur la modification du microbiote déjà installé mais sur les effets des pesticides sur l'installation du microbiote chez les abeilles naissantes, en partant du fait que ces dernières sont exposées aux pesticides dès leur naissance.

II. Résultats et discussion

Partie 1 : Les mélanges d'un insecticide, d'un fongicide et d'un herbicide sont-ils toxiques et induisent-ils des perturbations physiologiques systémiques chez les abeilles d'hiver ?

Avant-propos

La période hivernale parait une période critique pour la survie des colonies d'abeilles. Des pertes hivernales de colonies dépassant les 20% du cheptel sont observées à l'échelle européenne ainsi qu'à l'échelle mondiale. Durant la période hivernale, pouvant durer jusqu'à six mois, la ponte et le butinage sont suspendus et les sources d'alimentation se limitent au miel et au pain d'abeilles stockés dans la colonie avant l'arrivée de l'hiver. Les études montrent que les stocks de miel et de pain d'abeilles sont contaminés par une large gamme de pesticides et que cette contamination persiste pendant l'hiver. Ainsi, les abeilles se trouvant dans la ruche, sont exposées, pendant une longue période, aux pesticides appartenant à des classes ou des familles identiques ou différentes. C'est pour cette raison que la première étape de la thèse a consisté en l'étude des effets des mélanges de pesticides sur les abeilles d'hiver, afin de vérifier s'il existe un lien entre la contamination des matrices de la ruche par les mélanges de pesticides et les pertes hivernales de colonies. La première approche consiste à exposer les abeilles d'hiver aux trois pesticides d'intérêt, seuls et en mélanges binaires et ternaires, dans le but de déterminer si ces pesticides interagissent et forment des combinaisons toxiques augmentant les mortalités des abeilles exposées. La deuxième approche consiste à savoir si les interactions entre les pesticides conduisent à une réponse locale ou à une réponse systémique se manifestant par des perturbations physiologiques dans différents compartiments biologiques de l'abeille.

Ces travaux ont fait l'objet d'une première publication :

Ecotoxicology and Environmental Safety 203 (2020) 111013

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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Mixtures of an insecticide, a fungicide and a herbicide induce high toxicities and systemic physiological disturbances in winter *Apis mellifera* honey bees

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Article 1

L'article figure intégralement en annexe à la fin du mémoire.

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Résumé

De nombreux pesticides, provenant des traitements phytosanitaires et des traitements des parasites infectant les abeilles, sont fréquemment détectés dans les matrices de la ruche. Par conséquent, les abeilles d'hiver, qui ont une longue durée de vie, pourraient être exposées à ces pesticides plus longtemps que les abeilles d'été. Dans cette étude, les abeilles ont été exposées par voie orale à l'insecticide imidaclopride, au fongicide difénoconazole et à l'herbicide glyphosate, seuls ou en mélanges binaires et ternaires, à des concentrations environnementales (0 (témoins), 0,1, 1 et 10 µg/L) pendant 20 jours. La survie des abeilles a été considérablement réduite après l'exposition à ces 3 pesticides individuellement et en mélanges. D'une manière générale, les mélanges ont eu un impact plus élevé que les pesticides seuls, avec une mortalité maximale de 52.9%, observée après 20 jours d'exposition au mélange binaire insecticide-fongicide à la concentration de 1 µg/L. Les analyses effectuées sur les abeilles survivantes ont montré que les mélanges de pesticides ont un effet systémique sur la physiologie des abeilles. Cela est révélé par la modulation de la glutathion-S-transférase dans les têtes, les intestins moyens et les abdomens, l'acétylcholinestérase dans les têtes, la glucose-*6*-phosphote déshydrogénase dans les abdomens et la phosphatase alcaline dans les intestins moyens. Ces enzymes sont impliquées, respectivement, dans la détoxication des xénobiotiques, le système nerveux, les défenses contre le stress oxydant, le métabolisme et l'immunité. Ces résultats montrent l'importance d'étudier les effets des cocktails de xénobiotiques, en se fondant sur des niveaux d'exposition bas et réalistes, ainsi que l'importance de développer des essais à long terme afin de détecter les interactions létales et sublétales des pesticides chez les abeilles domestiques et les autres insectes pollinisateurs.

Mots-clés : Abeilles d'hiver, mélanges de pesticides, synergie, effet cocktail, état physiologique

Abstract

Multiple pesticides originating from plant protection treatments and the treatment of pests infecting honey bees are frequently detected in beehive matrices. Therefore, winter honey bees, which have a long life span, could be exposed to these pesticides for longer periods than summer honey bees. In this study, winter honey bees were exposed through food to the insecticide imidacloprid, the fungicide difenoconazole and the herbicide glyphosate, alone or in binary and ternary mixtures, at environmental concentrations (0 (controls), 0.1, 1 and 10 µg/L) for 20 days. The survival of the honey bees was significantly reduced after exposure to these 3 pesticides individually and in combination. Overall, the combinations had a higher impact than the pesticides alone with a maximum mortality of 52.9% after 20 days of exposure to the insecticide-fungicide binary mixture at 1 µg/L. The analyses of the surviving bees showed that these different pesticide combinations had a systemic global impact on the physiological state of the honey bees, as revealed by the modulation of head, midgut and abdomen glutathione-*S*-transferase, head acetylcholinesterase, abdomen glucose-*6*-phosphate dehydrogenase and midgut alkaline phosphatase, which are involved in the detoxification of xenobiotics, the nervous system, defenses against oxidative stress, metabolism and immunity, respectively. These results demonstrate the importance of studying the effects of chemical cocktails based on low realistic exposure levels and developing long-term tests to reveal possible lethal and adverse sublethal interactions in honey bees and other insect pollinators.

Keywords: winter honey bee, pesticide mixtures, synergy, cocktail effects, physiological state

1. Introduction

Despite the 45% global increase in managed honey bee colonies since 1961 (Aizen and Harder, 2009; Faostat, 2018), regional colony losses have been reported in different areas, such as the United States of America (USA) and Europe. In the USA, 31.3% of colonies were lost between 2007 and 2008, while in central Europe, a significant decrease of 25% took place between 1985 and 2005 (Potts et al., 2010; Vanengelsdorp et al., 2008). The reduction in managed beehives is accompanied by a global decrease in the number and diversity of other animal pollinators (Ollerton, 2017). It has been attributed to multiple factors, including the decline in diversity and abundance of flowers, the lack of natural habitat, the presence of parasites and pathogens and exposure to pesticides (Goulson et al., 2015; vanEngelsdorp and Meixner, 2010).

Field surveys have confirmed a transfer from crops to beehive matrices of applied pesticides belonging to the three main classes of insecticides, fungicides and herbicides (Piechowicz et al., 2018b; Pohorecka et al., 2012; Skerl et al., 2009). Scientists were interested in knowing the effects of insecticides on honey bees, as these products are considered the most potentially dangerous pesticides to beneficial insects (Brandt et al., 2016; Decourtye et al., 2004b; Glavan and Bozic, 2013; Gregorc and Ellis, 2011; Guez et al., 2001; Kessler et al., 2015; Yang et al., 2008). Fungicides and herbicides are considered harmless to honey bees due to their low acute toxicity. Nevertheless, an increasing number of studies are addressing their actual effects (Christen et al., 2019b; Cousin et al., 2013; Jaffe et al., 2019; Ladurner et al., 2005; Moffett et al., 1972). In beehive matrices, the phytopharmaceutical products of three main classes can coexist with acaricides used to control infestation by *Varroa destructor* (Chauzat et al., 2009; Chauzat et al., 2006; Mullin et al., 2010). Therefore, honey bees could be continuously exposed to mixtures of pesticides that may exhibit similar or completely different modes of action.

Despite the high probability of honey bee exposure to mixtures of pesticides, only a few studies have focused on their effects on honey bees, and most of them were restricted to the interactions between insecticides (pyrethroids and neonicotinoids) and fungicides (ergosterol biosynthesis inhibitor (EBI) family) (Bjergager et al., 2017; Colin and Belzunces, 1992; Iwasa et al., 2004; Meled et al., 1998; Schmuck et al., 2003; Thompson et al., 2014a; Zhu et al., 2017a; Zhu et al., 2017b). Effects varied from no effects to synergism, depending on the pesticides used, the method and duration of exposure, and the concentrations in food. Therefore, there is a large gap in the assessment of pesticide risk in the registration procedure because the mixtures were never investigated, and further studies are urgently needed in this field.

The losses of honey bee colonies are mostly seen at the end of the winter season (Genersch et al., 2010b; Guzmán-Novoa et al., 2010), with approximately 20 to 30% losses in Canada, Europe and the USA (van der Zee et al., 2012). During this period, beehive tasks are performed by a specific category of workers known as winter honey bees. These honey bees can survive up to 6 months (Free and Spencer-Booth, 1959), and they rely on the consumption of stored honey and bee bread for survival, exposing them to pesticides for a relatively long period.

Imidacloprid (insecticide), difenoconazole (fungicide) and glyphosate (herbicide) are among the pesticides that are frequently detected in beehive matrices (Berg et al., 2018; Chauzat et al., 2011; Mullin et al., 2010). Imidacloprid, together with its metabolite 6-chloronicotinic acid, was the most abundant pesticide in beehive matrices in French apiaries, with a mean concentration of 0.7 µg/kg in honey and 0.9 µg/kg in pollen (Chauzat et al., 2011). However, concentrations of 0.14-0.275 µg/kg in honey, 1.35 µg/kg in pollen and 3-5.09 µg/kg in wax comb were found in other studies (Lambert et al., 2013; Lopez et al., 2016; Nguyen et al., 2009). Imidacloprid belongs to the neonicotinoid family and acts as an agonist of the nicotinic acetylcholine receptors, leading to the disruption of the nervous system through impaired cholinergic neurotransmission (Casida and Durkin, 2013). Glyphosate is the most dominant herbicide worldwide. Its use has increased 15-fold since the introduction of genetically engineered glyphosate-tolerant crops in 1996 (Benbrook, 2016), and it was detected in beehive matrices at concentrations ranging between 17 to 342 µg/kg in honey and 52.4 to 58.4 µg/kg in beebread (Berg et al., 2018; El Agrebi et al., 2020; Rubio et al., 2015). It acts by inhibiting the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS), an enzyme necessary for the biosynthesis of aromatic amino acids in plants and some microorganisms, which leads to cell death (Amrhein et al., 1980). Difenoconazole, a curative and preventive fungicide of the triazole family, is authorized for use during full bloom. It has been found at mean concentrations of 0.6 µg/kg in honey, 43 µg/kg in pollen, 270 µg/kg in beebread and 1 µg/kg in wax comb (Kubik et al., 2000; Lopez et al., 2016). It belongs to the ergosterol biosynthesis inhibitor (EBI) fungicides and acts by inhibiting the demethylation of lanosterol (Zarn et al., 2003).

To understand the effects of pesticide mixtures on winter honey bees, we conducted a study investigating the effects of the insecticide imidacloprid, the fungicide difenoconazole and the herbicide glyphosate alone or in combinations in winter bees orally exposed at concentrations found in honey and pollen (Berg et al., 2018; Chauzat et al., 2011; Kubik et al., 2000; Nguyen et al., 2009; Thompson et al., 2019). Attention was focused on survival and physiology. The effects on physiological functions were assessed by analyzing the modulation of five physiological markers involved in the nervous system, detoxification, oxidative stress, metabolism and immunity.

2. Materials and Methods

2.1. Reagents

Triton X-100, monosodium phosphate (NaH_2PO_4), sodium chloride (NaCl), pepstatin A, leupeptin, aprotinin, trypsin, antipain, 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW284C51), 4-nitrophenyl acetate (*p*-NPA), ethanol, disodium phosphate (Na_2HPO_4), monopotassium phosphate (KH_2PO_4), disodium ethylenediaminetetraacetate dihydrate (EDTA), reduced L-glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), acetonitrile (CH_3CN), acetylthiocholine iodide (AcSCh), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), sodium bicarbonate ($NaHCO_3$), tris base, D-glucose-6-phosphate disodium salt hydrate

(G6P), magnesium chloride hexahydrate ($MgCl_2.6H_2O$), β-nicotinamide adenine dinucleotide phosphate hydrate (β-$NADP^+$), 4-nitrophenyl phosphate bis(tris) salt (*p*-NPP), sodium hydroxide (NaOH), dimethyl sulfoxide (DMSO) and hydrochloric acid (HCl) were obtained from Sigma Aldrich (Saint Quentin Fallavier, France). Imidacloprid (CAS No 138261-41-3), difenoconazole (CAS No 119446-68-3) and glyphosate (CAS No. 1071-83-6) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Protein solution (Bee Food) was purchased from Remuaux Ltd (Barbentane, France).

2.2. Honey bees

Honey bees were gathered in February 2018 from three colonies of the experimental apiary of the Abeilles & Environnement (Bees & Environment) research unit of INRAE (Avignon, France). The colonies were continuously checked for their health status. The honey bees were mixed together, slightly anesthetized with carbon dioxide and then placed, in groups of 30 honey bees, in plastic cages (6 x 8.5 x 10 cm) with a sheet of filter paper placed on the bottom and replaced daily to maintain hygiene. The honey bees were placed in the dark in incubators at 30°C ± 2°C and 60% ± 10% relative humidity. During the first day, the bees were fed water and candy (Apifonda®) *ad libitum*. The following day, the few dead bees were removed and replaced, and the chronic exposure to pesticides for 20 days was begun.

2.3. Chronic exposure to pesticides

The bees were exposed to the insecticide imidacloprid (I), the fungicide difenoconazole (F) and the herbicide glyphosate (H) individually or in combination. Imidacloprid, difenoconazole and glyphosate were prepared either alone or in binary mixtures (imidacloprid + glyphosate (IH), imidacloprid + difenoconazole (IF), and glyphosate + difenoconazole (HF)) or in a ternary mixture (imidacloprid + glyphosate + difenoconazole (IHF)) at concentrations of 0.1, 1 and 10 µg/L for each substance (equivalent to 0.083, 0.813 and 8.130 µg/kg, calculated with a sucrose solution density of 1.23 ± 0.02 (n=10)) in a 60% (w/v) sucrose solution containing a 0.1% (v/v) final concentration of DMSO. The treatments were abbreviated as follows: 0.1 µg/L: I0.1, F0.1, H0.1, IH0.1, IF0.1, HF0.1 and IHF0.1; 1 µg/L: I1, F1, H1, IH1, IF1, HF1 and IHF1; and 10 µg/L: I10, F10, H10, IH10, IF10, HF10 and IHF10. The primary mother solutions of the individual pesticides were prepared in 100% DMSO. These primary solutions were used to generate the mother solutions of the individual pesticides or were mixed to obtained the mother solutions of the pesticide mixtures. The mother solutions of the pesticides were prepared by serial dilution of the primary mother solutions to obtain 1% (v/v) DMSO and stored at -20°C. The sucrose solutions used for exposure to pesticides were prepared daily by 10-fold dilution of the mother pesticide solutions in sucrose solution to obtain final concentrations of 60% (m/v) sucrose, 1% (m/v) proteins and 0.1% (v/v) DMSO. The pesticide concentrations were checked by GC-MS/MS according to two analytical methods with RSD < 10% (Paradis et al., 2014; Wiest et al., 2011).

The control bees were fed a sucrose solution devoid of pesticides. For each modality of exposure (including the controls), 14 cages of 30 bees were used. Each day, the bee mortality and food consumption were recorded, the dead bees were discarded, and the filter paper placed at the bottom of the cage was replaced. For the analysis of the physiological markers, the bees were sampled 10 and 20 days after the beginning of chronic exposure.

2.4. Survival rate and food consumption

In each cage, the survival rate was recorded daily and expressed as a ratio of the initial population. Every morning, the dead bees were removed for sanitary considerations.

Food consumption was recorded for 20 days by measuring the food consumed daily by the bees in each cage. Individual daily food consumption was calculated by dividing the food consumed per cage by the number of bees that remained alive each day in each cage.

2.5. Choice of physiological markers

The effects of the pesticide combinations on honey bee physiology were assessed by analyzing the modulation of five physiological markers. The markers were chosen to distinguish the systemic and tissue-specific actions of the pesticides alone and in combination. The following two markers common to the three biological compartments (head, midgut and abdomen) were analyzed: CaE-3 and GST. In contrast, one specific physiological marker was chosen in each compartment as follows: AChE in the head, G6PDH in the abdomen and ALP in the midgut. These five markers have been found to be relevant in assessing the effects of pesticides on honey bees in different biological compartments (Badiou-Beneteau et al., 2013; Badiou-Beneteau et al., 2012; Boily et al., 2013; Carvalho et al., 2013; Kairo et al., 2017a; Zhu et al., 2017a; Zhu et al., 2017b).

2.6. Tissue preparation and marker extraction

At days 10 and 20, the surviving bees were sampled. To avoid animal suffering, the bees were anesthetized with carbon dioxide, the heads were separated from the rest of the body using a scalpel, and the midguts were obtained by pulling the stinger. The heads, midguts and abdomens (with the intestinal tract removed) were placed in 2 mL microfuge tubes, weighed and stored at -80°C until analysis. For each treatment modality and each type of tissue, 3 tissues were used and pooled to prepare the sample. From this sample, the tissues were homogenized to prepare a single tissue extract. Seven tissue extracts (7 × 3 tissues) were prepared (n=7) for each treatment modality. Each sample was assayed in triplicate. The tissues were homogenized in the extraction medium [10 mM sodium chloride, 1% (w/v) Triton X-100, 40 mM sodium phosphate pH 7.4 and

protease inhibitors (2 µg/ml of pepstatin A, leupeptin and aprotinin, 0.1 mg/ml soybean trypsin inhibitor and 25 units/ml antipain)] to make 10% (w/v) extracts. Homogenization was achieved by grinding tissues with a high-speed Qiagen TissueLyser II at 30 Hz for 5 periods of 30 seconds at 30 second intervals. The extracts were centrifuged at 4°C for 20 min at 15000 × $g_{av.}$ and the supernatants were kept on ice for further enzyme assays. Carboxylesterase para (CaE-3) and glutathione-*S*-transferase (GST) were extracted from the head, midgut and abdomen; acetylcholinesterase (AChE) from the head; glucose-*6*-phosphate dehydrogenase (G6PDH) from the abdomen; and alkaline phosphatase (ALP) from the midgut.

2.7. Enzyme assays

CaE-3 was assayed in a medium containing the tissue extract, 10 µM BW284C51 (acetylcholinesterase inhibitor), 0.1 mM *p*-NPA as the substrate and 100 mM sodium phosphate pH 7.0. The reaction was monitored at 410 nm (Badiou-Beneteau et al., 2012; Gomori, 1953; Renzi et al., 2016). GST was assayed at 340 nm by measuring the conjugation of GSH to CDNB. The extract was incubated in a medium containing 1 mM EDTA, 2.5 mM GSH as the cosubstrate, 1 mM CDNB as the substrate and 100 mM disodium phosphate pH 7.4 (Carvalho et al., 2013). AChE was assayed at 412 nm in a medium containing the tissue extract, 1.5 mM DTNB, 0.3 mM AcSCh as the substrate and 100 mM sodium phosphate pH 7.0 (Belzunces et al., 1988a). G6PDH was measured by following the formation of NADPH at 340 nm in a medium containing the tissue extracts, 1 mM G6P as the substrate, 0.5 mM $NADP^+$ as the coenzyme, 10 mM $MgCl_2$ and 100 mM Tris-HCl pH 7.4 (Renzi et al., 2016). ALP was assayed at 410 nm in a medium containing the tissue extract, 20 µM $MgCl_2$, 2 mM *p*-NPP as the substrate and 100 mM Tris-HCl pH 8.5 (Bounias et al., 1996). All reactions started after adding the substrate, and the activity was assessed by determining the initial velocity of the enzymatic kinetics, which corresponded to the slope of the tangent at the origin. All enzymatic reactions were followed using a TECAN F500 spectrophotometer.

2.8. Mode of interaction between pesticides

The interaction ratio (IR) was used to define the mode of interaction between pesticides (additive, antagonistic and synergistic) (Colin and Belzunces, 1992; Piggott et al., 2015):

$$\mathrm{IR} = \frac{(Mix - C)}{\sum_{n=0}^{2-3}(P_n - C)}$$

where *Mix* represents the crude mortality of the mixture (binary or ternary), *C* the mortality of the control, and (*Mix* - *C*) the mortality of the pesticide mixture corrected by the control mortality. $\sum_{n=0}^{2-3}(P_n - C)$ represents the sum of the mortalities induced by each pesticide (*n*) in the mixture corrected by the control mortality, which corresponds to the theoretical expected mortality of the

mixture. A value of IR = 1 reflects a pure additive effect. However, considering the variation in the effects, an IR is considered equal to 1 when $0.95 \leq IR \leq 1.05$. When IR > 1, the interaction is synergistic. For IR < 1, three cases were distinguished: (i) when the mortality of the mixture was lower than the mortality of the lowest toxic substance alone, the interaction was considered purely antagonistic. (ii) When the toxicity of the mixture was higher than the mortality of the most toxic substance but below the expected mortality, the interaction was considered subadditive. In this case, it was not possible to speak in terms of antagonism because the effect of the mixture was higher than the effect of each substance. (iii) When the effect of the mixture was between the effect of the least toxic substance and the effect of the most toxic substance, the interaction was also considered subadditive. In this case, it was also not possible to speak in terms of antagonism because, compared to the most toxic substance, antagonism could be considered, but compared to the least toxic substance, synergy could also be considered. (iv) The effect of the mixture was judged independent when the mixture induced a mortality similar to that of each pesticide.

2.9. Statistical analyses

The statistical analyses were performed using R software (Rstudio Version 1.1.463). The bee survival was analyzed by the Kaplan-Meier method (log-rank test), followed by a post hoc test to compare survival and treatments. The effects of the treatments on food consumption were investigated by comparing the individual cumulative sucrose consumption during the exposure period using the Kruskal-Wallis test, followed by pairwise comparisons using the Wilcoxon rank sum test with a Benjamini-Hochberg correction. The effects of the treatments on the physiological markers were determined by ANOVA, followed by Tukey's HSD test, when the data followed a normal distribution or a Kruskal-Wallis test, followed by a post hoc Dunn test (with Benjamini-Hochberg correction), when the data followed a non-normal distribution.

3. Results

3.1. Honey bee survival

Exposure to pesticides significantly decreased the survival rate of honey bees at 20 days, except for I0.1, I10 and F0.1, for which no significant difference from the control (20.0 ± 2.7%) was observed ($p > 0.05$) (Fig. 1A, 1B, 1C and Table S1). Based on mortality rates, the toxicities of pesticides could be ranked as follows: at 0.1 µg/L, H = IF (28.1%) < IHF (35.4%) < IH (43.3%) < HF (49.1%). At 1 µg/L, I (33.3%) < F (34.3%) < H (35.2%) < HF (36.2%) < IH (38.1%) < IHF (43.3%) < IF (52.9%). At 10 µg/L, HF (28.1%) < H (30.0%) < F (34.3%) < IF (41.0%) < IHF (43.3%) < IH (45.7%).

Based on the interaction ratio (IR), which corresponds to the ratio between the obtained mortality of the mixture and the expected mortality (sum of the obtained mortalities of the substances in the mixture), the interaction effects between the pesticides could be grouped into 5 different categories (Table S1): additive, synergistic, subadditive, antagonistic and independent effects. (i) A synergistic effect was observed for all the binary mixtures and the ternary mixture at 0.1 µg/L and for IF1 and IH10. (ii) An additive effect was observed for IF10. (iii) A subadditive effect was observed for IH1, IHF1 and IHF10. (iv) An independent effect was observed for HF1. (v) An antagonistic effect was observed for HF10. The five most toxic pesticide mixtures were ranked as follows based on mortality rates: IF10 (41.0%) < IHF1 (43.3%) = IHF10 = IH0.1 (43.3%) < IH10 (45.7%) < HF0.1 (49.1%) < IF1 (52.9%).

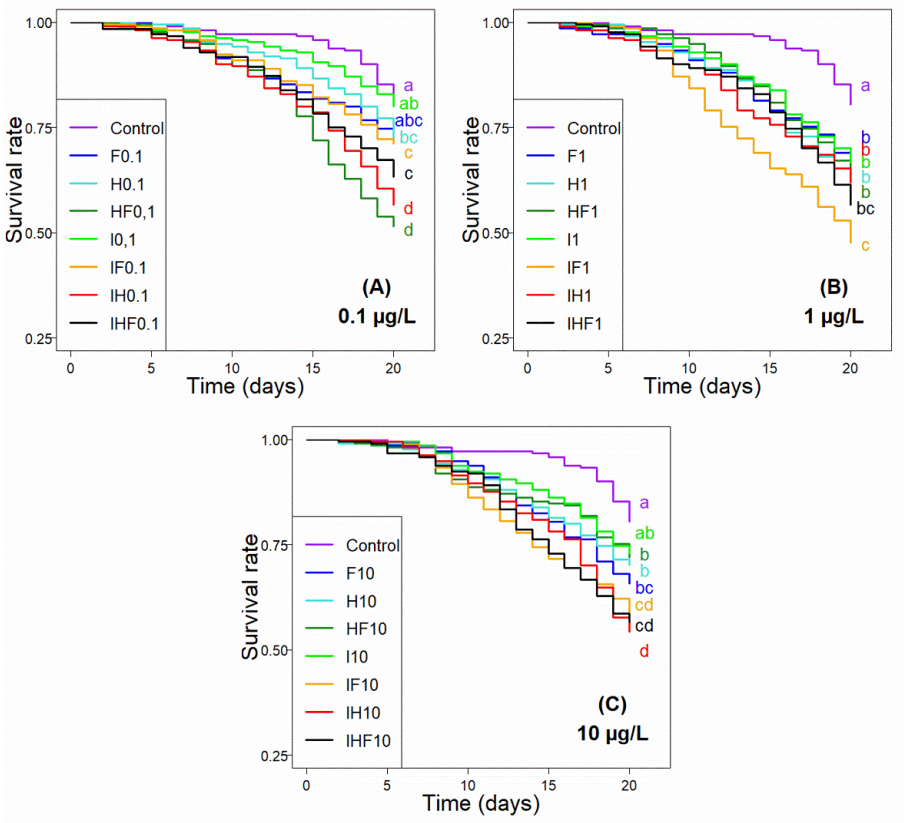

Fig. 1. Effects of pesticides alone or in combination on honey bee longevity

For 20 days, winter honey bees were fed sucrose solutions containing no pesticides (Control), difenoconazole (F), glyphosate (H), glyphosate + difenoconazole (HF), imidacloprid (I), imidacloprid + difenoconazole (IF), imidacloprid + glyphosate (IH) or imidacloprid + glyphosate + difenoconazole (IHF), at 0.1 µg/L (A), 1 µg/L (B) and 10 µg/L (C). The data represent the proportion of surviving honeybees exposed to these pesticides. Numbers after the abbreviations of each treatment refer to the concentrations of the pesticides in the sucrose solution. Treatments with different letters are significantly different ($p < 0.05$).

3.2. Effects of exposure to pesticides on food consumption behavior

Food consumption was monitored daily. In general, at the end of the exposure period, it appeared that the food consumption was higher in the exposed bees (Fig. 2 and Table S2). This higher consumption was significant for all exposure conditions except F1, I1, F10 and I10 for pesticides alone, and HF10 and IHF10 for the mixtures. The five highest individual cumulative consumption levels were ranked as follows: H0.1 (831.4 mg/bee) < IF10 (834.3 mg/bee) < IF1 (840.3 mg/bee) < HF0.1 (851 mg/bee) < IH0.1 (862.7 mg/bee) (control = 672.4 ± 33.0 mg/bee). At 0.1 µg/L, the bees exposed to imidacloprid alone or in IF, IH or IHF exhibited a cumulative food consumption of 759.7, 792.6, 862.7 and 781.9 mg/bee, respectively. Therefore, on the basis of a food density of 1.23 ± 0.02 (n = 10) and pesticide concentrations, each honey bee ingested 62, 64, 70 and 63 pg of imidacloprid, which corresponded to ca. 1/60, 1/58, 1/53 and 1/58 of the imidacloprid LD_{50} (LD_{50} = 3.7 ng/bee (Schmuck et al., 2001)). At 1 µg/L, the bees exposed to imidacloprid alone or in IF, IH or IHF exhibited a cumulative food consumption of 719.3, 840.3, 804.2 and 758.4 mg/bee, respectively. Therefore, each honey bee ingested 584, 682, 653 and 615 pg of imidacloprid, which corresponded to ca. 1/6, 1/5, 1/6 and 1/6 of the imidacloprid LD_{50}. At 10 µg/L, the bees exposed to imidacloprid alone or in IF, IH and IHF exhibited a cumulative food consumption of 749.3, 834.3, 794.1 and 702.5 mg/bee, respectively. Therefore, each honey bee ingested 6081, 6770, 6445 and 5701 pg of imidacloprid, respectively, which corresponded to ca. 1/0.6, 1/0.6, 1/0.6 and 1/0.7 of the imidacloprid LD_{50}. The $LD_{50\ values}$ of difenoconazole and glyphosate are equal to or higher than 100 µg/bee (National Center for Biotechnology Information). Therefore, for difenoconazole and glyphosate at 0.1, 1 and 10 µg/L, each honey bee ingested $1/1.6x10^6$, $1/1.7x10^5$ and $1/1.8x10^4$ of the LD_{50}, respectively (Table S2).

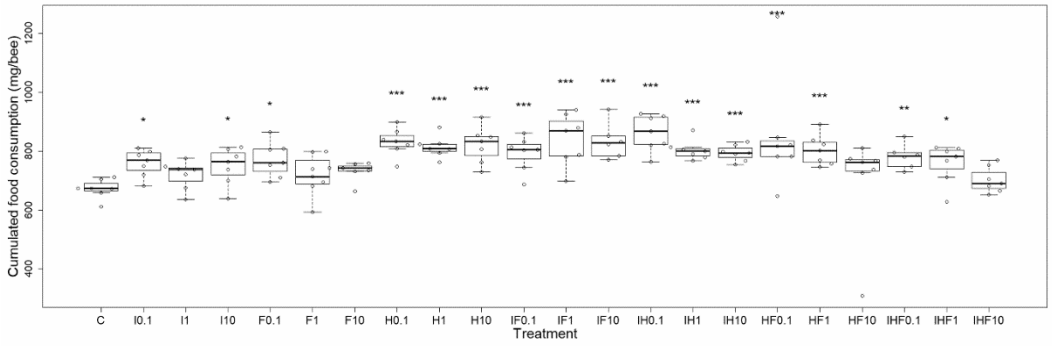

Fig. 2. Effects of pesticides alone or in combination on food consumption

For 20 days, winter honey bees were fed sucrose solutions containing no pesticide (C, control), difenoconazole (F), glyphosate (H), glyphosate + difenoconazole (HF), imidacloprid (I), imidacloprid + difenoconazole (IF), imidacloprid + glyphosate (IH) or imidacloprid + glyphosate + difenoconazole (IHF), at 0.1 µg/L, 1 µg/L, and 10 µg/L. Food consumption was followed during the 20 days of exposure by measuring the food consumed daily by the bees alive in each cage. Box plots represent the cumulated individual consumption (mg/bee) for 7 cages of 30 bees per treatment. Statistical analyses were performed using the Kruskal-Wallis test followed by pairwise comparisons using the Wilcoxon rank sum test with the Benjamini-Hochberg correction. The numbers after the abbreviations of each treatment refer to the concentrations of the pesticides in the sucrose solution. Asterisks indicate significant differences from the control group (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

3.3. Effect of exposure to pesticides on the physiological status of honey bees

The physiological status of the honey bees was examined by studying the modulation of physiological markers in different compartments to distinguish the local from the systemic effects of the pesticides (Table 1). The responses of the honey bee markers to the exposure to the pesticides alone or in combination were analyzed after 10 and 20 days of chronic exposure to concentrations of 0.1 µg/L and 1 µg/L (Fig. 3, Fig. 4, Table S3 and Table S4). The lowest concentrations were chosen because they are particularly environmentally relevant. To render the data comparable, the enzymatic activities are expressed as percentages of the control values (Zhu et al., 2017a).

Table 1. Distribution of common and specific physiological markers across honey bee tissues

	Head	Abdomen	Midgut
Common markers	CaE-3	CaE-3	CaE-3
	GST	GST	GST
Specific markers	AChE	G6PDH	ALP

Repartitioning of physiological markers across honey bee compartments. The following three tissues were investigated: head, abdomen and midgut. In each tissue, 1 specific marker (AChE in the head, G6PDH in the abdomen and ALP in the midgut) and 2 common markers (CaE-3 and GST) were considered.

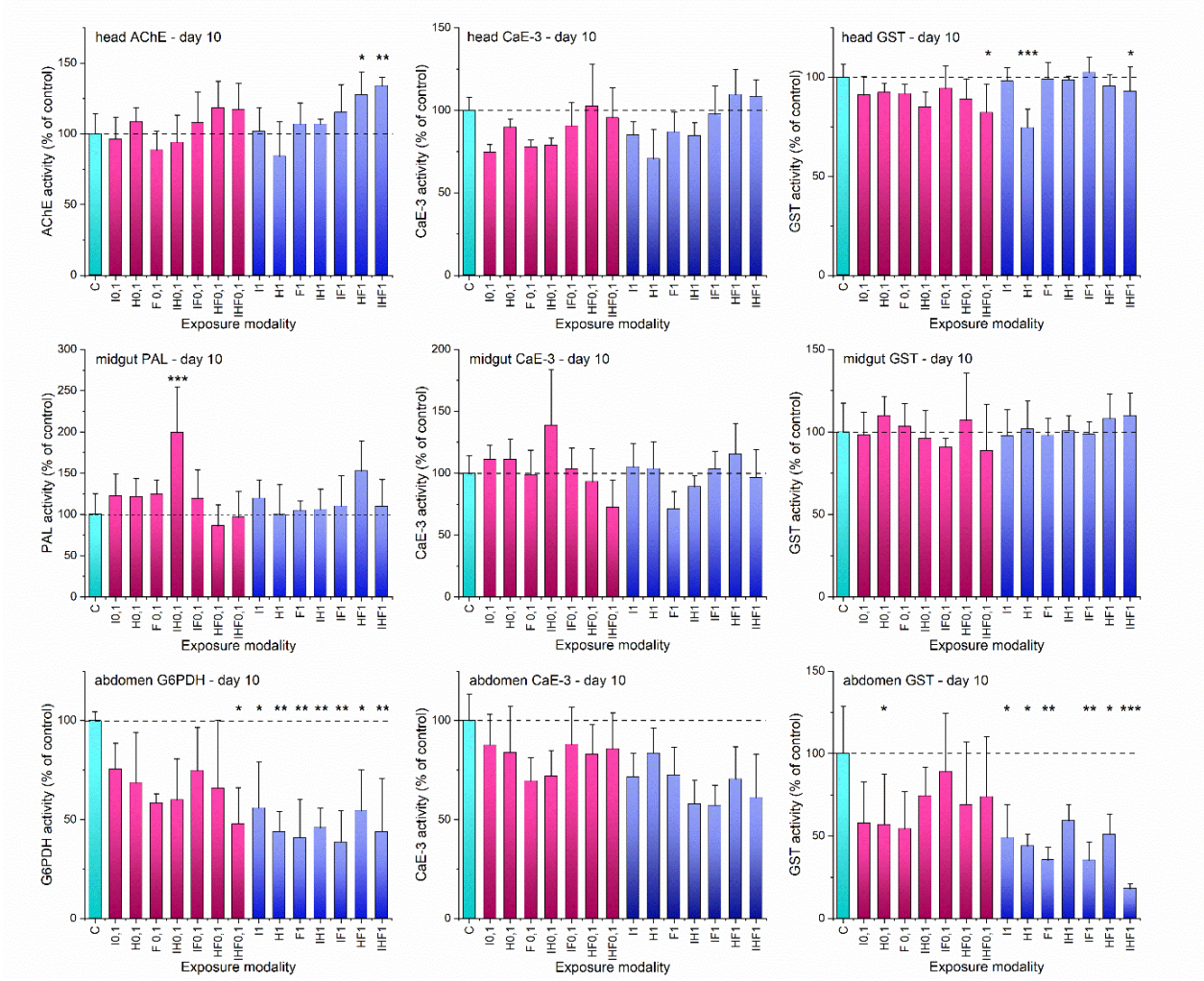

Fig. 3. Physiological impacts of pesticides alone or in combination in winter bees after 10 days of exposure

For 20 days, winter honey bees were fed sucrose solutions containing no pesticides (C, control), imidacloprid (I), glyphosate (H), difenoconazole (F), imidacloprid + glyphosate (IH), imidacloprid + difenoconazole (IF), glyphosate + difenoconazole (HF), or imidacloprid + glyphosate + difenoconazole (IHF). The impact of the exposure to pesticides on the physiology of the surviving honey bees at day 10 was investigated through an analysis of 2 common markers in the head, abdomen and midgut (GST and CaE-3) and 3 specific markers (AChE in the head, G6PDH in the abdomen and ALP in the midgut). To make the data comparable, the enzymatic activities were expressed as percentages of the control values. Numbers after the abbreviation of each treatment refer to the concentration of the pesticide in the sucrose solution. The exposure modalities above and below the dashed horizontal line indicate increases and decreases in enzymatic activity, respectively, compared to the control (C). Asterisks indicate significant differences from the control group (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

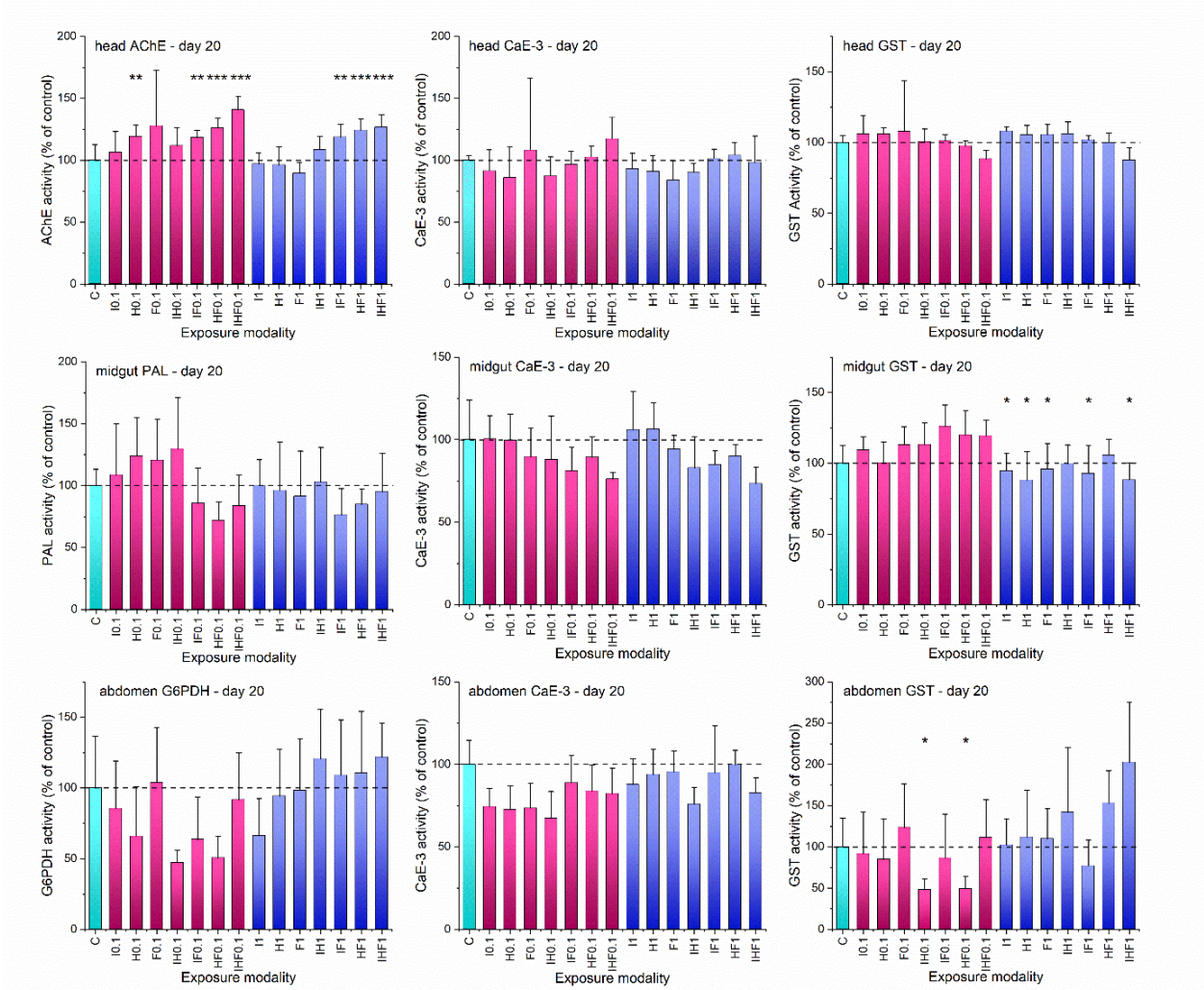

Fig. 4. Physiological impacts of pesticides alone or in combination in winter bees after 20 days of exposure

For 20 days, winter honey bees were fed sucrose solutions containing no pesticides (C, control), imidacloprid (I), glyphosate (H), difenoconazole (F), imidacloprid + glyphosate (IH), imidacloprid + difenoconazole (IF), glyphosate + difenoconazole (HF), or imidacloprid + glyphosate + difenoconazole (IHF). The impact of the exposure to pesticides on the physiology of the surviving honey bees at day 20 was investigated through an analysis of 2 common markers in the head, abdomen and midgut (GST and CaE-3) and 3 specific markers (AChE in the head, G6PDH in the abdomen and ALP in the midgut). To make the data comparable, the enzymatic activities were expressed as percentages of the control values. Numbers after the abbreviation of each treatment refer to the concentration of the pesticide in the sucrose solution. The exposure modalities above and below the dashed horizontal line indicate increases and decreases in the enzymatic activity, respectively, compared to the control (C). Asterisks indicate significant differences from the control group (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

At 0.1 µg/L, head, midgut and abdomen CaE-3 and midgut GST were not modulated by all types of exposure at day 10 and day 20. Head AChE was not modulated at day 10. However, at day 20, its activity was 119% of the control activity (127.5 ± 16.0 mUA.min^{-1}.mg of tissue^{-1}) for H, 126% for HF and 141% for IHF. Head GST, abdomen G6PDH, and midgut ALP underwent modulation at day 10. For IHF, these modulations corresponded to a decrease in head GST (82% of control activity (115.3 ± 7.5 mUA.min^{-1}.mg of tissue^{-1})) and a decrease in abdomen G6PDH (48% of control activity (2.07 ± 0.53 mUA.min^{-1}.mg of tissue^{-1})). For IH, midgut ALP increased to 199% of the control activity (10.86 ± 2.75 mUA.min^{-1}.mg of tissue^{-1}). Conversely, no modulation was observed at day 20 for any of these latter enzymes. A decrease in abdomen GST was observed at 10 and 20 days. At 10 days, GST decreased to 57% of the control activity (116.1 ± 33.3 mUA.min^{-1}.mg of tissue^{-1}) for H. At day 20, GST decreased to 48% of the control activity (83.0 ± 28.7 mUA.min^{-1}.mg of tissue^{-1}) for IH and 49% for HF.

At 1 µg/L, head, midgut and abdomen CaE-3 and midgut ALP were not modulated for all types of exposure at day 10 and day 20. Head and abdomen GST underwent modulation at day 10. Head GST decreased to 75% of the control activity (115.3 ± 7.5 mUA.min^{-1}.mg of tissue^{-1}) for H and 93% for IHF. Abdomen GST decreased for all types of exposure except IH: 49% of the control activity for I; 44% for H; 36% for F; 35% for IF; 51% for HF and 18% for IHF (116.1 ± 33.3 mUA.min^{-1}.mg of tissue^{-1} for the control). Conversely, head and abdomen GST were not modulated at day 20. Abdomen G6PDH decreased at day 10 for all types of exposure: 56% of the control activity for I; 44% for H; 41% for F; 46% for IH; 38% for IF; 55% for HF and 44% for IHF (12.1 ± 0.5 mUA.min^{-1}.mg of tissue^{-1} for the control). However, no modulation was observed at day 20. Midgut GST was not modulated at day 10 but was modulated at day 20. Its activity decreased with all exposure types except IH and HF: 95% of the control activity for I; 88% for H; 96% for F; 93% for IF and 88% for IHF (147.9 ± 18.8 mUA.min^{-1}.mg of tissue^{-1} for the control). At day 10, head AChE increased to 128% of the control activity (127.7 ± 18.5 mUA.min^{-1}.mg of tissue^{-1}) for HF and 134% of the control activity for IHF. At day 20, the activity of AChE increased to 124% of the control (127.5 ± 16.0 mUA.min^{-1}.mg of tissue^{-1}) for HF, 127% of the control for IHF and 119% of the control for IF.

When comparing the dose effect of each type of exposure on physiological markers (comparison of the effects at 0.1 and 1 µg/L), no dose effect could be observed for I alone. The effects of H on all markers were similar at both concentrations except for AChE at day 20 and head GST at day 10 (H0.1 > H1). F had the same effect on all markers at both concentrations except for AChE at day 20 (F0.1 > F1). The effect of IH on CaE-3, ALP, and abdomen GST was not similar at both concentrations. The effect of IH on head CaE-3 at day 10 and on abdomen CaE-3 and GST at day 20 was lower at 0.1 µg/L than at 1 µg/L. Conversely, the effect of IH on midgut CaE-3 at days 10 and 20 and on abdomen CaE-3 and midgut ALP at day 10 was higher at 0.1 µg/L than at 1 µg/L. The effect of IF on midgut GST at day 20 was higher at 0.1 µg/L than at 1 µg/L. Depending on the concentration, the IF mixture modulated abdomen GST at day 10 (IF0.1 > IF1) and abdomen G6PDH at day 10 (IF0.1 > IF1). The effect of HF was dose-dependent only on the activity of GST in the abdomen at day 20 (HF0.1 < HF1). The effect of the ternary mixture IHF

on abdomen GST at day 10 and on midgut GST at day 20 was higher at 0.1 µg/L than at 1 µg/L (IHF0.1 > IHF1) (Table S5).

4. Discussion

Honey bees that emerge at the end of the summer are considered winter bees. These bees can live up to 6 months (Free and Spencer-Booth, 1959) and, therefore, are chronically exposed to pesticide residues throughout the winter. In this study, the mixtures induced relatively high toxicity even though the winter honey bees were exposed for only 20 days to these three pesticides, alone or in binary and ternary mixtures, at concentrations equal to or even less than the environmental concentrations detected in beehive matrices. Thus, determining the effect of these pesticides on colony winter survival is highly important.

4.1. Pesticide combinations are more toxic to honeybees than individual pesticides

In this study, these three pesticides alone or in combination affected the survival of winter honey bees at all tested exposure concentrations, except for I0.1, I10 and F0.1. Concerning imidacloprid, the toxicity was less pronounced than that previously observed at the same concentrations on summer bees, where 50% mortality was reached after 8 days of chronic exposure at all concentrations (Suchail et al., 2001). In contrast, imidacloprid toxicity was much more pronounced than that observed in young summer bees after 14 days of exposure at 1 µg/L (Gonalons and Farina, 2018). The differences in imidacloprid toxicity could be attributed to seasonal variations (Decourtye et al., 2003b; Meled et al., 1998; Piechowicz et al., 2016), genetic differences (Smirle and Winston, 1987), the age of the bees or the exposure duration.

Herbicides and fungicides were considered nontoxic to honey bees for a long time. Concentrations of imidazole fungicides and glyphosate up to 0.084 and 35 mg/L, respectively (Zhu et al., 2017a), were shown to be nonlethal. However, in this study, chronic exposure to glyphosate and difenoconazole (except for F0.1) was lethal. All pesticide combinations alter honey bee survival and are more toxic than pesticides alone, except HF10, which exhibits an antagonistic effect. Thus, the tier approach implemented in the pesticide registration procedure, which is first based on acute toxicity, shows great limits in detecting pesticides toxic to bees.

4.2. Increased concentrations of pesticides are not always linked to increased toxicity

In terms of dose-effect relationships, in general, it appears that the highest concentration was not the most dangerous, and the highest mortalities were observed at the intermediate concentration of 1 µg/L. This bell-shaped non-monotonic dose response relationship (NMDR) (high response at intermediate doses and lower responses at low and high doses) was previously observed for

imidacloprid and glyphosate (Boily et al., 2013; Suchail et al., 2001; Vazquez et al., 2018). Three main hypotheses might explain this profile (Lagarde et al., 2015). The first is the plurality of molecular targets, i.e., each xenobiotic has several molecular targets of different affinities that may induce opposite effects across the range of the tested concentrations. The second hypothesis is the metabolic hypothesis (Suchail et al., 2001), which proposes that detoxification enzymes are induced at high but not at low concentrations. This hypothesis is consistent with the action of glyphosate, whose main metabolite, aminomethylphosphonic acid (AMPA), was shown to be nontoxic to honey bees (Blot et al., 2019). However, the metabolic hypothesis is not consistent with the action of imidacloprid because all metabolites were shown to be toxic to honey bees after chronic exposure (Suchail et al., 2001). The third hypothesis is receptor desensitization, where at high concentrations, numerous receptors are bound to xenobiotics, leading to a downregulation phenomenon (Lagarde et al., 2015).

The mixture of EBI fungicides with imidacloprid or glyphosate was shown in different studies to have no synergistic action (Iwasa et al., 2004; Thompson et al., 2014a; Zhu et al., 2017b) or to induce a synergistic effect (Biddinger et al., 2013). However, these studies were based on acute contact exposure. Therefore, it is not possible to directly compare these results with those of our study in which the mixtures induced an increase in mortality after chronic oral exposure. On the other hand, in two studies based on chronic oral exposure, the imidacloprid-fungicide and/or imidacloprid-glyphosate mixture did not show a synergistic or additive effect (Gonalons and Farina, 2018; Zhu et al., 2017a). The differences in the mixture effects between the different studies could be attributed to multiple factors: (i) The age of exposed honey bees, with newly emerged honey bees in the studies of Gonalons and Farina (2018) and Zhu et al. (2017b), and adult honey bees in our study. (ii) The duration of exposure, which did not exceed 14 days in the studies of Gonalons and Farina (2018) and Zhu et al. (2017b) but was 20 days in our study. (iii) The type of exposure, with the active ingredient in our study and in the study of Gonalons and Farina (2018) and with the formulated products in the study of Zhu et al. (2017b). (iv) Seasonal variability, which could be reflected by the use of winter honey bees in our study and summer or spring honey bees in the two previously cited studies. (v) The concentrations of the active ingredients constituting the mixtures, which were lower in our study when compared to the studies of Zhu et al. (2017b) and Gonalons and Farina (2018).

In this study, all binary mixtures had a differential effect on mortality in terms of both dose dependence and number of substances present in the mixture. Regarding the differential dose effect, HF induced a synergistic effect at 0.1 µg/L, an independent effect at 1 µg/L and an antagonistic effect at 10 µg/L. IF induced a synergistic effect at 0.1 and 1 and an additive effect at 10 µg/L. IH induced a synergistic effect at 0.1 and 10 µg/L and a subadditive effect at 1 µg/L. The ternary mixture induced a subadditive effect at 1 and 10 µg/L and a synergistic effect at 0.1 µg/L. Interactions between substances can occur not only through the primary biological targets responsible for the expected effect (insecticide, herbicide or fungicide) and common metabolic pathways, if they exist in the honey bee, but also through secondary targets responsible for non-intentional effects. Because primary and secondary targets may have different affinities for these substances, the effects induced could depend on the internal body concentration and, therefore,

the exposure level. Hence, substances may interfere by blocking or activating metabolic pathways triggered by the substances in the mixtures, which explains why the nature and importance of the effects vary with the doses (Lagarde et al., 2015). However, at 0.1 µg/L, the mortality induced by IHF was lower than those induced by IH and IF, leading us to conclude that increasing concentration or number of substances does not always increase the toxicity of a mixture. This finding exemplifies that the toxicity of a mixture is not merely the sum of the toxicity of the substances or the basic sum of the individual modes of actions.

4.3. Pesticides modulate feeding behavior through an increase in food consumption

Bees exposed to imidacloprid, difenoconazole and glyphosate, alone or in mixtures, consume more food than unexposed bees. Different hypotheses could explain this high consumption. (i) A higher food consumption could be triggered by energetic stress due to an increase in intermediary metabolism induced by the pesticides or the spoliation of energetic resources as has been shown for pyrethroids (Bounias et al., 1985). (ii) Honey bees could display a preference for sucrose solutions containing glyphosate and imidacloprid, as previously shown (Kessler et al., 2015; Liao et al., 2017). In contrast, a study has shown a decrease in food consumption after exposure to mixtures of the formulated products of imidacloprid with tetraconazole and of imidacloprid with glyphosate (Zhu et al., 2017a). This finding suggests that the decrease in food consumption could be attributed to adjuvants present in the formulated products that might have a repellent feeding effect. However, the effect on food consumption could also depend on the concentration of the pesticides to which honey bees are exposed. In our study, the presence of pesticides elicited a higher food consumption, whereas in the study conducted by Zhu et al. (2017b), at higher concentrations, the pesticides elicited a lower food consumption. Thus, active substances, adjuvants or both could induce concentration-dependent effects on food consumption depending on their affinities to the biological target.

The honey bees received a cumulative dose of imidacloprid equivalent to 1/60, 1/6 and 1/0.6 of the LD_{50} at 0.1, 1 and 10 µg/L, respectively. However, for glyphosate and difenoconazole, the cumulative quantity ingested was, at least, equivalent to $1/1.52x10^6$, $1/1.57x10^5$ and $1/1.65x10^4$ of the LD_{50} at 0.1, 1 and 10 µg/L. Despite cumulative exposure ratios of difenoconazole and glyphosate at least 10 000 times less than the LD_{50}, these two pesticides caused significant increases in mortality except for F0.1. Therefore, pesticides that are considered harmless to honey bees (high LD_{50}, superior to 100 µg/bee) can become dangerous even at very low concentrations after long-term exposure. This highlights the importance of an in-depth revision of the current risk assessment schemes used in the pesticide registration procedure (Sgolastra et al., 2020).

4.4. Pesticides induce perturbations in the detoxification process, nervous system, defense against oxidative stress, metabolism and immunity

CaE-3, along with the other carboxylesterases, is involved in the metabolism of xenobiotics by catalyzing the hydrolysis of substrates containing amide, ester and thioester bonds. It is also involved in lipid metabolism (Badiou-Beneteau et al., 2012; Ross et al., 2010). In our study, head, midgut and abdomen CaE-3 were not significantly modulated by any type of exposure. However, the activity of this enzyme was reported to decrease after acute exposure to 2.56 ng bee^{-1} thiamethoxam (neonicotinoid) (Badiou-Beneteau et al., 2012) and at $LD_{50}/20$ of fipronil (Carvalho et al., 2013). Several studies have shown differential expression of carboxylesterases (CaEs) after exposure to pesticides (Badiou-Beneteau et al., 2012; Zhu et al., 2019; Zhu et al., 2017a; Zhu et al., 2017b). Thus, measuring only overall CaE activity with nonspecific substrates could mask the differential modulation of several isoforms, including CaE-3.

AChE is a neural enzyme hydrolyzing the neurotransmitter acetylcholine in cholinergic synapses (Badiou et al., 2007). AChE was found to be involved in learning and memory processes (Gauthier et al., 1992; Guez et al., 2010). Its activity was significantly increased for HF1 and IHF1 at day 10 and for IF, HF and IHF at 0.1 and 1 µg/L at day 20. Therefore, the increase in AChE activity is closely related to the duration of exposure and the concentrations of the pesticides forming the mixture. This reflects a delayed effect of the pesticide combinations on the nervous system and reveals the importance of studies on the effects of these pesticide combinations on the behavior and cognitive functions of honey bees.

Glyphosate increased AChE activity in the bees exposed to 0.1 µg/L. This finding contradicts the results showing that both newly emerged and adult honey bees exposed for up to 14 days during the summer period to glyphosate or its formulated product Roundup, at concentrations ranging from 2.5 to 10 ng/bee (Boily et al., 2013) and 35 mg/L, exhibit a decrease in AChE activity (Zhu et al., 2017a). The difference in the effect of glyphosate between our study and the previously cited studies could be attributed to seasonal variability. This hypothesis is supported by studies showing that the adverse effects of pesticides may be higher in summer bees than in winter bees. This higher sensitivity of summer bees has been shown in terms of the effects of imidacloprid on learning performance (Decourtye et al., 2003b) and the synergistic effect of the pyrethroid insecticide deltamethrin and the azole fungicide prochloraz (Meled et al., 1998). These alterations in AChE activities might explain, at least in part, the impairment of cognitive behaviors, sucrose responsiveness and olfactory learning observed in honey bees after exposure to glyphosate (Balbuena et al., 2015; Gonalons and Farina, 2018; Herbert et al., 2014).

GST is a multifunctional enzyme involved in protection against oxidative stress and is a phase II enzyme involved in the detoxification of xenobiotics. It can also contribute to phase I detoxification by sequestering toxicants (Berenbaum and Johnson, 2015; du Rand et al., 2015). GST activity was mainly decreased after exposure to pesticides in the head, abdomen and midgut. This decrease could hypothetically be due either to inhibition of this enzyme or to a downregulation by these pesticides. However, noncovalent inhibition could not be detected

because of the dilution of the tissue components during the step of tissue homogenization and the assay procedure (at least 1/200-fold final dilution). In addition, a covalent inhibition of GST by pesticides has never been reported, even with electrophilic pesticides such as organophosphorus insecticides or herbicides that include glyphosate. Thus, the decrease in GST activity, associated with the absence of inhibition, is consistent with GST downregulation, which is also consistent with the 4-fold downregulation of GST S1, which is responsible for fighting against oxidative stress, in the heads of honey bee larvae exposed to imidacloprid (Wu et al., 2017). Furthermore, no phase II metabolites in imidacloprid metabolism, including those that could be conjugated to glutathione, were found in the honey bee (Suchail et al., 2004). This could be explained either by an absence of conjugation with GST, by the production of GST conjugates at undetectable levels, or by drastic downregulation of GST by imidacloprid. Thus, the decrease in GST activity may indicate a decrease in the honey bee capacities to detoxify these pesticides and to fight against oxidative stress that takes place after exposure to imidacloprid and glyphosate (Contardo-Jara et al., 2009; Gauthier et al., 2018; Jasper et al., 2012; Lushchak et al., 2009).

G6PDH is the primary enzyme of the pentose phosphate pathway that generates NADPH and is involved, among other things, in the regeneration of reduced glutathione, which contributes to the fight against oxidative stress (Thomas et al., 1991). G6PDH activity decreased after 10 days of exposure to all modalities at 1 µg/L. However, it is improbable that this decrease is due to oxidative stress. Indeed, in the presence of oxidative stress, glyceraldehyde-3-phosphate dehydrogenase (GAPD) is inhibited (Chuang et al., 2005), which induces a deviation of glycolysis towards the pentose phosphate pathway and an increase in G6PDH activity (Nicholls et al., 2012; Renzi et al., 2016).

ALP is an enzyme of the digestive tract involved in adsorption and transport mechanisms through the gut epithelium (Vlahović et al., 2009) and in immune response (Chen et al., 2011). The activity of ALP was not modulated after 10 and 20 days of exposure. Thus, imidacloprid, glyphosate and difenoconazole did not affect the activity of ALP. This finding strongly contrasts with the results of other studies that showed a modulation of ALP in bees exposed to other pesticides, such as fipronil and spinosad, and following infection by Nosema (Carvalho et al., 2013; Dussaubat et al., 2012a; Kairo et al., 2017a). Thus, the apparent absence of ALP modulation in our study could reflect either an absence of effect or the occurrence of a compensatory phenomenon.

4.5. The effect of exposure to pesticides is systemic and tissue-specific

By comparing the dose effect of IH on CaE-3, it is possible to notice that for the same exposure duration, the effect of IH on CaE-3 at 0.1 and 1 µg/L differed among the biological compartments. For the modulations of CaE-3 at day 10, IH0.1 < IH1 in the head and IH0.1 > IH1 in the midgut and abdomen. For the modulations of CaE-3 at day 20, IH0.1 > IH1 in the gut and IH0.1 < IH1 in the abdomen. This complex profile of modulations was also found for both head and midgut GST after exposure to *Bt* spores and to *Nosema*-fipronil combination (Kairo et al.,

2017a; Renzi et al., 2016), thus confirming a spatially differential response due to the specificity of each tissue and to the occurrence of pesticide metabolism not only in the gut but also in other honey bee compartments (Suchail et al., 2004).

GST activity was modulated in the head, midgut and abdomen. In addition, AChE was modulated in the head, G6PDH in the abdomen and ALP in the midgut. These results indicate that the effects of the exposure to pesticides are not localized in the midgut (and in turn in the abdomen), which is considered the primary site of interaction with the ingested pesticide, but are spread across all biological compartments, leading to a systemic response that could explain the severe impact on honey bee survival.

The effects of the pesticides on physiological markers were determined in surviving bees after 10 and 20 days of daily exposure. The results at day 10 revealed a massive modulation of all physiological markers except CaE-3 and midgut GST. However, a less pronounced effect was detected at day 20 with a higher number of non-modulated enzymes (CaE-3, head GST, ALP and G6PDH were not modulated). This lower effect at day 20 suggests that the honey bee population at day 10 was composed of both sensitive and resistant individuals, while the population that survived until the twentieth day mainly contained honey bees that were more resistant to these pesticides alone or in combination. However, this hypothesis could be ruled out because the progression of mortality during this period was approximately linear, indicating that the honey bees were sensitive to the pesticides and were unable to compensate for the increase in exposure duration.

5. Conclusion

This study demonstrates that chronic exposure to insecticides, herbicides and fungicides, alone or in combination, may induce high toxicity via systemic action in winter honey bees and constitutes a threat to these workers in two ways. The first is a direct drastic effect on survival, with a mortality that exceeded 50% after only 20 days of exposure, which can endanger the colony. The second involves a systemic action of these pesticides that alters honey bee physiology through metabolism, immunity, the nervous system, detoxification and antioxidant defenses. A severe loss of the winter bee population may compromise colony development during the spring, which might explain the high winter losses encountered in many regions. If such cocktail effects occurred in summer bees, this would have drastic impacts on colonies that could largely explain the bee population decline, especially because summer bees are more susceptible to pesticides and pesticide combinations than winter bees.

This study also reveals that the standard 10-day chronic toxicity test, used during pesticide risk assessment procedures, may not always be reliable in detecting the potential toxicities of pesticides. In addition, this study highlights the difficulty in predicting the cocktail effects of pollutants because the toxicity of the mixture is not always directly linked to the number of substances or the exposure level.

Acknowledgments

This study was supported in part by the recurrent funding of the Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAE) and by the Agence Nationale de la Recherche (ANR) (grant ANR-15-CE34-0004-01). Hanine ALMASRI was supported by grants from Lebanese University (grant number 2364) and the PACA region. The authors thank Jacques Sénéchal and Alexandre Gorit, beekeepers of the UR 406 INRAE Research Unit, for their expert beekeeping.

Table S1. Effects of pesticide combinations on mortality

For 20 days, winter honey bees were exposed orally to control solution (C), imidacloprid (I), glyphosate (H), difenoconazole (F), imidacloprid + glyphosate (IH), imidacloprid + difenoconazole (IF), glyphosate + difenoconazole (HF), or imidacloprid + glyphosate + difenoconazole (IHF) at concentrations in food of 0.1 µg/L, 1 µg/L and 10 µg/L. The data represent the mortality rates (%) ± standard deviations (SD), the mortality corrected by the control and the expected mortality (which is the sum of the corrected mortalities of each pesticide alone). The interaction ratio (IR) is calculated by dividing the corrected mortality by the expected mortality. Numbers after the abbreviations of each treatment refer to the concentrations of the pesticides in the sucrose solution. Treatments with different letters are significantly different ($p < 0.05$).

Treatment	Mortality rate (%) ± SD	Corrected mortality	Expected mortality	IR	Effect	Statistical significance ($p < 0.05$)
C	20 ± 2.73	-	-	-	-	a
I0.1	20 ± 2.76	0	-	-	-	ab
F0.1	25.71 ± 3.05	5.71	-	-	-	abc
H0.1	28.1 ± 3.01	8.1	-	-	-	bc
IF0.1	28.1 ± 3.17	8.1	5.71	1.42	synergism	c
IH0.1	43.33 ± 3.42	23.33	8.1	2.88	synergism	d
HF0.1	49.05 ± 3.49	29.05	13.81	2.1	synergism	d
IHF0.1	35.4 ± 3.59	15.4	13.81	1.11	synergism	c
I1	33.33 ± 3.32	13.33	-	-	-	b
F1	34.29 ± 3.27	14.29	-	-	-	b
H1	35.24 ± 3.29	15.24	-	-	-	b
IF1	52.86 ± 3.44	32.86	27.62	1.19	synergism	c
IH1	38.1 ± 3.35	18.1	28.57	0.63	sub-additivity	b
HF1	36.19 ± 3.31	16.19	29.52	0.55	independence	b
IHF1	43.31 ± 3.42	23.31	42.86	0.54	sub-additivity	bc
I10	27.14 ± 3.06	7.14	-	-	-	ab
F10	34.29 ± 3.27	14.29	-	-	-	bc
H10	30 ± 3.16	10	-	-	-	b
IF10	41.03 ± 3.66	21.03	21.43	0.98	addition	cd
IH10	45.71 ± 3.43	25.71	17.14	1.5	synergism	d
HF10	28.1 ± 3.1	8.1	24.29	0.33	antagonism	b
IHF10	43.33 ± 3.42	23.33	31.43	0.74	sub-additivity	cd

Table S2. Influence of exposure to pesticides on food consumption

For 20 days, winter honey bees were exposed orally to control solution (C), imidacloprid (I), glyphosate (H), difenoconazole (F), imidacloprid + glyphosate (IH), imidacloprid + difenoconazole (IF), glyphosate + difenoconazole (HF), or imidacloprid + glyphosate + difenoconazole (IHF) at concentrations in food of 0.1 µg/L, 1 µg/L and 10 µg/L. Food consumption was followed during the 20 days in the 7 cages of 30 bees per treatment by daily measurement of the food consumed by the bees remaining alive in each cage. The cumulated food consumption per bee is expressed in mg/bee ± standard deviations (SD). The cumulative quantities of ingested active substance (pg/bee) are calculated based on a food density of 1.23 ± 0.02 (n=10), and the relative ratios to the LD_{50} are calculated based on an LD_{50} of 3.7 ng/bee for imidacloprid and > 100 ng/bee for difenoconazole and glyphosate (National Center for Biotechnology Information; Schmuck et al., 2001). Numbers after the abbreviations of each treatment refer to the concentrations of the pesticides in the sucrose solution. Treatments with different letters are significantly different ($p < 0.05$).

Treatment	Cumulated food consumption/bee (mg/bee) ± SD	Statistical significance ($p < 0.05$)	*p* value	Cumulative quantity of substances ingested (pg/bee)	Relative ratio to the LD_{50} (LD_{50}/ Ingested)
C	672.4 ± 33.0	h	-	-	-
F0.1	771.2 ± 59.7	cdefg	0.024	63	F0.1: 1/1.6E+06
H0.1	831.4 ± 47.6	ab	0.013	68	H0.1: 1/1.5E+06
I0.1	759.7 ± 46.3	defg	0.017	62	I0.1: 1/6.E+01
HF0.1	851 ± 190.8	abcde	0.065	63	H0.1: 1/1.5E+06 F0.1: 1/1.5E+06
IF0.1	792.6 ± 58.4	abcde	0.017	64	I0.1: 1/5.8E+01 F0.1: 1/1.6E+06
IH0.1	862.7 ± 61.4	a	0.013	70	I0.1: 1/5.3E+01 H0.1: 1/1.4E+06
IHF0.1	781.9 ± 41.4	bcdef	0.013	63	I0.1: 1/5.8E+01 F0.1: 1/1.6E+06 H0.1: 1/1.6E+06
F1	717.3 ± 71.5	fgh	0.212	582	F1: 1/1.7E+05
H1	814.5 ± 36.0	abcd	0.013	661	H1: 1/1.5E+05
I1	719.3 ± 47.8	fgh	0.105	584	I1: 1/6.3E+00
HF1	804.2 ± 51.5	abcde	0.013	653	H1: 1/1.5E+05 F1: 1/1.5E+05
IF1	840.3 ± 87.7	abc	0.017	682	I1: 1/5.4E+00 F1: 1/1.5E+05
IH1	804.2 ± 33.6	abcde	0.013	653	I1: 1/5.7E+00 H1: 1/1.5E+05
IHF1	758.4 ± 67.5	cdefg	0.083	615	I1: 1/6.0E+00 F1: 1/1.6E+05 H1: 1/1.6E+05
F10	733.1 ± 31.9	fgh	0.050	5949	F10: 1/1.7E+04
H10	821.6 ± 61.6	abcd	0.013	6668	H10: 1/1.5E+04
I10	749.3 ± 62.6	efgh	0.105	6081	I10: 1/6.1E-01
HF10	698.3 ± 174.4	efgh	0.083	5667	H10: 1/1.8E+04 F10: 1/1.8E+04
IF10	834.3 ± 61.2	abc	0.013	6770	I10: 1/5.5E-01 F10: 1/1.5E+04
IH10	794.1 ± 27.7	abcde	0.013	6445	I10: 1/5.8E-01 H10: 1/1.6E+04
IHF10	702.5 ± 43.6	gh	0.514	5701	I10: 1/6.5E-01 F10: 1/1.8E+04 H10: 1/1.8E+04

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Table S3. Effects of pesticide combinations on physiological markers after 10 days of exposure

For 20 days, winter honey bees were exposed orally s to control sucrose solution (C), imidacloprid (I), glyphosate (H), difenoconazole (F), imidacloprid + glyphosate (IH), imidacloprid + difenoconazole (IF), glyphosate + difenoconazole (HF), or imidacloprid + glyphosate + difenoconazole (IHF) at concentrations in food of 0.1 µg/L, 1 µg/L and 10 µg/L. CaE-3 and GST were measured in the head (h), midgut (m) and abdomen (ab), and AChE, ALP and G6PDH were chosen as specific markers for the head, midgut and abdomen, respectively. On the 10th day, 7 samples of 3 tissues were collected for each treatment. For each treatment, the data represent the mean values of enzymatic activities expressed in milli-units of absorbance per minute and per mg of tissue (mAU.min^{-1}.mg of tissue^{-1}) ± standard deviations (SD). ANOVA or Kruskal-Wallis tests were applied to detect significant differences between treatments. Numbers after the abbreviations of each treatment refer to the concentrations of the pesticide in the sucrose solution. Treatments with different letters are significantly different ($p < 0.05$). Arrows indicate an increase or a decrease in activity relative to the control group.

Physiological marker (mAU.min^{-1}.mg of tissue^{-1}) ± SD at day 10	C	I0.1	H0.1	F0.1	IH0.1	IF0.1	HF0.1	IHF0.1	I1	H1	F1	IH1	IF1	HF1	IHF1
AChE$_{(h)}$	127.708 ± 18.473 cdef	122.950 ± 19.665 cdef	138.874 ± 12.426 bcde	113.097 ± 16.843 f	120.073 ± 24.521 def	137.874 ± 27.670 cdef	151.292 ± 23.897 abc	149.717 ± 23.406 abc	130.167 ± 21.009 cdef	107.484 ± 31.385 ef	136.59 ± 18.950 bdef	136.639 ± 4.215 bcdef	147.100 ± 25.060 abcd	162.862 ± 20.634 ab ↑	171.234 ± 7.724 a ↑
CaE-3$_{(h)}$	95.333 ± 7.679 ac	71.067 ± 4.437 ac	85.467 ± 4.700 ac	74.111 ± 4.225 ac	75.005 ± 4.419 c	86.305 ± 13.344 ac	97.702 ± 24.206 ab	90.981 ± 17.317 a	81.017 ± 7.687 ac	67.345 ± 17.143 ac	82.787 ± 11.619 a	80.634 ± 7.435 ab	93.138 ± 16.165 ac	104.473 ± 14.401 bc	103.421 ± 9.585 ac
GST$_{(h)}$	115.312 ± 7.513 cd	105.014 ± 10.578 ad	106.463 ± 5.316 bcd	105.873 ± 8.170 bcd	98.176 ± 8.663 ac	108.904 ± 13.124 bcd	102.537 ± 11.720 ad	94.716 ± 16.569 ab ↓	113.232 ± 7.545 bcd	86.046 ± 10.726 a ↓	114.372 ± 9.732 cd	113.809 ± 2.255 bcd	118.080 ± 8.972 d	110.187 ± 6.516 bcd	107.224 ± 14.226 ab ↓
ALP$_{(m)}$	10.856 ± 2.751 a	13.283 ± 2.915 a	13.179 ± 2.388 a	13.491 ± 1.857 a	21.617 ± 8.034 b ↑	12.950 ± 3.767 a	9.353 ± 2.755 a	10.499 ± 3.374 a	12.997 ± 2.375 a	10.818 ± 3.934 a	11.371 ± 1.238 a	11.491 ± 2.698 a	11.957 ± 4.001 a	16.565 ± 3.923 ab	11.911 ± 3.545 a
CaE-3$_{(m)}$	307.714 ± 43.726 ac	341.905 ± 36.122 ac	341.619 ± 51.368 ac	303.238 ± 61.711 ac	427.049 ± 138.173 c	318.456 ± 51.455 ac	286.768 ± 80.961 ab	222.830 ± 67.147 a	323.264 ± 58.611 ac	318.944 ± 67.010 ac	218.476 ± 42.885 a	274.223 ± 27.457 ab	318.569 ± 43.854 ac	354.740 ± 76.663 bc	296.618 8 ± 70.257 ac
GST$_{(m)}$	141.294 ± 24.620 a	138.881 ± 19.132 abcd	155.197 ± 16.488 abcd	145.903 ± 19.631 abcd	135.765 ± 23.814 abc	128.435 ± 7.334 ab	151.251 ± 40.423 abcd	125.136 ± 39.942 abc	138.004 ± 22.257 bcde ↓	143.855 ± 23.975 cde ↑	138.334 ± 14.601 de ↓	142.186 ± 13.251 abcd	139.288 ± 10.669 de ↓	152.640 ± 21.347 bcd ↑	155.200 ± 19.570 c ↑
G6PDH$_{(ab)}$	12.072 ± 0.529 a	9.106 ± 1.593 ab	8.283 ± 3.049 abc	7.050 ± 0.568 abc	7.233 ± 2.504 abc	9.024 ± 2.622 ab	7.954 ± 4.141 abc	5.765 ± 2.214 bc ↓	6.758 ± 2.783 bc ↓	5.300 ± 1.219 bc ↓	4.921 ± 2.356 c ↓	5.533 ± 1.195 bc ↓	4.640 ± 1.923 c ↓	6.581 ± 2.483 bc ↓	5.290 ± 3.266 bc ↓
CaE-3$_{(ab)}$	206.000 ± 27.222 ac	180.571 ± 31.771 ac	172.810 ± 48.460 ac	143.333 ± 23.981 ac	147.961 ± 26.810 c	181.269 ± 39.001 ac	170.936 ± 30.678 ab	176.762 ± 37.338 a	147.440 ± 24.459 ac	172.170 ± 25.952 ac	149.270 ± 28.761 a	119.388 ± 24.863 ab	117.576 ± 21.283 ac	145.190 ± 33.641 bc	125.931 ± 44.834 ac
GST$_{(ab)}$	116.079 ± 33.292 a	67.119 ± 29.160 abcd	66.089 ± 35.374 bcd ↓	63.154 ± 26.207 abcd	86.397 ± 20.000 abcd	103.447 ± 41.027 ab	80.018 ± 44.139 abcd	85.831 ± 42.144 abc	57.039 ± 22.978 bcde ↓	50.994 ± 8.476 cde ↓	41.223 ± 8.832 de ↓	68.860 ± 11.418 abcd	41.085 ± 12.831 de ↓	59.138 ± 14.473 bcd ↓	21.013 ± 3.594 e ↓

Table S4. Effects of pesticide combinations on physiological markers after 20 days of exposure

For 20 days, winter honey bees were exposed orally to control sucrose solution (C), imidacloprid (I), glyphosate (H), difenoconazole (F), imidacloprid + glyphosate (IH), imidacloprid + difenoconazole (IF), glyphosate + difenoconazole (HF), or imidacloprid + glyphosate + difenoconazole (IHF) at concentrations in food of 0.1 µg/L, 1 µg/L and 10 µg/L. CaE-3 and GST were measured in the head (h), midgut (m) and abdomen (ab), and AChE, ALP and G6PDH were chosen as specific markers for the head, midgut and abdomen, respectively. On the 20th day, 7 samples of 3 tissues were collected for each treatment. For each treatment, the data represent the mean values of enzymatic activities expressed in milli-units of absorbance per minute and per mg of tissue (mAU.min^{-1}.mg of tissue^{-1}) ± standard deviations (SD). ANOVA or Kruskal-Wallis tests were applied to detect significant differences between treatments. Numbers after the abbreviations of each treatment refer to the concentrations of the pesticide in the sucrose solution. Treatments with different letters are significantly different ($p < 0.05$). Arrows indicate an increase or a decrease in activity relative to the control group.

Physiological marker (mAU.min^{-1}.mg of tissue^{-1}) ± SD at day 20	C	I0.1	H0.1	F0.1	IH0.1	IF0.1	HF0.1	IHF0.1	I1	H1	F1	IH1	IF1	HF1	IHF1
AChE$_{(h)}$	127.537 ± 16.039 efgh	135.646 ± 21.575 defg	152.125 ± 11.888 bcd ↑	163.111 ± 56.839 bcde	142.502 ± 18.702 cdef	150.891 ± 7.739 bcd ↑	160.957 ± 9.997 ab ↑	179.507 ± 13.571 a ↑	123.760 ± 11.677 gh	122.723 ± 18.690 fgh	114.137 ± 11.351 h	138.625 ± 13.674 defg	151.579 ± 13.084 bcd ↑	158.549 ± 11.871 abc ↑	161.618 ± 12.820 abc ↑
CaE-3$_{(h)}$	80.666 ± 2.956 ab	73.722 ± 13.542 ab	69.238 ± 19.804 ab	87.238 ± 46.993 ab	70.351 ± 12.324 ab	77.798 ± 8.539 ab	82.763 ± 6.872 ab	94.614 ± 14.154 a	75.087 ± 9.980 ab	73.338 ± 10.166 ab	67.616 ± 12.399 b	72.640 ± 5.940 ab	81.571 ± 6.214 ab	83.866 ± 8.071 ab	79.226 ± 17.122 ab
GST$_{(h)}$	127.845 ± 6.593 abcd	135.803 ± 16.648 ab	135.373 ± 5.763 ^{a}	138.113 ± 45.383 abc	128.702 ± 11.366 abc	129.659 ± 5.591 abc	124.806 ± 4.464 bcd	113.284 ± 7.818 ^{d}	138.231 ± 3.472 ^{a}	134.959 ± 8.416 ab	135.062 ± 9.066 ab	135.660 ± 10.828 ab	130.397 ± 4.001 ab	127.980 ± 8.612 abc	111.889 ± 11.634 cd
ALP$_{(m)}$	10.992 ± 1.445	11.939 ± 4.538	13.617 ± 3.425	13.256 ± 3.649	14.273 ± 4.561	9.425 ± 3.115	7.894 ± 1.632	9.204 ± 2.713	11.028 ± 2.265	10.571 ± 4.286	10.044 ± 4.006	11.324 ± 3.057	8.388 ± 2.346	9.327 ± 1.376	10.470 ± 3.402
CaE-3$_{(m)}$	257.238 ± 62.026 ac	258.428 ± 36.272 ac	256.523 ± 40.551 ac	230.666 ± 44.9563 ^{ac}	226.306 ± 67.536 c	208.832 ± 36.331 ac	230.078 ± 31.975 ab	196.132 ± 9.838 ^{a}	272.718 ± 59.296 ac	273.806 ± 40.973 ac	242.512 ± 21.492 a	213.295 ± 48.694 ab	218.587 ± 21.795 ac	232.024 ± 17.181 bc	188.980 ± 25.088 ac
GST$_{(m)}$	147.854 ± 18.811 d	161.893 ± 13.724 ad	148.381 ± 21.688 ad	167.353 ± 18.675 ad	167.578 ± 22.297 bd	186.746 ± 22.036 cd	177.635 ± 24.994 ad	176.698 ± 16.011 bd	140.070 ± 18.228 abc ↓	130.258 ± 29.757 abc ↓	141.698 ± 27.035 ab ↓	147.652 ± 19.189 ad	137.104 ± 29.300 ab ↓	156.170 ± 16.689 ad	130.450 ± 17.557 a ↓
G6PDH$_{(ab)}$	10.523 ± 3.843 ab	9.003 ± 3.527 ab	6.929 ± 3.693 ab	10.925 ± 4.067 ab	4.961 ± 0.943 ^{a}	6.694 ± 3.170 ab	5.332 ± 1.597 ^{a}	9.661 ± 3.491 ^{ab}	6.980 ± 2.767 ab	9.930 ± 3.442 ab	10.337 ± 3.831 ab	12.706 ± 3.646 ab	11.476 ± 4.089 ab	11.633 ± 4.591 ab	12.829 ± 2.530 ^{b}
CaE-3$_{(ab)}$	231.619 ± 33.995 ac	172.666 ± 25.169 ac	168.555 ± 33.026 ac	170.222 ± 34.645 ac	155.727 ± 37.702 c	205.847 ± 38.146 ac	194.609 ± 36.622 ab	190.747 ± 35.692 a	203.628 ± 35.482 ac	217.500 ± 35.499 ac	221.306 ± 29.566 a	176.063 ± 23.425 ab	220.291 ± 66.062 ac	231.160 ± 19.872 bc	191.458 ± 21.622 ac
GST$_{(ab)}$	82.962 ± 28.692 abc	76.248 ± 41.716 bcd	70.458 ± 40.808 cd	102.570 ± 43.875 abc	39.853 ± 11.026 d ↓	71.716 ± 44.273 cd	40.825 ± 12.806 d ↓	92.416 ± 38.284 abc	85.019 ± 25.893 abc	92.674 ± 47.345 abc	91.227 ± 30.068 abc	117.722 ± 65.273 abc	64.019 ± 25.866 cd	127.148 ± 32.556 ab	168.153 ± 60.268 a

Table S5. Comparison of the effects of the pesticide concentration on the activity of the physiological markers.
H, F and the pesticide mixtures had different effects depending on the concentration. "0.1 < 1" corresponds to the markers having an activity lower at 0.1 µg/L than at 1 µg/L. "0.1 > 1" corresponds to the markers having an activity higher at 0.1 µg/L than at 1 µg/L.

	Physiological markers	
Type of exposure	0.1 < 1	0.1 > 1
H		AChE D20 GST head D10
F		AChE D20
IH	CaE-3 head D10 CaE-3 abdomen D20	
	GST abdomen D20	
		ALP midgut D10 CaE-3 midgut D10 CaE-3 midgut D20 CaE-3 abdomen D10
IF		GST midgut D20 GST abdomen D10 G6PDH abdomen D10
HF	GST abdomen D20	
IHF		GST midgut D20 GST abdomen D10

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Bilan

Cette étude a permis de mettre en évidence les effets létaux et sublétaux de l'imidaclopride, du difénoconazole et du glyphosate sur les abeilles d'hiver. Ces pesticides, seuls et en mélanges, présentent une toxicité élevée, bien que la durée d'exposition de 20 jours reste relativement courte par comparaison avec la durée d'exposition des abeilles d'hiver aux pesticides contaminants les matrices de la ruche. Ainsi, la multi-exposition des abeilles aux pesticides stockés dans les réserves de nourriture, pourrait représenter un des principaux facteurs contribuant aux pertes des colonies à la sortie de l'hiver, du fait que la colonie perd les ouvrières responsables de son démarrage au début du printemps.

D'une manière générale, la co-exposition des abeilles aux pesticides en mélanges binaires et ternaires, a conduit à une augmentation de la toxicité des pesticides avec l'apparition d'effets additifs et synergiques. Le mélange ternaire, aux trois concentrations étudiées, a causé moins de mortalité que quelques mélanges binaires. De plus l'augmentation de la concentration des pesticides n'a pas conduit à une augmentation de la toxicité. Ainsi, les résultats de cette étude montrent qu'il est difficile d'estimer la toxicité des interactions entre les pesticides du fait que l'augmentation du nombre de substances constituant le mélange n'entraine pas systématiquement une toxicité plus élevée. De plus, les effets les plus élevés ne sont pas systématiquement observés aux concentrations les plus élevées.

L'analyse des modulations des activités d'enzymes intervenant dans la détoxication, la lutte contre le stress oxydant, l'immunité et le système nerveux, révèle un impact élevé des pesticides sur la physiologie des abeilles. L'effet des différents pesticides sur l'état physiologique des abeilles est systémique, avec une réponse massive pas seulement limitée à l'intestin qui est le premier site d'interaction avec les pesticides ingérés. Cette réponse systémique pourrait expliquer les mortalités élevées qui ont eu lieu durant les 20 jours d'exposition aux pesticides. De plus, elle reflète de fortes perturbations physiologiques chez les abeilles exposées aux différents pesticides. De ce fait, il semble important de savoir si cet effet systémique est dû à des mécanismes communs aux trois pesticides ou à des mécanismes spécifiques à chaque pesticide.

Partie 2 : Effets conjoints de l'imidaclopride, du difénoconazole et du glyphosate sur l'équilibre oxydatif des abeilles d'hiver

Avant-propos

La première étude qui a été conduite, nous a permis de mettre en évidence la toxicité élevée de l'imidaclopride, du difénoconazole et du glyphosate, seuls et en mélanges, sur les abeilles d'hiver. Cette étude a permis aussi de mettre en évidence un effet systémique chez les abeilles exposées aux pesticides. Ainsi, il apparait crucial de chercher si l'effet systémique observé peut être lié à des mécanismes d'actions spécifiques de chaque pesticide, ou s'il est plutôt lié à des mécanismes généraux tels que les altérations du métabolisme, du système immunitaire, du système de détoxication et/ou de l'équilibre oxydatif. Ainsi, nous nous sommes intéressés à évaluer les effets de l'exposition chronique aux pesticides d'intérêt, seuls et en mélanges, sur la survie et l'équilibre oxydatif des abeilles d'hiver. Les abeilles ont été exposées chroniquement aux pesticides et à leurs mélanges aux concentrations environnementales de 0,1, 1 et 10 µg/L. De plus, la concentration de 0,01 µg/L a été ajoutée pour avoir une idée plus précise sur les effets des résidus de pesticides chez les abeilles d'hiver.

Article 2

Toxicity of the pesticides imidacloprid, difenoconazole and glyphosate alone and in binary and ternary mixtures to winter honey bees: effects on survival and antioxidative defenses

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Résumé

Les populations d'abeilles subissent un constant déclin et des pertes importantes de colonies à la fin de l'hiver. Afin d'expliquer les causes des pertes hivernales, nous avons étudié les effets de l'insecticide (I) imidaclopride, de l'herbicide (H) glyphosate et du fongicide (F) difénoconazole, seuls ou en mélanges binaires et ternaires, sur les abeilles d'hiver. Ces abeilles ont été exposées à une nourriture contaminée par les pesticides à des concentrations de 0 (témoins), 0,01, 0,1, 1 et 10 µg/L. L'attention a été focalisée sur les effets des pesticides sur la survie, la consommation de nourriture et le stress oxydant. Les effets des expositions aux pesticides sur le stress oxydant ont été évalués en déterminant les activités des enzymes faisant partie du système antioxydant (superoxyde dismutase (SOD), catalase (CAT), glutathion-S-transférase (GST), glutathion réductase (GR), glutathion peroxydase (GPox) et glucose-*6*-phosphate déshydrogénase (G6PDH)) dans trois compartiments biologiques (tête, abdomen et intestin moyen). Les dommages oxydatifs ont été aussi évalués à travers la quantification de la peroxydation lipidique et de la carbonylation des protéines. En général, aucun effet significatif sur la consommation alimentaire n'a été observé ; les mélanges de pesticides étaient plus toxiques que les substances seules, et les plus fortes mortalités ont été induites aux concentrations intermédiaires de 0,1 et 1 µg/L. La toxicité et le mode d'interaction n'étaient pas toujours liés au niveau d'exposition et au nombre de substances dans les mélanges. Les mélanges n'ont pas systématiquement induit d'effets synergiques, car des phénomènes d'antagonisme, de sub-additivité et d'additivité ont également été observés. Les pesticides testés, seuls ou en mélanges, ont déclenché un stress oxydatif important et systémique. Ce stress oxydatif pourrait expliquer en grande partie la toxicité des pesticides pour les abeilles domestiques.

Mots-clés : Abeilles domestiques, mélanges de pesticides, altérations physiologiques, stress oxydant

Abstract

Honey bee populations undergo constant decline and important losses after the winter season. To explain losses that could occur after the winter season, we studied the effects of the insecticide (I) imidacloprid, the herbicide (H) glyphosate and the fungicide (F) difenoconazole, alone and in binary and ternary mixtures, on winter honey bees that were orally exposed to food containing these pesticides at concentrations of 0 (control), 0.01, 0.1, 1 and 10 µg/L. Attention was focused on bee survival, food consumption and oxidative stress. The effects of exposures to pesticides on oxidative stress were assessed by determining the activity of enzymes involved in antioxidant defenses (superoxide dismutase (SOD), catalase (CAT), glutathione-*S*-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPox) and glucose-*6*-phosphate dehydrogenase (G6PDH)) in three compartments of the body (head, abdomen and midgut); oxidative damage reflected by both lipid peroxidation and protein carbonylation was also evaluated. In general, no significant effect on food consumption was observed; pesticide mixtures were more toxic than individual substances, and the highest mortalities were induced at the intermediate doses of 0.1 and 1 µg/L. The toxicity and mode of interactions were not always linked to the exposure level and the number of substances in the mixtures. Mixtures did not systematically induce synergistic effects, as antagonism, subadditivity and additivity were also observed. The tested pesticides, alone and in mixtures, triggered important, systemic oxidative stress that could largely explain pesticide toxicity to honey bees.

Keywords: honey bees, pesticide mixtures, physiological alterations, oxidative stress

1. Introduction

The honey bee *Apis mellifera* is a pollinator insect of agro-environmental and economic importance (Klein et al., 2007). In Europe, 84% of crop production depends on the foraging activity of bees (Williams, 1994). In 2009, the worldwide economic value of insect pollination for agriculture was estimated at €153 billion per year, which represented 9.5% of the value of the world agricultural production used for human food in 2005 (Gallai et al., 2009). However, despite the development of beekeeping, a constant decline in honey bee populations worldwide has been observed since the beginning of the 20th century (Klein et al., 2007; Neumann and Carreck, 2010; vanEngelsdorp and Meixner, 2010). This phenomenon is multicausal, and several factors that contribute to this decline have been identified (Moritz et al., 2010). During foraging, in a radius up to 12 km around the hive (Beekman and Ratnieks, 2000), honey bees are in contact with a large variety of environmental stressors, including pesticides and pathogens, and there seems to be a consensus that pesticides and pathogens represent the main contributors of colony decline (vanEngelsdorp and Meixner, 2010).

A large number of pesticide residues can be found in apicultural matrices such as honey, pollen and beeswax (Chauzat et al., 2009; Chauzat and Faucon, 2007; Chauzat et al., 2006; Chauzat et al., 2011; Kubik et al., 2000; Lambert et al., 2013; Mullin et al., 2010; Rubio et al., 2015). Pesticides have been shown to have strong impacts on all ecosystems, and many pesticides, namely, insecticides, have been shown to affect bees (Belzunces et al., 2012; Desneux et al., 2007). Pesticides may elicit both lethal and sublethal effects after acute or chronic exposure of bees either directly, during or after a plant protection treatment, or indirectly by the consumption of food (nectar and pollen) contaminated with pesticide residues (Belzunces et al., 2012; Desneux et al., 2007).

Pesticides can act not only alone but also in combination to induce synergistic effects (ChalvetMonfray et al., 1996; Colin and Belzunces, 1992; Gill et al., 2012; Meled et al., 1998; Papaefthimiou and Theophilidis, 2001; Pilling and Jepson, 1993). However, research on the synergistic action of pesticide mixture toxicity in honey bees is relatively scarce. The majority of studies are focused on possible synergistic effects on mortality in summer bees of pyrethroid insecticides and azole fungicides (ChalvetMonfray et al., 1996; Colin and Belzunces, 1992; Meled et al., 1998; Papaefthimiou and Theophilidis, 2001; Pilling and Jepson, 1993), neonicotinoid and pyrethroid insecticides (Gill et al., 2012), and neonicotinoid insecticides and azole fungicides (Iwasa et al., 2004; Schmuck et al., 2003; Spurgeon et al., 2016). However, substances belonging to the three main classes of pesticides, *i.e.,* herbicides, fungicides and insecticides, have been detected in bee hives (Lopez et al., 2016; Piechowicz et al., 2018a; Skerl et al., 2009). Thus, it is crucial to investigate the combined effects of mixtures of pesticides from different classes at environmentally relevant concentrations.

The present study investigated the effects of three pesticides (the insecticide imidacloprid, the fungicide difenoconazole and the herbicide glyphosate) alone and in binary and ternary mixtures. Imidacloprid is a neonicotinoid insecticide that acts as an agonist of nicotinic acetylcholine

receptors and induces hyperactivation of cholinergic neurons (Tomizawa and Casida, 2005). Difenoconazole is a triazole fungicide belonging to the ergosterol biosynthesis inhibitor fungicides (EBI fungicides). These fungicides alter the structure of the fungal cell membrane by inhibiting ergosterol synthesis (Guo et al., 2010). Glyphosate is an organophosphorus herbicide that blocks the synthesis of plant aromatic amino acids by inhibiting *5*-enolpyruvylshikimate-*3*-phosphate synthase (EPSPS), an enzyme involved in the shikimate pathway (Duke and Powles, 2008). This study was focused on winter bees because they maintain the colony during winter and are therefore important for the start of colony development during the spring. Moreover, their particular longevity makes them exposed during a long period of time. Here, we considered the effects of pesticides on bee longevity, food intake and physiology by exploring both oxidative damages and changes in antioxidant defenses.

2. Materials and Methods

2.1. Materials

Antipain, aprotinin, leupeptin, pepstatin A, soybean trypsin inhibitor, monobasic and dibasic sodium and potassium phosphates (NaH_2PO_4, KH_2PO_4, K_2HPO_4 and Na_2HPO_4), sodium chloride (NaCl), potassium chloride (KCl), Triton X-100, reduced L-glutathione (GSH), glucose-*6*-phosphate (G6P), NADPH, $NADP^+$, ethylenediaminetetraacetic acid (EDTA), xanthine, xanthine oxidase, nitroblue tetrazolium (NBT), hydrogen peroxide (H_2O_2), tert-butyl hydroperoxide (TBHP), glutathione reductase, 1-chloro-2,4-dinitrobenzene (CDNB), trishydroxymethyl-aminomethane base (Tris), sodium bicarbonate ($NaHCO_3$), hydrochloric acid (HCl), iron(III) chloride ($FeCl_3$), *4*-(*2*-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), *2*,*4*-dinitrophenylhydrazine (DNPH), nonidet P-40 (NP-40), magnesium chloride ($MgCl_2$), sodium dodecyl sulfate (SDS) and bovine serum albumin (BSA) were obtained from Sigma Aldrich® (Saint Quentin Fallavier, France). Imidacloprid (CAS No 138261-41-3), difenoconazole (CAS No 119446-68-3) and glyphosate (CAS No. 1071-83-6) (98% purity) were purchased from Cluzeau Info-Labo (Sainte-Foy-la-Grande, France). Anti-DNP antibody (clone 9H8.1) was obtained from Millipore™ (Guyancourt, France), and goat anti-mouse IgG HRP conjugate was obtained from Promega (Charbonnières, France). The Clarity™ Western ECL substrate was purchased from Bio-Rad (Roanne, France).

2.2. Honey bees

In January, *A. mellifera* honey bees were collected from two colonies, carefully monitored for their health status, located at the experimental apiary of the *Abeilles & Environnement* (Bees & Environment) Research Unit of Avignon INRAE Research Centre (southern France). Bees were slightly anesthetized with carbon dioxide, mixed and randomly distributed into laboratory cages (Pain type, 6 × 8.5 × 10 cm) in groups of 30 individuals per cage. The cages were then placed, in the darkness, in an incubator at controlled conditions (30°C ± 2°C; 60% ± 10% relative humidity)

until the end of the experiment. During the first 24 h of the experiment, the bees were provided with water and candy (Apifonda®) *ad libitum* and the dead honey bees were removed and replaced. To optimize hygiene conditions, a sheet of filter paper was placed on the bottom of the cages and replaced daily.

2.3. Exposure to pesticides

Honey bees were exposed for 24 h per day, during 16 days, by feeding a 60% (w/v) sucrose syrup containing 1% (v/v) of the Provita' Bee® (ATZ Dietetics, Mas-Cabardès, France) protein preparation and the insecticide imidacloprid, the fungicide difenoconazole and the herbicide glyphosate alone or in mixtures. For each pesticide, five concentrations were tested: 0 (control), 0.01, 0.1, 1 and 10 µg/L (equivalent to 0.0083 0.083, 0.813 and 8.130 µg/kg, respectively; calculated with a sucrose solution density of 1.23 ± 0.02 g/L (n=10)). Concentrations were consistent with the residual contamination found in honey, pollen and wax (Abdallah et al., 2017; Bridi et al., 2018; Chauzat et al., 2011; Johnson et al., 2010; Juan-Borras et al., 2016; Karise et al., 2017; Piechowicz et al., 2018c). Six experimental groups of exposure were investigated: the control group (C); the insecticide alone (I); the fungicide alone (F); the herbicide alone (H); the binary mixtures of insecticide + fungicide (IF), insecticide + herbicide (IH) and herbicide + fungicide (HF); and the ternary mixture of insecticide + herbicide + fungicide (IHF). For each group and at each exposure concentration, 14 replicates of 30 honey bees were exposed. Syrup consumption and mortality were recorded daily until the end of the experiment. For pesticide mixtures, all pesticides were used at the same concentration in the mixtures. The pesticide solutions were prepared in water and DMSO and stored at -20°C until use. The sucrose feed solutions were prepared daily and contained 60% (w/v) sucrose, pesticides (or no pesticides in the control) and 0.1% (v/v) DMSO. The pesticide concentrations were confirmed by GC-MS/MS following two analytical methods (Paradis et al., 2014; Wiest et al., 2011). For each concentration, the relative standard deviations (RSD) compared to the nominal concentrations were less than 10%.

2.4. Analysis of physiological life history traits

Variations in physiological life history traits were analyzed after 16 days of exposure to pesticides. The period of 16 days was chosen because some treatments drastically compromised bee survival, and it was necessary to have a sufficient number of living bees to conduct physiological analyses. Physiological traits were analyzed in surviving bees exposed to pesticides at concentrations of 0.1 and 1 µg/L. To avoid animal suffering, tissue samples were collected after anesthesia and decapitation of bees. For each bee, the head, midgut and abdomen (without the intestine) were sampled, weighed and stored at -80°C until analysis. The tissues were ground in extraction medium with Qiagen® TissueLyser II (30 Hz; 3 periods of 30 sec at 30 sec intervals) to make a 10% (w/v) extract. The extraction medium consisted of 10 mM NaCl, 1% (w/v) Triton

X-100 and 40 mM sodium phosphate at pH 7.4 and contained protease inhibitors (2 µg/mL antipain, leupeptin and pepstatin A; 25 units/mL aprotinin; and 0.1 mg/mL soybean trypsin inhibitor) (Belzunces et al., 1990). Tissue extracts were centrifuged at 4°C for 20 min at 15000 $g_{av.}$, and the supernatants were collected for analysis (Badiou-Beneteau et al., 2012). For each treatment, seven repetitions were performed and assayed in triplicate, and each sample corresponded to tissues pooled from three bees.

Physiological traits were spectrophotometrically assayed at 25°C on different organs of the same bees. The activities of glutathione-*S*-transferase (GST), catalase (CAT) and superoxide dismutase (SOD) were measured in both the head and midgut. The activities of glutathione reductase (GR) and glutathione peroxidase (GPox) were measured in the head, and the activity of glucose-*6*-phosphate dehydrogenase (G6PDH) was measured in the abdomen and midgut. Abdomen and midgut G6PDH activities were determined by continuously following the formation of NADPH at 340 nm. The reaction medium contained 10 mM $MgCl_2$, 1 mM glucose-*6*-phosphate, 0.5 mM $NADP^+$ and 100 mM Tris-HCl pH 7.4. Head and midgut GST activities were determined by measuring the conjugation of GSH to CDNB at 340 nm. The reaction medium contained 1 mM EDTA, 2.5 mM GSH, 1 mM CDNB and 100 mM Na/K phosphate at pH 7.4 (Habig et al., 1974). SOD activity was determined at 560 nm in a reaction medium containing 0.1 mM EDTA, 0.1 mM xanthine, 0.025 mM nitroblue tetrazolium (NBT), 8.33 mU/mL xanthine oxidase and 50 mM sodium phosphate/carbonate at pH 7.8. Head GPox activity was assayed using *tert*-butyl hydroperoxide (TBHP) as the substrate. The generated oxidized glutathione (GSSG) was reduced in the presence of NADPH by GR to generate GSH and $NADP^+$. The conversion of NADPH into $NADP^+$ was followed at 340 nm. The reaction medium contained 1 mM EDTA, 0.2 mM TBHP, 0.85 mM GSSG, 0.16 mM NADPH, 0.25 U/mL GR and 50 mM Na/K phosphate at pH 7.4. Head GR activity was determined at 340 nm by the conversion of NADPH to $NADP^+$. The reaction medium contained 1 mM EDTA, 0.85 mM GSSG, 0.16 mM NADPH and 50 mM Na/K phosphate at pH 7.4 (Dussaubat et al., 2012b). The decomposition of H_2O_2 by CAT was measured at 240 nm. The reaction medium contained 10 mM H_2O_2 and 100 mM sodium phosphate at pH 7.0 (Beers and Sizer, 1952). All enzymatic reactions were followed on TECAN F500 spectrophotometer.

2.5. Lipid peroxidation

Lipid peroxidation was assessed by the measurement of thiobarbituric acid-reactive substances (TBARS). The malondialdehyde (MDA) content determined with thiobarbituric acid was considered representative of overall lipid peroxidation (Armstrong and Browne, 1994). MDA was fluorometrically assayed with the TCA method (TBARS (TCA method) assay kit no 700870, Cayman Chemical, Michigan, USA) at λ_{exc} = 530 nm and λ_{em} = 550 nm. For each modality, nine extracts of three midguts per extract (n = 9) were assayed in triplicate.

2.6. Quantification of protein carbonylation

According to Paris et al. (2017), proteins were extracted on ice by crushing the midguts in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS, and 50 mM Tris-HCl at pH 8.0) supplemented with 1 mM PMSF to obtain a 10% (w/v) extract by means of a Eurostar digital IKA stirrer (Labortechnik). For each modality, 12 midguts (n=12) were assayed in triplicate. The extracts were incubated on ice for 15 min, vortexed every 5 min, and then centrifuged at 4°C for 15 min at 14000 $g_{av.}$. The supernatant was kept for analysis. The protein content was assayed with Bradford's method (Bradford, 1976) using the Coomassie Plus™ (Bradford) assay kit (Thermo Scientific, Rockford, USA). The measurements were performed in triplicate after a 1/40 dilution. To assess the carbonylation rate of proteins, carbonylated BSA was used as a standard according to Yoo and Regnier (2004): first, 10 mg of BSA was solubilized in 900 µL of solubilization buffer (250 mM ascorbic acid and 1 mM $FeCl_3$). Then, 100 µL of oxidation solution (100 mM KCl, 10 mM $MgCl_2$ and 50 mM HEPES at pH 7.4) was added. The reaction was stopped by adding EDTA (Requena et al., 2001). The quantification of protein carbonylation was performed according to Paris et al. (2017). Briefly, 18 µg of global proteins or the BSA standard was denatured in SDS, derivatized with DNPH and neutralized in Tris-base. The carbonylated proteins were slot-blotted on a polyvinylidene difluoride (PVDF) membrane with a slot blotter (PR 600 slot-blot, Hoefer). The membranes were incubated overnight at 4°C with diluted (1/2000) anti-DNP antibody (clone 9H8.1, Millipore™) and then for 1 hour at 25°C with the diluted (1/2500) secondary antibody coupled to horseradish peroxidase (goat anti-mouse IgG HRP conjugate, Promega). Detection was performed by chemiluminescence (Clarity™ Western ECL Substrate, Bio-Rad), and the signal was analyzed with a ChemiDOC™ MP system analyzer (Bio-Rad).

2.7. Statistical analysis

Statistics were performed using RStudio version 1.1.463 statistical software. Survival analyses were performed using the packages *survival* and *survminer*, and the Kaplan-Meier method was used followed by a post hoc test for comparison of survival between treatments. The Kruskal-Wallis test, followed by pairwise comparisons using the Wilcoxon rank test (with Benjamini-Hochberg correction), was used to compare the cumulative individual food consumption between treatments. The effects of treatments on enzymatic activities, lipid peroxidation and protein carbonylation were determined by ANOVA followed by Tukey's HSD test or by a Kruskal-Wallis test followed by post hoc Dunn's test (with Benjamini-Hochberg correction using the *agricolae* package). Principal component analyses (PCAs) were performed using the *FactoMineR* package to distinguish the different treatments according to their effects on physiological markers.

2.8. Mode of interaction between pesticides

The mode of interaction between pesticides (additive, antagonistic and synergistic) was evaluated by the interaction ratio (IR) (Table S1), which has been previously used to study synergies between pyrethroid insecticides and azole fungicides (Colin and Belzunces, 1992; Piggott et al., 2015):

$$\mathrm{IR} = \frac{(Mix - C)}{\sum_{n=0}^{2-3}(P_n - C)}$$

where *Mix* represents the crude mortality of the mixture, *C* is the mortality of the control, (*Mix* - *C*) is the mortality of the pesticide mixture (binary or ternary) corrected by the control mortality, and $\sum_{n=0}^{2-3}(P_n - C)$ represents the sum of the mortalities induced by each pesticide (*n*) in the mixture corrected by the control mortality, which corresponds to the theoretical expected mortality of the mixture. A value of IR = 1 reflects a pure additive effect. However, considering variations in the effects, an IR is considered = 1 when $0.95 \leq \mathrm{IR} \leq 1.05$. When IR > 1, the interaction is synergistic. For IR < 1, four cases were distinguished (Table S1): (i) When the mortality of the mixture was lower than the mortality of the lowest toxic substance alone, the interaction could be considered purely antagonistic. (ii) When the toxicity of the mixture was higher than the mortality of the most toxic substance but below the expected mortality, the interaction was considered subadditive. In this case, it was not possible to speak in terms of antagonism because the effect of the mixture was higher than the effect of each substance alone. (iii) When the effect of the mixture ranged between the effect of the least toxic substance and the effect of the most toxic substance, the interaction was also considered subadditive. In this case, it was also not possible to speak in terms of antagonism because compared to the most toxic substance, antagonism could be considered, but compared to the lowest toxic substance, synergy could also be considered. (iv) When the mixture induced a mortality similar to that of each pesticide, the effect of the mixture was considered independent (Table S1).

3. Results

3.1. Chronic toxicity of pesticides alone or in combination

Bees were exposed for 16 days to three pesticides at four different concentrations (0.01 µg/L, 0.1 µg/L, 1 µg/L and 10 µg/L), alone or in mixtures, and their survival rate was recorded every day (**Fig. 1**). In general, at all concentrations, the survival rate of the honey bees exposed to pesticides was significantly lower than that of the control, and the highest toxicities were observed at the intermediate concentrations of 0.1 and 1 µg/L. In addition, except for HF, the toxicity of the mixtures was higher than that of the individual pesticides. For each exposure condition, the highest toxicity, expressed as corrected mortality, was observed with IF (29.8%), IH (27.4%) and IHF (29.1%) at 0.01 µg/L; IHF (57.6%) at 0.1 µ/L; IH (46.2%) and IHF (40.5%) at 1 µg/L; and IF (20.9%), IH (32.1%) and IHF (21.9%) at 10 µg/L (Table S2).

The mode of interaction between pesticides was evaluated by the IR, which corresponds to the ratio between the effects induced by the mixture to the expected effects of the mixture, which is the sum of the effects induced by each component of the mixture alone (**Fig. 1** and Table S1). Antagonistic interactions between pesticides were observed in the binary mixtures containing the herbicide and fungicide (HF), with marked antagonism observed for HF0.01 and HF1, and slight antagonism observed for HF0.1 and HF10. Subadditive interactions were observed with the binary mixtures IF0.01 and IH0.1 and with the ternary mixture IHF0.01 and IHF10. Additive interactions were observed with the binary mixtures IH0.01 and IF0.1. Interestingly, synergistic interactions were observed for 6 out of the 16 mixtures, with the binary mixtures IF1, IH1, IF10 and IH10 and the ternary mixtures IHF 0.1 and IHF1 exhibiting slight synergy.

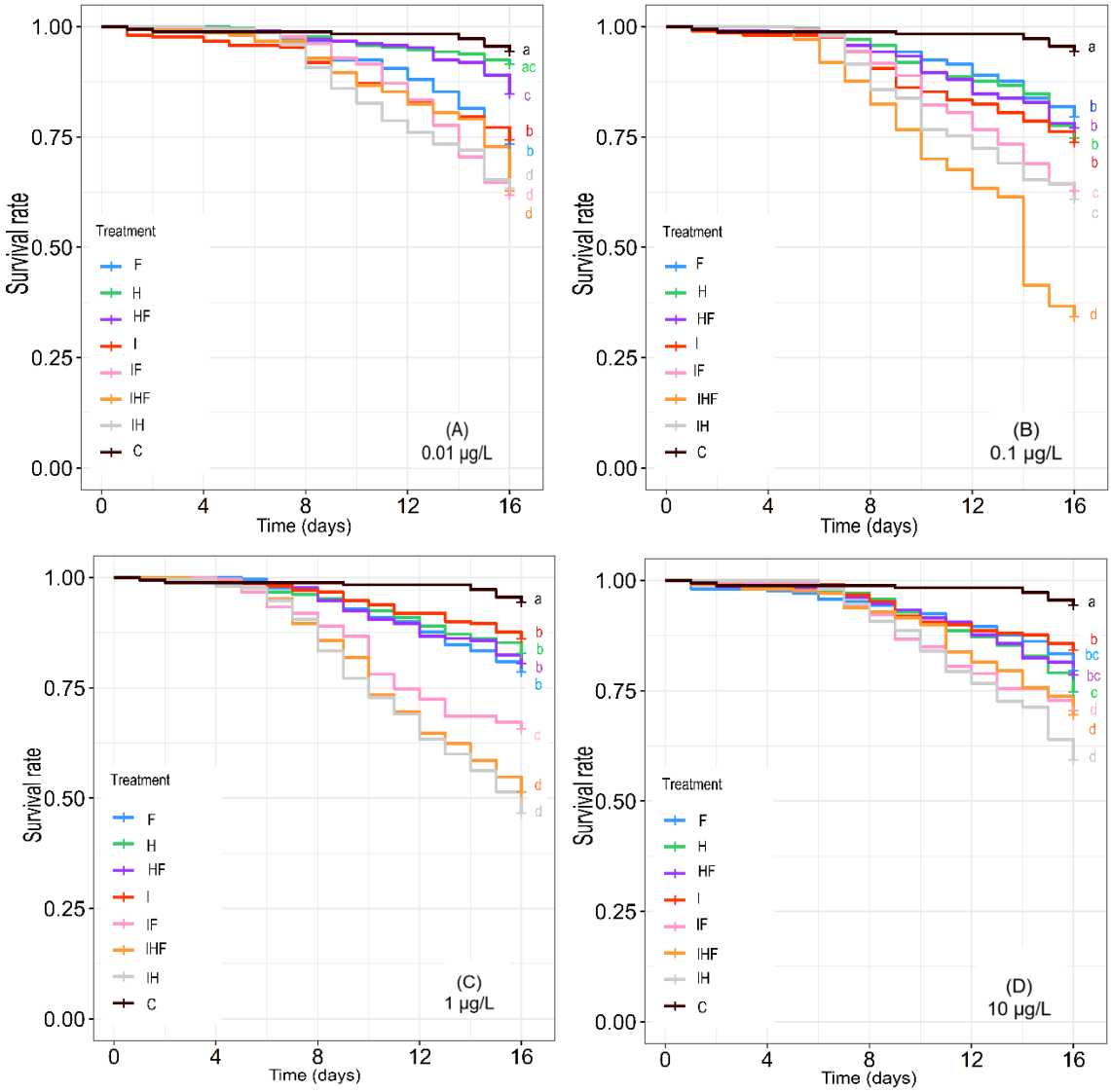

Fig. 1. Effects of pesticides alone or in mixtures on honey bee survival

Winter honey bees were orally exposed to food containing no pesticides (C), imidacloprid (I), difenoconazole (F), glyphosate (H), imidacloprid + difenoconazole (IF), imidacloprid + glyphosate (IH), glyphosate + difenoconazole (HF), or imidacloprid + glyphosate + difenoconazole (IHF) at concentrations of 0.01 µg/L (A), 0.1 µg/L (B), 1 µg/L (C) and 10 µg/L (D). The survival rate was followed until day 16 of exposure, at which bees were sampled for physiological analyses. The data represent the mean proportion of surviving honey bees. The mortalities from 14 replicates of 30 bees per treatment were analyzed using the Kaplan-Meier method followed by a post hoc test for comparison of survival between treatments. Data with different letters are significantly different ($p < 0.05$).

3.2. Effects of pesticides on feeding behavior

The influence of pesticide treatments on the feeding behavior of honey bees was followed by measuring the daily food consumption (**Fig. 2**). The individual cumulative food consumption was used to detect possible differences between treatments. As a general feature, honey bees exposed to pesticides consumed an equal amount of food compared to that consumed by unexposed bees, except for the bees of H0.01 that consumed relatively less food than controls (753.7 mg/bee and 852.3 mg/bee, respectively (Table S3)). When comparing the cumulative individual food consumption between different doses of the same treatment, honey bees exposed to glyphosate consumed significantly less food when exposed at 0.01 µg/L than when exposed at 10 µg/L (753.7 mg/bee and 917.3 mg/bee, respectively (Table S3)).

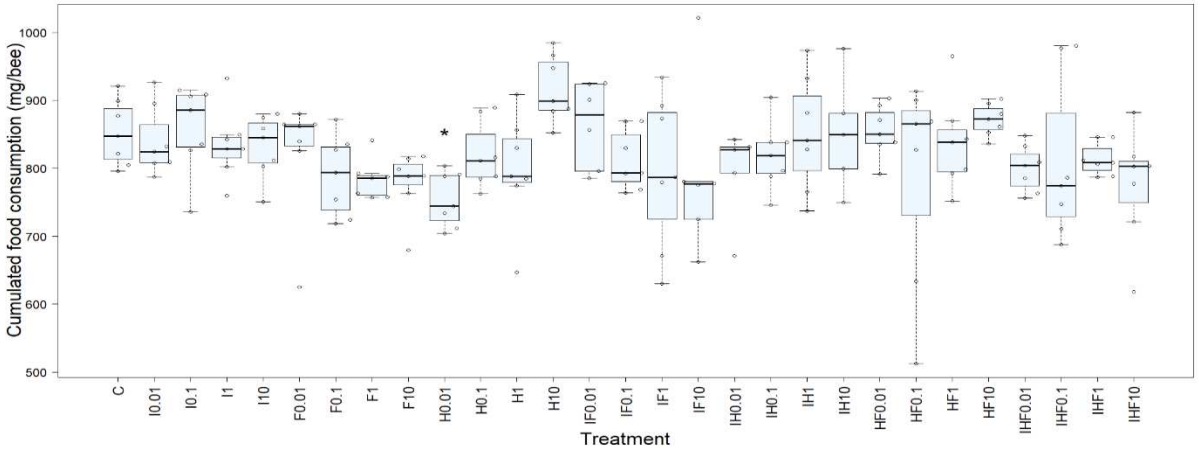

Fig. 2. Effect of exposure to pesticides on food consumption

Winter honey bees were orally exposed to food containing no pesticides (C), imidacloprid (I), difenoconazole (F), glyphosate (H), imidacloprid + difenoconazole (IF), imidacloprid + glyphosate (IH), glyphosate + difenoconazole (HF), or imidacloprid + glyphosate + difenoconazole (IHF) at concentrations of 0.01, 0.1, 1 and 10 µg/L. Food consumption was evaluated daily during the 16-day period. Box plots represent the cumulative individual consumption (mg/bee) at day 16 as determined from 14 cages of 30 bees per treatment. Statistical analyses were performed using the Kruskal-Wallis test followed by pairwise comparisons using the Wilcoxon rank sum test with Benjamini-Hochberg correction. Numbers after the abbreviations of each treatment refer to the concentrations of the pesticides in the food. Asterisks indicate significant differences from the control group (* $p \leq 0.05$).

3.3. Variations in physiological life history traits by pesticides

The variations in physiological life history traits were analyzed after 16 days of exposure to pesticides alone or in mixtures (**Figs. 3 and 4**). Changes at 0.1 and 1 µg/L were preferred for analysis because these groups exhibited the highest mortality rates, and the pesticide concentrations were environmentally relevant. Analyses were focused on oxidative stress by analyzing antioxidative defenses.

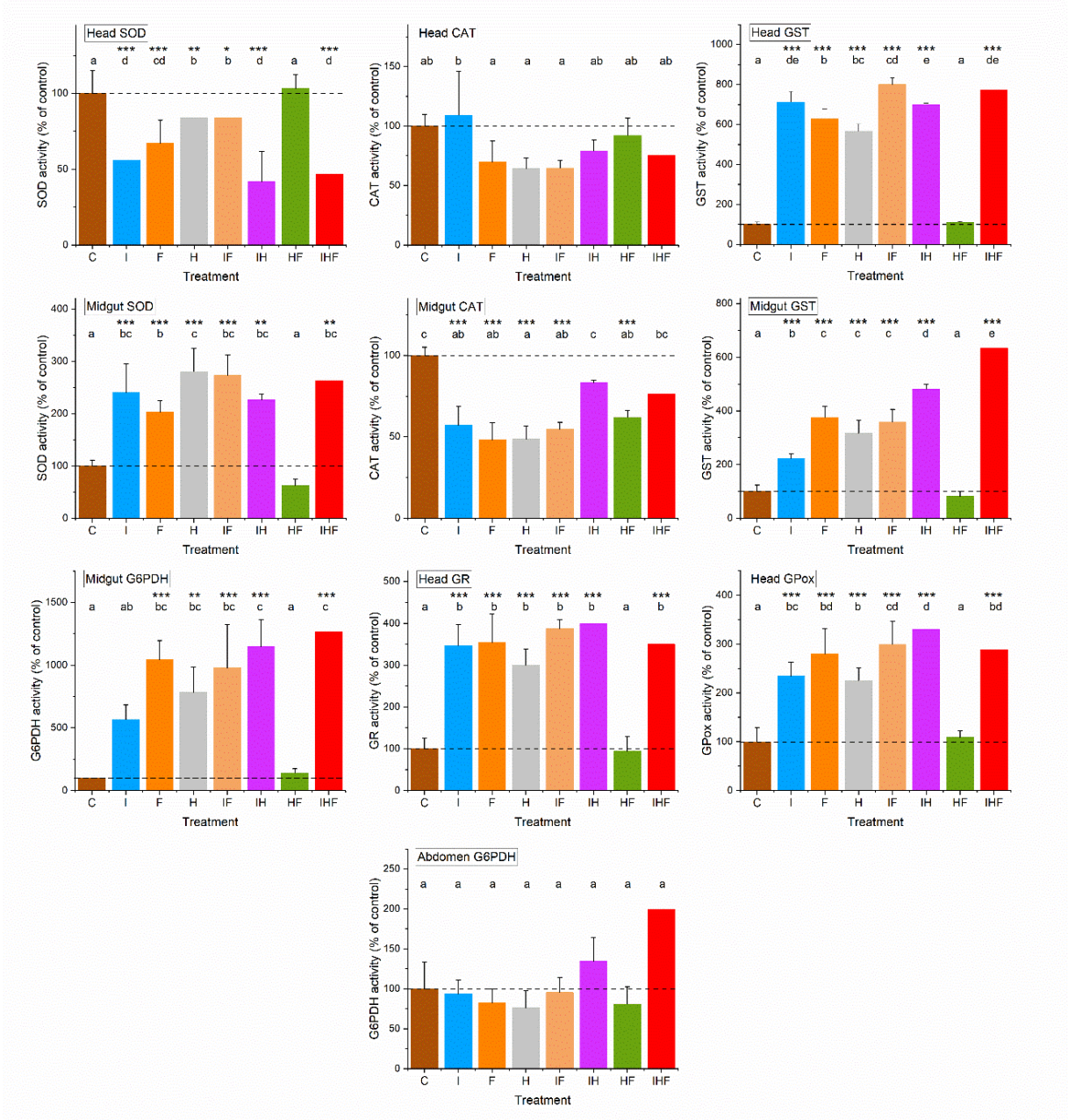

Fig. 3. Effects of exposure to pesticides at 1 µg/L on antioxidant defenses

Winter honey bees were orally exposed to food containing no pesticides (C), imidacloprid (I), difenoconazole (F), glyphosate (H), imidacloprid + difenoconazole (IF), imidacloprid + glyphosate (IH), glyphosate + difenoconazole (HF), or imidacloprid + glyphosate + difenoconazole (IHF) at a concentration of 1 µg/L. On day 16, enzymes involved in antioxidant defenses were assayed in the head, midgut and abdomen of bees. SOD, superoxide dismutase; CAT, catalase; GST, glutathione-*S*-transferase; G6PDH, glucose-*6*-phosphate dehydrogenase; GR, glutathione reductase; GPox, glutathione peroxidase. The data represent the means of tissue activities from 7 repetitions performed in triplicate and are expressed as percentages of the mean control value. Data with different letters are significantly different ($p < 0.05$). Asterisks indicate significant differences from the control group: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. The dotted lines indicate the control levels.

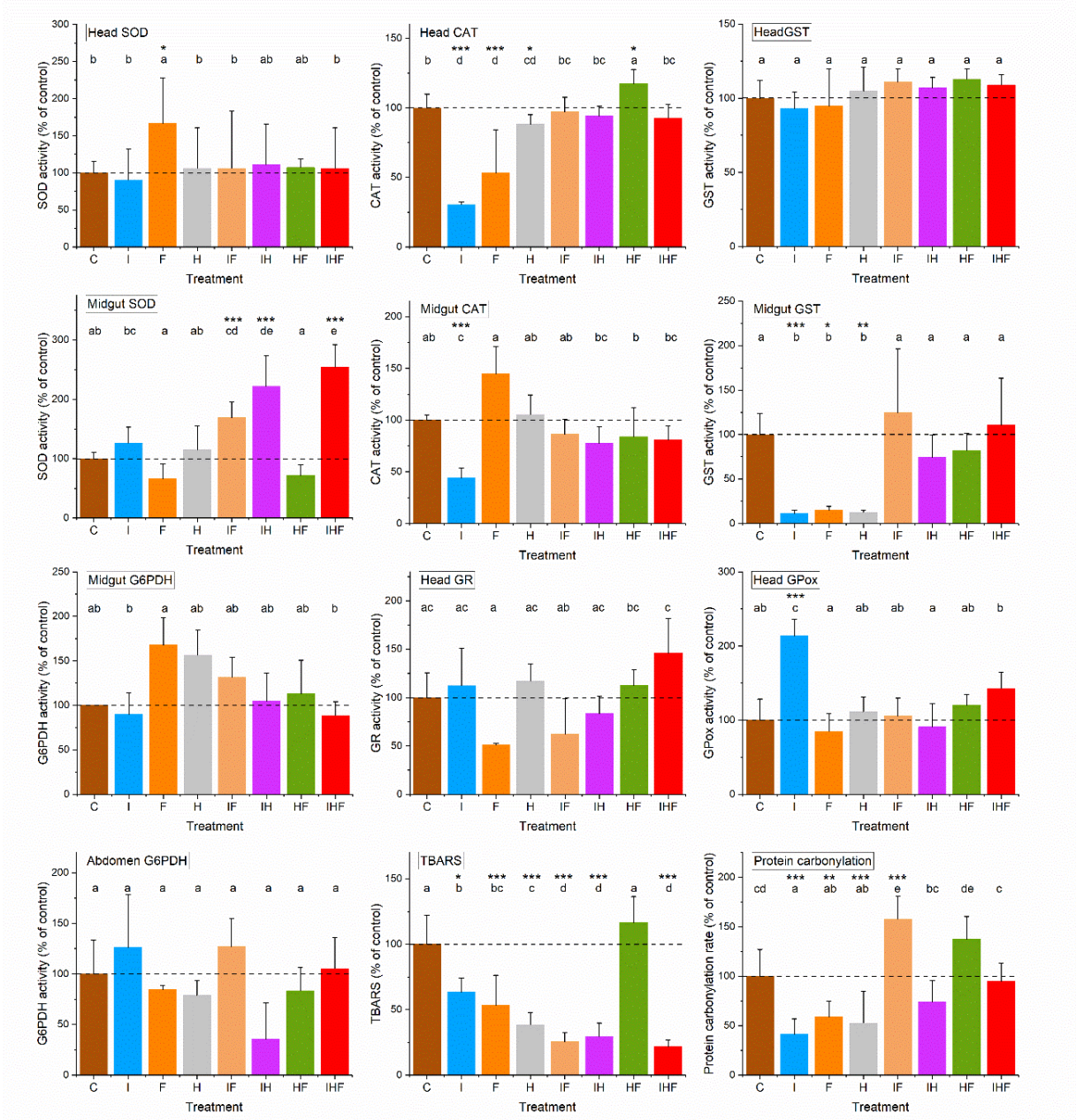

Fig. 4. Effects of exposure to pesticides at 0.1 µg/L on antioxidant defenses and oxidative damage

Winter honey bees were orally exposed to food containing no pesticides (C), imidacloprid (I), difenoconazole (F), glyphosate (H), imidacloprid + difenoconazole (IF), imidacloprid + glyphosate (IH), glyphosate + difenoconazole (HF), or imidacloprid + glyphosate + difenoconazole (IHF) at a concentration of 0.1 µg/L. On day 16, enzymes involved in antioxidant defenses were assayed in the head, midgut and abdomen of bees, and lipid peroxidation (TBARS) and protein carbonylation were assessed. SOD, superoxide dismutase; CAT, catalase; GST, glutathione-*S*-transferase; G6PDH, glucose-*6*-phosphate dehydrogenase; GR, glutathione reductase; GPox, glutathione peroxidase; TBARS, thiobarbituric acid-reactive substances. The data represent the means of tissue activities from 7 repetitions performed in triplicate and are expressed as percentages of the mean control value. Data with different letters are significantly different ($p < 0.05$). Asterisks indicate significant differences from the control group: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. The dotted lines indicate the levels of controls.

Generally, at 1 µg/L, there was a large change in enzyme activities involved in antioxidant defenses (**Fig. 3**) (Table S5). No change was observed in the activity of CAT in the head and G6PDH in the abdomen of honey bees. Decreased activity was observed for CAT in the gut in five out of seven exposure groups (excluding IH and IHF) and for SOD in the head of bees of all exposure groups except HF. For head and gut GST, GR, and GPox activities and gut SOD activity, an increase was observed in all exposure groups except HF. Decreased activity was also observed for gut G6PDH in almost all exposure groups except I and HF.

The bees exposed to pesticides at the concentration of 0.1 µg/L exhibited relatively complex changes in physiological life history traits (**Fig. 4**) (Table S4). Four categories of variations of enzyme activity were observed: (i) no change at all (head GST and GR activities and abdomen and gut G6PDH activities); (ii) increased activities (head SOD activity with F, head GPox activity with I and gut SOD activity with IF, IH and IHF); (iii) decreased activities (gut GST activity with I, F and H and gut CAT activity with I); and (iv) increased and decreased activity depending on exposure conditions (the CAT activity in the head decreased with I, F and H, and increased with HF). It appeared that the exposure cases for which the lowest number of antioxidant enzymes were affected corresponded to binary and ternary pesticide mixtures. However, in contrast with the concentration of 1 µg/L, for which a large change in antioxidant enzyme activities was observed, indications of oxidative stress were less obvious at the concentration of 0.1 µg/L, especially for the binary and ternary mixtures. Thus, at 0.1 µg/L, damage caused by oxidative stress was investigated by analyzing lipid peroxidation, reflected by TBARS, and protein oxidation, reflected by amino acid carbonylation. For TBARS, a decrease was observed with all exposure conditions, except with HF. For protein oxidation, a decrease was observed, except with IH, HF and IHF, which induced values similar to that of the control, and with IF, for which the carbonylation rate represented 158% of that of the control (28.2 ± 4.1% carbonylated proteins/mg of tissue for IF and 17.9 ± 4.8% carbonylated proteins/mg of tissue for the control (Table S6)).

PCA was conducted to distinguish the different treatments according to their effects on the 10 studied physiological markers (**Fig. 5A and 5C**). The correlation circles (**Fig. 5B and 5D**) indicate which enzymes had the largest influence on the determination of the physiological state of honey bees following exposure to each treatment. At 0.1 µg/L, the two axes accounted for 44.2% of the total dataset variation (**Fig. 5A and 5B**). Therefore, this PCA did not enable distinction of antioxidant enzyme activities. This complex representation was in accordance with our hypothesis of a relatively complex pattern of change in the physiological life history traits at an exposure level of 0.1 µg/L. At 1 µg/L (**Fig. 5C and 5D**), the two axes of the PCA accounted for 71.82% of the total dataset variation; therefore, this PCA sufficiently distinguished the activities of the enzymes. The enzymes were clearly separated into two groups; the first one was on the right of the correlation circle (**Fig. 5D**) and corresponded to the markers whose activities increased after exposure to pesticides (head GST, GR, and GPox activities and midgut SOD, GST and G6PDH). The second group was on the left of the correlation circle and corresponded to the enzymes exhibiting a decrease in activity after exposure (head SOD and midgut CAT). In the midgut, GST and G6PDH activities were positively correlated with each other. However,

these markers were independent of CAT activity in the same organ. In the head, GPox, GST and GR activities were positively correlated, while G6PDH activity was independent of that of CAT. The presence of these two clearly separated groups appeared to have the largest influence on distinguishing the control and HF treatments from the other treatments in the PCA plot at the exposure level of 1 µg/L **(Fig. 5C)**.

4. Discussion

Residues of pesticides, mainly acaricides and insecticides, have been detected in honey, pollen, wax comb and other bee colony matrices (Bridi et al., 2018; Chauzat et al., 2011; Mullin et al., 2010; Ostiguy et al., 2019; Ostiguy and Eitzer, 2014). More than 56 pesticides and metabolites of similar or completely different modes of action were found in pollen and wax comb with a mean of 1.1 to 8.7 detections per pooled pollen sample and a mean of 8 detections per wax sample (Mullin et al., 2010; Ostiguy et al., 2019). In addition, up to 8 different residues were found in a single honey sample with a mean of 2.9 residues per sample in honey of French apiaries (Lambert et al., 2013). Some studies focused on the effect of interactions between pyrethroid insecticides and fungicides (Colin and Belzunces, 1992; Meled et al., 1998; Pilling and Jepson, 1993; Thompson and Wilkins, 2003), neonicotinoid insecticides and fungicides (Iwasa et al., 2004; Schmuck et al., 2003; Thompson et al., 2014a), and different types of acaricides and fungicides (Johnson et al., 2013; Johnson et al., 2009). For the interaction of insecticides and EBI fungicides, the synergistic effect was mainly attributed to the blocking action of the fungicide on the metabolism of insecticides (Bjergager et al., 2017; Glavan and Bozic, 2013; Mao et al., 2011). In addition, it has been shown that pesticide mixtures mainly induce toxicity lower than the sum of the toxicities of each substance, even when the substances have a similar mode of action (Christen et al., 2017). Thus, the effects of the interactions between pesticides are unpredictable, not only for substances of different classes but also for substances exhibiting similar modes of action.

Imidacloprid, glyphosate and difenoconazole are frequently detected in beehive matrices (Chauzat et al., 2011; Lambert et al., 2013; Lopez et al., 2016; Nguyen et al., 2009; Thompson et al., 2019). Consequently, honey bees could be exposed to mixtures of these three pesticides year-round, including the winter season when a specific caste of workers, known as winter honey bees (living up to six months in the cold period), is present. These honey bees are responsible for the maintenance of the colony during winter and for resuming activity at the beginning of the spring period (Free and Spencer-Booth, 1959; Omholt and Amdam, 2004). This led us to investigate whether oral exposure of winter honey bees to environmental concentrations of imidacloprid, difenoconazole and glyphosate has effects on mortality and food consumption and

whether these effects could be aggravated when the active substances occur as binary or ternary mixtures.

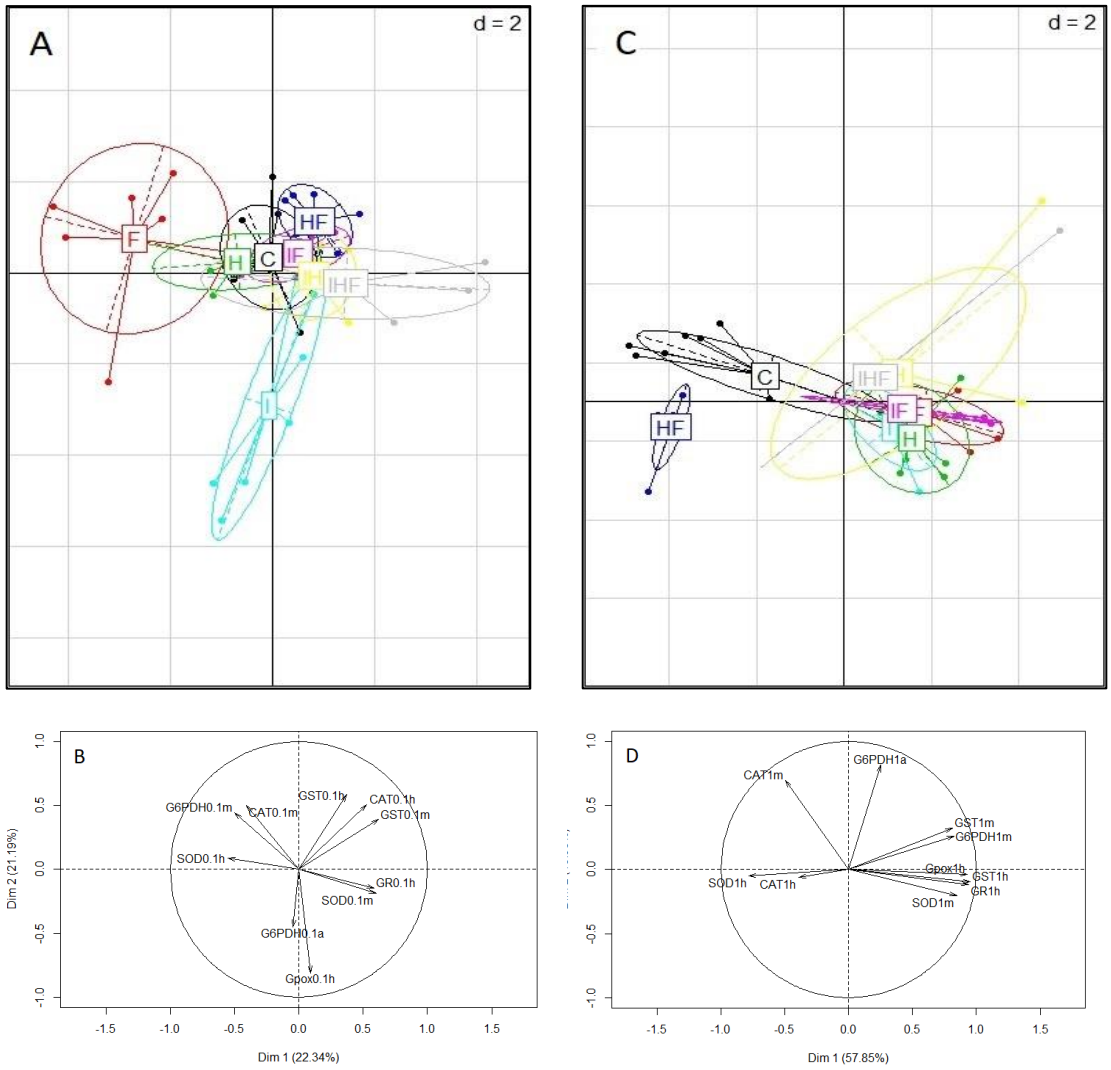

Fig. 5. Effects of exposure to pesticides on the physiological state of winter honey bees

Winter honey bees were orally exposed to food containing no pesticides (C), imidacloprid (I), difenoconazole (F), glyphosate (H), imidacloprid + difenoconazole (IF), imidacloprid + glyphosate (IH), glyphosate + difenoconazole (HF), or imidacloatprid + glyphosate + difenoconazole (IHF) at concentrations of 0.01, 0.1, 1 and 10 µg/L. On day 16, enzymes involved in antioxidant defenses were assayed. SOD, CAT and GST were measured in the head (h) and midgut (m). GPox and GR were measured in the head (h), and G6PDH was measured in the midgut (m) and abdomen (a). A multiple marker approach was performed to analyze the effects of these pesticides at 0.1 and 1 µg/L on oxidative stress. Principal component analyses (PCAs) (A and C) provide visual representations of the physiological states of honey bees exposed to the three pesticides individually or in binary and ternary mixtures at 0.1 µg/L (A) and 1 µg/L (C). The correlation circles (B and D) indicate the significance of the enzymes in the PCA representations in honey bees exposed to the pesticides individually or in binary and ternary mixtures at 0.1 µg/L (B) and 1 µg/L (D). SOD, superoxide dismutase; CAT, catalase; GST, glutathione-*S*-transferase; G6PDH, glucose-*6*-phosphate dehydrogenase; GR, glutathione reductase; GPox, glutathione peroxidase.

Our study revealed that toxicity is not always linked to the level of exposure to pesticides. For I, H and IF, the highest mortalities were observed at the lowest concentration of 0.01 µg/L. For F and all the other mixtures, the highest mortalities were observed at intermediate concentrations of 0.1 and 1 µg/L. This is in line with previous data showing that chronic exposures to glyphosate and imidacloprid have a stronger impact on honey bee survival at low concentrations than at high concentrations (Boily et al., 2013; Vazquez et al., 2018; Wegener et al., 2016). Thus, relatively high exposure levels are not systematically those that induce the highest toxicity, and low exposure levels may induce toxicity comparable to or higher than that induced at high exposure levels.

For a given pesticide mixture, the mode of interaction between pesticides strongly depends on the exposure level. Three-quarters of the pesticide mixture modalities induced subadditive, additive and synergistic effects (**Table S1**). IF induced a subadditive effect at 0.01 µg/L, an additive effect at 0.1 µg/L and a synergistic effect at 1 and 10 µg/L. IH induced a subadditive effect at 0.1 µg/L, an additive effect at 0.01 µg/L and a synergistic effect at 1 and 10 µg/L. The ternary IHF mixture induced a subadditive effect at 0.01 and 10 µg/L and a synergistic effect at 0.1 and 1 µg/L. The HF mixture was the only mixture that induced an antagonistic effect irrespective of the exposure concentration. Such a complex profile of interactions has been previously observed with mixtures associating EBI fungicides (prochloraz, propiconazole, fenbuconazole and myclobutanil) and the pyrethroid insecticide tau-fluvalinate (Johnson et al., 2013), for which EBI fungicides elicited a synergistic effect with tau-fluvalinate at doses of 1 and 10 nmol/bee but an antagonistic effect at a dose of 0.1 nmol/bee. This antagonist action could be linked to the effect of EBI fungicides on cytochromes P450 (CYP450), which are considered the primary enzymes for the detoxification of phytochemicals (Mao et al., 2009) and pesticides (Mao et al., 2011) in honey bees. EBI fungicides, including difenoconazole, are not only potential inhibitors but also inducers of CYP450 (Laignelet et al., 1989; Rivière, 1983; Snegaroff and Bach, 1989). Thus, the antagonistic effect of pesticide mixtures containing an EBI fungicide could be explained by the induction of detoxifying enzymes, which results in an increase in pesticide metabolism and a decrease in toxicity (Johnson et al., 2013). Such a mechanism of antagonism could be exemplified by piperonyl butoxide (PBO), an insecticide synergist that acts by inhibiting CYP450 (Tozzi, 1999) but that can also induce these enzymes (Willoughby et al., 2007), similar to EBI fungicides. This suggests that prolonged exposure or low-dose PBO and EBI fungicide could result in an induction of a number of genes coding for CYP450 (Hodgson and Levi, 1999; Willoughby et al., 2007). The induction of CYP450 would consequently increase the metabolism of imidacloprid and glyphosate. However, the metabolism of imidacloprid by CYP450 generates metabolites that have similar or even higher toxicities than that of imidacloprid (Suchail et al., 2004; Suchail et al., 2001), whereas the metabolism of glyphosate generates less toxic metabolites such as amino-methylphosphonic acid (AMPA) (Blot et al., 2019). Thus, the induction of CYP450 could explain both the increase in the toxicity of the IF mixtures and the antagonistic effects observed for the HF mixtures.

Exposure to pesticides, alone or in mixtures, did not modify the food consumption of honey bees. The absence of effects on food intake suggests that these pesticides do not exhibit particular

repellent or attractive properties, at least at the evaluated concentrations. For imidacloprid, this result is in accordance with the unchanged feeding behavior observed in summer bees exposed to imidacloprid for 10 days at concentrations ranging between 0.06 and 2 µg/L (Gauthier et al., 2018). However, this result contrasts with the increased consumption of food containing neonicotinoids (including imidacloprid) of bees submitted for 24 hours to a two-choice feeding assay (Kessler et al., 2015). These discrepancies in food consumption suggest that changes in food behavior are compensated for during long exposure periods. Lower food consumption has been observed with imidacloprid at a high concentration of 4.3 mg/L, but it could be due in part to the high exposure concentration and to the adjuvants of the product (Advise 2FL) used to prepare the feeding solution (Zhu et al., 2017a). In contrast, no modification of food consumption was observed in bees exposed to glyphosate. This confirms the results of studies in which newly emerged bees were exposed for 14 days at a high concentration of 35 mg/L, and winter and summer honey bees were exposed for 22 days to glyphosate at 0.21 and 1.08 g/kg (Blot et al., 2019; Zhu et al., 2017a); however, these results contrast with the higher preference of bees for food containing 10 µg/L glyphosate than for that containing 10 mg/L glyphosate (Liao et al., 2017).

One of the possible causes of the pesticide effects in honey bees is the disturbance of the pro-oxidative/antioxidative balance. However, this cause has been scarcely explored for pesticide mixtures. Under normal physiological conditions, the antioxidant/pro-oxidant balance is in equilibrium. Pro-oxidants are mainly reactive oxygen species (ROS) that are permanently produced at moderate concentrations during mitochondrial respiration or as signaling mediators and defense molecules (Barouki, 2006; Dupre-Crochet et al., 2013; Finkel, 2003). ROS can also be produced following exposure to toxicants, toxins, pollutants and radiation (Nathan and Cunningham-Bussel, 2013). Oxidative damage occurs in the case of ROS overproduction or when there is a deficit in the antioxidant system, leading to possible alterations of lipids, proteins and DNA (Kohen and Nyska, 2002). The antioxidant system is composed of nonenzymatic antioxidants, such as tocopherol and carotenoids, which could be diet-derived, and of antioxidant enzymes that can be modulated and act directly or indirectly on ROS (Corona and Robinson, 2006; Sies, 1993). Pesticides were previously reported to contribute to oxidative stress in plants and animals (Contardo-Jara et al., 2009; de Aguiar et al., 2016; Guilherme et al., 2010; Jasper et al., 2012; Lushchak et al., 2009; Malev et al., 2012; Nwani et al., 2010; Ortiz-Ordonez et al., 2011; Puértolas et al., 2010). Imidacloprid and glyphosate were shown to induce oxidative stress in honey bees (Gauthier et al., 2018; Gregore et al., 2018; Helmer et al., 2015; Jumarie et al., 2017; Vázquez et al., 2020). This led us to investigate whether imidacloprid, glyphosate and difenoconazole could induce oxidative stress and modulate antioxidative defenses and to determine whether these effects could be aggravated when honey bees were exposed to binary or ternary pesticide mixtures.

To assess the effect of the pesticides on oxidative stress, the activities of SOD, CAT, GPox, GR, GST, and G6PDH were measured in surviving honey bees after 16 days of chronic oral exposure to pesticides. These enzymes work to limit oxidative stress, and they were previously shown to be modulated in honey bees under the pressure of pesticides or biotic stressors such as *Nosema*

and *Bacillus thuringiensis* spores (Badiou-Beneteau et al., 2012; Carvalho et al., 2013; Kairo et al., 2017b; Li et al., 2017b; Nwani et al., 2010; Renzi et al., 2016). SOD, CAT and GPox are primary antioxidant enzymes that act directly on ROS. SOD transforms the highly reactive superoxide radical to the less reactive hydrogen peroxide and oxygen (Vaziri et al., 2003). CAT converts hydrogen peroxide into water and oxygen (Corona and Robinson, 2006). GPox also acts on hydrogen peroxide and other organic hydroperoxides and catalyzes their reduction using electrons provided by GSH (Michiels et al., 1994). GR and G6PDH are secondary antioxidant enzymes. GR converts oxidized glutathione into its reduced form GSH (Corona and Robinson, 2006). G6PDH acts in the pentose phosphate pathway and generates NADPH, leading indirectly to the regeneration of reduced GSH (Renzi et al., 2016). GST, which could be considered a primary antioxidant enzyme, also plays a role in phase II of the detoxification process. GST acts by conjugating GSH xenobiotics, which become more hydrophilic and therefore are transported outside of the organism (Berenbaum and Johnson, 2015; du Rand et al., 2015). GST also has a high affinity for lipid peroxidation products, that are produced during oxidative stress, and transform them into less toxic hydroxyl derivatives (Corona and Robinson, 2006; du Rand et al., 2015).

Honey bees exposed to pesticides at 1 µg/L exhibited large variations in antioxidant enzyme activities. As shown by PCA, the activities of GST and GPox in the midgut were positively correlated with each other, as well as the activities of GPox, GST and GR in the head. The activities of head and midgut GST and head GPox varied greatly and represented at least 566%, 223% and 225% of the control activities in all exposure groups, respectively, except for the HF exposure group, which did not exhibit different activities for these enzymes from those in the control group. A similar increase in GST activity was previously observed when honey bees were exposed to imidacloprid and other neonicotinoids, such as thiamethoxam (Badiou-Beneteau et al., 2012; Li et al., 2017b). The increase in GST and GPox activities strongly reflected the attempts of the organism to counteract the oxidative stress that took place following exposure to pesticides. In addition, an increase of GST activity may reflect an activation of the detoxification process through the conjugation of xenobiotics with glutathione (Berenbaum and Johnson, 2015). The activities of GST and GPox rely on the presence of reduced glutathione, which is under the control of GR, and GR uses NADPH as a reducer (produced in large part by G6PDH). However, an increase in the activity of GR in the head (at least 300% of that in the control) and G6PDH in the midgut (at least 782% of the control activity) was observed in almost all exposure conditions. In the midgut, the concomitant increases in GST and G6PDH activities correlated well because G6PDH generates the NADPH necessary for the reduction of oxidized glutathione into its reduced form for use by GST. Consequently, the activity of enzymes responsible for the destruction of peroxides (GST and GPox) increased in correlation with the increasing activities of enzymes (GR and G6PDH) responsible for the regeneration of cofactors (GSH and NADPH) necessary for the functioning of GST and GPox (**Fig. 6**).

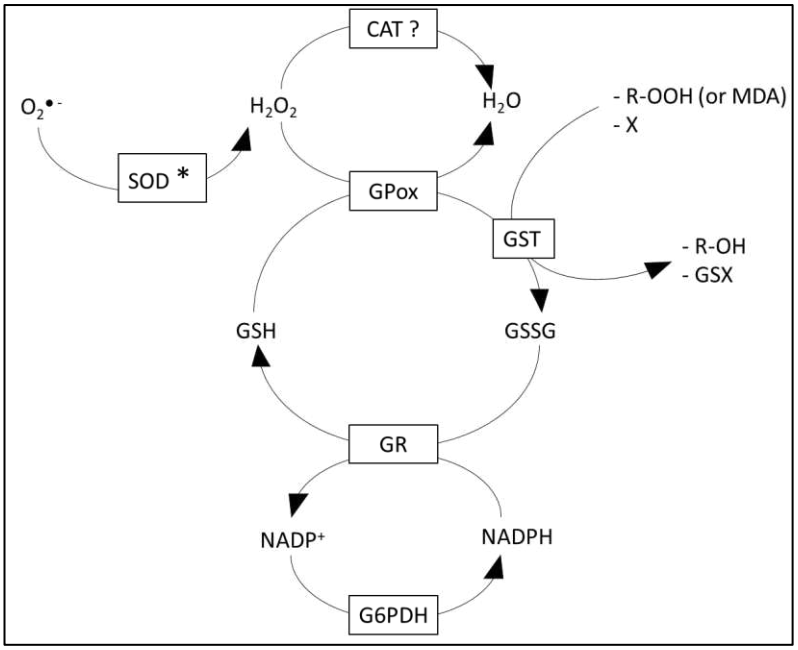

Fig. 6. Proposed mechanism underlying the specific antioxidant response of honey bees to exposure to imidacloprid, difenoconazole, and glyphosate individually or in binary and ternary mixtures (except the HF mixture) at 1 µg/L

The overproduction of reactive oxygen species (ROS), such as superoxide anion radicals ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), leads to the activation of enzymatic antioxidants (superoxide dismutase (SOD) in the midgut, glutathione peroxidase (GPox) in the head and glutathione-S-transferase (GST) in the head and midgut). The activities of GST and GPox rely on the presence of reduced glutathione (GSH) produced by glutathione reductase (GR) in the presence of NADPH, which is produced in large part by glucose-6-phosphate dehydrogenase (G6PDH). The proposed mechanism did not explain why the activity of catalase (CAT) decreased in the midgut (hence the question mark following CAT in the scheme). (*): SOD had a tissue-specific activity with an increase of activity in the midgut and decrease in the head. H_2O: water molecule; GSH: reduced glutathione; GSSG: oxidized glutathione; MDA: malondialdehyde; R-OOH, oxidized lipids; R-OH, detoxified lipids; X: xenobiotics; GSX: xenobiotics conjugated to glutathione.

In contrast to that at the dose of 1 µg/L, the change in antioxidant enzyme activities was more complex and less pronounced at 0.1 µg/L. This suggested either that the oxidative stress was less pronounced at 0.1 µg/L or that the honey bees were able to recover from some of the stress. To distinguish between these two hypotheses, Additional markers of oxidative stress were investigated in the exposure groups at 0.1 µg/L through the measurement of lipid peroxidation and protein carbonylation. In general, lipid peroxidation (except for HF) and protein carbonylation (except for IF, IH, HF and IHF) decreased in all exposure groups to below normal physiological rates. This indicated that the antioxidant systems were likely highly induced at the 0.1 µg/L exposure level to be able to reduce lipid and protein oxidations below the normal physiological rates.

Exposure to H and F alone induced well-pronounced variations in the antioxidant enzymes at 1 µg/L, and the levels of lipid peroxidation and protein carbonylation were below the physiological levels observed at 0.1 µg/L. However, the HF mixture induced the lowest variations in antioxidant enzyme activities at 0.1 and 1 µg/L, and the levels of lipid peroxidation and protein

carbonylation at 0.1 µg/L were similar to normal physiological levels. Therefore, the oxidative stress triggered by H and F was abolished when both pesticides are mixed together. In the mixture, the loss of the oxidative stress induced by H might be explained by the induction of CYP450 by difenoconazole (F) (Laignelet et al., 1989; Rivière, 1983; Snegaroff and Bach, 1989), leading to the detoxification of glyphosate and the reduction in oxidative stress and toxicity of the mixture. However, this hypothesis does not explain why, in the mixture, the oxidative stress induced by F was also inhibited. This exemplifies that the mechanism of action of a given mixture does not merely correspond to the sum of the mechanisms of action of each substance.

Changes in GR and GPox activities were observed in the head, while changes in CAT, SOD and GST activities were observed in both the head and midgut. Therefore, the effects of the pesticides were not restricted to the midgut, which is the primary site of oral exposure, but they were also extended to all biological compartments, leading to systemic oxidative stress that could compromise bee health. This systemic action not only reflected the distribution of the substances in the whole body, as already observed with imidacloprid (Suchail et al., 2004), but also showed that all tissues are sensitive to oxidative stress. In addition, for the same enzyme and the same type of exposure, physiological responses to pesticides may be tissue-specific. This was the case for SOD at an exposure level of 1 µg/L, whose activity decreased in the head and increased in the midgut, and for CAT and GST at exposure levels of 1 µg/L and 0.1 µg/L, respectively, whose activities were not modulated in the head but decreased in the midgut.

5. Conclusion

In the present study, we showed that chronic oral exposures to environmental concentrations of insecticides, fungicides and herbicides could negatively affect the survival of winter honey bees by systemic action. The toxicity of the pesticides highly increased when they occurred as mixtures, and the highest mortalities were recorded at intermediate exposure concentrations of 0.1 and 1 µg/L. Our data showed that the oxidative balance was severely disrupted by pesticides, both alone and in mixtures. The induction of oxidative stress could be one of the prevalent mechanisms that could explain the toxicity of pesticide mixtures. Hence, it is reasonable to propose that the adverse effects of exposures to pesticides on survival and oxidative stress could be aggravated by the cold and humid conditions of the winter season. Additionally, the presence of residues of numerous pesticides in beehive matrices (Ostiguy et al., 2019) could explain, at least in part, the increase in winter colony losses observed in many countries (Gray et al., 2019).

Acknowledgments

This study was supported in part by the recurrent funding of the Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAE) and by the Agence Nationale de la Recherche (ANR) (grant ANR-15-CE34-0004-01). Hanine ALMASRI was supported by

grants from Lebanese University (grant number 2364) and the PACA region. The authors would like to thank Jacques Sénéchal and Alexandre Gorit, who are beekeepers at the UR 406 INRAE Research Unit, for their expert beekeeping.

Table. S1. Modes of interaction of the different pesticide combinations and their effects on honey bee mortality

Winter honey bees were fed sucrose solutions containing no pesticides (C), difenoconazole (F), glyphosate (H), glyphosate + difenoconazole (HF), imidacloprid (I), imidacloprid + difenoconazole (IF), imidacloprid + glyphosate + difenoconazole (IHF) or imidacloprid + glyphosate (IH) at 0.01, 0.1, 1 and 10 µg/L for 16 days. The data represent the mean mortality rate (%) of 14 repetitions ± standard deviation (SD), which is the mortality corrected by the control and the expected mortality (which is the sum of the corrected mortality of each pesticide alone). The interaction ratio (IR) was calculated by dividing the corrected mortality by the expected mortality. Data with different letters are significantly different ($p < 0.05$).

Treatment		Mortality rate (%) ± SD	Corrected Mortality	Expected Mortality	IR	Statistical significance ($p < 0.05$)	Mode of Interaction
0 µg/L	C	8.57 ± 2.52	0.00	-	-	a	-
	I	27.62 ± 5.68	19.05	-	-	b	-
	H	18.38 ± 2.52	9.81	-	-	ac	-
	F	26.67 ± 3.56	18.10	-	-	b	-
0.01 µg/L	IF	38.33 ± 3.28	29.76	37.15	0.80	d	Sub-additive
	IH	36.00 ± 2.98	27.43	28.86	0.95	d	Additive
	HF	15.24 ± 3.71	6.67	27.91	0.24	c	Marked antagonism
	IHF	37.62 ± 5.44	29.05	46.96	0.62	d	Sub-additive
	I	26.19 ± 2.52	17.62	-	-	b	-
	H	23.33 ± 1.26	14.76	-	-	b	-
	F	20.48 ± 3.71	11.91	-	-	b	-
0.1 µg/L	IF	37.62 ± 2.62	29.05	29.53	0.98	c	Additive
	IH	35.24 ± 3.85	26.67	32.38	0.82	c	Sub-additive
	HF	22.86 ± 1.63	14.29	26.67	0.54	b	Slight antagonism
	IHF	66.19 ± 3.17	57.62	44.29	1.30	d	Marked synergism
	I	13.81 ± 1.78	5.24	-	-	b	-
	H	27.65 ± 2.52	19.08	-	-	b	-
	F	21.90 ± 3.17	13.33	-	-	b	-
1 µg/L	IF	35.24 ± 1.78	26.67	18.57	1.44	c	Marked synergism
	IH	54.76 ± 3.25	46.19	24.32	1.90	d	Marked synergism
	HF	19.05 ± 1.78	10.48	32.41	0.32	b	Marked antagonism
	IHF	49.05 ± 2.72	40.48	37.65	1.08	d	Slight synergism
	I	15.71 ± 2.62	7.14	-	-	b	-
	H	25.24 ± 3.71	16.67	-	-	c	-
	F	20.48 ± 2.30	11.91	-	-	bc	-
10 µg/L	IF	29.44 ± 1.72	20.87	19.05	1.10	d	Slight synergism
	IH	40.67 ± 2.98	32.10	23.81	1.35	d	Marked synergism
	HF	21.43 ± 2.30	12.86	28.58	0.45	bc	Slight antagonism
	IHF	30.48 ± 6.59	21.91	35.72	0.61	d	Sub-additive

Table. S2. Overall comparison of the effects of pesticides on mortalities

Winter honey bees were fed sucrose solutions containing no pesticides (C, control), imidacloprid (I), difenoconazole (F), glyphosate (H), glyphosate + difenoconazole (HF), imidacloprid + difenoconazole (IF), imidacloprid + glyphosate (IH) or imidacloprid + glyphosate + difenoconazole (IHF) at 0.01, 0.1, 1 and 10 µg/L for 16 days. The data represent the mean corrected mortality rate (%) of 14 repetitions ± standard deviation (SD). The significant differences between mortalities induced by the different treatments at the four different concentrations were determined. Numbers after the abbreviations of each treatment refer to the concentrations of the pesticides in the sucrose solution. Data with different letters are significantly different ($p < 0.05$).

Treatment	Corrected Mortality (%)	Statistical significance ($p < 0.05$)
C	0.00	a
I1	5.24	b
HF0.01	6.67	b
I10	7.14	b
H0.01	9.81	b
HF1	10.48	b
F0.1	11.91	bc
F10	11.91	bc
HF10	12.86	bc
F1	13.33	bc
HF0.1	14.29	bc
H0.1	14.76	bc
H10	16.67	bc
I0.1	17.62	bc
F0.01	18.10	bc
I0.01	19.05	bc
H1	19.08	bc
IF10	20.87	c
IHF10	21.91	cd
IH0.1	26.67	cd
IF1	26.67	cd
IH0.01	27.43	cd
IHF0.01	29.05	cd
IF0.1	29.05	cd
IF0.01	29.76	cd
IH10	32.10	cd
IHF1	40.48	d
IH1	46.19	de
IHF0.1	57.62	e

Table. S3. Effects of exposure to pesticides on honey bee food consumption

Winter honey bees were fed sucrose solutions containing no pesticides (C), difenoconazole (F), glyphosate (H), imidacloprid (I), glyphosate + difenoconazole (HF), imidacloprid + difenoconazole (IF), imidacloprid + glyphosate (IH), or imidacloprid + glyphosate + difenoconazole (IHF) at 0.01, 0.1, 1 and 10 µg/L for 16 days. Food consumption was evaluated during the 16 days by measuring the food consumed daily by the bees alive in each of the 14 cages per treatment. The cumulative food consumption is expressed in milligrams per bee (mg/bee) ± standard deviation (SD). Statistical analyses were performed using the Kruskal-Wallis test followed by pairwise comparisons using the Wilcoxon rank sum test with Benjamini-Hochberg correction. Asterisks indicate significant differences from the control group (* $p \leq 0.05$).

	Treatment	Cumulated food consumption ± SD (mg/bee)	Daily food consumption (mg/bee)	Statistical significance ($p < 0.05$)
0 µg/L	C	852.31 ± 48.19	53.27	abc
0.01 µg/L	F	822.97 ± 89.15	51.44	abcd
	H	753.71 ± 40.43	47.11	d* (p=0.0496)
	I	840.41 ± 51.13	52.53	abcd
	HF	854.67 ± 38.07	53.42	abc
	IF	864.53 ± 62.74	54.03	abc
	IH	792.96 ± 70.45	49.56	abcd
	IHF	799.77 ± 34.13	49.99	bcd
0.1 µg/L	F	789.16 ± 58.95	49.32	bcd
	H	819.34 ± 49.23	51.21	abcd
	I	859.14 ± 64.94	53.70	abc
	HF	788.80 ± 153.85	49.23	abcd
	IF	812.24 ± 44.42	50.77	abcd
	IH	818.51 ± 49.92	51.16	abcd
	IHF	809.04 ± 120.65	50.57	bcd
1 µg/L	F	783.40 ± 29.47	48.96	cd
	H	798.14 ± 82.04	49.88	bcd
	I	834.75 ± 52.66	52.17	abcd
	HF	836.89 ± 68.49	52.31	abcd
	IF	795.09 ± 113.68	49.69	abcd
	IH	851.14 ± 85.00	53.20	abcd
	IHF	813.25 ± 24.33	50.83	abcd
10 µg/L	F	778.52 ± 47.31	48.66	bcd
	H	917.31 ± 48.92	57.33	a
	I	831.87 ± 46.26	51.99	abcd
	HF	871.48 ± 23.42	54.47	ab
	IF	790.29 ± 122.04	49.39	bcd
	IH	851.09 ± 86.01	53.19	abcd
	IHF	774.66 ± 83.93	48.42	bcd

Table. S4. Effects of exposure to pesticides, alone or in combination, at 0.1 µg/L on physiological markers in winter honey bees

Winter honey bees were fed sucrose solutions containing no pesticides (C), difenoconazole (F), glyphosate (H), imidacloprid (I), glyphosate + difenoconazole (HF), imidacloprid + difenoconazole (IF), imidacloprid + glyphosate (IH), or imidacloprid + glyphosate + difenoconazole (IHF) at 0.01, 0.1, 1 and 10 µg/L for 16 days. A multiple marker approach was performed to study the effects of these pesticides at 0.1 µg/L on oxidative stress. SOD, CAT and GST activities were measured in the head (h) and midgut (m). GPox and GR were measured in the head (h), whereas G6PDH was measured in the midgut (m) and abdomen (a). Seven samples (n=7) of 3 tissues were collected in each treatment, and the mean value of enzymatic activity was calculated for each treatment. The enzymatic activity was expressed in milliunits of absorbance per minute and per g of tissue (mAU/min/g of tissue) ± standard deviation (SD). Data with different letters are significantly different ($p < 0.05$). Arrows indicate an increase (↑) or a decrease (↓) in activity relative to that in the control group.

Physiological marker (mAU/min/g of tissue)	Control	I [0.1 µg/L]	F [0.1 µg/L]	H [0.1 µg/L]	IF [0.1 µg/L]	IH [0.1 µg/L]	HF [0.1 µg/L]	IHF [0.1 µg/L]
$SOD_{(h)}$	7.288 ± 1.090 ^{b}	6.576 ± 3.067 ^{b}	12.184 ± 4.446 ^{a} ↑	7.733 ± 3.960 ^{b}	7.733 ± 5.636 ^{b}	8.119 ± 3.960 ^{ab}	7.809 ± 0.843 ^{ab}	7.733 ± 3.960 ^{b}
$CAT_{(h)}$	3.328 ± 0.331 ^{b}	1.012 ± 0.064 ^{d} ↓	1.769 ± 1.037 ^{d} ↓	2.940 ± 0.229 ^{cd} ↓	3.233 ± 0.350 ^{bc}	3.143 ± 0.223 ^{bc}	3.913 ± 0.337 ^{a} ↑	3.086 ± 0.331 ^{bc}
$GST_{(h)}$	13.000 ± 1.560	12.090 ± 1.430	12.350 ± 3.250	13.650 ± 2.080	14.430 ± 1.170	13.910 ± 0.910	14.690 ± 0.910	14.170 ± 0.910
$GR_{(h)}$	2.721 ± 0.700 ^{ac}	3.057 ± 1.049 ^{ac}	1.389 ± 0.048 ^{a}	3.195 ± 0.481 ^{ac}	1.701 ± 0.982 ^{ab}	2.268 ± 0.491 ^{ac}	3.067 ± 0.432 ^{bc}	3.969 ± 0.982 ^{c}
$GPox_{(h)}$	3.297 ± 0.938 ^{ab}	7.060 ± 0.733 ^{c} ↑	2.804 ± 0.794 ^{a}	3.675 ± 0.648 ^{ab}	3.481 ± 0.794 ^{ab}	2.998 ± 1.048 ^{a}	3.971 ± 0.464 ^{ab}	4.714 ± 0725 ^{b}
$SOD_{(m)}$	6.972 ± 0.768 ^{ab}	8.841 1.837 ^{bc}	4.585 ± 1.774 ^{a}	8.022 ± 2.807 ^{ab}	11.789 ± 1.837 ^{cd} ↑	15.472 ± 3.606 ^{de} ↑	5.026 ± 1.218 ^{a}	17.765 ± 2.625 ^{e} ↑
$G6PDH_{(m)}$	2.040 ± 0.000 ^{ab}	1.837 ± 0.495 ^{b}	3.422 ± 0.624 ^{a}	3.193 ± 0.578 ^{ab}	2.681 ± 0.465 ^{ab}	2.138 ± 0.640 ^{ab}	2.312 ± 0.765 ^{ab}	1.807 ± 0.319 ^{b}
$CAT_{(m)}$	0.997 ± 0.051 ^{ab}	0.441 ± 0.092 ^{c} ↓	1.443 ± 0.263 ^{a}	1.050 ± 0.188 ^{ab}	0.863 ± 0.139 ^{ab}	0.776 ± 0.158 ^{bc}	0.836 ± 0.283 ^{b}	0.803 ± 0.137 ^{bc}
$GST_{(m)}$	0.347 ± 0.082 ^{a}	0.039 ± 0.012 ^{b} ↓	0.053 ± 0.014 ^{b} ↓	0.044 ± 0.008 ^{b} ↓	0.432 ± 0.250 ^{a}	0.259 ± 0.087 ^{a}	0.286 ± 0.066 ^{a}	0.385 ± 0.181 ^{a}
$G6PDH_{(a)}$	22.789 ± 7.608	28.797 ± 11.885	19.267 ± 0.878	17.995 ± 3.349	29.004 ± 6.255	8.151 ± 8.151	19.000 ± 5.216	24.032 ± 7.031

Table. S5. Effects of exposure to pesticides, alone or in combination, at 1 µg/L on physiological markers in winter honey bees

Winter honey bees were fed sucrose solutions containing no pesticides (C), difenoconazole (F), glyphosate (H), imidacloprid (I), glyphosate + difenoconazole (HF), imidacloprid + difenoconazole (IF), imidacloprid + glyphosate (IH), or imidacloprid + glyphosate + difenoconazole (IHF) at 0.01, 0.1, 1 and 10 µg/L for 16 days. A multiple marker approach was performed to study the effects of these pesticides at 1 µg/L on oxidative stress. SOD, CAT and GST were measured in the head (h) and midgut (m). GPox and GR were measured in the head (h), whereas G6PDH was measured in the midgut (m) and abdomen (a). Seven samples (n=7) of 3 tissues were collected in each treatment, and the mean value of enzymatic activity was calculated for each treatment. The enzymatic activity was expressed in milliunits of absorbance per minute and per g of tissue (mAU/min/g of tissue) ± standard deviation (SD). Treatments with different letters are significantly different ($p < 0.05$). Arrows indicate an increase (↑) or a decrease (↓) in activity relative to that in the control group.

Physiological marker (mAU/min/g of tissue)	Control	I [1 µg/L]	F [1 µg/L]	H [1 µg/L]	IF [1 µg/L]	IH [1 µg/L]	HF [1 µg/L]	IHF [1 µg/L]
$SOD_{(h)}$	7.288 ± 1.090 ^{a}	4.081 ± 0.000 ^{d} ↓	4.897 ± 1.117 ^{cd} ↓	6.122 ± 0.000 ^{b} ↓	6.122 ± 0.000 ^{bc} ↓	3.061 ± 1.443 ^{d} ↓	7.529 ± 0.678 ^{a}	3.410 ± 0.000 ^{d} ↓
$CAT_{(h)}$	3.328 ± 0.331 ^{ab}	3.639 ± 1.224 ^{b}	2.143 ± 0.573 ^{a}	2.333 ± 0.284 ^{a}	2.154 ± 0.219 ^{a}	2.636 ± 0.313 ^{ab}	3.070 ± 0.488 ^{ab}	2.517 ± 0.000 ^{ab}
$GST_{(h)}$	13.000 ± 1.560	92.517 ± 6.908 ^{de} ↑	73.605 ±6.497 ^{b} ↑	81.519 ± 4.741 ^{bc} ↑	90.930 ± 4.100 ^{cd} ↑	104.082 ± 0.962 ^{e} ↑	14.318 ± 0.550 ^{a}	100.680 ± 0.000 ^{de} ↑
$GR_{(h)}$	2.721 ± 0.700 ^{a}	9.455 ± 1.369 ^{b} ↑	9.659 ± 1.825 ^{b} ↑	8.163 ± 1.053 ^{b} ↑	10.544 ± 0.589 ^{b} ↑	10.884 ± 0.000 ^{b} ↑	2.572 ± 0.969 ^{a}	9.523 ± 0.000 ^{b} ↑
$GPox_{(h)}$	3.297 ± 0.938 ^{a}	7.755 ± 0.912 ^{bc} ↑	9.244 ± 1.707 ^{bd} ↑	7.426 ± 0.871 ^{b} ↑	9.870 ± 1.558 ^{cd} ↑	10.877 ± 0.000 ^{d} ↑	3.595 ± 0.444 ^{a}	9.530 ± 0.000 ^{bd} ↑
$SOD_{(m)}$	6.972 ± 0.768 ^{a}	16.727 ± 3.866 ^{bc} ↑	14.142 ± 1.513 ^{b} ↑	19.551 ± 3.078 ^{c} ↑	19.040 ± 2.699 ^{bc} ↑	15.816 ± 0.721 ^{bc} ↑	4.384 ± 0.848 ^{a}	18.367 ± 0.000 ^{bc} ↑
$G6PDH_{(m)}$	2.040 ± 0.000 ^{a}	11.564 ± 2.356 ^{ab}	21.315 ± 3.086 ^{bc} ↑	15.963 ± 4.114 ^{bc} ↑	19.954 ± 7.080 ^{bc} ↑	23.469 ± 4.329 ^{c} ↑	2.892 ± 0.659 ^{a}	25.850 ± 0.000 ^{c} ↑
$CAT_{(m)}$	0.997 ± 0.051 ^{c}	0.570 ± 0.117 ^{ab} ↓	0.481 ± 0.104 ^{ab} ↓	0.486 ± 0.079 ^{a} ↓	0.545 ± 0.043 ^{ab} ↓	0.833 ± 0.014 ^{c}	0.618 ± 0.042 ^{ab} ↓	0.762 ± 0.000 ^{bc}
$GST_{(m)}$	0.347 ± 0.082 ^{a}	0.776 ± 0.061 ^{b} ↑	1.306 ± 0.143 ^{c} ↑	1.102 ± 0.163 ^{c} ↑	1.245 ± 0.163 ^{c} ↑	1.674 ± 0.061 ^{d} ↑	0.286 ± 0.061 ^{a}	2.204 ± 0.000 ^{e} ↑
$G6PDH_{(a)}$	22.789 ± 7.608	21.428 ± 3.818	18.877 ± 3.893	17.346 ± 4.929	21.768 ± 4.329	30.782 ± 26.696	18.448 ± 4.873	43.537 ± 0.000

Table. S6. Effects of exposure to pesticides, alone or in combination, at 0.1 µg/L on lipid peroxidation and protein carbonylation

Winter honey bees were fed sucrose solutions containing no pesticides (C), difenoconazole (F), glyphosate (H), imidacloprid (I), glyphosate + difenoconazole (HF), imidacloprid + difenoconazole (IF), imidacloprid + glyphosate (IH), or imidacloprid + glyphosate + difenoconazole (IHF) at 0.01, 0.1, 1 and 10 µg/L for 16 days. Lipid peroxidation and protein carbonylation were measured in the midguts of honey bees exposed to those pesticides at 0.1 µg/L. Nine samples of 3 midguts (n=9) were collected in each treatment for the measurement of lipid peroxidation, whereas 12 samples of 1 midgut (n=12) were collected for protein carbonylation. Lipid peroxidation and protein carbonylation were expressed in µmoles of malondialdehyde per mg of tissue (µmol of MDA/mg of tissue) and percentage of carbonylated proteins per mg of tissue (% of carbonylated proteins/mg of tissue) ± standard deviation (SD). Treatments with the different letters are significantly different ($p < 0.05$). Arrows indicate an increase (↑) or a decrease (↓) in activity relative to that in the control group.

Physiological marker	Unit	Control	I [0.1 µg/L]	F [0.1 µg/L]	H [0.1 µg/L]	IF [0.1 µg/L]	IH [0.1 µg/L]	HF [0.1 µg/L]	IHF [0.1 µg/L]
TBARS	µM MDA/mg of tissue	0.564 ± 0.126 [a]	0.358 ± 0.061 [b] ↓	0.302 ± 0.128 [bc] ↓	0.217 ± 0.053 [C] ↓	0.144 ± 0.040 [d] ↓	0.167 ± 0.057 [d] ↓	0.657 ± 0.112 [a]	0.122 ± 0.030 [d] ↓
Protein carbonylation	% of carbonylated proteins/mg of tissue	17.886 ± 4.847 [cd]	7.411 ± 2.754 [a] ↓	10.535 ± 2.822 [ab] ↓	9.418 ± 5.750 [ab] ↓	28.243 ± 4.088 [e] ↑	13.302 ± 3.769 [bc]	24.633 ± 4.086 [de]	17.028 ± 3.206 [c]

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Bilan

Les résultats de cette étude confirment que l'exposition chronique à l'imidaclopride, au glyphosate et au difénoconazole, à des concentrations environnementales, conduit à une toxicité élevée chez les abeilles d'hiver. La co-exposition des abeilles à ces pesticides en mélanges binaires et ternaires conduit principalement à des effets additifs et synergiques. Seul le mélange binaire difénoconazole-glyphosate a conduit à un effet antagoniste aux quatre concentrations. Cet effet antagoniste pourrait être lié à l'induction des CYP450 suite à l'exposition chronique au difénoconazole. Ainsi, une induction des CYP450 par le difénoconazole, lors d'une exposition chronique, augmenterait la métabolisation des pesticides et conduirait à l'apparition de métabolites plus toxiques dans le cas de l'imidaclopride et moins toxiques dans le cas du glyphosate. Ainsi, les modes d'interactions entre les pesticides peuvent varier en fonction des niveaux et des durées d'exposition qui influent sur le pouvoir inducteurs des fongicides azoles sur les CYP450, ce qui ajoute encore plus de complexité à la prédiction de la toxicité des mélanges de pesticides chez les abeilles.

Les résultats de cette étude montrent que l'équilibre oxydatif est fortement perturbé suite à l'exposition aux trois pesticides, seuls et en mélanges. Cela confirme que ces trois pesticides présentent chez l'abeille, bien qu'ils soient censés agir sur des cibles complétement différentes, au moins un mécanisme d'action commun qui est le stress oxydant. Ce mécanisme commun permet de mieux comprendre l'origine de la réponse systémique, la toxicité élevée et l'apparition des effets synergiques et additifs suite à l'exposition aux mélanges de pesticides.

Partie 3 : Une exposition chronique à un mélange insecticide-herbicide peut-elle sensibiliser les abeilles à un contact ultérieur à un fongicide utilisé en pulvérisation en pleine floraison ?

Avant-propos

Les résultats des deux études précédentes ont mis en évidence la forte toxicité de l'exposition chronique simultanée à l'imidaclopride, au glyphosate et au difénoconazole en mélanges binaires et ternaires, chez les abeilles d'hiver. Toutefois, l'exposition des abeilles aux pesticides ne se limite pas à l'exposition chronique. En effet, dès leurs premiers vols de butinage, les abeilles peuvent aussi être exposées aux pesticides par pulvérisation. Les fongicides sont les seuls pesticides autorisés pour une pulvérisation en pleine floraison du fait de leur faible toxicité aiguë.

Partant de ce constant, nous avons émis deux hypothèses.

(i) La première hypothèse était qu'une exposition aiguë des abeilles aux produits phytopharmaceutiques à base de difénoconazole pourrait avoir des effets négatifs retardés sur la survie des abeilles pulvérisées, ce qui pourrait expliquer la faible toxicité apparente des fongicides déterminée à très court terme.

(ii) La deuxième hypothèse était que l'exposition chronique à l'imidaclopride et au glyphosate pourrait sensibiliser les abeilles à un contact ultérieur au difénoconazole par pulvérisation.

Article 3

Toxicological status changes the susceptibility of the honey bee Apis mellifera to a single fungicidal spray application

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Résumé

Pendant toutes leurs étapes de vie, les abeilles sont exposées à des concentrations résiduelles de pesticides, tels que des insecticides, des fongicides et des herbicides, stockés dans les matrices de la ruche. Cependant, parmi les pesticides, les fongicides sont autorisés pendant la floraison des cultures en raison de leur faible toxicité aiguë apparente pour l'abeille domestique. Ainsi, une abeille qui aurait pu être préalablement exposée à des pesticides, via des aliments contaminés, peut être soumise à une pulvérisation fongicide lorsqu'elle amorce son premier vol à l'extérieur de la ruche. Dans cette étude, nous avons évalué l'effet d'une exposition aiguë à un fongicide sur des abeilles qui ont des statuts toxicologiques différents. Trois jours après l'émergence, les abeilles ont été soumises pendant 30 jours à une exposition chronique à l'insecticide imidaclopride et à l'herbicide glyphosate, individuellement et en mélange binaire, à des concentrations environnementales de 0,01 et 0,1 µg/L dans les aliments (0,0083 et 0,0803 µg/kg). Sept jours après le début de l'exposition chronique aux pesticides (10 dix jours après l'émergence), les abeilles ont été soumises à une pulvérisation avec le fongicide difénoconazole à la dose homologuée sur le terrain. Les résultats ont montré une baisse significative et retardée de la survie lorsque les abeilles mellifères étaient traitées avec le fongicide. La toxicité du fongicide a augmenté lorsque les abeilles étaient exposées de manière chronique au glyphosate à la concentration la plus faible, elle a diminué lorsque les abeilles étaient exposées à l'imidaclopride et n'était pas modifiée lorsque les abeilles étaient exposées au mélange binaire, quelle que soit la concentration. Les abeilles exposées à ces associations de pesticides ont montré des perturbations physiologiques révélées par la modulation de plusieurs traits de cycle de vie principalement impliqués dans le métabolisme, et ce, même lorsqu'il n'a pas été observé d'effet sur la toxicité du fongicide avec les autres pesticides. Ces résultats montrent que la toxicité des substances actives peut être mal estimée dans les tests conduits dans le cadre de l'homologation des pesticides, avant leur mise sur le marché, en particulier pour les fongicides.

Mots-clés : Fongicide, glyphosate, imidaclopride, mortalité, statut toxicologique

Abstract

During all their life stages, bees are exposed to residual concentrations of pesticides, such as insecticides, herbicides and fungicides, stored in beehive matrices. Fungicides are authorized for use during crop blooms because of their low acute toxicity to honey bees. Thus, a bee that might have been previously exposed to pesticides through contaminated food may be subjected to fungicide spraying when it initiates its first flight outside the hive. In this study, we assessed the effects of acute exposure to the fungicide in bees with different toxicological statuses. Three days after emergence, bees were subjected to chronic exposure to the insecticide imidacloprid and the herbicide glyphosate, either individually or in a binary mixture, at environmental concentrations of 0.01 and 0.1 µg/L in food (0.0083 and 0.083 µg/kg) for 30 days. Seven days after the beginning of chronic exposure to the pesticides (ten days after emergence), the bees were subjected to spraying with the fungicide difenoconazole at the registered field dosage. The results showed a delayed significant decrease in survival when honey bees were treated with the fungicide. Fungicide toxicity increased when honey bees were chronically exposed to glyphosate at the lowest concentration, decreased when they were exposed to imidacloprid and did not significantly change when they were exposed to the binary mixture regardless of the concentration. Bees exposed to all of these pesticide combinations showed physiological disruptions, revealed by the modulation of several life history traits related mainly to metabolism, even when no effect of the other pesticides on fungicide toxicity was observed. These results show that the toxicity of active substances may be misestimated in the pesticide registration procedure, especially for fungicides.

Key words: Fungicide; glyphosate; imidacloprid; difenoconazole; honey bee survival; toxicological status

1. Introduction

Agricultural crops worldwide potentially face approximately 70 000 deleterious species, including insects, mites, plant pathogens and weeds (Pimentel 2009), causing crop yield losses of 30% on average (Oerke 2006). The use of pesticides to combat pests has drastically increased since the 1960s, as this approach is considered economically profitable, resulting in a return of $4 per dollar invested in pesticide control (Pimentel 2009). Despite the positive direct effects of pesticides in controlling pests, they induce economic and environmental damage resulting from the development of pesticide resistance in pest species and increases in negative impacts on non-target species, including both natural enemies and pollinators such as honey bees (Popp 2011).

Honey bees are exposed to pesticides during all of their life stages. At the larval stage and after emergence, honey bees feed on stored honey and bee bread. These substances are

frequently contaminated by residues of pesticides such as insecticides, fungicides and herbicides, leading to chronic exposure to these pesticides (Ostiguy et al. 2019; Piechowicz et al. 2018; Wintermantel et al. 2019). Countless studies have shown sublethal effects of insecticides on honey bees (Belzunces et al. 2012; Meikle et al. 2020). The effects of herbicides have been less well investigated, but an increasing number of studies have reported that these pesticides are toxic to honey bees because they impair key functions such as metabolism and foraging activity and negatively impact the gut microbiota (Dornelles and Oliveira 2014; Gonalons and Farina 2018; Motta et al. 2020). Honey bees rarely encounter only a single pesticide in beehive matrices (Kanga et al. 2019; Mullin et al. 2010), and combinations of pesticides, such as insecticides along with herbicides or fungicides, may induce synergistic and additive toxicities (Gonalons and Farina 2018; Johnson et al. 2013; Wang et al. 2019; Wang et al. 2020).

In addition to chronic exposure to residual concentrations of pesticides, honey bees may be acutely exposed to high concentrations of pesticides (mainly fungicides) during their foraging flights. Fungicides are authorized for use during full bloom, and many foraging crops require at least one fungicide application during the blooming period to provide feasible management of fungal diseases (Xavier et al. 2020). Based on the standard test methods for the determination of acute oral and contact exposure to pesticides, fungicides are considered safe to honey bees, with a median lethal dose (LD50) higher than 100 µg/bee (Stanley et al. 2015). In these tests, toxicity is generally based on mortality observed 48 hours after a single exposure to a pesticide, and the assessment may be prolonged to 96 hours after exposure if mortality continues to rise (OCDE 1998; Rortais et al. 2017). However, delayed long-term acute exposures to fungicides are underexamined. In addition, the toxic effects of fungicides are not restricted to their impacts on survival. Fungicides affect larval development and mortality (Mussen et al. 2004), reduce the number of workers in the hive, perturb hive thermoregulation (Meikle et al. 2017), reduce pollen consumption and ATP levels and increase virus titers in honey bees (Degrandi-Hoffman et al. 2015; Simon-Delso et al. 2014).

In this study, we focused on determining the acute toxicity and long-term effects of a single spray application of a fungicide preparation on honey bees. We aimed to study the influence of the past exposure of individuals on the toxicity induced by fungicides to determine whether the toxicological status of honey bees may significantly modulate the toxicity of fungicides. Thus, we mimicked an environmental situation in which honey bees were chronically exposed to pesticides through food from the time of their emergence. Then, beginning at an age at which they could initiate their first outside flight, the bees were exposed to spraying with a fungicide at a registered field dosage. Emerging bees were first subjected to chronic exposure to an insecticide, imidacloprid, and an herbicide, glyphosate, either alone or in a mixture at environmental concentrations (0.01 and 0.1 µg/L in food). The bees were then exposed to the fungicide when they were 10 days old, when they can initiate their first flight outside the hive (Requier et al. 2020). Attention was focused on the effect of these exposure combinations on survival, food consumption and metabolism by analyzing the variations in six life history traits. In this protocol, we intentionally omitted the chronic treatments applied alone because,

in the toxicity tests used in the pesticide registration procedure, the history of the bees is not known. We aimed only to determine the extent to which the toxicological status of the bees could modulate the apparent toxicity of fungicides to elucidate toxicological impacts for the pesticide registration.

Imidacloprid is a neonicotinoid insecticide that disrupts the insect nervous system by acting as an agonist of nicotinic acetylcholine receptors (Taillebois et al. 2018). It is among the most frequently detected residues in honey samples. It is detected at concentrations ranging from 0.14 to 0.7 µg/kg in honey and at a mean concentration of 0.9 µg/kg in pollen (Chauzat et al. 2011; Lambert et al. 2013; Nguyen et al. 2009). Glyphosate is an herbicide that inhibits the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase, which is essential for the synthesis of some aromatic amino acids in plants (Duke and Powles 2008). It is detected in 27% of honey specimens, at concentrations ranging from 64 to 118 µg/kg, and in bee bread, at concentrations ranging from 52.4 to 58.4 µg/kg (Berg et al. 2018; El Agrebi et al. 2020; Rubio et al. 2015). Score® 250 EC is a phytopharmaceutical fungicide preparation containing difenoconazole. This fungicide belongs to the triazole fungicides, which are among the most widely used and ubiquitous fungicides worldwide. Triazoles are active substances that block the conversion of lanosterol into ergosterol in fungi by inhibiting 14α-demethylase (Zhang 2018). The Score fungicide is applied a broad-spectrum preventive and curative treatment in fruit trees such as apricot and peach trees and in potato, sugar beet, lettuce, asparagus and tomato; its use is authorized during the flowering period at label doses of 0.3 to 0.5 L per hectare (equivalent to 75 to 125 g of difenoconazole per hectare) (Anses 2014). Due to the extensive use of difenoconazole during all plant developmental stages, it is frequently detected in beehive matrices at concentrations ranging from 2.8 to 6.7 µg/kg in honey and at a mean concentration of 270 µg/kg in beebread (Abdallah et al. 2017; Kubik et al. 2000).

2. Materials and Methods

2.1. Materials

Imidacloprid (CAS No 138261-41-3) and glyphosate (CAS No. 1071-83-6) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Score® 250 EC was purchased from Syngenta France S.A.S. Bee food® Protein solution was purchased from Remuaux Ltd. (Barbentane, France). The Bee Boost® (PseudoQueen) pheromone preparation was purchased from Intko Supply Ltd. (Vancouver, Canada).

2.2. Honey bees

The study was conducted on emergent Apis mellifera honey bees (less than 24 hours old) collected from brood frames from three beehives that were continuously checked for their health status in the experimental apiary of the Abeilles & Environnement Research Unit (Bee & Environment Research Unit) of INRAE (Avignon, France). Emergent honey bees were collected, in mid- April 2018, directly from brood frames devoid of adult bees and placed in an incubator at 33 ± 2°C with 60 ± 5% relative humidity. The emerging bees from the three

beehives were mixed together and randomly distributed in groups of 30 honey bees house in plastic cages (6 × 8.5 × 10 cm). A small piece of Bee Boost® (PseudoQueen), releasing queen mandibular pheromone, and a small wax foundation sheet were deposited on the top of the cage to mimic the hive environment. For hygienic purposes, a sheet of filter paper was placed on the bottom of each cage and changed daily. During the first two days post-emergence, the honey bees were fed water, pollen and candy ad libitum, and the few dead bees were removed and replaced with bees of the same age set aside for this purpose.

2.3. Chronic exposure to pesticides

At the beginning of the third day after emergence, water, pollen and candy were removed from the cages and replaced with a 60% (w/v) sucrose solution containing 0.1% (v/v) dimethyl sulfoxide (DMSO) and 1% (v/v) Bee Food® protein solution, to which imidacloprid and glyphosate were added, either alone or in binary mixtures, at concentrations of 0 (control), 0.01 and 0.1 µg/L. These two concentrations were equivalent to 0.0083 and 0.083 µg/kg, respectively, calculated according to a sucrose solution density of 1.23 ± 0.02 (n=10). They were chosen because imidacloprid and glyphosate are frequently found in honey and pollen at these contamination levels (Bridi et al. 2018; Karise et al. 2017; Pareja et al. 2019). Chronic exposure was maintained until day 30 after emergence. Each treatment consisted of 14 cages (n = 14) with 30 honey bees per cage. The 10X working pesticide solutions were prepared in 1% (v/v) DMSO via the serial dilution of primary mother solutions and were stored at -20°C. The 10X working solutions were diluted 10-fold in a 66.67% (w/v) sucrose solution to obtain a feeding syrup containing 60% sucrose, 0.1% DMSO, and 1% (v/v) Bee Food® protein solution, plus or minus the pesticides at the desired concentrations. The working and feeding solutions were checked by GC-MS/MS and LC-MS/MS according to two analytical methods, with RSD < 10% (Oulkar et al. 2017; Paradis et al. 2014).

2.4. Exposure to fungicide spraying

Honey bees were exposed to Score® 250 EC by spraying in a Potter-type tower at an application rate of 0.3 L/ha (75 g/ha), which is the application rate recommended for use on peach, apricot and cherry trees to fight fungal diseases such as Monilia spp. during flowering. The Potter-type tower mimics agricultural spray application during which foragers are exposed by contact to pesticides at the field application rate. The formulation was freshly prepared before spraying using tap water to mimic the method performed by the farmers when treating their crops. Spray application was performed 10 days after emergence on honey bees that were chronically exposed to imidacloprid or glyphosate, either alone or in a binary mixture, for 1 week. The honey bees in each cage were lightly anesthetized with CO2 and placed together on a 200 cm2 Plexiglas disc. The disc was immediately subjected to rotation at 23 rpm to achieve homogenous spraying (Colin and Belzunces 1992; Poquet et al. 2014). Deposition was previously calibrated to achieve a rate of 2.14 ± 0.14 µL/cm2 (214 L/ha). The calibration accuracy was checked after every three spraying events. The bees that were exposed to the fungicide by spray application without being chronically exposed to the other pesticides were designated group F. The bees that were chronically exposed to the insecticide

(imidacloprid) at 0.01 or 0.1 µg/L and then to the fungicide were designated groups I0.01F and I0.1F, respectively. The bees that were chronically exposed to the herbicide (glyphosate) at 0.01 or 0.1 µg/L and then to the fungicide were designated groups H0.01F and H0.1F, respectively. The bees that were chronically exposed to the insecticide-herbicide mixture at 0.01 or 0.1 µg/L and then to the fungicide were designated groups IH0.01F and IH0.1F, respectively. Control honey bees (C) were fed a 60% sucrose solution containing 0.1% (v/v) DMSO and 1% (v/v) Bee Food® protein solution from the third day after emergence onward. Then, ten days after emergence, they were lightly anesthetized with CO2 and sprayed with tap water using the Potter-type tower.

2.5. Survival and food consumption

The number of dead bees was recorded daily at 8:30 a.m. until the end of the chronic exposure period. Dead bees were removed, and the sheet of filter paper was replaced to maintain hygienic conditions. The sucrose solutions were replaced with freshly prepared solutions, and individual food consumption was calculated by dividing the total daily food consumed per cage by the number of honey bees remaining alive each day. To obtain an accurate measurement of food consumption, an evaporation control was included.

2.6. Choice of physiological markers

The effects of the three pesticides on bee physiology were assessed by investigating the activity of six life history traits, mainly related to metabolism. These physiological markers are relevant for assessing the physiological perturbations induced by pesticides in different honey bee compartments (Almasri et al. 2020; Badiou-Beneteau et al. 2012; Carvalho et al. 2013; Degrandi-Hoffman et al. 2015; Kairo et al. 2017; Nicodemo et al. 2014). Glyceraldehyde-3-phosphate dehydrogenase (GA3PDH) was analyzed in the head, abdomen and thorax. Carboxylesterases (CaE-2, CaE-3) were analyzed in the abdomen and midgut. Glucose-6-phosphate dehydrogenase (G6PDH) was analyzed in the head. Lactate dehydrogenase (LDH) was analyzed in the head and midgut, and adenosine triphosphate (ATP) was analyzed in the thorax (Table 1).

GA3PDH plays an important role in energetic metabolism. It catalyzes the sixth step of glycolysis through the reversible conversion of glyceraldehyde-3-phosphate into 1,3-biphosphoglyceric acid with nicotinamide adenine dinucleotide (NAD+) as a cosubstrate (Nicholls et al. 2012). CaEs are multifunctional enzymes with different isoforms involved in the detoxification of xenobiotics, such as pesticides, and in lipid metabolism (Badiou-Beneteau et al. 2012; Ross et al. 2010). G6PDH is involved in metabolism and antioxidant defense. It catalyzes the first step of the pentose phosphate pathway, thus generating precursors for nucleotide synthesis and nicotinamide adenine dinucleotide phosphate (NADPH); NADPH is in turn used to generate reduced glutathione (GSH), which plays an important role in protection against oxidative damage (Efferth et al. 2000). LDH is a metabolic

enzyme involved in the regeneration of NAD+ by catalyzing the reduction of pyruvate into lactate under anaerobic conditions. NAD+ is used by GA3PDH during glycolysis (Tornheim 2018). ATP is the molecular unit of the intracellular energy currency, and it plays a role in signal transduction involving kinases and adenylate cyclase (Dunn and Grider 2020).

Table 1. Distribution of common and specific physiological markers across honey bee tissues

Head	Abdomen	Midgut	Thorax
GA3PDH	GA3PDH	LDH	GA3PDH
G6PDH	CaE-3	CaE-3	ATP
LDH	CaE-2	CaE-2	

2.7. Tissue preparation and enzyme extraction

On days 20 and 30 after emergence, the surviving bees were sampled. Their heads, abdomens and midguts were extracted according to Almasri et al. (2020). Briefly, to avoid animal suffering, the bees were first anesthetized with carbon dioxide, and their heads were separated from the rest of their bodies using a scalpel. Then, their midguts were obtained by pulling on the stinger, and the abdomen was recovered. The heads, midguts and abdomens (devoid of the intestinal tract) were separately placed in 2 mL microfuge tubes, weighed and stored at −80°C until analysis. For each treatment and each type of tissue, 3 tissue specimens were pooled to prepare the sample. Seven samples (7 × 3 tissues) were prepared (n = 7) for each treatment. During enzymatic analyses, each sample was assayed in triplicate. For each sample, the pooled tissues were homogenized in the extraction medium to prepare a 10% (w/v) tissue extract using a high-speed Qiagen TissueLyser II operated at 30 Hz in 5 periods of 30 s with 30 s intervals. The extraction medium consisted of 10 mM NaCl, 1% (w/v) Triton X-100, 40 mM sodium phosphate, pH 7.4, and protease inhibitors (2 µg/mL pepstatin A, leupeptin and aprotinin, 0.1 mg/mL soybean trypsin inhibitor and 25 units/mL antipain). The extracts were then centrifuged at 4°C for 20 min at 15 000 × gav, and the supernatants were kept on ice for further enzyme assays.

2.8. Analysis of physiological life history traits

Physiological traits were spectrophotometrically assayed at 25°C in the head, thorax, midgut and abdomen tissues of the same bees. GA3PDH was assayed on the basis of the conversion of 1,3-bisphosphoglyceric acid (1,3-BPG) into gylceraldehyde-3-phosphate (GA3P). In this reaction, 3-phosphoglyceric acid (3-PGA) is converted into 1,3-BPG by phosphoglycerate kinase (PGK), and 1.3-BPG is converted into glyceraldehyde-3-phosphate (GA3P) by

GA3PDH in the presence of reduced nicotinamide adenine dinucleotide (NADH), whose transformation into its oxidized form (NAD+) is followed at 340 nm. The reaction medium contained 7 mM 3-PGA, 120 µM NADH, 2 mM magnesium sulfate (MgSO4), 1.2 mM ATP, 4 mM L-cysteine-HCl neutralized with sodium bicarbonate (NaHCO3), 1 mM ethylenediaminetetraacetic acid (EDTA), 5 units.mL−1 3-phosphoglycerate kinase (3-PGK), and 80 mM triethanolamine buffer, pH 7.6 (Kairo et al. 2017; Renzi et al. 2016). CaE-2 and CaE-3 were monitored according to their specific respective substrates β–naphthyl acetate (β-NA) and p–nitrophenyl acetate (p-NPA) at 515 and 410 nm, respectively (Badiou-Beneteau et al. 2012). G6PDH activity was determined by following the formation of the reduced form of NADP+ (NADPH) at 340 nm. The reaction medium contained 10 mM magnesium chloride (MgCl2), 0.5 mM nicotinamide adenine dinucleotide phosphate (NADP+), 1 mM glucose-6-phosphate (G6P) and 100 mM Tris-HCl pH 7.4 (Renzi et al. 2016). LDH activity was determined by measuring the regeneration of NAD+ at 340 nm. The reaction medium contained 0.2 mM NADH, 5 mM disodium ethylenediaminetetraacetate dihydrate (EDTA), 2 mM sodium pyruvate and 50 mM triethanolamine, pH 7.6 (Al-Lawati et al. 2009; Bergmeyer and Gawehn 1978). ATP concentrations were quantified using an ATPliteTM assay kit (PerkinElmer®, MA, USA) by comparing luminescence values to a seven-point standard curve (0.01-2 µM).

2.9. Statistical analysis

Statistics were performed using RStudio version 1.1.463 statistical software. Survival analyses were performed using the packages survival and survminer (Kassambara and Kosinski 2018; Therneau 2015), and the Kaplan-Meier method followed by a post hoc test was used for the comparison of survival between treatments. The Kruskal-Wallis test followed by pairwise comparisons using the Wilcoxon rank test (with Benjamini-Hochberg correction) was employed to compare the cumulative individual food consumption between treatments. The effects of the treatments on enzymatic activities were determined by ANOVA followed by Tukey's HSD test when the data followed a normal distribution or by the Kruskal-Wallis test followed by the post hoc Dunn's test (with Benjamini-Hochberg correction using the agricolae package (de Mendiburu 2013)) when the data followed a nonnormal distribution. Principal component analyses (PCAs) were performed using the FactoMineR package to compare the different treatments according to their effects on physiological markers.

3. Results

3.1. Effects of exposure to pesticides on honey bee survival

The results regarding the toxicity of difenoconazole, either alone or preceded by chronic exposure to imidacloprid and glyphosate, are summarized in Table S1. The mortality recorded under all treatments was higher than that in the control group, which was below 15% at 30 days after emergence. The spray application of difenoconazole (F) induced a cumulative mortality of 37.8%. The I0.01F and I0.1F treatments induced toxicities lower than that induced

by F. The IH0.01F and IH0.1F treatments induced a toxicity identical to that induced by F. Chronic exposure to glyphosate followed by the spray application of difenoconazole (H0.01F and H0.1F) induced toxicities higher than that induced by F, but the difference was only significant under H0.01F, which induced mortality exceeding 49% after 30 days (Fig. 1 and Table S1).

3.2. Effects of exposure to pesticides on food consumption

The effects of the pesticide treatments on the feeding behavior of the honey bees were determined by comparing individual cumulative food consumption between the different treatments (Fig. 2 and Table S2). The honey bees exposed to the different pesticide treatments consumed an equal amount of food to the control group. When we compared the cumulative food consumption between honey bees that were chronically exposed to the pesticides at 0.01 µg/L followed by the spray application of difenoconazole, we noted significantly higher food consumption in the honey bees that were chronically exposed to glyphosate at 0.01 µg/L than in those subjected to the other treatments (Fig. 2 and Table S2).

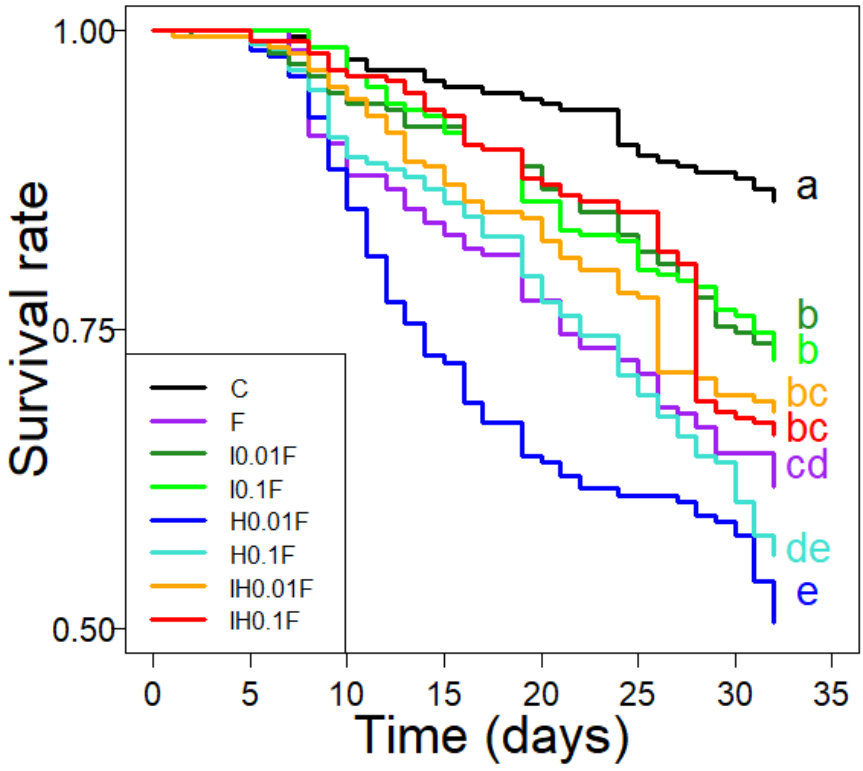

Fig. 1. Effect of chronic exposure to pesticides on the susceptibility of honey bees exposed to difenoconazole

Three days after emergence, honey bees were chronically exposed to food containing no pesticides (C), imidacloprid (I), glyphosate (H) or a binary mixture of imidacloprid + glyphosate (IH) at 0.01 or 0.1 µg/L. On the 10th day after emergence, honey bees were exposed to the fungicide Score® 250 EC (F) via spray application at a field application rate of 0.3 L/ha (75 g/ha). The survival rate was followed for 30 33 days . The data represent the mean proportion of surviving honey bees. The mortalities were analyzed from 7 replicates of 30 bees per treatment using the Kaplan-Meier method followed by a post hoc test for the comparison of survival between

treatments. The numbers after the abbreviations for each treatment refer to the concentrations of the pesticides in the feeding solution. Treatments with different letters are significantly different ($p < 0.05$).

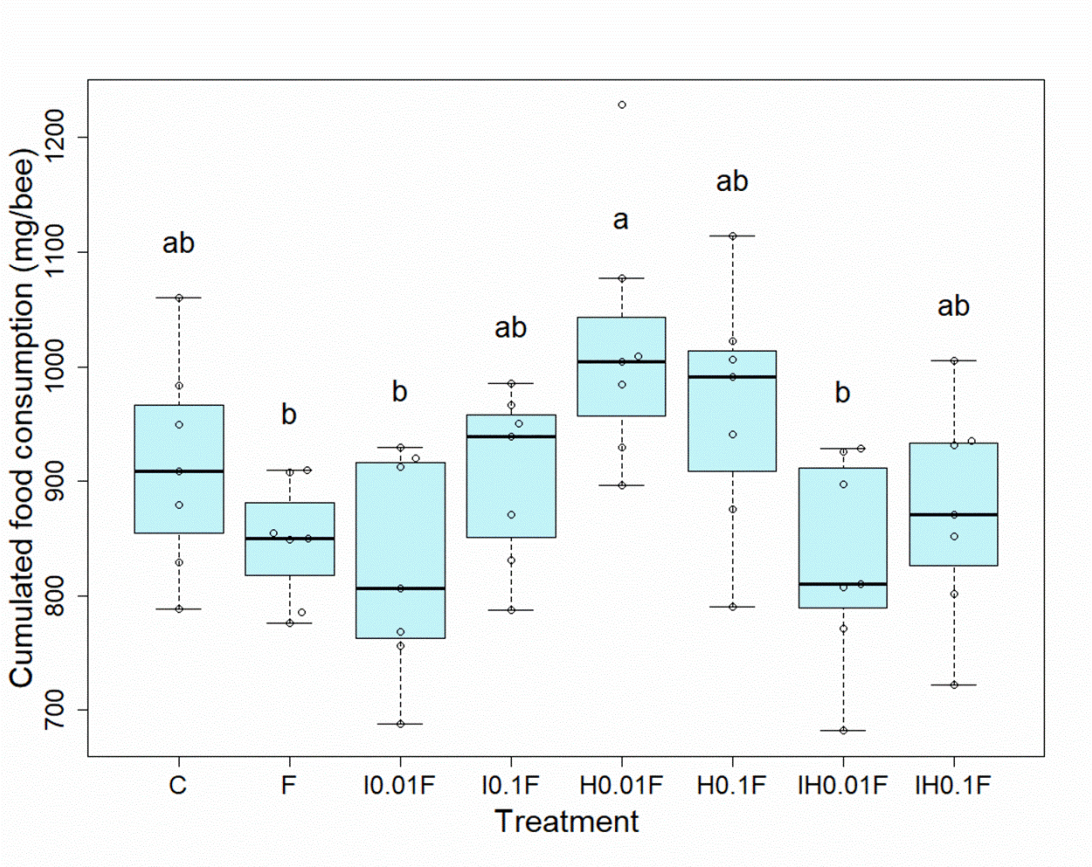

Fig. 2. Effect of exposure to pesticides on food consumption

Three days after emergence, honey bees were chronically exposed to food containing no pesticides (C), imidacloprid (I), glyphosate (H) or a binary mixture of imidacloprid + glyphosate (IH) at 0.01 or 0.1 µg/L. On the 10th day after emergence, honey bees were exposed to the fungicide Score® 250 EC (F) via spray application at a field application rate of 0.3 L/ha (75 g/ha). Box plots represent the cumulative individual consumption (mg/bee) at day 30 determined from 7 cages of 30 bees per treatment. Statistical analyses were performed using the Kruskal-Wallis test followed by pairwise comparisons using the Wilcoxon rank sum test with Benjamini-Hochberg correction. The numbers after the abbreviations for each treatment refer to the concentrations of the pesticides in the feeding solution. Treatments with different letters are significantly different ($p < 0.05$).

3.3. Physiological effects

To determine the physiological effects of chronic exposure to pesticides followed by the spray application of difenoconazole, we examined the modulation of six biomarkers mainly involved in metabolism. The responses of the physiological markers were determined at 20 and 30 days after emergence, which corresponded to 10 and 20 days, respectively, after the spray application of difenoconazole. The concentration of 0.01 µg/L was chosen because a higher mortality was recorded when this concentration was used than when the concentration applied was 0.1 µg/L. The enzymatic activities recorded on days 20 and 30 were expressed as percentages of their respective control values to render the data comparable (Fig. 3, Fig. 4, Table S3 and Table S4). GA3PDH showed changes on days 20 and 30 in the heads, abdomens and thoraxes. Head GA3PDH activity increased to 127% of the control activity under I0.01F at day 20 and decreased to 81.2% under H0.01F at day 30. At day 20, abdomen GA3PDH

activity decreased to 18.3% and 42% of the control activity under I0.01F and IH0.01F, respectively. At day 30, abdomen GA3PDH activity increased to 748.6% of the control activity under H0.01F. At day 20, thorax GA3PDH activity increased to 130.8% of the control activity under the I0.01F and decreased to 57.6% of the control activity under IH0.01F. At day 30, the activity of this enzyme increased to 110.6% and 104% of the control activity under H0.01F and IH0.01F, respectively. LDH activity showed no change in the head at day 20. However, it increased under all exposure modalities at day 30, with 191.6% of the control activity observed under F, 207.7% under I0.01F, 266.8% under H0.01F and 221.8% under IH0.01F. LDH activity showed no change in the midgut at days 20 and 30. CaE-2 activity showed no change in the abdomen at days 20 and 30 or in the midgut at day 20. However, at day 30, it increased in the midgut to 124.3% of the control activity under F. CaE-3 activity showed no change in the abdomen at day 30. However, it increased to 160% of the control activity under H0.01F at day 20. In the midgut, CaE-3 activity showed no change at day 20, but it decreased to 65.5% of the control activity under H0.01F at day 30. G6PDH activity showed no change at day 30, but it decreased to 68.1% of the control activity at day 20 under IH0.01F. At day 20, ATP level decreased to 21.6% and 34.9% of the control level under F and I0.01F, respectively. However , at day 30, it increased to 227% of the control level under IH0.01F.

PCA was conducted to differentiate the treatments based on their effects on the physiological markers (Fig. 5A and 5C). The correlation circles obtained at days 20 and 30 were not sufficient to distinguish the physiological activity levels, as the sum of the two axes on the two sampling dates did not exceed 42% (Fig. 5B and 5D). The results of PCA at day 20 (Fig. 5A) indicated similarity of the physiological status of the honey bees under the different treatments 20 days after the beginning of chronic exposure, while the results of PCA at day 30 (Fig. 5C) indicated the separation of I0.01F and IH0.01F from the other treatments.

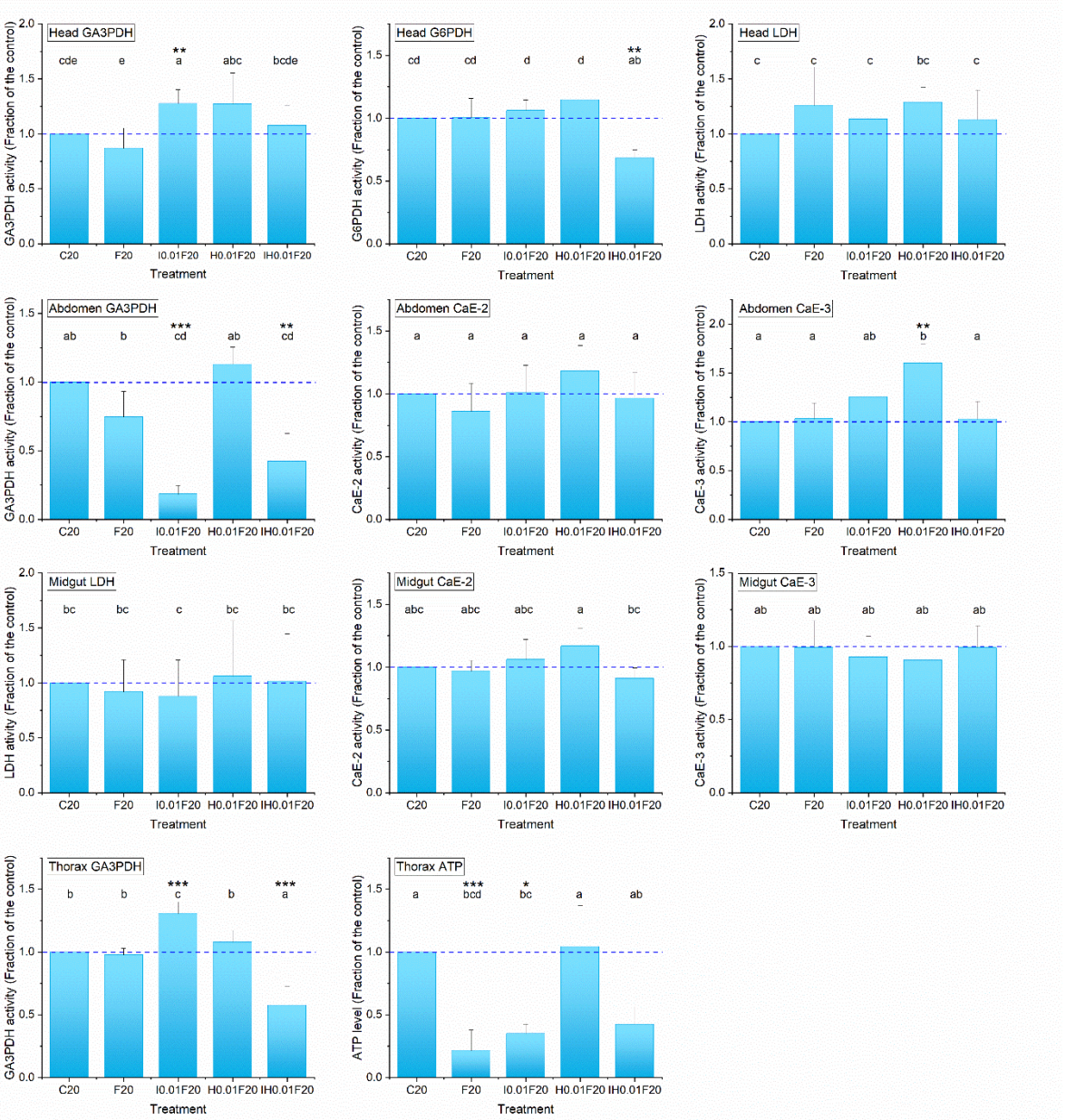

Fig. 3. Physiological effects of exposure to pesticides in honey bees at day 20

Three days after emergence, honey bees were chronically exposed to food containing no pesticides (C), imidacloprid (I), glyphosate (H) or a binary mixture of imidacloprid + glyphosate (IH) at 0.01 or 0.1 µg/L. On the 10th day after emergence, honey bees were exposed to the fungicide Score® 250 EC (F) via spray application at a field application rate of 0.3 L/ha (75 g/ha). Glyceraldehyde-3-phosphate dehydrogenase (GA3PDH) was analyzed in the head (h), abdomen (a) and thorax (t). Carboxylesterases (CaE-2, CaE-3) were analyzed in the abdomen and midgut (m). Glucose-6-phosphate dehydrogenase (G6PDH) was analyzed in the head. Lactate dehydrogenase (LDH) was analyzed in the head and midgut, and adenosine triphosphate (ATP) was analyzed in the thorax. The data represent the mean tissue activities from 7 repetitions (n = 7) performed in triplicate at day 20 and are expressed as percentages of the mean control value. Data with different letters are significantly different ($p < 0.05$). Asterisks indicate significant differences from the control group: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. The dotted lines indicate the levels in controls.

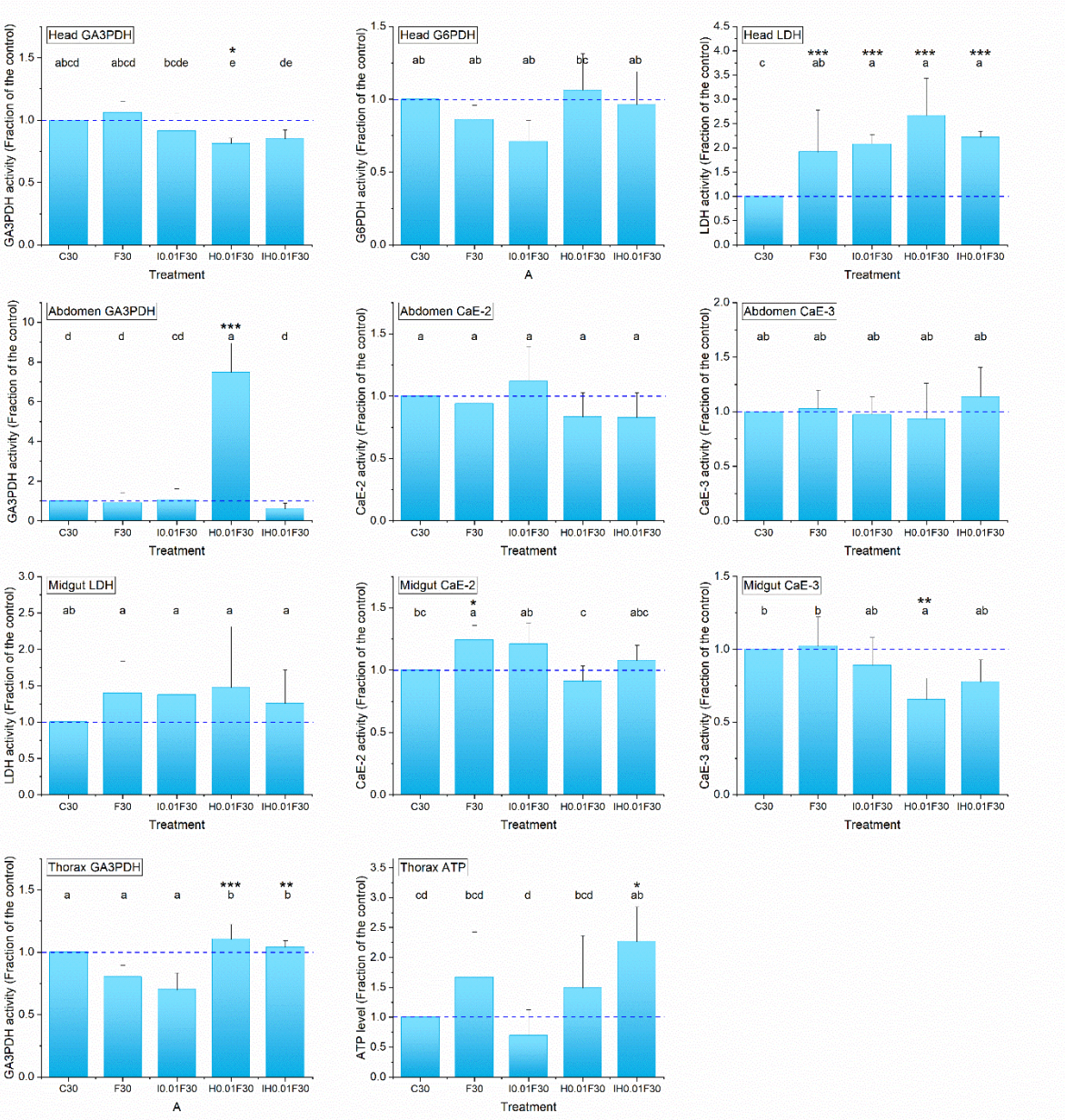

Fig. 4. Physiological effects of exposure to pesticides in honey bees at day 30

Three days after emergence, honey bees were chronically exposed to food containing no pesticides (C), imidacloprid (I), glyphosate (H) or a binary mixture of imidacloprid + glyphosate (IH) at 0.01 or 0.1 µg/L. On the 10th day after emergence, honey bees were exposed to the fungicide Score® 250 EC (F) via spray application at a field application rate of 0.3 L/ha (75 g/ha). Glyceraldehyde-3-phosphate dehydrogenase (GA3PDH) was analyzed in the head (h), abdomen (a) and thorax (t). Carboxylesterases (CaE-2, CaE-3) were analyzed in the abdomen and midgut (m). Glucose-6-phosphate dehydrogenase (G6PDH) was analyzed in the head. Lactate dehydrogenase (LDH) was analyzed in the head and midgut, and adenosine triphosphate (ATP) was analyzed in the thorax. The data represent the means of tissue activities from 7 repetitions (n = 7) performed in triplicate at day 30 and are expressed as percentages of the mean control value. Data with different letters are significantly different ($p < 0.05$). Asterisks indicate significant differences from the control group: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. The dotted lines indicate the levels in controls.

4. Discussion

Honey bees are exposed to a wide variety of fungicides during their foraging activity, especially when farmers treat their crops in the blooming period to fight fungal diseases (Fisher et al. 2017; Xavier et al. 2020). Despite reports on the frequent acute exposure of foragers to fungicides and their persistence in hive environments (Blaga et al. 2020; Piechowicz et al. 2018), the majority of studies on fungicides have focused on their potential to increase the toxicity of other pesticides, such as neonicotinoids and pyrethroids (Biddinger et al. 2013; Colin and Belzunces 1992; Manning et al. 2017; Wade et al. 2019; Zhu et al. 2017a), instead of studying their individual effects. Our study of the effect of a single spray application of difenoconazole at the recommended field application rate revealed delayed toxicity. Mortality started to occur 3 days after spray application and continued to rise, reaching 23.4% (corrected mortality) 20 days after spray application, which is a far-from-negligible effect. To the best of our knowledge, no previous studies have evaluated the possible delayed toxicity of difenoconazole, particularly in its Score® 250 EC form, to honey bees. Studies on other triazole fungicides, such as propiconazole, have failed to demonstrate significant toxicity to honey bees (Ladurner et al. 2005; Stanley et al. 2015). However, these studies were based on an observation period not exceeding 72 hours following topical propiconazole application. Thus, considering the relatively high and delayed toxicity of difenoconazole, it is legitimate to hypothesize that the toxicity of all fungicides is likely underestimated. This emphasizes the importance of further studies aimed at investigating the long-term effects of acute exposure to pesticides, especially to reveal the actual toxicity of fungicides.

In this study, we clearly show that the toxicological status of bees influences the apparent toxicity of pesticides. Prior chronic exposure situations have induced three clear cases of interaction between the fungicide and the other pesticides: (i) toxicity lower than that of the fungicide alone, for an insecticide; (ii) toxicity higher than that of the fungicide alone, for an herbicide; and (iii) no change in fungicide toxicity a pesticide mixture. However, the past exposure and toxicological status of bees are not known when performing toxicity tests for pesticide registration procedures. Thus, these three cases would have resulted in the underestimation of fungicide toxicity, overestimation of fungicide toxicity or no effect on the toxicity estimation, respectively, regardless of the effects that the prior chronic exposures would have been able to induce. This highlights the importance of not using only one or a few toxicological studies in the registration dossier to assess the acute toxicity of pesticides to bees before assigning a first tier value. This is particularly true if we consider that very large variations in the estimates of acute toxicity can be observed, independent of the quality of the experimenter. These variations could be attributed in part to genetic differences in the tested bees. However, considering the huge variation in the 48-hour contact LD50 values of substances such as the pyrethroid insecticide deltamethrin (1.5 to 67 ng/bee) or the nicotinoid insecticide imidacloprid (6.7 to 102 ng/bee) (Atkins et al. 1981; European commission 2002; Nauen et al. 2001; Suchail et al. 2000), the toxicological past of an individual could also play a role in the observed heterogeneity of pesticide toxicity.

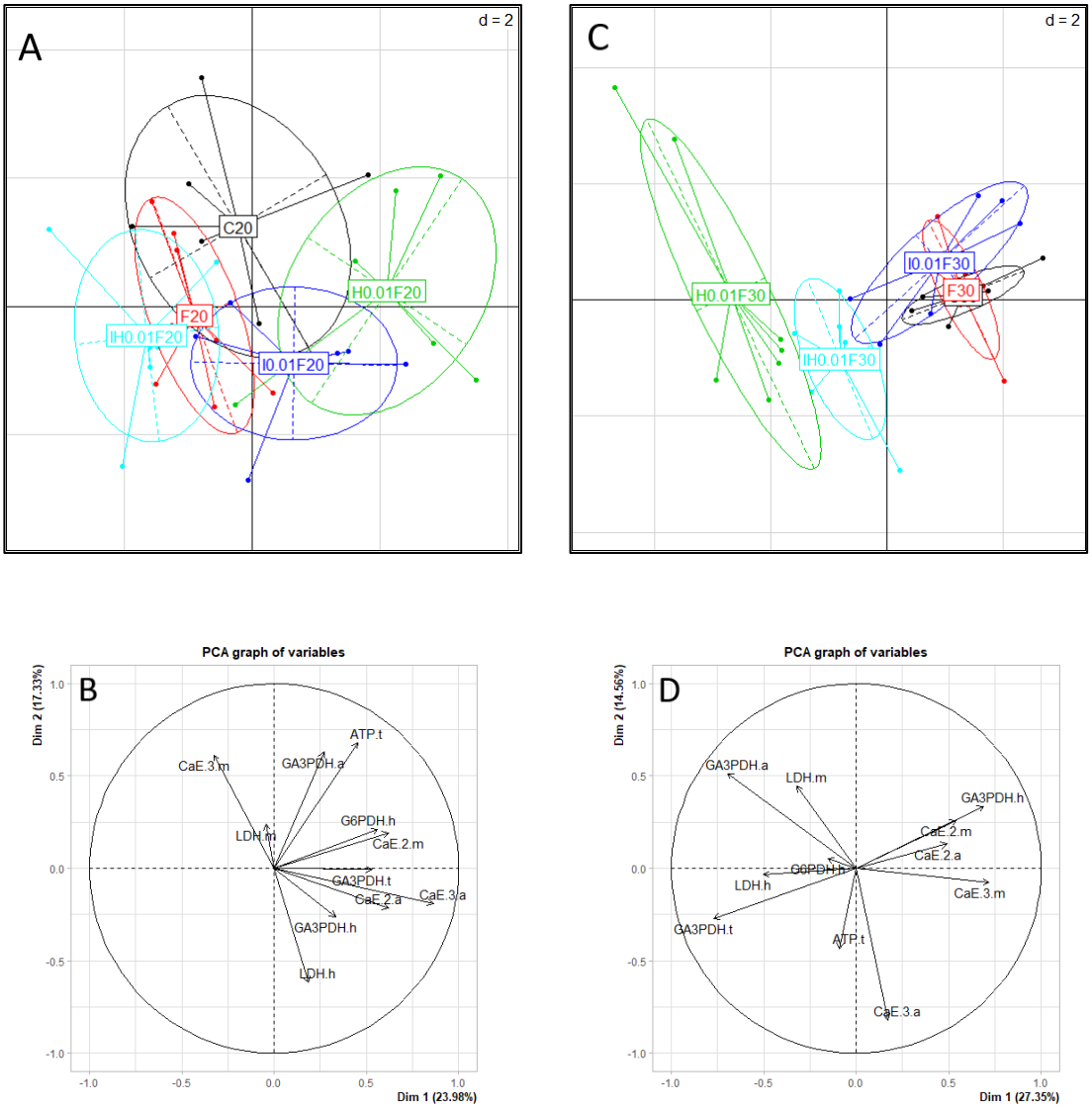

Fig. 5. Physiological effects of pesticides in honey bees exposed to a concentration of 0.01 µg/L

Three days after emergence, honey bees were chronically exposed to food containing no pesticides (C), imidacloprid (I), glyphosate (H) or a binary mixture of imidacloprid + glyphosate (IH) at 0.01. On the 10th day after emergence, honey bees were exposed to the fungicide Score® 250 EC (F) via spray application at a field application rate of 0.3 L/ha (75 g/ha). Glyceraldehyde-3-phosphate dehydrogenase (GA3PDH) was analyzed in the head (h), abdomen (a) and thorax (t). Carboxylesterases (CaE-2, CaE-3) in the abdomen and midgut (m). Glucose-6-phosphate dehydrogenase (G6PDH) in the head. Lactate dehydrogenase (LDH) in the head and midgut and adenosine triphosphate (ATP) in the thorax. Principal component analyses (PCAs) (A and C) provide visual representations of the physiological states of honey bees exposed to pesticides at day 20 (A) and day 30 (C). The correlation circles (B and D) indicate the significance of the enzymes in the PCA representations in honey bees exposed to the pesticides at day 20 (B) and day 30 (D).

This study on the influence of the toxicological status of honey bees on the toxicity of difenoconazole revealed several unexpected and interesting findings. First, chronic exposure to the insecticide imidacloprid at both tested doses in association with difenoconazole resulted in toxicity than that induced by the fungicide alone. This result is very surprising considering that neonicotinoids, including imidacloprid and azole fungicides, have been shown to induce synergistic interactions (Schmuck et al. 2003; Thompson et al. 2014; Zhu et al. 2017b). Second, for the herbicide glyphosate, the association of chronic exposure with difenoconazole induced the greatest effect at the lowest dose. Third, exposure to a greater number of substances did not consistently cause higher toxicity because no modulation of difenoconazole toxicity was observed when the binary mixture was used, regardless of the concentration. These results demonstrate that the effects induced by mixtures of toxic substances are difficult to predict; they do not vary in a predicable manner according to the dose or number of substances involved, and they result from complex interactions that may increase or decrease the toxicity of the substances.

The mechanism by which difenoconazole toxicity is differentially influenced by imidacloprid and glyphosate might involve the metabolism of xenobiotics. In this study, chronic exposure to glyphosate made honey bees more susceptible to difenoconazole, whereas imidacloprid made honey bees less susceptible, and the binary imidacloprid-glyphosate mixture did not affect difenoconazole toxicity. This complex type of interaction could be linked to the inhibition of cytochrome P450 (CYP450) enzymes involved in the metabolism of glyphosate and imidacloprid by difenoconazole (Berenbaum and Johnson 2015). The inhibition of CYP450 can lead to the accumulation of glyphosate instead of its less toxic metabolite aminomethylphosphonic acid (AMPA) (Blot et al. 2019) and, hence, to a high impact of the glyphosate-difenoconazole interaction on mortality. Conversely, the accumulation of imidacloprid instead of its toxic metabolites (5-OH-imidacloprid and an olefin derivative) moderates imidacloprid-difenoconazole toxicity (Suchail et al. 2001). The absence of an effect of the binary mixture on difenoconazole toxicity could be due to a compensation mechanism mediating a trade-off between an increase in toxicity associated with glyphosate and a decrease in toxicity associated with imidacloprid. Therefore, the presence of pesticides at residual concentrations in the hive environment during early developmental stages could result in two situations dramatic for honey bees. The first situation would involve adult honey bees, which would be more susceptible to fungicidal spray application during foraging. The second situation would result in honey bees being less susceptible to the fungicide during the toxicity tests applied in the registration procedure for pesticides, which would lead to an underestimation of fungicide toxicity.

The modulation of physiological life history traits may indicate the involvement of metabolic disruption in the modulation of fungicide toxicity according to the toxicological status of honey bees. Overall, the results of the present study revealed some modulation of all studied physiological markers in honey bees exposed to difenoconazole either alone or associated with prior chronic exposure to glyphosate and/or imidacloprid (individually or in a binary mixture). The spray application of difenoconazole alone induced delayed metabolic changes

in honey bees, reflected by a decrease in ATP levels in the thorax at day 20 and increases in LDH in the head and CaE-2 in the midgut at day 30. These results are consistent with previous findings showing the alteration of honey bee thermoregulation by difenoconazole via the modulation of energetic metabolism, possibly by inhibiting ATPases (Olivari et al. 1991; Vandame and Belzunces 1998). Difenoconazole and other triazole fungicides, such as propiconazole, also alter the levels of metabolites such as lactate and acetate, which are associated with energy metabolism in fish (Souders et al. 2019; Tabassum et al. 2016; Teng et al. 2018).

Difenoconazole induced physiological alterations that were more pronounced when the bees were chronically exposed to imidacloprid and glyphosate, either individually or in a binary mixture, from the time of emergence. The increase in the negative physiological impacts of difenoconazole resulting from chronic exposure to pesticides was expected because both glyphosate and imidacloprid are known to induce metabolic disruptions and oxidative stress in honey bees and other animals (Avigliano et al. 2014; Burchfield et al. 2019; Nicodemo et al. 2014; Powner et al. 2016).

The differences in the effects of the different treatments on the physiological markers between day 20 and day 30 after emergence allow the estimation of the effects linked to honey bee aging. The difference in the effects of treatments associated with honey bee aging was examined via PCAs at days 20 and 30. At day 20, the analysis groups all of the treatments together, whereas at day 30, H0.01F and IH0.01F were clearly separated from the other treatments, reflecting distinct physiological conditions. At day 20, I0.01F had the greatest physiological impact on the honey bees, mainly through the disruption of metabolism revealed by the modulation of GA3PDH and LDH in the head, GA3PDH and ATP in the thorax, and GA3PDH in the abdomen. At day 30, H0.01F and IH0.01F had the greatest impacts on honey bee physiology, resulting in a higher number of affected enzymes than in the control and to the other treatments. At day 30, the major changes in physiological markers observed in honey bees exposed to treatments H0.01F and IH0.01F coincided with higher mortalities under these treatments. This may reflect a strong correlation between the observed lethal effects and the metabolic alterations. Thus, the interference between pesticides and metabolism could induce symptoms similar to nutrient deficiency (Degrandi-Hoffman et al. 2015). This phenomenon could disrupt key physiological functions that rely on carbohydrate oxidation, such as flight (Thompson and Suarez 2009) and honey bee thermoregulation, which essential at both the individual and colony levels (Heinrich and Esch 1994).

5. Conclusion

This study demonstrates that a single spray application of a fungicide at a registered field dosage induces delayed toxicity that compromises the survival of foragers. The toxicological status of individuals may change the susceptibility of bees to the fungicide by increasing, decreasing or not affecting the toxicity of the fungicide. The effects of prior exposure to different pesticides on the susceptibility of bees to a fungicide are not directly linked to the

concentration or the number of substances to which bees are exposed. The fungicide difenoconazole elicits delayed metabolic disruptions that are more pronounced when honey bees are exposed to residual concentrations of other pesticides, such as imidacloprid and glyphosate. These findings emphasize the importance of putting more effort toward adopting new risk assessment approaches that take into consideration the delayed and long-term effects of acute exposures to fungicides at their field application rates. It appears necessary to extend the period during which the effects are observed to more than 96 hours and to include several studies in the pesticide registration dossier to consider possible coexposure situations that could lead to an underestimation of the toxicity of fungicides.

Acknowledgments

This study was supported in part by the recurrent funding of the Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAE) and by the Agence Nationale de la Recherche (ANR) (grant ANR-15-CE34-0004-01). Hanine ALMASRI was supported by grants from Lebanese University (grant number 2364) and the PACA region. The authors would like to thank Jacques Sénéchal and Alexandre Gorit, who are beekeepers at the UR 406 INRAE Research Unit, for their expert beekeeping.

Table S1. Effect of the chronic exposure to pesticides on the susceptibility of honey bees exposed to difenoconazole

Three days after emergence, honey bees were chronically exposed to a food containing no pesticides (C), imidacloprid (I), glyphosate (H) or the binary mixture imidacloprid + glyphosate (IH) at 0.01 and 0.1 µg/L. On the 10th day after emergence, honey bees were exposed to a spray application of the fungicide Score® 250 EC (F) at the field rate of 0.3 L/ha (75 g/ha). The data represent the mortality rates (%) ± standard deviations (SD) and the mortality corrected by the control (C). Numbers after the abbreviations of each treatment refer to the concentrations of the pesticides in the feeding solution. Treatments with different letters are significantly different ($p < 0.05$).

Treatment	Mortality rate (%) ± SD	Corrected mortality (%)	Statistical significance ($p < 0.05$)
C	14.4 ± 0.9	-	a
F	37.8 ± 1.9	23.4	cd
I0.01F	27.1 ± 1.7	12.7	b
I0.1F	27.6 ± 1.8	13.2	b
H0.01F	49.4 ± 2.1	35.0	e
H0.1F	43.8 ± 2.2	29.4	de
IH0.01F	31.9 ± 2.0	17.5	bc
IH0.1F	35.0 ± 2.2	20.6	bc

Table S2. Effect of exposure to pesticides on food consumption

Three days after emergence, honey bees were chronically exposed to a food containing no pesticides (C), imidacloprid (I), glyphosate (H) or the binary mixture imidacloprid + glyphosate (IH) at 0.01 and 0.1 µg/L. On the 10th day after emergence, honey bees were exposed to a spray application of the fungicide Score® 250 EC (F) at the field rate of 0.3 L/ha (75 g/ha). Food consumption was followed during 30 days in 7 cages of 30 bees per treatment by daily measurement of the food consumed and corrected by the bees remaining alive in each cage. The cumulated food consumption per bee is expressed in mg/bee ± standard deviations (SD). The cumulative quantities of ingested active substance (pg/bee) are calculated on the basis on a food density of 1.23 ± 0.02 (n = 10), and the relative ratios to the LD_{50} are calculated based on an LD_{50} of 3.7 ng/bee for imidacloprid and > 100 ng/bee for glyphosate (National Center for Biotechnology Information; Schmuck et al., 2001). Numbers after the abbreviations of each treatment refer to the concentrations of the pesticides in the sucrose solution. Treatments with different letters are significantly different ($p < 0.05$).

Treatment	Cumulated food consumption ± SD (mg/bee)	Daily food consumption ± SD (mg/bee)	Cumulated quantity of substance ingested (pg/bee)	Relative ratio to the LD_{50} (LD_{50}/Ingested)	Statistical significance ($p < 0.05$)
C	914.14 ± 92.72	30.47 ± 3.1	-	-	ab
F	847.45 ± 52.33	28.25 ± 1.74	-	-	b
I0.01F	825.84 ± 95.38	27.53 ± 3.18	6.70	1/552.1	b
I0.1F	904.25 ± 75.10	30.14 ± 2.5	73.39	1/50.4	ab
H0.01F	1018.54 ± 109.49	33.95 ± 3.65	8.27	$1/(1.21\times10^{7})$	a
H0.1F	962.67 ± 105.69	32.9 ± 3.52	78.13	$1/(1.28\times10^{6})$	ab
IH0.01F	831.70 ± 91.07	27.72 ± 3.04	6.75	I: 1/548.2 H: $1/(1.48\times10^{7})$	b
IH0.1F	873.94 ± 94.05	29.13 ± 3.14	71.00	I: 1/52.17 H: $1/(1.41\times10^{6})$	ab

Table S3. Physiological effects of exposure to pesticides in honey bees at day 20

Three days after emergence, honey bees were chronically exposed to a food containing no pesticides (C), imidacloprid (I), glyphosate (H) or the binary mixture imidacloprid + glyphosate (IH) at 0.01 and 0.1 µg/L. On the 10th day after emergence, honey bees were exposed to a spray application of the fungicide Score® 250 EC (F) at the field rate of 0.3 L/ha (75 g/ha). Glyceraldehyde-3-phosphate dehydrogenase (GA3PDH) was analyzed in the head (h), abdomen (a) and thorax (t). Carboxylesterases (CaE-2, CaE-3) was analyzed in the abdomen and midgut (m). Glucose-6-phosphate dehydrogenase (G6PDH) was analyzed in the head. Lactate dehydrogenase (LDH) was analyzed in the head and midgut and adenosine triphosphate (ATP) in the thorax. On the 20th day, 7 samples of 3 tissues were collected for each treatment. For each treatment, the data represent the mean values of enzymatic activities expressed in milli-units of absorbance per minute and per mg of tissue (mAU.min-1.mg of tissue-1) ± standard deviations (SD). ATP levels were expressed in nM.mg of tissue-1 ± SD. ANOVA or Kruskal-Wallis tests were applied to detect significant differences between treatments. Treatments with different letters are significantly different ($p < 0.05$). Arrows indicate an increase or a decrease in activity relative to the control group (C).

		Activity of the physiological markers ± SD				
	Unit	C20	F20	I0.01F20	H0.01F20	IH0.01F20
$GA3PDH_{(h)}$	mAU.min^{-1}.mg of tissue^{-1}	308.207 ± 59.801^{cde}	268.311 ± 56.121^{e}	393.042 ± 39.049^{a}↑	392.010 ± 87.044^{abc}	332.469 ± 56.379^{bcde}
$G6PDH_{(h)}$	mAU.min^{-1}.mg of tissue^{-1}	5.141 ± 0.986^{cd}	5.158 ± 0.790^{cd}	5.449 ± 0.436^{d}	5.895 ± 0.620^{d}	3.503 ± 0.349^{ab}↓
$LDH_{(h)}$	mAU.min^{-1}.mg of tissue^{-1}	10.267 ± 1.475^{c}	12.937 ± 3.586^{c}	11.648 ± 2.204^{c}	13.223 ± 1.375^{bc}	11.601 ± 2.764^{c}
$GA3PDH_{(a)}$	mAU.min^{-1}.mg of tissue^{-1}	193.799 ± 10.211^{ab}	144.586 ± 35.962^{b}	35.467 ± 12.231^{cd}↓	218.883 ± 24.349^{ab}	81.444 ± 39.581^{cd}↓
$CaE\text{-}2_{(a)}$	mAU.min^{-1}.mg of tissue^{-1}	210.381 ± 55.186^{a}	181.000 ± 46.976^{a}	212.229 ± 46.112^{a}	249.018 ± 42.153^{a}	202.861 ± 42.983^{a}
$CaE\text{-}3_{(a)}$	mAU.min^{-1}.mg of tissue^{-1}	148.190 ± 54.950^{a}	152.444 ± 24.297^{a}	185.714 ± 47.475^{ab}	237.000 ± 28.726^{b}↑	152.222 ± 26.772^{a}
$LDH_{(m)}$	mAU.min^{-1}.mg of tissue^{-1}	11.473 ± 2.727^{bc}	10.546 ± 3.347^{bc}	10.089 ± 3.774^{c}	12.161 ± 5.806^{bc}	11.647 ± 4.952^{bc}
$CaE\text{-}2_{(m)}$	mAU.min^{-1}.mg of tissue^{-1}	705.04 ± 82.750^{abc}	683.810 ± 58.860^{abc}	747.281 ± 114.349^{abc}	823.175 ± 98.685^{a}	641.342 ± 59.303^{bc}
$CaE\text{-}3_{(m)}$	mAU.min^{-1}.mg of tissue^{-1}	213.048 ± 37.178^{ab}	211.238 ± 39.028^{ab}	197.286 ± 30.142^{ab}	192.778 ± 27.375^{ab}	210.921 ± 32.179^{ab}
$GA3PDH_{(t)}$	mAU.min^{-1}.mg of tissue^{-1}	562.612 ± 37.216^{b}	550.000 ± 28.410^{b}	735.900 ± 51.537^{c}↑	607.558 ± 52.209^{b}	323.957 ± 83.834^{a}↓
$ATP_{(t)}$	nM. mg of tissue^{-1}	10.961 ± 3.096^{a}	2.364 ± 1.808^{bcd}↓	3.823 ± 0.840^{bc}↓	11.438 ± 3.568^{a}	4.639 ± 1.559^{ab}

Table S4. Physiological effects of exposure to pesticides in honey bees at day 30

Three days after emergence, honey bees were chronically exposed to a food containing no pesticides (C), imidacloprid (I), glyphosate (H) or the binary mixture imidacloprid + glyphosate (IH) at 0.01 and 0.1 µg/L. On the 10th day after emergence, honey bees were exposed to a spray application of the fungicide Score® 250 EC (F) at the field rate of 0.3 L/ha (75 g/ha). Glyceraldehyde-3-phosphate dehydrogenase (GA3PDH) was analyzed in the head (h), abdomen (a) and thorax (t). Carboxylesterases (CaE-2, CaE-3) was analyzed in the abdomen and midgut (m). Glucose-6-phosphate dehydrogenase (G6PDH) was analyzed in the head. Lactate dehydrogenase (LDH) was analyzed in the head and midgut and adenosine triphosphate (ATP) in the thorax. On the 30th day, 7 samples of 3 tissues were collected for each treatment. For each treatment, the data represent the mean values of enzymatic activities expressed in milli-units of absorbance per minute and per mg of tissue (mAU.min-1.mg of tissue-1) ± standard deviations (SD). ATP levels were expressed in nM.mg of tissue-1 ± SD. ANOVA or Kruskal-Wallis tests were applied to detect significant differences between treatments. Treatments with different letters are significantly different ($p < 0.05$). Arrows indicate an increase or a decrease in activity relative to the control group (C).

		Activity of the physiological markers ± SD				
	Unit	C30	F30	I0.01F30	H0.01F30	IH0.01F30
$GA3PDH_{(h)}$	mAU.min^{-1}.mg of tissue^{-1}	363.276 ± 59.538^{abcd}	385.508 ± 33.182^{abcd}	332.067 ± 52.054^{bcde}	295.234 ± 15.981^{e}↓	309.379 ± 25.172^{de}
$G6PDH_{(h)}$	mAU.min^{-1}.mg of tissue^{-1}	3.800 ± 0.518^{ab}	3.275 ± 0.369^{ab}	2.697 ± 0.560^{ab}	4.039 ± 0.947^{bc}	3.656 ± 0.868^{ab}
$LDH_{(h)}$	mAU.min^{-1}.mg of tissue^{-1}	11.343 ± 3.250^{c}	21.735 ± 9.736^{ab}↑	23.566 ± 2.261^{a}↑	30.260 ± 8.716^{a}↑	25.158 ± 1.384^{a}↑
$GA3PDH_{(a)}$	mAU.min^{-1}.mg of tissue^{-1}	28.146 ± 9.910^{d}	25.502 ± 13.791^{d}	29.506 ± 15.683^{cd}	210.714 ± 41.543^{a}↑	17.123 ± 7.678^{d}
$CaE\text{-}2_{(a)}$	mAU.min^{-1}.mg of tissue^{-1}	225.183 ± 43.211^{a}	211.689 ± 23.052^{a}	251.846 ± 61.983^{a}	187.333 ± 44.185^{a}	186.134 ± 45.409^{a}
$CaE\text{-}3_{(a)}$	mAU.min^{-1}.mg of tissue^{-1}	183.944 ± 15.287^{ab}	188.905 ± 31.200^{ab}	178.762 ± 30.166^{ab}	171.905 ± 60.261^{ab}	209.369 ± 49.138^{ab}
$LDH_{(m)}$	mAU.min^{-1}.mg of tissue^{-1}	15.738 ± 5.664^{ab}	21.971 ± 6.980^{a}	21.603 ± 9.462^{a}	23.173 ± 13.212^{a}	19.730 ± 7.268^{a}
$CaE\text{-}2_{(m)}$	mAU.min^{-1}.mg of tissue^{-1}	662.874 ± 55.179^{bc}	824.011 ± 77.522^{a}↑	802.579 ± 112.051^{ab}	603.472 ± 81.762^{c}	714.881 ± 80.764^{abc}
$CaE\text{-}3_{(m)}$	mAU.min^{-1}.mg of tissue^{-1}	237.088 ± 26.100^{b}	242.256 ± 47.339^{b}	210.657 ± 45.519^{ab}	155.215 ± 34.482^{a}↓	184.102 ± 35.546^{ab}
$GA3PDH_{(t)}$	mAU.min^{-1}.mg of tissue^{-1}	517.175 ± 36.927^{a}	414.122 ± 47.849^{a}	361.376 ± 69.825^{a}	572.036 ± 60.353^{b}↑	537.530 ± 27.919^{b}↑
$ATP_{(t)}$	nM.mg of tissue^{-1}	2.025 ± 0.348^{cd}	3.373 ± 1.547^{bcd}	1.399 ± 0.884^{d}	3.015 ± 1.774^{bcd}	4.595 ± 1.170^{ab}↑

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Bilan

Cette étude a permis de mettre en évidence les effets négatifs, sur un pollinisateur, de la pulvérisation des fongicides en pleine floraison. Le produit à base de difénoconazole, induit une toxicité retardée pour les abeilles, avec des mortalités qui dépassent les 35% 20 jours après l'exposition aiguë, et des perturbations au niveau du métabolisme. L'exposition chronique des abeilles au glyphosate, à la plus faible concentration, rend les abeilles plus sensibles au difénoconazole, alors que l'imidaclopride rend les abeilles moins sensibles et le mélange binaire imidaclopride-glyphosate n'a pas d'effet sur la toxicité du difénoconazole. Ainsi, la présence des pesticides dans les matrices apicoles, à des concentrations résiduelles environnementale, pourrait augmenter les effets nocifs de la pulvérisation du fongicide en pleine floraison. D'autre part, elle pourrait aussi rendre les abeilles moins sensibles au fongicide, ce qui pourrait conduire à une sous-estimation de la toxicité des fongicides lors des tests de toxicité effectués avant la mise sur le marché.

Le fongicide provoque des perturbations métaboliques qui apparaissent sur le long-terme et qui sont plus prononcées lorsque les abeilles sont co-exposées à des concentrations résiduelles d'imidaclopride et de glyphosate. Les perturbations métaboliques observées peuvent affecter plusieurs fonctions fondamentales chez les abeilles telles que les capacités de vol et de thermorégulation, du fait que ces deux fonctions dépendent principalement du métabolisme des glucides.

Actuellement, pour l'obtention d'une autorisation de mise sur le marché des produits phytopharmaceutiques, des tests spécifiques sont conduits pour chaque pesticide. Ces tests suivent des critères précis et sont élaborés par des organisations telles que l'Organisation de Coopération et de Développement Économiques (OCDE) et l'Organisation Européenne et méditerranéenne pour la Protection des Plantes (OEPP). Les tests consistent à évaluer les effets létaux qui apparaissent durant les 48 heures qui suivent l'exposition aiguë par voie orale ou par contact au pesticide d'intérêt. Si les mortalités continuent à s'accroitre au-delà des 48 heures, le test peut s'étaler pour une durée maximale de 96 heures (OCDE, 1998). Toutefois, les résultats de notre étude montrent qu'une durée de 96 heures n'est pas suffisante pour détecter les effets létaux d'un pesticide. De plus, la sensibilité au pesticide peut varier en fonction du statut toxicologique des abeilles, d'où l'importance d'adopter de nouveaux tests qui prennent en considération les potentielles sensibilisations par d'autres stresseurs et la possibilité d'effets létaux et sublétaux retardés induits par les pesticides.

Partie 4 : Les effets d'une exposition à des stresseurs identiques, avec les mêmes durées et niveaux d'exposition, peuvent-ils être modulés par la séquence des expositions des abeilles à ces stresseurs et par l'infection par *Nosema* ?

Avant-propos

Les organismes vivants peuvent être exposés à plusieurs facteurs de stress tels que les pesticides et les agents pathogènes. L'interaction entre ces deux facteurs est connue sous le nom d'interaction toxico-pathologique. Cette interaction apparait par exemple chez les abeilles infectées par *N. ceranae* et exposées aux insecticides tels que les néonicotinoïdes et les phényles pyrazoles. L'interaction *Nosema*-insecticide affecte la survie des abeilles et perturbe l'état physiologique des abeilles (Alaux et al., 2010a; Aufauvre et al., 2012; Doublet et al., 2014; Retschnig et al., 2014; Vidau et al., 2011). Toutefois, les effets des interactions *Nosema*-fongicide, *Nosema*-herbicide et *Nosema*-mélange de pesticides sont beaucoup moins étudiés que les interactions *Nosema*-insecticide. Ainsi, nous nous sommes intéressés à étudier les effets de l'interaction entre *Nosema* d'une part, et le glyphosate et le difénoconazole, seuls et en mélanges, d'autres part. Un autre facteur, très peu étudié, qui pourrait jouer un rôle dans la détermination du niveau d'interaction entre les facteurs de stress est la séquence d'exposition aux stresseurs (Aufauvre et al., 2012; Johnson et al., 2013). Ainsi, afin d'évoluer l'effet de la séquence d'exposition aux pesticides, les abeilles ont été exposées au glyphosate et au difénoconazole d'une façon séquentielle et d'une façon chevauchante.

Deux hypothèses sont émises concernant les effets de l'interaction *Nosema*-pesticides.

(i) La première hypothèse est que l'interaction *Nosema*-pesticides pourrait conduire à un effet antagoniste si l'infection par *Nosema* induit les mécanismes de détoxication ou bien si les pesticides ont un effet négatif sur le développement de *Nosema*. En effet, certains fongicides de la famille des triazoles pourraient être efficaces dans le traitement de la microsporidiose (Rossi et al., 1999).

(ii) La deuxième hypothèse est que l'interaction *Nosema*-pesticides pourrait conduire à un effet additif ou synergique si l'infection pas *Nosema* entraine des perturbations physiologiques qui rendraient les abeilles plus sensibles aux pesticides. Ces perturbations incluraient, entre autres, un stress énergétique et des altérations de l'intégrité du tissu épithélial intestinal qui constitue le premier site de protection contre les pesticides ingérés.

Article 4

Article soumis au journal *Ecotoxicology & Environmental Safety* le 23/10/2020

Physiological effects of the interaction between *Nosema ceranae* and sequential and overlapping exposure to glyphosate and difenoconazole in the honey bee *Apis mellifera*

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Résumé

Les agents pathogènes et les polluants, tels que les pesticides, sont des facteurs de stress potentiels pour tous les organismes vivants, y compris les abeilles domestiques. Les herbicides et les fongicides sont parmi les pesticides les plus répandus dans les matrices apicoles et leurs interactions avec *Nosema ceranae* n'est pas bien étudiée. Dans cette étude, les interactions entre *N. ceranae*, l'herbicide glyphosate et le fongicide difénoconazole ont été étudiées dans le cadre d'une exposition séquentielle et chevauchante aux deux pesticides à une concentration de 0,1 µg/L dans la nourriture. Lors de l'exposition séquentielle, les abeilles émergentes ont été exposées à l'herbicide du jour 3 au jour 13 après leur émergence et au fongicide du jour 13 au jour 23. Dans l'expérience d'exposition chevauchante, les abeilles ont été exposées à l'herbicide du jour 3 au jour 13 et au fongicide du jour 7 au jour 17. L'infection par *Nosema*, durant les premières heures qui suivent l'émergence, a fortement affecté la survie des abeilles et a provoqué une mortalité beaucoup plus élevée que celle induite par les pesticides seuls ou en mélange. L'exposition chevauchante aux deux pesticides a entraîné une mortalité plus élevée que celle causée par l'exposition séquentielle ou individuelle. L'exposition chevauchante, et non l'exposition séquentielle, aux pesticides a augmenté de manière synergique l'effet négatif de *N. ceranae* sur la longévité des abeilles. La combinaison de *Nosema* et des pesticides a eu un fort impact sur les marqueurs physiologiques du système nerveux, de la détoxication, des défenses antioxydantes et de l'immunité sociale des abeilles.

Mots-clés : pesticides, agents pathogènes, *Nosema ceranae*, exposition séquentielle, interactions toxico-pathologiques

Abstract

Pathogens and pollutants, such as pesticides, are potential stressors to all living organisms, including honey bees. Herbicides and fungicides are among the most prevalent pesticides in beehive matrices, and their interaction with *Nosema ceranae* is not well understood. In this study, the interactions between *N. ceranae*, the herbicide glyphosate and the fungicide difenoconazole were studied under combined sequential and overlapping exposure to the pesticides at a concentration of 0.1 µg/L in food. In the sequential exposure experiment, newly emerged bees were exposed to the herbicide from day 3 to day 13 after emerging and to the fungicide from day 13 to day 23. In the overlapping exposure experiment, bees were exposed to the herbicide from day 3 to day 13 and to the fungicide from day 7 to day 17. Infection by *Nosema* in early adult life stages (a few hours post emergence) greatly affected the survival of honey bees and elicited much higher mortality than was induced by pesticides either alone or in combination. Overlapping exposure to both pesticides induced higher mortality than was caused by sequential or individual exposure. Overlapping, but not sequential, exposure to pesticides synergistically increased the adverse effect of *N. ceranae* on honey bee longevity. The combination of *Nosema* and pesticides had a strong impact on physiological markers of the nervous system, detoxification, antioxidant defenses and social immunity of honey bees.

Key words: pesticides, pathogens, *Nosema ceranae*, sequential exposure, toxico-pathological interactions

1. Introduction

All living organisms are subjected to multiple stressors from anthropogenic (e.g., pollutants) and natural (e.g., pathogens) sources (Holmstrup et al., 2010). These environmental stressors can act alone or through interactions involving mixtures of pollutants, associations of pathogens, associations of pollutants and pathogens or complex combinations of pathogens and mixtures of pollutants (Feldhaar and Otti, 2020; Shahid et al., 2019). Concerning pollutants, pesticides are of particular concern for wildlife because they exhibit intrinsic expected toxicity designed for plant protection that can elicit adverse lethal and sublethal effects (Goulson et al., 2015). Among wild species, the honey bee is considered a beneficial species and is the most economically valuable pollinator of crop monocultures worldwide (Klein et al., 2007; McGregor, 1976; Roubik, 2002).

Honey bees can be exposed to pesticides belonging to different classes (mainly herbicides, insecticides and fungicides) either directly, during treatment for plant protection or via contact with treated plant parts, or indirectly, by the consumption of contaminated pollen, honey, guttation drops or water (Girolami et al., 2012; Girolami et al., 2009; Krupke et al., 2012; Tapparo et al., 2011). After plant treatments, contaminated nectar and pollen can be transferred to the hive, leading to the accumulation of numerous pesticide residues in the beehive matrices, along with acaricides used for the treatment of *Varroa* infestation (Böhme et al., 2018; Chauzat et al., 2011; Kanga et al., 2019; Lambert et al., 2013; Wiest et al., 2011). In addition, farmers increasingly use tank mixing for the spraying of several pesticides together to enhance the performance of the active substances and reduce pesticide application time and cost (Tornisielo et al., 2013). For example, in the UK, approximately 6 different products are applied in a single spraying event in soft fruits, orchards and vegetable crops (Fryday et al., 2011). Thus, honey bees could be subjected to simultaneous, sequential or overlapping exposure to pesticides. Simultaneous exposure occurs via the ingestion of food contaminated by several pesticides, by contact with plant parts contaminated with different pesticide residues or during plant treatment with a mixture of pesticides. Sequential and overlapping exposure may be observed when consecutive treatments are applied to a crop, when bees travel from one crop to another during foraging, when a migratory beekeeping strategy is applied, when bees consume honey or pollen successively contaminated with different pesticides or when bees are exposed to pesticides outside the hive after having consumed contaminated food (Luttik et al., 2017).

Throughout their life stages, honey bees are subjected to attacks by parasitic mites, scavengers, viruses, bacteria and fungi or microsporidia such as *Nosema* spp. (Genersch et al., 2010a). The interactions between pathogens and pesticides have been exploited to increase the toxicity and reduce the doses of pesticides used to kill pests and minimize the ecological impacts on nontarget species and humans (Ahmed et al., 2020; Baker et al., 2020; Ericsson et al., 2007; Paula et al., 2013; Purwar and Sachan, 2006). However, a combination of pathogens and pesticides could have negative impacts on beneficial species such as honey bees (Coulon et al., 2020; Pochini and Hoverman, 2017). Several studies have shown a significant correlation between virus loads and contamination by pesticides such as acaricides (Locke et al., 2012), insecticides (Coulon et al., 2020; Di Prisco et al., 2013) and fungicides (Simon-Delso et al., 2014) and between pesticides

(mainly insecticides) and *Nosema* spp. (Alaux et al., 2010a; Aufauvre et al., 2012; Aufauvre et al., 2014; Vidau et al., 2011). The microsporidium *Nosema ceranae* is an obligate intracellular parasite that colonizes the epithelial cells of the honey bee gut. It is frequently associated in certain regions with colony losses (Fries, 2010; Fries et al., 2006).

The main effects of *N. ceranae* infection are decreased honey bee survival (Aufauvre et al., 2012; Vidau et al., 2011), hormonal disruption associated with alterations in vitellogenin and juvenile hormone levels (Alaux et al., 2010a; Antunez et al., 2009; Dussaubat et al., 2010), energetic and nutritional stress (Aliferis et al., 2012; Mayack and Naug, 2010) and reduced immunocompetence (Aufauvre et al., 2014). On the other hand, herbicides and fungicides are among the pesticides that are frequently detected in beehive matrices (Lambert et al., 2013; Lopez et al., 2016; Mullin et al., 2010). The predominantly used herbicide is glyphosate (Benbrook, 2016). It inhibits 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS), an enzyme essential for the biosynthesis of aromatic amino acids in plants and some microorganisms (Amrhein et al., 1980; Bode et al., 1984). More than 80% of honey samples of different origins have been shown to contain glyphosate at concentrations ranging between 17 and 342 µg/kg. Glyphosate is also detected in bee bread at levels around 50 µg/kg (Berg et al., 2018; El Agrebi et al., 2020; Pareja et al., 2019; Rubio et al., 2015; Thompson et al., 2019). Among fungicides, azole fungicides, including difenoconazole, exhibit broad-spectrum antifungal activity. They are applied in both preventive and curative treatments due to their systemic properties (Hof, 2001). Their mode of action is based on the inhibition of the fungal lanosterol 14α-demethylase, which is responsible for the transformation of lanosterol into ergosterol, an essential constituent of the cytoplasmic membrane of fungi (Ji et al., 2000). Difenoconazole is present in honey, pollen, bee bread and wax at mean concentrations of 0.6, 43, 270 and 1 µg/kg, respectively (Kubik et al., 2000; Lopez et al., 2016). Thus, emerging honey bees could be exposed to glyphosate and difenoconazole after emergence through the consumption of contaminated bee bread and honey. However, because fungicides and herbicides show low acute toxicity (LD_{50} > 100 µg/bee), their application is allowed during the flowering period, causing particularly high exposure of honey bees through the consumption of contaminated pollen.

While the understanding of the effect of exposure to a single pesticide has increased (Christen et al., 2019a; Hesselbach and Scheiner, 2018; Nicodemo et al., 2014), there is a remaining gap in the assessment of the effects of sequential or overlapping exposure to multiple pesticides (EFSA, 2012), particularly when pesticides are associated with pathogens. Thus, the objectives of this study were to investigate the potential differences between the toxicity induced by sequential and overlapping exposure to pesticides and to determine whether the interactions between pesticides and the pathogen *N. ceranae* depend on the mode of exposure. The study was conducted on emerging bees orally exposed to pesticides to assess the sensitivity of the bees during the first stage of their adult life. The considered pesticides were the herbicide glyphosate and the fungicide difenoconazole. Attention was focused on the effect of exposure to this pesticide combination on survival, *Nosema* development, food consumption and some key physiological systems by analyzing the variations in eight life history traits that can reveal impairment in the integrity of the nervous system, immunity, defenses against oxidative stress and metabolism.

2. Materials and Methods

2.1. Materials

Triton X-100, monosodium phosphate (NaH_2PO_4), sodium chloride (NaCl), sodium bicarbonate ($NaHCO_3$), Tris-potassium phosphate (K_3PO_4), pepstatin A, leupeptin, aprotinin, trypsin, antipain, monopotassium phosphate (KH_2PO_4), glucose, horseradish peroxidase, o-dianisidine dihydrochloride, disodium phosphate (Na_2HPO_4), disodium ethylenediaminetetraacetate dihydrate (EDTA), reduced L-glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), acetylthiocholine iodide (AcSCh), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), dithiothreitol (DTT), D-glucose-*6*-phosphate disodium salt hydrate (G6P), D-fructose-6-phosphate dipotassium salt (F6P), 3,4-dihydroxy-L-phenylalanine (L-DOPA), Tris base, magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), β-nicotinamide adenine dinucleotide phosphate hydrate (β-$NADP^+$), 4-nitrophenyl phosphate di(Tris) salt (p-NPP), hydrogen peroxide (H_2O_2) and a Cytochrome c oxidase Assay Kit (CYTOCOX1) were obtained from Sigma Aldrich® (Saint Quentin Fallavier, France). Difenoconazole (IUPAC name 1-[2-[2-chloro-4-(4-chloro-phenoxy)-phenyl]-4-methyl[1,3]dioxolan-2-ylmethyl]-1H-1,2,4-triazole; CAS No. 119446-68-3) and glyphosate (IUPAC name [N-(phosphonomethyl)glycine]; CAS No. 1071-83-6) (98% purity each) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). A protein solution (bee food), wax foundation sheets and pollen, certified by Ecocert, were purchased from Remuaux Ltd. (Barbentane, France). Candi fondant Apifonda was purchased from Icko Apiculture (Bollène, France). The pheromone Bee Boost® (PseudoQueen) was purchased from Intko Supply Ltd (Vancouver, Canada).

2.2. Honey bees

The experiment was performed on emerging *Apis mellifera* honey bees (≤ 24 h old) obtained from brood frames of six beehives from the experimental apiary of the *Abeilles & Environnement* Research Unit (Bee & Environment Research Unit of INRAE (Avignon, France)) in July 2018. The colonies were continuously checked to examine their health status. Frames of sealed broods were collected and placed in incubators at 33 ± 2°C with 60 ± 5% relative humidity. Honey bees were collected directly on the brood frames after emergence; bees from different brood frames were mixed together and randomly distributed into plastic cages (6 × 8.5 × 10 cm) in groups of 30 honey bees. The cages were then placed in the dark in an incubator at 30 ± 2°C with 60 ± 5% relative humidity. To mimic the hive environment, a small piece of Bee Boost® (PseudoQueen), releasing a queen mandibular pheromone, and a small wax foundation sheet were deposited on the top of the cage. To maintain hygiene, a sheet of filter paper was placed on the bottom of each cage and replaced daily.

2.3. Infection with *N. ceranae* and treatment with pesticides

N. ceranae spore production and identification were carried out according to Rousel et al. (2015) and Paris et al. (2017) (Paris et al., 2017; Roussel et al., 2015) a few days before infection. Infection by *N. ceranae* was performed two hours after the emergence of honey bees. Honey bees were immobilized using sterile forceps and fed individually with 2 µL of a 40% (w/v) sucrose solution containing 100,000 *Nosema* spores. This dose is ten times higher than that required to observe infection in 100% of honey bees (Forsgren and Fries, 2010). During the two days following emergence, honey bees were fed with water, candy and pollen *ad libitum,* and the few dead bees were removed and replaced with infected or uninfected honey bees according to the treatment. Each treatment consisted of seven replicates (n=7 cages with 30 honey bees per cage). On the third day, chronic exposure to pesticides was initiated by replacing water, candy and pollen with a 60% (w/v) sucrose solution containing a 0.1% (v/v) final concentration of DMSO, pesticides (the herbicide glyphosate (H) and/or the fungicide difenoconazole (F)) at 0.1 µg/L or containing no pesticides for the controls. The concentration of 0.1 µg/L is equivalent to 0.083 µg/kg, calculated according to a sucrose solution density of 1.23 ± 0.02 (n=10). The duration of exposure to each pesticide was ten days. The exposure was either sequential or overlapping, with a 4-day coexposure period, starting with H, followed by F (**Fig. 1**). The treatments in which honey bees were infected by *N. ceranae* are all given identifiers starting with "N". The groups that had received only H at day 3 were designated H3 and N.H3, where the bees in the latter group were also infected with *Nosema*. The groups that received only F at day 7 were designated F7 and N.F7, where the bees in the latter group were also infected with *Nosema*. The groups that received only F at day 13 were designated F13 and N.F13, where the bees in the latter group were also infected with *Nosema*. The groups that received H at day 3 followed by overlapping exposure to F at day 7 were designated H3.F7 and N.H3.F7, where the bees in the latter group were also infected with *Nosema*. The groups that received H at day 3 followed by a sequential exposure to F at day 13 were designated H3.F13 and N.H3.F13, where the bees in the latter group were also infected with *Nosema*. Stock solutions of pesticides were prepared by dissolving the active compound in water for glyphosate or in 100% (v/v) DMSO for difenoconazole. The stock solutions were subjected to 1/10th cascade dilutions to obtain 10X stock solutions in 1% DMSO. The stock solutions were diluted 1:10 (v/v) in sucrose syrup to obtain a final concentration of 60% sucrose, 0.1% (v/v) DMSO, 1% (v/v) Bee Food® protein solution and 0.1 µg/L glyphosate, difenoconazole, or both for overlapping exposure. The pesticide concentrations of the stock and feeding solutions were checked according to Paradis et al. (2014) for difenoconazole and Oulkar et al. (2017) for glyphosate (Oulkar et al., 2017; Paradis et al., 2014). For each pesticide solution, the relative standard deviations (RSD) of the measured concentrations compared to the nominal concentrations were less than 10%.

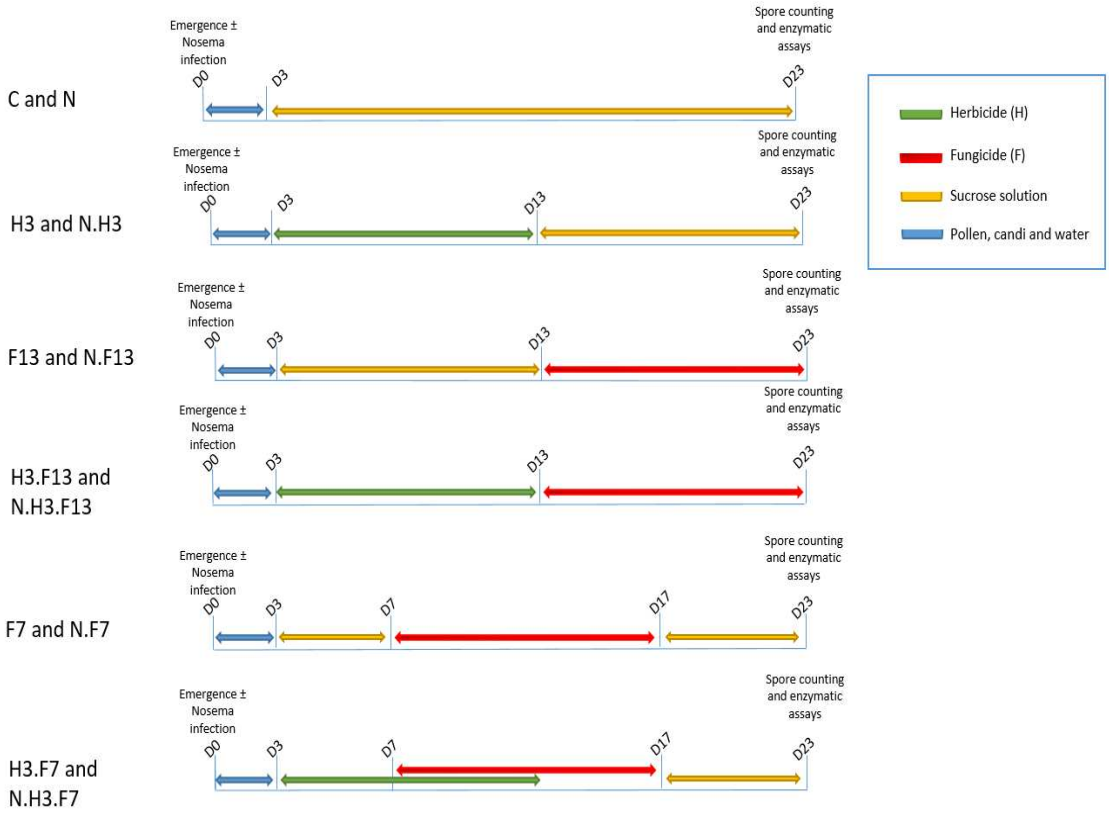

Fig. 1. Experimental design: analysis of physiological life history traits and spore loads following infection by *N. ceranae* and/or sequential or overlapping exposure to an herbicide (H) and a fungicide (F).

Twelve experimental conditions were tested: six uninfected and six infected with *N. ceranae*, with seven replicates per experimental condition (n=7 cages). At emergence (D0), half of the honey bees were infected with *Nosema* (N) and fed pollen, candy and water for 2 days. On the third day (D3), the honey bees in treatments H3 and N.H3 were exposed to H for 10 days. The honey bees in the treatments F7 and N.F7 were exposed for 10 days to F from day seven (D7), while those in treatments F13 and N.F13 were exposed for 10 days to F from day 13 (D13). The honey bees in treatment H3.F7 and N.H3.F7 were exposed to H at D3 and to F at D7. The honey bees in treatment H3.F13 and N.H3.F13 were exposed to H at D3 and to F at D13.

2.4. Survival and food consumption

Mortality and food consumption were recorded daily until the end of the experiment. The dead bees were counted at 08:00 am and discarded to preserve hygienic conditions. Individual food consumption was assessed by measuring the weight of the feeder daily and correcting the consumed food by the bees remaining alive. To estimate the cumulative dose ingested by the bees, the volume of the food ingested was calculated on the basis of a sucrose density of 1.23 ± 0.02.

2.5. Choice of physiological life history traits

The physiological effects induced by *Nosema* and pesticides were assessed by investigating the activity of eight physiological life history traits related to neural activity (acetylcholinesterase (AChE) and cytochrome c oxidase (COx)), immunity (glucose oxidase (GOx), phenoloxidase (POx) and alkaline phosphatase (ALP, also involved in metabolism)), and defense against oxidative stress and detoxification (glutathione-*S*-transferase (GST), glucose-*6*-phosphate dehydrogenase (G6PDH) and catalase (CAT)).

The physiological markers were assessed in tissues in which they are relevant and where their biological activity is particularly high. AChE, GOx and COx were assessed in the head (Alaux et al., 2010a; Armengaud et al., 2000; Belzunces et al., 1988b); CAT and ALP in the midgut (Badiou-Beneteau et al., 2012; Carvalho et al., 2013); POx and G6PDH in the abdomen (Alaux et al., 2010a; Renzi et al., 2016); and GST in the head, abdomen and midgut (Almasri et al., 2020) (**Table 1).**

AChE is involved in learning and memory processing (Gauthier et al., 1992; Guez et al., 2010) through the hydrolysis of the neurotransmitter acetylcholine in cholinergic synapses (Badiou et al., 2007). COx is the terminal enzyme in the respiratory electron transport chain (Lemberg, 1969). Variations in COx activity in the head reflect changes in neuronal cell respiratory activity, which indicate perturbations in the insect nervous system potentially linked with learning deficiencies (Bennett et al., 1996; Decourtye et al., 2004a). GOx is thought to confer a form of social immunity. It is secreted in the hypopharyngeal gland and is responsible for the antimicrobial properties of honey through the production of gluconic acid and hydrogen peroxide (Bucekova et al., 2014). POx contributes to the innate immune system through the activation of the melanization process to seal wound sites and encapsulate parasites and pathogens (Gonzalez-Santoyo and Cordoba-Aguilar, 2012). ALP is involved in immune function, intestinal adsorption and transport mechanisms and metabolism (Chen et al., 2011; Vlahović et al., 2009). GST transforms the peroxidation products of biological molecules formed during oxidative stress into less toxic hydroxyl derivatives and participates in the detoxification process via the conjugation of xenobiotics to reduced glutathione (GSH), making them more hydrophilic (Qin et al., 2013; Yan et al., 2013). G6PDH regenerates NADPH through the pentose phosphate pathway to indirectly promote the formation of GSH (Efferth et al., 2006). CAT transforms hydrogen peroxide (H_2O_2, a toxic reactive oxygen species (ROS)) into water and oxygen, directly leading to the reduction of oxidative stress (Felton and Summers, 1995).

Table 1: Distribution of common and specific markers in honey bee tissues

	Head	Abdomen	Midgut
Common marker	GST	GST	GST
	GOx	POx	ALP
Specific markers	AChE	G6PDH	CAT
	COx	-	-

2.6. Tissue homogenization and analysis of physiological life history traits

The changes in physiological life history traits were analyzed in surviving honey bees 23 days after emergence (equivalent to a maximum 20-day exposure period). To avoid animal suffering when sampling the tissues, the honey bees were first anesthetized with CO_2, the head was immediately separated from the thorax, and the midgut was pulled out from the abdomen. Then, the heads, abdomens (devoid of the intestinal tract) and midguts were placed in 2 mL tubes, weighed and stored at -80°C until analyses. For each treatment, seven repetitions (n=7 samples) of pooled tissues from three bees per sample were analyzed, and each sample was assayed in triplicate during the measurement of enzymatic activity. The tissues were homogenized using a high-speed Qiagen Tissuelyzer II at 30 Hz in five periods of 30 sec at 30 sec intervals after the addition of the extraction medium [10 mM sodium chloride, 1% (w/v) Triton X-100, 40 mM sodium phosphate, pH 7.4 and protease inhibitors (2 µg/mL of pepstatin A, leupeptin and aprotinin, 0.1 mg/mL soybean trypsin inhibitor and 25 units/mL antipain)] to produce 10% (w/v) tissue extracts. After homogenization, the extracts were centrifuged at 4°C for 20 min at 15,000 x $g_{av.}$ and the supernatants were kept on ice for further enzyme assays. The heads used for the measurement of COx were subjected to similar tissue grinding and centrifugation procedures, but the tissues were homogenized in buffer containing 2 mM EDTA, 5 mM DTT, 1 mM F6P, 3.5 mM G6P, 0.5% (v/v) Triton X-100 and 25 mM Tris-potassium phosphate, pH 7.8 (Suarez et al., 2005).

GOx was measured by following the formation of oxidized o-dianisidine at 430 nm in medium containing the head extract, 100 mM glucose, 2.5 units peroxidase, 0.3 mM o-dianisidine and 125 mM monopotassium phosphate, pH 7.0 (Kairo et al., 2017a). AChE was measured at 412 nm in medium containing the head extract, 1.5 mM DTNB, 0.3 mM AcSCh and 100 mM sodium phosphate, pH 7.0 (Belzunces et al., 1988b). COx was measured in the head extract by following the decrease in absorbance at 550 nm resulting from the conversion of ferrocytochrome c into ferricytochrome c. The Cytochrome c Oxidase Assay Kit (CYTOCOX1®) from Sigma Aldrich was used to measure cytochrome c oxidase activity. Briefly, cytochrome c was previously reduced into ferrocytochrome c at 25°C for 20 min in the presence of 0.5 mM DTT. The degree of reduction was considered suitable for the enzyme assay when the A_{550}/A_{565} ratio was between 10 and 20. The reaction medium contained 10 µM ferrocytochrome c, 5 mM KCl, 25 µM DTT

and 50 mM Tris-HCl, pH 7.8. POx was measured by following the transformation of L-DOPA into melanin at 490 nm in medium containing the abdominal extract, 0.4 mg/mL L-DOPA, 20 mM NaCl and 10 mM monosodium phosphate, pH 7.2 (Kairo et al., 2017a). G6PDH was measured by following the formation of NADPH at 340 nm in medium containing the abdominal extracts, 1 mM G6P, 0.5 mM $NADP^+$, 10 mM $MgCl_2$ and 100 mM Tris-HCl, pH 7.4 (Renzi et al., 2016). ALP was measured at 410 nm by following the formation of p-nitrophenol in medium containing the midgut extract, 20 µM $MgCl_2$, 2 mM p-NPP and 100 mM Tris-HCl, pH 8.5 (Badiou-Beneteau et al., 2012). CAT was measured by following the decomposition of H_2O_2 in medium containing the midgut extract, 10 mM H_2O_2 and 100 mM sodium phosphate, pH 7.0 (Beers and Sizer, 1952). GST was measured at 340 nm in the head, abdomen and midguts in medium containing the extract, 1 mM EDTA, 2.5 mM GSH, 1 mM CDNB and 100 mM sodium phosphate, pH 7.4 (Badiou-Beneteau et al., 2012).

2.7. *Nosema ceranae* spore count

To determine the effect of different types of pesticide combinations on the infection success of *Nosema*, 24 honey bees per treatment modality were anesthetized, and their whole intestinal tracts were dissected after head removal to avoid animal suffering. Three extracts of 8 guts were produced to quantify the number of *Nosema* spores. The guts were supplemented with 4 mL of distilled water and placed in Bioreba extraction bags. The guts were homogenized using a Bioreba ball-bearing head. The spore concentration in the homogenates was determined by counting the number of spores in a hematocytometer chamber (Fries et al., 2006).

2.8. Statistical analysis

Statistical analyses were performed using RStudio version 1.1.463 statistical software. Survival analyses were performed using the packages *survival* and *survminer* (Kassambara and Kosinski, 2018; Therneau, 2015), and the Kaplan-Meier method was used, followed by a post hoc test for the comparison of survival between treatments. The Kruskal-Wallis test, followed by pairwise comparisons using the Wilcoxon rank test (with Benjamini-Hochberg correction), was used to compare the cumulative individual food consumption between treatments. The effects of the treatments on enzymatic activities were determined by ANOVA followed by Tukey's HSD test when the data followed a normal distribution or by a Kruskal-Wallis test followed by a post hoc Dunn's test (with *Benjamini-Hochberg* correction using the *agricolae* package (de Mendiburu, 2013)) when the data followed a non-normal distribution.

3. Results

3.1. Effects of *Nosema* and the sequence of exposure to pesticides on honey bee survival

The effects of the sequence of exposure to pesticides on honey bee survival were assessed in both bees infected with *Nosema* and uninfected bees (**Fig. 2 and Table S1**). The analysis of survival revealed that the mortality rates of uninfected honey bees exposed to individual pesticides (H3, F7, and F13) were not significantly different from the mortality rates of control honey bees (C). No effect of sequential exposure to the herbicide followed by the fungicide was observed (H3.F13 versus H3, F13 and C). The day on which the bees started to be exposed to the fungicide did not significantly influence the toxicity of difenoconazole (F13 versus F7). Differences in mortality rates were observed only in H3 (17.7%) and H3.F13 (15.2%), which induced mortality rates significantly higher than that induced by F7 (7.6%).

The mortality rates of all *Nosema*-infected groups were significantly higher than those of their uninfected counterparts (C versus N; H3 versus N.H3; F7 versus N.F7; F13 versus N.F13; H3.F7 versus N.H3.F7 and H3.F13 versus N.H3.F13), revealing a strong adverse effect of *Nosema* on honey bee survival. Based on the observed mortality rates, the toxicity of the *Nosema* pesticide treatments could be ranked as follows: N.H3.F13 (43.8%), N (45.2%), N.H3 (52.4%), N.F7 (52.9%), N.F13 (54.3%), N.H3.F7 (62.9%). However, upon *Nosema* infection, overlapping exposure to the herbicide and the fungicide (N.H3.F7) not only induced significantly higher toxicity than that observed in the controls (N or C) but also induced higher toxicity than that induced by sequential exposure (N.H3.F13) (**Fig. 2 and Table S1**).

3.2. Effects of Nosema and the sequence of exposure to pesticides on food consumption.

The effect of sequential and overlapping exposure to pesticides on potential energetic stress was assessed through the daily monitoring of food consumption in bees infected with *Nosema* and uninfected bees (**Fig. 3** and **Table S2**). In general, honey bees from the *Nosema*-infected and uninfected groups consumed similar amounts of food. In addition, no significant difference in food consumption was observed between the honey bees exposed to glyphosate and difenoconazole either individually or in combination. The only difference in food consumption found among uninfected bees was between F13 and H3.F7, while the only differences among infected bees were found between N.F13 or N.H3.F13 and N.H3 and between N and N.H3. On the basis of a food density of 1.23 ± 0.02 (n = 10) and a pesticide concentration of 0.1 µg/L, honey bees ingested a cumulative dose of glyphosate over 10 days ranging from 18.8 to 26.7 ng/bee, which corresponded to $1/3.8x10^6$ to $1/5.3x10^6$ of the glyphosate LD_{50}, while the cumulative dose of difenoconazole ranged from 18.3 to 26.7 ng/bee, which corresponded to $1/3.8x10^6$ to $1/5.5x10^6$ of the difenoconazole LD_{50} (LD_{50} of both pesticides ≥ 100 µg/bee) (National Center for Biotechnology Information).

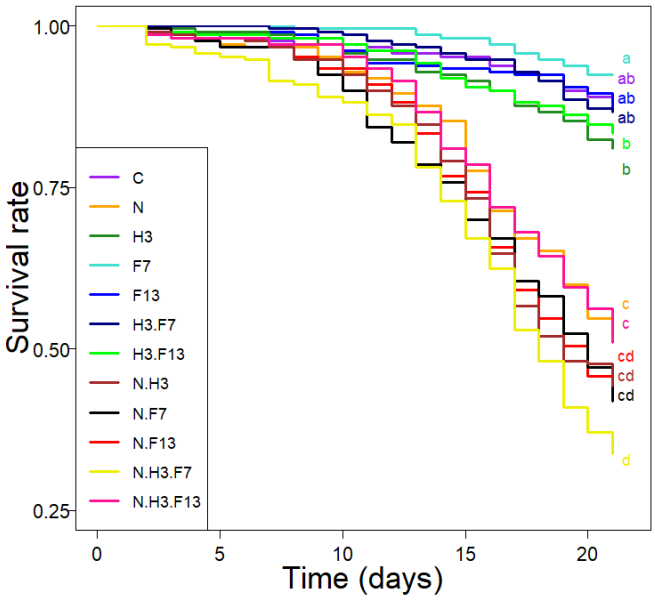

Fig. 2. Effects of *N. ceranae*-pesticide interactions on honey bee survival

Emerging honey bees were infected with *N. ceranae* (N) and then exposed to glyphosate (H) and/or difenoconazole (F) at 0.1 µg/L. H3 corresponds to honey bees exposed to H for 10 days starting at day 3, F7 corresponds to honey bees exposed to F for 10 days starting at day 7, and F13 corresponds to honey bees exposed to F for 10 days starting at day 13. C corresponds to control honey bees that were neither infected nor exposed to pesticides. The data represent the mean proportion of surviving honey bees during 23 days after emergence. The mortalities recorded from 7 replicates of 30 bees per treatment were analyzed using the Kaplan-Meier method followed by a post hoc test for the comparison of survival between treatments. Data with different letters are significantly different ($p < 0.05$).

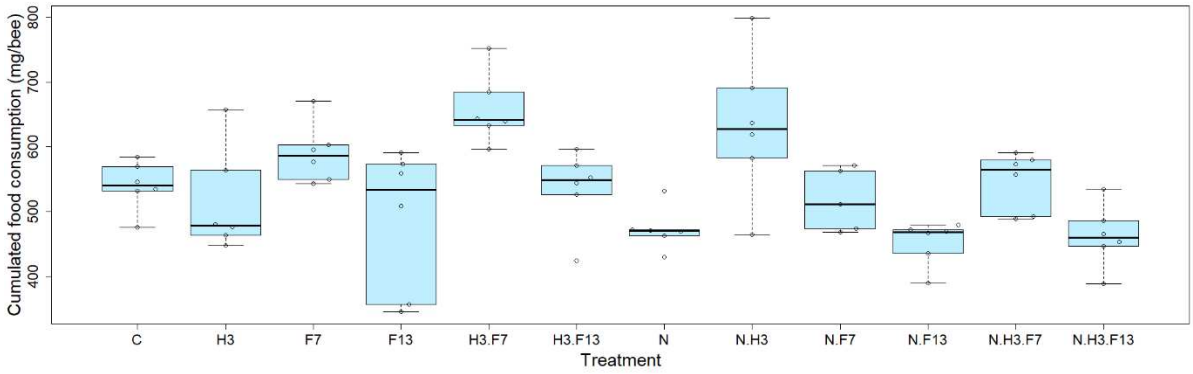

Fig. 3. Effects of *N. ceranae*-pesticide interactions on honey bee food consumption

Emerging honey bees were infected with *N. ceranae* (N) and then exposed to glyphosate (H) and/or difenoconazole (F) at 0.1 µg/L. H3 corresponds to honey bees exposed to H for 10 days starting at day 3, F7 corresponds to honey bees exposed to F for 10 days starting at day 7, and F13 corresponds to honey bees exposed to F for 10 days starting at day 13. C corresponds to control honey bees that were neither infected nor exposed to pesticides. Food consumption was evaluated daily for 23 days. Box plots represent the cumulative individual consumption (mg/bee) at day 23 as determined from 7 cages of 30 bees per treatment. Statistical analyses were performed using the Kruskal-Wallis test followed by pairwise comparisons using the Wilcoxon rank sum test with Benjamini-Hochberg correction.

3.3. Effects of the sequence of exposure to pesticides on the *Nosema* spore load

The number of spores present in the honey bee gut reflects the infection success of *N. ceranae*. *Nosema* spores were not detected in uninfected honey bees. However, in the groups infected with *N. ceranae*, the spore count showed a tendency to be higher in bees exposed to both pesticides either alone or in combination than in unexposed honey bees, although this difference was not significant ($18.5x10^6$ for N, $19.9x10^6$ for N.F7, $21.4x10^6$ for N.H3.F13, $21.7x10^6$ for N.F13, $22.8x10^6$ for N.H3 and $24.6x10^6$ for N.H3.F7) (**Table S3**).

3.4. Physiological effects of exposure to *Nosema* and pesticides

To detect the physiological effects induced by *Nosema*, the sequence of exposure to pesticides and *Nosema*-pesticide interactions, eight physiological markers were analyzed in the honey bee head, abdomen and midgut. To compare the enzymatic activities of the markers obtained at day 23 for each of the 11 treatment modalities, they were expressed as percentages of the control values (**Fig. 4 and Table S4**).

Head AChE increased to 134% of the control (C) activity (114.6 ± 5.0 mAU.min^{-1}.mg of tissue^{-1}) for H3.F13. There was no significant difference in head AChE activity between the control and *Nosema*-infected bees (N). However, the activity increased in all infected bees exposed to pesticides, regardless of the modality of exposure (152% of control activity for N.H3, 141% for N.F7, 130% for N.F13, 135% for N.H3.F7 and 128% for N.H3.F13).

Head GOx increased to 336% and 301% of the activity in the control (C) (1.2 ± 0.5 mAU.min^{-1}.mg of tissue^{-1}) in H3.F7 and H3.F13, respectively. However, the activity decreased to 31% of control activity in N.F13.

Head COx increased to 301% of the activity in the control (C) (46.5 ± 8.8 mAU.min^{-1}.mg of tissue^{-1}) and to 560% of the activity in *Nosema*-infected bees (N) (25.0 ± 12.9 mAU.min^{-1}.mg of tissue^{-1}) only in N.F13. All other treatments did not induce a significant alteration of COx activity.

Head GST did not undergo significant alteration. Abdomen GST increased to 311% of control activity (25.7 ± 33.3 11.5 mAU.min^{-1}.mg of tissue^{-1}) for F7 and 237% for N.H3.F7. Midgut GST decreased after overlapping (H3.F7 and N.H3.F7) and sequential (H3.F13 and N.H3.F13) exposure to glyphosate and difenoconazole in both infected and uninfected honey bees. Expressed as a percentage of control (C) activity (131.0 ± 12.2 mAU.min^{-1}.mg of tissue^{-1}), GST activity was 69% for H3.F7, 79% for H3.F13, 66% for N.H3, 75% for N.H3.F7 and 71% for N.H3.F13. Under exposure to a single pesticide, a decrease was observed only for N.H3, in which GST activity represented 66% of the control activity.

Abdomen POx activity was altered only under N.F13, showing a decrease to 43% of that in the control (7.7 ± 2.1 mAU.min^{-1}.mg of tissue^{-1}). For abdomen G6PDH, no significant change was observed.

Midgut ALP was not altered, regardless of the treatment modality. The activity of midgut CAT decreased in the bees of all groups infected with *Nosema*. Expressed as a percentage of control activity (1.0 ± 0.2 mAU.min^{-1}.mg of tissue^{-1}), CAT activity was 64% in N and 51% in N.H3, 67% in N.F7, 79% in N.F13, 71% in N.H3.F7 and 59% in N.H3.F13.

The analysis of the effects of infection by *Nosema* on the modulation of physiological markers under exposure to pesticides revealed different types of modulation. Infection with *Nosema* elicited increases in the activity of (i) AChE in H3, F7 and H3.F7; (ii) head COx in F13; and (iii) abdomen GST in H3.F7. Infection with *Nosema* elicited decreases in the activity of (i) head GOx in F7, F13, H3.F7 and H3.F13; (ii) head GST in F13 and H3.F7; (iii) abdomen POx in F13; (vi) abdomen GST in F7; (v) midgut GST in H3; and (vi) midgut CAT in H3, F7, H3.F7 and H3.F13.

In general, overlapping and sequential exposure to glyphosate and difenoconazole induced similar changes in the physiological markers. However, in uninfected bees, sequential exposure induced a greater change in AChE activity than did overlapping exposure (H3.F13 > H3.F7). In infected bees, overlapping exposure induced a greater change in abdomen GST activity than did sequential exposure (N.H3.F7 > N.H3.F13).

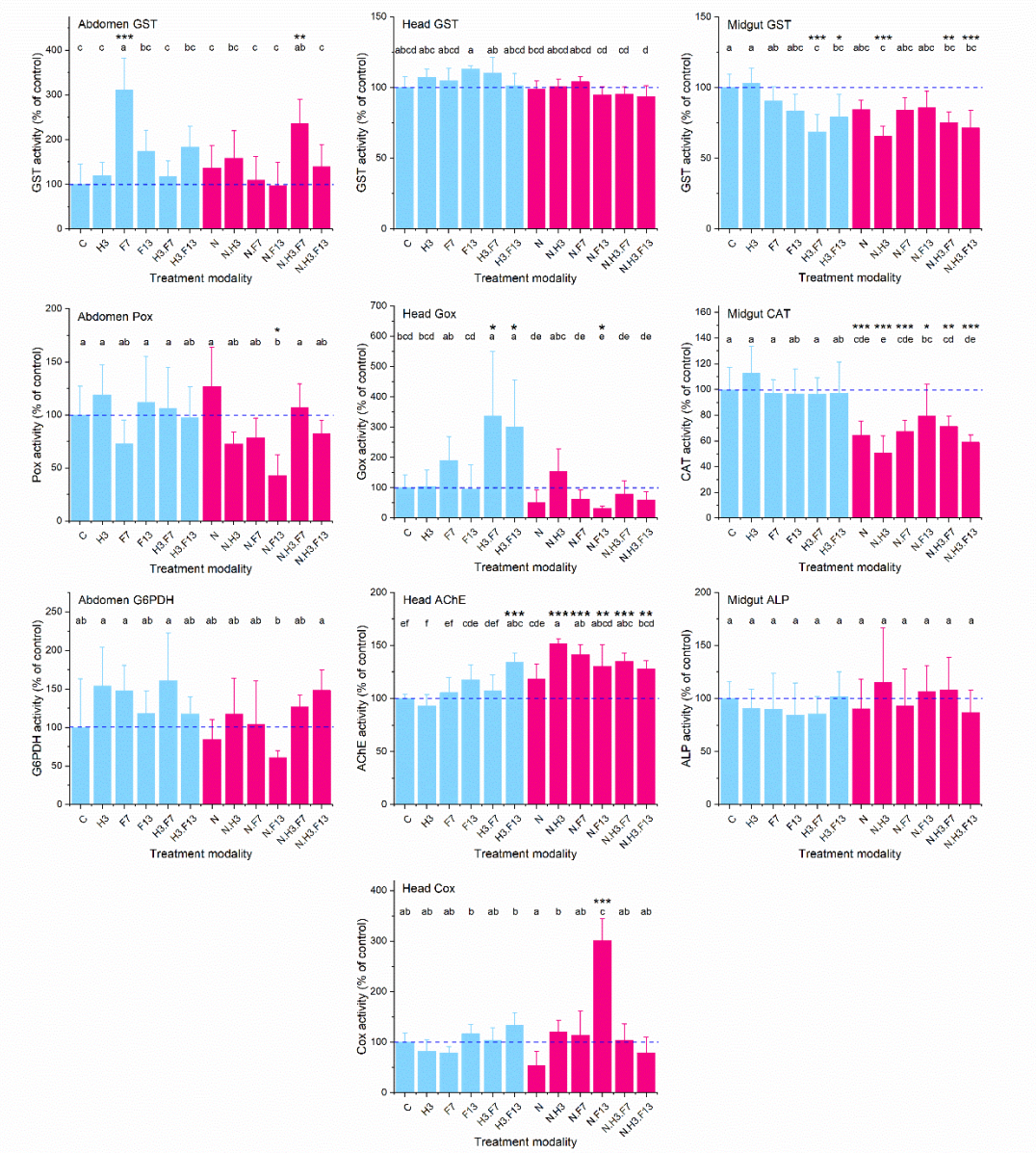

Fig. 4. Effects of *N. ceranae*-pesticide interactions on physiological markers of honey bees

Emerging honey bees were infected with *N. ceranae* (N) and then exposed to glyphosate (H) and/or difenoconazole (F) at 0.1 µg/L. H3 corresponds to honey bees exposed to H for 10 days starting at day 3, F7 corresponds to honey bees exposed to F for 10 days starting at day 7, and F13 corresponds to honey bees exposed to F for 10 days starting at day 13. C corresponds to control honey bees that were neither infected nor exposed to pesticides. A multiple marker approach was applied at day 23 after emergence, which corresponded to the maximum exposure period, to study the effects of the *N. ceranae*-pesticide interaction on the nervous system (AChE and COx), immune system (GOx and POx), oxidative stress and detoxification system (GST, CAT and G6PDH) and metabolism (ALP). GST was measured in the head, midgut and abdomen. AChE, GOx and COx were measured in the head. CAT and ALP were measured in the midgut. G6PDH and POx were measured in the abdomen. To make the data comparable, the enzymatic activities were expressed as percentages of the control values. The exposure modalities above and below the dashed horizontal line indicate increases and decreases in enzymatic activity, respectively, compared to that in the control (C). Data with different letters are significantly different ($p < 0.05$). Asterisks indicate significant differences from the control group (C): * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

4. Discussion

Infection with *N. ceranae* is the main factor influencing honey bee survival. A significant decrease in survival was observed in all *Nosema*-infected groups, confirming that this pathogen is able to reduce honey bee survival if infection occurs in the early stages of the life of adult bees (Aufauvre et al., 2012; Aufauvre et al., 2014; Dussaubat et al., 2012a). The exposure of infected honey bees to glyphosate or difenoconazole individually did not increase the adverse effect of *Nosema*. This result is in accordance with those of previous studies showing no significant effect of glyphosate on *Nosema* infection (Blot et al., 2019). However, to the best of our knowledge, no study has yet been conducted to examine the interactions between *N. ceranae* and azole fungicides. Concerning the effect of sequential exposure, exposure to glyphosate first and then difenoconazole (N.H3.F13) did not alter the effect of *Nosema* on longevity. In contrast, there was a synergistic adverse interaction effect on longevity of overlapping exposure to the pesticides (N.H3.F7) and *Nosema*. This could be due to the effect of the detoxification system of infected honey bees on the sequence of exposure. Infection by *Nosema* induces the overexpression of genes encoding cytochrome P450 (CYP450) monooxygenases (Dussaubat et al., 2012a), which are enzymes involved in the metabolism of xenobiotics (Mao et al., 2009; Mao et al., 2011). Hence, since the metabolism of glyphosate leads to nontoxic metabolites (Blot et al., 2019), the overexpression of CYP450 may increase the metabolism and decrease the toxicity of glyphosate. Azole fungicides, including difenoconazole, are known to be strong inhibitors of CYP450s involved in pesticide metabolism and are able to induce synergistic effects in association with insecticides (Colin and Belzunces, 1992; Johnson et al., 2013; Thompson and Wilkins, 2003). Thus, under overlapping exposure, difenoconazole may enhance the toxicity of glyphosate by inhibiting its metabolism, which is less likely under sequential exposure.

The cumulative ingested doses of glyphosate and difenoconazole were more than one million times less than their respective LD_{50} values. In all cases of exposure, honey bees consumed similar amounts of food, confirming that neither glyphosate nor difenoconazole exhibited an attractive or a repellent effect, at least at the concentration of 0.1 µg/L. These results do not support the hypothesis that an increase in pesticide toxicity in bees infected by *N. ceranae* could result from an increase in pesticide intake (Alaux et al., 2010a). The absence of an effect of glyphosate on food consumption was previously reported in summer honey bees exposed to this herbicide at concentrations of 0.21 and 1.08 g/kg (Blot et al., 2019). In addition, infection by *N. ceranae* did not result in an increase in food consumption, which is in accordance with the results of a previous study (Aufauvre et al., 2012). This confirms that the increase in food consumption is not a pertinent key symptom of infection by *N. ceranae* despite the dependency of this parasite on the energetic resources of the host (Liu, 1984) and the increase in energetic stress following infection (Martin-Hernandez et al., 2011; Mayack and Naug, 2009).

Glyphosate and difenoconazole, either alone or in combination, did not have an effect on *Nosema* proliferation success in gut epithelial cells, even in the sequential N.H3.F7 treatment, which elicited the highest mortality. The absence of an effect of the sequential N.H3.F7 treatment on the *Nosema* count confirms that the strongest adverse effects induced by the interaction between

Nosema and pesticides are not necessarily due to enhanced *Nosema* proliferation, as was previously shown for *Nosema*-fipronil and *Nosema*-thiacloprid interactions (Vidau et al., 2011). Thus, these results confirm that the *Nosema* spore count is not a suitable indicator of the mechanism involved in adverse *Nosema*-pesticide interactions (Collison et al., 2016).

The *Nosema*-pesticide interaction induced an impairment of the nervous system of honey bees. This was revealed by (i) an increase in AChE activity in all *Nosema* pesticide treatments. This enzyme is involved in learning and memory processes in insects (Gauthier et al., 1992), and the perturbation of its activity by pesticides alters the motor functions and behavior of honey bees (Williamson et al., 2013). In addition, the increase in AChE activity under all *Nosema*-pesticide treatments clearly shows that the influence of *Nosema* on honey bee physiology extends beyond the site of action of the parasite. (ii) An increase in COx activity was under the N.F13 treatment, reflecting an increase in neuronal cell respiratory activity that could be linked to learning deficiencies (Bennett et al., 1996; Decourtye et al., 2004a). Therefore, the interactions of *Nosema* with pesticides could contribute over time to colony collapse due to alterations in the behavior, foraging performance and homing flights of honey bees.

Infection by *Nosema* did not significantly alter GOx activity, which is in accordance with the results of two other studies (Alaux et al., 2010a; Kairo et al., 2017a). However, when *Nosema* was associated with difenoconazole exposure initiated 10 days after infection (modality N.F13), a decrease in GOx activity below the physiological level was observed. In addition, infection by *Nosema* abolished the increase in GOx activity induced by overlapping (H3.F7) and sequential (H3.F13) exposure to glyphosate and difenoconazole. Thus, these results suggest that infection by *N. ceranae* tends to suppress the protective immune effect generated by the induction of GOx, possibly as a strategy of the parasite to protect itself from the deleterious impact of H_2O_2 produced by this enzyme.

Globally, *Nosema* infection does not affect the melanization process. This conclusion was supported by the absence of changes in phenoloxidase activity in all *Nosema*-infected groups in our study except for N.F13 and by two other studies (Alaux et al., 2010a; Kairo et al., 2017a; Vázquez et al., 2020). The absence of an effect of *Nosema* on phenoloxidase activity is not correlated with the downregulation of the serine protease SP22 and SP40 genes in *Nosema*-infected honey bees (Aufauvre et al., 2014). These two genes are involved in the activation of prophenoloxidase pathways (Kanost and Clarke, 2005), and a decrease in their expression should result in a decrease in POx activity, which was not observed. Therefore, the alteration of the expression of POx-regulating genes does not always reflect changes at the phenotypic level, probably because of possible posttranslational modifications and regulation. However, honey bees do not rely solely on the melanization process to combat pathogens. A humoral immune response can also be achieved through antimicrobial peptides (AMPs). *N. ceranae* infection was previously reported to downregulate several AMP-coding genes (Antunez et al., 2009; Aufauvre et al., 2014; Chaimanee et al., 2012). Thus, as in the case of POx, the downregulation of AMP genes might not reflect a decrease in AMP levels in honey bee hemolymph, making it difficult to predict the effect of *Nosema* on the humoral immune response.

One way in which pesticides could increase the susceptibility of honey bees to pathogenic infection (Aufauvre et al., 2012) is by impairing immunocompetence (i.e., the ability to mount a functional immune response) (Collison et al., 2016; Wilson-Rich et al., 2009). In our study, neither glyphosate nor difenoconazole, either individually or under sequential or overlapping exposure, decreased honey bee immunocompetence, as they did not impact POx activity or, hence, melanization. Such an absence of an effect of glyphosate on the humoral response was previously reported in honey bee larvae, in which an absence of changes in prophenoloxidase-activating enzyme (PPOact) and several AMP genes was found (Gregorc et al., 2012). However, the large decrease in POx activity elicited by the interaction between *Nosema* and difenoconazole exposure starting at day 13 (N.F13) showed that difenoconazole can weaken the immune defenses of bees infected by *Nosema*. Thus, it appears that pesticides can accentuate the physiological weakening elicited by infectious agents by impairing the immune system. In addition, the fact that N.F13, but not N.F7 ($p = 0.967$), decreased POx activity reveals that the period of exposure may be critical for the impairment of immune defenses.

The effects of *Nosema*, glyphosate and difenoconazole on antioxidant defenses strongly depend on the type of stressor and appear to mainly be tissue specific. Neither glyphosate nor difenoconazole affected the activity of antioxidant enzymes. However, glyphosate has been reported to induce oxidative stress in adult honey bees at higher concentrations under identical exposure durations (Helmer et al., 2015; Jumarie et al., 2017). This suggests that glyphosate and difenoconazole may cause oxidative stress after a quantity of pesticides exceeding a certain threshold is ingested, which depends on the level and duration of exposure. Combined exposure to glyphosate and difenoconazole (H3.F7 and H3.F13) had a stronger impact on oxidative stress than either pesticide applied individually. This was reflected in the decrease in GST activity in the midguts of honey bees exposed to both pesticides, independent of the exposure sequence. The decrease in GST activity could lead to increases in oxidative damage and the toxicity of xenobiotics, as GST transforms lipid peroxidation products into less toxic hydroxyl derivatives and participates in the detoxification of xenobiotics (du Rand et al., 2015). Therefore, the detoxification system could become overwhelmed, and the oxidative balance could be impaired by combined exposure to several pesticides, which may lead to an increase in the pathogenicity of *Nosema* (Goblirsch, 2018). In the midgut, *N. ceranae* did not affect GST activity, which contrasts with the increase in activity observed in infected honey bees 7 and 10 days after infection (Dussaubat et al., 2012a; Vidau et al., 2011). Moreover, midgut CAT activity decreased 23 days after infection under the six exposure modalities involving infection compared with the results of their uninfected counterparts (Control versus N; H3 versus N.H3; F7 versus N.F7; H3.F7 versus N.H3.F7; H3.F13 versus N.H3.F13). This result contrasts with the increase in midgut CAT gene expression observed 7 days after infection (Dussaubat et al., 2012a). The differences in the changes in midgut CAT and GST observed between this study and previously published studies (Dussaubat et al., 2012a; Vidau et al., 2011) might be linked to the changes in gene expression and enzymatic activities that occur during honey bee aging (Aufauvre et al., 2014). This could be explained by an increase in the production of reactive oxygen species (ROS) upon infection by *Nosema* and coexposure to glyphosate and difenoconazole. The increase in ROS triggers an increase in antioxidant defenses to protect the host from the potential harmful

effect of ROS. A battery of enzymatic and nonenzymatic antioxidants are activated, which could explain the upregulation of the expression and the increases in the activities of CAT and GST in the midguts of infected honey bees observed during the first few days after infection (Corona and Robinson, 2006; Ha et al., 2005; Sies, 1993). The activation of these enzymes reduces the levels of ROS markers (soluble peroxides and protein carbonylation) (Paris et al., 2017). A decrease in the level of ROS, which serve as substrates for antioxidant enzymes, induces decreases antioxidant enzymes via a retro-control mechanism, which is in accordance with the decrease in CAT in the midguts 23 days after infection and the return to the normal physiological levels of CAT in the head, G6PDH in the abdomen and GST in the midgut.

5. Conclusion

The present study demonstrates that overlapping, but not sequential, exposure to an herbicide (glyphosate) and a fungicide (difenoconazole) at environmental concentrations synergistically increases the adverse effect of *Nosema* on honey bee longevity. Either alone or under overlapping and sequential exposure, glyphosate and difenoconazole induce disruptions in the nervous system, immunity, detoxification system and antioxidant defenses, particularly when they interact with *N. ceranae*. These findings reveal that the physio-pathological state of the honey bee should be considered a key variable in the assessment of pesticide toxicity in the registration procedure of phytopharmaceuticals.

Acknowledgments

This study was supported in part by the recurrent funding of the Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAE) and by the Agence Nationale de la Recherche (ANR) (grant ANR-15-CE34-0004-01). Hanine ALMASRI was supported by grants from Lebanese University (grant number 2364) and the PACA region. The authors thank Jacques Sénéchal, beekeeper of the UR 406 INRAE Research Unit, for his expert beekeeping.

Table. S1: Effect of Nosema-pesticide interactions on honeybee mortality

Emerging honeybees were infected by N. ceranae (N), then exposed to glyphosate (H) and/or difenoconazole (F) at 0.1 µg/L. H3 corresponds to honeybees exposed to H for 10 days starting at day 3, F7 corresponds to honeybees exposed to F for 10 days starting at day 7 and F13 corresponds to honeybees exposed to F for 10 days starting at day 13. C corresponds to control honeybees uninfected by N. ceranae and unexposed to pesticides. The data represent the mean mortality (%) of 7 repetitions ± standard deviation (SD) and the corrected mortality which is the mortality corrected by the control. Data with different letters are significantly different ($p < 0.05$).

	Mortality rate (%) ± SD	Corrected mortality (%)	Statistical significance ($p < 0.05$)
C	10.48 ± 2.52		ab
H3	17.70 ± 3.00	7.22	b
F7	7.62 ± 1.78	0	a
F13	9.52 ± 1.63	0	ab
H3.F7	12.86 ± 1.78	2.38	ab
H3.F13	15.24 ± 2.62	4.76	b
N	45.24 ± 3.25	34.76	c
N.H3	52.38 ± 1.26	41.90	cd
N.F7	52.86 ± 4.66	42.38	cd
N.F13	54.29 ± 4.66	43.81	cd
N.H3.F7	62.86 ± 3.56	52.38	d
N.H3.F13	43.81 ± 1.92	33.33	c

Table. S2: Effect of Nosema-pesticide interactions on food consumption

Emerging honeybees were infected by N. ceranae (N), then exposed to glyphosate (H) and/or difenoconazole (F) at 0.1 µg/L. H3 corresponds to honeybees exposed to H for 10 days starting at day 3, F7 corresponds to honeybees exposed to F for 10 days starting at day 7 and F13 corresponds to honeybees exposed to F for 10 days starting at day 13. C corresponds to control honeybees uninfected by N. ceranae and unexposed to pesticides. Food consumption was evaluated during the 23 days by measuring the food consumed daily by the bees alive in each of the 7 cages per treatment. The cumulative food consumption and the daily food consumption are expressed in milligrams per bee (mg/bee) ± standard deviation (SD). The relative ratios to the LD_{50} are calculated based on an $LD_{50} \geq 100$ ng/bee for difenoconazole and glyphosate (National Center for Biotechnology Information). Statistical analyses were performed using the Kruskal-Wallis test followed by pairwise comparisons using the Wilcoxon rank sum test with Benjamini-Hochberg correction. Data with different letters are significantly different ($p < 0.05$).

	Treatment	Cumulated food consumption ± SD (mg/bee)	Daily food consumption (mg/bee)	Relative ratio to the LD_{50} (LD_{50}/ Ingested)	Statistical significance ($p < 0.05$)
Uninfected	C	540.31 ± 37.46	27.02 ± 1.87	-	abcd
	H3	514.88 ± 80.53	25.74 ± 4.03	$1/(4.8 \times 10^6)$	bcd
	F7	589.84 ± 46.23	29.50 ± 2.31	$1/(4.2 \times 10^6)$	abc
	F13	488.91 ± 110.23	24.45 ± 5.51	$1/(5.0 \times 10^6)$	cd
	H3.F7	657.94 ± 53.89	32.90 ± 2.69	$1/(3.8\times 10^6)$	a
	H3.F13	535.75 ± 59.94	26.79 ± 3.00	$1/(4.6 \times 10^6)$	abcd
Infected	N	472.69 ± 33.19	23.63 ± 1.66	-	cd
	N.H3	632.10 ± 111.68	31.61 ± 5.59	$1/(3.9 \times 10^6)$	ab
	N.F7	517.22 ± 48.18	25.87 ± 2.41	$1/(4.8 \times 10^6)$	bcd
	N.F13	452.03 ± 34.33	22.60 ± 1.72	$1/(5.5 \times 10^6)$	d
	N.H3.F7	547.02 ± 44.78	27.36 ± 2.24	$1/(4.5 \times 10^6)$	abcd
	N.H3.F13	462.32 ± 48.07	23.12 ± 2.40	$1/(5.3 \times 10^6)$	d

National Center for Biotechnology Information, N. C. f. B., PubChem Database. HSDB : 8370, Source=HSDB, https://pubchem.ncbi.nlm.nih.gov/source/hsdb/837 (accessed on Nov. 5, 2019).

Table. S3: Effect of exposure to pesticides on N. ceranae spore production

Emerging honeybees were infected by N. ceranae (N), then exposed to glyphosate (H) and/or difenoconazole (F) at 0.1 µg/L. H3 corresponds to honeybees exposed to H for 10 days starting at day 3, F7 corresponds to honeybees exposed to F for 10 days starting at day 7 and F13 corresponds to honeybees exposed to F for 10 days starting at day 13. C corresponds to control honeybees uninfected by N. ceranae and unexposed to pesticides. The spore production in the abdomen was assessed at 23 days post infection. The data represents the mean number of spore/honeybee (× 106spores/bee ± standard deviation (SD)) from 24 honeybees per treatment.

Treatment	Spore numeration ($\times 10^6$ spores/bee ± SD)
N	18.5 ± 3.9
N.H3	22.8 ± 8.0
N.F7	19.9 ± 4.7
N.F13	21.7 ± 5.3
N.H3.F7	24.6 ± 3.0
N.H3.F13	21.4 ± 3.2

Table S4: Effects of Nosema-pesticide interaction on physiological markers of honeybees

Emerging honeybees were infected by N. ceranae (N), then exposed to glyphosate (H) and/or difenoconazole (F) at 0.1 µg/L. H3 corresponds to honeybees exposed to H for 10 days starting at day 3, F7 corresponds to honeybees exposed to F for 10 days starting at day 7 and F13 corresponds to honeybees exposed to F for 10 days starting at day 13. C corresponds to control honeybees uninfected by N. ceranae and unexposed to pesticides. A multiple marker approach was performed to study the effects of the N. ceranae-pesticides interaction on the nervous system (AChE and Cox), immune system (Gox and Pox), oxidative stress and detoxification system (GST, CAT and G6PDH) and metabolism (ALP). GST was measured in the head (h), midgut (m) and abdomen (a). While the other markers were measured in only one compartment as indicated in the table 1. Seven samples (n=7) of 3 tissues were collected in each treatment, and the mean value of enzymatic activity was calculated for each treatment. The enzymatic activity was expressed in milliunits of absorbance per minute and per mg of tissue (mAU/min/mg of tissue) ± standard deviation (SD). Data with different letters are significantly different ($p < 0.05$). Arrows indicate an increase (↑) or a decrease (↓) in activity relative to that in the control group.

Physiological marker (mAU/min/mg of tissue)	C	H3	F7	F13	H3.F7	H3.F13	N	N.H3	N.F7	N.F13	N.H3.F7	N.H3.F13
hAChE	114.627 ± 4.952 ef	106.512 ± 12.336 f	120.923 ± 16.556 ef	135.146 ± 15.861 cde	123.287 ± 16.726 def	153.555 ± 10.433 abc ↑	135.546 ± 16.146 cde	173.986 ± 4.82 a ↑	162.094 ± 10.796 ab ↑	149.412 ± 23.147 abcd ↑	154.627 ± 9.028 abc ↑	146.695 ± 9.043 bcd ↑
hGST	89.038 ± 6.724 abcd	95.574 ± 5.052 abc	93.088 ± 7.962 abcd	100.472 ± 2.378 a	98.272 ± 9.784 ab	90.146 ± 7.555 abcd	87.811 ± 5.236 bcd	89.549 ± 4.581 abcd	92.548 ± 3.073 abcd	84.487 ± 5.102 cd	84.617 ± 4.608 cd	83.466 ± 6.723 d
hGOx	1.210 ± 0.498 bcd	1.263 ± 0.648 bcd	2.295 ± 0.954 ab	1.153 ± 0.961 cd	4.068 ± 2.583 a ↑	3.642 ± 1.870 a ↑	0.627 ± 0.486 de	1.858 ± 0.900 abc	0.760 ± 0.370 de	0.377 ± 0.111 e ↓	0.945 ± 0.547 de	0.718 ± 0.323 de
hCOx	46.481 ± 8.762 ab	38.160 ± 10.701 ab	36.692± 5.687 ab	54.337± 8.676 b	48.414 ± 11.544 ab	62.054± 11.517 b	24.986 ± 12.865 a	55.894 ± 10.564 b	52.841 ± 22.562 ab	139.956± 19.982 C ↑	48.289 ± 14.962 ab	36.644 ± 14.582 ab
mCAT	0.985 ± 0.168 a	1.113 ± 0.204 a	0.956 ± 0.104 a	0.952 ± 0.188 ab	0.947 ± 0.127 a	0.957 ± 0.239 ab	0.633 ± 0.110 cde ↓	0.498 ± 0.132 e ↓	0.663 ± 0.086 cde ↓	0.780 ± 0.245 bc ↓	0.702 ± 0.077 cd ↓	0.580 ± 0.056 de ↓
mGST	131.004 ± 12.236 a	135.208 ± 13.909 a	118.616 ± 13.501 ab	109.442 ± 15.226 abc	89.752 ± 16.488 c ↓	103.666 ± 21.012 bc ↓	110.728 ± 8.498 abc	86.164 ± 9.145 c ↓	110.078 ± 11.594 abc	112.620 ± 14.924 abc	98.566 ± 9.690 bc ↓	93.614 ± 16.490 bc ↓
mALP	10.874 ± 1.756	9.877 ± 1.954	9.808 ± 3.679	9.178 ± 3.293	9.303 ± 1.788	11.040 ± 2.558	9.831 ± 3.002	12.542 ± 5.551	10.088 ± 3.817	11.602 ± 2.612	11.752 ± 3.320	9.467 ± 2.304
aG6PDH	4.606 ± 2.906 ab	7.072 ± 2.320 a	6.778 ± 1.526 a	5.454 ± 1.323 ab	7.417 ± 2.827 a	5.413 ± 1.031 ab	3.885 ± 1.172 ab	5.413 ± 2.125 ab	4.778 ± 2.624 ab	2.805 ± 0.400 b	5.827 ± 0.721 ab	6.826 ± 1.206 a
aGST	25.721 ± 11.505 c	30.935 ± 7.285 c	80.039 ± 18.414 a ↑	44.601 ± 12.323 bc	30.071 ± 9.027 c	47.156 ± 11.954 bc	35.089 ± 12.916 c	40.710 ± 15.754 bc	28.079 ± 13.717 c	25.098 ± 13.366 c	60.889 ± 13.739 ab ↑	35.750 ± 12.922 c
aPOx	7.651 ± 2.065 a	9.113 ± 2.155 a	5.571 ± 1.714 ab	8.570 ± 3.305 a	8.116 ± 2.959 a	7.457 ± 2.233 ab	9.699 ± 2.817 a	5.548 ± 0.866 ab	6.015 ± 1.393 ab	3.287 ± 1.490 b ↓	8.204 ± 1.698 a	6.291 ± 0.956 ab

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Bilan

Cette étude a permis de mettre en évidence les effets synergiques de l'interaction entre *Nosema* et le glyphosate et le difénoconazole. Les effets de cette interaction dépendent de la séquence d'exposition des abeilles aux pesticides. Ainsi l'exposition chevauchante, mais non séquentielle, au glyphosate et au difénoconazole, aggrave les effets létaux causés par *Nosema*. Le stress énergétique induit par *Nosema* est supposé produire une augmentation de la consommation de la nourriture contaminée par les pesticides chez les abeilles infectées, conduisant ainsi à l'effet synergique observé suite à l'interaction *Nosema*-pesticide. Cependant, cette hypothèse n'est pas confirmée puisque les abeilles infectées ou non par *Nosema* consomment la même quantité de nourriture. De plus, la charge sporale n'apparait pas comme un indicateur pertinent du mécanisme impliqué dans l'interaction *Nosema*-pesticide.

En dehors, des effets létaux induits par l'association *Nosema*-pesticides, les abeilles survivantes pourraient présenter une altération de la capacité à assurer leurs fonctions spécifiques à l'intérieur et à l'extérieur de la colonie. En effet, ces abeilles présentent de fortes altérations de plusieurs fonctions physiologiques y compris l'activité neurale, l'immunité sociale et les défenses antioxydantes. Ces perturbations à l'échelle individuelle remettent en question l'avenir de la colonie car les associations pesticides-pathogènes constituent un phénomène très fréquent dans les colonies d'abeilles.

Partie 5 : L'exposition chronique des abeilles à l'imidaclopride, au difénoconazole et au glyphosate, seuls ou en mélange ternaire, a-t-elle un effet sur l'établissement du microbiote intestinal?

Avant-propos

L'exposition des abeilles par voie orale à l'imidaclopride, au difénoconazole et au glyphosate, seuls et en mélanges, conduit à des mortalités élevées et des effets sublétaux incluant des perturbations du métabolisme, de l'immunité, du stress oxydant, du système nerveux et du système de détoxication. L'intestin constitue le premier site de contact avec les pesticides ingérés, il est en plus caractérisé par une communauté microbienne spécifique. Cette communauté microbienne joue un rôle prépondérant dans la stimulation du système immunitaire de l'abeille, dans la digestion et la synthèse de vitamines. Des récentes études ont montré que l'imidaclopride et le glyphosate perturbent les communautés bactériennes intestinales des abeilles (Blot et al., 2019; Motta et al., 2020; Motta and Moran, 2020; Motta et al., 2018; Rouze et al., 2019). Cependant, les concentrations auxquelles les abeilles ont été exposées dans ces études étaient beaucoup plus élevées que celles rencontrées par les abeilles dans les matrices de la ruche. D'autre part, à notre connaissance, il n'existe pas d'études sur les effets des fongicides azoles et des mélanges de pesticides sur le microbiote intestinal. Ainsi, nous avons essayé de savoir si une perturbation du microbiote intestinal pouvait expliquer, du moins en partie, les mortalités élevées et les perturbations physiologiques induites par une exposition aux trois pesticides d'intérêt.

Article 5

Mild chronic exposure to pesticides alters physiological markers of honey bee health without perturbing the core gut microbiota

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Résumé

L'intestin de l'abeille est le siège d'un microbiote spécialisé. Ce microbiote joue un rôle essentiel dans la santé de l'abeille, en stimulant le système immunitaire et en intervenant dans la digestion. Toutefois, l'intestin constitue également le principal site d'exposition à une grande variété de pesticides ingérés par les abeilles durant leurs vols de butinage et en se nourrissant de miel et de pain d'abeilles stockés dans la ruche. Des études récentes ont montré que l'exposition au glyphosate peut avoir des effets négatifs sur des membres spécifiques du microbiote intestinal des ouvrières. Toutefois, ces études ont exposé les abeilles à des concentrations élevées de glyphosate et n'ont pas évalué les interactions possibles avec d'autres pesticides. Dans cette étude, nous avons exposé chroniquement des abeilles émergentes à l'imidaclopride, au glyphosate et au difénoconazole, seuls et en mélange ternaire, à la concentration environnementale de 0,1 µg/L de nourriture. Nous avons ensuite étudié les effets de ces expositions sur l'établissement du microbiote intestinal, l'état physiologique, la longévité et la consommation de nourriture. Ainsi, les principales espèces bactériennes n'ont pas été affectées par l'exposition aux trois pesticides à la concentration environnementale. Des effets négatifs ont été observés, mais ils ont été limités à quelques espèces bactériennes transitoires. Cependant, en l'absence du microbiote de base (core microbiota), les pesticides ont induit une perturbation physiologique en altérant directement le système de détoxication, les défenses antioxydantes et le métabolisme de l'hôte. Notre étude indique qu'une exposition aux pesticides, même modérée, peut directement altérer l'homéostasie physiologique des abeilles émergentes, surtout si l'individu est dysbiotique. Cela souligne l'importance d'un établissement précoce d'une communauté bactérienne intestinale saine pour renforcer les défenses naturelles de l'abeille à miel contre les facteurs de stress xénobiotiques.

Mots-clés : *Apis mellifera*, glyphosate, imidaclopride, fongicides azole, microbiote intestinal, état physiologique

Abstract

The honey bee gut hosts a specialized microbiota that plays a critical role in bee health through the stimulation of the immune system and an involvement in digestion. However, the gut also constitutes the primary site of exposure to a wide variety of pesticides ingested by honey bees during foraging, and feeding on stored honey and beebread. Recent studies highlighted that exposure to glyphosate can have negative effects on specific members of the core gut microbiota of honey bee workers. However, these studies exposed bees to relatively high glyphosate concentrations and did not assess the possible interactions with other pesticides. Here, we chronically exposed newly emerged honey bees to an environmental concentration of imidacloprid, glyphosate and difenoconazole, individually and in ternary mixture. We studied the effects of these exposures on the establishment of the gut microbiota, the physiological status, the longevity, and food consumption of the host. The core bacterial species were not affected by the exposure to the three pesticides at these environmental concentrations. Negative effects were observed but they were restricted to few transient non-core bacterial species. However, in the absence of the core microbiota, the pesticides induced physiological disruption by directly altering the detoxification system, the antioxidant defenses, and the metabolism of the host. Our study indicates that even mild exposure to pesticides can directly alter the physiological homeostasis of newly emerged honey bees and particularly if the individuals are dysbiotic (i.e. mostly lack the core microbiota). This highlights the importance of an early establishment of a healthy gut bacterial community to strengthen the natural defenses of the honey bee against xenobiotic stressors.

Keywords: *Apis mellifera*, glyphosate, imidacloprid, azole fungicides, gut microbiota, physiological state

1. Introduction

Through the production of honey, wax, royal jelly, pollen and venom, honey bees constitute a source of income for more than 600 000 beekeepers in Europe (European Commission, 2016). Honey bees also provide, along with other pollinators, ecosystem and agricultural services through the pollination of wild flora and crops used for human consumption (Greenleaf and Kremen, 2006; Klein et al., 2007; Ollerton, 2017; Williams, 1994). Despite the vital importance of honey bees, the number of managed honey bee colonies has decreased in Europe and North America in the last few decades (Potts et al., 2010; Vanengelsdorp et al., 2008). The intense development of agriculture and the appearance of several parasites threatening honey bee health have increased the risk of exposure of honey bees to pesticides and jointly contributed to a steady decline in the number of honey bee colonies (Ellis et al., 2010; Haber et al., 2019; Le Conte et al., 2010; Schreinemachers and Tipraqsa, 2012).

Honey bees can be exposed during foraging to a wide variety of pesticides, such as insecticides, herbicides and fungicides, the three main classes of pesticides used worldwide. These pesticides can be transferred into the colony where they can contaminate at residual concentrations the beehive matrices such as honey, beebread and wax (Chauzat et al., 2009; Juan-Borras et al., 2016; Kasiotis et al., 2014; Mullin et al., 2010). The exposure of honey bees to pesticides could have lethal and sublethal effects. For example, beside their high acute toxicities, neonicotinoid insecticides, such as imidacloprid, are able to impair the cognitive functions, the immune system, the energetic metabolism as well as the detoxification and the antioxidant systems of honey bees (Alptekin et al., 2016; Brandt et al., 2016; Gregore et al., 2018; Schneider et al., 2012; Tesovnik et al., 2020b). Herbicides and fungicides have a low acute toxicity to honey bees. Nevertheless, they can induce adverse sublethal effects. For example, the herbicide glyphosate affects the oxidative balance, the cognitive functions and the larval development of honey bees (Farina et al., 2019; Vazquez et al., 2018; Vázquez et al., 2020). Fungicides, such as those belonging to the azole family that includes difenoconazole, also have negative effects on honey bees. However, the majority of the studies on toxicity of fungicides has focused on their ability to induce synergistic effects together with other pesticides such as pyrethroid and neonicotinoid insecticides (Kretschmann et al., 2015; Wang et al., 2020).

The effect of pesticides on the host gut microbiota has recently benefited from a growing interest, as the gut constitutes the primary site of interaction with ingested pesticides (Yang et al., 2019; Yuan et al., 2019). The honey bee gut harbors a specific bacterial community of low taxonomic complexity dominated by eight to ten bacterial phylotypes (Bonilla-Rosso and Engel, 2018; Corby-Harris et al., 2014; Martinson et al., 2011; Moran et al., 2012), five of which represent the core gut microbiota found in every honey bee worker throughout the planet (Kwong and Moran, 2016). Besides these bacterial species, other less abundant species could also be present (Corby-Harris et al., 2014; Kešnerová et al., 2020). Increasing evidence suggests that the gut microbiota has a direct effect on honey bee health by defending the host from pathogens (Forsgren et al., 2010; Killer et al., 2014), activating the innate immune system (Schwarz et al., 2015), digesting some food components (Engel et al., 2012; Kešnerová et al.,

2017), neutralizing dietary toxins, and biosynthesizing nutrients (Kwong et al., 2014; Lee et al., 2015).

The effects of pesticides on the honey bee gut microbiota were evaluated by several recent studies (Blot et al., 2019; Liu et al., 2020; Motta et al., 2020; Motta and Moran, 2020; Rouze et al., 2019; Syromyatnikov et al., 2020; Yang et al., 2019). However, several gaps still exist to understand the effect of the pesticides on the establishment of the gut microbiota. For example, studies on the effect of glyphosate during and after gut colonization were based on the exposure of honey bees to glyphosate at concentrations found in the worst-case scenarios under semi-field experiments (Thompson et al., 2014b). These concentrations were at least five times higher than those encountered by emerged honey bees in the beehive matrices (Blot et al., 2019; Motta et al., 2020; Motta and Moran, 2020; Motta et al., 2018), which did not exceed 342 µg/kg in honey and 58.4 µg/kg in beebread (Berg et al., 2018; El Agrebi et al., 2020; Rubio et al., 2015). Secondly, recent studies concerning the effect of imidacloprid on gut microbiota did not focus on determining its effect on the early gut colonization (Raymann et al., 2018; Rouze et al., 2019). Thirdly, the effect of triazole fungicides on the bee gut microbiota has not been investigated, in spite of their frequent uses in agriculture and their frequent detections in the beehive matrices (Abdallah et al., 2017; Blaga et al., 2020; Kubik et al., 2000; Lopez et al., 2016; Pettis et al., 2013). Moreover, the studies have focused mainly on the effect of a single pesticide on the gut microbiota. Therefore, we lack knowledge about the potential synergistic effects of mixtures of different pesticides, as they often occur in combination in agricultural landscapes and in the beehive matrices (Abdallah et al., 2017; Mullin et al., 2010; Piechowicz et al., 2018c).

In the present study, we investigated the effect of chronic exposure at an environmental concentration of 0.1 µg/L to imidacloprid, difenoconazole and glyphosate, individually and in ternary mixture, on the early gut colonization and physiology of newly emerged honey bees. The concentration of 0.1 µg/L was chosen because it corresponds to the lowest concentration at which it was shown that imidacloprid, difenoconazole, and glyphosate can interact when they occur as mixtures (Almasri et al., 2020). We performed quantitative PCR (qPCR) and 16S rRNA gene amplicon sequencing to assess the effects of the three pesticides on the total load and composition of the gut microbiota. In addition, we quantified the effects of pesticides and gut colonization on the physiological status of honey bees by studying the modulation of five physiological markers: glutathione-*S*-transferase (GST), glucose-*6*-phosphate dehydrogenase (G6PDH), lactate dehydrogenase (LDH), alkaline phosphatase (ALP) and phenoloxidase (POx). These physiological markers are involved in the detoxification system, oxidant defenses, metabolism, and immunity. Therefore, their alterations reflect perturbations in the key physiological functions and in the normal development of honey bees. Overall, our results show that the three pesticides and their ternary mixture at 0.1 µg/L did not affect the total bacterial load and the abundance of core bacterial species. However, they induce changes in the key physiological functions, which become more pronounced when the core gut bacterial community could not establish.

2. Materials and methods

2.1. Materials

Honey bees were obtained from five healthy looking colonies located in the experimental apiary of Abeilles & Environnement (Bees & Environment) Research Unit at Avignon INRAE Research Centre (South of France). To rear newly emerged honey bees lacking core gut bacteria, tan-colored dark-eyed pupae were removed from the brood combs using sterile forceps and were allowed to emerge in sterile plastic boxes. The plastic boxes were kept for 2 days at 34.5 ± 2°C under 85% relative humidity. Newly emerged honey bees were then distributed in sterile plastic cages (6 × 8.5 × 10 cm; 30 bees per cage) by mixing bees from all five hives into each cage.

2.2. The pesticides

The pesticides considered in this study were the insecticide imidacloprid (CAS No 138261-41-3), the fungicide difenoconazole (CAS No. 119446-68-3) and the herbicide glyphosate (CAS No. 1071-83-6) (98% purity each). They belong to the three main classes of pesticides used worldwide. These active substances were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). They were used either alone or in a ternary mixture (Mix) at a final concentration of 0.1 µg/L, which is consistent with the residual concentrations of these pesticides in honey (Kubik et al., 2000; Nguyen et al., 2009; Rubio et al., 2015). Mother pesticide solutions were previously prepared in sterilized water (glyphosate) or DMSO (imidacloprid and difenoconazole) and stored at -20°C. Working 10X pesticide solutions were prepared in 1% (v/v) DMSO and were stored at -20°C before their dilution in 66.7% sterilized sucrose solution to obtain feeding solutions containing 0.1 µg/L pesticides, 60% (w/v) sucrose and 0.1% (v/v) DMSO.

Imidacloprid is a neonicotinoid insecticide that disrupts the nervous system of insects by acting as an agonist to acetylcholine receptors (Mullins, 1993). It was detected at concentrations of 1.35 µg/kg in pollen and 0.14 to 0.275 µg/kg in honey (Lambert et al., 2013; Nguyen et al., 2009). Glyphosate [N (phosphonomethyl)glycine] is among the most widely used pesticides (Pollak, 2007), it is a herbicide that prevents the production of essential amino acids in plants through the inhibition of the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) present also in some microorganisms (Amrhein et al., 1980; Cao et al., 2012). Glyphosate residues were detected in beebread at concentrations ranging between 52.4 and 58.4 µg/kg and in honey at concentrations ranging between 17 to 342 µg/kg (Berg et al., 2018; El Agrebi et al., 2020; Rubio et al., 2015). Difenoconazole is an ergosterol biosynthesis inhibitor fungicide, it inhibits the lanosterol 14 alpha-demethylase leading to the depletion of ergosterol which is a vital constituent of the fungi cell wall (Hitchcock et al., 1990). It was frequently detected in honey and pollen at concentrations of 0.6 and 43 µg/kg respectively (Kubik et al., 2000).

2.3. Preparation of gut homogenate for bacterial inoculation of newly emerged bees

To obtain a single gut homogenate for subsequent bacterial colonization of experimental bees, we dissected the guts (hindgut, ileum and midgut, excluding the crop) of 15 forager honey bees, collected at the entrance of five hives, and homogenized them in sterile PBS (1 mL sterile PBS per gut) using the Qiagen® TissueLyser II (30 Hz for 3 periods of 10 sec, at 10 sec intervals). The homogenates were then pooled and 1/3rd volume of 80% (v/v) glycerol solution was added to produce a final glycerol concentration of 20% (v/v). The homogenate was aliquoted in sterile Eppendorf tubes and stored at -80°C until use.

2.4. Experimental colonization of honey bees and exposure to pesticides

Twenty-four hours after the emergence of adult honey bees, the bees present in half of the experimental cages were colonized from the gut homogenate. To achieve this, a 2-mL vial containing 300 µL of the gut homogenate was added to each cage. In the vial, the gut homogenate was diluted ten times with sterile PBS and mixed (1:1, v/v) with 50% (w/v) sterile sucrose solution. The bees were allowed to feed on this solution for 3 days. Microbiota-depleted honey bees (MD) were kept under the same conditions and fed sterile sucrose solution, diluted in PBS (1:1, v/v) lacking the gut homogenate.

Newly emerged bees, colonized (colonized bees; CL) or not (microbiota-depleted; MD) with the gut homogenate, were exposed or not (control) to the three pesticides (imidacloprid, glyphosate and difenoconazole) either individually or in a ternary mixture (Mix), in a two by four factorial design. At the beginning of the fourth day post emergence, honey bees were fed for 5 consecutive days with a sterile sucrose solution (60% (w/v)) containing or not (Control, C) pesticides and 0.1% (v/v) DMSO. Four replicates of 30 honey bees per treatment modality were made. During the experiment, the bees fed ad libitum with 60% (w/v) sterilized sucrose solution and gamma-irradiated bee pollen, which was obtained following the protocol of Emery et al. (2017). Cages were kept in an incubator at 30 ± 2°C under 60% relative humidity until the end of the experiment.

For each cage, the daily survival rate and food consumption were recorded, starting on the first day of chronic exposure to pesticides, and dead bees were daily removed for sanitary considerations. Nine days after emergence, bees were anesthetized with CO_2, decapitated, and the guts were extracted for analysis. For the gut microbiota analyses, we dissected 4 guts from honey bees collected from 4 cages per treatment (n=16 per treatment). Each gut was placed into a sterile 2 mL Eppendorf tube, flash frozen in liquid nitrogen then stored at -80°C. Thus, 16 samples of one honey bee worker were analyzed per treatment modality (resulting in a total of 160 experimental samples). For the analysis of physiological life history traits, the heads, midguts and abdomens (with the intestinal tract removed) were separately sampled. Three

tissues were pooled together to form a sample, weighed and stored at -80°C until analysis. For each treatment, seven repetitions (n = 7 samples of three pooled tissues per sample) were analyzed and each sample was assayed in triplicate for enzymatic activity.

2.5. DNA extraction from honey bee gut tissue

Total DNA was extracted from each dissected gut using the FastPure Bacteria DNA Isolation Mini Kit (Vazyme Biotech Co., Ltd (China)). Each gut was put in 1 mL PBS and bead beated twice, for 45 s at 6 m/s, with a Fast-Prep24TM5G homogenizer (MP Biomedicals). A fraction of 330 µL of each homogenate was then transferred to new sterile 1.5 mL tubes and centrifuged for 1 min at 10000 rpm. The pellet was supplemented with 180 µL of lysozyme (prepared according to the manufacturer's instructions) and placed in a water bath for two hours at 37°C. We then followed the rest of the manufacturer's protocol. The resulting dry pellet was resuspended in 50 µL of sterile water. For each batch of DNA extractions, two blank extractions (extractions in which no experimental tissue was added to the reagents; eight total blank extractions) were also performed and served to identify and exclude bacterial contaminants present in laboratory reagents during 16S rRNA gene amplicon sequencing (see below).

2.6. Quantitative PCR for the determination of absolute abundance

Total bacterial loads in experimental honey bee guts was quantified by quantitative PCR (qPCR) assays with universal 16S rRNA gene primers (F: AGGATTAGATACCCTGGTAGTCC; R: YCGTACTCCCCAGGCGG; Kešnerová et al., 2017). The 16S rRNA gene copy numbers were normalized against the host actin gene (amplified with primers F: TGCCAACACTGTCCTTTCTG and R: AGAATTGACCCACCAATCCA; (Zufelato et al., 2004)) as previously described (Kešnerová et al., 2020; Kešnerová et al., 2017). Briefly, qPCR was performed on a StepOnePlus instrument (Applied Biosystems) in 96-well plates. Each sample was amplified in triplicate, in a final volume of 10 µL containing 0.4 µL of each forward and reverse primer (5 µM), 5 µL of 2 × SYBR® Select Master Mix, 3.2 µL of MilliQ water and 1 µL of extracted DNA. The PCR program consisted of an initial denaturation step at 50°C for 2 min, followed by 95°C for 2 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. Melting curves were generated after each run at 95°C for 15 s and 60°C for 1 min and increments of 0.3°C until reaching 95°C for 15 s. Each plate contained a positive and negative control. The absolute quantity of each target was determined based on the standard curves of serial dilutions of plasmids (pGEM®-T Easy vector; Promega) containing the target sequence (Kešnerová et al., 2020). The number of bacterial cells per gut were determined by first calculating 'raw' copy numbers of each target in 1 µL of DNA from the *cycle quantification* (Cq) value and the standard curve using the formula $n = E^{(\text{intercept} - Cq)}$ (Gallup, 2011). These values were multiplied by the elution volume of the DNA extractions to obtain calculations per gut. We next

normalized the bacterial 16S rRNA gene copies against the median number of actin gene copies, dividing by the 'raw' actin copy number for the given sample and multiplying by the median number of actin gene copies across all samples. Finally, these values were divided by four, as this roughly represents the mean number of 16S rRNA gene loci present across honey bee gut symbionts (Kešnerová et al., 2017). Normalization with the actin gene was performed to reduce the effect of gut size variation and extraction efficiency.

2.7. 16S rRNA gene amplicon sequencing

The V4 region of the 16S rRNA gene was amplified using primers 515F-Nex (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA) and 806R-Nex (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAAT), containing the Illumina adapter sequences for Nextera XT indexes and the primers of the V4 region of the 16S rRNA gene (Caporaso et al., 2011), as described in the Illumina 16S metagenomic sequencing library preparation guide (https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf) and by Kešnerová et al. (2020). The first PCR step was performed in a total volume of 25 µL, using 12.5 µL of 2× Phanta Max Master Mix (Vazyme, Nanjing, China), 5 µL of MilliQ water, 2.5 µL of each primer (5 µM), and 2.5 µL of template DNA. The PCR program consisted of an initial denaturation step at 98°C for 30 s, followed by 25 cycles of 98°C for 10 s, 55°C for 20 s and 72°C for 20 s, and a final extension step at 72°C for 5 min. Amplifications were verified by 2% agarose gel electrophoresis. PCR products were purified with Clean NGS purification beads in a 1:0.8 ratio of PCR products to beads, and eluted in 27 µL of 10 mM Tris-HCl pH 8.5. A second PCR step was then performed to append unique dual indexes to each sample. The PCR was performed in a total volume of 25 µL, using 12.5 µL of 2× Phanta Max Master Mix (Vazyme, Nanjing, China), 5 µL of MilliQ water, 2.5 µL of Nextera XT index primers 1 and 2 (Nextera XT Index kit, Illumina) and 2.5 µL of template DNA. The PCR program consisted of an initial denaturation step at 95°C for 3 min followed by eight cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s, and a final extension step at 72°C for 5 min. The final libraries were purified using Clean NGS purification beads in a 1:1.1 ratio of PCR product to beads, and eluted in 27.5 µL of 10 mM Tris-HCl pH 8.5. The amplicon concentrations, including the negative PCR controls, were then quantified by PicoGreen and pooled in equimolar concentrations (with the exception of the negative controls and the blank extractions, which were pooled in equal volumes instead). We verified that the final pool was of the right size using a Fragment Analyzer (Advanced Analytical) and performed sequencing on an Illumina MiSeq sequencer, producing 2 × 250 bp reads, at the Genomic Technology Facility of the University of Lausanne.

2.8. Processing of 16S rRNA gene amplicon-sequencing data

We obtained a total of 13,976,585 raw sequences across 160 honey bee gut samples, two negative PCR controls, two mock community samples and 16 blank DNA extractions. Raw sequencing data were quality filtered with Trimmomatic (Bolger et al., 2014) using LEADING:3, TRAILING:3, SLIDINGWINDOW:4:15, and MINLEN:180. The quality-filtered data were analyzed with the Divisive Amplicon Denoising Algorithm 2 (DADA2) pipeline ("dada2" package version 1.14.1 in R) (Callahan et al., 2016). All functions were run using the recommended parameters (https://benjjneb.github.io/dada2/tutorial.html) except that at the filtering step we truncated the F and R reads after 232 and 231 bp, respectively. We then set randomize=TRUE and nbases=3e8 at the learnErrors step. The SILVA database (version 132) was used for taxonomy assignments of the identified amplicon-sequence variants (ASVs). Any ASV classified as mitochondria, chloroplast or Eukaryota ("phyloseq" package version 1.30.0 (McMurdie and Holmes, 2013), "subset_taxa" function) were removed. We then used both the "prevalence" and "frequency" methods (method = "either") in the R package "decontam" v.1.6.0 (Davis et al., 2018) to identify and remove contaminants introduced during wet lab procedures, using the negative PCR controls and the blank samples as reference, which allowed us to identify and filter out 109 such ASVs. After removing negative, blank and mock samples, the final dataset consisted of 6,524,951 reads belonging to 288 ASVs across the 160 experimental samples.

2.9. Statistical analyses of combined 16S rRNA gene amplicon-sequence and qPCR data

To calculate absolute bacterial abundances of each ASV, the proportion of each ASV in each sample inferred from MiSeq data were multiplied by the total 16S rRNA gene copy number of each sample as measured by qPCR. Bray-Curtis dissimilarities and weighted and unweigthed UniFrac distances were calculated after aligning all ASV sequences with DECIPHER v.2.14.0 and building a phylogenetic tree with Phangorn v.2.5.5 (Schliep, 2011). ADONIS and ANOSIM tests were then ran to assess differences in community structure between microbiota and pesticide treatments overall, and for pesticide treatment differences within each microbiota treatment individually.

To test for differences between pesticide treatments for each individual ASV within each microbiota treatment, we used a permutation approach (referred to as Permutation T-Test) as done in (Kešnerová et al., 2020). Briefly, we randomized the values of the calculated copy numbers for each ASV 10,000 times and computed the *t* values for the tested effect for each randomized dataset. The *p* values corresponding to the effects were calculated as the proportion of 10,000 *t* values that were equal or higher than the observed one. Pairwise comparisons between individual treatment groups were performed by Tukey's HSD using "multcomp" package (Hothorn et al., 2008) using *glht* function on the model. *P* values were adjusted using the Bonferroni method.

2.10. Survival and food consumption

Mortality and food consumption were followed daily during the whole experiment. Dead bees were counted at 8 am and removed for hygienic considerations. Individual food consumption was assessed by measuring the weight of the feeder daily. The food consumed was corrected by the bees remaining alive. An evaporation control was included to accurately calculate the food consumed by the bees.

2.11. Choice of physiological life history traits

The physiological effects induced by pesticides were assessed by investigating the activity of GST, G6PDH and LDH in the head, abdomen (with the intestinal tract removed) and midguts, and the activity of ALP and POx in the midguts. GST and G6PDH are involved in the detoxification process and in the protection against oxidative stress. GST acts through the reduction of hydroperoxides into alcohols and the conjugation of reduced glutathione (GSH) to xenobiotics such as pesticides (du Rand et al., 2015). G6PDH yields NADPH, which is essential for cytochrome P450 (CYP450) catalysis (Xiao et al., 2015). NADPH is also involved in the anti-oxidative defenses through the regeneration of GSH from its oxidized form (Renzi et al., 2016). LDH is involved in the energy metabolism in insects, precisely in the glycolytic pathway. Under anaerobic conditions, it catalyzes a reversible reduction of pyruvate into lactate using NADH as a cofactor (Singh et al., 2017). ALP is a metabolic enzyme involved in the adsorption and transport mechanism through the gut epithelium; it is also involved in the immune response (Bates et al., 2007; Coleman, 1992; Vlahović et al., 2009). POx plays a role in the constitutive immune response of insects through catalysis of the melanization process involved in sealing wounds and encapsulation of foreign bodies such as parasites and pathogens (Collison et al., 2016; Kanost and Gorman, 2008).

2.12. Analysis of physiological life history traits

For the analyses of physiological markers, heads, abdomens and midguts were mixed with an extraction medium to make 10% (w/v) extract. The extraction medium consisted of 10 mM sodium chloride (NaCl), 1% (w/v) Triton X-100 and 40 mM sodium phosphate pH 7.4, and contained protease inhibitors (2 µg/mL antipain, leupeptin and pepstatin A, 25 units/mL aprotinin and 0.1 mg/mL soybean trypsin inhibitor) (Belzunces et al., 1990). The extract was grinded using the Qiagen® TissueLyser II (30 Hz for 5 periods of 30 sec, at 30 sec intervals), then centrifuged at 4°C for 20 min at 15000 g_{av}. The supernatant was then collected for analysis.

The physiological markers of each repetition were spectrophotometrically assayed in triplicate at 25°C. LDH activity was determined by measuring the regeneration of nicotinamide adenine

dinucleotide (NAD^+) at 340 nm. The reaction medium contained 0.2 mM of the reduced form of nicotinamide adenine dinucleotide (NADH), 5 mM disodium ethylenediaminetetraacetate dihydrate (EDTA), 2 mM sodium pyruvate and 50 mM triethanolamine pH 7.6 (Al-Lawati et al., 2009; Bergmeyer and Gawehn, 1978). GST activity was determined by measuring the conjugation of GSH to 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm. The reaction medium contained 1 mM EDTA, 1 mM CDNB, 2.5 mM GSH and 100 mM Na/K phosphate pH 7.4 (Habig et al., 1974). G6PDH activity was determined by following the formation of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm. The reaction medium contained 10 mM magnesium chloride ($MgCl_2$), 0.5 mM nicotinamide adenine dinucleotide phosphate ($NADP^+$), 1 mM glucose-*6*-phosphate (G6P) and 100 mM Tris-HCl pH 7.4 (Renzi et al., 2016). ALP was determined by following the formation of p-nitrophenol at 410 nm. The reaction medium contained 2 mM p-nitrophenyl phosphate (*p*-NPP), 20 µM $MgCl_2$ and 100 mM Tris-HCl pH 8.5 (Bounias et al., 1996). POx was measured by following the transformation of 3,4-dihydroxy-L-dihydroxyphenylalanine (L-DOPA) into melanin at 490 nm. The reaction medium contained 20 mM NaCl, 0.4 mg/mL L-DOPA, and 10 mM monosodium phosphate pH 7.2 (Alaux et al., 2010a).

2.13. Statistical analyses

Statistical analyses of survival, food consumption and effects of the treatments on enzymatic activities were performed using R software (Rstudio Version 1.1.463). We determined the effects of pesticide treatments and gut colonization on survival using the packages *survival* and *survminer* (Kassambara and Kosinski, 2018; Therneau, 2015), and the Kaplan-Meier (log-rank test) method followed by post hoc test. The Kruskal-Wallis test was used to determine the effect of the different treatments on food consumption by comparing the individual cumulative sucrose consumption during the exposure period. To test the effects of the different treatments on the physiological markers, we performed an ANOVA followed by Tukey's HSD test, when the data followed a normal distribution, or a Kruskal-Wallis test followed by post hoc Dunn's test (with Benjamini-Hochberg correction using the *agricolae* package (de Mendiburu, 2013)), when the data followed a non-normal distribution. In addition, a cluster analysis (hierarchical clustering results) was performed using PermutMatrix software (Caraux and Pinloche, 2004). Each measure was normalized according to the colonized control treatment, and unweighted pair group method with arithmetic mean (UPGMA) was conducted to determine Euclidian distances to be used as the linkage rule for clusters.

3. Results

3.1. Effect of pesticide treatments on gut microbiota loads and community composition

To test the influence of chronic exposure to pesticides on early gut colonization, we performed our experiment on newly emerged honey bees, which we colonized (CL) or not (MD) with a

gut homogenate and exposed for five consecutive days to low concentrations (0.1 µg/L) of pesticides.

Gut bacterial loads were significantly different and up to three times higher in CL compared to MD groups (Wilcoxon rank sum tests, p=1e-12), and while the CL bees harbored a core gut microbiota, MD bees were only colonized by opportunistic bacteria, the majority of which are known to typically reside in the hive environment **(Fig. 1A and Fig. 1B)**. ADONIS tests based on Bray-Curtis dissimilarities and ANOSIM tests based on weighted and unweighted UniFrac distances of MiSeq data normalized by qPCR, showed a statistically significant difference between CL and MD honey bees (p=0.001 for all tests). In addition, a principal component analyses revealed that the samples were significantly separated according to the gut colonization status **(Fig. 1D)**.

Average total bacterial abundances were similar in all comparisons of pesticide-treated and control bees in both CL and MD groups (ANOVA, p=0.4 for MD and p=0.56 for CL) **(Fig. 1C)**. ADONIS and ANOSIM tests did not show any statistical difference between pesticide treatments in CL honey bees (all p>0.05), while a significant pesticide treatment effect was observed in MD bees (all p < 0.025). However, size effects were small (ADONIS Bray-Curtis dissimilarities, R^2 = 0.10; ANOSIM weighted UniFrac, R=0.076; ANOSIM unweighted UniFrac, R=0.038) and a significant Betadisper test (p=0.002) suggested that pesticide treatments in MD were not homogeneous in their multivariate dispersions. We nevertheless assessed pesticide treatment effects on 16S rRNA gene copy numbers for all individual ASVs by means of Permutation T-Test. In total, 42 ASVs had significant treatment effects **(Fig. 2A)**, ten of which were common between CL and MD groups. In MD and CL groups respectively, 24 and 10 ASVs had significant pesticide treatment effects **(Fig. 2B and Fig. 2C)**. None of these ASVs belonged to the core gut microbiota, representing changes in opportunistic transient bacteria of relatively low abundance and facultative presence across individual bee guts.

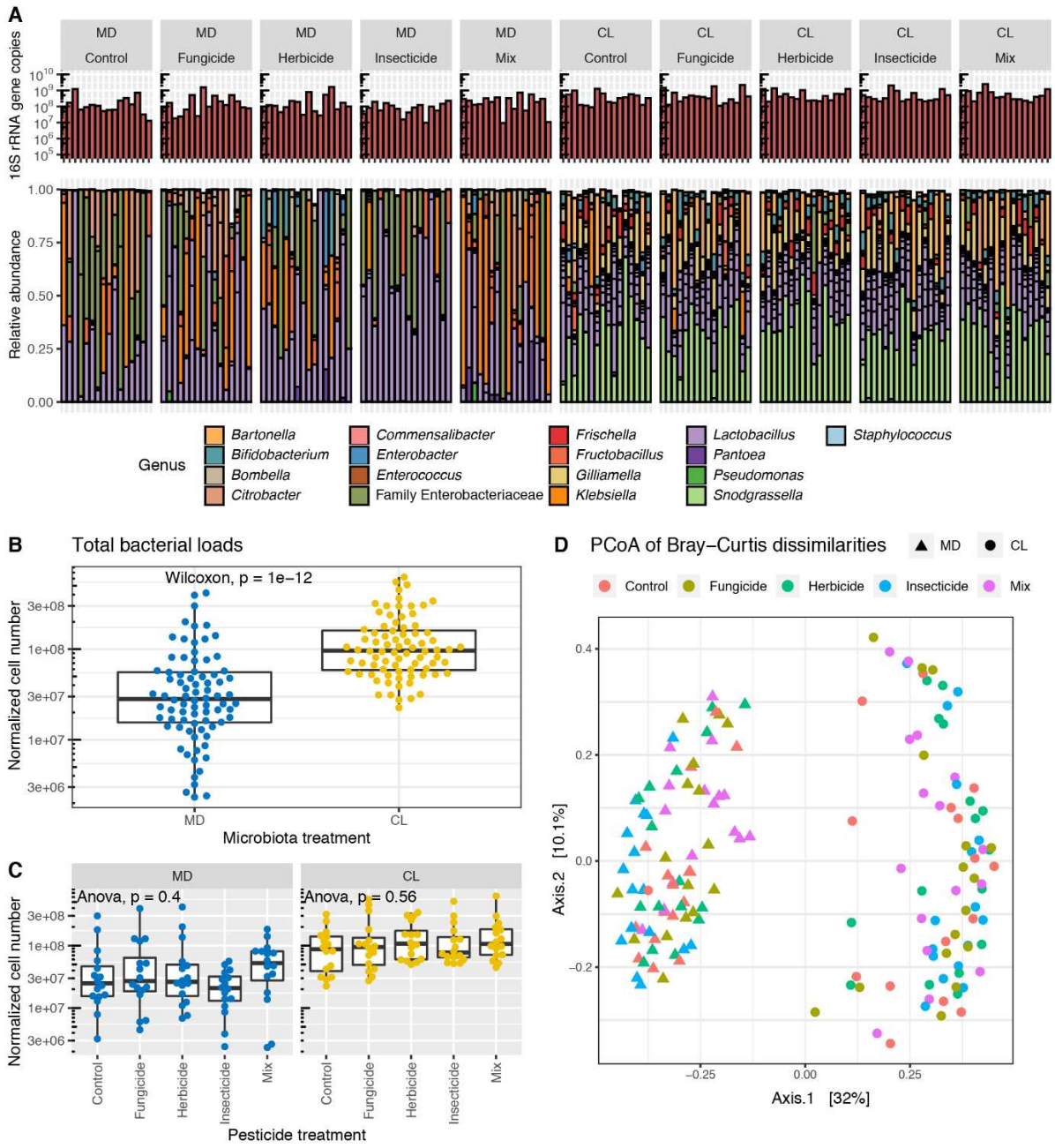

Fig. 1. Effects of pesticides on the establishment of gut microbiota

Gut community composition of colonized (CL) and microbiota-depleted (MD) honey bees following exposure to the fungicide (difenoconazole), herbicide (glyphosate) or insecticide (imidacloprid), alone or in a ternary mixture (Mix). A: Stacked bar plots show the relative abundance of gut bacterial genera in control and pesticide-treated honey bees. Each column represents an individual bee. B: Boxplots of total bacterial 16S rRNA gene copies estimated by qPCR in CL and MD bees. C: Boxplots of total bacterial 16S rRNA gene copies in control bees and in bees exposed to the different pesticides, reported separately by gut microbiota colonization treatment. D: Principal component analysis of Bray-Curtis dissimilarities based on amplicon-sequence data normalized by total bacterial loads as obtained by qPCR.

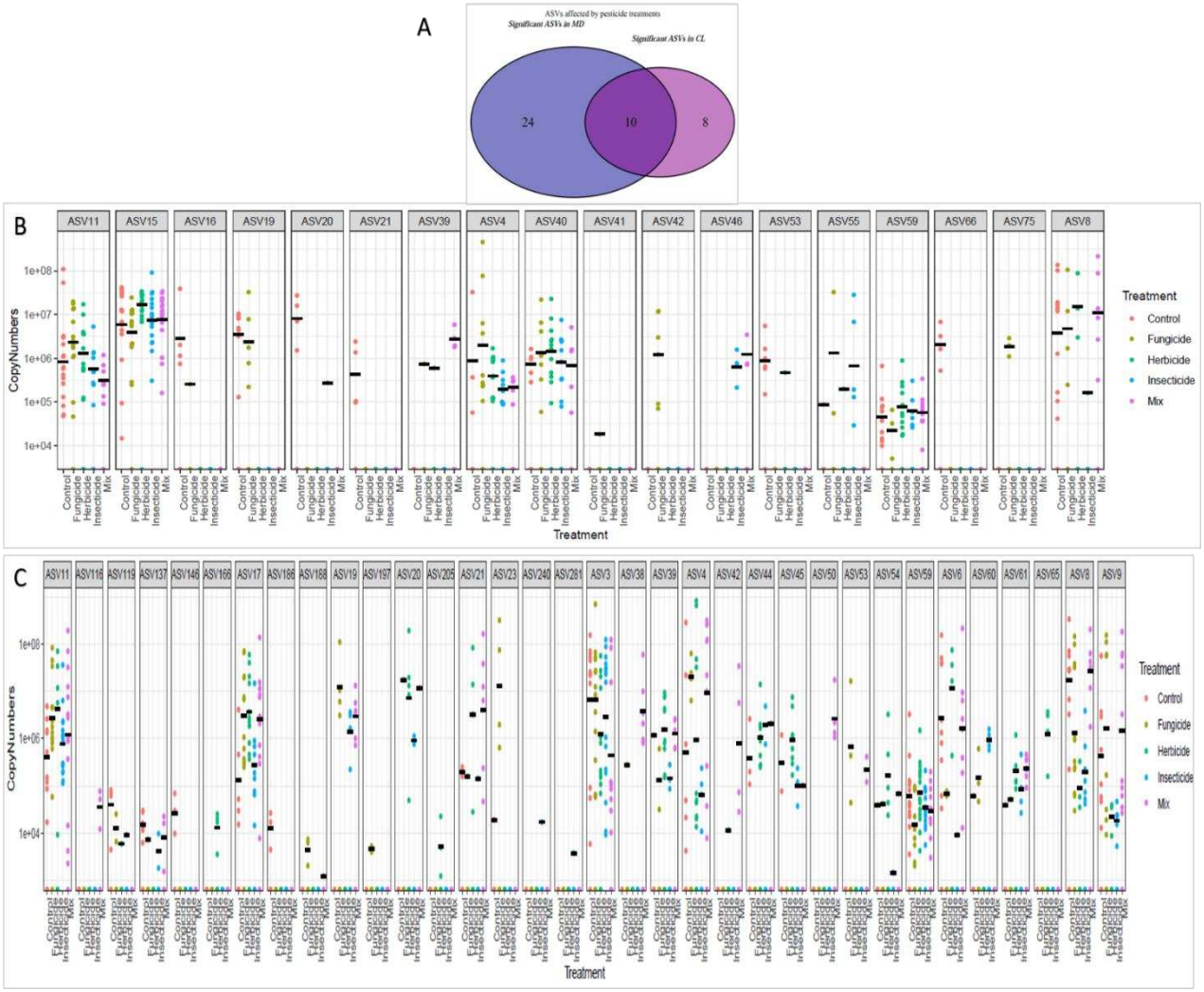

Fig. 2. Effects of pesticides on the establishment of gut microbiota

Changes in absolute abundance of specific amplicon-sequence variants (ASVs) in colonized (CL) and microbiota-depleted (MD) honey bees following exposure to the fungicide (difenoconazole), herbicide (glyphosate) and insecticide (imidacloprid), alone and in a ternary mixture (Mix). A: Venn diagram showing the distribution of common and exclusive significant ASVs in CL and MD groups. B and C: ASVs with statistically significant differences in permutation t tests in colonized (B) and microbiota-depleted (C) honey bees.

3.2. Physiological effects of pesticides

The effects of imidacloprid, difenoconazole and glyphosate individually and in ternary mixture on the physiological status of honey bees were determined by studying the modulations of five physiological markers in the head, abdomen and midgut **(Fig. 3) (Table S1 and S2)**. In CL honey bees, GST in the head and abdomen, G6PDH in the head, abdomen and midgut, LDH in the abdomen and midgut and ALP and POx in the midgut were not modulated following exposure to pesticides. However, the fungicide increased the activity of LDH in the head ($p < 0.01$) and decreased the activity of GST in the midgut ($p < 0.05$) **(Table S1 and S2-A)**. In MD honey bees, GST in the head and abdomen, G6PDH in the head, abdomen and midgut, LDH,

ALP and POx in the midgut were not modulated following exposure to pesticides. However, the fungicide increased the activity of LDH in the head ($p < 0.001$) and abdomen ($p < 0.05$) and GST in the midgut ($p < 0.05$). In addition, the herbicide and the Mix increased the activity of LDH in the head ($p < 0.05$ for Herbicide and $p < 0.001$ for Mix) and GST in the midgut ($p < 0.05$ for Herbicide and $p < 0.01$ for Mix) **(Table S1 and S2-B)**.

3.3. Effect of gut colonization on physiological markers

To detect the potential impact of gut colonization on the physiological status of the experimental bees, we compared enzymatic activities of CL and MD bees exposed to the same pesticide treatments **(Fig. 3) (Table S1 and S3)**. In control unexposed honey bees (MD.Control and CL.Control), GST in the midgut was the only enzyme differently modulated between CL and MD honey bees with a higher activity following gut colonization ($p < 0.01$). In honey bees exposed to the insecticide, LDH in the head and abdomen were modulated differently based on the gut colonization status. LDH activity was lower in the head and higher in the abdomen of CL compared to MD honey bees ($p < 0.001$ for head LDH and $p < 0.05$ for abdomen LDH). In honey bees exposed to the fungicide, the activity of LDH in the head was lower in CL honey bees compared to MD ($p < 0.01$). In honey bees exposed to the herbicide, the activities of G6PDH in the head and LDH in the abdomen were higher in CL compared to the MD honey bees ($p < 0.05$ for head G6PDH and $p < 0.01$ for abdomen LDH). However, the activity of LDH in the head was lower in CL honey bees ($p < 0.05$). In honey bees exposed to the ternary mixture, the activities of GST in the abdomen was higher in CL honey bees compared to the MD ones ($p < 0.05$) and the activity of LDH in the head was lower in CL honey bees ($p < 0.001$).

The hierarchical cluster analyses showed a tendency of the pesticide treatments to group according to the gut colonization status. In addition, physiological markers were not grouped together by body compartment. Only head LDH and midgut G6PDH were distant from the other enzymes, due to an overall increase of their activities in all treatments compared to those of CL.Control **(Fig. 4 and Fig. S1)**.

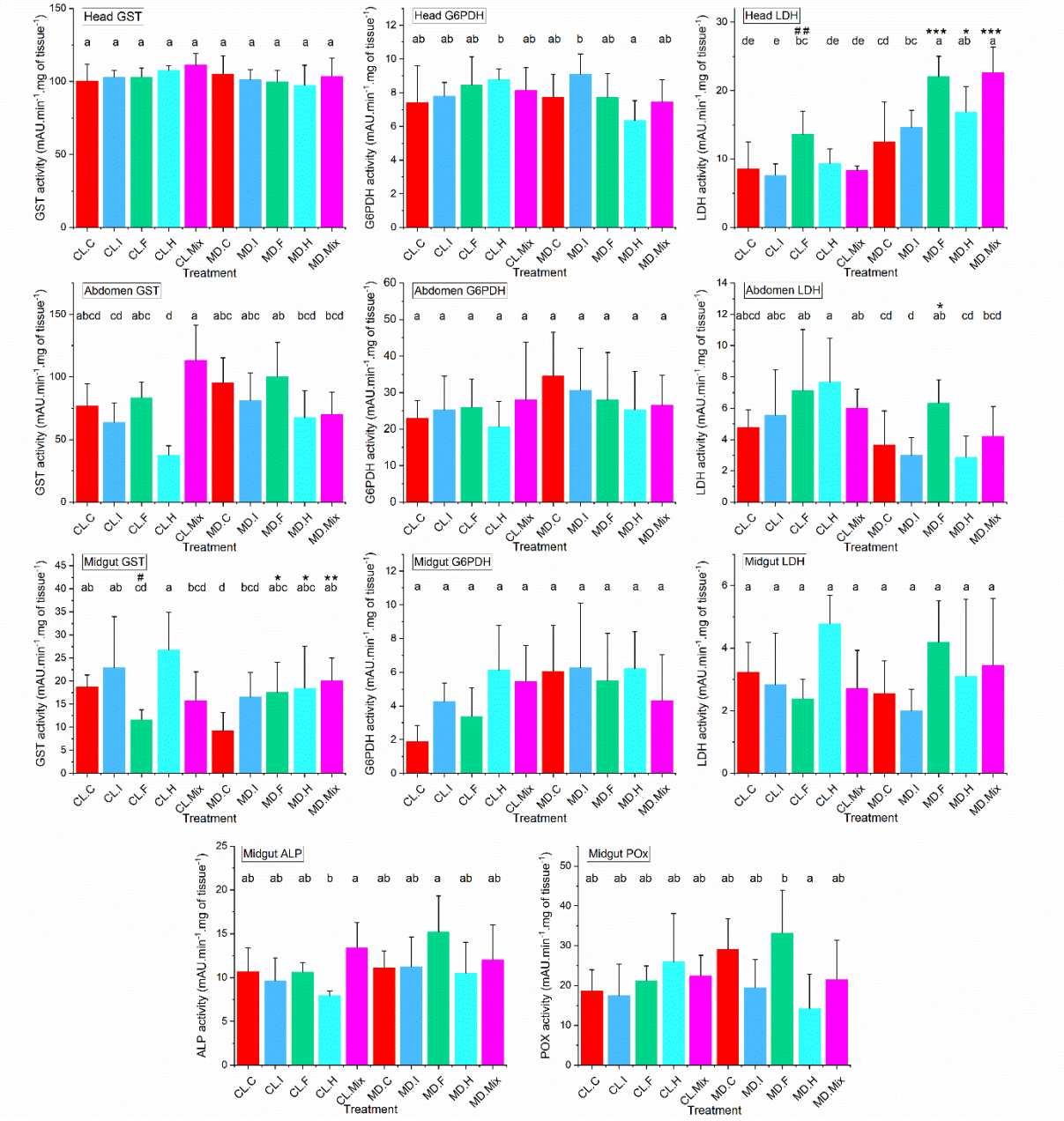

Fig. 3. Physiological impacts of pesticides in colonized (CL) and microbiota-depleted (MD) honey bees

For five days, colonized (CL) and microbiota-depleted (MD) newly emerged honey bees were fed sucrose solutions containing no pesticides (C, Control), imidacloprid (I, Insecticide), glyphosate (H, Herbicide), difenoconazole (F, Fungicide) or the ternary mixture of these pesticides (Mix) at the concentration of 0.1 µg/L in food. The impact of the exposure to pesticides on the physiology of the surviving honey bees at day five was investigated through an analysis of three common markers in the head, abdomen and midgut (GST, G6PDH and LDH) and two specific markers in the midgut (ALP and POx). ANOVA or Kruskal-Wallis tests were applied to detect significant differences between treatments. Treatments with different letters are significantly different ($p < 0.05$). # indicates a significant difference in the marker levels between colonized honey bees exposed to pesticides and their colonized control (CL.C). * indicate a significant difference in the marker levels between microbiota-depleted honey bees exposed to pesticides and their control (MD.C) (* or #: $p \leq 0.05$; ** or ##: $p \leq 0.01$; *** or ###: $p \leq 0.001$).

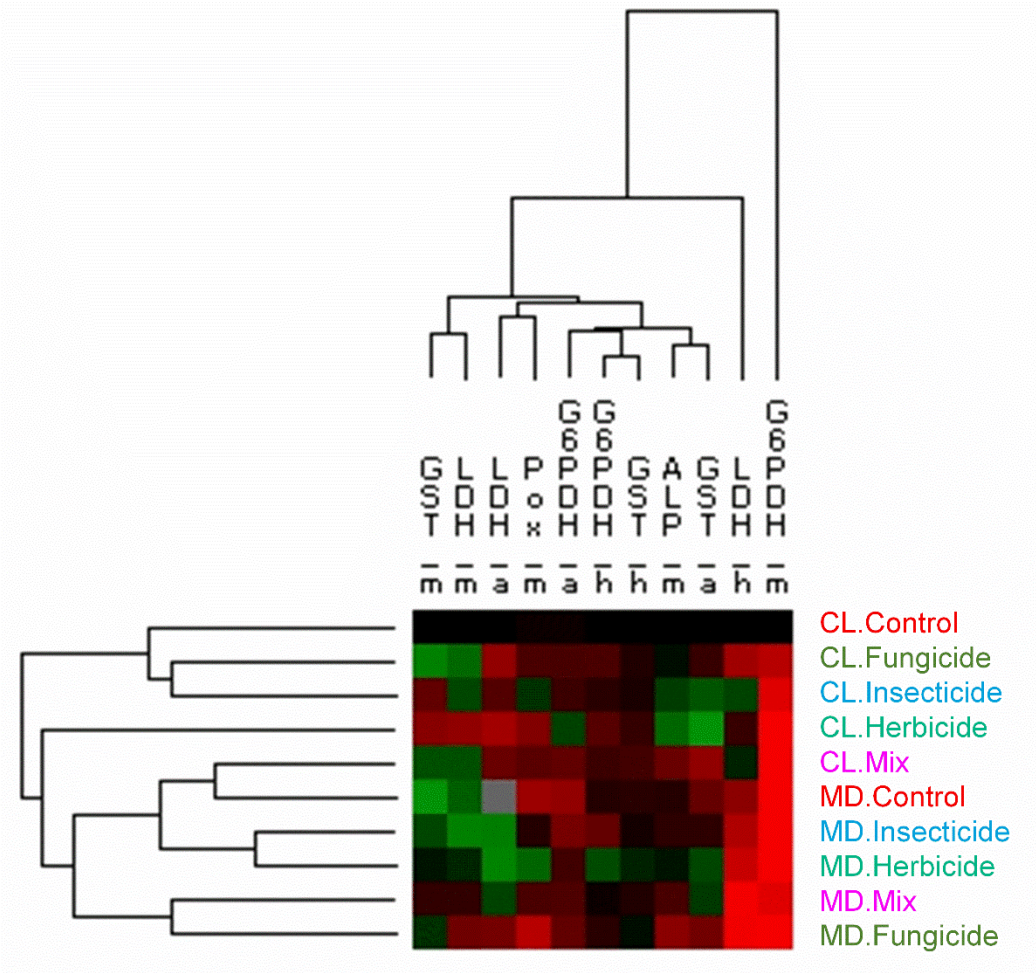

Fig. 4. Effects of the pesticides and gut colonization on the physiological state of honey bees

The levels of physiological markers in colonized (CL) and microbiota-depleted (MD) newly emerged honey bees exposed or not to pesticides was submitted to a cluster analysis to assess, with an integrative approach, the effect of pesticide treatments and gut colonization status on the physiological markers analyzed in the head (h), abdomen (a) and midgut (m). Colonized (CL) and microbiota-depleted (MD) honey bees were fed sucrose solutions containing no pesticides (Control), imidacloprid (Insecticide), glyphosate (Herbicide), difenoconazole (Fungicide) or the ternary mixture (Mix) at concentration of 0.1 µg/L in food. The distance measured was Euclidian distance with UPGMA as the linkage rule for clusters. Data normalization was required to convert the mean of each treatment to the rate of variation compared with the average of the control (CL.Control). The intensity of modulation is illustrated by the range of colors, with green and red indicating respectively a decrease and an increase of the mean enzymatic activity in each treatment, by comparison with the mean value in the CL.Control. Black indicates no change by comparison with the mean value of CL.Control.

3.4. Effect of exposure to pesticide and gut colonization on honey bee survival

The survival rate was recorded during the five days of exposure to pesticides. No differences in survival rates had been detected between the unexposed honey bees (CL.Control and MD.Control) and the bees exposed to the different pesticide treatments. In addition, no differences in survival rates had been detected between the CL and the MD honey bees following exposure to a common pesticide treatment **(Fig S2)**.

3.5. Effect of exposure to pesticide and gut colonization on food consumption

The influence of pesticide treatments and gut colonization on the feeding behavior of honey bees was followed by measuring the daily food consumption. Honey bees exposed to all pesticide treatments consumed an equal amount of food. No differences in food consumption was observed between CL and MD honey bees exposed to a similar pesticide treatment **(Fig. S3)**.

4. Discussion

Numerous studies have revealed an impact of several pesticides on the gut microbiota of honey bees. The majority of these studies have focused on the effect of a single pesticide and used concentrations higher than those encountered by honey bees under realistic conditions. Bees are unlikely to be exposed to single pesticides while feeding in the beehive or foraging, as herbicides, insecticides and fungicides are often used in combination to improve crop yields and are detected simultaneously in the beehive matrices (Kanga et al., 2019; Lambert et al., 2013; Mullin et al., 2010; Tornisielo et al., 2013). It is thus important to assess the potential synergistic effects between different combinations of agrochemicals. Our study provides a first attempt to determine the effects of several pesticides individually and in mixture at environmental realistic concentrations on the early establishment of the gut microbiota and key physiological functions in honey bees. Our study shows that chronic exposure to low doses of imidacloprid, difenoconazole and glyphosate, individually and in ternary mixture, can directly affect the physiology of honey bee workers without disrupting their core gut microbiota. Notably, the differences between the effects of the pesticide treatments were more marked in microbiota-depleted bees than in colonized bees. In addition, we found that the overall effects significantly differed between microbiota-depleted and colonized bees, suggesting that the core gut microbiota plays a role in the bees' physiological resilience to the action of pesticides.

To our knowledge, this is the first study about the effect of triazole fungicides on the establishment of the honey bee gut microbiota. However, previous studies on the effect of azole fungicides on other organisms and ecosystems, revealed their capacity to disrupt the gut microbiota of female rats, and to stimulate or inhibit soil bacterial proliferation depending on

the fungicide active ingredient (Xu et al., 2014; Yang et al., 2011). In addition, despite the high occurrence of pesticides as a mixture in the beehive residues, there is also a lack of data about their potential effects on honey bee gut. Therefore, further studies are needed to understand the effect of azole fungicides and the different pesticide combinations on the honey bee gut microbiota during and after gut colonization.

Recent studies have focused on the effects of oral exposure to glyphosate on the honey bee gut microbiota, showing changes in community structure, with marked shifts in total abundance of specific symbionts (*Snodgrassella* and *Lactobacillus* Firm 4 in particular) (Blot et al., 2019; Motta et al., 2020; Motta and Moran, 2020; Motta et al., 2018). However, in spite of testing a range of herbicide concentrations, these concentrations were, to the best of our knowledge, much higher than those found in honey and pollen samples in colonies all around the world. The doses of glyphosate used in the studies of Motta et al. (2018, 2020) and Motta and Moran (2020) ranged from 0.01 mM (1691 µg/L) to 1 mM (169070 µg/L). They relied in their choice of glyphosate concentrations on those measured in water sources (Coupe et al., 2012; Howe et al., 2004; Wagner et al., 2013) and on the results of a study performed by Thompson et al. (2014) under semi-field conditions. In this latter study, honey bee colonies were placed in insect proof glasshouses planted with *phacelia* and pulverized with glyphosate formulation during *phacelia* full bloom. Then, the residues of glyphosate were extracted from larvae, pollen, nectar and sucrose solution for seven days after treatment. Therefore, this study is a worst-case realistic exposure and the detected concentrations were at least five times higher than the highest residual concentration detected in honey and pollen in several other studies (Berg et al., 2018; El Agrebi et al., 2020; Pareja et al., 2019; Rubio et al., 2015; Thompson et al., 2019). The absence of an effect of glyphosate on the early gut colonization in our study could hence be linked to the low residual concentration (0.1 µg/L) to which newly emerged honey bees were exposed. This hypothesis is confirmed by the results of Motta and Moran (2020), who have found that the effects of glyphosate on gut microbiota increased from lower to higher concentrations, with an absence of effects at 0.01 mM (1.69 mg/L). This dose-response relationship is also confirmed by Dai et al. (2018) who found a high impact of glyphosate on the gut microbiota of honey bee larvae exposed at the highest dose of 20 mg/L but not at 0.8 and 4.0 mg/L. The glyphosate concentration used in our study might be considered too low to induce any effect. However, glyphosate, at this concentration can induce a chronic toxicity to bees through lethal and physiological effects, especially when it is associated with other pesticides (Almasri et al., 2020).

The absence of an effect of imidacloprid on early gut colonization in our study is consistent with the results of Raymann et al. (2018), who reported an absence of effect on established gut microbiota following a three days exposure to 500 µg/L of imidacloprid. However, neonicotinoids may have a negative impact on the gut microbiota when honey bees are exposed to them for a long exposure period. This is supported by two independent studies. In the first study it was observed that the relative abundance of *Lactobacillus* spp. and *Bifidobacterium* spp. significantly decreased in winter and summer worker bees exposed to imidacloprid at 3.5 µg/kg for 18 days (Rouze et al., 2019). In the second study, a decrease in the absolute abundance

of total bacteria was found after a 7-day chronic exposure of middle-aged honey bees to thiacloprid, an insecticide belonging to the neonicotinoid family, at 200 to 2000 µg/L (Liu et al., 2020).

In our experiment, the bacterial load was three-fold lower in microbiota-depleted bees than in colonized ones. This difference appears much smaller than the 100-fold difference reported in a study that explicitly quantified the bacterial loads in colonized and non-colonized bees (Kešnerová et al., 2017). This was of no particular concern for our study because our main aim was not to quantify physiological differences between MD and CL bees but rather the effects of exposures to pesticides. However, our results highlight the importance of always controlling the gut colonization status of microbiota-depleted bees at least by means of qPCR, an endeavor that is not always pursued even when the aim is to assess the impact of gut bacteria on the host physiology (Leger and McFrederick, 2020; Zheng et al., 2017).

There was no variation in the cumulated food consumption between the different pesticide treatments in colonized and microbiota-depleted honey bees. Therefore, bees had ingested an equal amount of pesticides in all treatments and these pesticides did not exhibit any attractive or repellent effect. The chronic exposure to the three pesticides and their ternary mixture did not affect bees' survival. This may be due to the relatively short duration of exposure of five days. In a previous study, an exponential increase in mortality was observed six to eight days after the beginning of the chronic exposure to the same pesticides in winter honey bees (Almasri et al., 2020). Gut colonization did not elicit a direct effect on bee survival, as both colonized and microbiota-depleted honey bees exhibited a similar survival rate. The absence of a negative effect on survival is in agreement with Zheng et al. (2017).

We found no major effect of pesticides on the oxidative system, metabolism and immunity of colonized honey bees. Only the fungicide increased the LDH activity in the head and decreased the GST activity in the midgut. The short duration of exposure to imidacloprid and glyphosate may explain the absence of an effect of these two pesticides, which have both been reported in previous studies to induce oxidative stress in honey bees (Gregore et al., 2018; Helmer et al., 2015; Vázquez et al., 2020).

Our results show differences in the modulation of physiological markers between colonized and microbiota-depleted honey bees exposed to the same pesticide treatments. They suggest that, in the presence of the core gut microbiota, bees have increased resilience to oxidative stress and improved detoxification of xenobiotics. Therefore, in the long-term, the core gut microbiota could decrease the toxicity of the ingested pesticides and increase host survival. This was reflected by a higher activity in CL bees of G6PDH in the head of herbicide treated bees, GST in the abdomen of ternary-mixture-treated bees, and GST in the midgut of control bees. G6PDH and GST are both involved in the detoxification process and fight against oxidative stress (Corona and Robinson, 2006; Efferth et al., 2006; Xiao et al., 2015). The role of specific core gut members in detoxification and oxidative balance was previously documented through the upregulation of esterase FE4-like, cytochrome Cyp6bd1 and multicopper oxidase 1 (MCO1)

genes in *F. perrarra* compared to honey bees inoculated with *S. alvi*. The esterase FE4-like gene is involved in the detoxification process and development of insecticide resistance in peach-potato aphids (Field et al., 1989). Moreover, this enzyme is involved in the oxidative response during adverse stress such as exposure to imidacloprid and paraquat in *Apis cerana cerana* (Ma et al., 2018). The Cyp6b family plays a significant role in the development of insecticide resistance in *Culex pipiens pallen* (Zou et al., 2019). MCO1 is indirectly involved in the regulation of oxidative stress through the catalysis of the oxidation of ferrous iron (Fe^{2+}) into ferric iron (Fe^{3+}) (Lang et al., 2012).

Early gut colonization of honey bees did not seem to have an effect on the melanization response as POx had a similar activity in colonized and microbiota-depleted honey bees. However, we did expect an increase in the activity of this enzyme in CL bees due to the upregulation of serine protease activation cascade and serine protease inhibitors in the presence of *F. perrara*, which was only detected in CL bees. This bacterium, through the activation of the previously cited genes, leads to the formation of the scab phenotype that corresponds to a melanization process in the pylorus and hence should involve activation of POx (Emery et al., 2017).

The differences in LDH activity between colonized and microbiota-depleted honey bees were tissue-specific. For a same pesticide treatment, with imidacloprid or glyphosate, head LDH activity was lower in colonized honey bees than in microbiota-depleted ones, while abdomen LDH activity was higher in colonized honey bees. The existence of differences in the LDH activity between colonized and microbiota-depleted honey bees is another evidence that the gut microbiota influences the host metabolism (Engel et al., 2012; Habineza et al., 2019; Lee et al., 2015; Zheng et al., 2017).

The hierarchical cluster analyses showed a distinct physiological status in honey bees colonized with the normal gut microbiota compared to those deprived of their core microbial species. The microbiota effects on host physiology were not limited to the midgut but they were also present in the head and abdomen. Therefore, it is becoming increasingly clear that the microbiota exerts a systemic effect on its host rather than being localized in the gut. This is also reflected by the interference of the gut microbiota with the abundance of apidaecin in the honey bee haemolymph (Kwong et al., 2017a), and its suggested effects on the honey bee nervous system by modulating the sugar intake through the increase in insulin sensitivity (Zheng et al., 2017).

In conclusion, our work showed that exposure to pesticide concentrations far lower than previously tested, individually and in ternary mixture, did not affect the early gut bacterial colonization by core members of the honey bee microbiota. However, these exposures directly induced changes in physiological markers broadly associated with honey bee health. These results are thus in agreement with previous studies raising concern about widespread pesticide use in agricultural landscapes damaging pollination services and insect populations more in general.

Acknowledgments

This study was supported in part by the recurrent funding of the Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAE) and by the Agence Nationale de la Recherche (ANR) (grant ANR-15-CE34-0004-01). Hanine ALMASRI was supported by grants from Lebanese University (grant number 2364) and PACA Region, and by a scholarship from Avignon Université (Bourse Perdiguier). Joanito Liberti was supported by the European Union's "Horizon 2020" research and innovation programme under the Marie Skłodowska-Curie grant agreement BRAIN (No 797113). Philipp Engel was supported by a Young Investigator grant (RGY0077/2016) from the International Human Frontier Science Program Organization (HFSP), a Starting Grant (MicroBeeOme) from European Research Council (ERC), and a Swiss National Science Foundation grant (No 31003A_160345). The authors thank Sylvie Tchamitchian for her technical assistance in this study, Jacques Sénéchal for its expert beekeeping and Weixin Ke for her help during DNA extraction.

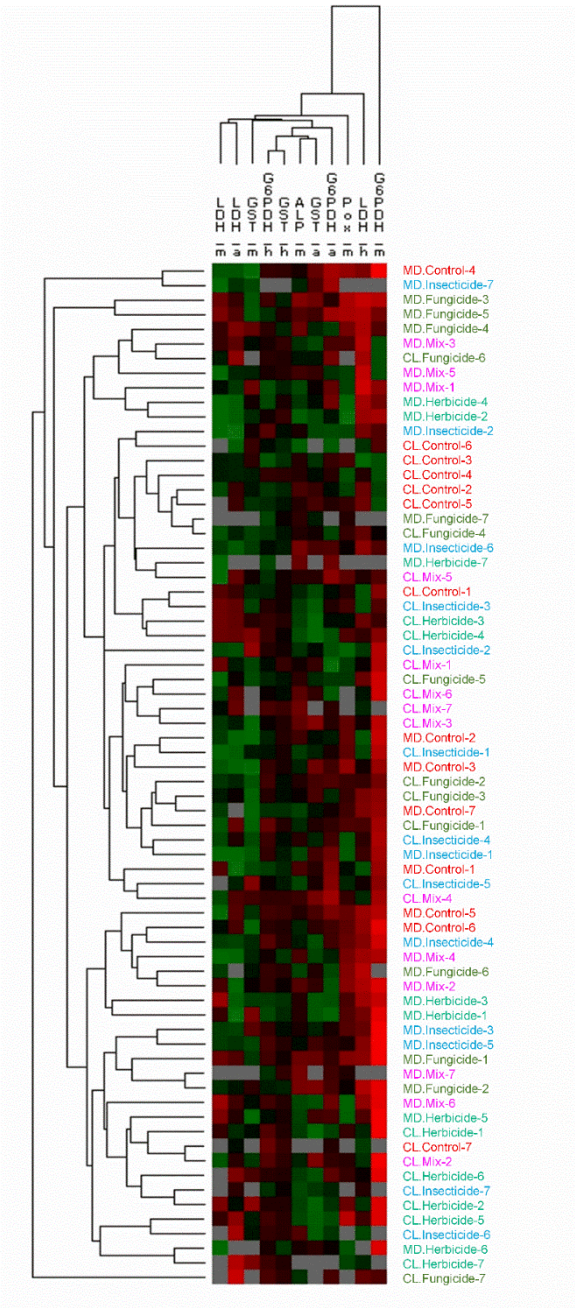

Fig. S1. Effects of the pesticides and gut colonization on the physiological state of newly emerged honey bees.

The Cluster analysis represents an interactive approach to assess the effect of pesticide treatments and gut colonization status on the physiological markers analyzed in the head (h), abdomen (a) and midgut (m) of the honey bee. Colonized (CL) and microbiota-depleted (MD) honey bees were fed sucrose solutions containing no pesticides (Control), imidacloprid (Insecticide), glyphosate (Herbicide), difenoconazole (Fungicide) or the ternary mixture (Mix) at concentrations of 0.1 µg/L in food. Euclidian distances were determined with UPGMA to serve as the linkage rule for clusters. Data normalization was required to convert each treatment to the rate of variation compared with the average of controls (CL.Control). The intensity of modulation is illustrated by the range of colors, with green and red indicating respectively a decrease and an increase of the mean enzymatic activity of each treatment, in comparison with the mean value in the CL.Control. Black indicates no change in comparison with the mean value of CL.Control.

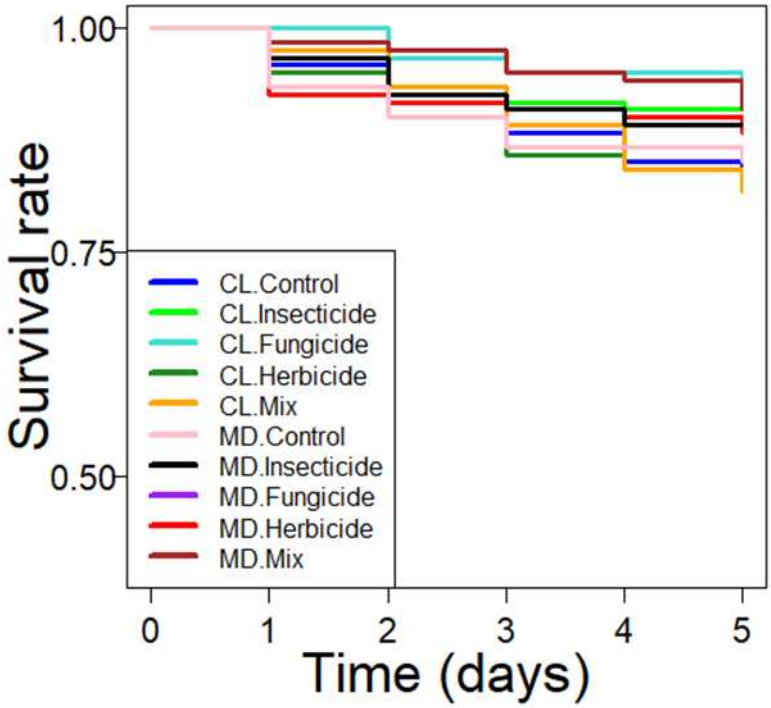

Fig. S2. Effects of pesticides on the longevity of colonized and microbiota-depleted honey bees

Microbiota-depleted (MD) and gut colonized (CL) honey bees were fed for five days sterile sucrose solutions containing no pesticides (Control) or imidacloprid (Insecticide), glyphosate (Herbicide), difenoconazole (Fungicide) alone or as a ternary mixture (Mix) at 0.1 µg/L in food. The data represent the proportion of surviving honeybees exposed to the different treatments. No significant differences in the survival rates were observed between the different treatments (Kaplan-Meier method (log-rank test), followed by a post hoc test).

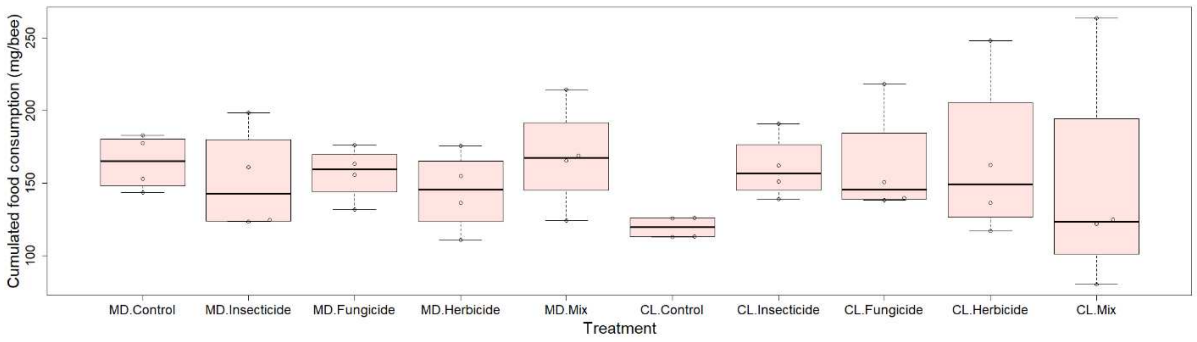

Fig. S3. Effects of pesticides the food consumption of colonized and microbiota-depleted honey bees

Microbiota-depleted (MD) and gut colonized (CL) honey bees were fed for five days sterile sucrose solutions containing no pesticides (Control) or imidacloprid (Insecticide), glyphosate (Herbicide), difenoconazole (Fungicide) alone or as a ternary mixture (Mix) at 0.1 µg/L in food. Box plots represent the cumulated individual consumption (mg/bee) for 4 cages of 30 bees per treatment. Statistical analyses were performed using the Kruskal-Wallis test followed by pairwise comparisons using the Wilcoxon rank sum test with the Benjamini-Hochberg correction. No differences in food consumption had been observed between the different treatments.

Table S1. Effects of pesticide combinations and gut colonization on the physiological state of newly emerged honey bees

Microbiota-depleted and colonized honeybees were generated. Two days after their emergence, honeybees were fed for five days sterile sucrose solutions containing no pesticides (Control) or imidacloprid (Insecticide), glyphosate (Herbicide), difenoconazole (Fungicide) alone or as a ternary mixture (Mix) at 0.1 µg/L. GST, G6PDH and LDH were measured in the head (h), abdomen (a) and midgut (m). ALP and POx were chosen as specific markers in the midgut (m). On the 7th day, 7 samples of 3 tissues were collected in each treatment. For each treatment, the data represent the mean values of enzymatic activities expressed in milli absorbance units of per minute and per mg of tissue (mAU.min-1.mg of tissue-1) ± standard deviations (SD). ANOVA or Kruskal-Wallis tests were applied to detect significant differences between treatments. Treatments with different letters are significantly different ($p < 0.05$). Red arrows indicate an increase (↑) and a decrease (↓) in the enzymatic activity of colonized honey bees exposed to pesticides relative to their control (CL.Control). Blue arrows indicate an increase (↑) and a decrease (↓) in the enzymatic activity of microbiota-depleted honey bees exposed to pesticides relative to their control (MD.Control).

Table S1 continued

	CL					MD				
	Activity of physiological markers (mAU.min^{-1}.mg of tissue^{-1})					Activity of physiological markers (mAU.min^{-1}.mg of tissue^{-1})				
	CL.Control	CL.Insecticide	CL.Fungicide	CL.Herbicide	CL.Mix	MD.Control	MD.Insecticide	MD.Fungicide	MD.Herbicide	MD.Mix
$GST_{(h)}$	100,196 ± 11,811	102,654 ± 4,968	103,080 ± 6,262	107,650 ± 3,301	111,278 ± 7,867	105,116 ± 12,334	101,222 ± 6,872	99,842 ± 7,812	97,362 ± 13,622	103,620 ± 12,387
$G6PDH_{(h)}$	7,401 ± 2,186 ^{ab}	7,804 ± 0,808 ^{ab}	8,457 ± 1,673 ^{ab}	8,787 ± 0,637 ^{b}	8,130 ± 1,353 ^{ab}	7,728 ± 1,377 ^{ab}	9,116 ± 1,183 ^{b}	7,744 ± 1,381 ^{ab}	6,350 ± 1,170 ^{a}	7,442 ± 1,336 ^{ab}
$LDH_{(h)}$	8,605 ± 3,930 ^{de}	7,625 ± 1,654 ^{e}	13,638 ± 3,333 ^{bc} ↑	9,362 ± 2,145 ^{de}	8,328 ± 0,664 ^{de}	12,493 ± 5,812 ^{cd}	14,652 ± 2,487 ^{bc}	22,081 ± 2,942 ^{a} ↑	16,848 ± 3,750 ^{ab} ↑	22,606 ± 3,767 ^{a} ↑
$GST_{(a)}$	76,907 ± 17,688 ^{abcd}	63,622 ± 15,387 ^{cd}	83,433 ± 12,188 ^{abc}	37,766 ± 7,316 ^{d}	113,362 ± 28,068 ^{a}	95,244 ± 19,998 ^{abc}	81,410 ± 21,713 ^{abc}	100,440 ± 27,080 ^{ab}	67,387 ± 21,651 ^{bcd}	69,943 ± 17,983 ^{bcd}
$G6PDH_{(a)}$	23,034 ± 4,770	25,270 ± 9,206	25,991 ± 7,705	20,724 ± 6,792	28,092 ± 15,750	34,531 ± 11,958	30,678 ± 11,400	28,079 ± 12,866	25,357 ± 10,428	26,499 ± 8,217
$LDH_{(a)}$	4,766 ± 1,146 ^{abcd}	5,569 ± 2,882 ^{abc}	7,137 ± 3,890 ^{ab}	7,675 ± 2,789 ^{a}	6,000 ± 1,207 ^{ab}	3,667 ± 2,157 ^{cd}	3,005 ± 1,123 ^{d}	6,319 ± 1,478 ^{ab} ↑	2,870 ± 1,353 ^{cd}	4,204 ± 1,914 ^{bcd}
$GST_{(m)}$	18,783 ± 2,548 ^{ab}	22,895 ± 11,099 ^{ab}	11,664 ± 2,141 ^{cd} ↓	26,742 ± 8,207 ^{a}	15,795 ± 6,201 ^{bcd}	9,261 ± 3,999 ^{d}	16,536 ± 5,324 ^{bcd}	17,616 ± 6,465 ^{abc} ↑	18,459 ± 9,137 ^{abc} ↑	20,104 ± 4,893 ^{ab} ↑
$G6PDH_{(m)}$	1,892 ± 0,936	4,277 ± 1,096	3,374 ± 1,717	6,128 ± 2,655	5,457 ± 2,126	6,064 ± 2,722	6,277 ± 3,817	5,515 ± 2,793	6,213 ± 2,191	4,319 ± 2,738
$LDH_{(m)}$	3,229 ± 0,956	2,833 ± 1,653	2,381 ± 0,630	4,772 ± 0,916	2,710 ± 1,219	2,553 ± 1,042	2,006 ± 0,682	4,191 ± 1,328	3,100 ± 2,456	3,457 ± 2,140
$ALP_{(m)}$	10,650 ± 2,733 ^{ab}	9,659 ± 2,547 ^{ab}	10,626 ± 1,093 ^{ab}	7,949 ± 0,519 ^{b}	13,419 ± 2,858 ^{a}	11,095 ± 1,968 ^{ab}	11,241 ± 3,408 ^{ab}	15,204 ± 4,129 ^{a}	10,532 ± 3,491 ^{ab}	12,023 ± 3,983 ^{ab}
$POx_{(m)}$	18,720 ± 5,220 ^{ab}	17,467 ± 7,882 ^{ab}	21,278 ± 3,661 ^{ab}	25,978 ± 12,079 ^{ab}	22,427 ± 5,183 ^{ab}	29,154 ± 7,654 ^{ab}	19,543 ± 7,005 ^{ab}	33,236 ± 10,748 ^{b}	14,281 ± 8,520 ^{a}	21,442 ± 9,915 ^{ab}

Table S2A. Effect of pesticide treatments on the physiological state of colonized honey bees

After emergence, honey bees were colonized or not from the gut homogenate. Two days after their emergence, honeybees were fed for five days sterile sucrose solutions containing no pesticides (Control) or imidacloprid (Insecticide), glyphosate (Herbicide), difenoconazole (Fungicide) alone or as a ternary mixture (Mix) at 0.1 µg/L. The enzymatic activities were compared between colonized (CL) honey bees exposed to the different pesticides and the control (CL.Control). Three groups were distinguished: (i) Enzymes exhibiting an activity similar to that of the control (No modulation). (ii) Enzymes exhibiting an activity higher than that of the control in at least one pesticide treatment. (iii) Enzymes exhibiting an activity lower than that of the control in at least one pesticide treatment.

No modulation	Increase of activity	Decrease of activity
Head GST	Head LDH (CL.Fungicide)	Midgut GST (CL.Fungicide)
Head G6PDH	-	-
Abdomen GST	-	-
Abdomen G6PDH	-	-
Abdomen LDH	-	-
Midgut G6PDH	-	-
Midgut LDH	-	-
Midgut ALP	-	-
Midgut POx	-	-

Table S2B. Effect of pesticide treatments on the physiological state of microbiota-depleted honeybees

After emergence, honey bees were colonized or not from the gut homogenate. Two days after their emergence, honeybees were fed for five days sterile sucrose solutions containing no pesticides (Control) or imidacloprid (Insecticide), glyphosate (Herbicide), difenoconazole (Fungicide) alone or as a ternary mixture (Mix) at 0.1 µg/L. The enzymatic activities were compared between microbiota-depleted honey bees exposed to the different pesticides and the control (MD.Control). Two groups were distinguished: (i) Enzymes exhibiting an activity similar to that of the control. (ii) Enzymes exhibiting an activity higher than that of the control.

No modulation	Increase of enzymatic activity
Head GST	Head LDH: (MD.Fungicide, MD.Herbicide, MD.Mix)
Head G6PDH	Abdomen LDH: (MD.Fungicide)
Abdomen GST	Midgut GST: (MD.Fungicide, MD.Herbicide, MD.Mix)
Abdomen G6PDH	-
Midgut G6PDH	-
Midgut LDH	-
Midgut ALP	-
Midgut POx	-

Table S3. Global effect of gut colonization on physiological markers

After emergence, honey bees were colonized or not from the gut homogenate. Two days after their emergence, honeybees were fed for five days sterile sucrose solutions containing no pesticides (Control) or imidacloprid (Insecticide), glyphosate (Herbicide), difenoconazole (Fungicide) alone or as a ternary mixture (Mix) at 0.1 µg/L. The enzymatic activities of the physiological markers were compared between microbiota-depleted (MD) and colonized (CL) honey bee exposed to the same pesticides.

Activity higher in colonized bees than microbiota-depleted bees	Activity lower in colonized bees than microbiota-depleted bees
Head G6PDH - CL.Herbicide > MD.Herbicide	Head LDH - CL.Insecticide < MD.Insecticide - CL.Fungicide < MD.Fungicide - CL.Herbicide < MD.Herbicide - CL.Mix < MD.Mix
Abdomen GST - CL.Mix > MD.Mix	-
Abdomen LDH - CL.Insecticide > MD.Insecticide - CL.Herbicide > MD.Herbicide	-
Midgut GST - CL.Control > MD.Control	-

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Bilan

Les résultats de cette étude montrent que l'exposition des abeilles à l'imidaclopride, au glyphosate et au difénoconazole, seuls ou en mélange ternaire à la concentration de 0,1 µg/L de nourriture, ne produit aucun effet sur l'installation du microbiote intestinal. Cette étude est, à notre connaissance, la première à évaluer les effets de l'imidaclopride et des fongicides azoles sur l'installation du microbiote intestinal. Cependant, le glyphosate est connu pour perturber les communautés bactériennes durant et après leurs établissements dans l'intestin des abeilles. Ces études sont fondées sur des concentrations bien supérieures aux concentrations environnementales et 16000 fois plus élevées que la concentration utilisée dans notre étude. Ainsi, notre étude révèle l'importance d'utiliser des doses environnementales afin d'évaluer les effets des pesticides sur les abeilles. Toutefois, notre étude s'est limitée à cinq jours d'exposition chronique, d'où des recherches supplémentaires seraient souhaitables afin d'évaluer l'effet d'une plus longue exposition chronique sur le microbiote intestinal durant et après son installation.

Bien que l'exposition de cinq jours aux trois pesticides et au mélange ternaire n'ait pas eu d'effet sur la survie des abeilles et sur le microbiote intestinal, des perturbations physiologiques systémiques étaient observées chez ces abeilles. Ces perturbations ont été plus prononcées chez les abeilles dépourvues de leur microbiote, confirmant ainsi l'importance du microbiote intestinal dans l'intégrité physiologique des abeilles.

III. Synthèse et perspectives

Synthèse et perspectives

Les travaux réalisés durant cette thèse révèlent le danger que présente les mélanges de pesticides sur les abeilles et la complexité des études sur les mélanges de stresseurs. L'imidaclopride, le glyphosate et le difénoconazole, seuls et en mélanges, présentent une toxicité élevée chez les abeilles d'hiver. La forte toxicité des pesticides, ne semble pas liée directement à l'augmentation de leur niveau d'exposition, confirmant ainsi que les pesticides peuvent avoir une relation dose-réponse non-monotones (DRNM), même chez des organismes non mammifères (Lagarde et al., 2015).

La co-exposition des abeilles d'hiver aux mélanges binaires et ternaires, conduit à une augmentation de la toxicité, par rapport aux substances seules, avec l'apparition d'effets synergiques, additifs et sub-additifs. Seul le mélange glyphosate-difénoconazole conduit à un effet antagoniste. Notre hypothèse pour expliquer l'apparition de cet effet antagoniste, était que le difénoconazole provoque l'induction des CYP450, ce qui aboutit à une augmentation de la détoxication du glyphosate et la diminution de sa toxicité. Ainsi, il serait intéressant de vérifier cette hypothèse en évaluant les effets des trois pesticides sur l'expression des CYP450. Chez l'abeille domestique, les CYP450 représentent une famille multigénique comprenant 46 gènes dont plusieurs appartiennent principalement aux familles CYP6 et CYP9 connues pour participer à la détoxication des pesticides (Mao et al., 2011). Ainsi, il pourrait être envisagé de déterminer l'expression de ces gènes afin de détecter une possible induction qui pourrait expliquer l'effet antagoniste du mélange glyphosate-difénoconazole. De plus, des dosages avec des substrats fluorogènes peuvent être conduits pour évaluer l'activité des CYP450 pour relier des modulations géniques à des modulations phénotypiques. Ces substrats permettraient aussi d'étudier l'activité effectrice (inhibitrice ou activatrice) des pesticides d'intérêt sur les CYP450 (Lennard et al., 2007; Zhu et al., 2017a).

Les travaux de thèse montrent que les pesticides, seuls et en mélanges, induisent des effets physiologiques qui révèlent une action systémique, avec une réponse générale dans tous les compartiments de l'abeille. Cet effet systémique est dû, au moins en partie, à un mécanisme d'action commun entre les trois pesticides, le stress oxydant. Ce stress est reflété par la modulation des activités des enzymes faisant partie du système antioxydant et par la présence de dommages oxydatifs sous forme de protéines et de lipides oxydés. Par ailleurs, il serait intéressant de déterminer si ces pesticides, seuls ou en mélanges, peuvent aussi altérer d'autres grandes fonctions physiologiques, comme la thermorégulation, par exemple. En effet, la thermorégulation est une fonction majeure chez l'abeille. Elle est indispensable à l'intégrité du vol lors de la recherche de la nourriture, et de l'exécution de nombreuses tâches à l'intérieur de la ruche telles que l'élevage du couvain et la survie pendant l'hiver (Human et al., 2006; Stabentheiner et al., 2003). Les études montrent que les néonicotinoïdes et les fongicides azoles sont tous les deux impliqués dans les altérations de la thermorégulation des abeilles. L'exposition aiguë aux néonicotinoïdes tels que le thiaméthoxame, conduit à une altération de la thermorégulation chez l'abeille africaine *Apis mellifera scutellata* (Tosi et al., 2016). De plus, l'imidaclopride conduit à une altération de la thermorégulation chez les bourdons *Bombus*

impatiens et *Bombus terrestris* (Crall et al., 2018; Potts et al., 2018). Le difénoconazole et le prochloraze induisent aussi une hypothermie chez les abeilles domestiques. Cette hypothermie est potentialisée par les insecticides pyréthrinoïdes, et pourrait être due à l'interaction entre les insecticides pyréthrinoïdes et les fongicides azoles sur des cibles communes, les ATPases (Vandame and Belzunces, 1998). Ainsi, il y existe une forte probabilité que la thermorégulation soit altérée en présence de l'imidaclopride et du difénoconazole et que l'altération soit plus prononcée quand ces pesticides sont en mélanges.

Les fongicides et les herbicides ont été longtemps considérés comme faiblement toxiques pour les organismes non-cibles, du fait de leur faible toxicité déterminée par des tests conduits dans le cadre de l'homologation des pesticides, avant leur mise sur le marché. Ces tests sont fondés sur l'évaluation des effets létaux qui apparaissent durant les 48 à 96 h qui suivent l'exposition aiguë par voie orale ou de contact au pesticide d'intérêt. Cependant, nos études montrent que les herbicides et les fongicides peuvent être toxiques. En effet, l'exposition aiguë par pulvérisation d'un produit phytopharmaceutique à base de difénoconazole (Score® 250 EC), présente une toxicité élevée qui apparait au-delà des 96 h qui suivent l'exposition. De plus, l'exposition chronique des abeilles au glyphosate et au difénoconazole conduit aussi à des mortalités élevées. La toxicité du difénoconazole, appliqué par pulvérisation, varie en fonction du statut toxicologique des abeilles ; elle augmente chez les abeilles exposées chroniquement au glyphosate, diminue chez les abeilles exposées chroniquement à l'imidaclopride et n'est pas modifiée par le mélange binaire glyphosate-imidaclopride. Ainsi, les résultats des études conduites dans le cadre de cette thèse vont dans le sens de résultats d'études récentes qui révèlent des effets létaux retardés et des effets sublétaux chez les organismes non cibles exposés aux herbicides et aux fongicides (Avigliano et al., 2014; de Castro Marcato et al., 2017; Marinho et al., 2020; Seide et al., 2018). De plus, ces résultats confirment l'importance d'adopter de nouvelles approches d'évaluation des risques qui prennent en considération les effets retardés et à long terme des expositions aiguës et chroniques aux fongicides et aux herbicides. Il apparaît aussi nécessaire de cumuler plusieurs études, dans le dossier d'enregistrement des pesticides, pour examiner les éventuelles situations de co-exposition qui pourraient conduire à une sous-estimation de la toxicité des pesticides.

L'association des stresseurs biotiques et abiotiques peut conduire à des effets synergiques sur l'état physiologique des abeilles allant jusqu'à compromettre la survie de ces dernières. L'interaction entre *Nosema* et les insecticides est connue pour induire des effets synergiques (Alaux et al., 2010a; Doublet et al., 2014). Par ailleurs, les travaux de cette thèse ont permis de mettre en évidence la présence de synergies entre *Nosema* et les pesticides d'autres classes, tels que les herbicides et les fongicides. L'effet synergique dépend de la séquence d'exposition aux pesticides car il apparait uniquement quand l'infection par *Nosema* est suivie d'une exposition chevauchante au glyphosate et au difénoconazole. Toutefois, le stress énergétique qui caractérise l'infection par *Nosema*, ne semble pas être responsable de l'effet synergique observé, puisque l'infection ne conduit pas à une augmentation de la consommation de la nourriture contaminée par les pesticides. De plus, l'interaction entre *Nosema*, le glyphosate et le difénoconazole n'augmente pas les capacités de production de spores chez *Nosema*, qui aurait pu en partie

expliquer l'effet synergique, mais conduit à des perturbations de plusieurs fonctions physiologiques y compris les défenses antioxydantes et l'immunité sociale.

Les résultats des études conduites dans cette thèse, ont montré une perturbation du système nerveux suite à l'exposition chronique au glyphosate et aux mélanges de pesticides chez les abeilles d'hiver et suite à l'association de *Nosema* avec le glyphosate et le difénoconazole, seuls et en mélanges. Ainsi, il serait intéressant de vérifier si les perturbations du système nerveux, induites par les interactions entre les pesticides et les interactions entre les pesticides et *Nosema*, affecteraient l'intégrité fonctionnelle comportementale des abeilles. Cette dernière pourrait être évaluée par des épreuves d'exploration fonctionnelle telles que le réflexe d'habituation, qui nécessite l'intégrité du système olfactif et des fonctions cognitives, des tests d'apprentissage et de mémoire olfactive, qui permettent de tester les atteintes des phases d'acquisition, de consolidation et de rappel de l'information.

L'imidaclopride, le glyphosate et le difénoconazole, seuls et en mélanges, ne présentent pas d'effet sur l'établissement du microbiote intestinal des abeilles émergentes. Cependant, il serait intéressant de déterminer si ces trois pesticides, seuls et en mélanges, aux mêmes niveaux d'exposition environnementaux et sur une durée d'exposition plus longue, possèdent un effet sur le microbiote intestinal après son installation. De plus, il serait utile de comparer les effets des trois pesticides, sur l'abondance totale et relative du microbiote intestinal des abeilles d'hiver et des abeilles butineuses d'été, du fait que le microbiote diffère entre ces abeilles (Kešnerová et al., 2020).

L'intestin moyen est un organe primordial pour la santé des abeilles puisqu'il est le site de digestion et d'absorption de nutriments et le siège primaire d'une protection immunitaire (Kwong et al., 2017a). De plus, il est le site de contact avec les xénobiotiques ingérés et de nombreux agents pathogènes tels que *Nosema*. L'imidaclopride, le glyphosate et les fongicides azoles (le myclobutanil) sont connus pour altérer l'épithélium intestinal des larves d'abeilles en causant plus de 60% de mortalité cellulaire dans ce tissu (Gregorc and Ellis, 2011). Ainsi, les perturbations du tissu épithélial intestinal pourraient ainsi être l'une des causes de la toxicité élevée de ces trois pesticides observée dans nos études. Aussi, il serait pertinent de savoir si les expositions chroniques des abeilles adultes à ces trois pesticides, aux niveaux d'exposition environnementaux utilisés dans nos études, peuvent altérer le tissu épithélial intestinal et si ces altérations sont plus prononcées quand les trois pesticides coexistent ensemble, en présence ou non de *Nosema*.

Afin de détecter les effets des pesticides sur le tissu épithélial intestinal, il est possible d'évaluer les effets sur la régénération des cellules somatiques intestinales par les cellules souches. Ces cellules souches se trouvent à la base de l'épithélium intestinal et sont responsables de la régénération des entérocytes. Ces dernières assurent la sécrétion d'enzymes digestives et l'absorption des substances digérées (Caccia et al., 2019). Le taux de régénération des cellules somatiques intestinales pourrait être déterminé en utilisant des techniques telles que le marquage immuno-histochimique de l'analogue de la thymidine, le 5-bromo-2-désoxyuridine (BrdU)

(Forkpah et al., 2014). De plus, il serait possible d'effectuer un marquage des cellules épithéliales intestinales au moyen de la méthode TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) afin de détecter le taux des cellules en apoptose suite à l'ingestion des pesticides (Gregorc and Ellis, 2011; Martin-Hernandez et al., 2017). Enfin, l'intégrité de la membrane péritrophique pourrait refléter les effets des pesticides sur le tissu épithélial intestinal. Cette membrane tapisse l'épithélium de l'intestin moyen des insectes et constitue une barrière efficace contre les particules alimentaires abrasives, les xénobiotiques, les toxines et les agents pathogènes (Abedi and Brown, 1961; Barbehenn, 1999; Hegedus et al., 2009). L'intégrité de la membrane péritrophique pourrait être déterminée par le marquage de la membrane avec le Bleu de Méthylène Fuchsine Basique (Garcia-Gonzalez and Genersch, 2013).

Dans notre protocole expérimental, les abeilles sont uniquement nourries, avec du sirop contaminé par les pesticides et ne peuvent pas choisir d'autres sources d'alimentation ; ces expériences sont appelées « no-choice experiments ». Cependant, dans les conditions naturelles, les abeilles ont le choix de se nourrir de miel et de pain d'abeilles d'origines différentes. Ainsi, les abeilles peuvent préférer ou éviter la consommation de certains pesticides, ce qui augmente ou diminue, respectivement, l'exposition au pesticide et, de ce fait, la toxicité induite par ces derniers. Par exemple, les abeilles évitent la consommation de pollen des cultures traitées par le prothioconazole (fongicide triazole) et préfèrent le pollen des cultures non traitées (Jaffe et al., 2019). De plus, elles évitent la consommation de nourriture contaminée par le prochloraze (fongicide imidazole) (Liao et al., 2017) et operculent le pollen riche en fongicides tels que le chlorothalonil (vanEngelsdorp et al., 2009). Cependant, les abeilles peuvent aussi présenter une préférence pour la nourriture contaminée par le glyphosate et les néonicotinoïdes (Kessler et al., 2015; Liao et al., 2017). D'où l'importance d'études supplémentaires, appelées « choice-experiment », qui permettent aux abeilles de choisir entre plusieurs sources de nourriture, contaminée ou non par des pesticides. Cela permettrait de déterminer si les abeilles préfèrent ou évitent la consommation de nourriture contaminée par l'imidaclopride, le glyphosate et le difénoconazole, seuls et en mélanges, afin d'évaluer le risque induit par une exposition des abeilles à ces pesticides en conditions naturelles.

Les expériences en cagette sont largement utilisées dans les études comportementales et toxicologiques sur les abeilles domestiques. Elles offrent un environnement plus contrôlable que les expériences en colonies, sous tunnel et en plein champs et permettent de constituer une première étape pour caractériser les effets possibles des pesticides. Cependant, elles pourraient ne pas permettre de mettre en évidence la réponse au stress au niveau de la colonie. Cette dernière forme un superorganisme avec des interactions sociales entre les castes reproductrices et les ouvrières. Elle est caractérisée par une capacité de résilience qui se traduit, entre autres, par une modulation de l'activité de ponte de la reine et par un polyéthisme d'âge qui varie suivant les besoins de la colonie (cf. A.3.2). Les interactions sociales, entre les individus au sein de la colonie, impliquent que les effets d'un facteur de stress tel qu'un pesticide ou un agent pathogène, peuvent être plus visibles chez les individus qui n'ont pas eux-mêmes rencontré directement le facteur de stress. Par exemple, le glyphosate et l'imidaclopride affectent tous les deux les glandes hypopharyngiennes des abeilles (Faita et al., 2018; Hatjina et al., 2013). Ces glandes produisent

la gelée royale qui est l'aliment exclusif des reines au stade larvaire et adulte. Ainsi, l'imidaclopride et le glyphosate, en modifiant la composition de la gelée royale, pourraient affecter les reines et agir sur la santé de la colonie sur le long terme. C'est pour cette raison que l'étude des effets de l'imidaclopride, du glyphosate et du difénoconazole au niveau colonial donnerait une information plus représentative de la réalité. Les colonies d'abeilles pourraient être placées en conditions de terrains semi-contrôlées (sous tunnels) et exposées aux trois pesticides, seuls et en mélanges, à travers du sirop contaminé pendant un mois. Elles seraient ensuite placées en conditions environnementales réelles correspondant à la pratique apicole, dans différents contextes agro-environnementaux, puis évaluées pendant une période d'au moins un an en considérant, au cours du temps, la survie de la colonie et les mortalités hivernales, la production de miel, le développement du couvain, la longévité de la reine et l'état sanitaire de la colonie.

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IV. Annexe

Ecotoxicology and Environmental Safety 203 (2020) 111013

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Mixtures of an insecticide, a fungicide and a herbicide induce high toxicities and systemic physiological disturbances in winter *Apis mellifera* honey bees

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ARTICLE INFO

Keywords:
Winter honey bee
Pesticide mixtures
Synergy
Cocktail effects
Physiological state

ABSTRACT

Multiple pesticides originating from plant protection treatments and the treatment of pests infecting honey bees are frequently detected in beehive matrices. Therefore, winter honey bees, which have a long life span, could be exposed to these pesticides for longer periods than summer honey bees. In this study, winter honey bees were exposed through food to the insecticide imidacloprid, the fungicide difenoconazole and the herbicide glyphosate, alone or in binary and ternary mixtures, at environmental concentrations (0 (controls), 0.1, 1 and 10 μg/L) for 20 days. The survival of the honey bees was significantly reduced after exposure to these 3 pesticides individually and in combination. Overall, the combinations had a higher impact than the pesticides alone with a maximum mortality of 52.9% after 20 days of exposure to the insecticide-fungicide binary mixture at 1 μg/L. The analyses of the surviving bees showed that these different pesticide combinations had a systemic global impact on the physiological state of the honey bees, as revealed by the modulation of head, midgut and abdomen glutathione-*S*-transferase, head acetylcholinesterase, abdomen glucose-6-phosphate dehydrogenase and midgut alkaline phosphatase, which are involved in the detoxification of xenobiotics, the nervous system, defenses against oxidative stress, metabolism and immunity, respectively. These results demonstrate the importance of studying the effects of chemical cocktails based on low realistic exposure levels and developing long-term tests to reveal possible lethal and adverse sublethal interactions in honey bees and other insect pollinators.

1. Introduction

Despite the 45% global increase in managed honey bee colonies since 1961 (Aizen and Harder, 2009; Faostat, 2008), regional colony losses have been reported in different areas, such as the United States of America (USA) and Europe. In the USA, 31.3% of colonies were lost between 2007 and 2008, while in central Europe, a significant decrease of 25% took place between 1985 and 2005 (Potts et al., 2010; Vanengelsdorp et al., 2008). The reduction in managed beehives is accompanied by a global decrease in the number and diversity of other animal pollinators (Ollerton, 2017). It has been attributed to multiple factors, including the decline in diversity and abundance of flowers, the lack of natural habitat, the presence of parasites and pathogens and exposure to pesticides (Goulson et al., 2015; vanEngelsdorp and Meixner, 2010).

Field surveys have confirmed a transfer from crops to beehive matrices of applied pesticides belonging to the three main classes of insecticides, fungicides and herbicides (Piechowicz et al., 2018; Pohorecka et al., 2012; Skerl et al., 2009). Scientists were interested in knowing the effects of insecticides on honey bees, as these products are considered the most potentially dangerous pesticides to beneficial insects (Brandt et al., 2016; Decourtye et al., 2004; Glavan and Bozic, 2013; Gregorc and Ellis, 2011; Guez et al., 2001; Kessler et al., 2015; Yang et al., 2008). Fungicides and herbicides are considered harmless to honey bees due to their low acute toxicity. Nevertheless, an increasing number of studies are addressing their actual effects (Christen et al., 2019; Cousin et al., 2013; Jaffe et al., 2019; Ladurner et al., 2005; Moffett et al., 1972). In beehive matrices, the phytopharmaceutical products of three main classes can coexist with acaricides used to control infestation by *Varroa destructor* (Chauzat et al., 2006, 2009; Mullin et al., 2010). Therefore, honey bees could be continuously exposed to mixtures of pesticides that may exhibit similar or completely different modes of action.

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https://doi.org/10.1016/j.ecoenv.2020.111013
Received 9 March 2020; Received in revised form 9 June 2020; Accepted 6 July 2020
Available online 21 July 2020

Despite the high probability of honey bee exposure to mixtures of pesticides, only a few studies have focused on their effects on honey bees, and most of them were restricted to the interactions between insecticides (pyrethroids and neonicotinoids) and fungicides (ergosterol biosynthesis inhibitor (EBI) family) (Bjergager et al., 2017; Colin and Belzunces, 1992; Iwasa et al., 2004; Meled et al., 1998; Schmuck et al., 2003; Thompson et al., 2014; Zhu et al., 2017a, 2017b). Effects varied from no effects to synergism, depending on the pesticides used, the method and duration of exposure, and the concentrations in food. Therefore, there is a large gap in the assessment of pesticide risk in the registration procedure because the mixtures were never investigated, and further studies are urgently needed in this field.

The losses of honey bee colonies are mostly seen at the end of the winter season (Genersch et al., 2010; Guzmán-Novoa et al., 2010), with approximately 20–30% losses in Canada, Europe and the USA (van der Zee et al., 2012). During this period, beehive tasks are performed by a specific category of workers known as winter honey bees. These honey bees can survive up to 6 months (Free and Spencer-booth, 1959), and they rely on the consumption of stored honey and bee bread for survival, exposing them to pesticides for a relatively long period.

Imidacloprid (insecticide), difenoconazole (fungicide) and glyphosate (herbicide) are among the pesticides that are frequently detected in beehive matrices (Berg et al., 2018; Chauzat et al., 2011; Mullin et al., 2010). Imidacloprid, together with its metabolite 6-chloronicotinic acid, was the most abundant pesticide in beehive matrices in French apiaries, with a mean concentration of 0.7 μg/kg in honey and 0.9 μg/kg in pollen (Chauzat et al., 2011). However, concentrations of 0.14–0.275 μg/kg in honey, 1.35 μg/kg in pollen and 3–5.09 μg/kg in wax comb were found in other studies (Lambert et al., 2013; Lopez et al., 2016; Nguyen et al., 2009). Imidacloprid belongs to the neonicotinoid family and acts as an agonist of the nicotinic acetylcholine receptors, leading to the disruption of the nervous system through impaired cholinergic neurotransmission (Casida and Durkin, 2013). Glyphosate is the most dominant herbicide worldwide. Its use has increased 15-fold since the introduction of genetically engineered glyphosate-tolerant crops in 1996 (Benbrook, 2016), and it was detected in beehive matrices at concentrations ranging between 17 and 342 μg/kg in honey and 52.4–58.4 μg/kg in beebread (Berg et al., 2018; El Agrebi et al., 2020; Rubio et al., 2015). It acts by inhibiting the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS), an enzyme necessary for the biosynthesis of aromatic amino acids in plants and some microorganisms, which leads to cell death (Amrhein et al., 1980). Difenoconazole, a curative and preventive fungicide of the triazole family, is authorized for use during full bloom. It has been found at mean concentrations of 0.6 μg/kg in honey, 43 μg/kg in pollen, 270 μg/kg in beebread and 1 μg/kg in wax comb (Kubik et al., 2000; Lopez et al., 2016). It belongs to the ergosterol biosynthesis inhibitor (EBI) fungicides and acts by inhibiting the demethylation of lanosterol (Zarn et al., 2003).

To understand the effects of pesticide mixtures on winter honey bees, we conducted a study investigating the effects of the insecticide imidacloprid, the fungicide difenoconazole and the herbicide glyphosate alone or in combinations in winter bees orally exposed at concentrations found in honey and pollen (Berg et al., 2018; Chauzat et al., 2011; Kubik et al., 2000; Nguyen et al., 2009; Thompson et al., 2019). Attention was focused on survival and physiology. The effects on physiological functions were assessed by analyzing the modulation of five physiological markers involved in the nervous system, detoxification, oxidative stress, metabolism and immunity.

2. Materials and methods

2.1. Reagents

Triton X-100, monosodium phosphate (NaH_2PO_4), sodium chloride (NaCl), pepstatin A, leupeptin, aprotinin, trypsin, antipain, 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW284C51), 4-nitrophenyl acetate (*p*-NPA), ethanol, disodium phosphate (Na_2HPO_4), monopotassium phosphate (KH_2PO_4), disodium ethylenediaminetetraacetate dihydrate (EDTA), reduced L-glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), acetonitrile (CH_3CN), acetylthiocholine iodide (AcSCh), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), sodium bicarbonate ($NaHCO_3$), tris base, D-glucose-6-phosphate disodium salt hydrate (G6P), magnesium chloride hexahydrate ($MgCl_2.6H_2O$), β-nicotinamide adenine dinucleotide phosphate hydrate (β-$NADP^+$), 4-nitrophenyl phosphate bis(tris) salt (*p*-NPP), sodium hydroxide (NaOH), dimethyl sulfoxide (DMSO) and hydrochloric acid (HCl) were obtained from Sigma Aldrich (Saint Quentin Fallavier, France). Imidacloprid (CAS No 138261-41-3), difenoconazole (CAS No 119446-68-3) and glyphosate (CAS No. 1071-83-6) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Protein solution (Bee Food) was purchased from Remuaux Ltd (Barbentane, France).

2.2. Honey bees

Honey bees were gathered in February 2018 from three colonies of the experimental apiary of the Abeilles & Environnement (Bees & Environment) research unit of INRAE (Avignon, France). The colonies were continuously checked for their health status. The honey bees were mixed together, slightly anesthetized with carbon dioxide and then placed, in groups of 30 honey bees, in plastic cages (6 × 8.5 × 10 cm) with a sheet of filter paper placed on the bottom and replaced daily to maintain hygiene. The honey bees were placed in the dark in incubators at 30 °C ± 2 °C and 60% ± 10% relative humidity. During the first day, the bees were fed water and candy (Apifonda®) *ad libitum*. The following day, the few dead bees were removed and replaced, and the chronic exposure to pesticides for 20 days was begun.

2.3. Chronic exposure to pesticides

The bees were exposed to the insecticide imidacloprid (I), the fungicide difenoconazole (F) and the herbicide glyphosate (H) individually or in combination. Imidacloprid, difenoconazole and glyphosate were prepared either alone or in binary mixtures (imidacloprid + glyphosate (IH), imidacloprid + difenoconazole (IF), and glyphosate + difenoconazole (HF)) or in a ternary mixture (imidacloprid + glyphosate + difenoconazole (IHF)) at concentrations of 0.1, 1 and 10 μg/L for each substance (equivalent to 0.083, 0.813 and 8.130 μg/kg, calculated with a sucrose solution density of 1.23 ± 0.02 (n = 10)) in a 60% (w/v) sucrose solution containing a 0.1% (v/v) final concentration of DMSO. The treatments were abbreviated as follows: 0.1 μg/L: I0.1, F0.1, H0.1, IH0.1, IF0.1, HF0.1 and IHF0.1; 1 μg/L: I1, F1, H1, IH1, IF1, HF1 and IHF1; and 10 μg/L: I10, F10, H10, IH10, IF10, HF10 and IHF10. The primary mother solutions of the individual pesticides were prepared in 100% DMSO. These primary solutions were used to generate the mother solutions of the individual pesticides or were mixed to obtained the mother solutions of the pesticide mixtures. The mother solutions of the pesticides were prepared by serial dilution of the primary mother solutions to obtain 1% (v/v) DMSO and stored at −20 °C. The sucrose solutions used for exposure to pesticides were prepared daily by 10-fold dilution of the mother pesticide solutions in sucrose solution to obtain final concentrations of 60% (m/v) sucrose, 1% (m/v) proteins and 0.1% (v/v) DMSO. The pesticide concentrations were checked by GC-MS/MS according to two analytical methods with RSD <10% (Paradis et al., 2014; Wiest et al., 2011). The control bees were fed a sucrose solution devoid of pesticides. For each modality of exposure (including the controls), 14 cages of 30 bees were used. Each day, the bee mortality and food consumption were recorded, the dead bees were discarded, and the filter paper placed at the bottom of the cage was replaced. For the analysis of the physiological markers, the bees were sampled 10 and 20 days after the beginning of chronic exposure.

2.4. Survival rate and food consumption

In each cage, the survival rate was recorded daily and expressed as a ratio of the initial population. Every morning, the dead bees were removed for sanitary considerations.

Food consumption was recorded for 20 days by measuring the food consumed daily by the bees in each cage. Individual daily food consumption was calculated by dividing the food consumed per cage by the number of bees that remained alive each day in each cage.

2.5. Choice of physiological markers

The effects of the pesticide combinations on honey bee physiology were assessed by analyzing the modulation of five physiological markers. The markers were chosen to distinguish the systemic and tissue-specific actions of the pesticides alone and in combination. The following two markers common to the three biological compartments (head, midgut and abdomen) were analyzed: CaE-3 and GST. In contrast, one specific physiological marker was chosen in each compartment as follows: AChE in the head, G6PDH in the abdomen and ALP in the midgut. These five markers have been found to be relevant in assessing the effects of pesticides on honey bees in different biological compartments (Badiou-Beneteau et al., 2012, 2013; Boily et al., 2013; Carvalho et al., 2013; Kairo et al., 2017; Zhu et al., 2017a, 2017b).

2.6. Tissue preparation and marker extraction

At days 10 and 20, the surviving bees were sampled. To avoid animal suffering, the bees were anesthetized with carbon dioxide, the heads were separated from the rest of the body using a scalpel, and the midguts were obtained by pulling the stinger. The heads, midguts and abdomens (with the intestinal tract removed) were placed in 2 mL microfuge tubes, weighed and stored at −80 °C until analysis. For each treatment modality and each type of tissue, 3 tissues were used and pooled to prepare the sample. From this sample, the tissues were homogenized to prepare a single tissue extract. Seven tissue extracts (7 × 3 tissues) were prepared (n = 7) for each treatment modality. Each sample was assayed in triplicate. The tissues were homogenized in the extraction medium [10 mM sodium chloride, 1% (w/v) Triton X-100, 40 mM sodium phosphate pH 7.4 and protease inhibitors (2 μg/mL of pepstatin A, leupeptin and aprotinin, 0.1 mg/mL soybean trypsin inhibitor and 25 units/mL antipain)] to make 10% (w/v) extracts. Homogenization was achieved by grinding tissues with a high-speed Qiagen TissueLyser II at 30 Hz for 5 periods of 30 s at 30 s intervals. The extracts were centrifuged at 4 °C for 20 min at 15 000 × gav. and the supernatants were kept on ice for further enzyme assays. Carboxylesterase para (CaE-3) and glutathione-*S*-transferase (GST) were extracted from the head, midgut and abdomen; acetylcholinesterase (AChE) from the head; glucose-6-phosphate dehydrogenase (G6PDH) from the abdomen; and alkaline phosphatase (ALP) from the midgut.

2.7. Enzyme assays

CaE-3 was assayed in a medium containing the tissue extract, 10 μM BW284C51 (acetylcholinesterase inhibitor), 0.1 mM *p*-NPA as the substrate and 100 mM sodium phosphate pH 7.0. The reaction was monitored at 410 nm (Badiou-Beneteau et al., 2012; Gomori, 1953; Renzi et al., 2016). GST was assayed at 340 nm by measuring the conjugation of GSH to CDNB. The extract was incubated in a medium containing 1 mM EDTA, 2.5 mM GSH as the cosubstrate, 1 mM CDNB as the substrate and 100 mM disodium phosphate pH 7.4 (Carvalho et al., 2013). AChE was assayed at 412 nm in a medium containing the tissue extract, 1.5 mM DTNB, 0.3 mM AcSCh as the substrate and 100 mM sodium phosphate pH 7.0 (Belzunces et al., 1988). G6PDH was measured by following the formation of NADPH at 340 nm in a medium containing the tissue extracts, 1 mM G6P as the substrate, 0.5 mM $NADP^+$ as the coenzyme, 10 mM $MgCl_2$ and 100 mM Tris-HCl pH 7.4 (Renzi et al., 2016). ALP was assayed at 410 nm in a medium containing the tissue extract, 20 μM $MgCl_2$, 2 mM *p*-NPP as the substrate and 100 mM Tris-HCl pH 8.5 (Bounias et al., 1996). All reactions started after adding the substrate, and the activity was assessed by determining the initial velocity of the enzymatic kinetics, which corresponded to the slope of the tangent at the origin. All enzymatic reactions were followed using a TECAN F500 spectrophotometer.

2.8. Mode of interaction between pesticides

The interaction ratio (IR) was used to define the mode of interaction between pesticides (additive, antagonistic and synergistic) (Colin and Belzunces, 1992; Piggott et al., 2015):

$$\mathrm{IR} = \frac{(Mix - C)}{\sum_{n=0}^{2-3}(P_n - C)}$$

where *Mix* represents the crude mortality of the mixture (binary or ternary), *C* the mortality of the control, and (*Mix* - *C*) the mortality of the pesticide mixture corrected by the control mortality. $\sum_{n=0}^{2-3}(P_n - C)$ represents the sum of the mortalities induced by each pesticide (*n*) in the mixture corrected by the control mortality, which corresponds to the theoretical expected mortality of the mixture. A value of IR = 1 reflects a pure additive effect. However, considering the variation in the effects, an IR is considered equal to 1 when $0.95 \leq \mathrm{IR} \leq 1.05$. When IR > 1, the interaction is synergistic. For IR < 1, three cases were distinguished: (i) when the mortality of the mixture was lower than the mortality of the lowest toxic substance alone, the interaction was considered purely antagonistic. (ii) When the toxicity of the mixture was higher than the mortality of the most toxic substance but below the expected mortality, the interaction was considered subadditive. In this case, it was not possible to speak in terms of antagonism because the effect of the mixture was higher than the effect of each substance. (iii) When the effect of the mixture was between the effect of the least toxic substance and the effect of the most toxic substance, the interaction was also considered subadditive. In this case, it was also not possible to speak in terms of antagonism because, compared to the most toxic substance, antagonism could be considered, but compared to the least toxic substance, synergy could also be considered. (iv) The effect of the mixture was judged independent when the mixture induced a mortality similar to that of each pesticide.

2.9. Statistical analyses

The statistical analyses were performed using R software (Rstudio Version 1.1.463). The bee survival was analyzed by the Kaplan-Meier method (log-rank test), followed by a post hoc test to compare survival and treatments. The effects of the treatments on food consumption were investigated by comparing the individual cumulative sucrose consumption during the exposure period using the Kruskal-Wallis test, followed by pairwise comparisons using the Wilcoxon rank sum test with a Benjamini-Hochberg correction. The effects of the treatments on the physiological markers were determined by ANOVA, followed by Tukey's HSD test, when the data followed a normal distribution or a Kruskal-Wallis test, followed by a post hoc Dunn test (with Benjamini-Hochberg correction), when the data followed a non-normal distribution.

3. Results

3.1. Honey bee survival

Exposure to pesticides significantly decreased the survival rate of honey bees at 20 days, except for I0.1, I10 and F0.1, for which no significant difference from the control (20.0 ± 2.7%) was observed ($p >$

0.05) (Fig. 1A, 1B, 1C and Table S1). Based on mortality rates, the toxicities of pesticides could be ranked as follows: at 0.1 μg/L, H = IF (28.1%) < IHF (35.4%) < IH (43.3%) < HF (49.1%). At 1 μg/L, I (33.3%) < F (34.3%) < H (35.2%) < HF (36.2%) < IH (38.1%) < IHF (43.3%) < IF (52.9%). At 10 μg/L, HF (28.1%) < H (30.0%) < F (34.3%) < IF (41.0%) < IHF (43.3%) < IH (45.7%).

Based on the interaction ratio (IR), which corresponds to the ratio between the obtained mortality of the mixture and the expected mortality (sum of the obtained mortalities of the substances in the mixture), the interaction effects between the pesticides could be grouped into 5 different categories (Table S1): additive, synergistic, subadditive, antagonistic and independent effects. (i) A synergistic effect was observed for all the binary mixtures and the ternary mixture at 0.1 μg/L and for IF1 and IH10. (ii) An additive effect was observed for IF10. (iii) A subadditive effect was observed for IH1, IHF1 and IHF10. (iv) An independent effect was observed for HF1. (v) An antagonistic effect was observed for HF10. The five most toxic pesticide mixtures were ranked as follows based on mortality rates: IF10 (41.0%) < IHF1 = IHF10 = IH0.1 (43.3%) < IH10 (45.7%) < HF0.1 (49.1%) < IF1 (52.9%).

3.2. Effects of exposure to pesticides on food consumption behavior

Food consumption was monitored daily. In general, at the end of the exposure period, it appeared that the food consumption was higher in the exposed bees (Fig. 2 and Table S2). This higher consumption was significant for all exposure conditions except F1, I1, F10 and I10 for pesticides alone, and HF10 and IHF10 for the mixtures. The five highest individual cumulative consumption levels were ranked as follows: H0.1 (831.4 mg/bee) < IF10 (834.3 mg/bee) < IF1 (840.3 mg/bee) < HF0.1 (851 mg/bee) < IH0.1 (862.7 mg/bee) (control = 672.4 ± 33.0 mg/bee). At 0.1 μg/L, the bees exposed to imidacloprid alone or in IF, IH or IHF exhibited a cumulative food consumption of 759.7, 792.6, 862.7 and 781.9 mg/bee, respectively. Therefore, on the basis of a food density of 1.23 ± 0.02 (n = 10) and pesticide concentrations, each honey bee ingested 62, 64, 70 and 63 pg of imidacloprid, which corresponded to ca. 1/60, 1/58, 1/53 and 1/58 of the imidacloprid LD_{50} (LD_{50} = 3.7 ng/bee (Schmuck et al., 2001)). At 1 μg/L, the bees exposed to imidacloprid alone or in IF, IH or IHF exhibited a cumulative food consumption of 719.3, 840.3, 804.2 and 758.4 mg/bee, respectively. Therefore, each honey bee ingested 584, 682, 653 and 615 pg of imidacloprid, which corresponded to ca. 1/6, 1/5, 1/6 and 1/6 of the imidacloprid LD_{50}. At 10 μg/L, the bees exposed to imidacloprid alone or in IF, IH and IHF exhibited a cumulative food consumption of 749.3, 834.3, 794.1 and 702.5 mg/bee, respectively. Therefore, each honey bee ingested 6081, 6770, 6445 and 5701 pg of imidacloprid, respectively, which corresponded to ca. 1/0.6, 1/0.6, 1/0.6 and 1/0.7 of the imidacloprid LD_{50}.

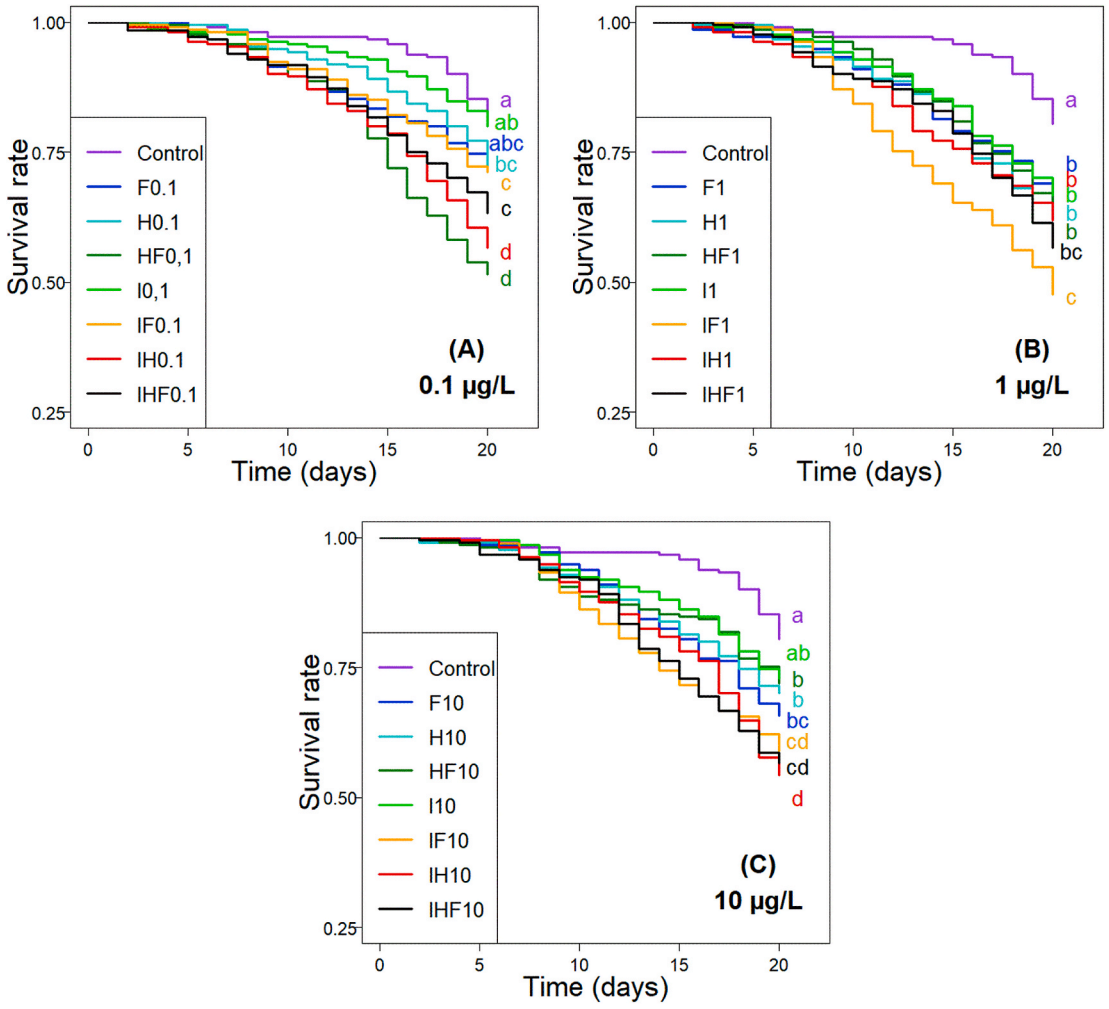

Fig. 1. Effects of pesticides alone or in combination on honey bee longevity. For 20 days, winter honey bees were fed sucrose solutions containing no pesticides (Control), difenoconazole (F), glyphosate (H), glyphosate + difenoconazole (HF), imidacloprid (I), imidacloprid + difenoconazole (IF), imidacloprid + glyphosate (IH) or imidacloprid + glyphosate + difenoconazole (IHF), at 0.1 μg/L (A), 1 μg/L (B) and 10 μg/L (C). The data represent the proportion of surviving honeybees exposed to these pesticides. Numbers after the abbreviations of each treatment refer to the concentrations of the pesticides in the sucrose solution. Treatments with different letters are significantly different ($p < 0.05$).

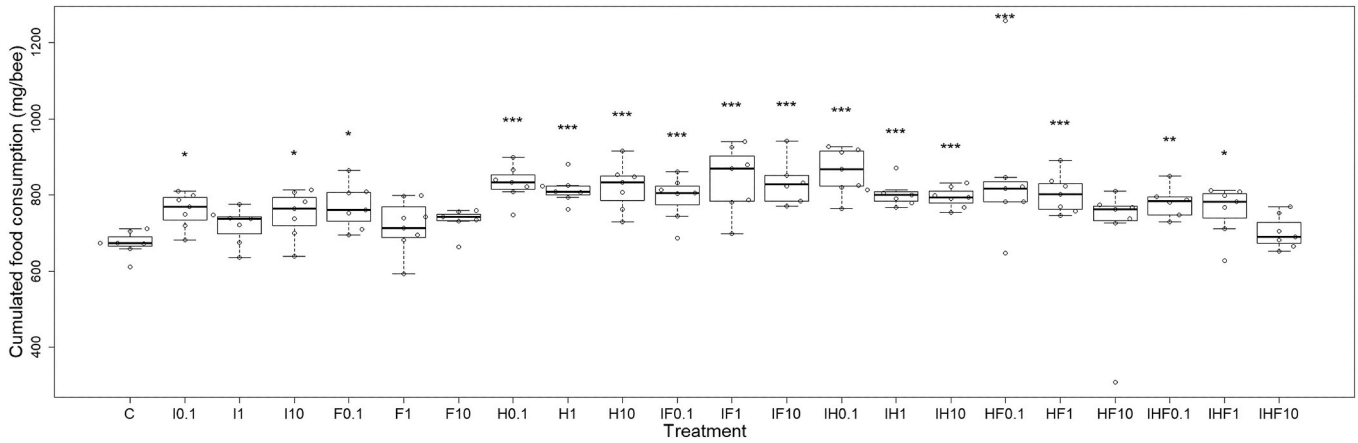

Fig. 2. Effects of pesticides alone or in combination on food consumption. For 20 days, winter honey bees were fed sucrose solutions containing no pesticide (C, control), difenoconazole (F), glyphosate (H), glyphosate + difenoconazole (HF), imidacloprid (I), imidacloprid + difenoconazole (IF), imidacloprid + glyphosate (IH) or imidacloprid + glyphosate + difenoconazole (IHF), at 0.1 μg/L, 1 μg/L, and 10 μg/L. Food consumption was followed during the 20 days of exposure by measuring the food consumed daily by the bees alive in each cage. Box plots represent the cumulated individual consumption (mg/bee) for 7 cages of 30 bees per treatment. Statistical analyses were performed using the Kruskal-Wallis test followed by pairwise comparisons using the Wilcoxon rank sum test with the Benjamini-Hochberg correction. The numbers after the abbreviations of each treatment refer to the concentrations of the pesticides in the sucrose solution. Asterisks indicate significant differences from the control group (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$).

The $LD_{50\ values}$ of difenoconazole and glyphosate are equal to or higher than 100 μg/bee (National Center for Biotechnology Information). Therefore, for difenoconazole and glyphosate at 0.1, 1 and 10 μg/L, each honey bee ingested at least $1/1.4 \times 10^6$, $1/1.6 \times 10^5$ and $1/1.5 \times 10^4$ of the LD_{50}, respectively (Table S2).

3.3. Effect of exposure to pesticides on the physiological status of honey bees

The physiological status of the honey bees was examined by studying the modulation of physiological markers in different compartments to distinguish the local from the systemic effects of the pesticides (Table 1). The responses of the honey bee markers to the exposure to the pesticides alone or in combination were analyzed after 10 and 20 days of chronic exposure to concentrations of 0.1 μg/L and 1 μg/L (Fig. 3, Fig. 4, Table S3 and Table S4). The lowest concentrations were chosen because they are particularly environmentally relevant. To render the data comparable, the enzymatic activities are expressed as percentages of the control values (Zhu et al., 2017a).

At 0.1 μg/L, head, midgut and abdomen CaE-3 and midgut GST were not modulated by all types of exposure at day 10 and day 20. Head AChE was not modulated at day 10. However, at day 20, its activity was 119% of the control activity (127.5 ± 16.0 mAU.min^{-1}.mg of tissue^{-1}) for H, 126% for HF and 141% for IHF. Head GST, abdomen G6PDH, and midgut ALP underwent modulation at day 10. For IHF, these modulations corresponded to a decrease in head GST (82% of control activity (115.3 ± 7.5 mAU.min^{-1}.mg of tissue^{-1})) and a decrease in abdomen G6PDH (48% of control activity (2.1 ± 0.5 mAU.min^{-1}.mg of tissue^{-1})). For IH, midgut ALP increased to 199% of the control activity (10.9 ± 2.8 mAU.min^{-1}.mg of tissue^{-1}). Conversely, no modulation was observed at day 20 for any of these latter enzymes. A decrease in abdomen GST was observed at 10 and 20 days. At 10 days, GST decreased to 57% of the control activity (116.1 ± 33.3 mAU.min^{-1}.mg of tissue^{-1}) for H. At day 20, GST decreased to 48% of the control activity (83.0 ± 28.7 mAU.min^{-1}.mg of tissue^{-1}) for IH and 49% for HF.

At 1 μg/L, head, midgut and abdomen CaE-3 and midgut ALP were not modulated for all types of exposure at day 10 and day 20. Head and abdomen GST underwent modulation at day 10. Head GST decreased to 75% of the control activity (115.3 ± 7.5 mAU.min^{-1}.mg of tissue^{-1}) for H and 93% for IHF. Abdomen GST decreased for all types of exposure except IH: 49% of the control activity for I; 44% for H; 36% for F; 35% for IF; 51% for HF and 18% for IHF (116.1 ± 33.3 mAU.min^{-1}.mg of tissue^{-1} for the control). Conversely, head and abdomen GST were not modulated at day 20. Abdomen G6PDH decreased at day 10 for all types of exposure: 56% of the control activity for I; 44% for H; 41% for F; 46% for IH; 38% for IF; 55% for HF and 44% for IHF (12.1 ± 0.5 mAU.min^{-1}.mg of tissue^{-1} for the control). However, no modulation was observed at day 20. Midgut GST was not modulated at day 10 but was modulated at day 20. Its activity decreased with all exposure types except IH and HF: 95% of the control activity for I; 88% for H; 96% for F; 93% for IF and 88% for IHF (147.9 ± 18.8 mAU.min^{-1}.mg of tissue^{-1} for the control). At day 10, head AChE increased to 128% of the control activity (127.7 ± 18.5 mAU.min^{-1}.mg of tissue^{-1}) for HF and 134% of the control activity for IHF. At day 20, the activity of AChE increased to 124% of the control (127.5 ± 16.0 mAU.min^{-1}.mg of tissue^{-1}) for HF, 127% of the control for IHF and 119% of the control for IF.

When comparing the dose effect of each type of exposure on physiological markers (comparison of the effects at 0.1 and 1 μg/L), no dose effect could be observed for I alone. The effects of H on all markers were similar at both concentrations except for AChE at day 20 and head GST at day 10 (H0.1 > H1). F had the same effect on all markers at both concentrations except for AChE at day 20 (F0.1 > F1). The effect of IH on CaE-3, ALP, and abdomen GST was not similar at both concentrations. The effect of IH on head CaE-3 at day 10 and on abdomen CaE-3 and GST at day 20 was lower at 0.1 μg/L than at 1 μg/L. Conversely, the effect of IH on midgut CaE-3 at days 10 and 20 and on abdomen CaE-3 and midgut ALP at day 10 was higher at 0.1 μg/L than at 1 μg/L. The effect of IF on midgut GST at day 20 was higher at 0.1 μg/L than at 1 μg/L. Depending on the concentration, the IF mixture modulated abdomen GST at day 10 (IF0.1 > IF1) and abdomen G6PDH at day 10 (IF0.1 >

Table 1
Distribution of common and specific physiological markers across honey bee tissues.

	Head	Abdomen	Midgut
Common markers	CaE-3 GST	CaE-3 GST	CaE-3 GST
Specific markers	AChE	G6PDH	ALP

Repartitioning of physiological markers across honey bee compartments. The following three tissues were investigated: head, abdomen and midgut. In each tissue, 1 specific marker (AChE in the head, G6PDH in the abdomen and ALP in the midgut) and 2 common markers (CaE-3 and GST) were considered.

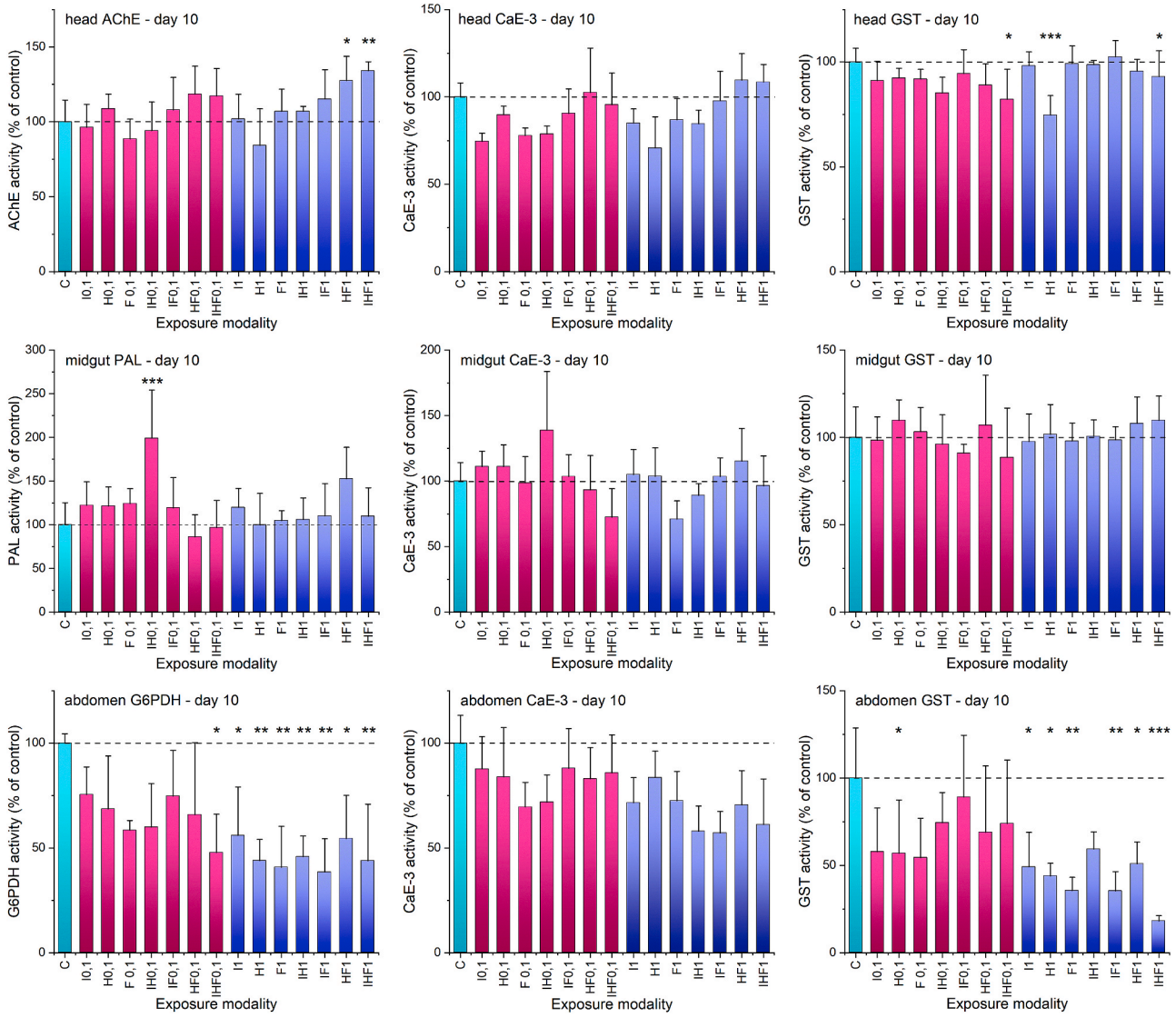

Fig. 3. Physiological impacts of pesticides alone or in combination in winter bees after 10 days of exposure. For 20 days, winter honey bees were fed sucrose solutions containing no pesticides (C, control), imidacloprid (I), glyphosate (H), difenoconazole (F), imidacloprid + glyphosate (IH), imidacloprid + difenoconazole (IF), glyphosate + difenoconazole (HF), or imidacloprid + glyphosate + difenoconazole (IHF). The impact of the exposure to pesticides on the physiology of the surviving honey bees at day 10 was investigated through an analysis of 2 common markers in the head, abdomen and midgut (GST and CaE-3) and 3 specific markers (AChE in the head, G6PDH in the abdomen and ALP in the midgut). To make the data comparable, the enzymatic activities were expressed as percentages of the control values. Numbers after the abbreviation of each treatment refer to the concentration of the pesticide in the sucrose solution. The exposure modalities above and below the dashed horizontal line indicate increases and decreases in enzymatic activity, respectively, compared to the control (C). Asterisks indicate significant differences from the control group (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$).

IF1). The effect of HF was dose-dependent only on the activity of GST in the abdomen at day 20 (HF0.1 < HF1). The effect of the ternary mixture IHF on abdomen GST at day 10 and on midgut GST at day 20 was higher at 0.1 µg/L than at 1 µg/L (IHF0.1 > IHF1) (Table S5).

4. Discussion

Honey bees that emerge at the end of the summer are considered winter bees. These bees can live up to 6 months (Free and Spencer-booth, 1959) and, therefore, are chronically exposed to pesticide residues throughout the winter. In this study, the mixtures induced relatively high toxicity even though the winter honey bees were exposed for only 20 days to imidacloprid, difenoconazole and glyphosate, alone or in binary and ternary mixtures, at concentrations equal to or even less than the environmental concentrations detected in beehive matrices. Thus, determining the effect of these pesticides on colony winter survival is highly important.

4.1. Pesticide combinations are more toxic to honeybees than individual pesticides

In this study, these three pesticides alone or in combination affected the survival of winter honey bees at all tested exposure concentrations, except for I0.1, I10 and F0.1. Concerning imidacloprid, the toxicity was less pronounced than that previously observed at the same concentrations on summer bees, where 50% mortality was reached after 8 days of chronic exposure at all concentrations (Suchail et al., 2001). In contrast, imidacloprid toxicity was much more pronounced than that observed in young summer bees after 14 days of exposure at 1 µg/L (Gonalons and Farina, 2018). The differences in imidacloprid toxicity could be attributed to seasonal variations (Decourtye et al., 2003; Meled et al., 1998; Piechowicz et al., 2016), genetic differences (Smirle and Winston,

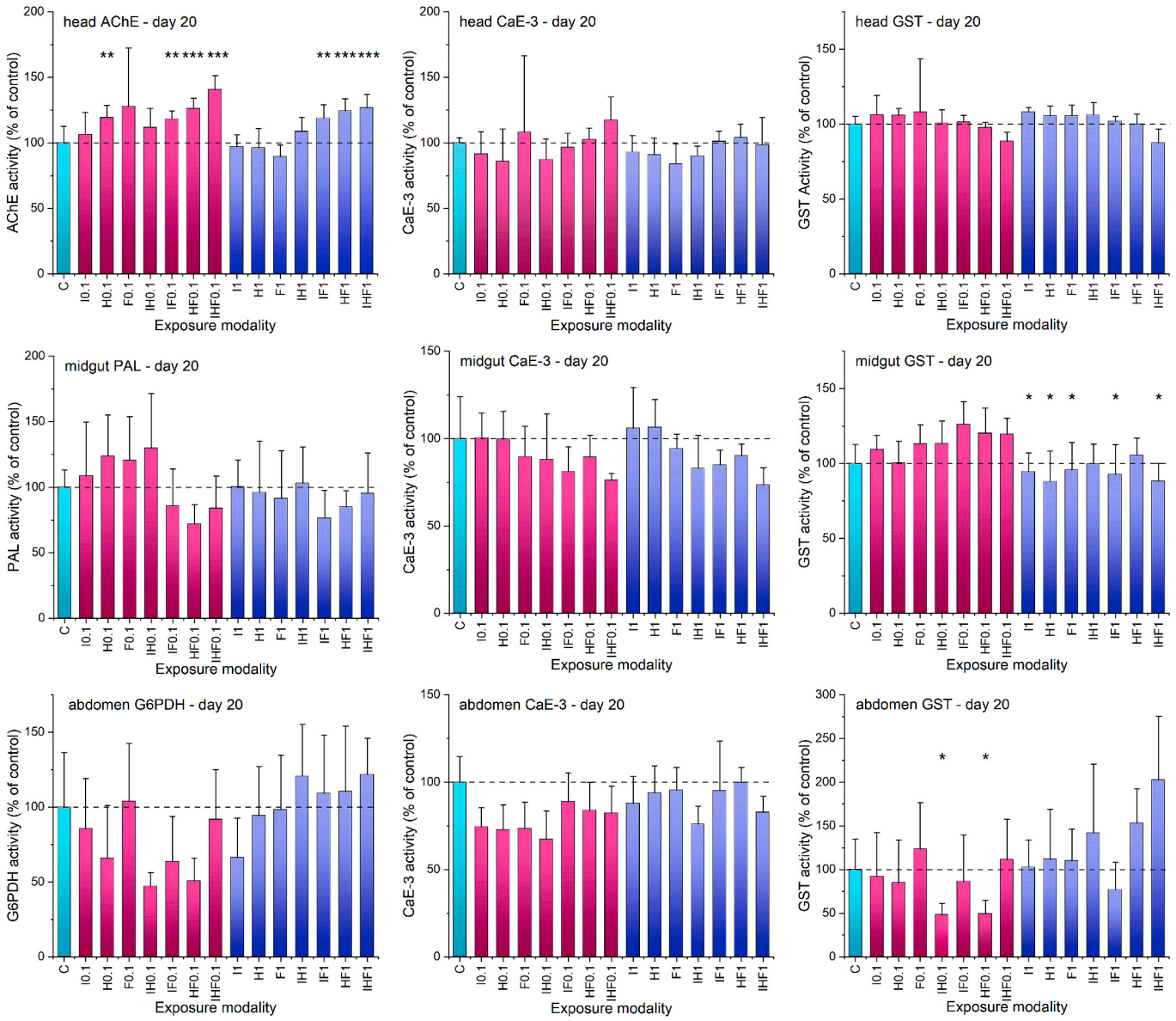

Fig. 4. Physiological impacts of pesticides alone or in combination in winter bees after 20 days of exposure. For 20 days, winter honey bees were fed sucrose solutions containing no pesticides (C, control), imidacloprid (I), glyphosate (H), difenoconazole (F), imidacloprid + glyphosate (IH), imidacloprid + difenoconazole (IF), glyphosate + difenoconazole (HF), or imidacloprid + glyphosate + difenoconazole (IHF). The impact of the exposure to pesticides on the physiology of the surviving honey bees at day 20 was investigated through an analysis of 2 common markers in the head, abdomen and midgut (GST and CaE-3) and 3 specific markers (AChE in the head, G6PDH in the abdomen and ALP in the midgut). To make the data comparable, the enzymatic activities were expressed as percentages of the control values. Numbers after the abbreviation of each treatment refer to the concentration of the pesticide in the sucrose solution. The exposure modalities above and below the dashed horizontal line indicate increases and decreases in the enzymatic activity, respectively, compared to the control (C). Asterisks indicate significant differences from the control group ($^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$).

1987), the age of the bees or the exposure duration.

Herbicides and fungicides were considered nontoxic to honey bees for a long time. Concentrations of imidazole fungicides and glyphosate up to 0.084 and 35 mg/L, respectively (Zhu et al., 2017a), were shown to be nonlethal. However, in this study, chronic exposure to glyphosate and difenoconazole (except for F0.1) was lethal. All pesticide combinations alter honey bee survival and are more toxic than pesticides alone, except HF10, which exhibits an antagonistic effect. Thus, the tier approach implemented in the pesticide registration procedure, which is first based on acute toxicity, shows great limits in detecting pesticides toxic to bees.

4.2. *Increased concentrations of pesticides are not always linked to increased toxicity*

In terms of dose-effect relationships, in general, it appears that the highest concentration was not the most dangerous, and the highest mortalities were observed at the intermediate concentration of 1 µg/L. This bell-shaped non-monotonic dose response relationship (NMDR) (high response at intermediate doses and lower responses at low and high doses) was previously observed for imidacloprid and glyphosate (Boily et al., 2013; Suchail et al., 2001; Vazquez et al., 2018). Three main hypotheses might explain this profile (Lagarde et al., 2015). The first is the plurality of molecular targets, i.e., each xenobiotic substance has several molecular targets of different affinities that may induce opposite effects across the range of the tested concentrations. The

second hypothesis is the metabolic hypothesis (Suchail et al., 2001), which proposes that detoxification enzymes are induced at high but not at low concentrations. This hypothesis is consistent with the action of glyphosate, whose main metabolite, aminomethylphosphonic acid (AMPA), was shown to be nontoxic to honey bees (Blot et al., 2019). However, the metabolic hypothesis is not consistent with the action of imidacloprid because all metabolites were shown to be toxic to honey bees after chronic exposure (Suchail et al., 2001). The third hypothesis is receptor desensitization, where at high concentrations, numerous receptors are bound to xenobiotics, leading to a downregulation phenomenon (Lagarde et al., 2015).

The mixture of EBI fungicides with imidacloprid or glyphosate was shown in different studies to have no synergistic action (Iwasa et al., 2004; Thompson et al., 2014; Zhu et al., 2017b) or to induce a synergistic effect (Biddinger et al., 2013). However, these studies were based on acute contact exposure. Therefore, it is not possible to directly compare these results with those of our study in which the mixtures induced an increase in mortality after chronic oral exposure. On the other hand, in two studies based on chronic oral exposure, the imidacloprid-fungicide and/or imidacloprid-glyphosate mixture did not show a synergistic or additive effect (Gonalons and Farina, 2018; Zhu et al., 2017a). The differences in the mixture effects between the different studies could be attributed to multiple factors: (i) The age of exposed honey bees, with newly emerged honey bees in the studies of Gonalons and Farina (2018) and Zhu et al. (2017b), and adult honey bees in our study. (ii) The duration of exposure, which did not exceed 14 days in the studies of Gonalons and Farina (2018) and Zhu et al. (2017b) but was 20 days in our study. (iii) The type of exposure, with the active ingredient in our study and in the study of Gonalons and Farina (2018) and with the formulated products in the study of Zhu et al. (2017b). (iv) Seasonal variability, which could be reflected by the use of winter honey bees in our study and summer or spring honey bees in the two previously cited studies. (v) The concentrations of the active ingredients constituting the mixtures, which were lower in our study when compared to the studies of Zhu et al. (2017b) and Gonalons and Farina (2018).

In this study, all binary mixtures had a differential effect on mortality in terms of both dose dependence and number of substances present in the mixture. Regarding the differential dose effect, HF induced a synergistic effect at 0.1 μg/L, an independent effect at 1 μg/L and an antagonistic effect at 10 μg/L. IF induced a synergistic effect at 0.1 and 1 and an additive effect at 10 μg/L. IH induced a synergistic effect at 0.1 and 10 μg/L and a subadditive effect at 1 μg/L. The ternary mixture induced a subadditive effect at 1 and 10 μg/L and a synergistic effect at 0.1 μg/L. Interactions between substances can occur not only through the primary biological targets responsible for the expected effect (insecticide, herbicide or fungicide) and common metabolic pathways, if they exist in the honey bee, but also through secondary targets responsible for non-intentional effects. Because primary and secondary targets may have different affinities for these substances, the effects induced could depend on the internal body concentration and, therefore, the exposure level. Hence, substances may interfere by blocking or activating metabolic pathways triggered by the substances in the mixtures, which explains why the nature and importance of the effects vary with the doses (Lagarde et al., 2015). However, at 0.1 μg/L, the mortality induced by IHF was lower than those induced by IH and IF, leading us to conclude that increasing concentration or number of substances does not always increase the toxicity of a mixture. This finding exemplifies that the toxicity of a mixture is not merely the sum of the toxicity of the substances or the basic sum of the individual modes of actions.

4.3. Pesticides modulate feeding behavior through an increase in food consumption

Bees exposed to imidacloprid, difenoconazole and glyphosate, alone or in mixtures, consume more food than unexposed bees. Different hypotheses could explain this high consumption. (i) A higher food consumption could be triggered by energetic stress due to an increase in intermediary metabolism induced by the pesticides or the spoliation of energetic resources as has been shown for pyrethroids (Bounias et al., 1985). (ii) Honey bees could display a preference for sucrose solutions containing glyphosate and imidacloprid, as previously shown (Kessler et al., 2015; Liao et al., 2017). In contrast, a study has shown a decrease in food consumption after exposure to mixtures of the formulated products of imidacloprid with tetraconazole and of imidacloprid with glyphosate (Zhu et al., 2017a). This finding suggests that the decrease in food consumption could be attributed to adjuvants present in the formulated products that might have a repellent feeding effect. However, the effect on food consumption could also depend on the concentration of the pesticides to which honey bees are exposed. In our study, the presence of pesticides elicited a higher food consumption, whereas in the study conducted by Zhu et al. (2017b), at higher concentrations, the pesticides elicited a lower food consumption. Thus, active substances, adjuvants or both could induce concentration-dependent effects on food consumption depending on their affinities to the biological target.

The honey bees received a cumulative dose of imidacloprid equivalent to 1/60, 1/6 and 1/0.6 of the LD_{50} at 0.1, 1 and 10 μg/L, respectively. However, for glyphosate and difenoconazole, the cumulative quantity ingested was, at least, equivalent to $1/1.4 \times 10^6$, $1/1.5 \times 10^5$ and $1/1.5 \times 10^4$ of the LD_{50} at 0.1, 1 and 10 μg/L. Despite cumulative exposure ratios of difenoconazole and glyphosate at least 10 000 times less than the LD_{50}, these two pesticides caused significant increases in mortality except for F0.1. Therefore, pesticides that are considered harmless to honey bees (high $LD_{50,}$ superior to 100 μg/bee) can become dangerous even at very low concentrations after long-term exposure. This highlights the importance of an in-depth revision of the current risk assessment schemes used in the pesticide registration procedure (Sgolastra et al., 2020).

4.4. Pesticides induce perturbations in the detoxification process, nervous system, defense against oxidative stress, metabolism and immunity

CaE-3, along with the other carboxylesterases, is involved in the metabolism of xenobiotics by catalyzing the hydrolysis of substrates containing amide, ester and thioester bonds. It is also involved in lipid metabolism (Badiou-Beneteau et al., 2012; Ross et al., 2010). In our study, head, midgut and abdomen CaE-3 were not significantly modulated by any type of exposure. However, the activity of this enzyme was reported to decrease after acute exposure to 2.56 ng bee^{-1} thiamethoxam (neonicotinoid) (Badiou-Beneteau et al., 2012) and at $LD_{50}/20$ of fipronil (Carvalho et al., 2013). Several studies have shown differential expression of carboxylesterases (CaEs) after exposure to pesticides (Badiou-Beneteau et al., 2012; Zhu et al., 2017a, 2017b, 2019). Thus, measuring only overall CaE activity with nonspecific substrates could mask the differential modulation of several isoforms, including CaE-3.

AChE is a neural enzyme hydrolyzing the neurotransmitter acetylcholine in cholinergic synapses (Badiou et al., 2007). AChE was found to be involved in learning and memory processes (Gauthier et al., 1992; Guez et al., 2010). Its activity was significantly increased for HF1 and IHF1 at day 10 and for IF, HF and IHF at 0.1 and 1 μg/L at day 20. Therefore, the increase in AChE activity is closely related to the duration of exposure and the concentrations of the pesticides forming the mixture. This reflects a delayed effect of the pesticide combinations on the nervous system and reveals the importance of studies on the effects of these pesticide combinations on the behavior and cognitive functions of honey bees.

Glyphosate increased AChE activity in the bees exposed to 0.1 μg/L. This finding contradicts the results showing that both newly emerged and adult honey bees exposed for up to 14 days during the summer period to glyphosate or its formulated product Roundup, at concentrations ranging from 2.5 to 10 ng/bee (Boily et al., 2013) and 35 mg/L, exhibit a decrease in AChE activity (Zhu et al., 2017a). The difference in

the effect of glyphosate between our study and the previously cited studies could be attributed to seasonal variability. This hypothesis is supported by studies showing that the adverse effects of pesticides may be higher in summer bees than in winter bees. This higher sensitivity of summer bees has been shown in terms of the effects of imidacloprid on learning performance (Decourtye et al., 2003) and the synergistic effect of the pyrethroid insecticide deltamethrin and the azole fungicide prochloraz (Meled et al., 1998). These alterations in AChE activities might explain, at least in part, the impairment of cognitive behaviors, sucrose responsiveness and olfactory learning observed in honey bees after exposure to glyphosate (Balbuena et al., 2015; Gonalons and Farina, 2018; Herbert et al., 2014).

GST is a multifunctional enzyme involved in protection against oxidative stress and is a phase II enzyme involved in the detoxification of xenobiotics. It can also contribute to phase I detoxification by sequestering toxicants (Berenbaum and Johnson, 2015; du Rand et al., 2015). GST activity was mainly decreased after exposure to pesticides in the head, abdomen and midgut. This decrease could hypothetically be due either to inhibition of this enzyme or to a downregulation by these pesticides. However, noncovalent inhibition could not be detected because of the dilution of the tissue components during the step of tissue homogenization and the assay procedure (at least 1/200-fold final dilution). In addition, a covalent inhibition of GST by pesticides has never been reported, even with electrophilic pesticides such as organophosphorus insecticides or herbicides that include glyphosate. Thus, the decrease in GST activity, associated with the absence of inhibition, is consistent with GST downregulation, which is also consistent with the 4-fold downregulation of GST S1, which is responsible for fighting against oxidative stress, in the heads of honey bee larvae exposed to imidacloprid (Wu et al., 2017). Furthermore, no phase II metabolites in imidacloprid metabolism, including those that could be conjugated to glutathione, were found in the honey bee (Suchail et al., 2004). This could be explained either by an absence of conjugation with GST, by the production of GST conjugates at undetectable levels, or by drastic downregulation of GST by imidacloprid. Thus, the decrease in GST activity may indicate a decrease in the honey bee capacities to detoxify these pesticides and to fight against oxidative stress that takes place after exposure to imidacloprid and glyphosate (Contardo-Jara et al., 2009; Gauthier et al., 2018; Jasper et al., 2012; Lushchak et al., 2009).

G6PDH is the primary enzyme of the pentose phosphate pathway that generates NADPH and is involved, among other things, in the regeneration of reduced glutathione, which contributes to the fight against oxidative stress (Thomas et al., 1991). G6PDH activity decreased after 10 days of exposure to all modalities at 1 µg/L. However, it is improbable that this decrease is due to oxidative stress. Indeed, in the presence of oxidative stress, glyceraldehyde-3-phosphate dehydrogenase (GAPD) is inhibited (Chuang et al., 2005), which induces a deviation of glycolysis towards the pentose phosphate pathway and an increase in G6PDH activity (Nicholls et al., 2012; Renzi et al., 2016).

ALP is an enzyme of the digestive tract involved in adsorption and transport mechanisms through the gut epithelium (Vlahović et al., 2009) and in immune response (Chen et al., 2011). The activity of ALP was not modulated after 10 and 20 days of exposure. Thus, imidacloprid, glyphosate and difenoconazole did not affect the activity of ALP. This finding strongly contrasts with the results of other studies that showed a modulation of ALP in bees exposed to other pesticides, such as fipronil and spinosad, and following infection by Nosema (Carvalho et al., 2013; Dussaubat et al., 2012; Kairo et al., 2017). Thus, the apparent absence of ALP modulation in our study could reflect either an absence of effect or the occurrence of a compensatory phenomenon.

4.5. The effect of exposure to pesticides is systemic and tissue-specific

By comparing the dose effect of IH on CaE-3, it is possible to notice that for the same exposure duration, the effect of IH on CaE-3 at 0.1 and 1 µg/L differed among the biological compartments. For the modulations of CaE-3 at day 10, IH0.1 < IH1 in the head and IH0.1 > IH1 in the midgut and abdomen. For the modulations of CaE-3 at day 20, IH0.1 > IH1 in the gut and IH0.1 < IH1 in the abdomen. This complex profile of modulations was also found for both head and midgut GST after exposure to *Bt* spores and to *Nosema*-fipronil combination (Kairo et al., 2017; Renzi et al., 2016), thus confirming a spatial differential response due to the specificity of each tissue and to the occurrence of pesticide metabolism not only in the gut but also in other honey bee compartments (Suchail et al., 2004).

GST activity was modulated in the head, midgut and abdomen. In addition, AChE was modulated in the head, G6PDH in the abdomen and ALP in the midgut. These results indicate that the effects of the exposure to pesticides are not localized in the midgut (and in turn in the abdomen), which is considered the primary site of interaction with the ingested pesticide, but are spread across all biological compartments, leading to a systemic response that could explain the severe impact on honey bee survival.

The effects of the pesticides on physiological markers were determined in surviving bees after 10 and 20 days of daily exposure. The results at day 10 revealed a massive modulation of all physiological markers except CaE-3 and midgut GST. However, a less pronounced effect was detected at day 20 with a higher number of non-modulated enzymes (CaE-3, head GST, ALP and G6PDH were not modulated). This lower effect at day 20 suggests that the honey bee population at day 10 was composed of both sensitive and resistant individuals, while the population that survived until the twentieth day mainly contained honey bees that were more resistant to these pesticides alone or in combination. However, this hypothesis could be ruled out because the progression of mortality during this period was approximately linear, indicating that the honey bees were sensitive to the pesticides and were unable to compensate for the increase in exposure duration.

5. Conclusion

This study demonstrates that chronic exposure to insecticides, herbicides and fungicides, alone or in combination, may induce high toxicity via systemic action in winter honey bees and constitutes a threat to these workers in two ways. The first is a direct drastic effect on survival, with a mortality that exceeded 50% after only 20 days of exposure, which can endanger the colony. The second involves a systemic action of these pesticides that alters honey bee physiology through metabolism, immunity, the nervous system, detoxification and antioxidant defenses. A severe loss of the winter bee population may compromise colony development during the spring, which might explain the high winter losses encountered in many regions. If such cocktail effects occurred in summer bees, this would have drastic impacts on colonies that could largely explain the bee population decline, especially because summer bees are more susceptible to pesticides combinations than winter bees.

This study also reveals that the standard 10-day chronic toxicity test, used during pesticide risk assessment procedures, may not always be reliable in detecting the potential toxicities of pesticides. In addition, this study highlights the difficulty in predicting the cocktail effects of pollutants because the toxicity of the mixture is not always directly linked to the number of substances or the exposure level.

Declaration of competing 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.

CRediT authorship contribution statement

Hanine Almasri: Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Daiana Antonia Tavares:** Investigation. **Maryline Pioz:** Formal analysis,

Data curation. **Déborah Sené:** Investigation. **Sylvie Tchamitchian:** Investigation, Resources. **Marianne Cousin:** Writing - review & editing. **Jean-Luc Brunet:** Writing - review & editing, Supervision. **Luc P. Belzunces:** Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Acknowledgments

This study was supported in part by the recurrent funding of the Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAE) and by the Agence Nationale de la Recherche (ANR) (grant ANR-15-CE34-0004-01). Hanine ALMASRI was supported by grants from Lebanese University (grant number 2364) and the PACA region. The authors thank Jacques Sénéchal and Alexandre Gorit, beekeepers of the UR 406 INRAE Research Unit, for their expert beekeeping.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2020.111013.

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