Contributions à une meilleur évaluation de la toxicité des pesticides chez l'abeille domestique (Apis mellifera)

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Contributions à une meilleure évaluation de la toxicité des pesticides chez l'abeille mellifère (*Apis mellifera*)

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ANSES : Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail

EFSA : Autorité européenne de sécurité des aliments (*European Food and Safety Authority*)

Exposition chronique : exposition à un facteur de stress ou une substance qui se produit à long terme (plusieurs jours à plusieurs mois)

Exposition aiguë : exposition à un facteur de stress ou une substance qui a lieu une seule fois ou durant une courte durée (de quelques secondes à quelques jours)

NOEC : concentration ne produisant pas d'effet observable (*No Observed Effect Concentration*)

NOEDD : dose sans effet observable (*No Observed Effect Dietary Dose*)

OCDE/OECD : Organisation de Coopération et de Développement Economiques (*The Organisation for Economic Co-operation and Development)*

OEPP/EPPO : Organisation Européenne et méditerranéenne pour la Protection des Plantes (*European and Mediterranean Plant Protection Organization*)

PEC : concentration prévisible d'une substance dans l'environnement (*Predicted Environmental Concentration*)

PNEC : pour une substance donnée, concentration maximale sans risque pour l'environnement (*Predicted No-Effect Concentration*)

US EPA : agence américaine de protection de l'environnement (*United State Environment Protection Agency*)

CHAPITRE I :

INTRODUCTION GENERALE

I. L'exposition des abeilles mellifères aux pesticides

A. L'abeille mellifère : un pollinisateur social

L'abeille mellifère, *Apis mellifera* (Hymenoptera : Apidae), est un insecte eusocial vivant en colonies composées de plusieurs milliers d'individus (entre 20 000 et 50 000), eux-mêmes différenciés en trois castes : la reine (la seule femelle fécondée), les ouvrières et les mâles ou faux-bourdons (Winston, 1987). Au sein de la colonie, les ouvrières sont organisées selon un polyéthisme d'âge, c'est-à-dire qu'elles effectuent différentes tâches de travail en fonction de leur âge, hormis la reproduction. Parmi ces différentes tâches, les activités de soin au couvain réalisées par les nourrices et de collecte de nourriture réalisées par les butineuses en sont les principales. Ainsi les butineuses ramènent à la colonie les ressources nécessaires à son développement et à sa survie, comme l'eau, le nectar, le pollen et la propolis (Beekman and Ratnieks, 2000). Le nectar des fleurs, contenant entre 5 et 80% de sucres (saccharose, fructose, glucose), représente la source d'énergie principale des abeilles mellifères, et permet, suite à sa transformation par les abeilles, l'élaboration du miel (Winston, 1987). Le pollen, principalement composé de protéines (6-28% in Winston (1987)) est l'élément indispensable au développement physiologique des abeilles nourrices en favorisant notamment le développement des glandes hypopharyngiennes (Standifer, 1967; Hagedorn and Moeller, 1968; Standifer *et al.*, 1970 ; DeGrandi-Hoffman *et al.*, 2010 ; Omar *et al.*, 2017), où est synthétisée la nourriture larvaire (gelée royale).

L'exceptionnelle capacité de communication entre les butineuses, au travers de leur danse (Tautz, 2009), leur permet de localiser efficacement les ressources florales disponibles dans leur aire de butinage. Cette dernière peut s'étendre jusqu'à de grandes distances de la ruche, certaines butineuses pouvant parcourir jusqu'à 10km, ce qui permet un accès à diverses ressources florales. Cependant, dans la majorité des cas, les butineuses restent à une distance moyenne de 5km autour de la ruche (Beekman and Ratnieks, 2000).

A travers leur activité de butinage, les butineuses contribuent à la pollinisation d'une grande partie des espèces florales, et donc à leur reproduction. En effet, les insectes, incluant les abeilles mellifères, réalisent la majeure partie de la pollinisation de près de 90% des plantes à fleurs dans le monde (Kremen *et al.*, 2007 ; Ollerton, 2017). Sur le plan écologique, cette pollinisation par les insectes est primordiale pour maintenir des populations diverses de plantes et par conséquent participe au maintien de la biodiversité. Sur le plan économique, la

pollinisation par les insectes augmente de manière significative le rendement des plantes cultivées destinées à l'alimentation humaine. Ce service écosystémique a notamment été estimé à plus de 150 milliards d'euros par an (Gallai *et al.*, 2009). Les abeilles mellifères sont considérées comme étant les pollinisateurs des monocultures les plus rentables économiquement (McGregor, 1976) et la production de cultures de fruits, graines et noix peut diminuer de plus de 90% pendant leur absence (Southwick and Southwick, 1992). L'abeille mellifère présente également un fort intérêt économique de par les valeurs médicales et nutritionnelles des produits de la ruche, tels que le miel, la cire, la gelée royale et la propolis, pour (Viuda-Martos *et al.*, 2008 ; Denisow and Denisow-Pietrzyk, 2016 ; García, 2018).

Cependant, les abeilles mellifères, en récoltant les ressources alimentaires sur les fleurs, peuvent s'exposer à un panel de produits phytopharmaceutiques et autres contaminants (métaux lourds, biocides) en milieu agricole (Johnson *et al.*, 2010 ; Krupke *et al.*, 2012). En effet, les produits phytopharmaceutiques, qui font partie des pesticides, ont été développés pour protéger les cultures contre les organismes nuisibles (insecticides, fongicides) ou empêcher la croissance de plantes indésirables (herbicides). Il arrive aussi que la flore sauvage en bordure de champs se retrouve contaminée par ces produits (Botías *et al.*, 2015 ; Long and Krupke, 2016). La contamination par les produits phytopharmaceutiques peut se produire sur le terrain (exposition des butineuses) mais également à la ruche lorsque les ressources contaminées (ex. pollen et nectar) sont ramenées et stockées dans la ruche, exposant ainsi les autres abeilles adultes et le couvain. Le nectar et le pollen peuvent en effet contenir des polluants environnementaux tels que les métaux lourds (Bromenshenk *et al.*, 1991 ; Hladun *et al.*, 2013), des pesticides systémiques (Bonmatin *et al.*, 2001; Schmuck *et al.*, 2001), ou des pesticides suite à des pulvérisations agricoles ou une dérive de ces applications sur la flore adventice (Krupke *et al.*, 2012). Les différentes voies d'expositions des abeilles mellifères aux pesticides seront détaillées par la suite (cf. Chapitre I. C. Les différentes voies d'exposition des abeilles mellifères aux pesticides, p : 13). Dans ce contexte, une grande diversité de pesticides présents dans l'environnement peuvent menacer les performances physiologiques ou comportementales et la survie des abeilles mellifères (Thompson 2003; Aliouane *et al.*, 2009 ; Blacquière *et al.*, 2012 ; Goulson 2013 ; Cabirol and Haase, 2019). A titre d'exemple, une étude sur le pollen collecté par les abeilles dans 23 États des États-Unis et une province canadienne, a recensé 98 résidus de pesticides différents, avec une moyenne de 7,1 résidus par échantillon de pollen (Mullin *et al.*, 2010). Similairement, une étude en France a identifié et quantifié des résidus de

16 insecticides et acaricides et de deux fongicides dans des échantillons de cire, issus des colonies d'abeilles mellifère (Chauzat and Faucon, 2007).

B. Les abeilles mellifères exposées à une diversité de pesticides

Près de 50% des pesticides utilisés en agriculture sont des herbicides, 29,5% sont des insecticides et 17,5% sont des fongicides (Arnab De *et al.*, 2014). Actuellement, les principales substances incriminées comme étant très toxiques pour les abeilles sont les insecticides, et notamment les trois grandes familles pyréthrinoïdes, phénylpyrazoles et néonicotinoïdes, qui sont efficaces à faibles doses contre les insectes dits nuisibles (pucerons, aleurodes, cicadelles) et possèdent des propriétés de diffusion à travers les végétaux (Casida and Quistad, 1998; Regnault-Roger *et al.*, 2005). La plupart des insecticides utilisés sont des neurotoxiques, agissant sur le système nerveux des organismes cibles, mais chaque famille chimique agit sur différents récepteurs spécifiques. A titre d'exemple, les néonicotinoïdes provoquent une hyperstimulation du système nerveux central des insectes en se liant sur les récepteurs nicotiniques à l'acétylcholine (nAChR) (Schmuck *et al.*, 2003 ; Jeschke *et al.*, 2011 ; Fairbrother *et al.*, 2014). Ce mode d'action les rend particulièrement efficaces pour le contrôle des insectes nuisibles notamment sur les cultures de coton, maïs, betterave ou colza (Elbert *et al.*, 2008). Hormis les insecticides ciblant le système nerveux, d'autres sont des régulateurs de croissance, tels que les inhibiteurs de synthèse de chitine, ou des molécules agissant sur le système respiratoire. Puisqu'ils ont comme cible les insectes, la plupart de ces insecticides présentent un risque élevé pour les abeilles, ce qui a été démontré maintes fois dans la littérature scientifique (Desneux *et al.*, 2007 ; Belzunces *et al.,* 2012 ; Kiljanek *et al.*, 2016 ; Alkassab and Kirchner, 2017 ; Botías and Sánchez-Bayo, 2018 ; Sponsler *et al.*, 2019). En plus de pouvoir tuer directement les abeilles, ils peuvent aussi affecter leur résistance aux parasites (thiaclopride, imidaclopride et clothianidine ; Brandt *et al.*, 2016), leur efficacité métabolique (clothianidine ; Cook, 2019), et de butinage (imidaclopride ; Colin *et al.*, 2019) ou encore leur capacité à revenir à la ruche (Henry *et al.*, 2012).

Outre les insecticides, d'autres pesticides tels que les herbicides et fongicides, même s'ils ne sont pas destinés à tuer les insectes, peuvent avoir des effets néfastes sur les abeilles (Cullen et al., 2019a; Rondeau and Raine, 2022). Les herbicides, bien qu'ils ciblent des voies physiologiques spécifiques des plantes, peuvent interférer avec les processus métaboliques et reproductifs des abeilles. A titre d'exemple, le glyphosate cible une voie métabolique présente dans les plantes mais celle-ci est également présente dans certaines bactéries du microbiome

intestinal des abeilles mellifères, pouvant ainsi le perturber (Motta *et al.*, 2018). D'autres effets des herbicides sur les abeilles ont été constatés (pour revue Sánchez-Bayo *et al.*, 2016 ; Cullen *et al.*, 2019). Néanmoins, le principal impact des herbicides sur les abeilles pourrait être leur effet négatif sur la disponibilité des plantes à fleurs, en réduisant les ressources nutritionnelles (Bohnenblust *et al.*, 2016; Freemark and Boutin, 1995).

Les fongicides sont largement utilisés en agriculture pour lutter contre les épidémies fongiques (USGS, 2015) puisqu'ils agissent sur de nombreuses fonctions cellulaires importantes pour la croissance des extrémités des hyphes (appareil végétatif filamenteux) des champignons (Casida, 2009). Leur utilisation en traitement de semences et/ou en application foliaire tout au long de la saison de croissance des plantes augmente les probabilités d'exposition potentielle des pollinisateurs. En conséquence, les fongicides ont largement été retrouvés dans la cire et le pollen des colonies d'abeilles mellifères (Mullin *et al.*, 2010). Bien qu'ils ne soient pas considérés comme présentant une toxicité aiguë pour les abeilles mellifères, ils peuvent tout de même nuire à leur survie, avec des effets largement observés sur le couvain plutôt que sur les adultes (Hillier *et al.*, 2013 ; Zhu *et al.*, 2014). A titre d'exemple, le captane, qui inhibe la division cellulaire de champignons, peut induire une mortalité larvaire et des problèmes de développement chez les abeilles domestiques (Mussen *et al.*, 2004) ; tandis que le boscalid peut entraîner une toxicité chronique et cumulative chez les abeilles adultes (Simon-Delso *et al.*, 2017).

C. Les différentes voies d'exposition des abeilles mellifères aux pesticides

La possibilité pour les abeilles d'être exposées aux pesticides dépend à la fois de la formulation du produit, de ses caractéristiques intrinsèques (physico-chimiques) et de la méthode d'application. L'ensemble de ces paramètres contribue à déterminer la persistance des produits dans l'environnement, leur distribution spatiale et la contamination éventuelle des ressources nutritionnelles des abeilles sous forme de résidus.

Tout d'abord, l'exposition peut se faire par un contact direct lorsque les produits phytosanitaires sont appliqués par pulvérisation aérienne dans les champs (Figure 1). Les abeilles butineuses peuvent ainsi se retrouver exposées directement aux gouttelettes de pesticides suite ou pendant l'application des produits dans les champs traités ou aux alentours. Elles peuvent également entrer en contact avec des surfaces des plantes traitées, lors de la collecte du pollen, nectar, et des gouttes d'eau et de guttation (Krupke *et al.*, 2012 ; Kasiotis *et al.*, 2014 ; Schmolke *et al.*,

2018). La contamination peut se produire également par la poussière dispersée pendant le semis de semences enrobées par des pesticides (Georgiadis *et al.*, 2011).

Un autre type d'exposition se fait par voie orale à travers l'ingestion de ressources contaminées (Figure 1) (Krupke *et al.*, 2012). Par exemple, les pesticides systémiques (appliqués en enrobage de semences) sont absorbés à travers les tissus foliaires des plantes pendant leur croissance et leurs résidus peuvent persister dans toutes les parties de la plante traitée (Laurent and Rathahao, 2003). Ainsi, ces pesticides contaminent sur de longues durées le nectar, le pollen et l'eau de guttation (Tapparo *et al.*, 2011 ; Krupke *et al.*, 2012 ; Bonmatin *et al.*, 2015). A titre d'exemple, de faibles quantités (3 µg/kg) d'imidaclopride ont été retrouvées dans le pollen de tournesols issues de semences traitées avec le Gaucho® (Bonmatin *et al.*, 2003) ; des résultats similaires ont été obtenus avec le maïs, où la contamination du pollen par l'imidaclopride était d'environ 2,1 µg/kg (Bonmatin *et al.*, 2005). De même, Wen *et al.* (2021) ont identifié 34 substances actives dans des échantillons de nectar de colza, tel que le chlorpyrifos détecté à des concentrations moyennes de 16,42 µg/kg. Par ailleurs, ces pesticides peuvent persister dans le sol et être absorbés par les cultures successives via les racines (Bonmatin *et al.*, 2003 ; Goulson, 2013 ; Wood and Goulson, 2017).

Ensuite, à l'issue de leur vol de butinage, les butineuses ramènent les ressources contaminées à la ruche, polluant ainsi les matrices par les résidus de pesticides (Figure 1) (Sanchez-Bayo and Goka, 2014). Ces derniers se retrouvent ainsi dans le pollen de la ruche, le pain d'abeille, le miel, la cire et la propolis (Lambert *et al.*, 2013 ; Bridi *et al.*, 2018 ; Calatayud-Vernich *et al.*, 2018 ; Kanga *et al.*, 2019). En France, une étude des pelotes de pollen récoltées par les abeilles, a montré la présence de 36 pesticides utilisés en agriculture, quelquefois à des concentrations inquiétantes telles que 487,2 µg/kg et 925 µg/kg, respectivement pour le tau-fluvalinate et le coumaphos (Chauzat *et al.*, 2006). Le pollen fraîchement stocké et le pain d'abeille sont ainsi considérés comme les sources de contamination les plus importantes dans la ruche pour les adultes et les larves (Orantes-Bermejo *et al.*, 2010 ; Krupke *et al.*, 2012).

Les abeilles ont aussi besoin d'eau pour la thermorégulation de la ruche et l'élevage du couvain (Schmaranzer, 2000). Or, des résidus de pesticides peuvent se retrouver dans l'eau suite à leur lessivage sur le sol ou encore à leur dérive vers les eaux de surface, devenant ainsi une voie d'exposition orale pour les abeilles (Belden *et al.*, 2007 ; de Souza *et al.*, 2020)

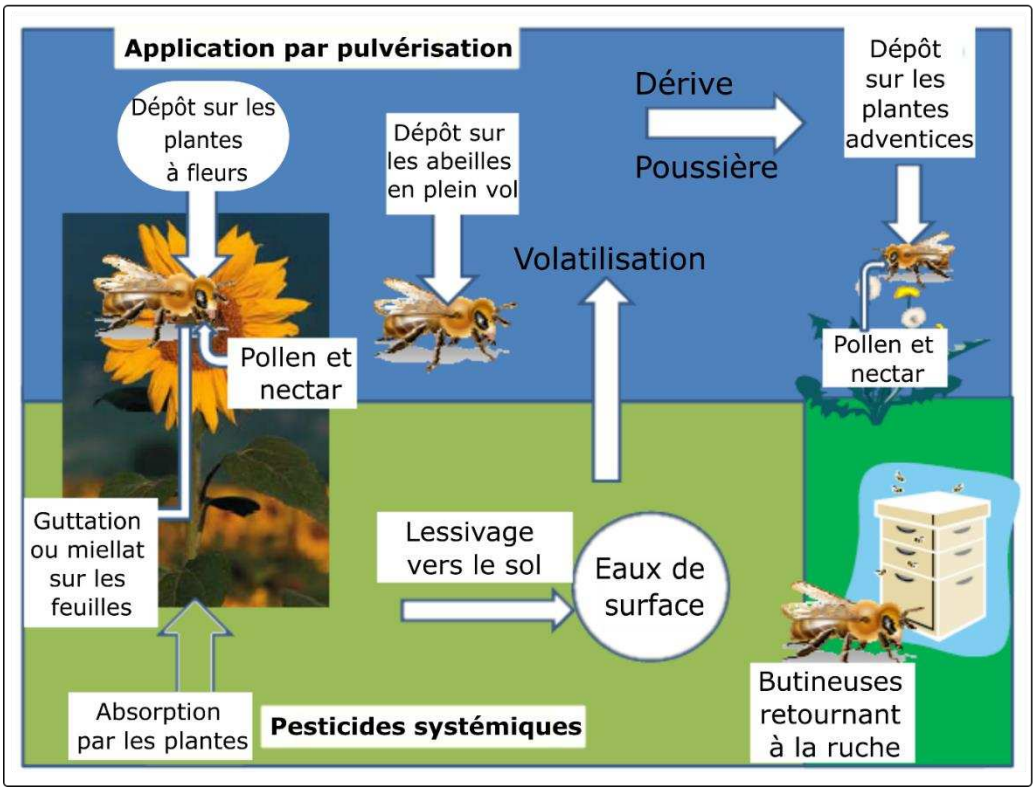

Figure 1. Les différentes voies d'exposition des abeilles aux pesticides. Modifié d'après EFSA (2012).

Enfin, outre les pesticides utilisés en agriculture, les abeilles sont également exposées aux acaricides utilisés par les apiculteurs pour lutter contre des parasites tels que l'acarien *Varroa destructor*. Dans ce cas, les abeilles peuvent être en contact direct avec des niveaux élevés de résidus présents dans la cire (Chauzat and Faucon, 2007; Herrera López *et al.*, 2016 ; Premrov Bajuk *et al.*, 2017), affectant les larves en développement (Zhu *et al.*, 2014). Il existe donc différentes voies par lesquelles les abeilles domestiques peuvent être exposées aux pesticides et à leurs résidus.

Toutes ces préoccupations relatives liées à l'exposition des abeilles aux pesticides ont conduit à la mise en place de tests réglementaires toxicologiques dans les processus d'autorisations des produits phytosanitaires avant leur mise sur le marché. Cependant, il a fallu plusieurs décennies avant que ces tests réglementaires voient le jour, bien que les premières intoxications des abeilles mellifères aux pesticides datent de plus d'un siècle.

II. Les premières intoxications et évaluations des risques liés à l'exposition aux pesticides ne datent pas d'hier

Lorsqu'un produit phytosanitaire est utilisé pour traiter une culture, il peut présenter des risques sur les espèces non cibles. Les abeilles mellifères en sont un exemple. Les premiers cas d'intoxications ont été observés aux Etats-Unis dans les années 1870, avec l'utilisation intensive de substances inorganiques tel que l'arsenic (Berenbaum, 2016; Fourche, 2017). Ce produit toxique, plus connu sous le nom de « Paris Green » ou « vert de Paris » a été le premier utilisé aux Etats-Unis pour lutter contre le doryphore sur les plants de pomme de terre. Son utilisation sur les vergers en fleurs contre le carpocapse du pommier a fait l'objet du 1er témoignage d'intoxication pour les abeilles en 1881 (Berenbaum, 2016; Fourche, 2017). Ces intoxications ont été établies par des études et des témoignages montrant que les produits à base d'arsenic étaient responsables de la mortalité des abeilles (Price, 1920; Shaw, 1941; Webster, 1894).

En France, ces produits à base d'arsenic ont été utilisés en agriculture dès le XIXe siècle mais en 1846, une ordonnance du roi a interdit la vente et l'emploi de ces produits dans le domaine de la production végétale et l'élimination des insectes (Figure 2; « Ordonnance du roi portant règlement sur la vente des substances vénéneuses », dans *Bulletin des lois du royaume de France*, 9e série, second semestre 1846, tome 33, p. 858-862 [article 10]). Bien que peu respectée, cette ordonnance ne sera modifiée qu'en septembre 1916 par arrêté (Fourche, 2017). A cette date, les composés à base d'arsenic sont devenus légalement utilisables à des fins agricoles. Cependant, pour les arbres fruitiers (pommiers, poiriers et pruniers), les traitements n'étaient pas autorisés pendant la floraison, mais possible après celle-ci. Cet arrêté a donc été le premier à vouloir protéger les abeilles. Cependant, cette interdiction dure peu de temps puisqu'en 1922, un nouvel arrêté élargit le nombre de cultures sur lesquelles l'emploi d'arsenic est autorisé et cela même pendant la floraison (Fourche, 2017). Suite à cela et à la généralisation des produits de synthèse après la seconde guerre mondiale, le nombre de cas d'intoxication et de pertes d'abeilles s'est généralisé. A partir des années 1930, la littérature apicole a publié de nombreux témoignages d'apiculteurs, déplorant la mortalité de leurs colonies d'abeilles suite à la pulvérisation d'arsenic sur les cultures en fleurs. Il faudra attendre 1947 pour voir apparaître des mentions interdisant l'emploi des insecticides sur les arbres fruitiers et les plantes visitées par les abeilles pendant la période de pleine floraison. A titre d'exemple, les organochlorés, dont le plus connu le DDT (dichlorodiphényltrichloroéthane), ont notamment été suspectés de

causer la mort de milliers de colonies dans les années 50 dans le bassin parisien (Louveaux, 1984), mais son interdiction n'est arrivée qu'en 1972 (Fournier, 2006). Depuis les années 1950, de nombreux arrêtés relatifs aux conditions d'utilisation des pesticides ont été promulgués afin de protéger les abeilles et autres insectes pollinisateurs (Figure 2). A titre d'exemple, en France, l'arrêté du 28 novembre 2003, interdit l'emploi des insecticides et acaricides durant la floraison des cultures ou au cours des périodes de production d'exsudats (https://www.legifrance.gouv.fr). Cependant, des dérogations à cette interdiction de traitement, appelées mention « Abeilles », peuvent être accordées à certains produits à condition que le traitement soit réalisé en dehors de la présence d'abeilles (ANSES, 2010).

Cet historique montre qu'il existe depuis plus d'un siècle une volonté de protéger les abeilles des pesticides, notamment pendant la période attractive de floraison des cultures. Cependant, malgré les restrictions, les intoxications ont persisté dans le temps en raison notamment des différentes dérogations aux interdictions de traitement et d'une réglementation de moins en moins protectrice au fil du XXème siècle, passant d'une interdiction complète des insecticides pendant la floraison au début du XXème à une autorisation possible sous conditions. Les derniers arrêtés autorisent les pesticides (insecticides, fongicides, acaricides) dont l'évaluation des risques réalisée par l'ANSES conclut à une autorisation d'utilisation pendant la période de floraison si les effets sont acceptables ou l'exposition négligeable. Dans ce cas, ils doivent être appliqués dans les 2 heures qui précèdent le coucher du soleil et dans les 3 heures qui suivent le coucher du soleil (Figure 2). Même si ces conditions visent à limiter davantage l'exposition des abeilles à l'impact direct des pesticides lors d'une pulvérisation, elles n'empêchent pas la contamination aux résidus de produits par voie orale, à travers l'ingestion des ressources alimentaires comme le nectar et le pollen, et par contact avec le pesticide présent sur les fleurs.

Figure 2. Evolution des arrêtés sur l'emploi des pesticides en France pour protéger les abeilles entre 1846 et 2021.

Pour réduire les risques d'intoxication, il a été important d'évaluer les effets des pesticides et le risque encouru par les abeilles couvert par la réglementation européenne. Celle-ci exige la présentation de données écotoxicologiques sur l'abeille domestique avant la mise sur le marché des pesticides depuis la directive européenne 91/414-CEE remplacée par le règlement européen No 1107/2009 (European Commission, 2009).

III. L'évaluation de la toxicité des pesticides chez l'abeille mellifère

A. Les tests réglementaires pour l'autorisation de mise sur le marché des pesticides

L'autorisation de mise sur le marché des pesticides se fait en deux étapes. La première consiste en l'approbation de la/les substance(s) active(s) qui entre(nt) dans la composition de la formulation de produit au niveau européen par l'EFSA. La seconde concerne la décision d'autorisation du produit formulé à l'échelle nationale par l'ANSES. Notamment, le règlement européen No 1107/2009, associé à des arrêtés réglementaires (cf. exemples à la fin de la partie III.A), a pour objectif une évaluation de l'efficacité et des risques vis-à-vis de la santé humaine, animale et de l'environnement des substances actives et pesticides dans leurs conditions d'emploi (European Commission, 2009).

Le règlement (UE) N° 284/2013 définit les test standards à conduire pour l'évaluation du risque des pesticides sur l'abeille mellifère (EFSA, 2013). Cette évaluation se fait de façon hiérarchisée selon trois niveaux de progression à partir de tests réalisés en laboratoire, en conditions semi-naturelles et plein champs (EPPO, 2010a), qui suivent les lignes directrices référentes de l'OEPP (EPPO, 2010b) et de l'OCDE (Figure 3).

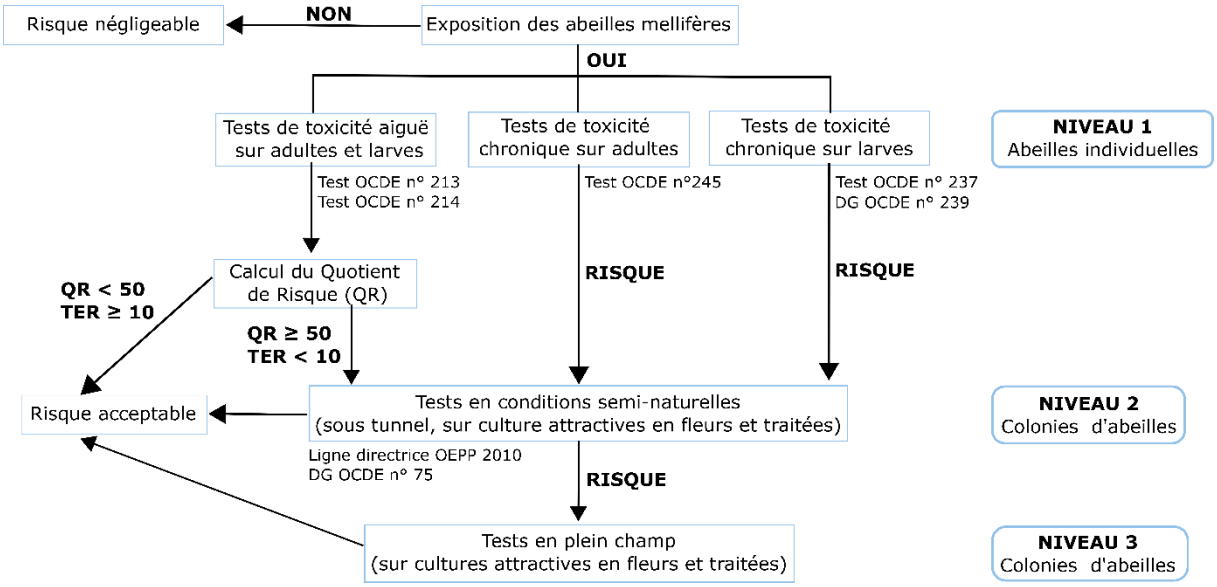

Figure 3. Schéma d'évaluation du risque des pesticides avant l'Autorisation de Mise sur le Marché.

Le premier niveau d'évaluation des risques est obligatoire et repose sur la mise en place de tests de toxicité des substances actives et des produits formulés en laboratoire suivant des protocoles standards officiels (Figure 3). Pour cela, sont requis des essais de toxicité aiguë (exposition par voie orale ou par contact) sur abeilles adultes (OECD N° 213, 1998a, N° 214 1998b) ainsi que des essais de toxicité chronique (exposition par voie orale) sur abeilles adultes (OECD N° 245, 2017) et larves (GD OECD N° 239, 2016, OECD N° 237 2013). Le test de toxicité aiguë permet de caractériser la dose moyenne létale (DL_{50}) tuant 50% des individus 48 à 96 heures (adultes et larves) après exposition unique. Les tests de toxicité chronique permettent de déterminer une dose ou concentration sans effets observés (NOEC pour *No Observed Effect Concentration* ou NOEDD pour *No Observed Effect Dietray Dose*) 10 jours après exposition répétée pour les adultes et jusqu'à l'émergence adulte pour les tests sur larves. Si un risque est identifié à l'issue de cette première étape (niveau 1), un second niveau d'évaluation est demandé, voire un troisième niveau où les produits formulés sont appliqués à la/les dose(s) d'application (g/ha) requise(s) pour l'utilisation par les agriculteurs. Ces niveaux supplémentaires requièrent des tests réalisés sous tunnel couvert (tests en conditions semi-naturelles pour le niveau 2), et en plein champ (tests en conditions naturelles pour le niveau 3) où les abeilles butinent la culture traitée (EPPO, 2010b). Les produits agrochimiques sont ainsi testés sur des colonies entières, dans des conditions d'expositions plus réalistes. Selon les études, les effets peuvent être suivis en mesurant la mortalité des abeilles, le développement des colonies, la taille des colonies (nombre d'abeilles), l'activité de butinage. Des analyses de résidus de pesticides dans les matrices apicoles (cire, miel, pollen, adultes et larves) peuvent également être réalisées. De

plus, pour les études de plein champ, des informations sur le nombre de colonies testées, la taille des parcelles ainsi que des recommandations sur le choix des cultures attractives à utiliser sont à prendre en compte. Enfin, des protocoles standards existent pour les études plus spécifiques évaluant les effets sur le développement du couvain sous tunnel (GD OECD N° 75, 2007) ou évaluant le taux de retour à la ruche en conditions naturelles (GD OECD N° 332, 2021).

A partir de ces tests de toxicité, une quantification du risque peut être menée en comparant la toxicité des pesticides à l'exposition environnementale. En effet, le risque correspond à la probabilité que l'introduction d'un contaminant (d'origine naturelle ou anthropique) dans un écosystème produise des effets néfastes sur les différents compartiments de cet écosystème (U.S. EPA, 1998). Pour une cible donnée, le risque dépend à la fois des propriétés intrinsèques de la substance considérée (= danger ou toxicité) et de la concentration ou de la dose à laquelle la cible est exposée (= exposition) (Caquet, 2012). Cette quantification du risque est la phase clé du processus d'évaluation des risques et permet la prise de décision d'autorisation de vente et d'utilisation d'un pesticide.

Chez l'abeille mellifère, la caractérisation du risque lié aux pesticides s'inscrit dans un schéma de prise de décision selon la ligne directrice de l'OEPP (EPPO, 2010a) et répond au principe d'évaluation uniforme des risques entre les Etats Membres du règlement (UE) No 546/2011. Des estimateurs de risque sont calculés au niveau 1 du processus d'évaluation des risques liés à l'exposition aux pesticides. Le quotient de risque (QR ou HQ : Hazard Quotient) est couramment utilisé pour évaluer le risque potentiel, après exposition unique, pour les abeilles mellifères exposées aux pesticides après pulvérisation foliaire sur les cultures. Il s'agit du ratio entre la dose d'application du produit phytopharmaceutique (g/ha) et la DL_{50} (µg/abeille). La valeur seuil de 50 est utilisée pour évaluer si le produit présente un risque minimal ($HQ < 50$) ou un risque potentiel ($HQ \geq 50$), nécessitant ainsi des expérimentations supplémentaires en conditions semi-naturelles et/ou en plein champ. Si le $HQ \geq 2500$, le produit est considéré comme présentant un risque élevé chez l'abeille et sera alors non autorisé.

Cependant, la caractérisation du risque par cette méthode n'est valable que pour les insecticides appliqués par pulvérisation sur les parties aériennes. Par ailleurs, l'estimation de l'exposition est approximative puisqu'elle n'utilise que les doses épandues à l'hectare. De plus, le HQ estime un niveau d'exposition des abeilles aux pesticides par une quantité appliquée par pulvérisation et donc ne prend pas en compte les produits systémiques dont les résidus restent dans les différentes parties de la plante pendant plusieurs jours, voire plusieurs mois, et dont les effets

peuvent être différés dans le temps. Enfin, seuls les effets létaux sont considérés. En 2010, l'OEPP a proposé une approche alternative, basée sur la méthode de Villa *et al.* (2000) et le ratio toxicité-exposition (TER : Toxicity Exposure Ratio) qui prend en compte à la fois les données d'exposition et les données de toxicité aiguë (EPPO, 2010a). Il permet de prendre en compte par exemple le comportement de la substance dans l'environnement et dans la plante jusqu'au pollen. Ce ratio correspond au rapport de la concentration prévue d'exposition (PEC : *Predicted Exposure Concentration*) sur la concentration prévue sans effets observables (PNEC : *Predicted No Effect Concentration*). Le PEC est obtenu à partir de la concentration résiduelle en produit estimée ou mesurée sur la plante entière, le nectar ou le pollen, et de l'estimation des quantités de nectar et pollen consommées par toutes les catégories d'abeilles (EFSA, 2012; Rortais *et al.*, 2005). Le PNEC est basé sur des données de toxicité en laboratoire pour une exposition aiguë ou une exposition chronique (DL_{50}, NOEC), auxquelles est associé un facteur d'incertitude prenant en compte la variabilité intra et inter-laboratoire et celles obtenues dans les conditions d'utilisation réelle. Ce facteur d'incertitude varie de 1 pour les tests en conditions de plein champ, à 10 pour les tests de laboratoires. Ce rapport PEC/PNEC est calculé pour toutes les catégories d'abeilles de la colonie (larves, ouvrières butineuses ou nourrices, reine), en utilisant tous les scénarii d'exposition. Il permet l'évaluation du risque à la fois à l'échelle de l'individu mais également de la colonie. Un risque minimal est identifié si ce ratio est supérieur ou égal à 10 ; à l'inverse, le produit est estimé comme présentant un risque potentiel si le ratio est inférieur à 10.

En France, ces dispositions réglementaires sont renforcées par des mesures de gestion de risque après la mise sur le marché des produits phytopharmaceutiques : l'arrêté du 20 novembre 2021 qui remplace celui du 28 novembre 2003 relatif aux conditions d'utilisation des traitements à usage agricole pendant la floraison des cultures attractives en vue de protéger les abeilles et autres insectes pollinisateurs ; l'arrêté du 13 janvier 2009 relatif aux conditions d'enrobage et d'utilisation des semences traitées et l'arrêté du 7 avril 2010 relatif à l'utilisation des mélanges extemporanés de produits phytopharmaceutiques (ANSES, 2018).

La surveillance des intoxications est également indispensable pour identifier les cultures et les traitements à risque car elle permet de mettre en place des mesures préventives *a posteriori*. Ainsi, dans les pays développés comme la France, des réseaux de surveillance plus ou moins établis collectent les données et informent les autorités qui prennent alors les mesures nécessaires (restrictions d'utilisation, information des usagers, retrait des autorisations de mise sur le marché par exemple) afin de réduire le risque pour les abeilles.

B. Des lacunes dans l'évaluation de la toxicité des pesticides

Malgré les tests réglementaires mis en place avant la mise sur le marché des produits phytopharmaceutiques, les procédures d'essai actuelles ne semblent pas suffisantes pour protéger les abeilles mellifères. En effet, actuellement le risque lié aux pesticides sur l'abeille domestique est principalement basé sur l'évaluation des effets létaux (calcul DL_{50}). Cependant, il convient d'accorder une attention particulière aux effets sublétaux, de plus en plus observés, notamment les performances comportementales et cognitives des abeilles telles que la capacité d'apprentissage, l'orientation, ou la recherche de nourriture (Decourtye *et al.*, 2003 ; Karahan *et al.*, 2015 ; Zhang *et al.*, 2020a). De plus, de faibles dose/concentrations préalablement identifiées comme non létales en laboratoire, peuvent affectées la survie des butineuses sur le terrain, contribuant à l'affaiblissement des colonies (Henry *et al.*, 2012). Enfin, la courte durée des tests (48h ou 10 jours) par rapport à la période de croissance des colonies d'abeilles (plusieurs mois) entrave encore plus la capacité à détecter les effets sublétaux significatifs et pertinents sur le terrain et au niveau colonial (Sgolastra *et al.*, 2020).

Par ailleurs, les pesticides n'induisent pas nécessairement une seule réponse mais une variabilité de réponses dont l'intensité peut varier en fonction de différents facteurs (Poquet *et al.*, 2016). En effet, si de nombreuses études montrent que la toxicité des pesticides dépend du type, de la dose, du mode et de la fréquence d'exposition (Belzunces *et al.*, 2012 ; Johnso, 2015 ; Godfray *et al.*, 2015), elle peut aussi être modulée par des facteurs endogènes (génotype, physiologie) et/ou exogènes (environnement) (Poquet *et al.*, 2016). Par exemple, les abeilles sont exposées à diverses conditions environnementales et à plusieurs facteurs de stress (agents pathogènes, disponibilité des ressources, conditions climatiques, exposition à d'autres pesticides, …), (Chauzat *et al.*, 2009 ; Mullin *et al.*, 2010 ; Cornman *et al.*, 2012 ; Ravoet *et al.*, 2013 ; Simon-Delso *et al.*, 2014) pouvant potentiellement influer sur leur sensibilité aux pesticides (effets additifs, synergiques, antagonistes). Or, les effets des pesticides sont actuellement évalués sur les abeilles par des tests standards non appropriés. Ainsi, afin de mieux évaluer la toxicité présentée par ces produits pour les abeilles et afin de développer une évaluation plus détaillée des risques pouvant en découler, il est nécessaire d'étudier cette variabilité ou modulation intraspécifique de la réponse.

Il existe un décalage entre les preuves récentes de toxicité des pesticides chez les abeilles et les tests réglementaires de toxicité nécessaires à l'approbation des pesticides avant leur mise sur le marché. Cet écart contribue à la controverse entre les parties prenantes (apiculteurs, industries

chimiques, industries agricoles, Organisations Non Gouvernementales…), les décideurs ou encore les scientifiques (Thompson and Maus, 2007; Sgolastra *et al.*, 2020 ; Durant, 2020). Il est donc nécessaire de combler ces lacunes et d'améliorer l'évaluation de la toxicité des pesticides sur les abeilles mellifères.

IV. Des nouvelles pistes pour améliorer l'évaluation de la toxicité des pesticides

A. Mieux évaluer la sensibilité des abeilles mellifères aux pesticides au niveau 1 de l'évaluation des risques

Les effets des pesticides sur les organismes dépendent de deux facteurs principaux : la toxicocinétique et la toxicodynamique (Tardif and Viau, 2003). La toxicocinétique fait référence au devenir de la molécule dans le corps de l'organisme et implique différents mécanismes : son absorption, sa distribution, sa biotransformation et son élimination. La toxicodynamique correspond à l'interaction entre le composé toxique, et la cible, ainsi qu'à ses effets sur l'organisme (par exemple, déficience physiologique, mortalité, … ; McCarty and Mackay, 1993). La manière dont un organisme traite le toxique (toxicocinétique) et son impact sur l'organisme (toxicodynamique), dépendent donc du profil physiologique de l'organisme (Tardif and Viau, 2003). Chez les abeilles, comme chez la plupart des organismes, le système de détoxification est impliqué dans la toxicocinétique des pesticides et permet de réduire le nombre de molécules toxiques avant qu'elles n'atteignent la cible et/ou après avoir été éliminées de la cible. Ce système réduit généralement la toxicité des xénobiotiques en faisant intervenir des enzymes qui les dégradent, et des transporteurs membranaires qui facilitent leur élimination. Il existe trois types d'enzymes : les enzymes de phase I, ou enzymes de fonctionnalisation, qui altèrent la structure de la molécule toxique et la rendent incapable d'agir sur sa cible (par exemple, les mono-oxygénases à cytochrome P450 (CYP450) et les carboxylestérases (CaEs)) ; les enzymes de phase II, enzymes de conjugaison ultérieure (par exemple, la glutathion-S-transférase (GST)), qui contribuent à la détoxication en se liant aux xénobiotiques pour permettre sa solubilisation et son excrétion; et les enzymes de phase III, enzymes transmembranaires comme les transporteurs ABC (ou ATP Binding Cassettes), qui utilisent l'adénosine triphosphate (ATP) pour transporter les molécules obtenues à travers la membrane cellulaire, et les excréter hors de l'organisme (Claudianos *et al.*, 2006 ; Mao *et al.*, 2011 ; Berenbaum and Johnson, 2015). Des changements dans le système de détoxification

(toxicocinétique) sont attendus tout au long du développement des abeilles (maturation physiologique et comportementale), ou au fur et à mesure que les abeilles vieillissent, ce qui suggère que la réponse aux pesticides peut être modulée tout au long de la vie de l'abeille (Poquet *et al.*, 2016).

La réponse aux pesticides peut également être affectée/modifiée par des interactions avec d'autres pesticides ou avec des agents pathogènes (Vidau *et al.*, 2011 ; Doublet *et al.*, 2015 ; Retschnig *et al.*, 2015). Il est donc important d'étudier les facteurs environnementaux et endogènes pouvant affecter la sensibilité des abeilles aux pesticides. En effet, les changements physiologiques (par exemple, le passage du statut de nourrices à butineuses) (Robinson, 2002), peuvent s'accompagner de modifications dans les fonctions impliquées dans la toxicocinétique et toxicodynamique des pesticides. Par exemple, en toxicologie, l'effet d'une dose est souvent lié au poids corporel de l'organisme : il a été constaté que les abeilles plus lourdes sont moins sensibles aux substances toxiques que les abeilles plus légères (Tahori *et al.*, 1969 ; Nogueira-Couto *et al.*, 1996). Les résultats récents de Pamminger (2021) confirment que le poids corporel des abeilles (abeilles mellifères, bourdons, abeilles solitaires) est un facteur prédictif de la sensibilité aiguë des abeilles par contact à une série d'insecticides. Par ailleurs, du fait des changements physiologiques lors du vieillissement des abeilles, il n'est pas surprenant d'observer une réponse aux pesticides dépendant de l'âge (Poquet *et al.*, 2016). Des études ont montré que les jeunes abeilles étaient moins sensibles à certains toxiques que des abeilles plus âgées (Bendahou *et al.*, 1997 ; Rinkevich *et al.*, 2015). Plus récemment, Zhu *et al.* (2020) a confirmé les effets de l'âge sur la sensibilité des abeilles mellifères à cinq insecticides, avec une plus forte mortalité observée chez les abeilles âgées. Enfin, concernant les facteurs environnementaux, il est bien établi que la quantité et/ou la qualité des ressources fournissent des nutriments qui modulent la réponse au stress chez les insectes (Simpson *et al.*, 2015). A titre d'exemple, des études chez les abeilles domestiques ont montré que l'ingestion de pollen affecte le niveau de transcription des CYP450 (Alaux *et al.*, 2011 ; Corby-Harris *et al.*, 2014) et que l'activité de la GST varie en fonction de la qualité du pollen ingéré (Di Pasquale *et al.*, 2013). Dans l'ensemble, de nombreuses études montrent donc que la réponse des abeilles à un pesticide donné varie en fonction de leur environnement et de leur état physiologique et/ou comportemental.

B. Mieux évaluer la sensibilité des abeilles aux pesticides au niveau 2 de l'évaluation des risques

L'évaluation de la toxicité des pesticides avant leur mise sur le marché est principalement basée sur l'évaluation des effets létaux (mortalité) avec comme principal critère la DL_{50}. Cependant, des effets significatifs peuvent se produire à des doses inférieures à celles utilisées dans les tests de laboratoire. Ainsi, avec la révision actuelle du principe d'évaluation du risque, l'EFSA (2013) a identifié le besoin de renforcer l'évaluation des effets des faibles doses (dites doses « sublétales ») de pesticides, c'est-à-dire n'entraînant pas directement la mort des individus. De nombreuses études ont montré qu'elles peuvent altérer des fonctions comportementales de l'abeille comme l'apprentissage et la mémorisation, la navigation et l'orientation (Bortolotti *et al.*, 2003 ; Decourtye *et al.*, 2004b ; El Hassani *et al.*, 2008 ; Decourtye *et al.*, 2009 ; Ciarlo *et al.*, 2012), ainsi que la reproduction (Kairo *et al.*, 2017b, 2017a, 2016), l'immunité (Aufauvre *et al.*, 2014), et potentiellement affecter la colonie (Woodcock *et al.*, 2017). Chez les abeilles mellifères, les effets des faibles doses ont été vérifiés lors d'essais sur le terrain en enregistrant le taux de retour à la ruche des abeilles exposées à une dose de thiaméthoxame, supposée sublétale sur la base des essais réglementaires. En effet, cette faible dose a provoqué un échec significatif du vol de retour des abeilles, ce qui affaiblit les colonies (Henry *et al.*, 2015, 2012).

Cette sous-estimation des risques liés aux faibles doses souligne la nécessité de compléter l'évaluation réglementaire actuelle des pesticides par des critères au niveau individuel qui soient plus fiables et plus pertinents que ceux mesurés lors des tests toxicologiques. Pour cela, des test complémentaires comme la mesure des performances comportementales (l'extension du proboscis, la communication par la danse, l'activité de butinage, le vol de retour à la ruche) se développent de plus en plus pour évaluer la toxicité des pesticides (Barascou *et al.*, 2021a).

Ces mesures de performances comportementales bénéficient notamment de l'émergence des nouvelles technologies de suivi, augmentant ainsi le taux d'acquisition et la quantité de données de suivi *in situ* et sur le long-terme (Kays *et al.*, 2015). Ainsi, les enregistrements des comportements tels que l'activité de butinage sont désormais plus rapides et plus faciles grâce aux outils informatiques et au marquage des individus. Par exemple, les compteurs d'abeilles et la méthode RFID (Radio-Frequency Identification) se révèlent très efficaces pour suivre automatiquement l'activité de vol de centaines d'abeilles dans les études toxicologiques (Decourtye *et al.*, 2011 ; Schneider *et al.*, 2012a ; Prado *et al.*, 2019 ; Colin *et al.*, 2019b).

En somme, des tests intégrant des mesures comportementales semblent prometteur pour évaluer la toxicité des doses et concentrations sublétales chez les abeilles. Cependant, des travaux sont nécessaires pour améliorer les tests de toxicité et évaluer la pertinence de diverses mesures comportementales comme métriques de la toxicité des pesticides.

CONTEXTE ET PROBLEMATIQUE DE LA THESE

L'abeille mellifère, en tant que pollinisateur majeur des cultures, est souvent exposée aux pesticides, d'autant plus que ces produits atteignent aussi la flore et la faune sauvage. Afin de protéger ces abeilles d'intoxications létales, des tests réglementaires de toxicité ont été développés et certains sont devenus un requis avant une éventuelle mise sur le marché de nouveaux pesticides. Cependant, ces tests standards ne tiennent pas en compte les disparités physiologiques et comportementales entre les individus composant les colonies d'abeilles mellifères, ce qui peut conduire à une évaluation erronée ou partielle de la toxicité des pesticides. Il est donc essentiel d'étudier la gamme de toxicité des pesticides en fonction du statut physiologique et comportemental de l'abeille. Enfin, si un risque est identifié à l'issue de cette première étape d'évaluation des risques liés à l'exposition aux pesticides, des tests sont requis en conditions semi-naturelles ou en plein champ. A cet effet, des mesures comportementales sont de plus en plus privilégiées car elles permettent d'identifier des effets sublétaux, elles améliorent la pertinence écologique des risques liés aux pesticides, et enfin, leur acquisition *in situ* est facilitée par l'émergence de nouvelles technologies. Néanmoins, il est nécessaire de continuer à améliorer les études comportementales à la fois au niveau individuel et colonial.

C'est dans ce contexte que se situent les objectifs principaux de ma thèse :

1. Le 1er objectif est de mieux évaluer les facteurs de variabilité ou de modulation des réponses aux pesticides.

2. Le 2nd objectif est de mieux évaluer les effets des pesticides, en utilisant la surveillance de l'activité de vol à l'échelle individuelle et coloniale, comme métrique de la toxicité des pesticides.

Afin de répondre à ces objectifs, j'ai focalisé mes études sur deux pesticides d'intérêt : l'insecticide sulfoxaflor et le fongicide azoxystrobine.

Le sulfoxaflor, neurotoxique appartenant à la famille des sulfoximines, une nouvelle classe d'insecticide en pleine expansion sur le marché mondial des pesticides, est un successeur potentiel des néonicotinoïdes (Brown *et al.*, 2016). En effet, il agit sur les récepteurs nicotiniques de l'acétylcholine (nAChR) des insectes (Zhu *et al.*, 2011 ; Watson *et al.*, 2011 ; Cutler *et al.*, 2013) et représente un outil important pour le contrôle des ravageurs piqueurs-suceurs qui résistent aux autres insecticides. Dans les grandes cultures, il est utilisé notamment

contre le puceron du coton et la mouche blanche de la patate douce (Babcock *et al.*, 2011). Malgré une interdiction récente en France de ce pesticide, il reste encore autorisé dans 18 États membres de l'UE (European Commission, dernier accès le 11 octobre 2020).

L'Azoxystrobine est un fongicide systémique à large spectre appartenant à la classe des strobilurines, largement utilisé en agriculture (Bartlett *et al.*, 2002). Il représente l'un des fongicides les plus fréquemment détectés dans le pollen récolté par les abeilles à proximité des champs de maïs (34 à 87,5% des échantillons polliniques) aux Etats-Unis (Long and Krupke, 2016). Il est utilisé dans les traitements en enrobage des semences pour les protéger contre les pathogènes du sol tels que les champignons *Fusarium spp.* (Broders *et al.*, 2007) en inhibant leur respiration mitochondriale.

Le chapitre II du manuscrit s'inscrit dans le 1er objectif de ma thèse et traite de la modulation physiologique de la sensibilité des abeilles mellifères aux pesticides. Il est composé de deux études : la 1ère porte sur la variabilité de réponse des abeilles mellifères aux pesticides selon leur caste comportementale (nourrices, butineuses) ; la 2ème étude aborde la modulation nutritionnelle de la sensibilité des abeilles aux pesticides.

Le chapitre III s'inscrit dans le 2ème objectif de ma thèse et traite de l'évaluation de la toxicité des pesticides à l'aide de la surveillance de l'activité de vol des abeilles à la fois à l'échelle individuelle et coloniale. Ce chapitre est divisé en trois sous parties : une revue de littérature résumant la pertinence des différentes mesures de performances comportementales et reproductives développées pour mieux évaluer la toxicité des pesticides ; une étude évaluant la toxicité chronique d'une dose unique de l'insecticide sulfoxaflor sur l'activité de vol des abeilles (au niveau individuel) ; et enfin une seconde étude porte sur le suivi de l'activité de vol des abeilles et des taux de mortalité au niveau colonial, en utilisant des compteurs d'abeilles.

CHAPITRE II :

MODULATION PHYSIOLOGIQUE DE LA SENSIBILITE DES ABEILLES AUX PESTICIDES

I. Modulation de la toxicité des pesticides selon la caste comportementale des abeilles ouvrières

A. Avant-propos

De nombreuses modifications physiologiques, notamment endocriniennes et métaboliques, ont lieu lors de la maturation comportementale des abeilles mellifères, en particulier le passage du stade nourrice à celui de butineuse (Robinson, 2002). Cela inclut des changements de poids, avec des butineuses qui peuvent peser jusqu'à deux fois moins que les nourrices (Vance *et al.*, 2009), ainsi qu'une activité de la glutathion S-transférase et des mono-oxygénases à cytochrome P450 plus élevée chez les butineuses (Mao *et al.*, 2015; Smirle and Robinson, 1989). De plus, ces deux types d'abeilles consomment des quantités différentes de nectar et sont donc potentiellement exposées à différents niveaux de pesticides.

Ces différences interindividuelles chez les abeilles mellifères pourraient conduire à une variabilité de réponses aux pesticides. Afin de caractériser au mieux le risque lié à une exposition aux pesticides, une approche déterministe peut être utilisée en comparant la toxicité du pesticide à l'exposition environnementale. Cette approche estime l'exposition réelle des abeilles individuelles en prenant en compte des différences existantes dans la consommation des matrices contaminées (nectar et pollen).

L'objectif de ce premier travail a donc été de déterminer la sensibilité des abeilles aux pesticides en fonction de la caste comportementale. Le risque potentiel présenté par une exposition à ces pesticides a par la suite été calculé, en intégrant la consommation de sirop contaminé respectivement pour les nourrices et butineuses, et la toxicité des pesticides.

Cette étude a été soumise dans *Environnemental Science and Pollution Research* (21/03/2022).

B. Article 1

Pesticide risk assessment: Honeybee workers are not all equal regarding the risk posed by exposure to pesticides

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Abstract

Toxicological studies in honeybees have long shown that a single pesticide dose or concentration does not necessarily induce a single response. Inter-individual differences in pesticide sensitivity and/or the level of exposure (*e.g.* ingestion of pesticide-contaminated matrices) may explain this variability in risk posed by a pesticide. Therefore, to better inform pesticide risk assessment for honeybees, we studied the risk posed by pesticides to two behavioral castes, nurse and forager bees, which are largely represented within colonies and which exhibit large differences in their physiological backgrounds. For that purpose, we determined the sensitivity of nurses and foragers to azoxystrobin (fungicide) and sulfoxaflor (insecticide) upon acute or chronic exposure. Azoxystrobin was found to be weakly toxic to both types of bees. However, foragers were more sensitive to sulfoxaflor than nurses upon acute and chronic exposure. This phenomenon was not explained by better sulfoxaflor metabolization in nurses, but rather by differences in body weight (nurses being 1.6 times heavier than foragers). Foragers consistently consumed more sugar syrup than nurses, and this increased consumption was even more pronounced with pesticide-contaminated syrup (at specific concentrations). Altogether, the stronger susceptibility and exposure of foragers to sulfoxaflor contributed to increases of 2 and 10-fold for the acute and chronic Risk Quotients, respectively, compared to nurses. In conclusion, to increase the safety margin and avoid an under-estimation of the risk posed by insecticides to honeybees, we recommend systematically including forager bees in regulatory tests.

Keywords: *Apis mellifera*, nurse, forager, pesticide sensitivity, pesticide metabolization, risk quotient

Introduction

Within the current regulatory framework for pesticide risk assessment, the effects of pesticides on honeybees are assessed by standard tests in a stepwise approach. In Tier 1, active substances or formulated products are tested on honeybees at different life stages (larvae and adults). This is the first mandatory step that includes acute toxicity tests after oral or contact exposure in adults (OECD, 1998b, 1998a) and a chronic oral toxicity test on adults (OECD, 2017). Next, a deterministic approach to characterize risk quantitatively can be used by comparing the pesticide toxicity to environmental exposure. For that purpose, a Risk Quotient (RQ) is generally calculated by dividing a point estimate of exposure by a toxicity end-point value (*e.g.* LD_{50} or chronic non-observable effect dose - NOED) (Thompson, 2021). If RQ values rise above determined levels of concern (*e.g.* 0.4 or 1 for acute and chronic exposure, respectively), then high-risk situations are identified and supplementary tests are required at a higher tier (semi-field and field tests) for regulatory decision making (Thompson, 2021).

However, a single pesticide dose or concentration does not necessarily induce a single response, as the level of the measured toxicity endpoint may vary depending on the physiological state of honeybees (Poquet *et al.*, 2016). Investigating this intraspecific variability or modulation of response is therefore important, notably at Tier 1, to better screen the risks posed by pesticides to honeybees. In this regard, some studies have shown that heavier honeybees are less sensitive to pesticides than lighter honeybees (Gerig, 1991; Nogueira-Couto *et al.*, 1996; Tahori *et al.*, 1969). In addition, sensitivity may depend on age with younger bees being more sensitive to certain pesticides, but less to others, than older bees (Bendahou *et al.*, 1997; Ladas, 1972; Mayland and Burkhardt, 1970; Rinkevich *et al.*, 2015; Zhu *et al.*, 2020). This is likely related to the tremendous changes in endocrine and metabolic activity occurring during age-related behavioral maturation (transition from nurse to forager tasks) (Robinson, 2002). For instance, foragers can weigh two times less than nurse bees (Vance *et al.*, 2009), and the activity of glutathione S-transferase, an enzyme involved in the detoxification pathways (Berenbaum and Johnson, 2015; Claudianos *et al.*, 2006), is significantly higher in forager bees than in nurses (Smirle and Robinson, 1989). As a result, pesticide sensitivity might strongly vary depending on the behavioral state of honeybee individuals. Confirmation of this hypothesis was given by Tosi and Nieh (2019) who found that foragers were consistently more susceptible to flupyradifurone (4-fold greater effect) than in-hive bees. Nevertheless, this intraspecific difference in pesticide sensitivity between behavioral castes, as well as the underlying mechanisms, has rarely been studied, although it could better inform pesticide risk assessment for honeybees.

In addition, and as mentioned earlier, the risk posed by pesticide to honeybees not only depends on the pesticide toxicity but also on the level of exposure, which includes both the environmental concentrations of pesticides and the level of ingestion or contact with pesticide-contaminated matrices (*e.g.* nectar). While differential consumption of pollen and nectar between behavioral castes of honeybees (Rodney and Purdy, 2020; Rortais *et al.*, 2005) is well-established, with, for instance, forager bees consuming more nectar than nurse bees, the consumption of nectar was also found to be affected by pesticide contamination. Indeed, honeybee foragers strongly preferred sugar solutions containing neonicotinoids (imidacloprid or thiamethoxam), glyphosate or chlorothalonil at specific concentrations, and avoided prochloraz at high concentrations (Kessler *et al.*, 2015; Liao *et al.*, 2017b). The mechanisms underlying this preference or avoidance of contaminated nectars are currently not known, but this phenomenon could potentially trigger an increase or decrease in the exposure to pesticides, which would lead to differences between behavioral castes.

Therefore, in order to better quantify the risks posed by pesticides on honeybees, intraspecific variability in bee sensitivity and exposure (consumption of contaminated food) to these chemicals needs to be considered in risk assessment tests. To investigate to what extent behavioral caste affects the risks posed by pesticides , we exposed nurse and forager bees either to sulfoxaflor, a new neurotoxic insecticide that shares the same mode of action with neonicotinoids, or to azoxystrobin, a fungicide widely used in agriculture and regularly found in bee-foraged food (Long and Krupke, 2016; Mullin *et al.*, 2010; Sanchez-Bayo and Goka, 2014). We then assessed the sensitivity of nurse and forager bees to both pesticides, as well as their consumption of contaminated food, and calculated the acute and chronic RQs. We finally investigated whether dissimilarities in pesticide sensitivity could be attributed to differences in detoxification capacities by measuring the residual concentrations of pesticides over time in both nurse and forager bees.

Materials and methods

1. Bee sampling

Experiments were conducted with honeybees obtained from a local apiary at the "*Institut National de la Recherche pour l'Agriculture, l'Alimentation et l'Environnement"* (INRAE) in Avignon (France). To determine whether pesticide sensitivity differs between bees of different behavioral castes, nurses and foragers were collected the same day from four different colonies. Nurses were identified by removing brood combs from colonies and detecting bees that dipped

their heads into several cells containing larvae. Foragers were identified as bees returning to the colony with pollen loads, thereby discarding bees performing orientation or cleansing flights.
To validate the nurse sampling method, we checked whether bees collected as nurses had more developed hypopharyngeal glands (HPG) than forager bees. Indeed, HPGs, where jelly is produced to feed larvae, the queen and drones (Crailsheim, 1992), are consistently more developed in nurse than forager bees (Knecht and Kaatz, 1990; Robinson *et al.*, 1992). For that purpose, 10 bees of each behavioral caste were sampled in all colonies and stored at -20°C. Glands from 10 bees per caste and colony were dissected in distilled water under a binocular magnifier (LEICA MZ 12). Pictures of each gland were taken with a digital camera (Toupcam^{TM}) and ToupView image-capturing software (v3.7.5660). Then, the gland development was assessed by measuring the maximum diameter of 10 to 15 randomly chosen ovoid acini per gland using ImageJ v1.53e (http://rsb.info.nih.gov/ij/index.html). The diameters of acini were significantly larger in nurse (80.34 ± 9.66 µm) than in forager bees (53.54 ± 9.45 µm; Kruskal-Wallis test, $\chi^2 = 1274.4$, $p < 0.001$), which validated our sampling method.
After collecting bees from the four different colonies, nurses and foragers were immediately placed in different cages (10.5 cm x 7.5 cm x 11.5 cm) (Pain, 1966) containing a feeding tube with a solution of 50 % (w/v) sucrose and brought back to the lab. They were then placed in an incubator under controlled conditions (28°C and 50-70 % relative humidity).

2. Nurse and forager bee sensitivity to pesticides

Acute toxicity tests

We assessed the sensitivity of nurse and forager bees to sulfoxaflor and azoxystrobin by exposing them to a range of pesticide doses and estimating the dose-response curve. We tested a control dose (0 ng/bee) and a total of five doses of azoxystrobin (1, 10, 20, 40, 60 µg/bee) and six doses of sulfoxaflor (1, 10, 25, 50, 100, 150 ng/bee). Each exposure to azoxystrobin or sulfoxaflor was tested alone. The doses were chosen to surround the theoretical LD_{50} reported by EFSA for each pesticide (LD_{50} EFSA: > 25 µg/bee for azoxystrobin and 146 ng/bee for sulfoxaflor (EFSA, 2014, 2010).
Stock solutions of sulfoxaflor (Techlab, France) and azoxystrobin (Sigma Aldrich, France) in acetone were previously aliquoted and conserved at −20°C. Bees were sugar-starved for 2 h and then fed with a solution of 50% (w/v) sucrose and azoxystrobin (10% acetone) or sulfoxaflor (1% acetone). For each dose, one cage of 10 bees per colony was used, except for one colony for which we had 2 cages per dose. Each treated cage received 100 µl of the solution laced with pesticides, which was fully consumed within 60 min (giving around 10 µl per bee).

Control groups were fed with pesticide-free sucrose solution (50% w/v sucrose, 1 or 10% acetone). The experiment was repeated twice for each colony (n = 10 cages per dose and behavioral caste). Mortality was recorded at 24 and 48h post-exposure.

To express the toxicity endpoint (median lethal dose – LD_{50}) in ng per g of bees, we determined the weight of nurse and forager bees. We did not weigh test bees but bees from the same colony and collected at the same time as the acute toxicity tests. Bees were frozen at -20°C, then individually placed in glass petri dishes, at room temperature for 1 hour. They were weighed and then dried at 60°C for 72 hours to measure their dry weight.

Chronic toxicity test and pesticide-contaminated syrup consumption

Nurse and forager bees were chronically exposed to a low and a high concentration of pesticides. Groups of 20 nurse or forager bees from the same four colonies were placed in different cages (n = 1 or 2 cages per colony giving n = 6 cages per pesticide concentration and behavioral caste). Bees were provided with a solution of 50 % (w/v) sucrose, 0.1 % acetone and azoxystrobin (0.2 or 2 µg/ml) or sulfoxaflor (0.02 or 0.2 µg/ml). Control groups were fed with pesticide-free sugar solution (50 % w/v sucrose, 0.1 % acetone). The concentrations were chosen based on pesticide residue data found in nectar. Depending on different application rates of sulfoxaflor and the crops, field studies reported levels of the neurotoxin ranging from 0.04 to 2.37 mg/kg in nectar (EPA, 2019). Residues of azoxystrobin have been found at high concentrations (up to 1.45 mg/kg) in nectar collected by honeybees, shortly after the application day (Schatz and Wallner, 2009). The chronic pesticide treatments were performed over 5 days and the syrup feeders were replaced every day. Since forager lifespan is on average 8 days (Prado *et al.* 2020), chronic toxicity tests were performed over 5 days to minimize the risk of natural forager mortality. For each cage, individual syrup consumption was assessed daily, by weighing feeders and dividing the consumed food by the number of remaining live bees. Dead bees were counted daily and removed over the 5-day period. The exact concentrations of sulfoxaflor and azoxystrobin were determined by liquid chromatography–tandem mass spectrometry (LC-MS/MS, see Barascou *et al.* 2021), giving low and high concentrations of 0.021 and 0.223 µg/ml for sulfoxaflor and 0.16 µg/ml and 1.46 µg/ml for azoxystrobin.

3. Residual concentrations of pesticides in nurse and forager bees

In order to investigate the potential mechanisms underlying the difference in pesticide sensitivity between nurses and foragers, we compared their residual pesticide concentrations at 2 and 8 h post-exposure. Groups of 60 bees were placed in cages. Bees were starved for 2 hours

and then fed with a concentration of 2 µg/ml of azoxystrobin or sulfoxaflor. Control groups were fed a sucrose solution only. Each cage received 600 µl of sucrose solution, giving a theoretical dose of 20 ng of pesticide per bee (19 ng of azoxystrobin and 23.5 ng of sulfoxaflor, based on the exact concentration of the tested solution, see above). Once solutions were all consumed, bees were provided with a 50 % sucrose solution (w/v). At 2 and 8h post-exposure, 50 bees per cage were respectively sampled on dry ice and stored at -80°C for later analysis (n = 5 cages per behavioral caste, pesticide and time point).

Pesticide concentrations were analyzed on pools of 25 bees (2 pools per cage giving n = 10 pools per behavioral caste, pesticide and time point). Each pool of bees was weighed and sulfoxaflor and azoxystrobin content were subsequently analyzed via LC-MS/MS. The QuEChERS method was used for the extraction of the active ingredients from samples, following the European Standard EN 15662 (see Barascou *et al.* (2021) for further method details). The limit of quantification for azoxystrobin and sulfoxaflor was 0.001 mg/kg and 0.01 mg/kg, respectively.

4. Statistical analysis

Data were analyzed using the statistical software R v3.3.3 (R Core Team, 2020). In the acute toxicity test, the LD_{50} values were calculated for each pesticide and each behavioral caste, by fitting a dose-response model to the data (*drm* function of the "drc" package) (Ritz *et al.*, 2015). Different models were compared based on the log likelihood value, Akaike's information criterion, and the estimated residual standard error. For sulfoxaflor and azoxystrobin data, the two-parameter (W1.2) and four-parameter Weibull models (W2.4) were shown to best describe the data analyzed for nurses and foragers, respectively, and were therefore used to calculate all of the dose–concentration response curves in the present study. The toxicity between nurses and foragers was compared by interpooling the 95% CI (confidence interval) limits of the LD_{50} values, considering the LD_{50} as different if the CI values did not overlap.

Variations in body weights between nurses and foragers were analyzed using a Kruskal-Wallis test, followed by Dunn's multiple comparison tests with the Benjamini–Hochberg correction. Syrup consumption between nurses and foragers, and among experimental groups in the chronic toxicity experiment was also analyzed using a Kruskal-Wallis test, followed by Dunn's multiple comparison test with the Benjamini–Hochberg correction. Survival data from the chronic toxicity tests were analyzed with a Cox proportional hazards regression model (*coxph* function of the survival package in R (Cox, 1970)).

In order to assess the potential risk posed to nurse and forager bees by an acute exposure to pesticide, a Risk Quotient (RQ) was calculated based on the exposure concentration, the caste-specific sucrose consumption values from the chronic toxicity experiment (mean consumption within 5 days), and acute toxicity data (LD_{50}):

$$RQ = \frac{\text{Exposure concentration (µg/kg)} \times \text{Consumption (kg/day)}}{\text{LD50 (µg/bee)}}$$

We similarly assessed the potential risk posed to nurse and forager bees by a chronic exposure to pesticide, by using the NOED from the chronic toxicity test:

$$RQ = \frac{\text{Exposure concentration (µg/kg)} \times \text{Consumption (kg/day)}}{\text{chronic 5-day oral NOED (µg/bee/day)}}$$

The acute and chronic RQ threshold levels of concern (LOC) are 0.4 and 1, respectively. If the RQ is less than 0.4 or 1, the risk posed by the pesticide is acceptable, but if the RQ is equal or greater than 0.4 or 1, the risk is not acceptable (Thompson, 2021).

Residual pesticide concentrations were compared between behavioral castes and time points using Wilcoxon rank test pairwise comparisons and a Bonferroni adjustment.

Results

Acute toxicity for nurse and forager bees

We recorded bee mortality rates at both 24 and 48 h following exposure to the different doses of pesticides (Table S1 and S2). We then calculated the LD_{50} of each pesticide by using the 48 h mortality data since additional mortality occurs between 24 and 48 h post-exposure. Only a slight overlap in the 95% CI values was observed between the dose-response curves of nurse and forager bees, and the calculated sulfoxaflor LD_{50} was lower for foragers (41.04 ng/bee,) than for nurse bees (54.40 ng/bee; Fig.1 and Table 1). Nurses were 1.6 times heavier (134.77 ± 24.24 mg) than foragers (85.81 ± 8.69 mg, Kruskal-Wallis test, $\chi^2 = 69.88$, $p < 0.001$; Table 1), and when the sulfoxaflor toxicity was expressed in ng per g of bees, a strong overlap was observed between the dose-response curves of nurses and foragers (Table 1).

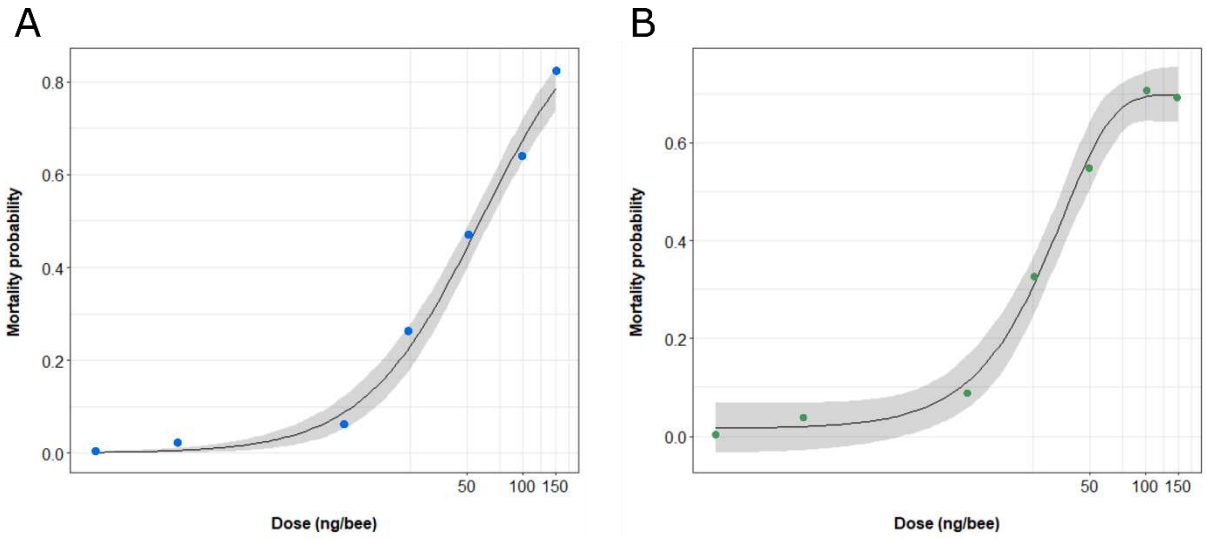

Figure 1. Dose-response relation resulting from oral exposure to sulfoxaflor in (A) nurse and (B) forager bees. Data show bee mortality at 48 h post-exposure (n = 10 bees per cage and 10 cages per dose). Dots represent the mean values of bee mortality for each tested dose. Grey areas show the confidence interval at 95%.

Tested azoxystrobin doses marginally increased bee mortality (up to 20% for the highest dose; Table S2). It was therefore not possible to determine an exact azoxystrobin LD_{50} for both foragers and nurses (LD_{50} values > 60 µg a.i./bee; Table 1).

Table 1. Oral median lethal dose (LD50) of sulfoxaflor and azoxystrobin in nurse and forager bees. N: total number of bees treated with one of the pesticides.

Bee caste	Pesticide	N	LD_{50} (95 % CI), ng/bee	LD_{50} (95 % CI), ng/g
Nurses	Sulfoxaflor	685	54.40 (47.47 – 61.31)	403.65 (352.23 – 454.92)
	Azoxystrobin	605	> 60 µg/bee	> 445.20 µg/g
Foragers	Sulfoxaflor	710	41.04 (33.50 – 49.28)	478.27 (390.40 – 574.29)
	Azoxystrobin	602	> 60 µg/bee	> 699.22 µg/g

Chronic toxicity for nurse and forager bees

Regardless of the pesticide and its concentration, forager bees consistently consumed more sugar solution than nurse bees (sulfoxaflor: Kruskal-Wallis test, $\chi^2 = 31.49$, $p < 0.001$ and azoxystrobin: $\chi^2 = 37.67$, $p < 0.001$; Fig. 2). Overall, forager bees ingested 1.4 times more syrup than nurse bees. Although we did not find any effect of pesticide concentration on sugar consumption by nurse bees (sulfoxaflor: $p = 0.38$ and azoxystrobin: $p = 0.528$), a significant effect was observed in forager bees (sulfoxaflor: $p = 0.024$ and azoxystrobin: $p < 0.01$; Fig. 2). Foragers exposed to 0.021 µg/ml of sulfoxaflor consumed more syrup (68.79 ± 19.76 mg/day)

than control foragers (53.90 ± 14.90 mg/day, Dunn's test, p = 0.022) but not for foragers exposed to 0.223 µg/ml of sulfoxaflor (p = 0.07). Similarly, foragers exposed to 1.46 µg/ml of azoxystrobin consumed more syrup (72.16 ± 26.71 mg/day) than bees exposed to 0.16 µg/ml of azoxystrobin (54.16 ± 14.11 mg/day, p < 0.01) and control bees (53.90 ± 14.90 mg/day, p < 0.01).

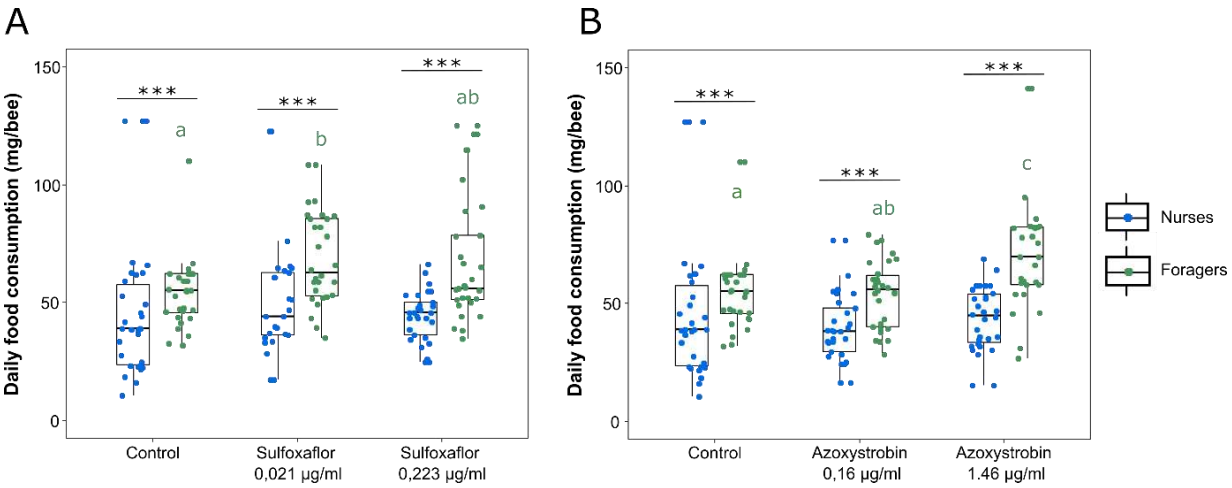

Figure 2. Individual syrup consumption according to pesticide treatments in nurse and forager bees. Daily individual consumption (mg/bee) is shown for foragers and nurses exposed to (A) sulfoxaflor and (B) azoxystrobin (n = 20 bees per cage and 6 cages per pesticide concentration and behavioral caste). Boxes indicate the first and third interquartile range with a line denoting the median. Whiskers include 90% of the individuals, beyond which circles represent outliers. Different letters and number of asterisks indicate significant differences between pesticide concentrations and between nurse and forager bees, respectively (Kruskal–Wallis tests followed by Dunn's multiple comparison test, *** denotes p < 0.001).

Chronic exposure to azoxystrobin (0.16 – 1.46 µg/ml) and sulfoxaflor (0.02 – 0.22 µg/ml) did not affect the survival of nurse bees (Cox model, p = 0.99; Fig. 3A). While we did not find any effect of both azoxystrobin concentrations and the lowest concentration of sulfoxaflor (0.021 µg/ml) on forager mortality, the highest concentration of sulfoxaflor (0.223 µg/ml) reduced their survival probability by around 50% within 5 days (Cox model, p < 0.001, Fig. 3B).

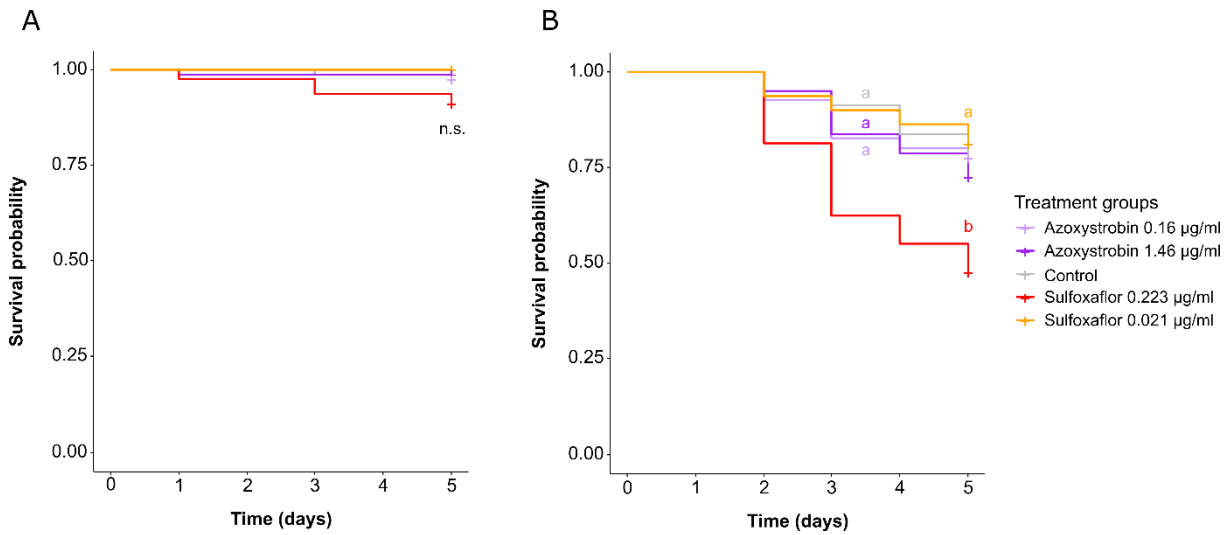

Figure 3. Chronic toxicity of azoxystrobin and sulfoxaflor on (A) nurse and (B) foragers bees. Data represent the survival probabilities of bees (n = 20 bees per cage and 6 cages per pesticide concentration and behavioral caste). Different letters indicate significant differences (Cox model).

Risk quotient for nurse and forager bees

Due to a higher level of exposure (consumption of pesticide-contaminated syrup) and a lower LD_{50}, the acute risk quotient (RQ) upon exposure to sulfoxaflor was twice as high for forager bees than for nurse bees (Table 2). Based on the tested field-relevant concentrations of sulfoxaflor, none of the acute RQ exceeded the theoretical threshold level of concern of 0.4 (Table 2). However, the acute RQ for foragers exposed to 0.223 µg/ml of sulfoxaflor was close to this threshold (RQ = 0.309; Table 2).

The chronic 5-day oral NOED of sulfoxaflor for nurse bees was 8.36 ng/bee/day (NOEC: 0.223 µg/ml). None of the chronic RQ for nurses exceeded the theoretical threshold LOC of 1 (Table 2). However, the chronic 5-day oral NOED for foragers was 1.22 ng/bee/day (NOEC: 0.021 µg/ml), leading to a chronic RQ above the theoretical threshold LOC of 1 (RQ = 10.373) and ten times higher than that of nurse bees (Table 2), when exposed to a sulfoxaflor concentration of 0.223 µg/ml.

The azoxystrobin LD_{50} could not be determined and therefore the acute RQ for both nurse and foragers bees was very low. The chronic RQ based on the higher concentrations of azoxystrobin with no-observed lethal effect (NOEC: 1.46 µg/ml) was calculated for both nurse (NOED: 89.28 ng/bee/day) and forager bees (NOED: 53.81 ng/bee/day). For both concentrations of azoxystrobin, none of the chronic RQ exceeded the theoretical threshold LOC of 1 (Table 2).

Table 2. Acute and chronic RQ for nurse and forager bees under different scenarios of exposure to the tested concentrations of sulfoxaflor and azoxystrobin.

	Nurses			Foragers		
	Consumption (mg/day ± SE)	Acute RQ	Chronic RQ	Consumption (mg/day ± SE)	Acute RQ	Chronic RQ
Sulfoxaflor 0.021 µg/ml	49.38 ± 21.84	0.016	0.105	68.79 ± 19.76	0.030	1
Sulfoxaflor 0.223 µg/ml	44.24 ± 10.68	0.154	1	67.21 ± 25.97	0.309	10.373
Azoxystrobin 0.16 µg/ml	39.52 ± 13.78	< 0.0001	0.099	54.16 ± 14.11	< 0.0001	0.082
Azoxystrobin 1.46 µg/ml	43.49 ± 13.49	< 0.0011	1	72.16 ± 26.71	< 0.0018	1

Residual concentrations of pesticides in nurse and foragers bees

Residues of azoxystrobin were detected at very low concentrations at 2 h post-exposure and could be quantified (above LOQ) in only 3 nurse samples (1.35 ± 0.54 ng/bee) and 6 forager samples (1.26 ± 0.60 ng/bee), demonstrating a high metabolization rate (amount of eliminated pesticide divided by the amount of pesticide to which bees were exposed; nurses: 92.89 ± 4.25 % and foragers: 93.37 ± 3.47 %). At 8h post-exposure, azoxystrobin concentrations were below the LOQ.

Regarding sulfoxaflor, the concentrations of residues found at 2 h post-exposure were lower in forager (metabolization rate = 36.30 ± 13.44 %) than in nurse bees (metabolization rate = 19.59 ± 10.99 %) (Wilcox test, $p < 0.01$; Fig. 4). However, this difference disappeared at 8 h post-exposure (Wilcox test, $p = 0.059$; Fig. 4), likely because sulfoxaflor residual concentrations decreased significantly between 2 and 8 h post-exposure in nurse bees (Wilcox test, $p = 0.036$; metabolization rate at 8 h = 33.31 ± 21.76 %) while it did not in forager bees (Wilcox test, $p = 0.059$; metabolization rate at 8 h = 49.80 ± 10.64 %; Fig. 4).

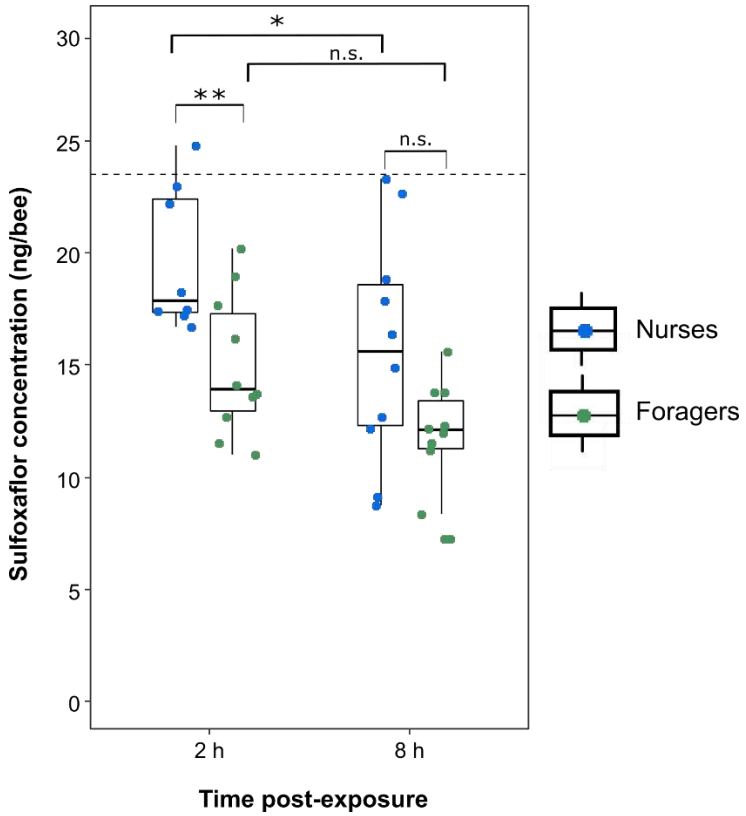

Figure 4. Residual concentrations of sulfoxaflor in nurse and forager bees. Data represent the pesticide concentrations in 10 pools of 25 bees per experimental condition. Boxes indicate the first and third interquartile range with a line denoting the median. Whiskers include 90% of the individuals, beyond which circles represent outliers. Asterisks indicate statistically significant differences between behavioral caste (ns: not significant; *p < 0.05, Wilcoxon test). The dotted lines represent the dose provided to bees (assuming an equal consumption of sugar syrup between bees).

Discussion

Responses to pesticides can be highly variable between species (Adams *et al.*, 2021; Azpiazu *et al.*, 2021; Sgolastra *et al.*, 2017; Spurgeon *et al.*, 2020; Uhl *et al.*, 2016), but also within species (Calow, 1996; Dahlgren *et al.*, 2012; Graves and Mackensen, 1965; Szabó *et al.*, 2021). This intraspecific variance might add another level of complexity to ecotoxicological studies, but also provides highly relevant information on the risk posed to populations by pesticides (Calow, 1996). Such levels of information are especially important for better understanding the risk associated with pesticide exposure in honeybees, which exhibit a high inter-individual variability in their physiological backgrounds.

In the sulfoxaflor acute toxicity test, a slight overlap of the dose-response curves was observed between the two behavioral castes, but in general, foragers were more sensitive to the insecticide as compared to nurse bees. Such results are consistent with a previous study, which showed that in LD_{50} tests foragers were more susceptible to another insecticide

(flupyradifurone) than in-hive bees (S Tosi and Nieh, 2019). This higher sensitivity of foragers was also confirmed at the chronic level, since exposure to the highest sulfoxaflor concentration significantly reduced the survival of foragers but not of nurse bees. An increase in bee sensitivity to insecticides as bees age has been reported in several studies (Bendahou *et al.*, 1997; Ladas, 1972; Mayland and Burkhardt, 1970; Zhu *et al.*, 2020). This phenomenon is somewhat in agreement with our results as forager bees are generally older than nurse bees. However, this age-dependent sensitivity is not always consistent as indicated by a Rinkevich *et al.* (2015) study, which showed that sensitivity to the insecticides naled (organophosphate) and phenothrin (pyrethroid) significantly increased and decreased with bee age, respectively. In addition, one still needs to be cautious with the interpretation of age-dependent effects because honeybee workers may have the same chronological age (i.e. time elapsed since adult emergence) but different biological ages, which refer to the changes in the physiological state that occur throughout its lifespan. For instance, within the colony, bees of the same chronological age might be specialized in different behavioral tasks and therefore have different physiological backgrounds (Robinson, 2002; Whitfield *et al.*, 2003). The biological age, determined by the behavioral specialization of bees (e.g. nurse vs forager) might therefore better reflect the biological state of bees and give information on their sensitivity to pesticides.

Further evidence of variation in honeybee sensitivity to pesticides is the comparison of sulfoxaflor LD_{50} values across studies. The oral LD_{50} (48h) reported by EFSA for in-hive bees was of 146 ng/bee (EFSA, 2014), which is almost three times the LD_{50} we found for nurse bees. This may be explained by differences in the genetic backgrounds leading to dissimilar responses to pesticides, but also to the large range of ages among the tested in-hive bees given that all age cohorts can normally be found on hive frames (Free, 1960; van der Steen *et al.*, 2012). Similarly, some inconsistencies can be observed in the forager LD_{50}, as indicated by the higher LD_{50} reported in a recent study (55.38 ng/bee vs 41.04 ng/bee in our study; (Azpiazu *et al.*, 2021). This difference might find its origin in the sampling procedure since we focused on pollen foragers, while Azpiazu *et al.* (2021) sampled all bees returning to the hive, which might include young bees performing orientation or cleansing flights in addition to forager bees, and is particularly reflected by the wider range of the LD_{50} 95 % CI (26.34 to 111.46 ng/bee) compared to our study (33.50 – 49.28 ng/bee).

An age-related increase in the sensitivity to herbicide and fungicide has also been previously described by Wahl and Ulm (1983). However, in our study, the fungicide azoxystrobin was found to be weakly toxic for both nurse and forager bees upon both acute and chronic exposure, confirming data from regulatory tests (> 25 µg/bee; EFSA 2010). This lack of effect might be

due to either the tested doses, which were limited to 60 µg/bee, because of the low solubility of azoxystrobin in solvent, or to the rapid metabolization of the fungicide (no trace of azoxystrobin could be found at 8 h post-exposure). Alternatively, azoxystrobin may be non-toxic or weakly toxic to honeybees given its targeting of fungi with a mode of action that might be too specific to affect insects.

How to explain the differences in sulfoxaflor sensitivity between nurse and forager bees? To survive toxic compounds, insects have developed detoxification mechanisms, which prevent their accumulation in organs and tissues (Lu *et al.*, 2021; Panini *et al.*, 2016; Smith, 1955). Accordingly, we expected a more efficient elimination of sulfoxaflor by nurse bees as compared to foragers. However, the analysis of sulfoxaflor residues showed that its concentration did not differ between nurse and forager bees at 8 h post-exposure. On the contrary, sulfoxaflor metabolization was stronger in foragers within 2 h post-exposure, suggesting faster sulfoxaflor elimination by forager bees in the very short-term. These results agree with a study that showed that the expression level of three genes encoding cytochrome P450 monooxygenases (involved in the detoxification pathway) was higher in forager bees than in nurses (Vannette *et al.*, 2015). This was later confirmed at the enzymatic level, in which cytochrome P450 monooxygenase activity gradually increased with bee age (Zhu *et al.*, 2020). However, we cannot exclude that in the long-term (> 8 h post-exposure), nurse bees are more efficient in eliminating sulfoxaflor than foragers. This is suggested by the improved ability of bees to metabolize pesticides after the consumption of pollen (Ardalani, 2021; Ardalani *et al.*, 2021; Barascou *et al.*, 2021c), which is essentially consumed by nurses. Lastly, given that the impact of pesticides depends not only on the fate of the molecule in the body, but also on its interaction with the biological target and consecutive effects on the organism, we could also expect that sulfoxaflor affected nurses and foragers in different manners. A recent study demonstrated that nurse and forager bees were affected in different ways by neonicotinoids at the gene expression level in the brain: while the expression of genes involved in cognition and development was predominantly affected in foragers, the expression of genes involved in metabolism was modified in nurses (Tsvetkov and Zayed, 2021). Although, it is not known how such effects might affect honeybee survival or performance, it could help to explain differences in pesticide sensitivity. But perhaps, the most reasonable explanation relies on the body weight difference, because for any given bee species, the heavier the individual bees are the less sensitive they are to a given dose of pesticide (Gerig, 1975; Nogueira-Couto *et al.*, 1996; Pamminger, 2021; Tahori *et al.*, 1969; Thompson and Hunt, 1999). When converted to ng/g of bee, the sulfoxaflor LD_{50} did not differ between nurses and foragers. However, the fold change in body weight was much stronger in favor of nurses (1.6

times heavier than foragers), which likely explains the higher sensitivity of foragers compared to nurses at the individual level.

We also noted a significantly higher consumption of sugar syrup by forager bees compared to nurses, and this propensity to consume more syrup was even more pronounced when it was laced with pesticide (at a specific concentration). This phenomenon confirms the often observed preferences of forager bees for sugar solutions containing pesticides (Kessler *et al.*, 2015; Liao *et al.*, 2017b), but also has the consequence of intensifying the risk posed by pesticides to foragers. Indeed, the higher consumption of pesticide-contaminated syrup (sulfoxaflor and azoxystrobin) combined with the stronger susceptibility to pesticide (sulfoxaflor) in foragers contributed to an increase by 2 and 10-fold of the acute and chronic RQ, respectively. The magnitude of RQ differences might however be lower in the case of pollen contamination by pesticides given that nurse bees can additionally consume on average 5-10 mg pollen/day (Brodschneider and Crailsheim, 2010; Pernal and Currie, 2000).

In conclusion, our results show that honeybee workers are not all equal regarding the risk posed by pesticides and that, depending on the honeybee behavioral caste, it might be under or over-estimated. The growing agreement across studies that foragers or old bees are more sensitive to insecticides than nurse or young bees, therefore suggests consistent inclusion of forager bees in regulatory tests should allow for an increase in the safety margin of pesticide risk assessment. However, further studies are needed to determine whether this caste-dependent variation in insecticide sensitivity also occurs and to what extent in response to a range of fungicides and herbicides.

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Supplementary information

Table S1. Bee mortality (%) at both 24 and 48 h following the acute exposure to sulfoxaflor.

		Doses (ng/bee)						
		0	**1**	**10**	**25**	**50**	**100**	**150**
Nurses	24 h	0	4.21	1.02	26	40.40	53.53	75.26
	48 h	0	4.12	1.02	28	45.45	59.59	84.54
Foragers	24 h	1	2	1.94	20.59	50.48	52.47	50.49
	48 h	2	4	7.77	32.35	56.31	70.30	69.31

Table S2. Bee mortality (%) at both 24 and 48 h following the acute exposure to azoxystrobin.

		Doses (ng/bee)					
		0	**1**	**10**	**20**	**40**	**60**
Nurses	24 h	3	0.98	0	1.96	14	17.82
	48 h	3	0.98	0	3.92	17	20.79
Foragers	24 h	0	0	3	1.98	12	14
	48 h	6	4	8	9.90	16	17

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C. Conclusion

Les résultats de cette étude confirment une différence de sensibilité au sulfoxaflor entre les nourrices et les butineuses chez les abeilles mellifères, avec une toxicité plus forte de l'insecticide pour les butineuses que pour les nourrices. Cela ne s'explique pas par une meilleure détoxification du sulfoxaflor chez les nourrices, mais par une différence de poids corporel entre les deux castes. De plus, les butineuses consomment plus de solution sucrée que les nourrices, les exposant davantage aux pesticides présents dans le sirop (ou nectar dans l'environnement). L'ensemble de ces observations conduit à un risque plus élevé du sulfoxaflor pour les butineuses.

Ces résultats suggèrent l'inclusion systématique des abeilles butineuses dans les tests réglementaires actuels d'évaluation des risques liés à l'exposition aux insecticides. Cela permettra de garantir une plus grande protection des abeilles mellifères, et d'éviter une sous-estimation du risque posé par les insecticides.

II. Modulation nutritionnelle de la sensibilité des abeilles aux pesticides

A. Avant-propos

L'étude précédente nous a permis de mettre en évidence une variabilité de la réponse des abeilles mellifères aux pesticides, avec une sensibilité plus faible des nourrices à un insecticide. Ces dernières consomment davantage de pollen que les autres abeilles de la colonie, ce qui contribue à la croissance des glandes hypopharyngiennes (lieu de synthèse de la nourriture larvaire) et à la constitution des réserves lipidiques (corps gras). La nutrition pollinique chez l'abeille mellifère est ainsi indispensable au développement physiologique des nourrices (Crailsheim *et al.* 1992 ; Keller *et al.* 2005a, b ; Brodschneider and Crailsheim 2010).

Cependant, dans l'environnement, les abeilles sont confrontées à des disparités dans le temps et dans l'espace de la quantité et qualité des pollens. Par exemple, la teneur en protéines du pollen peut varier de 2.5% à 61% selon les espèces florales (Roulston and Cane, 2000). Cette variation dans la qualité nutritive est connue pour avoir des effets sur la physiologie des abeilles et leur système immunitaire (Di Pasquale *et al.*, 2013). De plus, avec l'intensification des paysages agricoles, la disponibilité et la diversité des ressources sont réduites (Goulson *et al.* 2015 ; Requier *et al.* 2015 ; de Vere *et al.* 2017). Il a ainsi été constaté que des abeilles ayant accès à une nutrition pollinique ont une sensibilité moindre aux pesticides que des abeilles nourries sans pollen (Schmehl *et al.*, 2014). De plus, certains composés phytochimiques des pollens, comme la quercétine, peuvent stimuler le système de détoxification des abeilles (Liao *et al.*, 2020, 2017a). Ainsi, l'objectif de cette étude a été de déterminer et de quantifier les effets de la disponibilité et de la qualité du pollen sur la sensibilité des abeilles aux pesticides. Ces travaux ont fait l'objet d'une publication dans *Royal Society in Open Science* (Barascou *et al.*, 2021c) .

Pour réaliser ces expériences, une étude a été menée au préalable afin d'identifier des mélanges polliniques ayant des valeurs nutritives différentes pour les abeilles. Cette étude sur les besoins nutritionnels des abeilles mellifères (*Apis mellifera*) et trois autres espèces d'abeilles (le bourdon *Bombus terrestris*, les osmies *Osmia cornuta* et *bicornis*) a fait l'objet d'une publication dans *Frontier in Sustainable Food Systems* (Barraud *et al.* 2022 ; Annexe 1).

B. Article 2

Pollen nutrition fosters honeybee tolerance to pesticides

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Abstract

A reduction in floral resource abundance and diversity is generally observed in agro-ecosystems, along with widespread exposure to pesticides. Therefore, a better understanding on how the availability and quality of pollen diets can modulate honeybee sensitivity to pesticides is required. For that purpose, we evaluated the toxicity of acute exposure and chronic exposures to field realistic and higher concentrations of azoxystrobin (fungicide) and sulfoxaflor (insecticide) in honeybees provided with pollen diets of differing qualities (named *S* and *BQ* pollens). We found that pollen intake reduced the toxicity of the acute doses of pesticides. Contrary to azoxystrobin, chronic exposures to sulfoxaflor increased by 1.5 to 12-fold bee mortality, which was reduced by pollen intake. Most importantly, the risk of death upon exposure to a high concentration of sulfoxaflor was significantly lower for the *S* pollen diet as compared to the *BQ* pollen diet. This reduced pesticide toxicity was associated with a higher gene expression of vitellogenin, a faster sulfoxaflor metabolization, and a lower concentration of the phytochemical p-coumaric acid, known to upregulate detoxification enzymes. Thus, our study revealed that pollen quality can influence the ability of bees to metabolize pesticides and withstand their detrimental effects, providing another strong argument for the restoration of suitable foraging habitat.

Keywords: Bees, survival, pesticide residue, vitellogenin, p-coumaric acid, metabolization

Introduction

The availability of nutritive resources has long been acknowledged as a key ecological factor affecting the expression of several life-history traits (Simpson and Raubenheimer, 2012). Notably, the quantity and balance of macro- and micronutrients, as well as secondary metabolites, in the diet of insects, can determine their longevity and ability to respond to environmental pressures, such as xenobiotics (Simpson and Raubenheimer, 2012). For instance, variation in the protein:carbohydrate ratio can modulate their sensitivity to toxins (Deans *et al.*, 2017), and secondary metabolites may increase their tolerance to various pesticides by stimulating the production of detoxification enzymes (Johnson *et al.*, 2012; Terriere, 1984). In this context, the contribution of resource availability and quality to the overall health of honeybees (*Apis mellifera*), a major pollinator of crops and wild plants (Hung *et al.*, 2018), has received increased attention (Brodschneider and Crailsheim, 2010; Dolezal and Toth, 2018; Wright *et al.*, 2018). Indeed, like many organisms, their environment has rapidly changed under the influence of human activity. They are thus exposed to more frequent and diverse sources of stress, including pesticides, along with a reduction of floral resource abundance and diversity due to landscape simplification and habitat loss (Goulson *et al.*, 2015).

Among the different stress factors threatening honeybee colonies, pesticides have attracted most of the attention and debate (Durant, 2020; Sgolastra *et al.*, 2020; Storck *et al.*, 2017). The toxicity of a large range of pesticides has been documented (Aliouane *et al.*, 2009; Alkassab and Kirchner, 2017; Balbuena *et al.*, 2015; Belzunces *et al.*, 2012; Blacquière *et al.*, 2012; Johnson, 2015; Siviter *et al.*, 2018). Research on the modulation of pesticide toxicity by nutritional factors, while still in its infancy, could lead to a better understanding of the impact of pesticides on honeybee populations and the design of more supportive habitats. The amount of nutrients in nectar and pollen can indeed differ between plant species (6.3–85% for sugar concentration in nectars (Pamminger *et al.*, 2019), and 2.5- 61 % and 1-20% for protein and lipid contents in pollens, respectively (Roulston and Cane, 2000; Vaudo *et al.*, 2020). In addition, both pollen and nectar are nutritional sources of several amino acids, minerals, micronutrients and secondary metabolites (Palmer-Young *et al.*, 2019; Wright *et al.*, 2018). As a consequence, the quality of honeybee diets varies greatly over time and according to landscape features (Avni *et al.*, 2014; Requier *et al.*, 2015). Therefore, these variations in nutritional content may provide a basis for nutritional modulation of pesticide toxicity.

Confirmation of this hypothesis was tested for nectar by providing bees with limited access or access to low concentrations of sugar. The survival of bees was synergistically reduced by the

combination of poor nutrition and field-realistic exposure to neonicotinoids (−50%) (Tosi *et al.*, 2017b). However, most of data on the nutritional modulation of pesticide toxicity comes from studies that have tested pollen diets, likely because pollen is essential to the physiological development of bees (Abdulaziz, 2006; Brodschneider and Crailsheim, 2010; Haydak, 1970; Pernal and Currie, 2000). Wahl and Ulm (Wahl and Ulm, 1983) were the first to report that feeding bees with pollen increased their tolerance to pesticides. They notably found that pollen intake as well as the quality of pollen (protein content) increased the median lethal dose (LD_{50}) of several pesticides (Wahl and Ulm, 1983). Later, Schmehl *et al.* (Schmehl *et al.*, 2014) demonstrated that pollen intake reduced bee sensitivity to chronic exposure to chlorpyrifos compared to bees fed without pollen. At the same time, Archer *et al.* (Archer *et al.*, 2014) showed that bees having access to an artificial protein-rich diet were more able to withstand nicotine exposure than bees provided with a protein-poor diet. More recently, Crone and Grozinger (Crone and Grozinger, 2021) found that artificial and pollen diets characterized by different protein to lipid ratios can influence the survival time of bees chronically exposed to chlorpyrifos. Endpoints other than mortality rate have been used to assess the influence of pollen quality and availability on pesticide sensitivity in honeybees (development of feeding glands (Renzi *et al.*, 2016)), as well as in bumblebees (micro-colony performance, nest founding (Barraud *et al.*, 2020; Dance *et al.*, 2017; Leza *et al.*, 2018)) and *Osmia* (reproduction (Stuligross and Williams, 2020)), however, these studies generally reported a lack of interactions between these two factors.

Regarding the potential mechanisms underlying this nutritional modulation of pesticide sensitivity, pollen intake upregulates the expression of several xenobiotic-metabolizing cytochrome P450 genes (Schmehl *et al.*, 2014), as well as the activity of glutathione S-transferases (Di Pasquale *et al.*, 2013), which are involved in Phases I and II of the detoxification pathways, respectively (Berenbaum and Johnson, 2015; Claudianos *et al.*, 2006; Gong and Diao, 2017). More specifically, upon ingestion, several constituents of pollen, like the phytochemicals p-coumaric acid and quercetin, can upregulate the expression of cytochrome P450 genes (Mao *et al.*, 2013, 2011) and increase the survival rate of bees exposed to pesticides (Johnson *et al.*, 2012; Liao *et al.*, 2020, 2017a; Wong *et al.*, 2018). Such an effect on the detoxification capacity of bees was further confirmed by measuring pesticide metabolism. Ardalani *et al.* (Ardalani, 2021) found a reduction in the concentration of imidacloprid in honeybee bodies fed with quercetin, although no effect was observed on the reduction in the concentration of tebuconazole or tau-fluvalinate. Similarly, adding p-coumaric

acid to a sucrose diet led to faster coumaphos disappearance (Mao *et al.*, 2013). Overall, these studies indicate that pollen may influence the ability of bees to metabolize pesticides, which was recently confirmed (Ardalani *et al.*, 2021). Lastly, we cannot exclude an influence of pollen on the ability of bees to withstand the effects of pesticides given that the impact of pesticides depends not only on the fate of the molecule in the body (uptake, distribution, biotransformation, elimination), but also on its interaction with the biological target and effects at the physiological level. Due to its positive effect on bee longevity and on several molecular pathways and physiological functions (e.g., energy storage, immunocompetence, nutrient metabolism, protection against oxidative stress) (Alaux *et al.*, 2011; Castelli *et al.*, 2020; Jack *et al.*, 2016; Rutter *et al.*, 2019), pollen consumption might help bees to better tolerate the wide range of physiological impairments caused by pesticides, notably on nutrient metabolism, immunity, cell signalling and developmental processes (Aufauvre *et al.*, 2014; Christen *et al.*, 2018; Gao *et al.*, 2020; Schmehl *et al.*, 2014; Ye *et al.*, 2020).

In sum, it was found that pollen nutrition can influence the survival rate of pesticide-exposed bees and the metabolization of pesticides. However, the nutritional modulation of pesticide sensitivity and the underlying mechanisms have been rarely studied together. To further examine the influence of pollen on pesticide sensitivity and the underlying mechanisms, we provided bees with pollen of different qualities (protein, lipid, p-coumaric acid contents) or no pollen and then exposed them to either sulfoxaflor, a new neurotoxic insecticide that shares the same mode of action with neonicotinoids (Watson *et al.*, 2011; Zhu *et al.*, 2011), or azoxystrobin, an inhibitor of mitochondrial respiration in fungi and one of the most frequently detected fungicides in pollen collected by bees (34 to 87.5% of samples) (Long and Krupke, 2016). We then determined whether the survival of bees exposed to a single dose of pesticide (previously identified as the median lethal dose) or chronically to field realistic and higher concentrations of pesticides is affected by pollen intake and/or the quality of pollens. Finally, we investigated the mechanisms underlying the modulation of pesticide sensitivity by testing whether pollen consumption induces a decrease in the concentration of pesticides in bees and/or help to tolerate the detrimental effect of pesticides on bee vitality. The latter was determined by measuring the gene expression level of vitellogenin, a well-established marker of bee health and longevity (Amdam *et al.*, 2012; Seehuus *et al.*, 2006).

Material and methods

5. Pollen diet quality

In order to assess the influence of pollen intake and pollen quality on bee sensitivity to pesticides we used two pollen blends that differed regarding their nutritional properties: one predominantly composed of Brassicaceae (36%) and Quercus robur (35%) ("BQ pollen"), and the other primarily composed of Salix (89%) ("S pollen") (see Table S1 for the pollen species composition). They were purchased fresh from Abeille heureuse® (France). We analysed protein and lipid content and their ratio following protocols detailed in (Vaudo *et al.*, 2020). The BQ pollen had higher protein and lipid content (respectively 28.39 ± 0.72 % and 18.7±1.6 %, n = 9) than the S pollen (21.49 ± 1.05 % and 14.07±1.5 %, n = 9). The protein:lipid ratio was similar between pollen mixes (BQ pollen: 1.52 and S pollen: 1.53). We also determined the concentrations of both phytochemicals, p-coumaric acid and quercetin (see supplementary information). The p-coumaric acid concentrations reached 244.7 mg/kg (1491.6 µM) in the *BQ* pollen and 104.5 mg/kg (637 µM) in the *S* pollen. The level of quercetin was under the quantification limit of the analysis method for both pollens, i.e. below 10 mg/kg. The presence of pesticide residues in one extract of each pollen blend was determined by liquid chromatography–tandem mass spectrometry (LC-MS/MS) with a limit of quantification of 0.01 mg/kg and a limit of detection of 0.005 mg/kg following the European Standard EN 15662:2018 procedure. Only residues of 2,4-dimethylformamide (DMF, degradation products of amitraz) and tau-fluvalinate were detected in both pollen blends but were below the limit of quantification. These compounds used as chemical treatments against the honeybee parasite *Varroa destructor* are consistently found in pollens (47.4% and 88.3% of trapped pollens for amitraz and tau-fluvalinate, respectively; (Calatayud-Vernich *et al.*, 2019; Mullin *et al.*, 2010)) and are considered as relatively safe for honeybees with an oral LD_{50} of 75 µg/bee for amitraz (contact exposure) and 45 µg/bee for tau-fluvalinate (oral exposure) (U.S. EPA, n.d.). Both pollen blends were gamma irradiated to avoid parasite contamination and stored at -20°C.

1. Influence of pollen nutrition on honeybee sensitivity to pesticides

Experiments were performed at the Institut National de la Recherche Agronomique (INRAE) in a semi-urban area (Avignon, France, 43°540N-4°-520E) with honeybees (*Apis mellifera*) from our local apiary. To obtain one-day-old bees, brood frames containing late-stage pupae were removed from 8 to 10 colonies (depending on the experiments) and kept overnight in an incubator under controlled conditions (34°C, 50-70% relative humidity (RH)). The next day, newly-emerged bees (less than 1 day old) were collected, mixed and placed in cages (10.5 cm

x 7.5 cm x 11.5 cm) (Pain, 1966). To better simulate colony rearing conditions, cages were equipped with Beeboost® (Ickowicz, France), releasing one queen-equivalent of queen mandibular pheromone per day.

Caged bees were kept in an incubator (30°C and 50-70% RH) and provided with water and Candy (Apifonda ® + powdered sugar) *ad libitum*. Except of the control groups, bees were also provided with one of the fresh pollen blends (*BQ* or *S* pollens) via an open tube feeder for 7 days. To prevent a potential nutritive compensation of bees fed with one of the pollens, they were not provided with *ad libitum* pollen, but with a determined quantity of pollen each day, representing the minimal daily consumption of pollen: 4 mg/bee/day for the first two days, 5 mg/bee/day for the next two days, 3 mg/bee/day for the fifth day, and 2 mg/bee/day for the last two days, as described in Di Pasquale *et al.* (Di Pasquale *et al.*, 2013). If some bees died during the pollen-feeding period (7 days), pollen amount was adjusted to the number of surviving bees. Both pollen diets were fully consumed every day.

Acute single exposure

In the first experiment, we tested whether pollen intake and pollen quality could modify the sensitivity of bees to a single dose of pesticide previously identified as a LD_{50} (data not published). Groups of 20 one-day old bees were placed in cages, which were randomly assigned to the different experimental groups: control (sucrose solution only), *BQ* pollen, *S* pollen, azoxystrobin, sulfoxaflor, *BQ* pollen/azoxystrobin, *S* pollen/azoxystrobin, *BQ* pollen/sulfoxaflor, and *S* pollen/sulfoxaflor (n = 10 cages per experimental group).

On day 5, bees were sugar-starved for two hours and then fed with a solution of 50 % (w/v) sucrose and azoxystrobin (4600 µg/ml, 1.14 % acetone) or sulfoxaflor (3.67 µg/ml, 0.37 % acetone) accordingly to the experimental group. Sugar solutions were provided via a feeding tube with a hole at its extremity. Each treated cage received 200 µl of the solution laced with pesticides. Solutions were provided for 4 hours and all of them were consumed within this time period. Assuming that the bees equally consumed the solutions, pesticide treatments resulted in a theoretical exposure to 46 µg/bee of azoxystrobin and 36.7 ng/bee of sulfoxaflor, corresponding to the LD_{50} levels previously determined. Control groups were fed with pesticide-free sugar solution (50% w/v sucrose, 1% acetone). After exposure to pesticides, bees were provided with water and Candy (Apifonda ® + powdered sugar) *ad libitum*. Mortality was recorded 48 hours after exposure.

Stock solutions of sulfoxaflor (Techlab, France) and azoxystrobin (Sigma Aldrich, France) in acetone were previously aliquoted and conserved at -20°C. The exact concentrations were

confirmed with LC-MS/MS (see below) and resulted in 5746 µg/ml for azoxystrobin and 3.62 µg/ml for sulfoxaflor, which corresponds to a real exposure of 57.5 µg/bee and 36.2 ng/bee, respectively.

Chronic exposure

In the second experiment, bees were chronically exposed to two concentrations of pesticides: a concentration that was considered to be field realistic and a higher concentration representing a worst-case exposure scenario. Groups of 30 one-day old bees were placed in cages (n = 10 cages per experimental group) and treatment groups were provided with one of the pollen blends as described above. On day 5, caged bees were provided with a solution of 50 % (w/v) sucrose, 0.1% acetone and azoxystrobin or sulfoxaflor at either a low or high environmental concentration which corresponded to theoretical values of: 0.02 and 2 µg/ml for sulfoxaflor and 0.2 and 2 µg/ml for azoxystrobin according to the experimental group. Control groups were fed with pesticide-free sugar solution (50 % w/v sucrose, 0.1 % acetone). The concentrations were chosen based on pesticide residue data found in pollen and nectar. Different application rates of sulfoxaflor before or during flowering of cotton resulted in 6.6 to 13.8 ppb of sulfoxaflor in nectar and 7.7 to 39.2 ppb in pollen of cotton flowers (Jiang *et al.*, 2020). However, other field residue studies with cotton, buckwheat and phacelia reported higher levels of sulfoxaflor ranging from 0.05 to 1 ppm in nectar and from 0.22 to 2.78 ppm in pollen collected by honeybees during the flowering period (EPA, 2019; U.S. EPA., 2010). Azoxystrobin was found at levels ranging from 0.03 to 0.11 ppm in pollen collected by honeybees in North America (Mullin *et al.*, 2010). In France, these levels ranged from 0.01 to 1.9 ppm (Observatory of Pesticide Residue, ITSAP- Institut de l'Abeille, personal communication). The chronic pesticide treatments were performed over 10 days and the syrup feeders were replaced every day. For each cage, individual syrup consumption was assessed daily by weighting feeders and dividing the consumed food by the number bees remaining alive. The cumulated syrup consumption over the 10 days of exposure to pesticide was then determined. After exposure to pesticides, bees were provided with water and Candy (Apifonda ® + powdered sugar) *ad libitum*. Dead bees were counted daily and removed over a 16-day period (i.e., when the high sulfoxaflor concentration group reached 100% mortality). Following the chemical analyses (LC-MS/MS) the real concentrations of tested diets resulted in 0.02 and 2.35 µg/ml for sulfoxaflor and 0.22 and 1.90 µg/ml for azoxystrobin, respectively, for the low and high exposure rates.

1. Potential mechanisms underlying the nutritional modulation of pesticide sensitivity

In order to investigate the mechanisms underlying the beneficial effect of pollen on pesticide sensitivity, we compared the gene expression level of vitellogenin and the amount of pesticide among groups. Groups of 80 one-day old bees were placed in cages and, as above, fed with one of the pollen diets (n = 10 cages per experimental group). On day 5, bees were sugar-starved for 2 hours and then fed with the highest concentration of azoxystrobin and sulfoxaflor (2 µg/ml), or sugar solution only. Each cage received 800 µl of sugar solution. Solutions were provided for 4 hours and all of them were consumed within this time period, giving a theoretical dose of 20 ng of pesticide per bee (19 ng of azoxystrobin and 23.5 ng of sulfoxaflor based on the real concentration of the tested solution, see above). After exposure to pesticides, bees were provided with water and Candy (Apifonda ® + powdered sugar). At 8 and 24 hr post-exposure (i.e., once all the solutions were consumed), 25 and 35 bees per cage were respectively sampled on dry ice and stored at -80°C for later analysis.

Influence of pollen nutrition and pesticides on vitellogenin expression level

For each cage, the abdomens of 6 bees sampled at 24 hr post-exposure were pooled in groups of 3. Abdomen pools were homogenised in 800 µL of Qiazol reagent (Qiagen) with a Tissue Lyser (Qiagen) (4 x 30 s at 30 Hz). RNA extraction was then carried out as indicated in the RNeasyPlus Universal kit (Qiagen) with DNase treatment (Qiagen). RNA yields were measured with a Nanodrop (Thermo Scientific) and cDNA synthesis was carried out on 1 µg of RNA per sample using the High capacity RNA to cDNA Kit (Applied Biosystems®, Saint Aubin, France). cDNA samples were diluted ten-fold in molecular grade water. The expression level of vitellogenin was determined by quantitative PCR using a Step One-Plus Real-Time PCR System (Applied Biosystems) and the SYBR green detection method. Three µL of cDNA were mixed with 5 µL SYBR Green Master Mix, 1 µL of forward primer (10 µmol) and 1 µL of reverse primer (10 µmol) of the target gene. A dissociation stage for the subsequent melting curve analysis was included. All qPCR reactions were run in duplicate. The average cycle threshold values of vitellogenin were normalised to the geometric mean of the housekeeping genes actin and *RPS18*, which proved to have rather stable expression levels (Jeon *et al.*, 2020). We used sequences of primers previously published (Boncristiani *et al.*, 2012; Scharlaken *et al.*, 2008). The ΔCt value of each group was subtracted by the ΔCt value of the control group (sugar syrup only) to yield ΔΔCt.

Influence of pollen nutrition on pesticide detoxification

Pesticide concentrations were analysed on a pool of 25 bees per cage and time point in the following groups: sulfoxaflor, azoxystrobin, *BQ* pollen/sulfoxaflor, *BQ* pollen/azoxystrobin, *S* pollen/sulfoxaflor, *S* pollen/azoxystrobin.

Sulfoxaflor and azoxystrobin content were analysed via LC MS/MS. The QuEChERS method was used for the extraction of the active ingredients from samples, following the European Standard EN 15662. Briefly, samples were ground in liquid nitrogen and 2 g of the crushed sample were mixed with 15 mL of a 1:2 water and acetonitrile mixture and a bag containing 4 g of magnesium sulfate, 1 g of sodium chloride, 1 g of sodium citrate tribasic dihydrate, and 0.5 g of sodium citrate dibasic sesquihydrate. An aliquot of the supernatant was mixed with 900 mg of magnesium sulfate, 150 mg of PSA and 150 mg of C18-E. After centrifugation, 2 µL of extract were injected into an Accela 1250 ultra-high-performance liquid chromatography (UHPLC) system for sulfoxaflor or azoxystrobin detection. The UHPLC system was coupled to a TSQ Quantum Access MAX Triple-Stage Quadrupole Mass Spectrometer, equipped with a heated-electrospray ionisation (H-ESI) source working in positive polarity. The mobile phase used for the analysis consisted of 4mM ammonium formate in water and 4mM ammonium formate in MeOH, both containing 0.1% formic acid. The fragments analysed were at m/z 372.1; 329.1; 344.1 (products) generated by the ion at m/z 404.12 (parent, azoxystrobin), and the fragments at m/z 154.1 and 104.2 (products) generated by the ion at m/z 278.1 (parent, sulfoxaflor). Quantification was performed using acetamiprid as an internal standard. The limit of quantification (LOQ) for azoxystrobin and sulfoxaflor was 0.001 and 0.01 mg/kg, respectively.

2. Statistical analysis

Data were analysed using the statistical software R v3.3.3 (R Core Team, 2020). In the acute toxicity tests, the percentage of dead bees in each cage was determined and each cage was considered as a replicate. Since data were not normally distributed, the effect of pesticide and pollen treatments on bee mortality was analysed using Kruskal–Wallis, followed by Dunn's multiple comparison tests with Benjamini-Hochberg correction. Then, the epsilon-squared (ε^2) was computed to obtain a measure of effect size between experimental groups (*epsilonSquared* function of the *rcompanion* package (Mangiafico, 2021)). The interpretation values were as follows: $\varepsilon^2 < 0.01$: very small effect, $0.01 < \varepsilon^2 < 0.08$: small effect, $0.08 < \varepsilon^2 < 0.26$: medium effect, and $\varepsilon^2 \geq 0.26$: large effect (Cohen, 1988). Survival data from the chronic toxicity tests were analysed with a Cox proportional hazards regression model (*coxph* function of the *survival*

package in R (Cox, 1970)). Data were transformed in a survival table and the remaining bees were considered alive at day 16. The Cox model was used to calculate the Hazard Ratio (HR). The HR is defined as the ratio between the instantaneous risk in the treatment group (H_1) and the risk in the control group (H_0), occurring at a given time interval (Hoffman, 2019). The influence of experimental treatments on the cumulated syrup consumption, vitellogenin expression level and pesticide detoxification was analysed using Kruskal-Wallis, followed by Dunn's multiple comparison tests with Benjamini-Hochberg correction.

Results

1. Influence of pollen nutrition on honeybee sensitivity to pesticides

Acute single exposure

The dose of azoxystrobin significantly increased the mortality of bees that did not have access to pollen (Kruskal-Wallis test: $p < 0.05$, and Dunn post-hoc tests: $p < 0.05$; Fig.1A). Although, only 10% of bees were found dead 48 hours post-exposure (vs 0% in control groups), the size of the negative effect of azoxystrobin could be considered as medium ($\varepsilon^2 = 0.163$; Table 1). Azoxystrobin did not increase bee mortality in bees fed with either the *BQ* or *S* pollen diets (Dunn post-hoc tests: $p = 0.41$ and $p = 0.79$ for *BQ* and *S* pollen, respectively; Fig.1A). However, bee mortality did not differ between the different pollen diets (no pollen, *BQ* and *S* pollen) after exposure or not to azoxystrobin (Fig.1A).

Sulfoxaflor increased the mortality of bees over 48 hours (Kruskal-Wallis test, $p < 0.01$; Fig.1B). For instance, the dose of sulfoxaflor killed around 50% of the bees that did not ingest pollen (Fig.1B). However, sulfoxaflor toxicity was also reduced by pollen consumption. First, the sulfoxaflor-induced mortality was significantly lower in bees fed with *BQ* or *S* pollen diets than in bees fed without pollen (*BQ* pollen: $p < 0.05$ and *S* pollen: $p < 0.05$). Second, the sulfoxaflor toxicity was reduced by half in bees provided with pollen (*BQ* pollen: $\varepsilon^2 = 0.183$, *S* pollen: $\varepsilon^2 = 0.143$ – medium effect) as compared to bees fed without pollen ($\varepsilon^2 = 0.277$ – large effect; Table 1).

Ultimately, the two types of pollen diet did not differentially affect the acute toxicities of azoxystrobin and sulfoxaflor (*BQ* pollen vs *S* pollen: $p = 1.0$ for azoxystrobin and sulfoxaflor; Fig 1A and B).

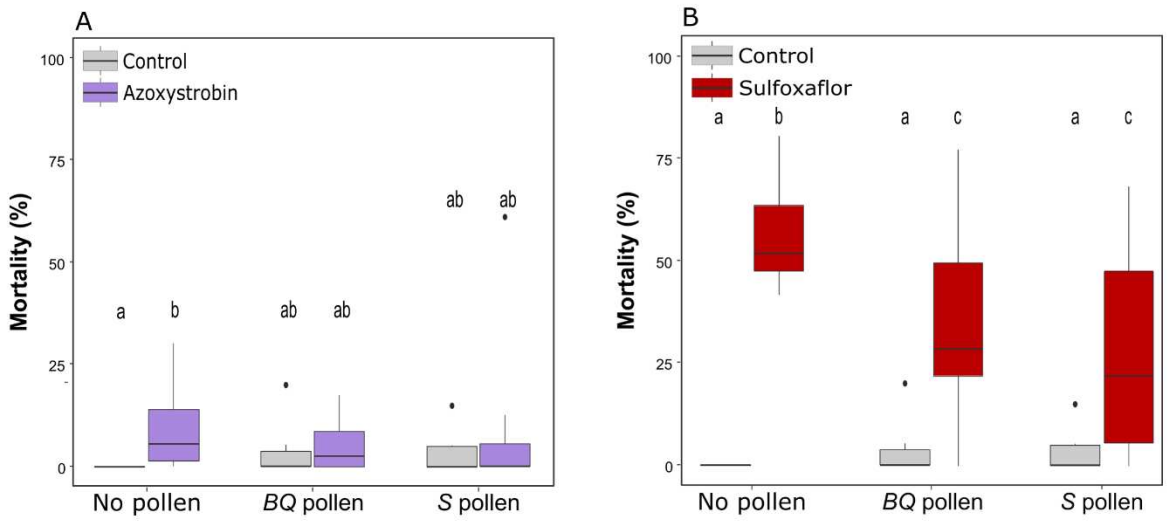

Figure 1. Acute toxicity of azoxystrobin (57.5 µg/bee) (A) and sulfoxaflor (36.2 ng/bee) (B) on bees fed with different pollen regimes. Data represent the 48 hr post-exposure mortality of bees (n = 20 bees per cage and 10 cages per modality). Boxes indicate the 1st and 3rd interquartile range with a line denoting the median. Whiskers include 90% of the individuals, beyond which circles represent outliers. Different letters indicate significant differences (Kruskal-Wallis tests followed by Dunn's multiple comparison test).

Chronic exposure

In the azoxystrobin experiment, we found that, regardless of exposure to pesticides, bees provided with pollen consumed more syrup than bees who did not received pollen (Kruskal-Wallis test: $p < 0.001$; Fig. 2A). However, besides the non-intoxicated bees who consumed less syrup than intoxicated bees in the *BQ* pollen groups, there was no difference in syrup consumption between pesticide treatments for a given pollen diet. In the sulfoxaflor experiment, the pollen effect on syrup consumption was only found in non-intoxicated bees: bees without pollen consumed less syrup than bees with pollen (Kruskal-Wallis test: $p < 0.001$; Fig. 2B). At the low and high concentration of sulfoxaflor, pollen diets did not affect syrup consumption. The main variation in syrup consumption was due to the high concentration of sulfoxaflor. Bees exposed to 2 ppm of sulfoxaflor consumed less syrup than bees exposed to 0 or 0.02 ppm of sulfoxaflor (although it was not significant for the BQ pollen groups).

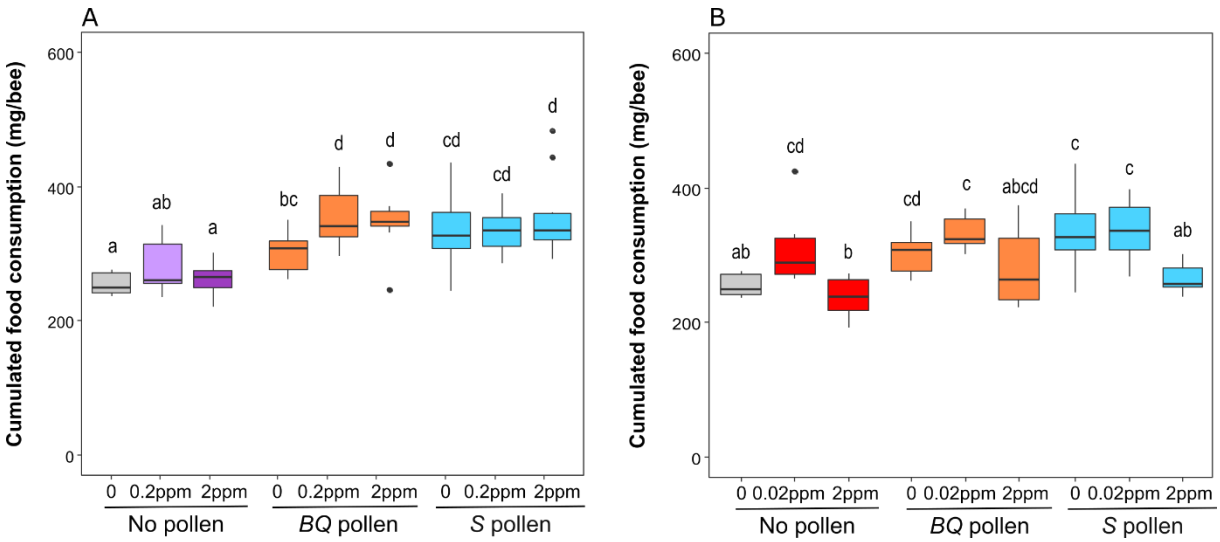

Figure 2. Individual syrup consumption according to pesticides and pollen feeding treatments. Cumulative individual consumption (mg/bee) are shown for bees exposed to azoxystrobin (A) and sulfoxaflor (B) (n = 30 bees per cage and 10 cages per experimental conditions). Boxes indicate the 1st and 3rd interquartile range with a line denoting the median. Whiskers include 90% of the individuals, beyond which circles represent outliers. Different letters indicate significant differences (Kruskal-Wallis tests followed by Dunn's multiple comparison test).

Chronic exposure to azoxystrobin (0–2 - 2ppm) did not affect bee survival whether bees consumed pollen or not (Cox model, $p = 0.17$; Fig.3A). However, both sulfoxaflor concentrations decreased bee survival (Cox model, $p < 0.001$, Fig.3B). While, in bees fed without pollen, the highest concentration of sulfoxaflor (2 ppm) caused 100% bee mortality within 16 days, the lowest concentration (0.02 ppm) reduced the survival probability by around 25%.

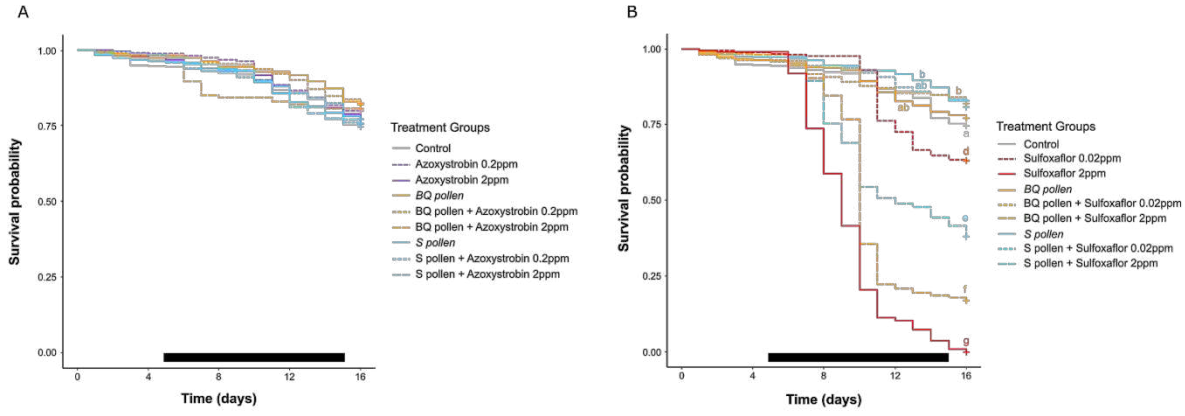

Figure 3. Chronic toxicity of environmentally-relevant concentrations of azoxystrobin (A) and sulfoxaflor (B) on bees fed with different pollen regimes. Data represent the survival probabilities of bees (n = 30 bees per cage and 10 cages per modality). Different letters indicate significant differences (Cox model) and the black bar represents the period of exposure to pesticides.

The survival probability of bees over 16 days was enhanced by the *S* pollen (no pollen, $p < 0.05$), but not by the *BQ* pollen ($p = 0.58$), although no difference in survival was found between the two pollen diets ($p = 0.14$; Fig. 3B). The survival probability of bees intoxicated with the low concentration of sulfoxaflor improved with pollen feeding (sulfoxaflor vs sulfoxaflor + *BQ* pollen: $p < 0.001$, sulfoxaflor vs sulfoxaflor + *S* pollen: $p < 0.001$), but no difference was found between the two pollen diets (sulfoxaflor + *S* pollen vs sulfoxaflor + *BQ* pollen: $p = 0.68$). As a consequence, if sulfoxaflor (0.02 ppm) increased the risk of death in bees fed without pollen (HR = 1.53), feeding bees *BQ* pollen or *S* pollen reversed this risk (*BQ* pollen: HR = 0.76; *S* pollen: HR = 1.07; Fig. 4).

Similarly, the consumption of pollen lowered the negative effect of the highest concentration of sulfoxaflor (sulfoxaflor vs sulfoxaflor + *BQ* pollen: $p < 0.001$ and sulfoxaflor vs sulfoxaflor + *S* pollen: $p < 0.001$). However, the improvement of bee survival was significantly higher when bees consumed the *S* pollen as compared to the *BQ* pollen (sulfoxaflor + *BQ* pollen vs sulfoxaflor + *S* pollen: $p < 0.001$; Fig. 3B). Overall, the consumption of *BQ* pollen and *S* pollen decreased the mortality risk by 2 and 2.5-fold, respectively (*BQ* pollen: HR = 5.72, *S* pollen: HR = 4.79) compared to bees fed without pollen (HR = 12.01; Fig. 4).

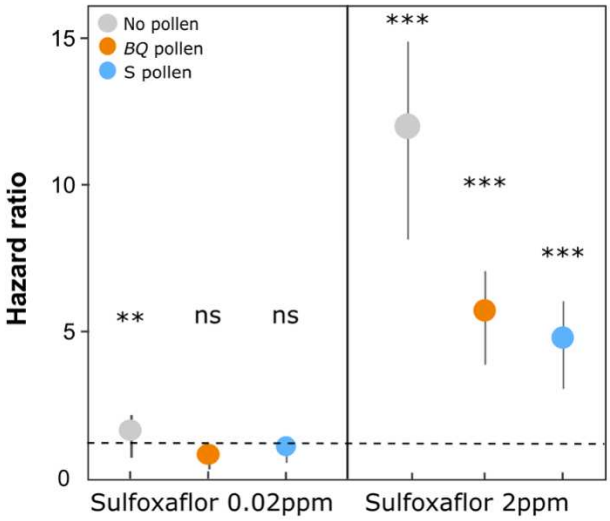

Figure 4. Hazard ratio for bees exposed to sulfoxaflor under different pollen feeding regimes. Bars indicate the 95 % confidence interval. Stars indicate statistically significant risks of death caused by the pesticide (* = $p < 0.05$; ** = $p < 0.01$ and *** = $p < 0.001$) and the dotted line represents HR=1.

2. Potential mechanisms underlying the nutritional modulation of pesticide sensitivity

Influence of pollen nutrition and pesticides on vitellogenin expression level

The expression level of vitellogenin was significantly affected by the different treatments (Kruskal-Wallis test, $p < 0.001$; Fig. 5A and B). In bees not exposed to pesticide, pollen feeding increased vitellogenin expression but the effect was significantly stronger with the *S* pollen (~ 30-fold) than with the *BQ* pollen (~ 8-fold, $p < 0.001$).

In all pollen treatments, we did not find any effect of azoxystrobin and sulfoxaflor exposure on vitellogenin expression levels (no pollen, *BQ* pollen and *S* pollen: $p = 1.0$). However, after both pesticide exposures, the level of vitellogenin remained significantly higher in bees fed with the *S* pollen as compared to bees who consumed the *BQ* pollen ($p < 0.05$).

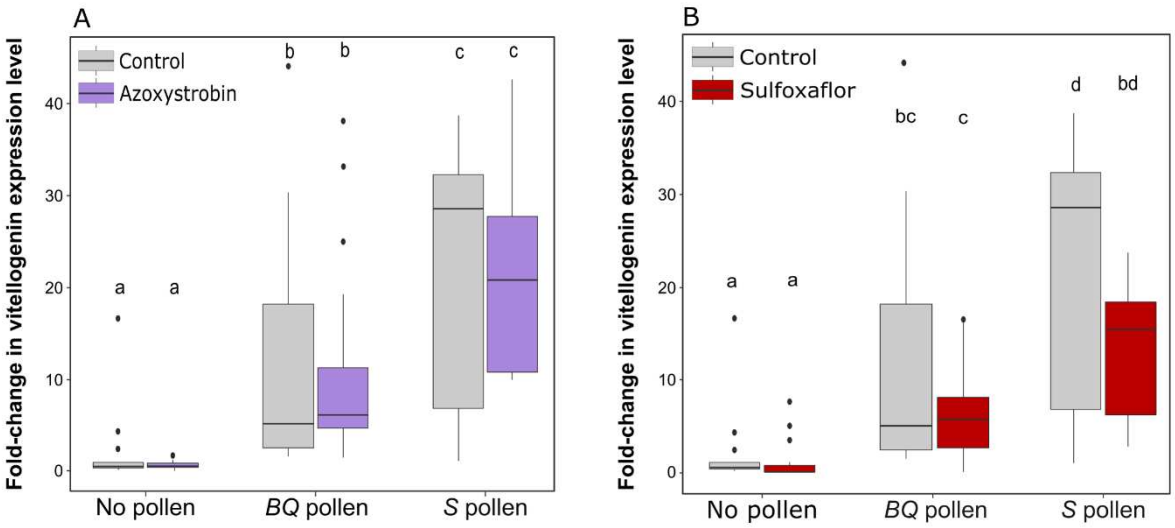

Figure 5. Gene expression levels of vitellogenin in response to pesticides and pollen feeding regimes. Vitellogenin expression levels are shown for bees exposed to azoxystrobin (2ppm) (A) and sulfoxaflor (2ppm) (B) and according to the pollen diets (n = 18-20 pools of 3 bees per experimental condition). Boxes indicate the 1st and 3rd interquartile range with a line denoting the median. Whiskers include 90% of the individuals, beyond which circles represent outliers. Different letters indicate significant differences (Kruskal-Wallis tests followed by Dunn's multiple comparison tests).

Influence of pollen nutrition on pesticide detoxification

Residues of azoxystrobin were detected at very low concentrations at 8 hr post-exposure in all treatment groups (Fig. 6A). No significant difference was observed between experimental groups (Kruskal-Wallis test, $p = 0.71$). Since azoxystrobin concentrations were close to the LOQ at 8 hr post-exposure, samples at 24 hr post-exposure were not analysed.

Regarding sulfoxaflor, the concentrations of residues found in bees 8 hr post-exposure was significantly different between experimental groups (Kruskal-Wallis test, $p < 0.01$; Fig. 6B). The maximum concentrations were found in bees fed only with sugar syrup (0.19 ± 0.02 mg/kg). Consumption of *S* pollen significantly decreased sulfoxaflor concentrations (0.13 ± 0.03 mg/kg) compared to bees who did not ingest pollen (1.5 times less, $p < 0.01$). The concentrations of sulfoxaflor in *BQ* pollen-fed bees were intermediate (0.17 ± 0.04 mg/kg) and did not differ from control ($p = 0.11$) and *S* pollen-fed bees ($p = 0.11$). For each treatment group, the concentration of sulfoxaflor significantly decreased between 8 and 24 hr post-exposure (Mann-Whitney test, $p < 0.001$ for each treatment group). It also differed between treatment groups at 24 hr post-exposure (Kruskal-Wallis test, $p < 0.001$). Sulfoxaflor concentration was more than two times lower in bees fed with the *S* or *BQ* pollen diets than in bees fed without pollen (*S* pollen: 0.04 ± 0.02 mg/kg, *BQ* pollen: 0.03 ± 0.02 mg/kg, and control: 0.10 ± 0.03 mg/kg; $p < 0.001$ for both diets). Finally, no difference in sulfoxaflor concentration was found between the two pollen diets at 24 hr post-exposure ($p = 0.63$).

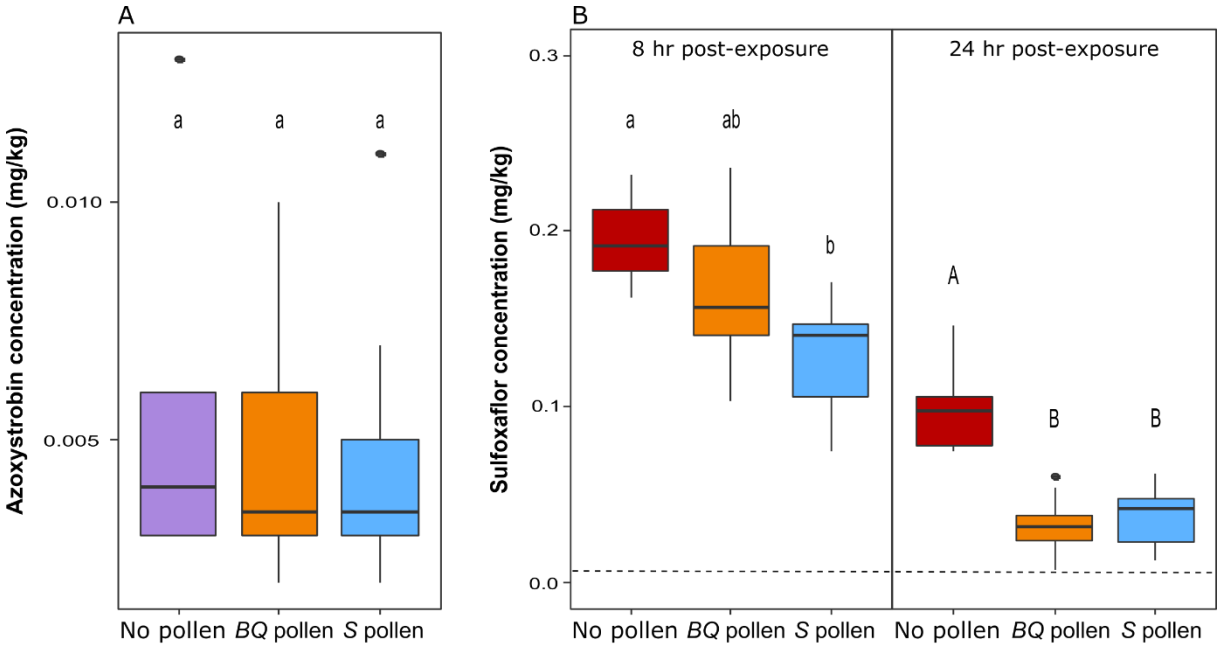

Figure 6. Concentration of (A) azoxystrobin and (B) sulfoxaflor in bees fed with different pollen regimes. Data represent the pesticide concentrations in 10 pools of 25 bees per experimental conditions. Boxes indicate the 1st and 3rd interquartile range with a line denoting the median. Whiskers include 90% of the individuals, beyond which circles represent outliers. Different letters indicate significant differences (Kruskal-Wallis tests followed by Dunn's multiple comparison tests) and the dotted line represents the limit of quantification.

Discussion

In the present study, we showed that pollen consumption, besides its well-known positive effect on honeybee longevity (Di Pasquale *et al.*, 2013; Schmidt *et al.*, 1987), can reduce the mortality risk caused by pesticides across different conditions of exposure. In addition, we found that the quality of pollen diets can substantially affect the toxicity of pesticides. These results may help to explain the variability of responses often observed at a given dose or concentration of pesticide (Poquet *et al.*, 2016).

Similar to Wahl and Ulm (Wahl and Ulm, 1983), the negative effect of an acute dose of pesticide was reduced by pollen consumption. The tested dose of azoxystrobin was found to be non-lethal (over 48 hr) to bees fed with pollen, while it slightly increased the mortality level of bees fed without pollen. Interestingly, the LD_{50} of azoxystrobin determined during preliminary assays appeared to be less toxic here, providing another example of response variability to pesticides. Experiments were performed in different years and with different colonies (bee genetics), which likely explain the different responses across LD_{50} experiments (Rinkevich *et al.*, 2015). Contrary to Wahl and Ulm (Wahl and Ulm, 1983), pollen quality did not influence the sensitivity of bees to the tested doses of pesticides. This lack of effect here might be due to the doses or the pollen diets we used. For instance, measurements should be repeated over a range of dosages to derive more general conclusions about a potential influence of pollen quality. It is also possible that the differences in our pollen diets were not strong enough to influence bee sensitivity to pesticides in the short-term (i.e. over 48 hours). Similar differences in the nutritional quality of pollens were previously found to affect the chronic susceptibility of honeybees to a parasitic infection (Di Pasquale *et al.*, 2013), suggesting that effects might rather be observed over the long-term as confirmed by our chronic exposure experiment.

In the chronic exposure experiment, pollen diets increased the consumption of sugar syrup. Such results are consistent with previous studies, which showed that in response to pollen nutrients, genes involved in carbohydrate metabolism are upregulated (Alaux *et al.*, 2011; Annoscia *et al.*, 2017). This may reflect a higher energy demand since pollen consumption stimulates tissue growth (e.g., hypopharyngeal glands and fat body) (Brodschneider and Crailsheim, 2010), which is an energetically costly process. However, this phenomenon was not observed in bees exposed to sulfoxaflor. Syrup consumption did not differ between pollen groups and thus bees provided with different pollen diets were exposed to similar amount of pesticides. Only bees exposed to the high concentration of sulfoxaflor (2 ppm) tended to consume less syrup. There is now strong evidence for preference or avoidance of sugar syrup

laced with pesticides, and this food choice was found to be dependent on pesticide concentration (Kessler *et al.*, 2015; Liao *et al.*, 2017b). Even though bees are capable of taste perception (de Brito Sanchez, 2011), the underlying mechanisms of food choice are not clearly known (Kessler *et al.*, 2015). In our experiments, bees were not provided with a food choice but it is possible that sulfoxaflor at high concentration gives syrup a bitter taste as previously found with high concentration of nicotine (Köhler *et al.*, 2012), which target nicotinic receptors like sulfoxaflor.

Survival data from the chronic exposure experiment further confirmed the beneficial effect of pollen on tolerance to pesticides: the risk of death upon exposure to the low and high sulfoxaflor concentrations disappeared or was significantly reduced by pollen feeding, respectively. This is in accordance with a previous study, which showed that bees fed over several days with a pollen-based diet exhibit reduced sensitivity to a daily exposure to chlorpyrifos (Schmehl *et al.*, 2014). Both pollen diets contained traces of DMF and tau-fluvalinate (below the LOQ), introducing possible interactive effects with the experimental pesticides (Johnson, 2015). However, this was not the case for azoxystrobin since no toxic effect were found on bee survival. Regarding sulfoxaflor, it may have increased its toxicity but to a small extent since survival upon exposure to sulfoxaflor remained lower in bees fed without pollen than in bees provided with pollen.

Interestingly, bees fed with the *S* pollen were less sensitive to the high sulfoxaflor concentration as compared to the *BQ* pollen diet. This suggests that the quality of pollen diets can also affect their capacity to tolerate chronic exposure to pesticides. The higher protective effect of *S* pollen might result from an improved physiological state. For instance, regardless of exposure to pesticides, the expression level of vitellogenin was much higher in bees fed with the *S* pollen as compared to bees provided with the *BQ* pollen. As a glycoprotein with antioxidant functions that protects bees from oxidative stress (Havukainen *et al.*, 2013; Park *et al.*, 2018), vitellogenin promotes bee longevity but may also have reduced the effects of sulfoxaflor. Indeed, a recent study found that sulfoxaflor increases the level of reactive oxygen/nitrogen species and thus significantly elevates oxidative stress in bees (Chakrabarti *et al.*, 2020). The higher vitellogenin expression level induced by the *S* pollen might have thus contributed to better protect bees from exposure to sulfoxaflor, assuming that changes in transcript levels translated into different protein levels. This latter point is supported by the significant decrease in haemolymph vitellogenin concentration upon inhibition of vitellogenin gene activity via RNA interference (Nelson *et al.*, 2007), and the concomitant change in the gene and protein expression of vitellogenin between nurses and foragers (Ament *et al.*, 2011; Fluri *et al.*, 1982).

We did not find any pesticide-induced differences in vitellogenin levels between bees, both in the presence or absence of a pollen diet. This suggests that the negative impact of pesticides on bee survival does not involve a decline in vitellogenin level, although we cannot eliminate long-term exposure effects. Reported effects of pesticides on vitellogenin in the literature have been contradictory across studies, ranging from no effects (acute exposure to fipronil and deltamethrin (Bordier *et al.*, 2017)), to increasing (chronic exposure to neonicotinoids (Christen *et al.*, 2016)) and also inhibitory effects (chronic exposure to imidacloprid (Abbo *et al.*, 2017). This indicates that effects on vitellogenin may be quite variable and possibly depend upon multiple factors, e.g. age of the bees, season, pesticide type and mode of exposure (acute, chronic).

In response to exposure to dietary toxins, organisms have developed elimination mechanisms (e.g., detoxification) that prevent their accumulation in organs and tissues. How the body is able to handle pesticides can therefore affect its pesticide sensitivity. The analysis of pesticide residues showed that azoxystrobin was eliminated much faster than sulfoxaflor (~100-fold difference between the 2 pesticide concentrations at 8 hr post-exposure), even though the same doses were given to bees. The mechanisms underlying this faster metabolization of azoxystrobin are not known, but enzymes from the detoxification pathways, like the cytochrome P450 monooxygenases, often exhibit substrate-specificity. For instance, cytochrome P450 members of the CYPQ9 family were found to be responsible for tau-fluvalinate metabolism (Mao *et al.*, 2011). Honeybees might thus possess cytochrome P450s that can dock and metabolize azoxystrobin better than sulfoxaflor. This rapid azoxystrobin metabolization might also contribute to its lower toxicity as compared to sulfoxaflor. However, since azoxystrobin is a fungicide we obviously cannot exclude that it is less efficient in reaching its biological target and/or has a different mode of action in insects.

Finally, sulfoxaflor concentration decreased more quickly in bees fed with the *S* pollen as compared to bees provided with the *BQ* pollen. This faster metabolization may participate in the reduced sulfoxaflor toxicity upon ingestion of the *S* pollen diet. Such results also confirm a recent study, which reported that some pollens are better than others in promoting pesticide metabolization (Ardalani *et al.*, 2021). Different pollens have different nutritional values, which generally translate into differences in bee physiology and longevity (Brodschneider and Crailsheim, 2010; Di Pasquale *et al.*, 2013; Omar *et al.*, 2017; Schmidt *et al.*, 1987; Standifer, 1967). Among the pollen nutrients that have positive effects on bee health, the amount of protein plays a substantial role (Brodschneider and Crailsheim, 2010; Frias *et al.*, 2016),

although it does not result systematically in healthier bees, especially regarding pathogen resistance (Di Pasquale *et al.*, 2013). Our study further indicates that the quality of pollen should not only be estimated based on protein content since the *S* pollen had a lower concentration of protein than the *BQ* pollen. For instance, the amount of other nutrients, such as amino acids, sterols, vitamins, minerals, nutrient ratio can also influence bee physiology and longevity (Corby-Harris *et al.*, 2018; de Groot, 1953; Moerman *et al.*, 2017; Stabler *et al.*, 2021; Vanderplanck *et al.*, 2018, 2014). More specifically, the macronutrients ratio in pollen may also influence the sensitivity of bees to pesticides. This was demonstrated by testing diets with modified protein to lipid ratios (P:Ls) and several pollen diets with different P:Ls (Crone and Grozinger, 2021). The pollen-induced differences in pesticide sensitivity reported in our study could not be explained by this nutritional factor since both pollen diets had similar P:Ls. However, several studies have shown that the pollen phytochemicals quercetin and p-coumaric acid, upon ingestion, can significantly enhance bee longevity (Bernklau *et al.*, 2019; Liao *et al.*, 2017a) and also tolerance to several pesticides (Liao *et al.*, 2020, 2017a; Wong *et al.*, 2018). However, the effects of these phytochemicals are concentration-dependant; lower concentrations tend to have a positive effect on honeybee longevity (p-coumaric acid at 5, 50 and 500 µM and quercetin at 12.5, 25 and 250µM), while higher natural concentrations (1000 µM) have no effects (Liao *et al.*, 2020, 2017a). Similarly, the reduced mortality risk upon exposure to pesticide was observed over a range of relatively low natural concentrations (5, 50 and 500 µM) for p-coumaric acid. Higher concentrations (1000 µM) increased or did not change the toxicity of pesticides (Liao *et al.*, 2020). Our results are therefore consistent with these data given that the *S* pollen, containing 637 µM of p-coumaric acid, was better in improving bee longevity and tolerance to sulfoxaflor when compared to the *BQ* pollen (1491.6 µM of p-coumaric acid). This former concentration might be more optimal for stimulating detoxification enzymes (Mao *et al.*, 2013) and thereby more quickly eliminating sulfoxaflor, as indicated by our results. It was not possible to quantify quercetin, but its concentration below 33.09 µM likely falls in the range of beneficial concentrations for both pollens, and therefore does not explain the differences in pollen quality.

In conclusion, our study demonstrated the modulation of pesticide toxicity by the nutritional state of worker honeybees. Pollen availability and quality, by modifying the physiological background of bees, can improve their ability to eliminate pesticides and withstand their detrimental effects (e.g., protective effect of vitellogenin against oxidative stress), as observed with the high concentration of sulfoxaflor. This nutritional modulation may cause a large range

of pesticide responses in the field, given that the abundance and composition of honeybee pollen diets can be highly variable across landscapes and seasons (Danner *et al.*, 2017; de Vere *et al.*, 2017; Elliott, 2021; Galimberti *et al.*, 2014; Kamo *et al.*, 2018; Requier *et al.*, 2015; Richardson *et al.*, 2015). A decline in resource availability and biodiversity in agro-ecosystems (Lichtenberg *et al.*, 2017) might therefore impair the bee's ability to deal with pesticides (Klaus *et al.*, 2021), giving another strong argument for the restoration of floral resource abundance and diversity in such habitats (introduction of extensive grasslands and flower strips, protection of semi-natural habitats) (Decourtye *et al.*, 2019; Scheper *et al.*, 2013). Further research is therefore needed to evaluate the influence of a larger range of pollens of different qualities on pesticide toxicity to better mitigate the impact of exposure to pesticides.

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Supplementary information

Methods for the quantification of p-coumaric acid and quercetin in pollen mixes

Each pollen mix (0.5 gr) was extracted with 5mL of EtOH in an ultrasonic bath. The extract solutions were filtered through PTFE syringe filters. Chromatographic analyses were carried out using an HPLC system (Waters Acquity UPLC) equipped with a photodiode-array detector (Waters Acquity PDA), and a reversed-phase column (Accucore C18, 100 x 2.1 mm i.d., 2.6 µm). The mobile phases were (A) 0.1% formic acid or 5 mM ammonium acetate (solvent for acid p-coumaric and quercetin respectively), and (B) acetonitrile. Injection volume was 20 µL. The flow-rate was set at 0.40 mL/min. and the temperature of the column at 30°C. Chromatographic separations were carried out using a gradient elution mode, performed as follows: 95% of (A) for 2 min, 95% of (A) to 5% in 10 min, 5% of (A) for 10 min, 5% of (A) to 95% in 0.1 min and then 95% (A) for 10 min. Spectra acquisition ranged from 190 to 790 nm and p-coumaric acid and quercetin were detected at 311 nm and 372 nm, respectively. Quantifications were calibrated using standard curves ranging from 1 to 150 mg/L, and the results were expressed as milligrams per kilograms of pollen.

Table S1: Pollen species composition of *S* pollen and *BQ* pollen blends.

Pollen blends	Pollen species	(%)
BQ pollen	*Brassicaceae*	36
	Quercus robur gr.	35
	Salix	17
	Malus/Pyrus f.	6
	Acer	2
	Carex	1
	Ranunculaceae	1
	Rhamnaceae	1
	Carpobrotus	<1
	Clematis	<1
	Juglans	<1
	Oleaceae	<1
	Poaceae	<1
	Robinia	<1
S pollen	*Salix*	89
	Malus/Pyrus.f	3
	Asteracae forma T.	2
	Quercus robur gr.	2
	Prunus f.	1
	Ranunculaceae	1
	Allophyllum	<1
	Betulaceae	<1
	Brassicaceae	<1
	Carex	<1
	Citrus	<1
	Crocus	<1
	Fragaria/Potentilla	<1
	Juglans	<1
	Liliaceae	<1
	Plantago	<1
	Populus	<1
	Quercus ilex	<1
	Robinia	<1

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C. Conclusion

Les résultats de cette étude révèlent que la nutrition pollinique et sa qualité peuvent influencer la capacité des abeilles à métaboliser les pesticides, comme nous l'avons observé pour le sulfoxaflor, ainsi que leur sensibilité à ces pesticides. La qualité de l'alimentation en pollen pourrait ainsi induire des variations dans la toxicité des pesticides et également dans les tests réglementaires d'évaluation des risques.

Cette étude fournit également un argument en faveur de la restauration des ressources florales dans les paysages agricoles afin d'accroître l'accès à une alimentation plus diversifiée en pollen et ainsi réduire la sensibilité des abeilles aux pesticides dans l'environnement. Une plus grande diversité des ressources florales pourrait également réduire l'exposition des abeilles aux pesticides à travers un butinage de ressources autres que celles des cultures (Klaus *et al.*, 2021).

CHAPITRE III :

LA SURVEILLANCE DE L'ACTIVITE DE VOL A L'ECHELLE INDIVIDUELLE ET COLONIALE COMME METRIQUE DE LA TOXICITE DES PESTICIDES

I. Revue

A. Avant-propos

Pour aborder ce troisième chapitre de thèse, j'ai rédigé une revue sur l'importance d'utiliser des mesures de performances comportementales et de reproduction pour mieux évaluer les risques liés à des expositions sublétales de pesticides. Cette revue propose un état des lieux de ces différentes mesures étudiées dans la littérature scientifique, des tests utilisés, et de leur niveau de standardisation, de simplicité et de pertinence écologique, qui sont des critères importants pour les tests de toxicité réglementaires.

Cette revue a été publiée dans *Chemosphere* (Barascou *et al.*, 2021a).

B. Article 3

Pesticide risk assessment in honeybees: toward the use of behavioral and reproductive performances as assessment endpoints

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Highlights

- There is a gap between new evidence of pesticide toxicity in honeybees and regulatory toxicological bioassays
- Current endpoints should be complemented with behavioral and reproductive endpoints
- We review such endpoints and discuss their possible use in pesticide risk assessment
- There is a need to translate toxicological research studies into regulatory test methods

Abstract

The growing gap between new evidence of pesticide toxicity in honeybees and conventional toxicological assays recommended by regulatory test guidelines emphasizes the need to complement current lethal endpoints with sublethal endpoints. In this context, behavioral and reproductive performances have received growing interest since the 2000s, likely due to their ecological relevance and/or the emergence of new technologies. We review the biological interests and methodological measurements of these predominantly studied endpoints and discuss their possible use in the pesticide risk assessment procedure based on their standardization level, simplicity and ecological relevance. It appears that homing flights and reproduction have great potential for pesticide risk assessment, mainly due to their ecological relevance. If exploratory research studies in ecotoxicology have paved the way toward a better understanding of pesticide toxicity in honeybees, the next objective will then be to translate the most relevant behavioral and reproductive endpoints into regulatory test methods. This will require more comparative studies and improving their ecological relevance. This latter goal may be facilitated by the use of population dynamics models for scaling up the consequences of adverse behavioral and reproductive effects from individuals to colonies.

Keywords: *Apis mellifera*; toxicology; hazard assessment; sublethal endpoints; regulatory test methods

1. Introduction

Plant protection products, also referred to as phytopharmaceutical products or pesticides, consist of active substances (with safeners or synergists) intended to protect plants against harmful organisms (*e.g.* insects, fungi) or prevent the growth of undesired plants (herbicides). Pesticides must be evaluated and approved by regulatory authorities before registration. Besides providing data on the physical and chemical properties, the mode of action and the efficacy, pesticide registration applications must include data on the product's fate in the environment, as well as its toxicity for humans and non-target organisms. Regulatory authorities can then provide an assessment of the risk presented by the pesticide and determine to what extent it is safe for human and environmental health.

As part of the overall risk assessment procedure, OECD (Organization for Economic Co-operation and Development) and EPPO (European and Mediterranean Plant Protection Organization) test guidelines require toxicological data on honeybees (*Apis mellifera*). Until very recently, honeybees were the only insect pollinator used for pesticide risk assessment and were considered a surrogate species for non-*Apis* bees (EPPO, 2010a, 2010b). Likely because they are relatively easy to rear, their biology has been well-studied, and they are one of the most important pollinators worldwide (Ghazoul, 2005; Klein *et al.*, 2007). Honey bees forage within large areas around their hives and are thus likely to be exposed to several pesticides either directly (collecting nectar, pollen and water) or indirectly (sharing food with nestmates: larvae and adult worker bees, queen and drones).

Over the last decades, progress in research has been made in evaluating the risk that pesticides pose to honeybees by assessing the toxicological effects. Extensive empirical data have therefore accumulated on pesticide effects, which have been compiled in several reviews (see for instance Alkassab and Kirchner, 2017; Belzunces *et al.*, 2012; Blacquière *et al.*, 2012; Cullen *et al.*, 2019; Desneux *et al.*, 2007; Johnson, 2015; Poquet *et al.*, 2016). Toxic effects vary depending on the dose/concentration, modes of action and exposure routes. More importantly, a new range of endpoints (biological parameters used to evaluate the toxicity of a chemical) have been used to identify sublethal effects (*e.g.* impairment of cognitive capacities, foraging activity, communication, and reproductive performances) (Decourtye *et al.*, 2003; Kairo *et al.*, 2017b; Karahan *et al.*, 2015; Zhang *et al.*, 2020b). In addition, small doses or concentrations that were believed to be sublethal in laboratory bioassays proved to be lethal in field conditions (*e.g.* Henry et al 2012).

These recent findings raise concerns about risk assessment procedures for pesticide registration, which mainly focus on lethal effects. First, while sublethal effects do not directly cause the death of individual bees, they may impair individual and colony performance, as well as their pollination services (Meikle *et al.*, 2016; Prado *et al.*, 2019; Tison *et al.*, 2017; Woodcock *et al.*, 2017). Second, exposures to pesticides, previously identified as nonlethal in lab assays, caused high mortality in natural conditions (Henry et al 2012). Therefore, current test procedures do not appear sufficient to protect honey bees. This growing gap between new evidence of pesticide toxicity (*e.g.* sublethal effects) in honeybees and the conservative toxicological bioassays needed for pesticide approval contribute to the controversy between stakeholders, policymakers, environmentalists and scientists (Durant, 2020; Sgolastra *et al.*, 2020; Storck *et al.*, 2017; Thompson and Maus, 2007). In order to fill this gap and better assess the threat of pesticides to honeybees, we emphasize the need to complement current endpoints (essentially based on LD_{50} - dose at which 50% of the individuals die) with sublethal endpoints. We focus on behavioral and reproductive endpoints, which have received increasing interest due to their ecological relevance and the technical advances made in their recording. Furthermore, pesticides can have low-dose effects on these endpoints.

We review such endpoints and discuss which ones could be considered for pesticide risk assessments based on several criteria, such as the possibility of standardization, their monitoring simplicity and their ecological relevance. Finally, we highlight the future challenges that need to be addressed to better integrate and use these endpoints in pesticide risk assessment.

2. The rise of behavioral and reproductive endpoints in response to the risks associated with low exposure to pesticide

In the current regulatory framework for honeybees, the effects of pesticides are assessed by standard regulatory tests, in a tiered approach (EFSA, 2013; EPPO, 2010a, 2010b). At a low tier, laboratory tests on active substances or formulated products are used on individual bees that are representative of different life stages (larval and adult). This is the first mandatory step that includes an acute toxicity test after oral or contact exposure in adults (OECD, 1998b, 1998a) and larvae (OECD, 2013), and a chronic toxicity test on adults over 10 days (OECD, 2017). For acute toxicity tests, the LD_{50} is determined. Then, if a risk is identified as a result of this first step, supplementary tests are required at a higher tier (semi-field and field tests). For instance, several key parameters of honeybee colony state may be measured, besides bee mortality, like foraging activity and honey and brood production (EPPO 2010a, 2010b). However, particular attention should be paid to sublethal effects to better identify risk, and

avoid situations where a pesticide might not undergo tests at a higher tier because of its supposedly low toxicity under current laboratory tests. We therefore need individual-level endpoints that are more reliable and relevant.

The primary endpoint of laboratory tests is the LD_{50}, which suggests that the dose-response for a given substance follows either a proportional or a threshold dose-response relationship. However, there are several lines of evidence for multiphasic relationships. For instance, Suchail *et al.* (2001) found that mortality rates in response to exposure to imidacloprid (neonicotinoid insecticide) increased for low doses, decreased for medium doses, and then increased in a dose-dependent manner for higher doses. This suggests that significant effects can occur at doses lower than the ones used in mortality bioassays (LD_{50} tests) or under the NOEL (No Observed Effect Level). Such effects may occur in environments contaminated by low doses of pesticide residues and are usually sublethal with adverse effects on development (Dai *et al.*, 2010), metabolism (Decourtye *et al.*, 2004a), immunity (Aufauvre *et al.*, 2014), behavior (Yang *et al.*, 2008), and reproduction (Kairo *et al.*, 2017a, 2016). This may be translated into potential risk to colonies. For instance, the immunosuppression caused by clothianidin has been shown to promote the replication of the deformed wing virus (Di Prisco *et al.*, 2013), a virus that has been frequently linked to colony losses (Grozinger and Flenniken, 2019).

Additionally, comparing effects observed in laboratory tests to field observations raised concerns about the underestimation of pesticide toxicity at sublethal doses, as illustrated by studies of aquatic populations of macroinvertebrates (Rasmussen *et al.*, 2012; Schäfer *et al.*, 2012) (*e.g.* for *Daphnia magna*, the level of exposure to pesticides was found to be 10 to 100 times more harmful than that predicted by the EU's "first tier" risk assessment). In honeybees, these concerns were verified in field tests by recording the homing success of bees exposed to a dose of thiamethoxam assumed to be sublethal based on regulatory tests (nearly four times lower than the LD_{50}). This sublethal dose caused significant failure in homing flight which could potentially decrease colony survival (Henry *et al.*, 2015, 2012). This discrepancy between the laboratory and the field could be explained by the differences between the safe, non-challenging conditions of the laboratory assays and the environmentally challenging conditions of the field.

The underestimation of risks related to low doses and the striking mismatch between laboratory and field tests highlight the need to complement the current regulatory evaluation of pesticides, essential for performing a preliminary screening of toxic effects, with sublethal and more ecologically-relevant tests. In this context, the measurement of behavioral and reproductive performances in ecotoxicity tests has become increasingly popular. Notably, a keen interest in

behavioral effects has recently been tracked in the US EPA ECOTOX database with records of 17 324 and 13 809 measurements for aquatic and terrestrial organisms, respectively (Ågerstrand *et al.*, 2020). As explained by Ågerstrand *et al.* (2020), the relevance of behavioral endpoints is driven by several advantages, which we believe are also valid for reproductive endpoints. They improve the ecological relevance of pesticide risk assessment because they add ecological realism to the test conditions and help connect organismal response to populations, which are generally the ultimate protection goal (Rudén *et al.*, 2017). These individual-level endpoints therefore should help predict effects on higher levels of biological organization (*i.e.* colonies for honeybees). In addition, pesticides can have low-dose effects on these endpoints, which can thus provide a more effective assessment of risks associated with low contamination levels of the environment as compared to the current mortality endpoints. Finally, regarding behavioral measurements, the emergence of new tracking technologies has increased the rate of acquisition and amount of *in situ* monitoring data (Kays *et al.*, 2015). More importantly, such technology enables us to improve the robustness and precision of measurements and thus decrease the variability of ecotoxicological data, which can be relatively high when obtained through basic observations. The improved reproducibility of toxicity tests is notably essential for the regulatory assessments of pesticides.

3. Overview of behavioral and reproductive performances as assessment endpoints in honeybees

The investigation of pesticide effects at sublethal doses or concentrations in honeybees began in earnest in the 2000s (Fig. 1A). Since then, behavioral and reproductive endpoints have been more frequently included in toxicological research studies. The goal here is not to provide a systematic review but rather an overview of the main individual-based behavioral and reproductive endpoints by describing *(i)* their biological interests, *(ii)* the measurement methodologies and *(iii)* the main observed effects. For that purpose, we focused on the most studied endpoints (used in more than five research studies) that were grouped into the seven following categories: grooming, locomotion, learning and memory, foraging, homing flight, waggle dance and reproduction (Fig. 1B).

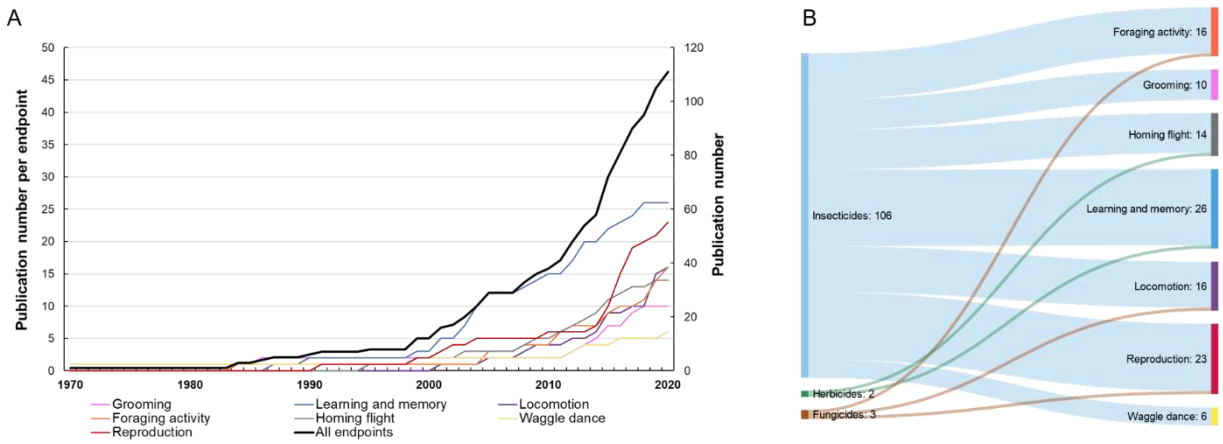

Figure 1. Bibliometric of honeybee toxicological research studies using individual-based behavioral and reproductive endpoints. (A) Cumulative number of publications per year on behavioral and reproductive endpoints. The Y-axis on the left refers to the number of publications per endpoints (colored lines) and the Y-axis on the right to total number of publications (thick black line). The X-axis refers to the years of publication. (B) Breakdown of pesticide types (left) and endpoints (right). The thickness of each line connecting a pesticide type to a given endpoint is proportional to the number of studies.

3.1. Grooming

The ability of worker bees to clean themselves (self-grooming) and their nestmates (allo-grooming) contributes to the prevention of the spread and multiplication of parasites and pathogens within the colony and is therefore related to colony health (Evans and Spivak, 2010). Bees notably groom themselves or another bee in order to remove parasites from their body with their legs and mandibles.

Since grooming mainly involves motor functions, neurotoxic pesticides have been hypothesized to alter the performance of these behaviors. Grooming behavior has traditionally been evaluated by marking and tracking bees in observation hives or petri dishes. Only two studies investigated the influence of pesticides on allo-grooming; both reported a lack of effect after exposure to imidacloprid, bioinsecticides (essential oil and geraniol; Santos *et al.*, 2018), thiacloprid and tau-fluvalinate (Retschnig *et al.*, 2015). Conversely, self-grooming performance appeared more sensitive to intoxication. While de Mattos *et al.* (2017) found a reduction in the duration of self-grooming after exposure to acaricides (coumaphos, amitraz and tau-fluvalinate), bees intoxicated with pesticides, such as neonicotinoids (imidacloprid, thiamethoxam, clothianidin, dinotefuran) (Williamson *et al.*, 2014), acetylcholinesterase inhibitors (Williamson *et al.*, 2013), permethrin (Cox and Wilson, 1984) and oxalic acid (Schneider *et al.*, 2012), showed an increased propensity to perform self-grooming. However, two toxicological studies found no effect of pyrethroids (including tau-fluvalinate and permethrin) (Oliver *et al.*, 2015), imidacloprid or bioinsecticides on self-grooming (Santos *et al.*, 2018).

3.2. Locomotion

Insecticides, such as pyrethroids and neonicotinoids, which target the voltage-sensitive sodium channels or nicotinic acetylcholine receptors, respectively, may directly impair neuronal transmission, muscle activation (Hirata, 2016), and therefore locomotor activity (walking, flying). Moreover, some pesticides, like fungicides, may reduce energy production (Degrandi-Hoffman *et al.*, 2015) and therefore affect locomotion, especially flight capacity, as flying is one of the most intense and energy-demanding physiological processes in insects (Dudley, 2000).

Walking locomotion experiments typically monitor the walking pattern of individual bees in standardized open field arenas and may reveal in-hive worker impairments during ongoing tasks in the colonies. Movement pattern is most often monitored by means of automated video tracking systems coupled with an ad-hoc image processing software that detects on a continuous basis the position of bees. Locomotion activity is typically indicated by the total covered distance or conversely by the length of time of immobility (Hesselbach and Scheiner, 2018), also termed prostration (Kadala *et al.*, 2019) or lethargy (Williams *et al.*, 2020), during a fixed period (typically between 3 to 15 min), and more recently, by circadian rhythms (Delkash-Roudsari *et al.*, 2020). More subtle metrics may document abnormal trembling or convulsive movements of legs and wings (Aliouane *et al.*, 2009), as well as the loss of the righting reflex (Oliver *et al.*, 2015). Experiments may be either conducted in complete darkness using infrared recording systems (Teeters *et al.*, 2012), or in the presence of a nearby light stimuli to explore the effect of exposure to pesticides on phototaxis (Charreton *et al.*, 2015; El Hassani *et al.*, 2008, 2005; Tosi and Nieh, 2017). Additionally, when placed vertically, the arena experiment may reveal a potential interaction with geotaxis (El Hassani *et al.*, 2008, 2005). Finally, more advanced versions of the experiments may introduce food or conspecifics into the arena to further implement food attraction or social interactions as a part of the walking pattern (Ingram *et al.*, 2015; Teeters *et al.*, 2012).

Flying locomotion is more difficult to document. It requires more sophisticated apparatus like flight mills, whereby the tested bees are tethered to a balanced arm and allowed to fly circularly around an axis (Tong *et al.*, 2019; Tosi *et al.*, 2017a). Alternatively, bees may be harnessed to a fixed support and exposed to air flow and optic flow stimuli in order to elicit flight (Liao *et al.*, 2019). Flight experiments typically document flight duration until exhaustion, total covered distance, as well as average flight velocity.

Most of the studies reported significant locomotion deficits in bees exposed to pesticides, particularly pyrethroids and neonicotinoids. Walking experiments typically reported increased

immobility (Aliouane *et al.*, 2009; El Hassani *et al.*, 2005), righting difficulties (Williamson *et al.*, 2014) and shorter walking distances (Charreton *et al.*, 2015; Ingram *et al.*, 2015). However, common patterns are difficult to identify across studies due to the broad variety of experimental designs and peculiarities related with bee age (Charreton *et al.*, 2015), topical vs. oral exposure (El Hassani *et al.*, 2008, 2005), exposure duration (Williams *et al.*, 2020) or timescale after exposure (Kadala *et al.*, 2019). Similarly, if a reduction in flying capacities was generally reported by experiments with flight mills (Liao *et al.*, 2019; Tong *et al.*, 2019; Tosi *et al.*, 2017a), depending on the type of exposure, opposite effects could also be induced on specific flight traits (Tong *et al.*, 2019).

3.3. Learning and memory

One commonality of nearly all studies investigating pesticide effects on learning and memory in honeybees is the use of a Pavlovian conditioning protocol, the olfactory conditioning of the "Proboscis Extension Response" (PER). Other paradigms for testing learning and memory (*e.g.*, habituation, visual learning, aversive learning) are available, but have been less used in toxicological studies (Armengaud *et al.*, 2002; Guez *et al.*, 2001; Lambin *et al.*, 2001; Muth and Leonard, 2019; Urlacher *et al.*, 2016). The olfactory PER conditioning protocol is notably useful for excluding confounding variables and experimental noise in learning assays. It enables one to reproduce, under laboratory conditions, the conditioning process occurring on the flower (Menzel and Müller, 1996). Whereas bees initially exhibit PER (unconditioned response) to antennal contact with sucrose (reward), they learn to initiate PER in response to an odor alone (conditioned response) when this contact was previously paired with this odor (Giurfa and Sandoz, 2012). Although Muth and Leonard (2019) proposed an original method of PER conditioning in free-flying bees, PER experiments are commonly carried out on restrained individuals in the laboratory. The PER protocol has proven very useful in addressing many aspects of bee learning, such as extinction learning, and stimulus generalization and discrimination (Giurfa and Sandoz, 2012).

Multiple authors have investigated the effects of pesticides with the PER assay with varying parameters (*e.g.* pesticide concentration, honeybee age, season, subspecies, and trial number, inter-trial interval and performance measures). Underlying these toxicological studies is that sublethal doses of neurotoxic pesticide affect the learning and memory of bees (Taylor *et al.*, 1987), thereby reducing individual foraging efficiency. This explains why, besides some studies on fungicide or insect-growth regulators, PER assays were mostly used to test insecticides with neurotoxic activity, (Abramson *et al.*, 2004; Decourtye *et al.*, 2004b). As of now, nearly thirty

pesticides have been tested with this bioassay; the majority of studies being performed with the active ingredient (rarely the 'ready to use' formulation of the farmer, except for (Abramson *et al.*, 2012; Tison *et al.*, 2017b)). By building on this strong background, Siviter *et al.* (2018) conducted a meta-analysis to quantify pesticide effects on learning and memory. They showed that both neonicotinoid and non-neonicotinoid pesticides cause significant decreases in learning and memory capacity at field-realistic exposure levels and under both chronic and acute exposure. The impacts at the adult stage of larval exposure remain relatively unexplored (Papach *et al.*, 2017; Yang *et al.*, 2012). However, Siviter *et al.* (2018) suggested that learning performances of bees could be more sensitive to pesticides when exposed during larval development.

3.4. Foraging activity

Foraging consists of repeated visits to different flowers for collecting pollen and nectar in an average radius of 1.5 to 5 km (up to 10 km if the floral resources are scarce) (Beekman and Ratnieks, 2000; Steffan-Dewenter and Kuhn, 2003). Typically performed by older bees foraging involves many complex cognitive functions such as learning (*e.g.* visual and olfactory stimuli of flowers, landscape characteristics), orientation and navigation (Dyer and Gould, 1981; Menzel, 1999), and is also energetically costly. Because foragers are usually the first bees to encounter pesticides while visiting flowers and can bring back pesticide-contaminated pollen and nectar to the hive, foraging is considered as the starting point of colony intoxication. For these reasons (frequent risk of exposure, behaviorally challenging tasks), the influence of pesticides on foraging has long been investigated.

To be able to record individual-based foraging activity, experiments are generally performed in a flight tunnel and with a feeder on which bees can forage. The frequency of visits to a feeder by forager bees (marked with a color dot on the thorax), as well as the time period between visits, can then be determined. Exposure to pesticides can be carried out in different ways, either by providing colonies with a pesticide-contaminated sugar solution (Ramirez-Romero *et al.*, 2005; Yang *et al.*, 2008) or individually by submitting the bees to an acute treatment (topical or oral route) (Guez *et al.*, 2005; Karahan *et al.*, 2015). A decrease in visit frequency and an increase in time period between visits were generally observed upon exposure to pesticides (Karahan *et al.*, 2015; Ramirez-Romero *et al.*, 2005; Yang *et al.*, 2008). However, depending on the dose, opposite effects could be observed. For instance, doses of 10 and 50 ng of methyl parathion induced a decrease and an increase in the frequency of visits to the feeder, respectively (Guez *et al.*, 2005). Only recently, the emergence of new technologies (Radio

Frequency Identification – RFID, optic bee counters) has enabled more detailed analysis of foraging activity and a move from non-challenging foraging activity in a flight tunnel, under controlled conditions, to field foraging activity (Decourtye *et al.*, 2011; Streit *et al.*, 2003). By recording, on a continuous basis, the activity of individual bees at the hive entrance (time of each exit and entrance of tagged bees), these technologies allow access to different individual foraging traits, such as the number and duration of trips (daily and total) or the age at the onset of foraging. Apart from an early study, which demonstrated a short-term reduction in foraging activity and longer foraging trips between the hive and a feeder after exposure to clothianidin and imidacloprid (Schneider *et al.*, 2012), no clear effect was observed on the daily number and duration of trips in the field when bees were exposed, to field-realistic doses of insecticides (Colin *et al.*, 2019b; Hesselbach *et al.*, 2020). However, a significant premature onset of foraging was often reported (Colin *et al.*, 2019b; Hesselbach *et al.*, 2020; Shi *et al.*, 2020). Precocious foraging was then accompanied by a reduction in lifespan and total foraging activity. The onset of foraging was delayed in bees exposed to mixtures of pesticides at very low doses (from 100 to 10 000 times lower than their LD_{50} values) (Prado *et al.*, 2019); such bees exhibited a lowered daily foraging activity. Scaling up to a higher tier of study, Henry *et al.*, (2015) investigated the foraging activity and mortality of bees originating from colonies placed near fields of rape oilseed treated with thiamethoxam. Although no modification in foraging traits was reported, an increased mortality rate, likely due to failure in homing flights, was observed. Finally, foraging efficiency was analyzed by Prado *et al.* (2019) by measuring the amount of nectar and pollen collected by bees (exposed to pesticide at emergence): no effect of exposure to pesticides was found on nectar foraging but individual foragers returned to the colonies with a lower amount of pollen. Morfin *et al.* (2019) also observed the impairment of pollen foraging in bees exposed to clothianidin at the larval stage. This higher sensitivity of pollen-foraging bees, as opposed to nectar foraging, has also previously been reported for bumblebees following exposure to pesticides (Feltham *et al.*, 2014; Gill *et al.*, 2012), and therefore seems to be a hallmark of stressed bees (Bordier *et al.*, 2018; Lach *et al.*, 2015).

3.5. Homing flight

Over the last thirty years, sublethal doses of pesticides have been suspected to affect orientation and homing flight of foragers. This concern originated with the depopulation of hives observed by beekeepers near fields sowed with seed-dressing-treated crops. Orientation performance of foragers exposed to low doses of an insecticide were initially investigated using a complex maze device (Decourtye *et al.*, 2009). This device relies on associative learning between a

visual mark to navigate in the maze and a reward of sugar solution (Zhang *et al.*, 1999). Indeed, a bee exposed to a pesticide during foraging trips may incorrectly learn and/or memorize visual patterns for navigation, causing disorientation and bee loss due to homing failure (Desneux *et al.*, 2007). These experiments are based on the use of limited cues whereas navigation in natural conditions relies on a combination of several guidance mechanisms and cognitive functions (see above). More realistic experimental situations were designed and showed an impact of insecticides on the homing performances of foragers by recording the flight activity between a feeder and the colony located 8 m away in semi-field conditions (Vandame *et al.*, 1995) or 500 m away in field conditions (Bortolotti *et al.*, 2003).

More recently, Matsumoto (2013) showed an increase in the proportion of homing failure of foragers, released 500 m away from the colony, when exposed to sublethal doses of neonicotinoid or pyrethroid insecticides. However, the number of individuals monitored simultaneously and the time span of the observations may present experimental difficulties in these studies using marked bees (paint or colored, numbered tags) (Decourtye *et al.*, 2011; Pahl *et al.*, 2011). The emergence of automatic tracking technology helped to solve these problems and study the effects of pesticides on free-ranging foragers in field conditions. Using RFID technology with continuous recording (48 hours), Henry *et al.* (2012) showed that foragers released 1 km away from their colony returned to the hive at a significantly lower rate when exposed to a sublethal dose of thiamethoxam. Such effects were confirmed with colonies placed near rape oilseed crop originating from seed-dressing treated with Cruiser®-containing thiamethoxam (Henry *et al.*, 2015). Foragers originating from these colonies and tracked with RFID microchips disappeared at a faster rate with increasing field exposure (*e.g.* increased mortality rate of 10% due to non-returning bees per 15 ha of treated crop in a radius of 1 km around the colony) and this excess mortality increased over time. The homing flight failure might be explained by impairments to flight capacity (*e.g.* motor functions) (Tosi *et al.*, 2017a) and cognitive functions, such as the retrieval of previously learned information (*e.g.* visual cues) (Fischer *et al.*, 2014; Tison *et al.*, 2016). These assumptions are further supported by the fact that negative effects on homing performances were more pronounced at lower temperatures (below 28°C) and in environments with a greater density of landmarks (*e.g.* hedges, forest borders) (Henry *et al.*, 2014). Finally, the health status of colonies and especially varroa infestation levels may also impact the homing performances of foragers. Experimental data and predictive modelling show a three times greater effect of thiamethoxam on foragers originating from colonies infested by 5 varroa mites per 100 bees as compared to foragers from varroa-free colonies (Monchanin *et al.*, 2019).

3.6. Waggle dance

When returning from a food rich area, successful foragers share information about its distance and direction through body movements to recruit naive bees (Dyer, 2002). The dancing bee notably performs repeated waggle dances (loops with a shape of a '8') if the food source is located at more than 50 meters away. In the waggle dance, composed of a waggle and return phase, the axis of the '8' represents the direction of the food source relative to the sun position, and the number of waggle runs inform receivers of its distance. Waggle dance performance therefore requires detecting and integrating into the central nervous system several environmental parameters linked to spatial information, which are then encoded into body movements. Pesticides, and particularly insecticides, that target the nervous system, would have an expected impact on forager ability to communicate food location.

To decipher potential effects of pesticides, several components of the waggle dance can be measured, such as the number of loops, the angle of the dance, the duration of the waggle run and the frequency of the vibrations produced by the bees (Dyer, 2002). The simplest methods for studying the waggle dance are visual observation or video recording of a colony in an observation hive. Using such techniques, a precursor study by Schricker and Stephen (1970) reported a pesticide effect on the waggle dance, with changes in the dance angle in a stepwise fashion (instead of linearly) in bees exposed to parathion. Then, three studies, investigating the toxic effect of imidacloprid on the waggle dance, found a reduced number of dance circuits (Eiri and Nieh, 2012) and decreased precision of directional information (*i.e.* larger variance in the angle of the waggle phase) (Kirchner, 1999; Zhang *et al.*, 2020a). A similar impairment of dance communication was found upon exposure to deltamethrin (Zhang *et al.*, 2020b). A more automatic recording of waggle dance, based on the measurements of electrical fields, was developed by Greggers *et al.* (2013). They found that dancing bees emit low- (movements of the abdomen, 16Hz) and high-frequency (buzzing of the wings, 230 Hz) electrical fields, which allowed them to determine the number of waggle runs (Greggers *et al.*, 2013). Using this technique, Tison *et al.* (2016) found a significant reduction in the number of waggle dances per hour in bees intoxicated by the neonicotinoid thiacloprid as compared to control bees.

3.7. Queen and drone fertility

As early as the 1960s, it was shown that exposure to chemicals can directly alter sexual behavior, mating success, sex ratio and fertility, notably through a decrease in spermatozoa viability and number. The pioneering study of Carson found that the organochlorine insecticide DDT (Dichlorodiphenyltrichloroethane) causes reproductive injuries in birds, leading to a

decline in their populations (Carson, 1962). Evidence for pesticide-induced reproductive toxicity in honeybees emerged 40 years later and was especially intriguing, given that the reproductive castes (queen and drones) are generally exposed to much lower concentrations of pesticides than worker bees. The queen and drones normally feed on food processed and supplied by workers, which can lead to exposure to concentrations below the limit of detection of analytical methods (Kairo *et al.*, 2016) that represents between 0.001 and 0.016 % of the original pesticide concentration found in the diet consumed by workers bees (Böhme *et al.*, 2018). However, they may be exposed during their development to higher levels of pesticides contained in the wax (Chauzat and Faucon, 2007; Mullin *et al.*, 2010).

An impairment of queen fertility could translate into either a temporary or permanent absence of egg laying, or the laying of unfertilized eggs (drones), leading to poor colony development or ultimately colony failure (Büchler *et al.*, 2013; Pettis *et al.*, 1991; Rangel *et al.*, 2013; vanEngelsdorp *et al.*, 2013). Queen fertility after pesticide exposure has been investigated at both the colony and individual level. Colony-level examination usually consists of spiking the colony food supplement with pesticide and then analyzing queen egg-laying rate, brood area, hive population and food stocks (Colin *et al.*, 2019a; Meikle *et al.*, 2016; Odemer and Rosenkranz, 2020; Woodcock *et al.*, 2017). Using this procedure, colonies that were chronically exposed to the neonicotinoids, imidacloprid, thiamethoxam and clothianidin, demonstrated a significant decrease in queen egg-laying followed by reduced brood production (Sandrock *et al.*, 2014; Wu-Smart and Spivak, 2016). Egg-laying reduction was notably associated with lower queen mobility (Wu-Smart and Spivak, 2016). Finally, colony foraging on rapeseed grown from seeds coated with thiamethoxam was followed by an increase in drone brood production (Henry *et al.*, 2015). Such an effect could reflect either a higher investment of colonies in reproduction or an impairment of egg fertilization, the latter suggesting damage to spermatozoa in queens. These impairments caused by pesticides did not trigger colony collapse, even if a greater number of supersedures were reported (Bendahou *et al.*, 1999; Sandrock *et al.*, 2014). A major drawback of colony-level experiments is separating the direct and indirect effects of the pesticide on the observed reduction in egg-laying. The pesticide may be acting directly on the queen or may be decreasing worker foraging performance, resulting in reduced colony resources essential to brood production.

An alternative or complementary approach is to determine the effects of pesticides directly on queen physiology and fertility (body weight, the structure and the development of ovaries, the quantity and viability of spermatozoa stored in queen spermatheca). Negative impacts on ovary weight and fertility impairments were observed in young queens exposed during their

development to coumaphos, fluvalinate and neonicotinoids via contaminated food (Haarmann *et al.*, 2002; Pettis *et al.*, 2004; Williams *et al.*, 2015). Exposure to pesticides at the larval stage through contaminated wax also resulted in reduced queen egg-laying rate and attractiveness to workers (Walsh *et al.*, 2020).

Regarding drones, the count and the viability rate of spermatozoa are two major biological parameters of fertility from which the quantity of spermatozoa available for queen fertilization can be deduced. Sperm count is determined using a microscopic cell count chambers or by flux cytometry analyses. The reproductive toxicity of pesticides in drones has also been investigated during the larval and adult stage. By supplying colonies with food contaminated with neonicotinoids during drone development (Ciereszko *et al.*, 2017; Straub *et al.*, 2016) or exposing drones during their development to beewax previously sprayed with miticides or agrochemicals (Fisher and Rangel, 2018), a consistent decrease in sperm viability and motility was found. To determine the reproductive toxicity of environmental concentration of the insecticide fipronil on adult drones, experiments were performed in semi-field conditions under insect-proof tunnels or in laboratory conditions. In semi- fields conditions, colonies foraged on a pesticide-contaminated feeder, whereas in laboratory conditions, drones were reared with worker bees in cages provided with pesticide-contaminated pollen. For all exposure methods, fipronil did not affect drone survival, maturity rates (number of drones that provided sperm after stimulation) and semen volumes, but significantly altered drone fertility by lowering spermatozoa concentration and viability, which reduced the amount of living sperm available for reproduction (Kairo *et al.*, 2017b, 2016).

4. Relevance of behavioral and reproductive performance as assessment endpoints

The goal of this section is to provide general insights into the reliability and relevance of the different behavioral and reproductive endpoints in regards to the risk assessment procedure, *i.e.* to determine to what extent each of them is appropriate for chemical regulations. For that purpose, we primarily relied on criteria cited for test method validation in a regulatory hazard assessment context: simplicity, sensitivity, robustness, reproducibility (OECD, 2005). Simplicity implies that the endpoint method is relatively easy, and time- and cost-effective, for use in a regulatory context. Sensitivity indicates whether the method used to assess the endpoint can accurately detect dose-dependent effects. Robustness refers to the stability of responses to minor variations in experimental procedures and reproducibility to the ability to duplicate the results across laboratories/studies. We grouped both robustness and reproducibility criteria into one: the standardization level. The more toxicological studies have used a given method, the

better we can evaluate its sensitivity and standardization level. Finally, we discussed the ecological relevance of endpoints, which indicates whether effects measured in individual bees can be related to consequences for colonies in field conditions.

4.1. Test endpoints with low standardization level and uncertain ecological relevance: grooming, waggle dance

The observation of bee behaviors has traditionally been conducted either directly by the observer or by means of video recording. If both methods are relatively easy to perform and do not require expensive materials, complexity in behavioral recording can arise from the targeted behavior itself. For instance, some behaviors may occur at a relatively low rate, making observations quite time-consuming to obtain enough sampling events, while others might be quite complex and require trained observers to record the full behavioral pattern. The waggle dance falls into both categories, which might explain why almost all toxicological studies focusing on the waggle dance tested only one dose or concentration. It is therefore currently not possible to determine whether pesticides can cause dose-dependent effects on the waggle dance. Similarly, few studies with grooming behavior as an endpoint used several doses or concentrations of pesticides. To our knowledge, only Williamson *et al.* (2014a; 2013) exposed bees to several sublethal doses of pesticides and reported effects on grooming in a dose-dependent manner. Regarding the robustness and reproducibility of methods, the waggle dance seems promising as revealed by three studies performed by different laboratories, which found similar effects of imidacloprid on waggle dance performance (Eiri and Nieh, 2012; Kirchner, 1999; Zhang *et al.*, 2020b). However, some inconsistencies across studies testing the same substance were found for grooming behaviors. For instance, bees exposed to low doses of tau-fluvalinate showed either a decrease in grooming (de Mattos *et al.*, 2017) or a lack of effect (Oliver *et al.*, 2015). Such a discrepancy might be due to variability in experimental conditions (duration of exposure, dose, size of bee groups…) rather than to the recording of the behavior itself, which is rather straightforward.

The main shortcoming for the use of these endpoints in pesticide risk assessment might be the limited extrapolation of effects on the consequences for the colonies. Grooming is essential to colony hygiene, but to what extent a decrease or increase in grooming performance of individual bees will affect colony development and survival, is largely unknown. Regarding the waggle dance, several studies have attempted to quantify the benefits of spatial information on colony foraging success. Schürch and Grüter (2014) found, via agent-based simulations, that spatial information was beneficial in the long-term in almost all ecological conditions, but it

remains to be empirically validated. In reality, in experimental studies, the benefits were often null or occurred only in very specific habitats. For instance, benefits were observed in Asian tropical habitats but not in temperate habitats (Dornhaus and Chittka, 2004), in the winter or in urban areas (Sherman and Visscher, 2002), and then, only in habitats with high floral species richness and dense floral patches (Donaldson-Matasci and Dornhaus, 2012). Overall, these studies suggest that pesticide-induced disoriented dances might not have a clear impact on foraging success of the colony.

4.2. Test endpoints with high standardization level but lower ecological relevance: learning/memory, locomotion

The high interest in PER conditioning for evaluating pesticide effects on learning and memory can be explained by its simplicity. The method benefits from 60 years of methodological and conceptual development (Giurfa and Sandoz, 2012), is cheap, does not require specific technical expertise, and provides fast results for a large number of individuals. Most importantly, after reviewing the dozens of toxicological studies that used PER conditioning and taking into account possible technical bias across laboratories, Siviter *et al.* (2018) found that size effects on both learning and memory were robust between studies. Therefore, PER conditioning seems to provide reliable data for a wide range of doses and application procedures, which makes it a serious endpoint candidate for chemical regulation policy. The main weakness of PER conditioning is its ecological relevance. Drawing general conclusions about the impacts in field conditions of poor learning performances measured in the laboratory is challenging (Decourtye and Pham-Delègue, 2002; Thompson, 2003). Since cognitive abilities can directly affect functional behaviors (see Morand-Ferron *et al.*, 2016 for a review), an impairment of cognitive capacities is expected to affect honeybee foraging performance. However, while reversal learning performance is associated with foraging experience (cumulated foraging time) (Cabirol *et al.*, 2018), it remains unclear whether and how bee cognitive abilities contribute to individual foraging success and efficiency, and ultimately to colony performance. In bumblebees no difference in the rate of food collection and the number of foraging trips per day was found between fast and slow learners (Evans *et al.*, 2017). There is a lack of studies using exposure methods that attempt to mimic field realistic scenarios. Therefore, the extrapolation of PER conditioning results to individual and colony foraging success is a key challenge that needs to be addressed in regard to its selection as an assessment endpoint.

Methods for analyzing potential locomotor disabilities upon exposure to pesticides also proved to be sensitive since several studies were able to detect dose-dependent effects on bee locomotion (*e.g.* significant differences between exposures differing by 2 to 10-fold in dose) (Ingram *et al.*, 2015; Tosi and Nieh, 2017; Williamson *et al.*, 2014). The robustness and reproducibility of methods is more difficult to assess due to a relatively low number of research studies. However, the recording of bee activity in an arena is rather straightforward: it simply requires a camera and data that can be analyzed in a semi-automatic way by using free software like ImageJ (Kadala *et al.*, 2019). The standardization of endpoints measured with the flight mill is also promising. This latter method was developed in the 1940s and is increasingly used in a broad range of scientific areas, such as invasion biology, population dynamics, and pest management, with over 400 publications recorded (Naranjo, 2019). In addition, there is a free online protocol for building a standard and affordable flight mill that can record the flight activity of up to 16 insects simultaneously (Attisano *et al.*, 2015). Tests using flight mills might need qualified staff but the recording and analysis can also be done in an automatic manner with sensors and customized scripts. The use of standard flight mills in honeybee toxicological research thus has the potential to provide robust data. However, as for PER conditioning, the ecological relevance of bee activity recorded in indoor conditions remains to be determined. Impairment of locomotion ability, due to alteration of either energetic metabolism (Liao *et al.*, 2019) or brain and muscle excitability (Kadala *et al.*, 2019), may affect the performance of foragers or in-hive bees (like nurse bees). For instance, flight mills have been largely used for better understanding migration in insects, and flight studies have been shown to support field observations (Minter *et al.*, 2018). The challenge though is to define how locomotion patterns obtained in lab-controlled conditions can be used to predict honeybee performance in the field and to what extent it could affect colonies.

4.3. Test endpoints with promising standardisation levels and/or ecological relevance: foraging, homing flight, queen and drone fertility

The evaluation of pesticide toxicity on foraging activity and homing flights has recently been boosted by the emergence of automatic tracking technologies. The major drawbacks of these bioassays are the cost of equipment and the need for specific technical expertise. However, the toxicity assays are usually performed in more field-realistic conditions than other behavioral endpoints, and both foraging activity and homing flights integrate a wide range of biological functions (learning/memory, orientation, locomotion, energetic metabolism…) with which pesticides with different modes of action may interfere. This may explain why these endpoints

are receiving growing interest despite the relative complexity of the measurements. A great majority of these studies have shown consistency in the results obtained (*e.g.* decrease in the number of visits to the feeder, precocious onset of foraging and mortality, and lowered homing success). It is still difficult to assess the robustness and reproducibility of methods used for recording foraging activity, because studies were mostly performed on different pesticides. However, a dose-dependent effect of sublethal doses of acetamiprid was found on the onset of foraging (Shi *et al.*, 2020), indicating that the recording of foraging activity with tracking technologies is a sensitive method.

Homing flight tests were identified as a useful method to assess the effects of sublethal doses of pesticides on honey bees in field conditions (EFSA, 2013, 2012). The method has been tested since 2015 with a dozen laboratories in different environments (Fourrier *et al.*, 2019). During five years of ring test, the majority of laboratories performed the test successfully. In addition, this method has proved to be sensitive because dose-effect responses have been found (Monchanin *et al.*, 2019). As a whole, the results show that the method is well standardized. The homing flight test, which was proposed to OECD standard protocols for pesticide risk assessment, is currently under evaluation. Even if it has been validated for only thiamethoxam, there is growing evidence for low-dose effects of others insecticides, fungicides and herbicides, on honeybee navigation, foraging activities and flight performances (*e.g.* Balbuena *et al.*, 2015; Bortolotti *et al.*, 2003; Liao *et al.*, 2019; Matsumoto, 2013; Prado *et al.*, 2019; Vandame *et al.*, 1995). Such endpoints have a high ecological relevance because of the obvious link between the loss of foragers and/or decreased foraging activity and colony development. The rate of forager loss and activity decline that could lead to a critical reduction of the colony size remains an open question. However, population dynamics models might help to fill this gap. For instance, effects on homing failure and foraging performances can be converted into death rate and foraging parameters (resource collection, age at the onset of foraging…), respectively, and implemented into models to investigate the consequences on colony development and survival.

The field of reproductive toxicity in honeybees has been growing recently. Multiple studies are now available that investigated the reproductive toxicity of the same compound and have revealed consistent results. For instance, both Haarmann *et al.* (2002) and Pettis *et al.* (2004) showed that exposure to coumaphos during development negatively affects queen weight at emergence. Moreover a series of studies using both Tier 1 (laboratory test) and Tier 2 (semi-field test) approaches to analyze the reproductive toxicity of fipronil found similar results. By first showing that drones reared under laboratory and semi-field conditions had similar semen quality (Ben Abdelkader *et al.*, 2014), they found consistent toxic effects of chronic exposure

to fipronil across different times of the year and among years, *i.e.* higher spermatozoa mortality and lower spermatozoa production (Kairo *et al.*, 2017b, 2017a, 2016). This could serve as a basis for the development of standardized methods for testing the reproductive toxicity of pesticides. If the methods using reproduction as assessment endpoints provide robust results, their sensitivity is less known. Besides Wu-Smart and Spivak (2016) who described a dose-dependent effect of imidacloprid on queen egg-laying rate, most studies tested a single dose or concentration of pesticide. This is likely due to the complex and time-consuming protocols. A clear strength of these reproductive endpoints in pesticide risk assessment is their great ecological relevance: impairment of queen and drone fertility can be readily extrapolated to consequences at the colony level. A decrease in queen egg-laying would generally translate into lower brood production and queen replacement by workers (supersedure) (Hendriksma *et al.*, 2004; Sandrock *et al.*, 2014); the latter being risky for the colony fate if it fails. In addition, smaller queens tend to mate with a lower number of drones and to have a lower sperm count in the spermatheca (Delaney *et al.*, 2011). Reproductive endpoints are especially interesting given that the risk associated with poor fertility has been quantified in the literature. For instance, it was found via artificial insemination that a sperm count lower than 4 million results in premature queen supersedures (Harizanis and Gary, 1984; Woyke, 1989c). Additionally, queens that are considered by beekeepers as failing (*e.g.* poor brood production) or that need to be changed have a lower sperm viability (median value of 55%) than healthy queens (92%) (Pettis *et al.*, 2016). Such observations indicate that environmental exposure to fipronil represents a high risk for honeybee colonies. Indeed, Kairo *et al.* (2016) found that queens inseminated with semen from drones exposed to an environmentally-relevant concentration of fipronil had 62% sperm viability and 3.14 million spermatozoa on average (vs 75% sperm viability and 4.62 million spermatozoa for control queens). They would thus fall into the category of failing queens.

4.4. Relevance of behavioural endpoints from the point of view of stakeholders

The relevance of some behavioral endpoints within the context of pesticide risk assessment was further evaluated in a participative session of a workshop organized by ITSAP-Institut de l'Abeille and ANSES in January 2020 with different European stakeholders: scientists, beekeeping representatives, regulators, contract laboratory representatives and manufacturers (23 participants). The representatives worked especially on the standardization levels of four behavioral endpoints and associated test methods (PER, waggle dance, foraging activity and homing flight) within the perspective of chemical regulation. The test methods were noted

according to the 5 assessment criteria (simplicity, sensitivity, reproducibility, robustness and ecological relevance) (Fig. 2). The test on foraging activity at a syrup feeder scored, on average, the best for all of the criteria. In particular, it was considered easier to implement and more robust than the homing flight test. The homing flight test was well noted for sensitivity, reproducibility of results and the best rated for ecological relevance. The test measuring the effects on social communication (waggle dance) received the lowest score (Fig. 2). While it was not possible to have the variability of the participants' responses, the results of this participative session reflect a reality observed by the participants, namely that there are no behavioral tests that respond perfectly to all of the proposed criteria, and that it is rather a trade-off between the different criteria that must be found.

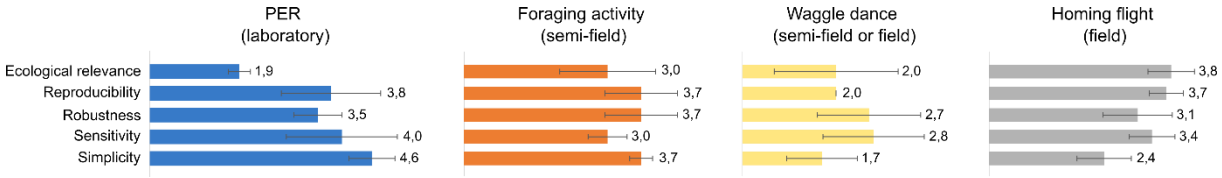

Figure 2. Average scores (± s.e.) assigned by all of the stakeholders to each of the four test methods for the five assessment criteria considered. For each of the test methods, score ranges from 0 (low) to 5 (high); n= 23 participants.

5. Challenges for better integrating behavioral and reproductive performances in pesticide risk assessment

5.1. Translation of toxicological research studies into regulatory test methods

Research studies performed by academics and testing methods used by governments, industries and independent laboratories generally share the same goal, which is identifying and determining potential hazards of pesticides. However, research studies are often hypothesis-driven or exploratory with relatively complex protocols, as opposed to testing methods that rely on simpler and highly standardized protocols. As a consequence, few research studies share the same methodological designs for the measurement of a given endpoint and the robustness and reproducibility of measurements are difficult to assess. Moving from an exploratory to a standard method therefore requires defining standard measurements (such as the LD_{50} in mortality tests), sharing full details of protocols, building a broader database and developing comparative studies. A larger number of tested doses/concentrations and replicates will notably help determine whether the method is reliable enough for chemical regulation.

Regarding behavioral endpoints, the standardization and analysis of measurements currently benefits from the emergence of new technologies. Labor-intensive and time-consuming recordings of behaviors are now faster and easier thanks to computational tools (*e.g.* machine

learning-based system) and electronic tagging of animals. In addition, such tools contribute to removing or reducing opportunities for human observation error. For instance, optic bee counters and RFID proved to be very efficient in automatically tracking the foraging and homing flights of hundreds of honeybees in toxicological studies. Most importantly they drastically reduced the measurement complexity of these highly ecologically-relevant endpoints, making them more accessible to regulatory test methods. Computer vision algorithms have also been developed to track and phenotype the communication and in-hive behaviors of honeybees. For example, by tracking thousands of bees the BeesBook system allows for the automatic detection and decoding of communication dances (Wario *et al.*, 2015). Similarly, the Bee Behavioral Annotation System (Blut *et al.*, 2017) and the technology developed by Gernat *et al.* (2018) enable the monitoring of honeybee worker social interactions (trophallaxis, antennation). The next step will be to test the efficiency and sensitivity of computer vision algorithms in detecting toxicological effects on bees, as recently performed by Siefert *et al.* (2020) for investigating the influence of neonicotinoids on nursing behavior. The improved phenotyping of behaviors that are, by definition, complex, dynamic and noisy will facilitate the potential integration of behavioral endpoints in pesticide risk assessment.

The evaluation of pesticide reproductive toxicity requires the implementation of strong human and technical expertise, both in beekeeping and in the laboratory, which can be limiting for regulatory test methods. Thus, the next step would be to simplify measurement methods. The recent development of cytometry flux methods to determine spermatozoa number and viability in either queen spermatheca or drones has led to more accurate and robust quantification (Pettis *et al.*, 2016; Tofilski *et al.*, 2012; Yániz *et al.*, 2020). Such endpoints notably have the advantage of communicating actual or future queen quality. Identifying in queens and drones the most important route of exposure to pesticides would also help to narrow down tests to the most relevant life stages (larva, adult) and modes of exposure (contact, oral).

5.2. Improve the extrapolation from effects on individuals to colony response

To improve the ecological relevance of behavioral and reproductive endpoints, causative links between individual effects and colony response are needed. However, to confidently detect an effect at the colony level, large colony sample size has to be used. This might explain why none of the field studies developed to assess neonicotinoid effects had the power to detect an effect on the size and the fate of the colony based on the EFSA standard (Franklin and Raine, 2019). Efforts are being made to lower variability inherent to semi-field and field methodologies and thus reduce the Minimum Detectable Difference (MDD) (Wang *et al.*, 2020). The use of

population dynamics models may help to integrate behavioral and reproductive endpoints into a risk assessment framework. The challenge of scaling up the consequences of adverse behavioral and reproductive effects from individuals to populations (or colonies) as a whole is known as the extrapolation problem (Forbes *et al.*, 2008). Population dynamics models are promising tools in that respect. By population models, Forbes *et al.* (2008) refer to mechanistic models that relate individual level responses to changes in population size and structure. Such models have been developed for honey bees (Becher *et al.*, 2014; Khoury *et al.*, 2013, 2011; Schmickl and Crailsheim, 2007; Torres *et al.*, 2015) and investigated as potential, useful tools to inform pesticide risk assessment because they allow for the joint implementation of mortality endpoints as well as a range of behavioral and reproductive endpoints. To date, most models have been used to assess the consequences of lethal pesticide effects at different individual developmental stages, including eggs, larvae, pupae, in-hive workers and ultimately foragers (*e.g.* Rumkee *et al.*, 2015; Schmolke *et al.*, 2019). Model users may also tune those mortality parameters, jointly with other sublethal endpoints, to simulate the consequences of pesticide exposure from individual response to risk of colony collapse. Two sublethal parameters may be readily implemented in the context of pesticide risk assessment, namely homing failure and queen egg-laying rate. Homing failure can be readily combined with natural forager death rate to assess its global effect on the colony dynamics (Bryden *et al.*, 2013; Henry *et al.*, 2012). Likewise, reduced egg laying rate is easily introduced into population dynamics models by simply tuning down the daily egg production to simulate impaired queen reproductive activity (Henry *et al.*, 2017; Rumkee *et al.*, 2015). Models are also useful tools to reveal how combined sublethal stresses at the individual scale can act in concert to exacerbate global colony collapse risks (Henry *et al.*, 2017). Still, additional sublethal parameters along with appropriate mathematical response functions need to be formally implemented into population models. Most importantly, their predictive power remains to be thoroughly tested with empirical data.

We do not suggest the use of an *in silico* approach as a substitute for empirical studies with colony level endpoints, but rather as a tool to narrow down the possible scenarios (e.g. level and duration of exposure) that might lead to the decline of several colony traits, such as brood and adult population size, honey production and colony collapse (Henry *et al.*, 2017). A modelling approach should also give insight into the effect size and time delay before the impact of exposure to pesticide on colony traits. This is particularly important since a pesticide might be considered safe for honeybee colonies if field experiments are not sufficiently well designed, i.e. small-scale experiments and/or short-term monitoring. Therefore, once a risk has been identified at the individual level, population dynamics models may represent a first step

screening process to design field experiments able to confidently detect potential changes at the colony level.

6. Conclusions

New endpoints need to be implemented at the screening level of pesticide risk assessment to consider the risk posed by sublethal doses and concentrations in honeybees. We showed here that several behavioral and reproductive endpoints are available thanks to the development of exploratory research studies in ecotoxicology. These endpoints are not all equally standardized and some are simpler than others. However, one of the most important criteria in the decision-making process of risk assessment relies on the ability of the endpoint to communicate information on the potential impact on the population. In this context, homing and reproductive performances have great potential. While measured at the individual level, both provide great insight into the risk at the colony level, since colony demography and dynamics, like for any population, are primarily governed by birth (fecundity) and death (survival) of individuals. However, for using behavioral and reproductive endpoints in pesticide regulation, further work is needed to develop standardized test protocols.

Around 95% of toxicological studies that used behavioral and reproductive endpoints to investigate the non-target effects of pesticides on honeybees have focused on insecticides (Fig. 1B). This is likely explained by the fact that insecticides are intended to affect insects and generally target the nervous system. In contrast, fungicides and herbicides are usually not considered as a threat to honeybees due to their high LD_{50} values (Zioga *et al.*, 2020). However, there is now growing evidence for lethal and sublethal effects induced by such pesticides (Cullen et al., 2019b). Notably, fungicides may affect honeybee energetic metabolism (Degrandi-Hoffman *et al.*, 2015), which can translate into impaired behavioral performance (Liao *et al.*, 2019; Prado *et al.*, 2019). In addition, in terms of tonnage, insecticide use is outweighed by fungicides and herbicides (Cullen et al., 2019b). There is thus a clear need for filling the gap on the evaluation of fungicide and herbicide toxicity. In this context, behavioral and reproductive endpoints are of great interest, especially given that field-realistic exposures to fungicides and herbicides are generally below their LD_{50} values (Ostiguy *et al.*, 2019; Prado *et al.*, 2019). Future research on fungicides and herbicides will notably have to screen a range of endpoints and investigate which ones are the most appropriate for use in regulatory risk assessment, as they might differ between pesticide type and family.

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C. Conclusion

Cette revue montre que de nombreuses mesures de performances comportementales, telle que l'activité de vol des abeilles, sont de plus en plus utilisées pour estimer la toxicité des pesticides, notamment suite à une exposition à des doses ou concentrations sublétales des produits agrochimiques. Ces mesures sont possibles grâce à l'émergence de nouvelles technologies, permettant un suivi continu de l'activité des abeilles sur le long terme.

II. Evaluation de la toxicité d'une dose sublétale de pesticide sur l'activité de vol des abeilles

A. Avant-propos

Lors des tests réglementaires d'exposition aiguë, la toxicité des pesticides est le plus souvent évaluée sur le court terme et sur une durée fixe (par exemple, la détermination de la DL_{50} se fait sur 48 heures), ce qui peut constituer une sous-évaluation de la toxicité du pesticide en question. Il en est de même pour la plupart des tests comportementaux, qu'ils soient réalisés en laboratoire, en conditions semi-naturelles ou naturelles. Les performances comportementales des abeilles après exposition à une dose unique de pesticide sont généralement mesurées sur le court-terme (Barascou *et al.*, 2021a; OECD, 1998a, 1998b). Il existe ainsi très peu d'information sur la toxicité potentiellement chronique d'une exposition aiguë (Schneider *et al.* 2012b ; Shi *et al.* 2020).

L'objectif de cette partie a donc été de déterminer si une exposition à une dose unique de pesticide peut avoir des effets chroniques ou retardés sur les abeilles mellifères. Pour cela, nous avons mesuré l'activité de vol des abeilles, une mesure qui est apparue comme pertinente pour évaluer la toxicité des pesticides (article 4). L'étude a été réalisée uniquement avec l'insecticide sulfoxaflor, identifié comme à risque pour les abeilles dans le chapitre I.

Ces travaux ont fait l'objet d'une publication dans *Science of The Total Environment* (Barascou *et al.*, 2021b).

B. Article 4

Delayed effects of a single dose of a neurotoxic pesticide (sulfoxaflor) on honeybee foraging activity

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Highlights

- Young honeybees were exposed to a sublethal dose of sulfoxaflor (16 or 60 ng).
- Both doses of sulfoxaflor reduced the daily flight activity and the total number of flights.
- Sulfoxaflor effects were delayed and emerged when bees transitioned to foraging activity.
- Time-to-effect measurements are needed to evaluate pesticide toxicity in honeybees.

Abstract

Pesticide risk-assessment guidelines for honeybees (*Apis mellifera*) generally require determining the acute toxicity of a chemical over the short-term through fix-duration tests. However, potential long-lasting or delayed effects resulting from an acute exposure (e.g. a single dose) are often overlooked, although the modification of a developmental process may have life-long consequences. To investigate this question, we exposed young honeybee workers to a single sublethal field-realistic dose of a neurotoxic pesticide, sulfoxaflor, at one of two amounts (16 or 60 ng), at the moment when they initiated orientation flights (preceding foraging activity). We then tracked in the field their flight activity and lifespan with automated life-long monitoring devices. Both amounts of sulfoxaflor administered reduced the total number of flights but did not affect bee survival and flight duration. When looking at the time series of flight activity, effects were not immediate but delayed until foraging activity with a decrease in the daily number of foraging flights and consequently in their total number (24 and 33 % less for the 16 and 60 ng doses, respectively). The results of our study therefore blur the general assumption in honeybee toxicology that acute exposure results in immediate and rapid effects and call for long-term recording and/or time-to-effect measurements, even upon exposure to a single dose of pesticide.

Keywords: Ecotoxicology, bees, acute exposure, sublethal effects, pesticide risk assessment

Introduction

Within the framework of the pesticide risk assessment procedure, test guidelines require toxicological data on honeybees (*Apis mellifera*), a major pollinator of crops (Garibaldi *et al.*, 2013) and wild plants (Hung *et al.*, 2018). The first tier assessment relies on acute and chronic toxicity tests measuring the survival rate of bees over the duration of the tests, i.e. 48 hours and 10 days for acute and chronic exposure, respectively (OECD 1998a, b, 2013; OECD 2017). Thus, long-term mortality risks are often neglected in pesticide risk assessment, especially when effects on bee mortality occur not during but after chronic exposure to pesticides (i.e. after 10 days) (Dechaume Moncharmont *et al.* 2003; Rondeau *et al.* 2015). In addition, there is growing evidence for long-term sublethal effects upon chronic exposure to pesticides, such as the impairment of bee behavior, physiology or colony performance (Al Naggar and Baer, 2019; Colin *et al.*, 2019b; Faucon *et al.*, 2005; Hesselbach *et al.*, 2020; Prado *et al.*, 2019; Sandrock *et al.*, 2014; Traynor *et al.*, 2021). These data therefore show that time-to-effect experiments rather than fixed-duration tests are required for evaluating chronic toxicity (Bommuraj *et al.*, 2021; Rondeau *et al.*, 2015; Simon-Delso *et al.*, 2018).

Similarly, long-lasting or delayed effects induced by an acute exposure have often been neglected. For instance, in the acute toxicity tests, mortality rate is measured over 48 hours and to a maximum of 96 h if the mortality increases by more than 10% after the first 24 h (OECD, 1998b, 1998a). Moreover, sublethal effects have been generally assayed immediately or over the days following acute exposure to pesticides (Barascou *et al.* 2021). In fact, relatively little is known about the occurrence of long-lasting or delayed effects upon acute exposure (S. Schneider *et al.*, 2012; Shi *et al.*, 2020). After exposure to a single dose, a rapid reduction in pesticide concentration is generally observed in honeybees, which contributes to shortening the exposure to the toxic compound (Ardalani, 2021; Barascou *et al.*, 2021c). Relatively short-term effects can thus be expected; however, some pesticides may cause in-depth changes in bee physiology. For instance, neurotoxic insecticides, by targeting the neurotransmission pathways of insects, impact the neural plasticity, and brain function and structure of bees (Cabirol and Haase, 2019). Thus, single pesticide exposure may lead to life-long impacts if they inhibit or modify a physiological and/or developmental process in the bee brain.

In honeybees, foraging activity is preceded by orientation flights, during which individuals develop highly complex cognitive capacities essential to navigation and homing (Capaldi and Dyer, 1999; Degen *et al.*, 2016). The experience accumulated during this pre-foraging stage then positively influences their foraging capacities and lifespan (Prado *et al.*, 2020). We

therefore hypothesized that a perturbation of this neurocognitive process by a neurotoxin could trigger chronic effects. To test this hypothesis we exposed bees at 7-days old, the median age of first orientation flights (Prado *et al.*, 2020; Requier *et al.*, 2020), to two sublethal doses of sulfoxaflor and tracked their flight activity and lifespan with automated life-long monitoring devices (optic bee counters) (Prado *et al.*, 2020, 2019). We expected a modification of bee behavioral performance, given that sulfoxaflor is a new sulfoximine-based insecticide that shares a mode of action with neonicotinoids as selective agonists of nicotinic acetyl choline receptors (nAChRs) (Sparks *et al.*, 2013; Zhu *et al.*, 2011), and nAChRs play a central role in honeybee cognition (Gauthier and Grünewald, 2012) .

Materials and methods

Experimental setup

Experiments were performed in a peri-urban area (Avignon, France, 43°540N-4°-520E) with honeybees (*Apis mellifera*) from INRAE livestock. Newly emerged bees were collected from 3 colonies and marked with a data-matrix barcode (3 mm of diameter) glued on the thorax (Sader®). They were then released into a colony equipped with a bee counter, which consists of a camera that monitors the hive entrance and image analysis software that detects and registers the activity of bees (direction: in or out of the hive, and time of activity: day, hour, minute, and second) (Prado *et al.*, 2020). The experiment was repeated five times in 2019 (2 in May, 2 in June and 1 in July) using three different colonies equipped with bee counters (2 colonies were used twice). Each time, between 500 and 800 bees were introduced into colonies (see Table S1 for details). Colonies consisted of five-frames with similar population size (around 10 000 adult worker bees) and resource storage (honey and pollen). They were all treated against the parasite *Varroa destructor* the previous year (Apistan).

Exposure to sulfoxaflor

Tagged bees were allowed to develop normally for 7 days after their introduction into colonies. They were then collected and randomly assigned to experimental groups (n = 64-126 bees per experimental group; Table S1). Bees were individually fed with 2 µL of a solution of 30% (w/v) sucrose, 0.1 % acetone and sulfoxaflor at 5 µg/ml or 25 µg/ml, which corresponded to a theoretic exposure of 10 and 50 ng of sulfoxaflor/bee and to the ~LD_{50}/15 and LD_{50}/3 reported by EFSA for in-hive (i.e. young) bees (146 ng/bee) (EFSA, 2014). Control bees were individually fed with pesticide-free syrup (30% (w/v) sucrose and 0.1% acetone).

The doses were chosen based on pesticide residue data found in pollen and nectar. Depending on the application rates and the crops, field residue studies reported levels of sulfoxaflor ranging

from 0.04 to 2.37 mg/kg and from 0.15 to 2.78 mg/kg respectively in nectar and pollen collected by bees (EPA, 2019). Considering that young bees consume between 35 and 60 mg of nectar and 6 and 10 mg of pollen per day (Rodney and Purdy, 2020; Rortais *et al.*, 2005), we could reasonably assume an exposure to sulfoxaflor between 1.4 and 142 ng in nectar and between 0.9 and 27.8 ng in pollen.

After exposure, bees were placed in plastic cages (10.5 cm × 7.5 cm × 11.5 cm) (Pain, 1966) and kept for 60 min in the dark to allow them to ingest the sucrose solution before releasing them into their host colony. Individual flight behavior was recorded continuously from day 1 (introduction date) to day 45.

Stock solutions of sulfoxaflor (Techlab, France) were prepared with acetone, aliquoted and conserved at -20°C. The exact concentrations were checked with LC-MS/MS (European Standard EN 15662:2018 procedure) and resulted in 8 µg/ml and 30 µg/ml for the prepared sulfoxaflor concentrations, which corresponded to a real exposure of 16 ng/bee and 60 ng/bee, respectively.

Measuring bee life-history traits

After exposure to the treatments, we tracked 1521 bees overall (3 experimental groups x 5 replicates). Bees without at least one exit and entrance sequence (i.e. a flight sequence) were excluded from analyses, thus longevity and behavioral data were successfully recorded for 1108 bees. This loss was attributable to the loss of the tag number, the ejection of some tagged bees by nestmates, or an early death of individuals (just after re-introduction). Among the 1108 tracked bees, we obtained data for 407 control bees, and 376 and 325 bees exposed to 16 and 60 ng of sulfoxaflor, respectively (Table S1).

For the analysis of flight activity, exit-entrance sequences shorter than 2 sec or longer than 180 min were excluded (Prado *et al.*, 2020; Requier *et al.*, 2020). The last detection for each barcoded bee was used to calculate bee survival over the 45 days of the experiment. The age at onset of foraging (AOF) was computed for each bee using the *aof* function developed in the *aof* R-package (Requier and Rebaudo, 2020).

Data analysis

Data were analyzed using the statistical software R v4.0.3 (R Core Team, 2020). Survival analyses were performed with the Kaplan-Meier method, using the *survival* package (Therneau, 2021) and followed by a log-rank test for the comparison of survival between treatments. Data were transformed in a survival table and the remaining bees were considered alive at day 45.

Variations in flight activity in response to treatments, flight experience (flight activity before pesticide exposure) and age (fixed factors) were analyzed using GLMM fitted by Penalized Quasi-Likelihood (*glmmPQL* function), using the *MASS* R-package (Venables and Ripley, 2002). The total number of flights, the daily number of flights and the daily duration of flights over the 45 days were fitted with a Quasi-poisson error distribution due to over-dispersion in the data. The total duration of flight activity (log_{10} transformed) and the AOF were fitted with a Gaussian error distribution, using the *glmmPQL* function. We introduced a quadratic term (age^2) into our daily flight activity models to account for a non-linear pattern of the observed relationship between daily flight activity and bee age. Multiple comparisons between levels of treatment were performed using the Tukey method in the *glht* function of the *multcomp* package (Hothorn *et al.*, 2008). Variations in the total number and duration of flights were analyzed with treatment and flight experience as fixed factors, and replicate as a random factor. Variations in the daily activity (number and duration of flights) of each bee were analyzed with treatment and bee age as fixed factors, and replicate and bee identity as random factors. Effects of treatment on the AOF were investigated with replicate as a random factor. The replicate variable was considered as a random factor in the models given that this variable accounted for seasonal variability between bee cohort introductions performed between May and July.

Results

Bee survival

Bee survival did not significantly differ between experimental groups, demonstrating that the tested doses were non-lethal (Kaplan–Meier: p = 0.1; log Rank test, control *vs.* dose 16 ng: p = 0.56; control *vs.* dose 60 ng: p = 0.19, and dose 16 ng *vs.* dose 60 ng: p = 0.12; Fig. S1).

Total flight activity

At day 7 (day of exposure to sulfoxaflor), the percentage of bees that had already performed their first flight did not differ between experimental groups (19.10 ± 0.01 % control bees, 18.74 ± 0.02 % and 14.81 ± 0.001 % bees exposed to 16 or 60 ng of sulfoxaflor, respectively; $\chi2 = 0.0012$, df = 2, P = 0.999). As expected, the total flight activity of bees before pesticide exposure did not differ between experimental groups (Table S2 and S3).

However, the total number of flights was significantly affected by the exposure to sulfoxaflor; bees exposed to 16 ng and 60 ng of sulfoxaflor made significantly fewer trips over the 45 days (28.06 ± 40.21 days and 21.25 ± 24.88 days, respectively) than control bees (33.23 ± 64.22 days; Table 1; Fig. 1A). No difference in the number of flights was observed between the two pesticides doses (Table S4). The total number of flights after exposure to the pesticide treatment

was not affected by flight experience (Table 1; Fig. 1A). The total duration of flight activity was not affected by exposure to sulfoxaflor (16 ng: 53 166.8 ± 6 3681.79 sec, 60 ng: 47 441.21 ± 58 916.81 sec and control: 54 117 ± 65 037.63 sec), but it was negatively related to flight experience (Table 1; Fig 1B). The sulfoxaflor exposure of 16 ng balanced out this negative relationship, although the size effect of this interaction was rather small (Table 1; Fig. 1B).

Table 1. Results of the generalized mixed effect models assessing the effects of pesticide treatments and flight experience on the total flight activity. Intercept represents the control bees. Flight experience corresponds to the flight activity of bees before exposure to pesticide.

Parameters	**Estimate**	**Standard error**	***p*-value**
Total number of flights			
Intercept	3.5824	0.1938	**< 0.001**
Flight experience (number of flights before pesticide exposure)	-0.1401	0.0736	0.0572
Dose 16 ng	-0.3391	0.1262	**< 0.01**
Dose 60 ng	-0.5145	0.1509	**< 0.001**
Flight experience × Dose 16 ng	0.1510	0.0840	0.0725
Flight experience × Dose 60 ng	0.1345	0.1178	0.2537
Total duration of flight activity (sec.)			
Intercept	4.2666	0.1120	**< 0.001**
Flight experience (duration of flight activity before pesticide	-0.0001	0.00003	**< 0.01**
Dose 16 ng	0.00334	0.06016	0.9568
Dose 60 ng	-0.0138	0.06050	0.832
Flight experience × Dose 16 ng	0.0001	0.00004	**0.0339**
Flight experience × Dose 60 ng	0.0001	0.00005	0.2898

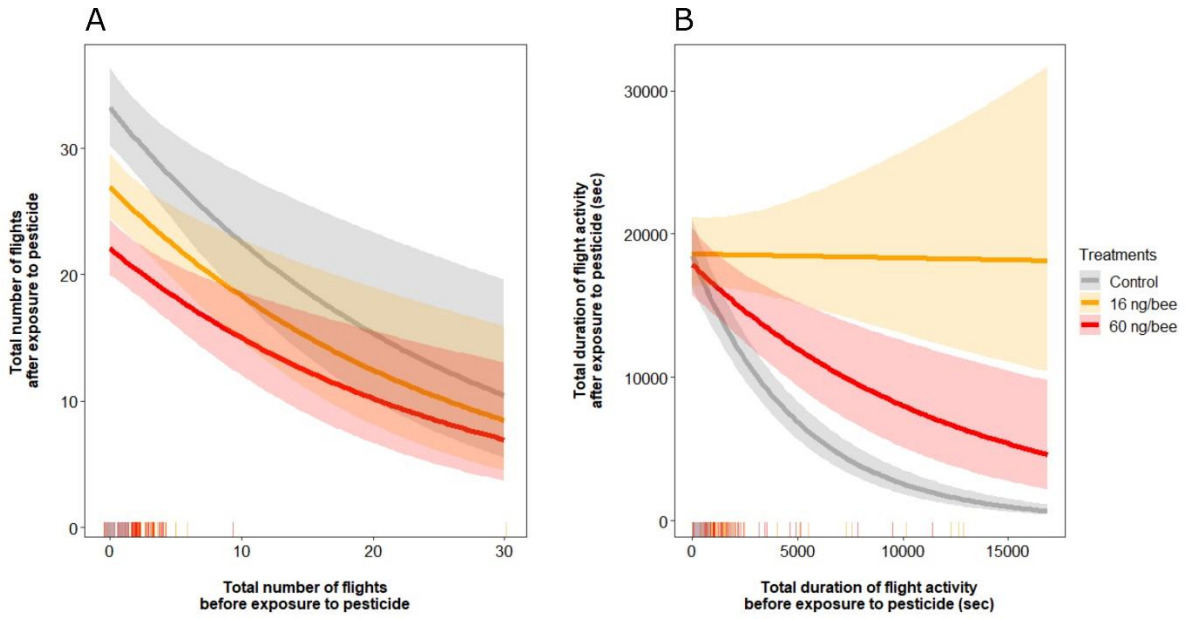

Figure 1. Total flight activity in response to pesticide treatments and flight experience.
(A) Total number of flights and (B) total duration of flight activity (sec.) over 45 days (n = 407 control bees, n = 376 and 325 bees exposed to 16 or 60 ng of sulfoxaflor, respectively). Thick lines represent the model predictions with shaded areas indicating ½ standard error. Colored marks show the distribution of raw data along the horizontal axis.

Time series of flight activity

Overall, the daily number of flights was significantly lower in bees exposed to the lower or higher dose of sulfoxaflor compared to control bees (Table 2; Fig. 2A), and differed between the sulfoxaflor doses, with bees exposed to the higher dose performing less daily flights than bees exposed to the lower dose (Table S5). The daily number of flights also changed according to the age of bees. This relationship was significantly affected by both doses of sulfoxaflor, although the effect was less pronounced for the lower dose (Table 2; Fig. 2A). Young bees belonging to the different treatment groups performed a similar number of daily flights, but their daily activity started to differentiate once they reached the AOF (age at onset of foraging). This latter did not differ between treatment groups (14.71 ± 5.27 for control bees, 14.92 ± 5.02 and 14.11 ± 4.47 for bees exposed to 16 or 60 ng of sulfoxaflor, respectively; GLMM, dose 16 ng: $p = 0.703$ and dose 60 ng: $p = 0.261$; Table S6). Consequently, the number of foraging flights (i.e. flights performed after the AOF) was lower in sulfoxaflor-exposed bees as compared to control bees (32.60 ± 51.19) but did not differ between the two doses (24.75 ± 27.93 and 21.62 ± 22.29, respectively for bees exposed to 16 or 60 ng of sulfoxaflor; GLMM, dose 16 ng: $p < 0.01$ and dose 60 ng: $p < 0.005$; Table S7).

The daily duration of flights was not affected by treatments (Table 2; Fig 2B). Only bee age had an effect. Similarly, to the daily number of flights, the duration increased with age up to the age of 30-days old and then declined (Table 2; Fig 2B).

Table 2. Results of the generalized mixed effect models assessing the effects of pesticide treatments and age on the time series of flight activity. Intercept represents the control bees. Age2 represents a quadratic term to take into account a non-linear pattern of the observed relationship between daily flight activity and bee age.

Parameters	Estimate	Standard error	*p*-value
Time series of flight number			
Intercept	-1.9041	0.1516	**< 0.001**
Age (days)	0.3471	0.0168	**< 0.001**
Dose 16 ng	0.4730	0.2033	**0.0202**
Dose 60 ng	1.0872	0.2170	**< 0.001**
Age × Dose 16 ng	-0.0545	0.0230	**0.018**
Age × Dose 60 ng	-0.1393	0.0248	**< 0.001**
Age^2	-0.0083	0.0004	**< 0.001**
Age^2 × Dose 16 ng	0.0012	0.0006	0.0555
Age^2 × Dose 60 ng	0.0036	0.0007	**< 0.001**
Time series of flight duration (sec.)			
Intercept	3.9608	0.1836	**< 0.001**
Age (days)	0.2996	0.0141	**< 0.001**
Dose 16 ng	-0.0811	0.2047	0.6920
Dose 60 ng	0.3517	0.2128	0.0987
Age × Dose 16 ng	0.0140	0.0201	0.4861
Age × Dose 60 ng	-0.0235	0.0212	0.2691
Age^2	-0.0047	0.0003	**< 0.001**
Age^2 × Dose 16 ng	-0.0005	0.0005	0.2859
Age^2 × Dose 60 ng	0.0004	0.0005	0.3779

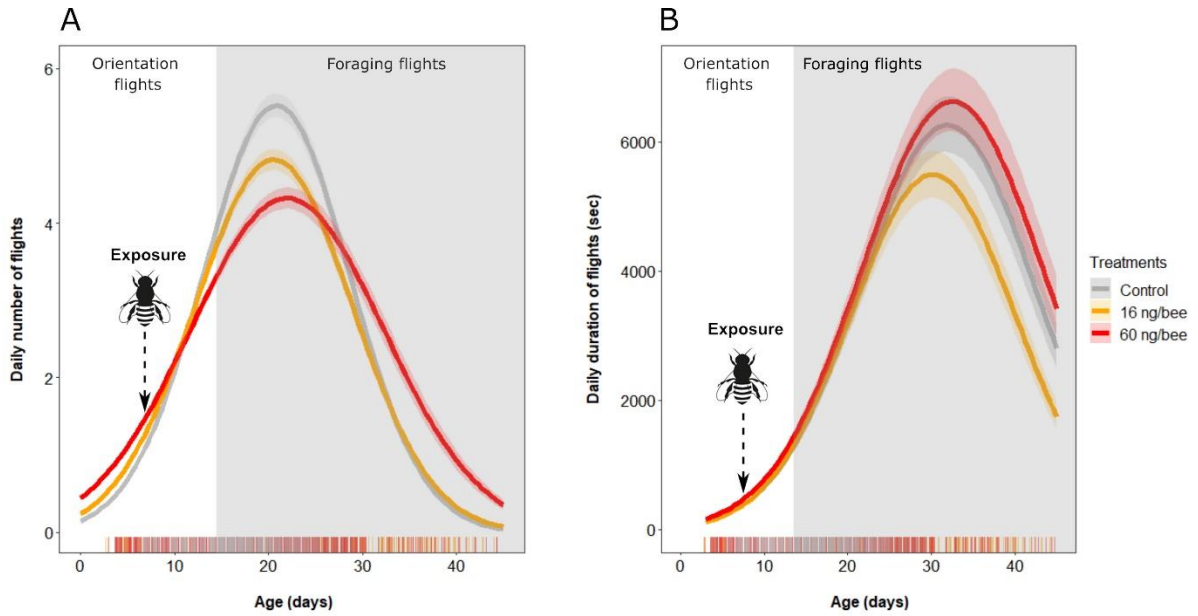

Figure 2. Time series of flight activity in response to pesticide treatments. (A) Number and (B) duration of daily flights (sec.) over bee lifetime (n = 407 control bees, n = 376 and 325 bees exposed to 16 or 60 ng of sulfoxaflor, respectively). Thick lines represent the model predictions with shaded areas indicating ½ standard error and the dashed line represents the day of exposure to pesticides. The transition to foraging activity started on average at 14.5 days. Colored marks show the distribution of raw data along the horizontal axis.

Discussion

In the present study, we showed that acute exposure in honeybees does not necessarily cause short-term effects but can generate delayed effects, as previously found in others insects (Beketov and Liess, 2008; Wolz *et al.*, 2021). Long-term effects following exposure to a single dose of pesticide have been previously described in honeybees. However, contrary to our study, latency effects were not investigated and effects were only reported at doses that significantly increased honeybee mortality (Schneider *et al.* 2012; Shi *et al.* 2020). The sulfoxaflor doses we tested (16 and 60 ng/bee) were below the LD50 determined for in-hive bees (146 ng/bee) (EFSA, 2014) and proved to be non-lethal since no mortality increase was found as compared to control groups over the 45 days of monitoring. Recently, a lower LD50 has been reported for forager bees (55 ng/bee) (Azpiazu *et al.*, 2021). The lack of toxicity of a similar dose in our experiment seems to confirm that in-hive bees are less sensitive to insecticides than forager bees (S. Tosi and Nieh, 2019), although this might not be a general rule as it could depend on the pesticide type (Rinkevich *et al.*, 2015). Another possible explanation for the non-toxicity of the doses in our study is that we monitored bee survival in field conditions. Control honeybees live less long in field conditions than in the laboratory, and therefore the risk of death of

experimental groups relative to control groups tend to be reduced in field conditions as compared to the laboratory (Alaux *et al.*, 2014).

Although both doses of sulfoxaflor significantly reduced the number of flights performed by bees, the total duration of flight activity was not affected, as would be expected from a lower number of flights but of similar duration. This might be explained by the natural variation in the duration of flights combined with the fact that the effect of sulfoxaflor on daily activity only emerged at about the time bees transitioned to foraging activity, i.e. a week after exposure to the pesticide. Interestingly, bees that had longer flight activity before exposure had shorter activity afterwards, suggesting that bees have a defined time-budget for their flight activity. We are cautious in our interpretation, because bees were experimentally manipulated during their activity; this work remains to be investigated with non-manipulated free-flying bees. This relationship between flight experience and the duration of flight activity after treatment was affected by the lower sulfoxaflor dose, which resulted in bees flying for a rather fixed amount of time regardless their flight experience. The underlying mechanisms are not known but the effect was quite marginal and driven by a few bees.

An increase in oxidative stress and cell apoptosis, as well as a modification of immunocompetence, have been described upon sublethal exposure to sulfoxaflor (Al Naggar and Paxton, 2021; Chakrabarti *et al.*, 2020; Li *et al.*, 2021). However, the behavioral impairments we observed are more in line with an impact on bee cognition. Since sulfoxaflor and neonicotinoids have a similar mode of action (agonist of nAChRs), and neonicotinoids are known to negatively influence bee behavior through impact on cognition (Belzunces *et al.* 2012; Siviter *et al.* 2018), we can reasonably assume that sulfoxaflor also affected bee behavioral performance through cognitive impairments. In our experiments, bees were exposed to sulfoxaflor when they initiated their orientation flights. It is therefore possible that sulfoxaflor affected their learning abilities, explaining why bees made later and fewer foraging flights. By testing the olfactory conditioning performances of honeybee foragers, Siviter *et al.* (2019) did not find any negative effects of acute sulfoxaflor exposure on learning and memory. However, besides the lower doses (0.05 to 2.5 ng), the tests were performed immediately after exposure to sulfoxaflor. The response to sulfoxaflor we found took more than a few days to develop and could therefore be considered delayed. The latency of response to pesticide may depend on many factors, such as the rates of absorption and distribution and the speed of action at the target site. But it may also depend on the physiological process that is affected. Interestingly, by analyzing the effect of imidacloprid (neonicotinoid) on habituation of the proboscis

extension reflex, Guez *et al.* (2001) found a contrasting effect between 7- and 8-day-old bees. This effect seemed to be associated with a differential expression of two subtypes of nAChRs during bee behavioral maturation i.e. transition from nurse to forager tasks, (Guez *et al.*, 2003). Later, it was found that during their behavioral maturation, young bees exhibit massive changes in brain gene expression, which are essentially completed at 8 days old (Whitfield *et al.*, 2006) and coincide with structural brain changes (Farris *et al.*, 2001). Altogether these studies provide strong evidence for developmental change in the neurophysiological state of 1-week old bees which might explain the effects of sulfoxaflor. By affecting this neurodevelopmental process essential to behavioral maturation, a single dose of neurotoxin might cause intrinsic changes in the bee brain and therefore impair future foraging performances.

Whilst much work remains to investigate the occurrence of long-term effects after acute exposure, our findings blur the general assumption in honeybee toxicology that acute exposure results in immediate and short-term effects. Therefore, long-term recording and/or time-to-effect measurements are needed, even upon exposure to a single dose of pesticide, to avoid a potential underestimation of pesticide toxicity.

Competing interests

The authors declare that they have no competing interests.

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Author Contributions

L.B., Y.L.C and C.A. conceived the study. L.B., D.S., D.C. and C.A. conducted the experiments. L.B., C.A. and F.R. analyzed the data, Y.L.C. and C.A. contributed to reagents. L.B, C.A and F.R. wrote the manuscript. All authors read and reviewed the manuscript.

Supplementary information

Figure S1. Survival probability of bees exposed to pesticide treatments. Data represent the survival probabilities of bees from day 7 to 45 with a 95% confidence interval. (n = 407 control bees, n = 376 and n = 325 bees exposed to 16 or 60 ng of sulfoxaflor, respectively).

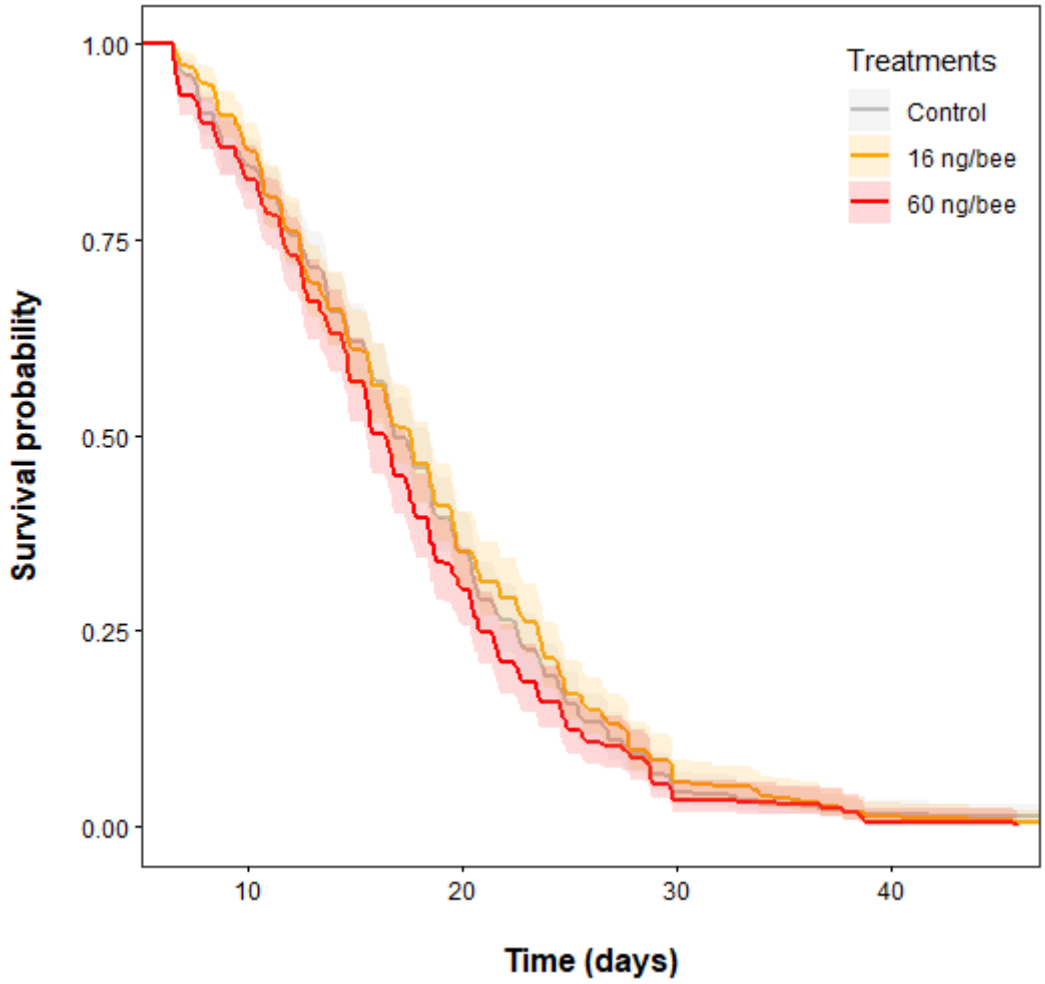

Table S1. Number of bees per cohort and treatment.

Cohorts	Treatments	Number of tagged bees	Number of bees exposed to treatments	Number of analyzed bees per treatment
May (1)	Control	600	118	97
	16 ng/bee		90	70
	60 ng/bee		122	79
May (2)	Control	600	83	60
	16 ng/bee		102	76
	60 ng/bee		91	33
June (1)	Control	500	115	103
	16 ng/bee		120	82
	60 ng/bee		126	91
June (2)	Control	800	125	96
	16 ng/bee		110	98
	60 ng/bee		120	93
July	Control	800	64	51
	16 ng/bee		69	50
	60 ng/bee		66	29

Table S2. Results of the generalized mixed effect models assessing the variation in flight experience before pesticide treatments. Intercept represents the control bees.

Parameters	Estimate	Standard error	*p*-value
Total number of flights before treatment			
Intercept	-0.235	0.1844	0.2095
Dose 16 ng	-0.0306	0.1221	0.8018
Dose 60 ng	-0.0424	0.1337	0.7514
Total duration of flight activity (sec.) before treatment			
Intercept	1072.004	144.5752	**< 0.001**
Dose 16 ng	-137.1147	199.3991	0.492
Dose 60 ng	-150.7959	212.3794	0.478

Table S3. Results of the Tukey post-hoc tests assessing the variation in flight experience before pesticide treatments.

Comparisons	Estimate	Standard error	z	*p*-value
Total number of flights before treatment				
Dose 16 ng vs control	0.031	0.122	0.251	0.966
Dose 60 ng vs control	-0.042	0.133	-0.317	0.946
Dose 60 ng vs Dose 16 ng	-0.073	0.134	-0.546	0.848
Total duration of flights (sec.) before treatment				
Dose 16 ng vs control	-137.11	198.88	-0.689	0.769
Dose 60 ng vs control	-150.80	211.83	-0.712	0.756
Dose 60 ng vs Dose 16 ng	-13.68	213.04	-0.064	0.998

Table S4. Results of the Tukey post-hoc tests assessing the effects of pesticide treatments on the total flight activity.

Comparisons	Estimate	Standard error	z value	*p*-value
Total number of flights				
Dose 16 ng vs control	-0.339	0.126	-2.695	**0.02**
Dose 60 ng vs control	-0.515	0.151	-3.419	**< 0.01**
Dose 60 ng vs Dose 16 ng	-0.175	0.153	-1.143	0.486
Total duration of flights (sec.)				
Dose 16 ng vs control	0.003	0.062	0.054	0.998
Dose 60 ng vs control	-0.014	0.065	-0.213	0.975
Dose 60 ng vs Dose 16 ng	-0.017	0.066	-0.259	0.964

Table S5. Results of the Tukey post-hoc tests assessing the effects of pesticide treatments on the daily flight activity.

Comparisons	Estimate	Standard error	z value	*p*-value
Daily number of flights				
Dose 16 ng vs control	0.4730	0.2032	2.328	0.0520
Dose 60 ng vs control	1.0872	0.2168	5.014	**< 0.001**
Dose 60 ng vs Dose 16 ng	0.6142	0.2127	2.887	**0.01**
Daily duration of flights (sec.)				
Dose 16 ng vs control	-0.0811	0.2046	-0.396	0.917
Dose 60 ng vs control	0.3517	0.2127	1.654	0.223
Dose 60 ng vs Dose 16 ng	0.4328	0.2146	2.017	0.108

Table S6. Results of the Tukey post-hoc tests assessing the effects of pesticide treatments on the AOF.

Comparisons	Estimate	Standard error	z value	*p*-value
Dose 16 ng vs control	0.1542	0.4033	0.382	0.923
Dose 60 ng vs control	-0.4769	0.4226	-1.128	0.496
Dose 60 ng vs Dose 16 ng	-0.6311	0.4315	-1.462	0.309

Table S7. Results of the Tukey post-hoc tests assessing the effects of pesticide treatments on the total foraging flight activity.

Comparisons	Estimate	Standard error	z value	*p*-value
Dose 16 ng vs control	-0.299	0.113	- 2.651	**0.022**
Dose 60 ng vs control	-0.395	0.124	- 3.186	**< 0.01**
Dose 60 ng vs Dose 16 ng	-0.096	0.133	- 0.726	0.747

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C. Conclusion

Les résultats de cette étude montrent que l'exposition à une seule dose de sulfoxaflor a diminué l'activité de butinage des abeilles mellifères, ces dernières ayant effectué jusqu'à 33 % en moins de vols de butinage que les abeilles non exposées au pesticide. Ce phénomène est apparu une semaine après l'exposition à l'insecticide. Ainsi, cette étude montre qu'une exposition aiguë chez les abeilles mellifères ne provoque pas nécessairement des effets à court terme mais peut générer des effets retardés, observables sur l'activité de vol des butineuses.

Des tests à plus long terme (supérieurs à 48h) sont ainsi recommandés pour déterminer la toxicité d'une dose unique de pesticide.

III. Evaluation de la toxicité d'un pesticide sur l'activité de vol des abeilles à l'échelle coloniale

A. Avant-propos

Les procédures d'évaluation des risques liés à une exposition aux pesticides ont pour objectif la protection des colonies d'abeilles. Dans ce contexte, la quantification des taux de mortalités à l'échelle de la colonie est devenue une mesure de référence pour évaluer les effets dus à l'exposition aux pesticides et fixer des objectifs de protection des colonies d'abeilles (EFSA *et al.*, 2020). Les informations sur les taux de mortalité des abeilles peuvent ensuite être utilisées pour déterminer des seuils d'effets acceptables, c'est-à-dire ne causant pas de réduction inacceptable de taille des colonies (EFSA, 2013).

Il est donc crucial d'obtenir des données de mortalité à l'échelle coloniale et d'en extraire des scénarii de risque pour les colonies suite à une exposition à un pesticide. C'est dans cette optique que nous avons utilisé pour la première fois des compteurs d'abeilles pour i) évaluer la toxicité d'un pesticide sur l'activité de vol de la population de la colonie et les pertes quotidiennes d'abeilles, et ii) identifier le risque consécutif pour les colonies en comparant les taux de mortalité des abeilles à ceux ayant été identifiés comme conduisant à une diminution non-négligeable de la taille des colonies (EFSA, 2013). Cette étude doit représenter une preuve de concept de l'utilisation des compteurs d'abeilles pour évaluer la toxicité des pesticides à l'échelle coloniale.

Cette étude a été rédigée sous forme d'article pour soumission dans un journal à comité de lecture internationale.

B. Article 5

Assessing the risk posed by a pesticide on honeybee colonies by using a real-time monitoring of daily activity and bee loss rates

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Article en préparation.

Abstract

Information on honeybee foraging performances at colony-level and especially bee loss rates are crucial for evaluating the magnitude of effects due to pesticide exposure and therefore ensuring that protection goals for honeybee colonies are met (i.e. threshold of acceptable effects). However, current methods for monitoring of honeybee foraging activity and mortality are very approximate (visual records) or are time-limited and mostly based on single cohort analysis. Therefore, to assess the risk posed by an insecticide on honeybee colonies we monitored the colony-level flight activity and bee loss rates in real-time with the use of bee counters. After assessing the background activity and bee loss rates, we exposed colonies to two concentrations of sulfoxaflor (neurotoxic insecticide): a concentration that was considered to be field realistic and a higher concentration representing a worst-case exposure scenario. We did not find any effect of the field-realistic concentration on the flight activity and bee loss rate. However, a two-fold decrease in daily flight activity and a 10-fold increase in daily bee losses were detected in colonies exposed to the high sulfoxaflor concentration. When compared to the theoretical trigger values associated to the specific protection goal of 7% colony size reduction, such fold changes in daily bee losses were often found to be at risk for colonies. In conclusion, the real-time and colony-level monitoring of bee loss rates, combined with threshold values indicating which fold changes in bee loss rates start to be at risk for colonies, have great potential for improving regulatory pesticide risk assessments for honeybees under field conditions.

Keywords: pesticide risk assessment, sulfoxaflor, bee counter, trigger values, mortality rates

Introduction

Plant protection products, also referred to as pesticides, have been developed to protect crops against harmful organisms (*e.g.* insects, fungi) or prevent the growth of undesired plants (herbicides). However, the yield and/or quality of around 75% of globally important crop types also depend on pollination mediated by animals, especially bees, which visit more than 90% of the leading 107 global crop types (Klein et al., 2007). As a consequence, bees, including the honeybee (*Apis mellifera*), can be exposed to several pesticides recovered in pollen and nectar (Botías et al., 2015; Mullin et al., 2010; Pohorecka et al., 2012; Sanchez-Bayo and Goka, 2014; Zioga et al., 2020). There is therefore a clear need to provide an assessment of the risk presented by pesticides and determine to what extent there are safe for bees. For that purpose, OECD (Organization for Economic Co-operation and Development) and EPPO (European and Mediterranean Plant Protection Organization) test guidelines require toxicological data on honeybees (*Apis mellifera*). Indeed, they are relatively easy to rear, their biology has been well-studied, and they are one of the most important pollinators worldwide (Hung et al., 2018; Klein et al., 2007).

Within the current regulatory framework for pesticide risk assessment, the effects of pesticides on honeybees are assessed by standard tests in a stepwise approach. In the Tier 1, active substances or formulated products are tested on honeybees at different life stages (larvae and adults) (OECD, 2017, 2013, 1998b, 1998a). Then, a deterministic approach to characterize risk quantitatively can be used by comparing the pesticide toxicity to environmental exposure. If a risk is identified as a result of this first step, supplementary tests are required at a higher tier on colonies (semi-field and field tests) for the marketing authorization (MA). Higher tier tests can also be performed in post-MA studies monitoring adverse effects linked to the use of pesticides. A major issue is that the test guidance for the conduct of such semi-field and field studies (EPPO, 2010a) relies on measurements of several key colony parameters that are very approximate (Wang et al., 2020). For instance, the estimation of flight activity is based on the count for a few seconds of bees foraging on flowers near the hive, which represents a small snapshot of what happens over a day and does not take into account the daily changes in foraging activity according to the weather and others environmental factors (e.g. discovery of new food sources) (Winston, 1987). Then, mortality estimates rely mainly on the number of bees in dead bee traps in front of the hive and therefore excludes bees that died outside of traps. Counting the number of bees entering and exiting the hive can also be used to assess forager activity and bee losses since worker bees die in the field or are removed from the hive if dead

inside. To accurately determine these assessment endpoints, researchers in the past years have relied on individual tagging and detection of bees at the hive entrance. Notably, radiofrequency identification (RFID) tags coupled with detectors at the hive entrance and bee counters recording the activity of bees marked with data-matrix barcodes have been successfully used in several studies to automatically record individual bee activity and mortality in response to pesticide exposure (Bortolotti *et al.* 2003; Henry *et al.* 2012; Prado *et al.* 2019; Monchanin *et al.* 2019; Colin *et al.* 2019b, a; Hesselbach *et al.* 2020; Shi *et al.* 2020; Barascou *et al.* 2021a; Henry *et al.*, 2012; Monchanin *et al.*, 2019; Schneider *et al.*, 2012). If individual bee tracking is highly effective, it provides data on honeybee flight activity and mortality that are time-limited and based on a small portion of the bee population, as well as on a single age-cohort, excluding the fact that pesticide sensitivity may depend on age with younger bees being more sensitive to certain pesticides, but less to others, than older bees (Bendahou et al., 1997; Ladas, 1972; Mayland and Burkhardt, 1970; Rinkevich et al., 2015; Zhu et al., 2020).

This high uncertainty of current semi-field and field regulatory tests combined with the lack of tools and methods for the field assessment of bee activity and mortality at the colony-level represent a major gap for pesticide risk assessment, especially since the measurement of bee mortality has emerged as a benchmark for evaluating the magnitude of effects due to pesticide exposure (EFSA, 2013; EFSA et al., 2020). Indeed, specific protection goals (SPGs) have been established as the maximum permitted level of colony size reduction resulting from exposure to pesticides, and the level and duration of bee losses that would cause this specific decline in colony size are defined as trigger values. For instance, a reduction in colony size that does not exceed 7 % has been considered as negligible and therefore protective for honeybee colonies in the EFSA "Guidance document on the risk assessment of plant protection products on bees" (EFSA, 2013). Accordingly, it was determined by using a quantitative model of colony population dynamic (Khoury et al., 2011), that forager mortality should not be increased compared with controls by a factor of 1.5 for 6 days, a factor of 2 for 3 days or a factor of 3 for 2 days (EFSA, 2013).

We therefore aimed for the first time to assess *i)* the toxicity of a pesticide on the colony-level daily activity and bee loss and *ii)* the consecutive risk for colonies. For that purpose, we performed a real-time monitoring of colony population flight activity with an optic bee counter. We first identified the background activity and mortality and then exposed colonies to a field realistic concentration of sulfoxaflor or a higher concentration representing a worst-case exposure scenario. Sulfoxaflor is a new sulfoximine based insecticide that shares a mode of action with neonicotinoids as selective agonists of nicotinic acetyl choline receptors (nAChRs)

(Sparks et al., 2013). Since nAChRs play a central role in honeybee cognition and behavioral performances (Belzunces et al., 2012; Gauthier and Grünewald, 2012), and neonicotinoid effects on adult honeybees include impairments of learning and memory, navigation and orientation, and foraging behavior (see Blacquière *et al.* 2012; Wood and Goulson 2017 for reviews), we hypothesized that sulfoxaflor would decrease bee activity and increase bee losses. To finally assess the risk posed by sulfoxaflor to honeybee colonies, we compared the level and duration of bee losses to the theoretical trigger values associated to the SPG of 7% colony size reduction.

Materials and methods

Experimental setup

Experiments were performed in 2021 in a peri-urban area near Avignon (France, 43°540N-4°-520E) with honeybee colonies (*Apis mellifera*) from INRAE livestock. Each colony composed of five Dadant frames were selected and then randomly assigned to one of the different treatments (see below 'Exposure to sulfoxaflor'). The day before exposure to sulfoxaflor, we assessed the demographic state and the amount of honey and pollen stored in hives. For that purpose, each side of each frame, was visually inspected and the area covered by the number of bees, open and closed brood cells, and honey were reported in percentage (one full side = 100%). Percentage were then converted into numbers of adult bees, open and capped brood cells, and dm^2 of honey and pollen based on a previously determined coefficient for Dadant frames (Hernandez et al., 2020). Colony were visually inspected again at the end of exposure and 10 days post-exposure to sulfoxaflor by following the same methodology. Finally, colony overwintering survival was checked the following year (March 2022).

Exposure to sulfoxaflor

After the random assignment to treatment groups, colonies were left alone for four days. They were then exposed to one of the sulfoxaflor treatment. The sulfoxaflor concentrations were chosen based on pesticide residue data found in pollen and nectar. Depending on the application rates and the crops, field residue studies reported levels of sulfoxaflor ranging from 0.04 to 2.37 ppm and in nectar collected by bees and from 0.00447 to 19.8 ppm in the nectar of flowers (EPA, 2019). Thereby, colonies were provided with a solution of 50 % (w/v) sucrose, 0.1 % acetone and sulfoxaflor at a field realistic concentration (0.5 µg/ml) or a higher concentration representing a worst-case exposure scenario (2 µg/ml). Control groups were fed with pesticide-free sugar solutions (50 % w/v sucrose, 0.1 % acetone) and were used as environmental control, to determine whether differences in colony activity could be attributed to variation in field

conditions (e.g., climate, resources). Stock solutions of sulfoxaflor (Techlab, France) were diluted in acetone, aliquoted and conserved at -20°C. The exact concentrations were checked with LC-MS/MS (Barascou et al., 2021c) and resulted in 0.59 µg/ml and 2.37 µg/ml for the prepared sulfoxaflor concentrations. Colonies were given 500 ml of pesticide-free or sulfoxaflor-contaminated sugar solutions every two days, over a 10-days exposure period (giving a total of 2,500 ml of syrup). The experiment was repeated each month between April and September 2021 (except in August due to too high environmental temperatures and therefore low colony activity). Each month was composed of one colony per experimental treatment, excepted in April (no colony was exposed to the 0.59 µg/ml sulfoxaflor treatment) and June and July (two colonies were exposed to the 0.59 µg/ml sulfoxaflor treatment); giving a total of n = 5 colonies for the control and 2.37 µg/ml sulfoxaflor treatments, and n = 6 colonies for the 0.59 µg/ml sulfoxaflor treatment.

Colony-level monitoring of daily flight activity and homing flight rates

To assess the effect of sulfoxaflor on the daily flight activity and homing flight rates, we equipped each colony with an optic bee counter developed in our laboratory (patent IDDN.FR.001.130013.000.R.P.2010.000.31235; Crauser and Le Conte 2010). For each replicate, bee counters were assigned to a different treatment group, so that each bee counter monitored the activity of colonies exposed or not to sulfoxaflor (0.59 and 2.37 µg/ml).

The bee counter device consists of a camera that monitors the hive entrance and an image-analysis software that detects and registers in real-time the activity of all bees by counting the number of out-going and in-coming bees (Fig. 1). The hive entrance was modified and composed of eight passages narrow enough so that one bee can circulate in each tunnel and only with the back of the thorax facing the camera (see Dussaubat *et al.* 2013 for more details). The software runs in LabView environment for graphic programming and adjusts the frequency of images captured per second in order to reduce the chance of missing bees passing in front of the camera (error rate below 3 % determined by comparing visual counts of bees to bee counter counts).

The cumulated activity was recorded every 5 minutes and automatically saved with the time and date. From these bee entry and exit data, we determined the daily: *i)* flight activity (sum of the number of bee exits during a day) and *ii)* homing flight rates, calculated as follows: (number of entries - number of exits) / number of exits for each day (Odemer, 2022). When negative, the homing flight rate was assimilated to a loss rate. Positive values corresponded to a gain of

bees, likely from drifting behaviour since experimental colonies were located in the same apiary (two meters away from each other's).

The colony-level monitoring with bee counters started 4 days before the exposure to sulfoxaflor and ended at 10 days post-exposure. We therefore had data on the daily flight activity and homing flight rates for three time periods: pre-exposure period (4 days), during exposure (10 days), and post-exposure (10 days).

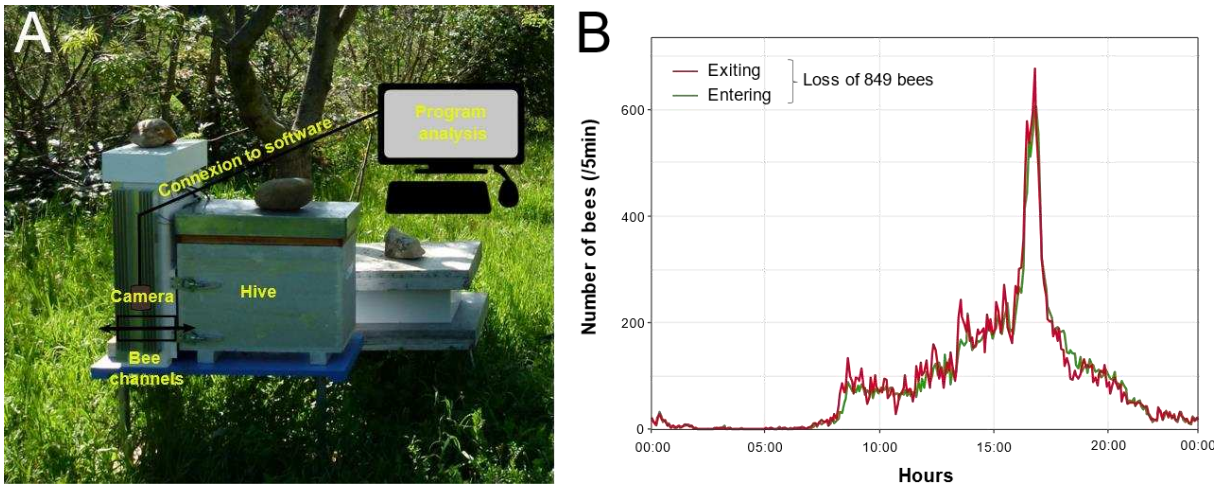

Figure 1. Optic bee counters in the field measuring the activity of a honeybee colony. (A) The optic bee counter with bee channels and the camera is as the entrance of the hive. The camera recording bee activity is directly connected to a computer and a program analysing in real-time bee traffic. (B) Number of bees exiting and entering the hive over a day during the experiment. During this day, the colony lost 849 bees, corresponding to a loss rate of 3.3 %.

Data analysis

Data were analysed using the statistical software R v4.0.3 (R Core Team, 2020).

Variations among treatment groups in the number of honeybees, open and capped brood cells, and the amount of honey and pollen before exposure to sulfoxaflor were analysed using Kruskal-Wallis tests. The influence of treatment groups on the cumulated syrup consumption was also analysed using a Kruskal-Wallis test.

The effects of sulfoxaflor were determined on both the daily flight activity of bees and homing flight rates and analysed using a Linear Mixed Model (LMM) fitted by maximum likelihood using the *lme4* package (Bates et al., 2015). Variations in daily flight activity (daily number of exits, as response variable) and in the homing flight rates ((number of entries - number of exits) / number of exits, as response variable) were fitted with a Gaussian error distribution using the *lmer* function. *P*-values for the t-test of fixed terms of the LMM were obtained through the *lmer* function of the *lmerTest* package (Kuznetsova et al., 2017). For each model, we selected the best one based on the second-order Akaike's Information Criterion (AICc) (Burnham and

Anderson, 2003) by using backward model selection from the full model. Variations in the daily flight activity and homing flight rates were analysed with treatment, pesticide exposure period and months as fixed factors, and colony replicates as random factor. For homing flight rates analysis, due to a heavy tailed distribution of the response variable and non-normal residues of the LMM, we used a bootstrap procedure to assess the significance of variables. 95% confidence intervals for model parameters have been estimated using the *lme4* R-package (*bootMer* function). We generated 10,000 bootstrapped samples from the model and refitted the model to these samples to obtain bootstrapped parameter estimates (Canty and Ripley, 2021; Davison and Hinkley, 1997). If bootstrapped confidence intervals around the parameter estimate in that model did not overlap with zero, we concluded on a significant effect.

In addition, we assessed for each treatment group the magnitude of sulfoxaflor effects on the daily flight activity and homing flight rates. For each each pair of pesticide exposure period (before vs during exposure; during vs after exposure; before vs after exposure), we calculated Hedges'g (g) or Glass's delta (Δ) as a measure of effect size (*hedges_g* or *glass_delta* functions of the *effectsize* package; Ben-Shachar *et al.* 2020). Glass's delta was used when the standard deviations were significantly different between groups, as it uses only the second group's standard deviation. The interpretation values were as follows: $0.2 > g$ or Δ: very small effect, $0.2 \leq g$ or $\Delta < 0.5$: small effect, $0.5 \leq g$ or $\Delta < 0.8$: medium effect, and g or $\Delta \geq 0.8$: large effect (Ben-Shachar et al., 2020). An effect size (g or Δ) was considered significant if the 95% confidence intervals (CI) of effect size did not overlap zero, and as negative or positive when it was lower or higher than 0, respectively (Ben-Shachar et al., 2020).

For each treatment group, variations in the number of honeybees, open and capped brood cells, and the amount of honey and pollen between the pre-exposure period (D4) and the post-exposure periods (D14 and D24) were analysed using Wilcoxon rank test pairwise comparisons.

Finally, we determined whether an increase in mortality rates (negative homing flight rates) exceeded or not the theoretical trigger values associated to the SPG for honeybees (.i.e. 7 % colony size reduction; (EFSA, 2013). For that purpose, we calculated the fold-changes in bee loss rates between the pre-exposure and exposure periods to sulfoxaflor. These values were determined for exposure lasting 2, 3 or 6 days and then compared to the theoretical trigger values (EFSA, 2013).

Results

Level of exposure to sulfoxaflor

We did not find any difference between treatment groups in the colony demographic state (number of bees: Kruskal-Wallis, p = 0.417, open brood cells: p = 0.309 and capped brood cells: p = 0.284; Fig. S1) and food storage (honey: p = 0.803 and pollen: p = 0.564) before exposure to sulfoxaflor. The cumulated syrup consumption did not differ either between treatment groups (control: 1 193± 761 ml, 0.59 µg/ml of sulfoxaflor: 1 343 ± 792 and 2.37 µg/ml of sulfoxaflor: 1 348 ± 659 ml, Kruskal-Wallis: p = 0.642; Fig. S2).

Colony-level effect of sulfoxaflor on the daily flight activity

The results of model selection regarding the effect of sulfoxaflor treatment on the daily flight activity are presented in Table S1 (LMM analysis). The explanatory variables in the best selected model were: treatment, exposure period and months.

The overall daily flight activity, including all three time periods (before exposure: BE, during exposure: DE and after exposure: AE), did not differ between months. We did not find either an effect of sulfoxaflor on the overall daily flight activity at both concentrations of sulfoxaflor (Table 1). However, there was a significant interaction effect between the treatment and the exposure period in control colonies and colonies exposed to 2.37 µg/ml of sulfoxaflor (Table 1 and Fig. 2). While the daily activity in colonies exposed to 0.59 µg/ml of sulfoxaflor did not change across the different periods of exposure (BE: 34 092 ± 18 666; DE: 35 018 ± 13 372 and AE: 36 086 ± 9 534 flights/day), it increased between the BE and AE periods in control colonies (BE: 27 287 ± 9 657; DE: 30 814 ± 12 260 and AE: 34 200 ± 11 401 flights/day). Honeybees colonies exposed to 2.37 µg/ml of sulfoxaflor made significantly fewer flights per day during (9 575 ± 7 395) and after (14 354 ± 8 695) than before exposure to the insecticide (23 038 ± 7 921) (Table 1, Fig. 2 and Fig.S3). As a matter of fact, the daily flight activity was reduced by half during the period of exposure to sulfoxaflor as compared to before exposure (DE vs BE: Δ = -1.76 – large effect; Fig. 3; Table S2). Then, colonies made around 5 000 more flights after exposure to sulfoxaflor than during the treatment period (AE vs DE: Δ = 0.59 – medium effect; Fig. 3; Table S2).

Table 1. Results of the generalized mixed effect models assessing the effects of pesticide treatments, the period of exposure and month on the daily flight activity of honeybee colonies. Intercept represents the control group and the period before exposure to sulfoxaflor in April.

Parameters	Estimate	Standard error	t-value	p-value
Intercept	26 464.899	6 182.069	**4.281**	**< 0.01**
0.59 µg/ml sulfoxaflor	7 001.899	5 449.945	1.285	0.219
2.37 µg/ml sulfoxaflor	-4 248.850	5 487.602	-0.774	0.450
During exposure	3 526.610	2 416.930	1.459	0.145
After exposure	**6 912.470**	**2 416.930**	**2.860**	**< 0.01**
May	2 047.631	7 084.529	0.289	0.779
June	-3 541.382	6 912.618	-0.512	0.621
July	5 532.833	6 911.513	0.801	0.444
September	74.674	7 083.792	0.011	0.992
0.59 µg/ml sulfoxaflor × During exposure	-2 993.225	3 296.384	-0.918	0.364
2.37 µg/ml sulfoxaflor × During exposure	**-16 989.990**	**3 418.055**	**-4.971**	**< 0.001**
0.59 µg/ml sulfoxaflor × After exposure	-53 11.219	360.014	-1.611	0.108
2.37 µg/ml sulfoxaflor × After exposure	**-15 653.744**	**360.002**	**-4.573**	**< 0.001**
Random effect: Colony	*7 377.1*	*9 135.1*	**-**	**-**

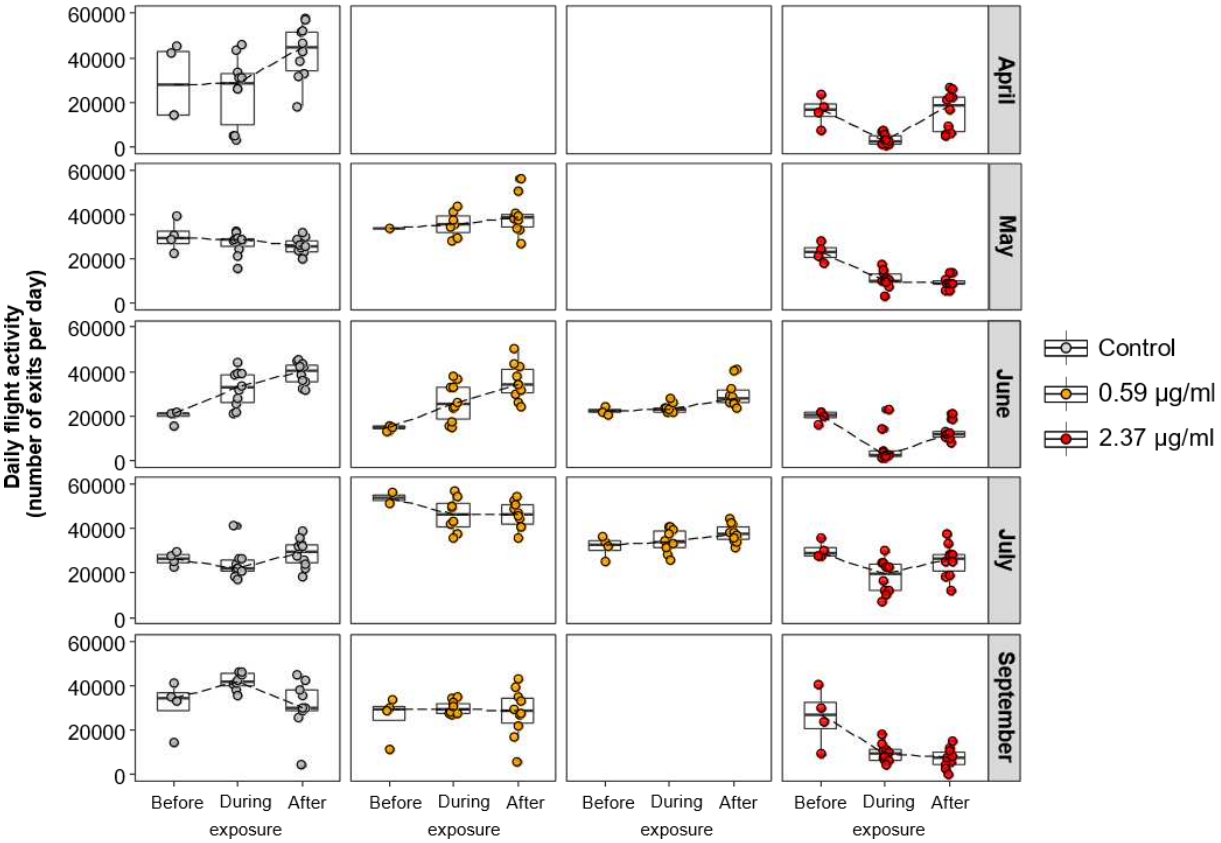

Figure 2. Daily flight activity in response to sulfoxaflor treatments. The experiment was repeated 5 times between April and September. Each plot represents the daily activity of a colony exposed or not to sulfoxaflor. The flight activity was recorded before, during and after exposure to sulfoxaflor. Box plots show the 1st and 3rd interquartile range with line denoting median. Whiskers encompass 90% of the individuals.

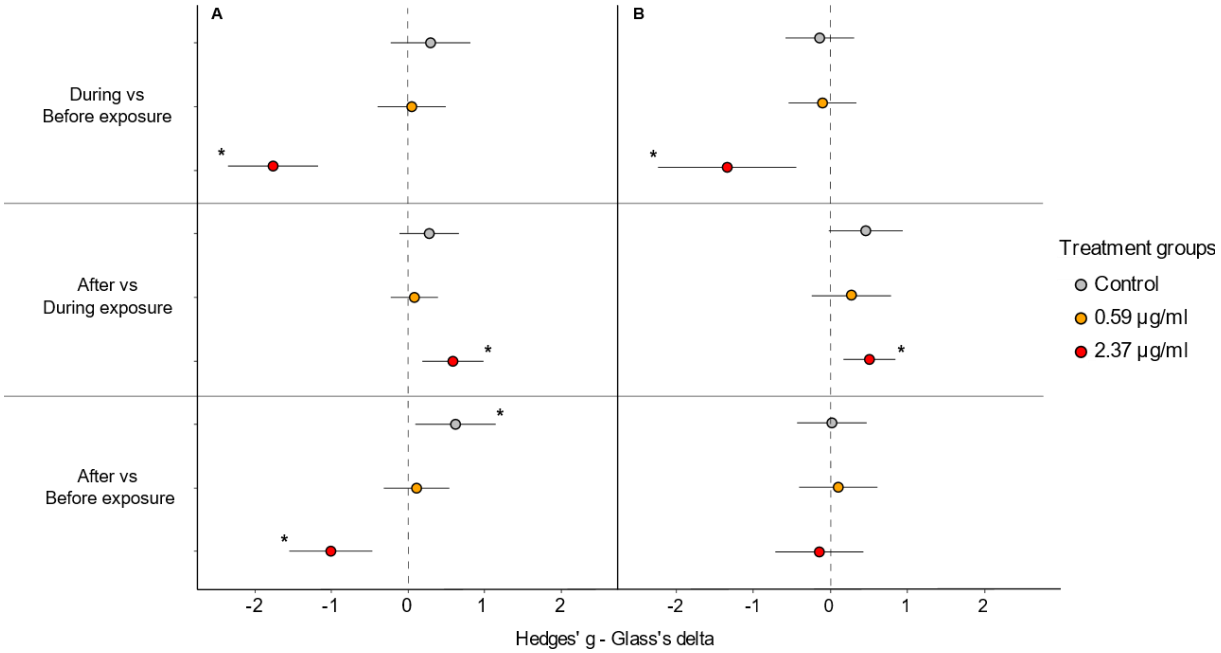

Figure 3. Effect size (hedges' g) and 95% confidence intervals (CI) of treatment effects on the daily (A) flight activity and (B) homing flight rates between the different periods of exposure. Asterisks indicate significant effect of treatment.

Colony-level effect of sulfoxaflor on the homing flight rates

The results of model selection regarding the effect of sulfoxaflor treatment on the homing flight rates are presented in Table S1 (LMM analysis). The explanatory variables in the best selected model were: treatment, exposure period and months.

The overall homing flight rates, including all three time periods (BE, DE and AE), differ significantly among the months: it was lower in May, June and July compared to April (Table 2). If none of the tested concentration affected the overall homing flight rates (i.e. BE, DE and AE pooled altogether; Table 2), a significant interaction effect between the treatment and the exposure period in colonies exposed to 2.37 µg/ml of sulfoxaflor was found (Table 2 and Fig. 4). While the homing flight rates in control colonies (BE: -2.48 ± 5.90 %; DE: -3.25 1.91 % ± and AE: -2.40 ± 2.66 %) and colonies exposed to 0.59 µg/ml of sulfoxaflor (BE: -1.89 ± 3.11 %; DE: -2.17 ± 2.08 % and AE: -1.64 ± 3.68 %) did not change across the different period of exposure, we found a significant increase in bee loss rates between the BE and DE periods in colonies exposed to 2.37 µg/ml of sulfoxaflor (Fig. 4, Table 2 and Fig. S3). A 10-fold increase in the loss rate was found between the period of exposure to sulfoxaflor (-5.97 ± 9.33 %) and the pre-exposure period (-0.54 ± 3.98 %; DE vs BE: Δ = -1.36 – large effect; Fig. 3; Table S3). After exposure to sulfoxaflor, the bee loss rate decreased and was significantly lower (-1.14 ± 5.07 %) than during the treatment period (AE vs DE: Δ = 0.52 – medium effect; Fig. 3; Table S3), and was comparable to the one observed before exposure to the insecticide (Fig. 3; Table S3).

Table 2. Parameter estimates and bootstrapped 95% confidence intervals of the linear mixed effect model assessing the effects of pesticide treatments, the period of exposure and month on the flight homing rates. Intercept represents the control group and the period before exposure to sulfoxaflor in April. Data in bold indicate a significant effect when the bootstrapped confidence intervals around the parameter estimate did not overlap with zero.

Parameters	Original estimate	Bootstrapped standard error	Lower C.I.	Upper C.I.
Intercept	-0.069	1.424	-2.898	2.724
0.59 µg/ml sulfoxaflor	1.357	1.533	-1.639	4.312
2.37 µg/ml sulfoxaflor	1.932	1.564	-1.231	4.981
During exposure	-0.767	1.175	-3.118	1.552
After exposure	0.077	1.175	-2.298	2.337
May	**-3.341**	**1.320**	**-5.887**	**-0.745**
June	**-2.534**	**1.289**	**-5.031**	**-0.020**
July	**-4.516**	**1.287**	**-7.035**	**-1.981**
September	-1.669	1.319	-4.229	0.936
0.59 µg/ml sulfoxaflor × During exposure	0.492	1.602	-2.677	3.727
2.37 µg/ml sulfoxaflor × During exposure	**-4.654**	**1.662**	**-7.921**	**-1.411**
0.59 µg/ml sulfoxaflor × After exposure	0.184	1.602	-2.897	3.418
2.37 µg/ml sulfoxaflor × After exposure	-0.695	1.664	-3.938	2.669
Random effect: Colony	*1.186*	*1.089*	*0.000*	*3.534*

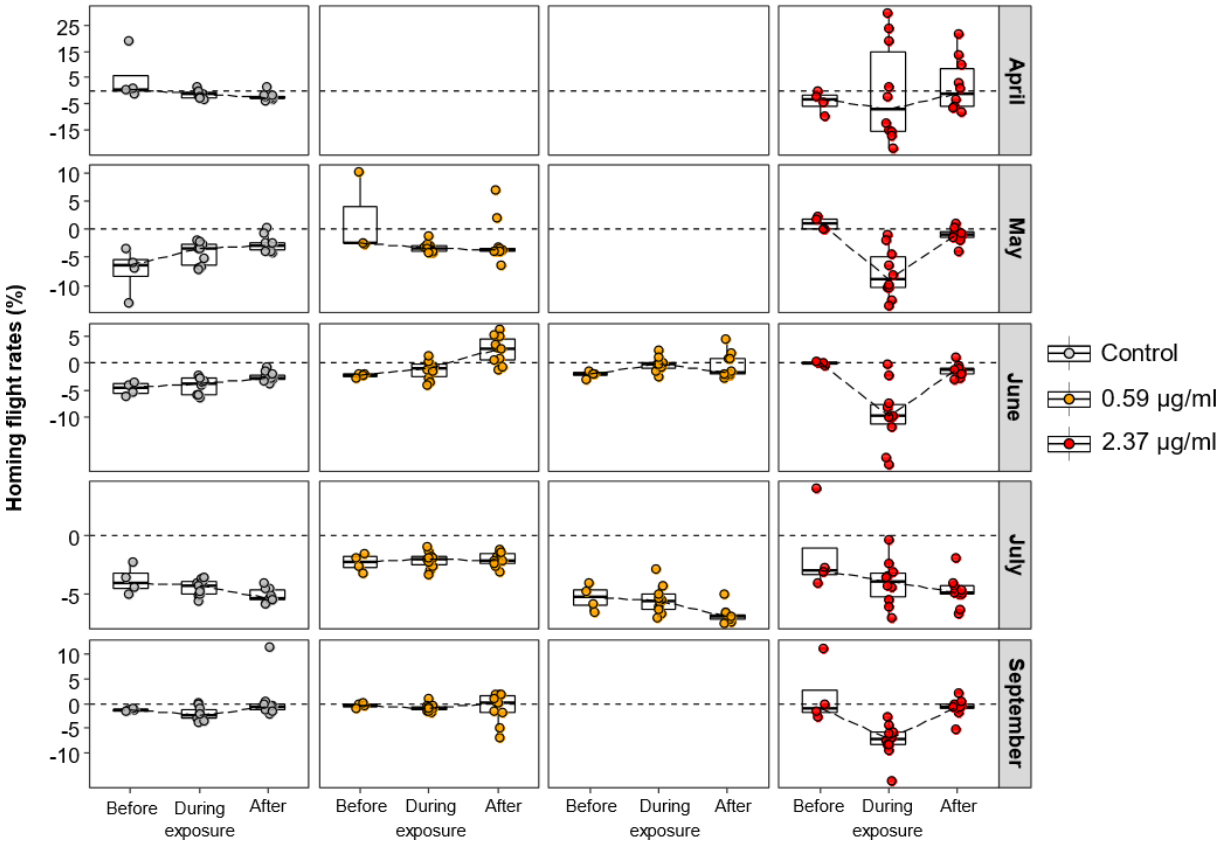

Figure 4. Daily homing flight rates in response to sulfoxaflor treatments. The homing flight rate was calculated as (Number of entries – Number of exits) / Number of exits) x 100. Values below and above the dashed line represent a loss and a gain of bees, respectively. The experiment was repeated 5 times between April and September. Each plot represents the daily homing flight rates in a colony exposed or not to sulfoxaflor. Data were recorded before, during and after exposure to sulfoxaflor. Box plots show the 1st and 3rd interquartile range with line denoting median. Whiskers encompass 90% of the individuals.

Finally, to determine whether this increase in bee losses caused by the exposure to 2.37 µg/ml of sulfoxaflor could be considered at risk or not for colonies, we compared the fold changes in bee losses to the theoretical trigger values considered to be protective for colonies, i.e. that cause a negligible reduction in colony size (EFSA 2013). We found that the fold changes in bee mortality were most of the time above these trigger values (Table 3). In some cases, homing flight rates before exposure to sulfoxaflor were positive or close to 0, but strongly negative afterwards indicating a major effect of sulfoxaflor, i.e. above the theoretical trigger values.

Table 3. Fold changes in daily homing flight rates between the pre-exposure and exposure periods to sulfoxaflor. Fold changes were determined for exposure lasting 2, 3 and 6 days. Values in bold represent the fold changes that exceeded the theoretical trigger values above which a non-negligible risk of colony size reduction has been identified (i.e. forager mortality should not be increased by a factor of 1.5 for 6 days or a factor of 2 for 3 days or a factor of 3 for 2 days). NA represent uncalculated values since some colonies prior to pesticide exposure exhibited positive homing flight rates (gain of bees).

Duration of effects (maximum protective fold-change)	Month	Homing flight rates -Before exposure	Homing flight rates -During exposure	Fold-change in bee losses
2 days (x3)	April	-4.07	-8.58	x2.11
	May	1.04	-3.63	NA
	June	0.03	-9.39	NA
	July	-1.42	-4.44	**x3.13**
	September	1.67	-3.59	NA
3 days (x2)	April	-4.07	-9.84	**x2.42**
	May	1.04	-3.08	NA
	June	0.03	-12.05	NA
	July	-1.42	-4.13	**x2.91**
	September	1.67	-4.90	NA
6 days (x1.5)	April	-4.07	-5.43	x1.33
	May	1.04	-6.12	NA
	June	0.03	-10.98	NA
	July	-1.42	-4.67	**x3.29**
	September	1.67	-7.56	NA

Colony development and survival

The number of adult bees, open and capped brood cells, as well as the amount of honey and pollen storage, measured before and after exposure to sulfoxaflor are shown for each month in Fig.S2. We did not find any effect of treatment (control, 0.59 µg/ml and 2.37 µg/ml of sulfoxaflor) on the number of adult bees and open brood cells, and on the amount of honey and pollen storage between the different periods of exposure (Table S4 and Table S5). However, the number of capped brood cells in honeybee colonies exposed to 2.37 µg/ml of sulfoxaflor was significantly lower 10 days post-exposure than before (Wilcox test: $p = 0.032$; Table S5). All colonies provided with sugar syrup only (n = 5 colonies) or with sugar syrup laced with 0.59 µg/ml of sulfoxaflor (n = 6 colonies) survived throughout the winter. However, three out of the five colonies exposed to 2.37 µg/ml of sulfoxaflor were found dead at the end of winter; these three colonies were exposed to sulfoxaflor in April, May and June, respectively.

Discussion

Within the frame of pesticide risk assessment procedures for honeybees, data on effects to colonies are required if some risks have been identified on individual bees. Such Tier 2 and 3 studies are considered more environmentally realistic but demand greater resources and can be more difficult to interpret. As a matter of fact, the high uncertainty and variability of ecotoxicological data under semi-field and field tests have contributed to some failures in detecting the risks associated to pesticides, such as neonicotinoids (Franklin and Raine 2019; Sgolastra *et al.* 2020). However, such issues could be partly resolved thanks to the recent technological advances in the monitoring of honeybee behaviors (Bromenshenk et al., 2015; Marchal et al., 2020; Meikle and Holst, 2015). Indeed, by performing an *in situ* and in real-time monitoring of colony daily flight activity, we were able to determine i) the background activity and consecutive homing rates, and then ii) to what extent they were affected by exposure to the neurotoxic insecticide sulfoxaflor for identifying risks for colonies.

We first assessed the effect of sulfoxaflor on the colony daily flight activity. Only control colonies, which were used as an environmental control, exhibited an increase in the daily flight activity between the pre- and post-exposure periods. This phenomenon might result from the colony growth and/or a higher resource need over the course of the experiment. However, we did not observe any increase in the number of adult bees or brood production during these two periods. This increase in colony activity might rather be a direct consequence of sugar syrup feeding, since forager bees have high sugar requirements to perform foraging flights (Rodney and Purdy, 2020), and providing sugar syrup to a colony generally may affect its foraging efforts (Free, 1965). While some monthly tendencies in the increase of activity were observed for colonies exposed to the lowest concentration of sulfoxaflor, the magnitude of effects was not significant. This suggests that at this concentration, sulfoxaflor prevented colonies from having the same dynamic than control colonies, and therefore triggered some minor but sublethal effects on colony activity. A stronger effect was however found at the highest sulfoxaflor concentration, with a reduction by half of the colony daily flight activity, likely due to the impairment of locomotion and foraging behaviours as previously described with neonicotinoids (Alkassab and Kirchner, 2018; C. W. Schneider et al., 2012). This confirms a recent study, which found a lower activity in colonies provided for 21 days with sugar solutions contaminated by the formulated product Closer ® (active ingredient: sulfoxaflor, 0.3 ppb) (El-Din et al., 2022). However, no change in flight activity was reported when colonies were not directly exposed to the pesticide (Tamburini *et al.* 2021). In this latter study, floral resources

were instead treated with the formulated product at the recommended maximum rate for spray applications (i.e. 0.4 L/ha), and effects started to be monitored 6 days after the application. This lack of effect might therefore reflect a lower level of exposure and corresponds to our low concentration treatment. Another difference in the experimental setup was that flight activity was estimated by counting the number of bees entering the hive within 1 min each day, which likely gives a partial estimate of colony activity since flight activity is strongly governed by environmental factors and can exhibit daily changes (Winston, 1987).

The colony-level assessment of bee losses is a key parameter used for evaluating the magnitude of effects due to pesticide exposure and setting protection goals for honeybee colonies (EFSA, 2013; EFSA et al., 2020). Indeed, colony demography and dynamic, like for any population, are strongly governed by the death rate of individuals. By monitoring the colony homing flight rates, we identified a bee loss rate of 2.77 ± 3.18 % for control colonies and of 1.90 ± 2.99 % for colonies exposed to the lowest concentration of sulfoxaflor. These values are lower but rather in accordance with a systematic review of the literature, which reported median values of daily background mortality rates of 3.5-4% for all honeybee workers (covering all behavioral roles; dataset mainly based on visual and RFID records) (EFSA, 2020). We then observed a monthly change in the overall mortality rates, with higher losses in May, June and July as compared to April. This change may be a consequence of a higher individual foraging activity during colony growth since the more time bees spend foraging, the lower is their survival probability (Prado et al., 2020). Finally, we detected a significant decrease in the homing flight rates upon exposure to the highest concentration of sulfoxaflor, while no effect was found in control and colonies exposed to the low pesticide concentration. Interestingly, the effect on bee mortality, as for the flight activity, were restricted to the period of exposure, which might be explained by the relatively rapid elimination of sulfoxaflor in honeybees (Barascou et al., 2021c). Such increase in bee mortality is consistent with previous semi-field studies, which showed adverse effects of sulfoxaflor on bee mortality after two spray applications of a crop (residues in flower ranging from 5 mg/kg on the first day of application to 0.1 mg/kg 7 days after application (Cheng et al., 2018)). In addition, if no effect of sulfoxaflor on learning and memory have been reported at doses ranging from 0.024 to 2.5 ng (Siviter *et al.* 2019), a recent study showed that honeybees fed with 26 ng of sulfoxaflor exhibited poor homing flight abilities: only 28% of them successfully came back to the colony compared to 75% for control bees (Capela et al., 2022). We could therefore reasonably assumed that the high concentration of sulfoxaflor impaired the homing ability as well as their flight or foraging capacities as often

observed with neonicotinoids (Bortolotti et al., 2003; Ma et al., 2019; Matsumoto, 2013; Monchanin et al., 2019).

In a 10 days chronic toxicity experiment performed with honeybees reared in cages, we previously found no lethal effects of sulfoxaflor at a concentration of 0.02 µg/ml, while at 2.35 µg/ml it significantly decreased bee survival (Barascou et al., 2021c). A preliminary experiment also showed that 0.1 µg/ml of sulfoxaflor was not toxic to bees (unpublished data). Altogether, these studies suggest a similar chronic toxicity in Tier 1 and Tier 2 toxicity tests. One difference might be the fold change in bee mortality since in laboratory conditions the hazard ratio for bees exposed to the high sulfoxaflor concentration was around 5, while in our current experiment this concentration trigger a 10-fold increase in bee losses. However, if mortality rates upon exposure to high sulfoxaflor concentration went up to 21.8% within a day, it rarely exceeded 13 % (8 on 50 records). This is in contradiction with a recent study suggesting that daily loss rates less than 13 % could be classified as normal (Ngo et al., 2021). Besides a few exceptions, our data suggest that mortality rates below 5 % could rather be considered as normal. Finally, it is interesting to note that in some cases, the homing flight rates were positive indicating a gain of bees for the colony. This was especially noticed during exposure to the high concentration of sulfoxaflor in the month of April. Such rates were quite high but were associated to a very low flight activity, which shows that the colony gained very few bees. This might be due to some drifting between colonies or to some bees that stayed outside overnight and entered the hive the next day.

Despite an increase in bee mortality, colony size was not impacted on the short-term. This could be explained by the lack of precision of the method used to evaluate the number of bees, since bees foraging in the field could not be included in the colony assessment. However, a significant decrease in the number of capped brood cells was found 10 days post-exposure. Since the capped brood stage lasts between 11 and 12 days, most of these individuals were under the larva stage during exposure to sulfoxaflor. The impact of sulfoxaflor might therefore be an indirect consequence of reduced foraging efforts (lower flight activity combined to a higher bee loss) and therefore less food resources for the colonies. A reduction in the storage of pollen, which is used by nurse bees to provision larvae with jelly, was notably found after 21 days of exposure to sulfoxaflor (El-Din et al., 2022). Although not significant, we could observe a similar trend with some colonies having no pollen storage at the end of the pesticide treatment. Finally, a non-mutually exclusive hypothesis would be a direct effect of sulfoxaflor on brood development due to the role of acetylcholine and its receptor on larval development (Grünewald and Siefert, 2019).

In order to have natural variations in colony state, we made the choice to not artificially homogenize their population size but rather to perform a random assignment of colonies to the different treatment groups. Although, there was no difference in colony state among these groups, colonies exposed to the high sulfoxaflor concentration tended to be initially smaller than the others. This was not associated to differences in the colony flight activity and daily bee losses, but we cannot exclude a reinforcement of effects due to a potentially smaller population. This will need to be confirmed by experimentally testing pesticide effects according to colony size.

Lastly, to determine whether the specific protection goals (SPGs) for honeybees were or not satisfied for the high sulfoxaflor concentration, we compared the observed fold changes in daily bee losses to the theoretical trigger values associated to the SPG of 7% colony size reduction. Such fold changes were often found to be above the SPG and therefore at risk for colonies. Some of them could not be determined due to positive homing flight rates before exposure to the pesticide, but the range of negative values during exposure to sulfoxaflor reflected even larger fold-changes. The risk posed by the bee loss rates was somehow confirmed by the winter loss of 3 out 5 of the colonies. Actually, by using model simulations of colony dynamics Rumkee *et al.* (2015) found that an increase in adult bee loss rates had a much greater impact on colony survival than mortality of bee larvae or reduction in egg-laying rate. In addition, bees might have stored some contaminated syrup and therefore prolong the exposure causing sublethal effects on the long-term rather than lethal effects as evidenced by the absence of increased mortality once the exposure was over. For instance, a reduction in pollen foraging activity has often been described upon exposure to pesticides in honeybees and bumblebees (Feltham et al., 2014; Gill et al., 2012; Prado et al., 2019), which might lead to carryover effects on colony survival (Requier et al., 2017). The fact that only colonies exposed in the spring died over the winter, but not the ones exposed in July and September, tends to support this hypothesis of carryover effects. Furthermore, a significant loss of foragers can lead to the early recruitment of nurse bees for foraging, and such precocious foragers were found to be less efficient than normal-age foragers: they have a lower lifespan, make fewer foraging trips and are much more likely to die during their first flights outside the hive (Barron, 2015).

In conclusion, real-time and longitudinal data on bee mortality, combined with threshold values indicating which fold changes in mortality rates start to be at risk for colonies, can contribute to improve pesticide risk assessment under field conditions. Indeed, while we did not find any major effects on the colony demographic state, bee loss rates highlighted a risk for colonies. In addition, bee counters can significantly reduce the measurement complexity of mortality rates

in the field and be relatively time-effective for use, which is essential for the regulatory assessments of pesticides. Last but not least, since the *in situ* monitoring of daily bee losses can be connected to consequences for colonies (the ultimate protection goal), this measured endpoint has a strong ecological relevance in pesticide risk assessment. All these criteria are important for test methods in chemical regulation. However, these trigger values for protecting bee colonies have been determined via *in silico* approaches (EFSA, 2013), and it remains unknown whether they correctly estimate effects on colonies. Future efforts should therefore be made to empirically link mortality rates to effects on colonies and determine mortality rates that are truly at risk for colonies.

Competing interests

The authors declare that they have no competing interests.

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Author Contributions

L.B., Y.L.C and C.A. conceived the study. L.B., D.S., D.C. and C.A. conducted the experiments. L.B, U.G., M.P., O.M. and C.A. analyzed the data. Y.L.C. and C.A. contributed to reagents. L.B, U.G. and C.A. wrote the manuscript. All authors read and reviewed the manuscript.

Supplementary information

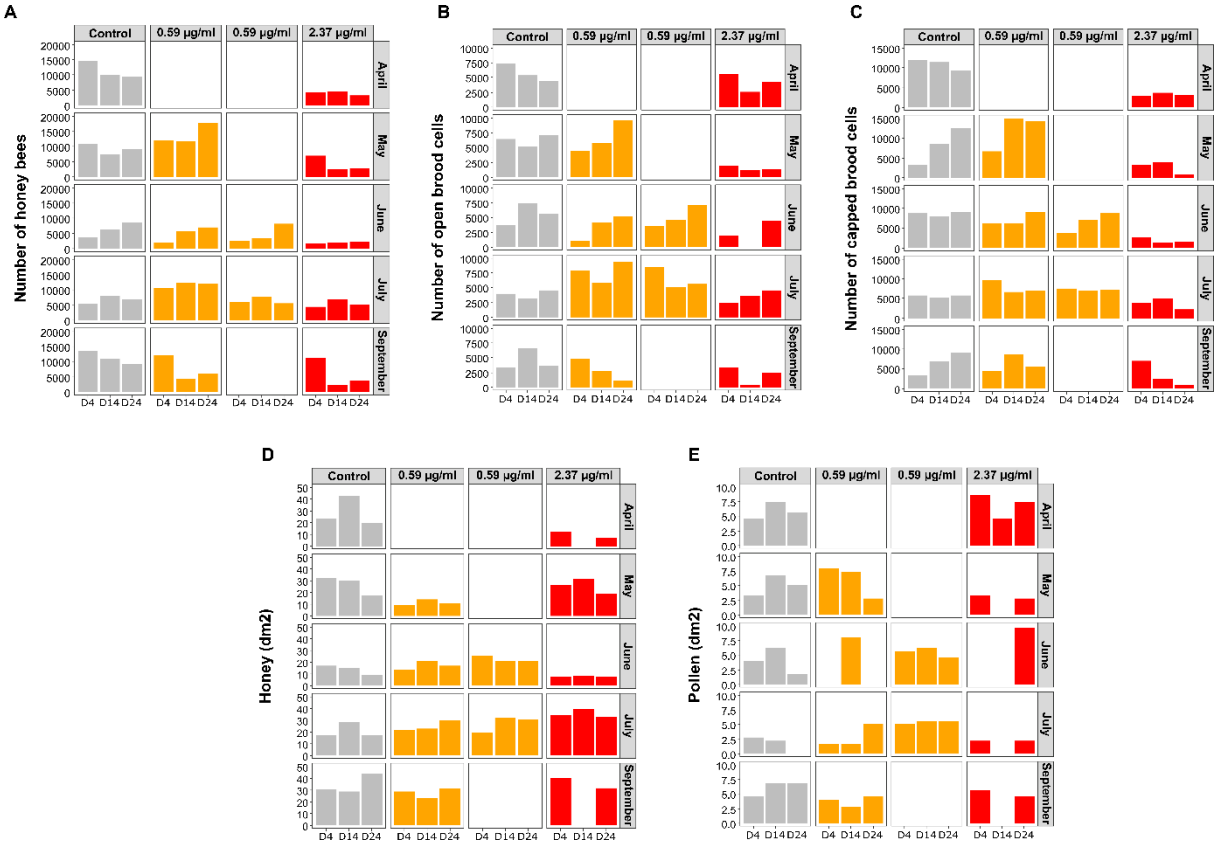

Figure S1. Number of honeybees (A), capped brood cells (B) and open brood cells (C), and amount of honey (D) and pollen stored (E) in colonies before (D4) and after exposure to sulfoxaflor (Day 4 and D24).

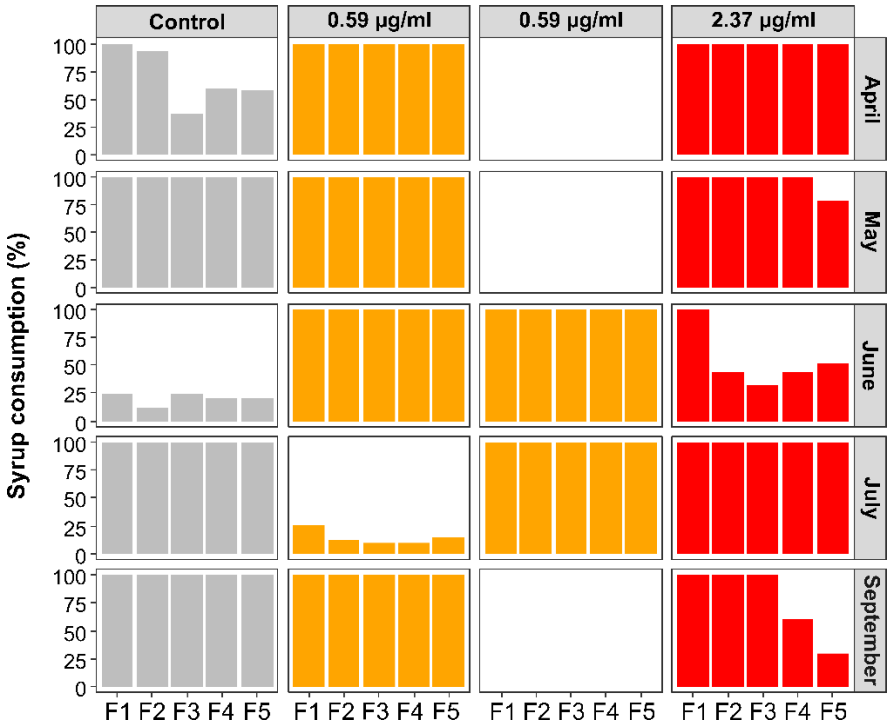

Figure S2. Percentage of syrup consumed by honeybee colonies in each treatment group. The experiment was repeated 5 times between April and September. Each colony were provided with 500 ml of pesticide-free or sulfoxaflor-contaminated sugar solution every two days over 10 days (5 feeding events – F1 to F5).

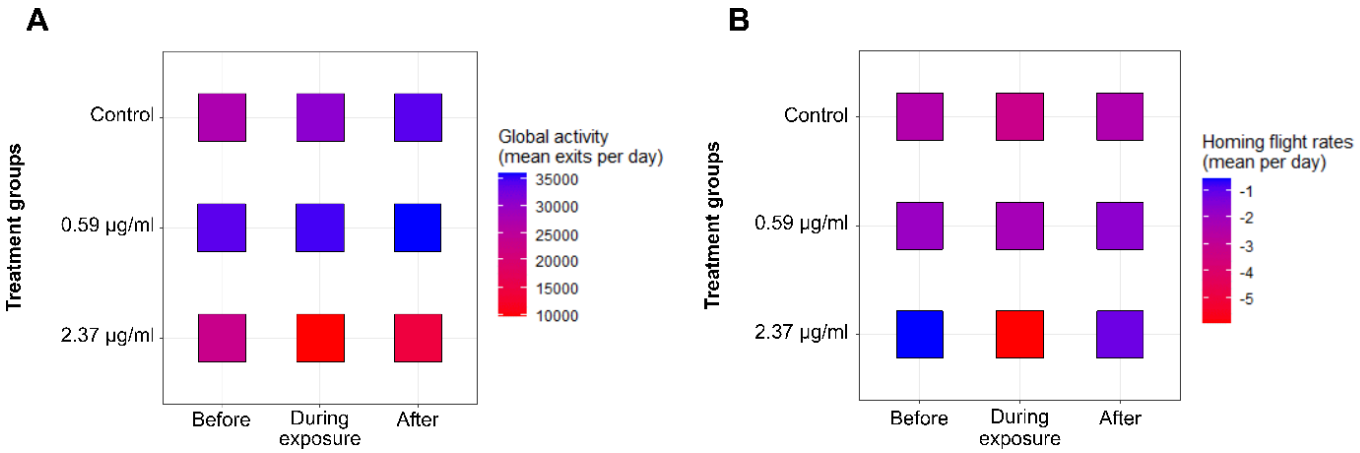

Figure S3. Global activity and homing flight rates in colonies exposed or not to 0.59 or 2.37 µg/ml of sulfoxaflor. Values were calculated for all months combined.

Table S1. Models fitted to investigate the effect of sulfoxaflor treatments on the daily flight activity and homing flight rates. For each model, the best one is in bold (lowest AICc).

Dependent variables	Fixed explanatory variables	Random explanatory variable	Df	AICc
Number of daily flights	**Treatment*exposure period + months**	**colony**	**15**	**7881.32**
	Treatment + exposure period + months treatment	colony	11	7973.81
	Treatment * exposure period	colony	11	7952.33
	Treatment + exposure period	colony	7	8044.82
	Treatment + month	colony	9	8010.66
	Exposure period + month	colony	9	8020.75
	Treatment	colony	5	8081.67
	Exposure period	colony	5	8094.24
	Month	colony	7	8057.61
	null	colony	3	8131.10
Daily homing flight rates	**Treatment*exposure period + months**	**colony**	15	**2233.89**
	Treatment + exposure period + months treatment	colony	11	2254.38
	Treatment * exposure period	colony	11	2245.21
	Treatment + exposure period	colony	7	2265.67
	Treatment + month	colony	9	2269.26
	Exposure period + month	colony	9	2258.35
	Treatment	colony	5	2280.54
	Exposure period	colony	5	2266.84
	Month	colony	7	2273.21
	null	colony	3	2281.70

Table S2. Effect size (Hedges' *g* or Glass's *delta*) of pesticide exposure on the daily flight activity. 95% CI, 95% confidence interval. Values in bold represent significant effects size when CIs do not include zero.

Comparison	Control [95% IC]	0.5ppm [95% IC]	2ppm [95% IC]
During versus Before exposure	0.30 [-0.22, 0.82]	0.05 [-0.40, 0.50]	**-1.76** [-2.35, -1.17]
After versus During exposure	0.28 [-0.11, 0.67]	0.08 [-0.23, 0.39]	**0.59** [0.19, 0.99]
After versus Before exposure	**0.62** [0.10, 1.15]	0.11 [-0.32, 0.54]	**-1.01**[-1.55, -0.47]

Table S3. Effect size (Hedges' *g* or Glass's *delta*) of pesticide exposure on the daily homing flight rates. Values in bold represent significant effects size when CIs do not include zero.

Comparison	Control [95% IC]	0.5ppm [95% IC]	2ppm [95% IC]
During versus Before exposure	-0.13 [-0.58, 0.32]	-0.09 [-0.53, 0.35]	**-1.36** [-2.24, 0.45]
After versus During exposure	0.44 [-0.04, 0.92]	0.26 [-0.26, 0.77]	**0.52** [0.18, 0.85]
After versus Before exposure	0.01 [-0.44, 0.47]	0.08 [-0.43, 0.59]	-0.15 [-0.72, 0.42]

Table S4. Comparisons of the number of honeybees, open and capped brood cells, and amount of honey and pollen stored in colonies before and at the end of the pesticide treatment (Day 4 and D14, respectively; Wilcoxon signed-rank tests).

Parameters	Treatment	Z	*p-value*
Number of honeybees	Control	14	0.841
	0.59 µg/ml of sulfoxaflor	9.5	0.772
	2.37 µg/ml of sulfoxaflor	16	0.528
Number of open brood cells	Control	10.5	0.753
	0.59 µg/ml of sulfoxaflor	7	0.886
	2.37 µg/ml of sulfoxaflor	18	0.295
Number of capped brood cells	Control	10	0.690
	0.59 µg/ml of sulfoxaflor	5	0.468
	2.37 µg/ml of sulfoxaflor	14.5	0.753
Area of honey/nectar (dm^2)	Control	11	0.841
	0.59 µg/ml of sulfoxaflor	6	0.686
	2.37 µg/ml of sulfoxaflor	7	0.310
Area of pollen (dm^2)	Control	5	0.141
	0.59 µg/ml of sulfoxaflor	9	0.885
	2.37 µg/ml of sulfoxaflor	19	0.205

Table S5. Comparisons of the number of honeybees, open and capped brood cells, and amount of honey and pollen stored in colonies before and 10 days post-exposure to the pesticide (Day 4 and D24, respectively; Wilcoxon signed-rank tests).

Parameters	Treatment	Z	*p-value*
Number of honeybees	Control	15	0.675
	0.59 µg/ml of sulfoxaflor	4	0.343
	2.37 µg/ml of sulfoxaflor	18	0.310
Number of open brood cells	Control	11	0.841
	0.59 µg/ml of sulfoxaflor	7	0.886
	2.37 µg/ml of sulfoxaflor	10.5	0.753
Number of capped brood cells	Control	5.5	0.172
	0.59 µg/ml of sulfoxaflor	5	0.468
	2.37 µg/ml of sulfoxaflor	**23**	**0.032**
Area of honey/nectar (dm^2)	Control	15	0.672
	0.59 µg/ml of sulfoxaflor	7	0.886
	2.37 µg/ml of sulfoxaflor	16.5	0.463
Area of pollen (dm^2)	Control	10	0.675
	0.59 µg/ml of sulfoxaflor	9	0.886
	2.37 µg/ml of sulfoxaflor	14.5	0.753

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C. Conclusion

Les résultats de cette étude montrent une diminution de l'activité de vol quotidienne et une augmentation des pertes d'abeilles butineuses dans les colonies exposées à la forte concentration de sulfoxaflor (2.37 µg/ml). Ces pertes quotidiennes d'abeilles ont été jugées à risque pour les colonies. Les compteurs d'abeilles se révèlent donc être un outil prometteur pour surveiller l'impact des pesticides au niveau colonial, notamment en améliorant la pertinence écologique des mesures (activités de butinage et taux de mortalités pouvant se répercuter directement sur la dynamique populationnelle).

Cet outil représenterait une avancée majeure pour la surveillance des colonies par les apiculteurs, les réseaux de surveillance épidémiologique, les agences d'évaluation du risque lié à l'usage de produits phytopharmaceutiques, et les laboratoires de recherche.

CHAPITRE IV :

DISCUSSION GENERALE ET PERSPECTIVES

I. Bilan des travaux de thèse

Par une approche intégrative combinant physiologie et comportement, les travaux réalisés durant cette thèse ont permis de répondre à deux objectifs principaux. Le premier objectif a été de mieux évaluer les facteurs de variabilité ou de modulation des réponses aux pesticides. Pour cela, deux facteurs potentiels de variabilité intraspécifique ont été étudiés : la caste comportementale et la disponibilité et qualité des ressources polliniques. Le second objectif a été de mieux évaluer les effets des pesticides, en utilisant la surveillance de l'activité de vol à l'échelle individuelle et coloniale, comme métrique de la toxicité des pesticides. Ce dernier objectif a été atteint à travers un suivi longitudinal de l'activité et performance de vol des abeilles et ainsi de leur taux de mortalité.

Mes premiers résultats montrent que les abeilles expriment une large gamme de réponses aux pesticides selon leur caste comportementale mais également selon leur état nutritionnel. Ces observations suggèrent de prendre en compte les butineuses dans les tests réglementaires de toxicité des pesticides au niveau 1 de l'évaluation des risques puisqu'elles s'avèrent être plus sensibles que les nourrices. De plus, la qualité de l'alimentation pollinique peut affecter la sensibilité des abeilles aux pesticides, en particulier à travers une élimination plus rapide du toxique. Par ailleurs, les résultats de mes études utilisant l'activité de vol comme métrique de la toxicité des pesticides, montrent qu'une exposition à une dose unique de pesticide peut avoir des effets retardés à l'activité de butinage. Enfin, la quantification de l'activité de vol à l'échelle des colonies s'avère être très prometteuse pour identifier les taux de pertes populationnelles suite à une exposition à un pesticide. Ainsi, elle permet de déterminer les risques consécutifs pour les colonies à travers la comparaison des taux de pertes à ceux ayant été identifiés comme conduisant à une diminution non-négligeable de la taille des colonies. Mes travaux de thèse, apportent ainsi des éléments à prendre en compte pour améliorer l'évaluation des risques présentés par les pesticides pour les abeilles mellifères. Les principaux résultats de ma thèse sont résumés dans la Figure 1 ci-dessous.

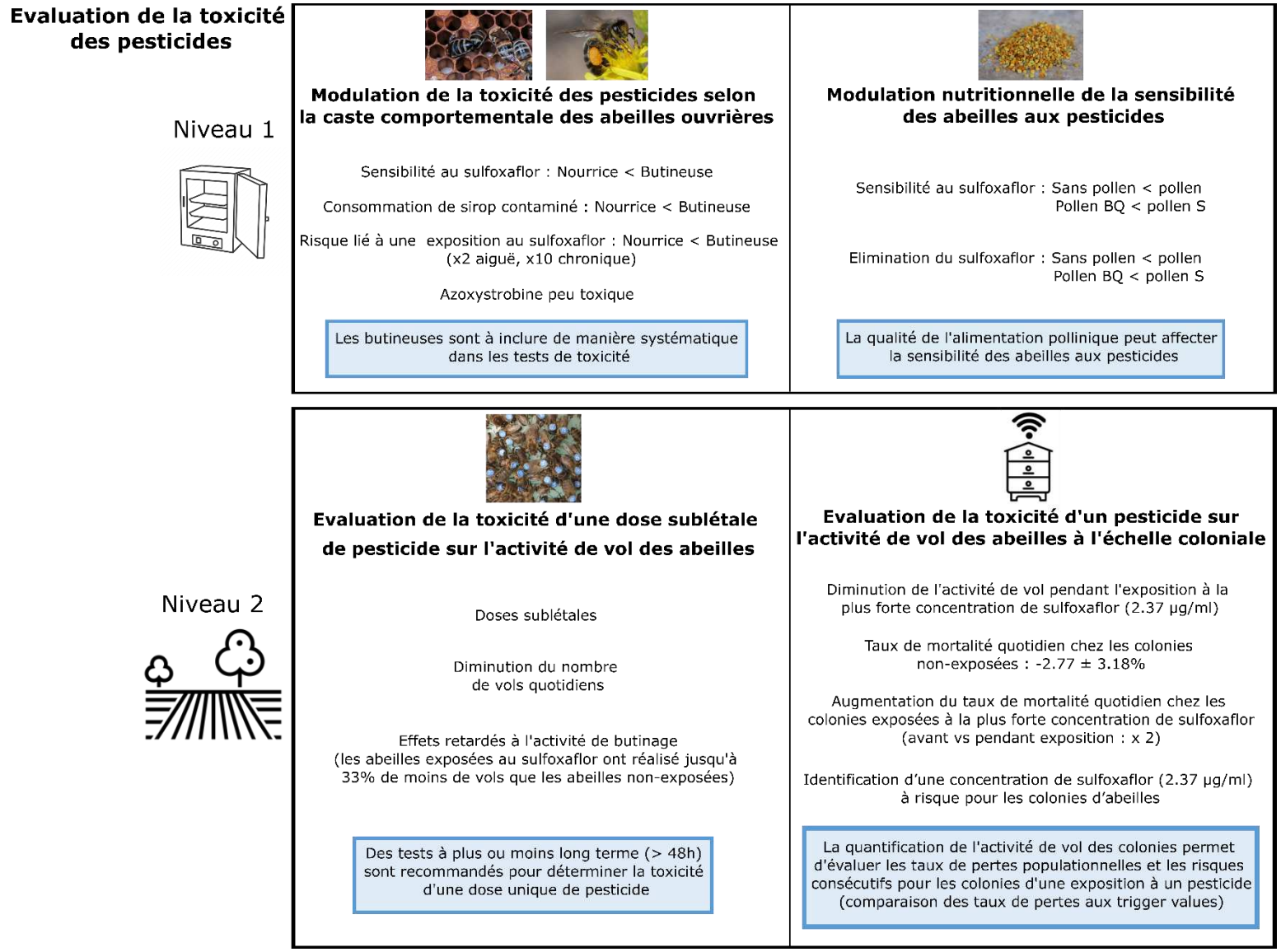

Figure 1. Schéma de synthèse des principaux résultats de mes travaux de thèse.

Après avoir rappelé les principaux résultats de mes travaux de thèse, la suite est de les discuter de façon plus intégrative en faisant des liens entre toutes les études. Tout d'abord, la faible toxicité de l'azoxystrobine observée dans mes expériences réalisées en conditions contrôlées de laboratoire est abordée. Plusieurs hypothèses basées sur le mode d'action du fongicide, la voie d'exposition utilisée dans les tests de toxicité ou encore sa métabolisation par les abeilles mellifères, sont discutées. A l'inverse, le sulfoxaflor présente une toxicité plus forte que l'azoxystrobine. L'évaluation de sa toxicité est également discutée au travers de plusieurs points : son mécanisme d'action, les concentrations testées dans mes études, les similitudes/différences d'effets observés entre le laboratoire et le terrain, ou encore la mesure de sa toxicité par les compteurs d'abeilles en conditions naturelles.

A. Evaluation de la toxicité de l'azoxystrobine

Les premières expériences menées en laboratoire ont montré une faible toxicité du fongicide azoxystrobine pour les abeilles mellifères. L'exposition aiguë à la dose maximale de 60 µg/abeille a entraîné un taux de mortalité de seulement 20% des abeilles et aucune DL_{50} n'a pu être déterminée pour les nourrices et butineuses (DL_{50} > 60 µg/abeille). Cela peut s'expliquer soit par la dose testée, pas assez haute car limitée à 60 µg/abeille en raison de la faible solubilité de l'azoxystrobine dans le solvant, soit par la métabolisation rapide du fongicide. En effet, les analyses de quantification de l'azoxystrobine, suite à une exposition aiguë, ont montré une élimination très rapide de la molécule (les quantités étant en dessous du seuil de détection 8 heures après l'exposition).

Des résultats similaires ont été obtenus lors de notre étude sur la modulation nutritionnelle de la sensibilité des abeilles à l'azoxystrobine. Lors de l'exposition aiguë, la dose d'azoxystrobine testée (57.5 µg/abeille), définie comme la DL_{50} par une étude antécédente à la nôtre (données non publiées), a causé un taux de mortalité de 10% chez les abeilles qui n'ont pas eu accès au pollen. De même lors des 16 jours d'exposition chronique, l'azoxystrobine n'a pas affecté la survie des abeilles ayant consommé ou non du pollen. Ainsi, nous avons principalement observé une absence de toxicité de l'azoxystrobine dans les tests de niveau 1 effectués au laboratoire lors d'expositions aiguës et chroniques. C'est pourquoi, les études menées sur le terrain (niveau 2) n'ont pas été réalisées avec ce fongicide mais uniquement avec le sulfoxaflor, insecticide que nous avons identifié comme à risque pour les abeilles mellifères.

Plusieurs hypothèses pourraient expliquer cette faible toxicité observée chez l'azoxystrobine. Tout d'abord, il s'agit d'un fongicide, de la famille des strobilurines, qui cible les champignons avec un

mode d'action bloquant la formation d'énergie au niveau des mitochondries (Bartlett *et al.*, 2002). Plus spécifiquement, l'azoxystrobine inhibe la respiration mitochondriale en se liant au site « Q_o » du cytochrome b (appartenant au complexe bc_1 ou complexe III), localisé dans la membrane mitochondriale interne des champignons et autres eucaryotes. Lorsque la molécule se fixe, elle bloque le transfert d'électrons entre le cytochrome b et c_1, ce qui perturbe ainsi le cycle énergétique au sein du champignon cible, en interrompant la production d'ATP (Adénosine TriPhospate) (Bartlett *et al.*, 2002). Ce mode d'action apparaît également efficace chez les invertébrés aquatiques puisque des effets néfastes du fongicide ont été démontrés dans plusieurs études (Bartlett *et al.* 2002b ; Friberg-Jensen *et al.* 2010 ; Kunz *et al.* 2017). L'étude de Wei *et al.* (2021) a notamment montré chez les chironomes (*Chironomus dilutus)* une régulation à la baisse de l'expression du cytochrome b après exposition à l'azoxystrobine, indiquant une perturbation des chaînes de transport d'électrons mitochondriales. Chez l'abeille mellifère, Christen *et al.* (2019) ont montré que des altérations mineures sur l'expression de gènes impliqués dans la phosphorylation oxydative pouvaient avoir des conséquences sur la production d'énergie et le comportement des abeilles. Cependant aucun effet létal n'a été observé.

Par ailleurs, les faibles quantités voire l'absence d'azoxystrobine dans le corps des abeilles 8 heures post-exposition, confortent l'idée d'une métabolisation extrêmement rapide du fongicide (en comparaison de celle du sulfoxaflor). A titre comparatif, une étude précédente a montré une dégradation moins rapide de l'imidaclopride chez les abeilles mellifères exposées une dose de 41 ng/abeille avec une demi-vie du toxique d'environ 22 heures (Schott *et al.*, 2017). Piechowicz *et al.* (2021) ont cependant observé une dégradation rapide du pyréthrinoïde λ-cyhalothrin, puisque la majorité de l'insecticide a été éliminé 6 heures après l'intoxication. La vitesse de métabolisation des pesticides est donc variable et pourrait dépendre de la spécificité du système de détoxification (Berenbaum and Johnson, 2015). A cet effet, l'azoxystrobine pourrait provoquer l'induction de mono-oxygénases du cytochrome P450 (CYP450), pour aboutir à une augmentation de la détoxification du fongicide et ainsi à la diminution de sa toxicité. En effet, chez les abeilles mellifères, les CYP450 représentent une famille multigénique dont certains gènes issus principalement des familles CYP6 et CYP9, participent à la détoxification des acaricides (Mao *et al.*, 2011). De plus, certaines enzymes CYP450, sont connues pour avoir une spécificité de substrat (Mao *et al.*, 2011). Ainsi, les abeilles pourraient posséder des CYP450 capables de fixer et de métaboliser plus particulièrement des molécules telles que l'azoxystrobine. Il serait ainsi intéressant de déterminer si l'azoxystrobine stimule l'expression de *cyp450* et de comparer ses effets sur le système de détoxification à celui d'autres pesticides, notamment ceux dégradés plus lentement.

Enfin, dans nos protocoles expérimentaux, les abeilles ont été uniquement exposées au pesticide par voie orale. Cependant, dans les conditions naturelles, les abeilles peuvent être exposées par contact direct, notamment lorsque les pesticides sont appliqués par pulvérisation aérienne dans les champs, comme c'est le cas pour le fongicide azoxystrobine. Il serait donc intéressant d'évaluer la sensibilité des abeilles mellifères à l'azoxystrobine, en les exposant directement par voie topique, et déterminer s'il ne devient pas plus toxique par cette voie d'exposition pour les abeilles. En effet, la toxicité de certains pesticides peut dépendre de la voie d'exposition (oral vs contact) avec parfois une plus forte toxicité observée lors d'une exposition topique. Par exemple, les insecticides pyréthrinoïdes sont en moyenne trois fois plus toxiques pour les abeilles mellifères par contact que par voie orale (Sanchez-Bayo and Goka, 2014).

B. Evaluation de la toxicité du sulfoxaflor

Les résultats de mes travaux de thèse montrent que le sulfoxaflor présente une plus forte toxicité que l'azoxystrobine. Ce qui peut s'expliquer par une meilleure interaction du sulfoxaflor avec sa cible (récepteurs nicotiniques à l'acétylcholine – nAChR), des effets plus délétères sur la physiologie et/ou comportement des abeilles, une moins bonne élimination du sulfoxaflor, ou bien une combinaison de deux ou trois de ces facteurs.

Le sulfoxaflor s'est révélé particulièrement plus toxique pour les butineuses que pour les nourrices (41.04 ng/abeille vs 54.40 ng/abeille). Comme expliqué dans le chapitre II (article 1), ces différences sont probablement dues à des disparités dans le poids plutôt que dans les capacités d'élimination du pesticide. De plus, la différence de consommation de nectar (ou sirop dans les expériences) en faveur des butineuses par rapport aux nourrices, peut se retrouver renforcée lorsque le nectar est contaminé par un pesticide (sulfoxaflor). Au final, une plus forte toxicité du sulfoxaflor ajoutée à une exposition plus élevée a conduit à un risque plus accru pour les butineuses. Ces résultats montrent qu'il est nécessaire de prendre en compte des paramètres telle que la consommation de la matrice contaminée, afin de mieux déterminer l'exposition réelle des abeilles mellifères, et en particulier d'inclure systématiquement les abeilles butineuses dans les tests réglementaires de toxicité des pesticides de niveau 1. Puisqu'un risque a été identifié à ce premier niveau d'évaluation, la toxicité du sulfoxaflor a été évaluée au niveau 2 sur le terrain.

Dans le test de toxicité de deux doses de sulfoxaflor (16 et 60 ng/abeille) en condition de terrain, il est apparu qu'elles n'ont pas diminué les probabilités de survie des abeilles, contrairement à ce qui a été observé en laboratoire avec des doses de 41.04 ng/abeille et 54.40 ng/abeille, induisant une mortalité de 50 % chez les nourrices et butineuses respectivement. Différentes hypothèses

peuvent expliquer cette différence d'effet entre le laboratoire et le terrain. La première repose sur une probabilité de survie moindre des abeilles en condition de terrain (élevées dans les colonies) qu'en conditions contrôlées de laboratoire (température constante, nourriture *ad libitum*, pas d'exposition à des parasites ou prédateurs, …). Ce phénomène a été observé et quantifié en comparant la probabilité de survie d'abeilles après exposition au parasite *Nosema ceranae* ou à une piqûre dans l'abdomen pour simuler un stress (Alaux *et al.*, 2014). Les abeilles, en particulier non-exposées à un facteur de stress, ont eu une longévité réduite en colonie par rapports aux abeilles élevées en conditions contrôlées de laboratoire, et les effets d'un stress ont été diminués voire ont disparu chez les abeilles élevées en colonies (Alaux *et al.*, 2014). Ceci s'explique probablement par une survie très largement réduite sur le terrain chez les abeilles témoins. Il est fort probable que l'effet du stress soit toujours présent mais réduit par rapport aux témoins en conditions de laboratoire, du moins pour les doses de sulfoxaflor utilisées et dans nos conditions expérimentales.

Par ailleurs, dans les études réalisées au laboratoire, les abeilles sont uniquement nourries avec du sirop contaminé par les pesticides et ne peuvent pas choisir d'autres sources d'alimentation ; il s'agit d'expériences appelées « no-choice experiments ». Cependant, dans les conditions naturelles, les abeilles peuvent se nourrir de miel et de pain d'abeilles d'origines différentes. Or, la consommation de ces ressources peut diminuer la toxicité des pesticides. En effet, plusieurs études ont montré un effet positif de l'alimentation pollinique sur la résistance des abeilles mellifères aux pesticides (Crone and Grozinger, 2021; Schmehl *et al.*, 2014; Wahl and Ulm, 1983), et mes travaux ont également révélé que la qualité des pollens peut influencer la capacité des abeilles à métaboliser les pesticides (Barascou *et al.*, 2021c). De même, le miel et ses composants sont connus pour augmenter l'expression des gènes de détoxification chez l'abeille mellifère (Mao *et al.*, 2013).

Dans les tests de niveau 2, un autre résultat marquant fut la diminution de l'activité quotidienne des vols et du nombre total de vols suite à une exposition à une seule dose de sulfoxaflor. Des effets à court-terme étaient plutôt attendus. Nos résultats sont cependant concordants avec l'étude de vol de retour de Capela *et al.* (2022) montrant que 28% des butineuses exposées à une dose de sulfoxaflor (26 ng/abeille) ne rentrent pas à la colonie. Une hypothèse possible pour expliquer ces résultats serait une perturbation du système nerveux par l'insecticide. En effet, le sulfoxaflor est un neurotoxique qui agit sur les nAChR, et a probablement un mode d'action similaire aux néonicotinoïdes (Sparks *et al.*, 2013). Ces derniers sont connus pour impacter négativement les performances cognitives et comportementales des abeilles (Belzunces *et al.* 2012 ; Siviter *et al.*

2018). Les néonicotinoïdes se lient aux nAChR 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 (Christen *et al.*, 2016). Des mesures *in situ* du système nerveux des pucerons *T. salignus* exposés au sulfoxaflor ont montré des effets néfastes de l'insecticide sur l'activité neuronale (Cutler *et al.*, 2013). En effet, un blocage rapide des potentiels d'action spontanés a été observé, comme dans le cas d'exposition aux néonicotinoïdes (imidaclopride et nitenpyram) (Cutler *et al.*, 2013). Par conséquent, cela confirme qu'au niveau des récepteurs et des neurones, le sulfoxaflor se comporte d'une manière similaire à l'imidaclopride chez les pucerons. Cependant, il est a noté que des différences dans la sélectivité et l'affinité du sulfoxaflor et de l'imidaclopride avec les nAChRs ont été observées chez le phasme morose (*Carausius morosus*) (Oliveira *et al.*, 2011). Nous pouvons tout de même supposer que le sulfoxaflor affecte l'activité de vol des abeilles par le biais de déficiences cognitives (ex. orientation, apprentissage) comme celles observées suite à une exposition aux néonicotinoïdes (Bortolotti *et al.*, 2003; Decourtye *et al.*, 2004a, 2004b; Fischer *et al.*, 2014; Jiang *et al.*, 2018; Ludicke and Nieh, 2020; Vandame *et al.*, 1995).

Dans cette étude, nous avons également constaté une réponse retardée du sulfoxaflor puisque les effets sur l'activité de vol sont apparus une semaine après l'exposition au pesticide. Ce délai de réponse peut dépendre des processus physiologiques affectés. En effet, pendant la maturation comportementale des abeilles mellifères, les jeunes abeilles présentent des changements dans l'expression des gènes du cerveau, essentiellement achevés vers l'âge de 8 jours (Whitfield *et al.*, 2006) et qui coïncident avec des changements structurels du cerveau (Farris *et al.*, 2001). Or, nous avons exposé nos abeilles à l'âge de 7 jours, ce qui concorde avec le moment où ces changements neurophysiologiques des abeilles ont lieu. Le sulfoxaflor, qui semble affecter les processus neuronaux, a pu provoquer des changements intrinsèques dans le cerveau des abeilles, altérant ainsi leurs performances de butinages ultérieures. Ces réponses retardées pourraient expliquer que les tests cognitifs réalisés dans certaines études ne détectent pas toujours des effets puisque la plupart sont réalisés presque immédiatement après l'exposition au pesticide. C'est le cas dans l'étude de Siviter *et al.* (2018), dans laquelle aucun effet négatif sur l'apprentissage et la mémoire des abeilles mellifères et des bourdons n'a été détecté une heure après exposition au sulfoxaflor. Même si dans cette étude les individus testés étaient des butineuses, nous ne pouvons exclure des effets différés au-delà d'une heure.

Lors des expositions chroniques une certaine cohérence a été observée dans la toxicité des différentes concentrations de sulfoxaflor testées en conditions de laboratoire et de terrain : les

concentrations de 0.02 µg/ml et 0.021 µg/ml de sulfoxaflor se sont révélés non toxiques pour les abeilles en condition de laboratoire et de terrain alors que celles à 2.35 µg/ml et 2.37 µg/ml ont induit une forte toxicité sur les abeilles dans les deux conditions de tests.

Notre étude en conditions naturelles a donc confirmé la toxicité de l'insecticide observée dans nos tests toxicité de niveau 1. Des taux de pertes importants d'abeilles ont été observés pendant l'exposition au pesticide, engendrant un risque pour la colonie entière sur le long terme avec trois colonies mortes sur cinq après l'hiver. Cela peut s'expliquer par un déséquilibre entre les abeilles de la ruche et les butineuses. En effet, si la ruche perd des butineuses, des abeilles plus jeunes sont recrutées pour les remplacer dans l'activité de butinage (Barron, 2015; Huang *et al.*, 1994). De nombreuses études ont montré que le butinage précoce des jeunes abeilles est une réponse commune à divers facteurs de stress tels la privation de pollen (Free, 1960; Janmaat and Winston, 2000) et l'exposition à des agents pathogènes (Higes *et al.* 2008 ; Dussaubat *et al.* 2013 ; Pettis *et al.* 2013). Cette réponse adaptative permet de remplacer rapidement les pertes de butineuses. Cependant, face à des facteurs de stress chronique, cela peut être problématique et mener à un effondrement de la colonie. Tout d'abord, Perry *et al.* (2015) ont montré que les butineuses précoces sont moins efficaces que les butineuses d'âge normal : elles vivent moins longtemps et sont plus susceptibles de mourir lors de leurs premiers vols à l'extérieur de la ruche. De plus, un début précoce de butinage réduit le nombre d'abeilles de la ruche qui s'occupent du couvain (Khoury *et al.* 2011 ; Barron 2015). D'autant plus que dans notre étude, les colonies entières ont été exposées et donc une mortalité des jeunes abeilles a pu avoir lieu, en plus de la mortalité des butineuses observées.

Les compteurs d'abeilles, en enregistrant de manière automatique et en temps réel l'activité de vol des abeilles permettent d'améliorer considérablement la robustesse et la précision de ces mesures. De plus, ce type d'outils permet de diminuer l'incertitude dans l'interprétation des données dans les tests d'évaluation des risques de terrain (niveau 2 et 3). Par ailleurs, les compteurs d'abeilles réduisent la complexité de la mesure des taux de mortalité sur le terrain. Enfin, les taux de mortalité quotidiens enregistrés en conditions naturelles et à l'échelle de la colonie apportent une forte pertinence écologique à l'évaluation des risques liés aux pesticides. Tous ces critères sont importants pour la validation des méthodes d'essai dans un contexte réglementaire d'évaluation des risques liés aux pesticides (OECD, 2005).

II. Perspectives

Mes travaux de thèse démontrant une sensibilité plus importante des butineuses à un insecticide que les nourrices s'accordent avec des études précédentes montrant une toxicité des insecticides augmentant avec l'âge des abeilles (Rinkevich *et al.* 2015 ; Tosi and Nieh 2019 ; Zhu *et al.* 2020). Il est donc primordial d'inclure les butineuses dans les tests réglementaires de toxicité de niveau 1. Cependant, des études complémentaires semblent nécessaires afin de déterminer si cette différence de sensibilité entre les castes d'ouvrières est généralisable à d'autres pesticides tels que les fongicides ou herbicides. En effet, ces deux autres familles de pesticides restent très utilisés en agriculture et de nombreuses études ont montré des effets de ces pesticides sur la mortalité des abeilles (Cullen et al., 2019b). De plus, les fongicides et herbicides affectent tous deux le métabolisme énergétique des abeilles (Mao *et al.* 2017 ; Cullen *et al.* 2019 ; Belsky and Joshi 2020 ; He *et al.* 2020). Ce qui, combiné aux différences dans le métabolisme énergétique des nourrices et butineuses (Bordier *et al.*, 2017 ; Huang *et al.*, 1994 ; Toth and Robinson, 2005), suggère des différences de sensibilité des nourrices et butineuses aux fongicides et herbicides.

De plus, l'étude sur la modulation nutritionnelle de la sensibilité des abeilles aux pesticides indique un large éventail de réponses aux pesticides sur le terrain, puisque l'abondance et la composition des régimes polliniques des abeilles mellifères sont très variables selon les paysages et les saisons. Cependant, dans notre expérience seulement deux pollens de qualités différentes ont été testés. D'autres recherches sont donc nécessaires pour évaluer l'influence d'une plus large gamme de pollen sur la toxicité des pesticides, en se focalisant en particulier sur des pollens avec des teneurs différentes en composés phytochimiques ou avec des ratios variables de protéines/lipides. En effet, l'ensemble de ces constituants du pollen sont connus pour affecter la sensibilité des abeilles aux pesticides (Liao *et al.* 2017 ; Bernklau *et al.* 2019 ; Vaudo *et al.* 2020 ; Crone and Grozinger 2021). Ces futures études pourraient ainsi permettre de prédire dans une certaine mesure la gamme de sensibilité des abeilles aux pesticides en fonction de la qualité des pollens.

Par ailleurs, chaque expérimentation développée dans cette thèse a été réalisée sur *Apis mellifera*, sans contrôle supplémentaire de la génétique de chaque abeille ou colonie testée. Or, la diversité génétique des populations d'abeilles peut influencer leur capacité d'adaptation à divers stress environnementaux, tels que les pesticides (Tahori *et al.* 1969 ; Ladas 1972 ; Suchail *et al.* 2000 ; Laurino *et al.* 2013 ; Sandrock *et al.* 2014), les virus (Desai and Currie, 2015) ou les agents pathogènes (Seeley and Tarpy, 2007 ; Tarpy, 2003). En particulier, Rinkevich *et al.* (2015) ont montré des différences de sensibilité aux pesticides entre des abeilles issues de plusieurs sous-

espèces (carniolienne, italienne et russe). Ce facteur génétique pourrait expliquer les différences de résultats obtenus dans nos expériences et ceux d'études précédentes. En effet, la DL_{50} de l'azoxystrobine déterminée lors d'une étude précédente (57.5 µg/abeille, données non publiées – communication personnelle) est apparue moins toxique pour les abeilles dans nos tests de toxicité (Article 2), et la DL_{50} du sulfoxaflor calculée pour les butineuses (41.04 ng/abeille) et nourrices (54.40 ng/abeille) se trouvent trois fois plus faible que celle reportée par l'EFSA (146 ng/abeille). Nous pouvons supposer que des différences dans les fonds génétiques entre les abeilles testées dans les études peuvent conduire à des réponses dissemblables aux pesticides. Il serait ainsi pertinent d'approfondir les études sur le rôle du patrimoine génétique sur la sensibilité des abeilles aux pesticides. Par exemple, des abeilles issues d'une génétique différente pourrait avoir des capacités de métabolisation des pesticides différentes, comme il a été montré que l'expression de gènes codant pour des cytochromes P450 diffère selon la souche génétique des abeilles (Derecka *et al.* 2013).

Les nouveaux outils technologiques sont efficaces pour suivre automatiquement et en continu l'activité de butinage et les vols de retour à la ruche des abeilles dans les études toxicologiques. A cet effet, le test de vol de retour a été identifié comme une méthode appropriée pour évaluer les effets de doses sublétales de pesticides sur les abeilles mellifères dans des conditions de terrain (EFSA, 2013, 2012), et a récemment été approuvé par l'OCDE pour l'évaluation des risques liés aux pesticides (OECD, 2021). Cependant, si la technologie associée à du marquage d'abeilles (ex. RFID) est très pertinente pour la surveillance des abeilles, elle fournit des données limitées dans le temps car basée sur une cohorte d'abeilles à la fois. Caractériser la mortalité des abeilles en temps réel à l'échelle de la colonie nécessiterait alors un marquage régulier et continu des abeilles dans le temps. Dans ce contexte, notre dernière étude renforce l'idée selon laquelle les compteurs d'abeilles sont prometteurs pour évaluer la toxicité des pesticides à l'échelle coloniale ainsi que les risques associés. L'objectif suivant serait de tester l'efficacité de ces compteurs en plein champs. Pour cela, les colonies d'abeilles munies d'un compteur pourraient être placées dans différents contextes agro-environnementaux afin de suivre la mortalité des abeilles et la relier à une exposition aux pesticides. Enfin, la dernière étape consiste à traduire l'augmentation des taux de mortalité en risque pour les colonies. A cette fin, des objectifs de protection spécifique (*specific protection goals* - SPG) peuvent être établis. Par exemple, une réduction de la taille des colonies qui ne dépasse pas 7 % a été considérée comme négligeable et donc protectrice pour les colonies d'abeilles (EFSA, 2013). Ainsi, la mortalité des butineuses ne doit pas être augmentée par rapport aux témoins d'un facteur 1,5 pendant 6 jours ou d'un facteur 2 pendant 3 jours ou d'un facteur 3

pendant 2 jours. Cependant, ces données de mortalité pour la protection des colonies d'abeilles ont été déterminées à partir d'un modèle de la dynamique populationnelle des colonies (Khoury *et al.*, 2011). Ainsi, il serait intéressant de lier de manière empirique les taux de mortalité mesurés par les compteurs d'abeilles à des effets sur les colonies, en les augmentant artificiellement par la capture d'abeilles butineuses. Cela permettra de déterminer les taux de mortalité à risque pour les colonies, c'est-à-dire engendrant une diminution de taille des colonies au-dessus de 7% ou 10 % ; un SPG de 10% étant en cours de validation par l'Union européenne ; EFSA 2021).

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ANNEXES

frontiers in Sustainable Food Systems

ORIGINAL RESEARCH
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Variations in Nutritional Requirements Across Bee Species

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With 2,000 species currently recorded in Europe, bees are a highly diversified and efficient group of pollinating insects. They obtain their nutrients from nectar and pollen of flowers. However, the chemical composition of these resources, especially of pollen (e.g., protein, lipid, amino acids, fatty acids, or sterol content), is highly variable among plant species. While it is well-known that bees show interspecific variation in their floral choices, there is a lack of information on the nutritional requirements of different bee species. We therefore developed original experiments in laboratory conditions to evaluate the interspecific variations in bee nutritional requirements. We analyzed the chemical content of eight pollen blends, different in terms of protein, lipid, amino acids, and sterols total concentration and profiles. Each pollen blend was provided to four different bee model species: honey bees (*Apis mellifera*), bumblebees (*Bombus terrestris*), mason bees (*Osmia bicornis* and *Osmia cornuta*). For each species, specific protocols were used to monitor their development (e.g., weight, timing, survival) and resource collection. Overall, we found that the nutritional requirements across those species are different, and that a low-quality diet for one species is not necessarily low-quality for another one. While honey bees are negatively impacted by diets with a high protein content (∼40%), bumblebees and mason bees develop normally on these diets but struggle on diets with a low total amino acid and sterol content, specifically with low concentrations of 24-methylenecholesterol and β-sitosterol. Overall, our study supports the need of conserving and/or introducing plant diversity into managed ecosystems to meet the natural nutritional preferences of bees at species and community level.

Keywords: bees, pollen, nutrition, bumble bee, honey bee, *Osmia*, nutrients

INTRODUCTION

With more than 2,000 species recorded in Europe (Rasmont et al., 2017), bees represent a highly diverse group of pollinators (Michener, 2007; Danforth et al., 2013). These species show a wide variability in various traits such as body size (i.e., from 0.3 mm to 4.5 cm in Europe), social behavior (e.g., cleptoparasitic, solitary, eusocial), nesting behavior (e.g., cavity- or soil-nesting), foraging strategies (e.g., pollen generalist or specialist), or phenology (e.g., uni- or bivoltine) (Michener, 2007; Michez et al., 2019). This diversity is crucial for the successful sexual reproduction of wild and domesticated plants, but it is also critical to

understand this variability to implement efficient conservation programs (Nieto et al., 2014). Indeed, bees are the dominant pollinators of crop and wild plants in most ecosystems, visiting more than 90% of crop varieties (Potts et al., 2016). Some generalist bee species have been domesticated and are now used for crop pollination like the western honey bee (*Apis mellifera*), the buff-tailed bumblebee (*Bombus terrestris*) (Banda and Paxton, 1991; Velthuis and Doorn, 2006), and a few solitary species (Gruber et al., 2011; Pitts-Singer and Cane, 2011). However, unmanaged species are still key pollinators as there are many genus-specific plant-pollinator interactions, linking wild plant diversity to wild bee diversity (Ollerton, 2017). Moreover, wild bees have been shown to increase crop production by up to twice as much as honey bees, underlining the importance of wild bees even in agro-ecosystems (Garibaldi et al., 2013; Weekers et al., 2022).

Losses and declines in managed and wild bee populations have been reported worldwide (Cameron et al., 2011; Goulson et al., 2015; Duchenne et al., 2020). Habitat loss and agricultural intensification, resulting in landscape simplification have been identified as important drivers of pollinator decline (Winfree, 2010; Persson et al., 2015; Vray et al., 2019). These factors can directly or indirectly affect the quality, the quantity and the diversity of floral resources and thus the food sources of bees (e.g., Roger et al., 2017b). This makes the abundance, distribution/availability, quality and diversity of these resources potentially a main proximal pressure explaining bee population trends (Roulston and Goodell, 2011; Vaudo et al., 2015).

Bees obtain their carbohydrate nutrient intake mainly from nectar, and their protein and lipid from pollen (Roulston and Cane, 2000; Nicolson, 2011). The chemical composition of pollen is highly variable across floral species, between 2–60 and 1–20% for protein and lipid contents, respectively (Roulston and Cane, 2000; Vaudo et al., 2020). Field and semi-field studies showed that this chemical composition can be related to bee health (e.g., honey bee *A. mellifera*: Alaux et al., 2010; Brodschneider and Crailsheim, 2010; Di Pasquale et al., 2013, mason bee *Osmia bicornis*, Bukovinszky et al., 2017). Generalist bees seem able to assess pollen chemical quality and balance multiple macronutrient resources when making foraging decisions (Vaudo et al., 2016, 2018; Kraus et al., 2019; Ruedenauer et al., 2020). Based on a large quantity and diversity of samples, Vaudo et al. (2020) showed that honey bees collected pollens between 1:1 and 2:1 protein to lipid (P:L) ratio. This species appears to occupy a different nutritional space compared to *Bombus impatiens* and *Osmia cornifrons*, which collect at P:L ratios of 4:1 and 2:9, respectively. Furthermore, to satisfy the food intake of colonies with numerous individuals, honey bees must collect large amounts of pollen. Therefore, honey bees collect pollen from generalist, open floral morphologies such as mass blooming trees (e.g., *Quercus* sp., *Salix* sp., *Prunus* sp.) and wild herbs with high production of pollen (e.g., Asteraceae), which may have a nutritional make up that falls in the lower P:L values (i.e., 1–3:1 P:L) (Vaudo et al., 2020). Bumblebees look much more picky in their choices, since many species mainly forage on Fabaceae pollen showing a high P:L ratio value (3.8 ± 0.5) (Leonhardt and Blüthgen, 2012; Wood et al., 2021). In contrast to honey bees and bumblebees, *Osmia cornifrons,* a solitary foraging bee with a short flight period, has mixed preferences for Rosaceae and Fabaceae pollen (Haider et al., 2014; Nagamitsu et al., 2018), with average P:L ratios of 1.6 ± 0.3 and 3.8 ± 0.5, respectively.

Regarding chemical profiles, particular lipids and proteins seem more also important in bee nutritional requirement. For example, sterols (e.g., β-sitosterol) are essential to synthetize ecdysteroid, involved in the molting of the larvae and the maturation of the ovaries of female imago. In case of sterol deficiency, a delay in molting can be observed (Regali, 1996). Additionally, a good amino acid balance is also crucial for the bee development (Moerman et al., 2016). They are involved in growth, survival, flight ability or in immunity (Regali, 1996; Carter et al., 2006; Moerman et al., 2016). Some amino-acids (methionine, lysine, threonine, histidine, leucine, isoleucine, valine, phenylalanine, tryptophan) and sterols (24-methylenecholestrol and β-sitosterol) cannot be synthetised by the bee and are therefore considered as essential, meaning that it is necessary to obtain them through pollen consumption (De Groot, 1953; Svoboda et al., 1978; Behmer and Nes, 2003).

Experimental studies in controlled conditions have confirmed that the nutritional quality of pollen (e.g., the concentration of protein, and lipids, sterols, and amino acids) can have an impact on the development and mortality of bumblebees (e.g., Taseï and Aupinel, 2008a; Vanderplanck et al., 2014; Moerman et al., 2016, 2017; Barraud et al., 2020; Carnell et al., 2020) and mason bees (Sedivy et al., 2011; Eckhardt et al., 2014). The floral diversity of pollen diet does not seem to be the major factor of quality, as bumblebees develop better on high-quality monofloral diets compared to low-quality polyfloral diets (Moerman et al., 2017; Carnell et al., 2020). The pattern for honey bees appears to be similar at an individual level, with pollen quality (reflected by protein content) having an impact on the physiology and survival of adult honey bees (Brodschneider and Crailsheim, 2010; Di Pasquale et al., 2013; Frias et al., 2016; Omar et al., 2017; Li et al., 2019).

Overall, these results suggest that a loss of a part of the plant community, especially the families covering specific physiological requirement (e.g., Fabaceae), is more likely to affect bumblebees and solitary bees than honey bees (Leonhardt and Blüthgen, 2012). The more generalized the foraging behavior of a particular bee species, the more likely it is to be able to switch to alternative host plants and persist in an area, even if those host plants are of a lower nutritional quality (Roger et al., 2017b). However, there are multiple studies evaluating and comparing the development of various generalist bee species in controlled conditions on the same pollen diets (Moerman et al., 2016), and no study considering a broad diversity of bee clades (e.g., different bee tribes or bee families).

To address these knowledge gaps, we evaluated the effect of 8 pollen mixes of different qualities on key life-history traits regulated by pollen consumption in four European bee species (2 Apidae species: *Apis mellifera* (Apini) and *Bombus terrestris* (Bombini); 2 Megachilidae species: *Osmia bicornis* and *O. cornuta*). We first conducted palynological and chemical analysis (total protein, total lipid, amino acid, and sterol content) on these pollen blends to characterize their quality

composition. We then developed experiments in controlled conditions and monitored the key life-history traits in bees fed with these pollen diets (e.g., survival for honey bees, brood production for bumblebees, and larva development for mason bees). We finally investigated which nutritional factors better explain bee health and development across the four species. Our hypothesis was that bee nutritional requirements are different across species.

MATERIALS AND METHODS

Bee Model Species

This study was conducted on four common pollen generalist bee species recorded in Europe which are foraging in the same habitat for part of the year (Michez et al., 2019). We selected the Western honey bee *Apis mellifera* (Hymenoptera, Apidae, Apini), a domesticated eusocial species; the buff tailed bumblebee *Bombus terrestris* (Hymenoptera, Apidae, Bombini), a wild social species (Rasmont et al., 2008); and two mason bees (*Osmia bicornis* and *O. cornuta*; Hymenoptera, Megachilidae, Osmiini), wild solitary species. They are commonly used as model species because of their easy management in laboratory conditions. Bumblebee colonies were provided by Biobest NV (Westerlo, Belgium); honey bees were obtained from local apiaries at the "*Institut National de la Recherche pour l'Agriculture, l'Alimentation et l'Environnement*" (INRAE) in Avignon (France) and the mason bees were provided by Wildbiene + Partner (Switzerland).

Characterization of Pollen Diets

Eight organic blends of honeybee-collected pollen were purchased from the company "Abeille heureuse" (France). Each pollen blend was gamma irradiated to avoid parasite infection, homogenized to reduce the risk of variation in palynological composition in each pollen treatment, and stored at −80°C before the experiment. In addition, a fraction of each pollen diet was lyophilized and stored at −20°C for palynological and chemical analyses (see below). Each pollen mix was named based on their palynological analysis, using the first letter of dominant pollen species (see **Table 1**).

TABLE 1 | Frequency (%) of most represented pollen species in the 8 pollen blend.

Pollen mixes	Dominant pollen species	Frequency (%)
C	Cistaceae: *Cistus ladanifer*	95
MS	Rosaceae: *Malus/Pyrus f.*	40
	Salicaceae: *Salix*	27
TSo	Asteraceae: *Taraxacum*	21
	Fabaceae: *Sophora*	17
QS	Fagaceae: *Quercus robur gr.*	51
	Salicaceae: *Salix*	29
BQ	*Brassicaceae*	36
	Fagaceae: *Quercus robur gr.*	35
SP	Salicaceae: *Salix*	43
	Rosaceae: *Prunus f.*	34
S	Salicaceae: *Salix*	89
ST	Salicaceae: *Salix*	64
	Asteraceae: *Taraxacum*	21

Pesticide Analyses

For each pollen diet, the presence of pesticide residues was determined by liquid chromatography–tandem mass spectrometry (LC-MS/MS) with a limit of quantification of 0.01 mg/kg and a limit of detection of 0.005 mg/kg following the EN 15662:2018 procedure. Residues of 2,4 dimethylformamidine (DMF, degradation products of amitraz) and tau-fluvalinate were detected in all pollen blends but were below the limit of quantification. These compounds used as chemical treatments against the honeybee parasite *Varroa destructor* are consistently found in pollens (47.4 and 88.3% of trapped pollens for amitraz and tau-fluvalinate, respectively; Mullin et al., 2010; Calatayud-Vernich et al., 2019) and are considered as relatively safe for honeybees with an oral LD50 of 75 μg/bee for amitraz (contact exposure) and 45 μg/bee for tau-fluvalinate (oral exposure) (US EPA, 2021).

Palynological Analyses

One gram of pollen sample was inserted and centrifuged in a 50 ml centrifuge tube and then dissolved in 20 ml of distilled water. Using a Pasteur pipette, a drop of sediment was placed on a microscope slide and spread out over an area of about 18 × 18 mm. After drying, the sediment was included in one drop of glycerine jelly and covered with the cover slip. Examination under the microscope were performed with 400X magnification. After a first general check to identify all the pollen types in the slide, a second read of the slide was carried out until 500 pollen grains were counted. Abortive, irregular, or broken pollen grains were still counted if they could be identified.

Recognition of pollen type was based on comparison between the observed pollen forms and those present in the CREA-AA collection of reference slides (built from anthers of identified plants). For each pollen type, the percentage of each species with respect to the total number of counted pollen grains was calculated.

Protein Analyses

Pollen protein concentration was measured using the Bradford assay according to Vaudo et al. (2020). We added 1.5 mL of 0.1 M NaOH to ∼1 mg of pollen sample (dry weight), and conducted the Bradford assay with the Bio-Rad Protein Assay Kit microassay 300 μL microplate protocol using bovine γ-globulin as the protein standard (Bio-Rad Laboratories, Inc., Hercules, CA). We used three technical replications for each biological replication and measured absorbance at 595 nm using a SpectraMax 190 spectrophotometer (Molecular Devices, LLC, Sunnyvale, CA). Protein concentrations were calculated using polynomial 2nd analysis from the protein standards.

Lipid Analyses

Pollen lipid concentrations were determined using a modified protocol from Van Handel and Day (1988). In 2.0 mL microcentrifuge tubes, we added 200 μL 2% sodium sulfate and 1.6 mL chloroform/methanol to ∼1 mg of each pollen sample (dry weight) before a 5 min centrifugation. Supernatant was transferred to a clean glass tube with 600 μL deionised water, and centrifuged for 5 min. We separated the top carbohydrate/water/methanol fraction and the remaining chloroform fraction was used for lipid analysis. The lipid/chloroform fraction was left overnight in a fume hood to completely evaporate the solvent. We added 200 μL sulfuric acid to the sample and heated at 100°C for 10 min. Then, 5 mL vanillin/phosphoric acid reagent was added. We used three 300 μL technical replications for each biological replication and measured absorbance at 525 nm. Lipid concentrations were calculated using polynomial 2nd analysis from vegetable oil standards. Pollen concentrations of protein and lipids are reported as μg nutrient/mg pollen, and subsequent P:L ratios were determined for each diet.

Amino Acids Analyses

For the analysis of total amino acids, 1 mL of hydrolysis solution (6N HCl, 0.1% phenol and 500 μM norleucine) was added to 3–5 mg (dry weight) of pollen (Vanderplanck et al., 2014) and then incubated for 24 h at 110°C. The hydrolysate was evaporated until dryness under vacuum in a boiling bath at 100°C. Afterwards, 1 mL of the sodium citrate buffer pH 2.2 was added into the tube. The sample solution was poured in an HPLC vial after filtration (0.2 μm filter), and each amino acid was measured separately with an ion-exchange chromatograph. A post-column ninhydrin reaction produced colored derivatives, which was monitored via a UV detector. For amino acid quantification, norleucine was used as internal standard. This analysis includes essential amino acids that bee cannot synthesize, as well as the non-essential ones. The essential amino acids were established by De Groot (1953) for honey bees; namely arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine.

Sterols Analyses

Before each analysis, pollen samples were divided into a minimum of three samples (i.e., 20 mg (dry weight) per analytical replicate). Sterols were quantified by GC-FID after extraction and purification according to the method described by Vanderplanck et al. (2011). The multi-step procedure can be summarized as follows: (i) saponification with 2M methanolic potassium hydroxide, (ii) extraction of the unsaponifiable portion with diethylether and several water washings, (iii) solvent evaporation, (iv) fractionation of the unsaponifiable portion by TLC, (v) trimethylsilylation of the sterols (scraped from the silicagel), and (vi) separation by GC. The total sterol content was determined considering all peaks above the limit of quantification [(LOQ); LOQ = 9.6 ng/1.2 μl injected] whose retention time were between cholesterol and betulin (internal standard). Individual sterols were quantified on the basis of peak areas from analyses. Under the present analytical conditions applied, campesterol and 24-methylenecholesterol co-eluted. Therefore, the results are pooled for these two compounds. Compounds were identified according to their retention times by comparison with those of sunflower oil as reference. The identifications were corroborated by GC-FID (Vanderplanck et al., 2011).

Bee Nutrition Studies

As the three genera (i.e., *Apis*, *Bombus*, and *Osmia*) show very different life cycles and behavior, they could not be tested following the same protocol in laboratory conditions. Thus, we developed different experimental setup for each of the three bee genera.

Honeybees (*Apis mellifera*)

In honeybees, pollen is mostly consumed by young adult bees. Its consumption enables the development of mandibular glands (Camilli et al., 2020) and especially hypopharyngeal glands (Crailsheim et al., 1992), where jelly is produced to feed larvae, the queen and drones (Crailsheim, 1992). We therefore tested the influence of pollen quality at the individual level on the fresh weight of individual heads, which is highly correlated to the volume of acini from the hypopharyngeal glands (Hrassnigg and Crailsheim, 1998). We also measured the survival rate of bees. Experiments were performed in the spring. To obtain 1-day-old bees, brood frames containing late-stage pupae were taken from eight healthy colonies normally treated against the parasitic mite *Varroa destructor* (*Apis mellifera ligustica* × *Apis mellifera mellifera*), and were placed overnight into an incubator under controlled conditions [34°C, 50–70% of relative humidity (RH)]. The next day, newly-emerged bees (<1 day old) were collected, mixed and groups of 40 bees were placed in cages (10.5 × 7.5 × 11.5 cm) (Pain, 1966). Caged bees, kept in an incubator (30°C and 50–70% RH), were provided *ad libitum* with water, candy (Apifonda® + powdered sugar) and one of the pollen diets ($n = 10$ cages per experimental group) (**Figure 1A**). Pollen diets were replaced every day for 10 days. To simulate as much as possible colony rearing conditions, caged bees were provided with a Beeboost® (Ickowicz, France), releasing one queen-equivalent of queen mandibular pheromone per day. Each day, pollen diets were weighed to determine the amount of pollen consumed per day and per bee. Pollen collection was corrected for evaporation, which was estimated by placing two samples of each pollen mixture in the same incubator for 24 h. Bee mortality was recorded every day for 44 days by counting and removing dead bees from cages. On day 7, 9 bees were sampled from each cage and stored at −80°C. The fresh weight of heads was then measured on individual bees ($n = 9$ bees per cage giving a total of 90 bees per experimental group).

Bumblebees (*Bombus terrestris*)

We tested the impact of pollen on bumblebees at micro-colony level. Such a method to test the nutritive value of pollen diets has been shown to be a good estimate of queenright colony development at least under laboratory conditions with food *ad libitum* (Taseï and Aupinel, 2008b). A total of five queen-right colonies of 100 *Bombus terrestris* workers were used to build up 80 queen-less micro-colonies of five workers, placed in plastic

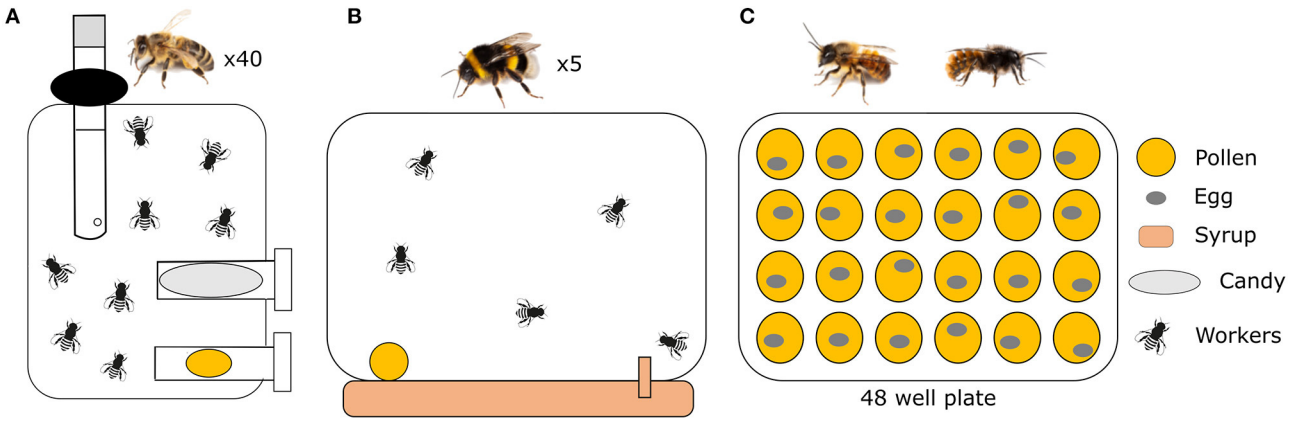

FIGURE 1 | Experimental set-ups for **(A)** honeybees, **(B)** bumblebees and **(C)** mason bees. Caged honeybees were provided with water, candy, and one of the pollen diets. Five bumblebees (workers) were placed in plastic boxes provided with pollen and syrup. Eggs of mason bees developed in cell culture plates of 48 wells filled with 400 mg of pollen.

boxes (8 × 16 × 16 cm) (**Figure 1B**). This number of individuals per micro-colony has been repeatedly used and is assumed to be the most reliable for assessing diet effects (Gradish et al., 2013; Moerman et al., 2016; Roger et al., 2017a; Vanderplanck et al., 2018; Klinger et al., 2019). Micro-colonies were then distributed in the different conditions (n = 10 micro-colonies for each experimental treatment). All micro-colonies were maintained in the same room in constant darkness at 26 ± 2°C with a relative humidity of 60–65%. They were manipulated under red light to minimize disturbance (Sadd, 2011) for a period of 28 days. Pollen diets were provided *ad libitum* to the micro-colonies as candies (mixed pollen with sugar syrup). New pollen candies were provided every 2 days, while the previous ones were weighed to assess the pollen collection. Pollen collection was corrected for evaporation by monitoring the weight of two samples of each diet placed in the rearing room for 48 h. To estimate the performance and development of bumblebee micro-colonies, we measured: (i) the total pollen and syrup collections, which can impact brood production and development (e.g., Plowright et al., 2008; Sutcliffe and Plowright, 2008); (ii) colony growth after 28 days of development [i.e., mass of individuals from all brood stages (eggs, larvae, pupae, non-emerged, and emerged males)] (Vanderplanck et al., 2014, 2018). For each micro-colony, all the measured parameters were divided by the total mass of the five workers to standardize the results and avoid potential effect of worker activities related to their size (i.e., consumption and brood care) (Cnaani and Hefetz, 1994). Additionally, we calculated the pollen efficacy as the mass of total offspring divided by the total pollen collection to estimate the colony performance.

Mason Bees (*O. cornuta* and *O. bicornis*)

We tested the impact of the pollen diet on the two species of mason bees at the larval stage. Standard mason bee nesting plates were installed close to the laboratory on the campus of the University of Mons (Belgium). A total of 1,000 individuals were released next to the nests. At regular time intervals, nests were opened and investigated for brood cell production. After 3 weeks, offspring were collected at the egg stage to avoid the consumption of the original pollen supply by the freshly emerged larvae. In the laboratory, cell culture plates of 48 wells were filled with 400 mg of prepared pollen (mixed pollen with sugar syrup) (**Figure 1C**). A fine brush was used to pick the egg from its original brood cell, and a single egg was placed cautiously onto each pollen provision (n = 35–40 eggs per treatment group). Plates were then placed into an incubator under controlled conditions (23°C, 60% RH). Developmental stage of larvae was assessed every day for 1 month and categorized into egg, larvae, feeding larvae, feeding and defecating larvae, spinning larvae, light cocoon, and cocoon. The time required to reach cocoon stage was used for the analyses. On average 90 days after cocoon development, each of them was taken out of the brood cells and weighed. Plates were then kept at 12°C for 4 days and at 4°C for ∼120 days to mimic hibernation. After 140 days, all cocoons were again kept at 12°C for 4 days before moving them into an incubator (25°C, 60% RH) to elicit emergence. After emergence, adults were weighed (fresh weight) and determined as male or female.

Statistical Analyses

Statistical analyses were run using statistical software R version 3.3.3 (R Core Team, 2017). To analyse the influence of pollen diets on honey bee survival, the number of dead bees per day and cage throughout the experiments were transformed into a survival table. A Cox proportional-hazards regression model was then used to compare the different diets, with R functions (*coxph*) and the package *survival* (Cox, 1970), considering the censored data of the bees that were alive at the end of the study. Pollen consumption and fresh weight of heads were analyzed using Kruskal–Wallis followed by Dunn's multiple comparison tests since data were not normally distributed.

For statistical analyses on bumblebees and *Osmia* data, two-way crossed analyses of variance (Two-Way crossed ANOVA) were conducted to evaluate the effect of diet. Since it is a parametric test, homoscedasticity (Bartlett test) and normality of the residuals (Shapiro test) were checked prior to the analyses.

When violation occurred, data were log- or z-transformed to normality of residuals ("ztransform"function, R-package "GenABEL," Lenth, 2009) prior to the test. Multiple pairwise comparisons were conducted using Tukey HSD *post-hoc* tests when ANOVA detected significant difference between pollen diets ($p < 0.05$).

Differences in nutritional content were assessed using either Kruskal–Wallis followed by Dunn's multiple comparison tests for proteins, lipids and P:L ratios, or perMANOVA for sterols and amino acids (Euclidean distance, 999 permutations, "adonis" command) after testing for multivariate homogeneity ("betadisper" command) (R-package vegan, Oksanen et al., 2020). Given the number of replicates, it was not possible to run a multiple pairwise comparisons on amino acids and sterol data. Differences were then visually assessed on UPGMA clusters using Euclidean distance and multiscale bootstrap resampling to calculate *p*-values for uncertainty in hierarchical cluster (R- package pvclust, Suzuki et al., 2019). Indicator compound analyses were performed to identify nutrients that were indicative of the groups defined based on the hierarchical cluster ("indval" command) (R-package labdsv, Roberts, 2019). All these analyses were conducted using data expressed as mg/g.

We analyzed the influence of each macro-nutrient in diets (i.e., proteins and lipids) on species performance using Response Surface Models (RSM). As it is standard for geometric analyses of nutrition, the models included the linear and quadratic components for protein and lipid intake as well as the interaction term between proteins and lipids as explanatory variables. Regarding the response variable, we used the proportion of individuals that survived for each diet treatment for *Apis mellifera*, the pollen efficacy for *Bombus terrestris* and the adult mass for *Osmia bicornis* and *O. cornuta*. As our response variables were measured in different units, we standardized each response variable to a mean of zero and a standard deviation of one using a Z transformation prior to analysis (≪ ztransform ≫ function from the GenABEL R-package; Ronnegard et al., 2016). We performed these analyses using the ≪ rsm ≫ function from the rsm R-package (Lenth, 2009), first considering lipid and protein content (RSM 1), then sterol and amino acid content (RSM 2).

RESULTS

Palynological Analyses of Pollen Blends

The pollen mixtures were analyzed to verify the indication of the dominant pollen (**Table 1**). Analyses confirmed the palynological origin indicated by the seller only for two out of eight samples (samples C, S). Only four out of eight samples showed the presence of a dominant pollen (samples C, MS, BQ, SP); the other samples were found to be more or less heterogeneous mixtures of three or more pollen (see **Supplementary Figure S1**).

Chemical Analyses of Pollen Blends

Pollen mixes had different chemical composition (**Table 2**, perMANOVA, $p < 0.001$). Protein content varies from 209.61 to 397.66 mg/g of pollen ($p < 0.001$). C and S pollen blends had the significantly lowest protein content compared to other mixes (209.61 ± 9.13 and 214.86 ± 10.5 mg/g, respectively, all $p < 0.03$), whereas TSo pollen mix had the highest protein content (397.66 ± 11.5 mg/g, all $p < 0.001$). SP pollen mix had the lowest lipid content (45.72 ± 2.42 mg/g, all $p < 0.001$) while ST and QS pollen mixes had the significantly highest lipid content (89.93 and 82.87 mg/g, respectively, all $p < 0.03$). P:L ratio ranged from 2.93 to 3.44 for ST, C and S pollen mixes, to 6.01–6.19 for SP and TSo diets (all $p < 0.001$).

Total and essential amino acid content ranged from 109.73 mg/g (SP diet) and 233.28 mg/g (SP diet) to 52.57 mg/g (C diet) and 125.04 mg/g (C diet), respectively. UPGMA analyses identified two clusters: one composed of C diet only (Cluster A, **Supplementary Figure S1A**) with a higher proline concentration ($p = 0.018$), and a second cluster composed of all other diets (Cluster B, **Supplementary Figure S1A**) with a higher content of every amino acid except proline (all $p < 0.017$). The different diets displayed concentrations of total sterol from 4.54 mg/g (C diet) to 14.38 mg/g (ST diet). UPGMA analyses identified two clusters: one composed of ST and SP diets (Cluster B, **Supplementary Figure S1B**) with a higher 24-Methylenecholesterol concentration compared to the second cluster composed of all other diets (Cluster A, **Supplementary Figure S1B**, $p = 0.021$). Other significant differences out of these clusters have been identified: higher concentrations of Cholesterol and δ7-Stigmasterol in TSo diet ($p = 0.024$ and 0.01, respectively), a higher concentration of Stigmasterol in BQ diet ($p = 0.007$) and higher concentration of δ7-Avenasterol in ST diet ($p = 0.007$). Despite the low concentrations of 24-Methylenecholesterol and δ5-Avenasterol observed in C diet compared to other diets (**Table 2**), they were not defined as significant by our analytical model, probably due to the lack of replicates. Those differences were therefore considered as tendencies.

Diet Effects on Bees

Honeybees (*Apis mellifera*)

Significant differences in bee survival between pollen diets were observed with the following order from the least to the most beneficial pollen: TSo < MS – BQ – C < SP < ST - QS – S (**Figure 2A**). Pollen diets were not consumed equally ($p < 0.01$, **Figure 2B**). Bees consumed significantly more of the QS and BQ than TSo and S pollen mixes. The head weight was also affected by the type of pollen ($p < 0.001$, **Figure 2C**). Bees fed with TSo pollen diet had a lighter head than bees fed with S, QS, BQ, or ST pollen mixes. After normalization to the amount of consumed pollen, head weights were the lowest for QS and BQ pollens and the highest for TSo and S pollen mixes ($p < 0.001$; **Supplementary Figure S2**).

Bumblebees (*Bombus terrestris*)

The brood production was impacted by the diet ($p < 0.01$). Microcolonies fed with MS and S diets were more developed, in term of total brood mass after 28 days, compared to those fed with *C* diet ($p = 0.026$ and $p = 0.022$, respectively, **Figure 3A**). Pollen efficacy was also influenced by the different pollen diets. Microcolonies with bumblebees fed with MS, TSo, SP, S, and ST pollen diets produced more brood per gram of pollen consumed in 28 days compared to those fed with C pollen diet (all $p < 0.04$,

TABLE 2 | Chemical composition and P:L ratios of the 8 pollen blends.

Chemicals (mg/g)	ST	C	S	QS	BQ	MS	SP	TSo
Protein content	263.73 ± 26.3^{b}	**209.61 ± 9.13^{a}**	**214.86 ± 10.5^{a}**	285.10 ± 12.4^{b}	283.89 ± 7.23^{b}	287.49 ± 9.00^{b}	274.94 ± 18.1^{b}	**397.66 ± 11.5^{c}**
Lipid content	**89.93 ± 3.20^{d}**	64.33 ± 1.15^{b}	62.63 ± 4.3^{b}	82.87 ± 2.75^{d}	74.98 ± 2.53^{c}	68.55 ± 2.74^{bc}	**45.72 ± 2.42^{a}**	64.24 ± 2.4^{b}
Protein:Lipid ratio	2.96^{a}	3.26^{ab}	3.44^{ac}	3.44^{ac}	3.79^{bc}	4.19^{c}	6.01^{d}	**6.19^{d}**
Total amino acids	184.05 ± 7.69	**125.04 ± 13.39**	181.35 ± 25.04	194.18 ± 5.39	174.54 ± 24.48	218.37 ± 17.07	**233.28 ± 16.01**	198.57 ± 25.42
Essential amino acids	88.66 ± 1.89	**52.57 ± 5.03**	86.46 ± 10.45	90.45 ± 3.28	85.63 ± 8.05	103.54 ± 5.54	**109.73 ± 7.48**	91.54 ± 12.3
Alanine	11.14 ± 0.21	**7.87 ± 0.97**	10.21 ± 1.28	10.87 ± 0.31	10.73 ± 1.27	12.79 ± 0.62	**13.1 ± 0.87**	11.28 ± 1.46
Arginine	10.53 ± 0.18	**5.94 ± 0.27**	12.43 ± 1.28	12.39 ± 0.09	11.48 ± 0.75	12.33 ± 0.73	**14.88 ± 0.76**	10.4 ± 1.18
Asparagine	18.79 ± 1.53	**10.4 ± 2.01**	20.05 ± 2.96	20.62 ± 0.46	17.46 ± 3.51	22.52 ± 2.4	**25.51 ± 1.58**	19.68 ± 2.41
Glutamate	19.97 ± 3.76	**11.3 ± 3.87**	22.88 ± 4.55	25.32 ± 1.64	18.3 ± 5.51	24.65 ± 5.3	**30.56 ± 3.8**	24.06 ± 4.45
Glycine	8.7 ± 0.33	**4.01 ± 1.51**	8.17 ± 1.03	8.43 ± 0.26	7.39 ± 1.43	9.14 ± 0.79	**10.31 ± 0.86**	8.73 ± 1.02
Histidine	**9.83 ± 1.34**	**5.11 ± 0.79**	6.22 ± 1.15	7.17 ± 0.15	7.22 ± 0.58	6.1 ± 2.09	7.54 ± 0.68	7.95 ± 1.11
Isoleucine	7.57 ± 0.09	**4.81 ± 0.3**	7.76 ± 0.96	8.16 ± 0.36	7.7 ± 0.86	10.16 ± 1.14	**10.36 ± 0.82**	8.6 ± 1.3
Leucine	13.69 ± 0.34	**9.34 ± 0.42**	13.99 ± 1.79	14.82 ± 0.9	13.61 ± 1.48	**18.4 ± 2.46**	18.33 ± 1.84	15.6 ± 2.8
Lysine	16.68 ± 0.39	**8.25 ± 1.71**	14.65 ± 1.25	14.71 ± 0.24	14.58 ± 1.59	16.29 ± 1.41	**17.77 ± 1.06**	14.55 ± 1.13
Methionine	4.19 ± 0.15	**2.84 ± 0.25**	4.53 ± 0.37	4.25 ± 0.22	4.23 ± 0.47	5.38 ± 0.16	**5.42 ± 0.35**	4.57 ± 0.42
Phenylalanine	8.48 ± 0.39	**5.35 ± 0.62**	8.61 ± 1.14	9.7 ± 0.73	8.72 ± 1.1	**12.42 ± 2.4**	11.83 ± 1.55	10.37 ± 2.08
Proline	14.2 ± 0.59	**28.41 ± 1.42**	11.52 ± 0.63	14.55 ± 0.15	14.8 ± 1.34	**20.59 ± 2.25**	17.19 ± 0.96	18.87 ± 1.56
Serine	9.31 ± 1.52	**5.09 ± 1.54**	9.4 ± 1.76	10.14 ± 0.4	8.52 ± 2.24	11.17 ± 1.69	**11.79 ± 1.14**	10.74 ± 1.32
Threonine	7.65 ± 0.3	**4.72 ± 0.67**	8.03 ± 1.07	8.38 ± 0.34	7.93 ± 1.18	9.39 ± 0.72	**10.13 ± 0.84**	8.48 ± 0.99
Tyrosine	6.69 ± 0.26	**3.95 ± 0.39**	6.9 ± 1.23	7.59 ± 0.62	6.57 ± 1.18	9.08 ± 1.51	**9.46 ± 0.96**	8.05 ±1.55
Valine	10.04 ± 0.19	**6.2 ± 0.17**	10.23 ± 1.49	10.87 ± 0.45	10.17 ± 0.98	13.07 ± 1.12	**13.47 ± 1.02**	11 ± 1.76
Total sterols	14.38 ± 1.42	**4.54 ± 2.06**	8.22 ± 2.09	6.31 ± 1.6	6.86 ± 2.06	6.51 ± 0.16	12.68 ± 4.59	7.45 ± 0.44
Cholesterol	0.14 ± 0.02	0.08 ± 0.02	0.1 ± 0.02	0.05 ± 0.03	0.06 ± 0.08	**0.04 ± 0**	0.06 ± 0.02	**0.52 ± 0.14**
24-Methylenechol/camp	**8.37 ± 1.21**	**1.43 ± 0.56**	2.48 ± 1.53	2.5 ± 0.76	3.21 ± 3.47	4.85 ± 0.15	10.26 ± 3.64	2.61 ± 1.71
Stigmasterol	0.11 ± 0.02	0.1 ± 0.08	0.1 ± 0.02	0.08 ± 0	**0.79 ± 1.44**	0.08 ± 0.01	**0.02 ± 0.02**	0.23 ± 0.1
β-Sitosterol	1.44 ± 0.1	**0.73 ± 0.5**	2.42 ± 0.48	**2.57 ± 0.66**	1.5 ± 1.3	**0.73 ± 0.04**	1.2 ± 0.46	2.28 ± 1.16
δ5-Avenasterol	0.96 ± 0.07	**2.15 ± 0.87**	1.41 ± 0.36	0.96 ± 0.17	1.27 ± 2.81	**0.75 ± 0.04**	1.05 ± 0.39	0.97 ± 0.5
δ7-Stigmasterol	0.24 ± 0.04	**0 ± 0**	0.02 ± 0.04	0.07 ± 0.05	**0 ± 0**	0.02 ± 0.01	0.03 ± 0.05	**0.61 ± 0.15**
δ7-Avenasterol	**3.12 ± 0.34**	**0.04 ± 0.04**	1.69 ± 0.25	0.08 ± 0.02	**0.04 ± 0.02**	0.05 ± 0	± 0.02	0.22 ± 0.03

Mean values are presented with their standard deviation. Maximum and minimum values for each chemical are in bold as indicative. Different letters indicate significant differences (Kruskal-Wallis tests followed by Dunn's multiple comparison tests, $p < 0.05$).

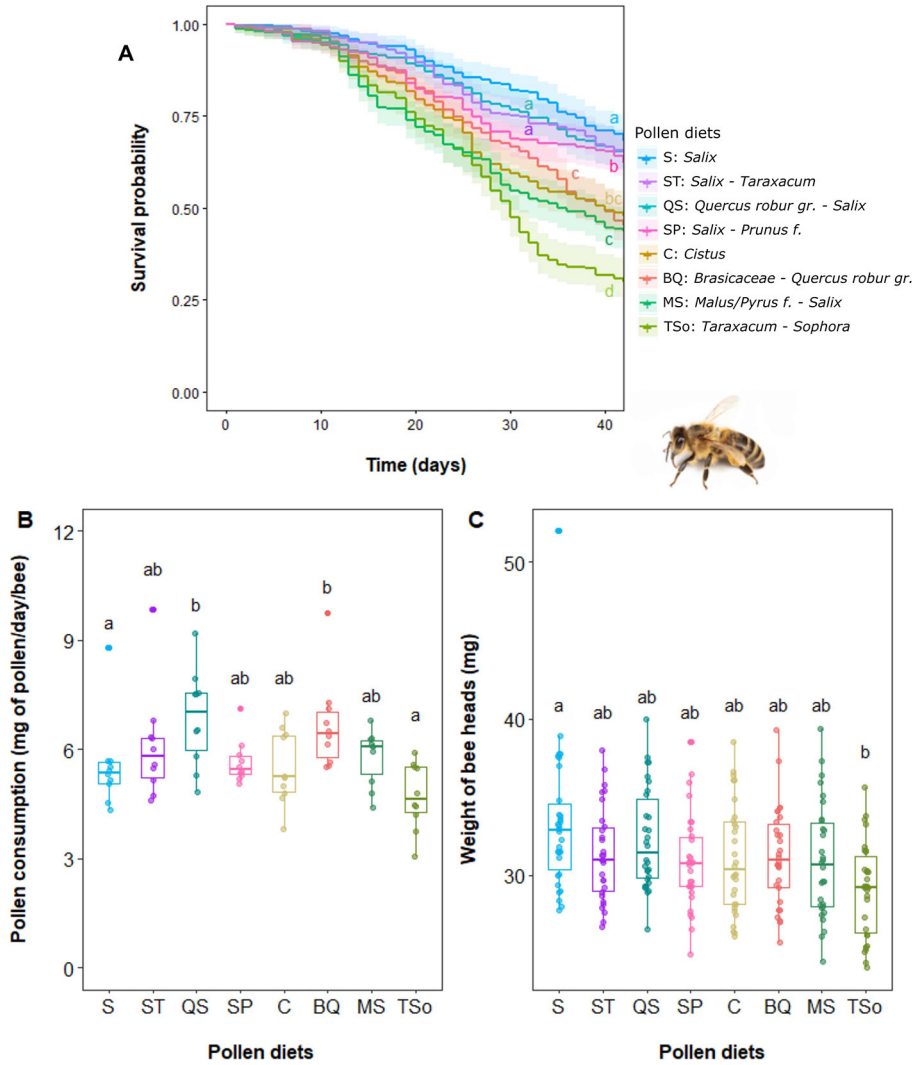

FIGURE 2 | Influence of pollen diets on *Apis mellifera*. **(A)** Survival probability ($n = 30$ bees per cage and 10 cages per pollen regime), **(B)** pollen collection and **(C)** head weight. Boxes indicate the 1st and 3rd interquartile range with line denoting median ($n = 30$ pools of 3 bees per pollen). Whiskers include 90% of the individuals, beyond which each outlier are represented by circles. Different letters indicate significant differences between pollen diets (Kruskal-Wallis tests followed by Dunn's multiple comparison tests: $p < 0.01$).

Figure 3B). The different pollen mixes had a limited impact on resources collection. Bumblebees consume more of the TSo diet than the SP diet ($p = 0.024$, **Figure 3C**). No significant differences were observed between other diets nor regarding syrup collection ($p > 0.05$, **Figures 3C,D**).

Mason Bees (*Osmia bicornis* and *O. cornuta*)

Osmia bicornis developed differently depending of their diet ($p < 0.001$). Bees fed with C diet had a significantly higher development time compared to any other diets (all $p < 0.027$), with ∼25 and 31 days required to reach cocoon stage for females and males, respectively (**Figure 4A**). In contrast, bees fed with BQ diet had the lowest development time (all $p < 0.004$), with 20 and 23 days required to reach cocoon stage for females and males, respectively (**Figure 4A**). Development time on other diets range from 22 to 27 days. Cocoons of larvae fed with C diet were significantly lighter compared to any other diets (all $p < 0.002$), with a mean weight of 0.118 ± 0.011 and 0.095 ± 0.012 g for females and males, respectively. Cocoons of larvae fed with MS diet were ∼29, 12, and 11% heavier compared to those fed with C, TSo or ST diet (all $p < 0.02$), respectively (**Figure 4C**). Adult weight showed similar differences (**Figure 4E**). The time required to reach cocoon stage for *Osmia cornuta* was significantly lower when fed with BQ diet compared to any other diets (all $p < 0.02$, **Figure 4B**). No significant differences were observed among the other diets (all $p > 0.05$). Cocoons of larvae fed with C diet were significantly lighter compared to any other diets (all $p < 0.021$), with a mean weight of 0.102 ± 0.005 and 0.094 ± 0.011 g for females and males, respectively. Cocoons of larvae fed with SP diet were ∼27% heavier compared to those fed with C diet, with

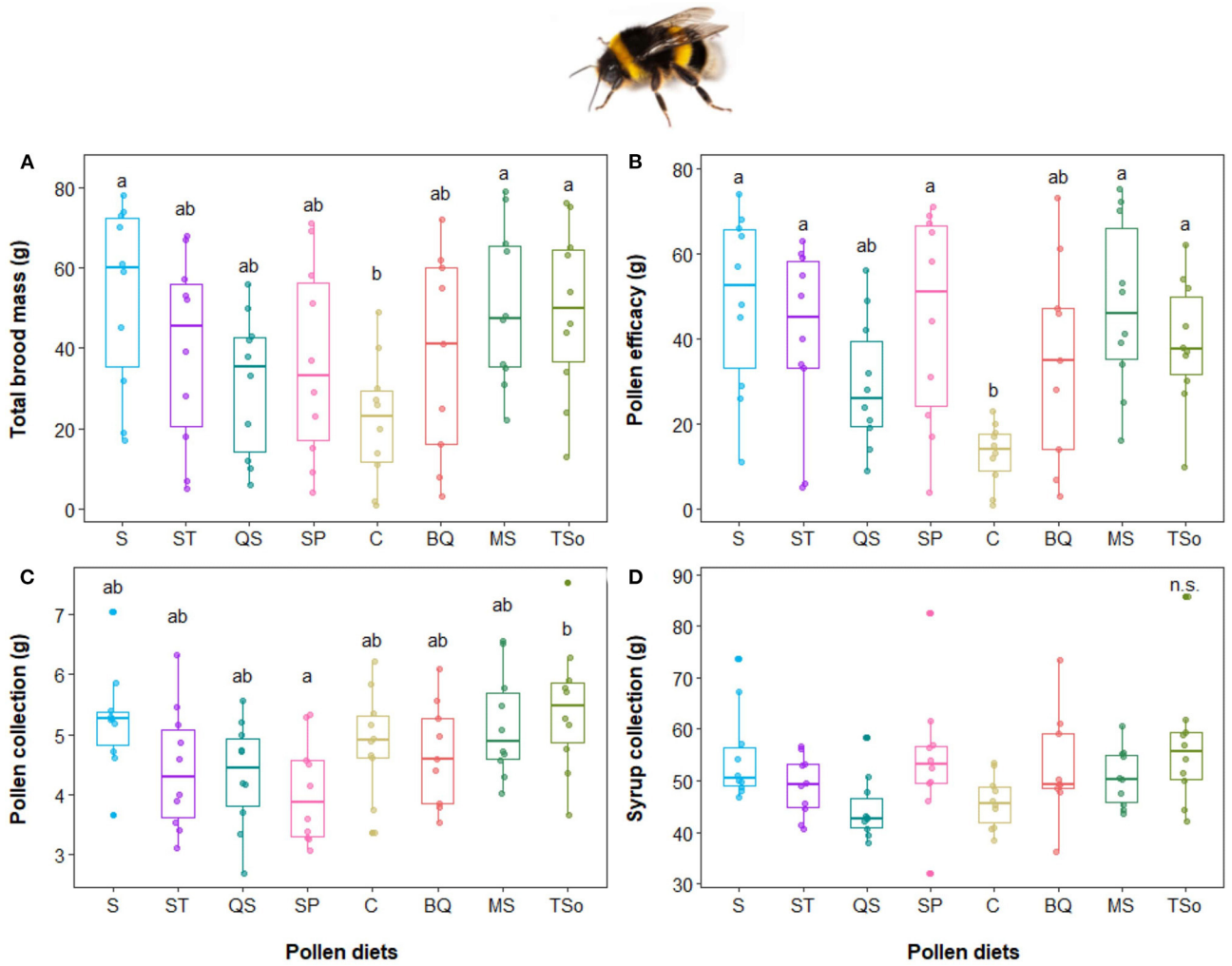

FIGURE 3 | Influence of pollen diets on *Bombus terrestris*. **(A)** Brood mass production (g), **(B)** pollen efficacy (ratio between brood mass and pollen collection), **(C)** pollen collection (g), and **(D)** syrup collection. Boxes indicate the 1st and 3rd interquartile range with line denoting median ($n = 10$ micro-colonies with 5 workers per pollen diet). Different letters indicate significant differences between pollen diets (ANOVA tests followed by Tukey's multiple comparison tests: $p < 0.05$).

a mean weight of 0.135 ± 0.012 and 0.115 ± 0.021 g for females and males, respectively. They were also significantly heavier than cocoons of larvae fed with QS, BQ, ST, and TSo diet (all $p < 0.003$, **Figure 4D**). Adult weight showed similar differences, but with no significant differences between C, Tso, and ST diets (all $p > 0.07$, **Figure 4F**).

Geometric Analyses

RSM analyses showed that *A. mellifera* survival variations between diets were not explained by total amino acids (AAT) or sterols (RSM 2, **Supplementary Table S2**), but rather by protein and lipid content (RSM 1, **Supplementary Table S2**). On the other hand, observed differences between diet-dependant pollen efficacies in *B. terrestris* were not explained by protein or lipid content (RSM 1, **Supplementary Table S2**), but rather by AAT and sterols (RSM 2, **Supplementary Table S2**). *O. bicornis* female mass variations were explained by the protein content only, while male mass was explained by proteins, AAT and sterols (RSM 1, **Supplementary Table S2**). Finally, *O. cornuta* adult mass variations between diets were explained in the same way as for *O. bicornis*: proteins for females (RSM 1, **Supplementary Table S2**); proteins, AAT and sterols for males (RSM 2, **Supplementary Table S2**).

DISCUSSION

Variation in Chemical Composition of Pollen Diets

Chemical composition of the tested diets was different, despite some observed similarities. First, none of the pollen diets had a protein content lower than 21%, which is already an average amount: ∼25 plant genera contain <15% of protein in pollen (Roulston and Cane, 2002). On the other hand, the protein-richest diet of our experiments contained nearly 40% of proteins

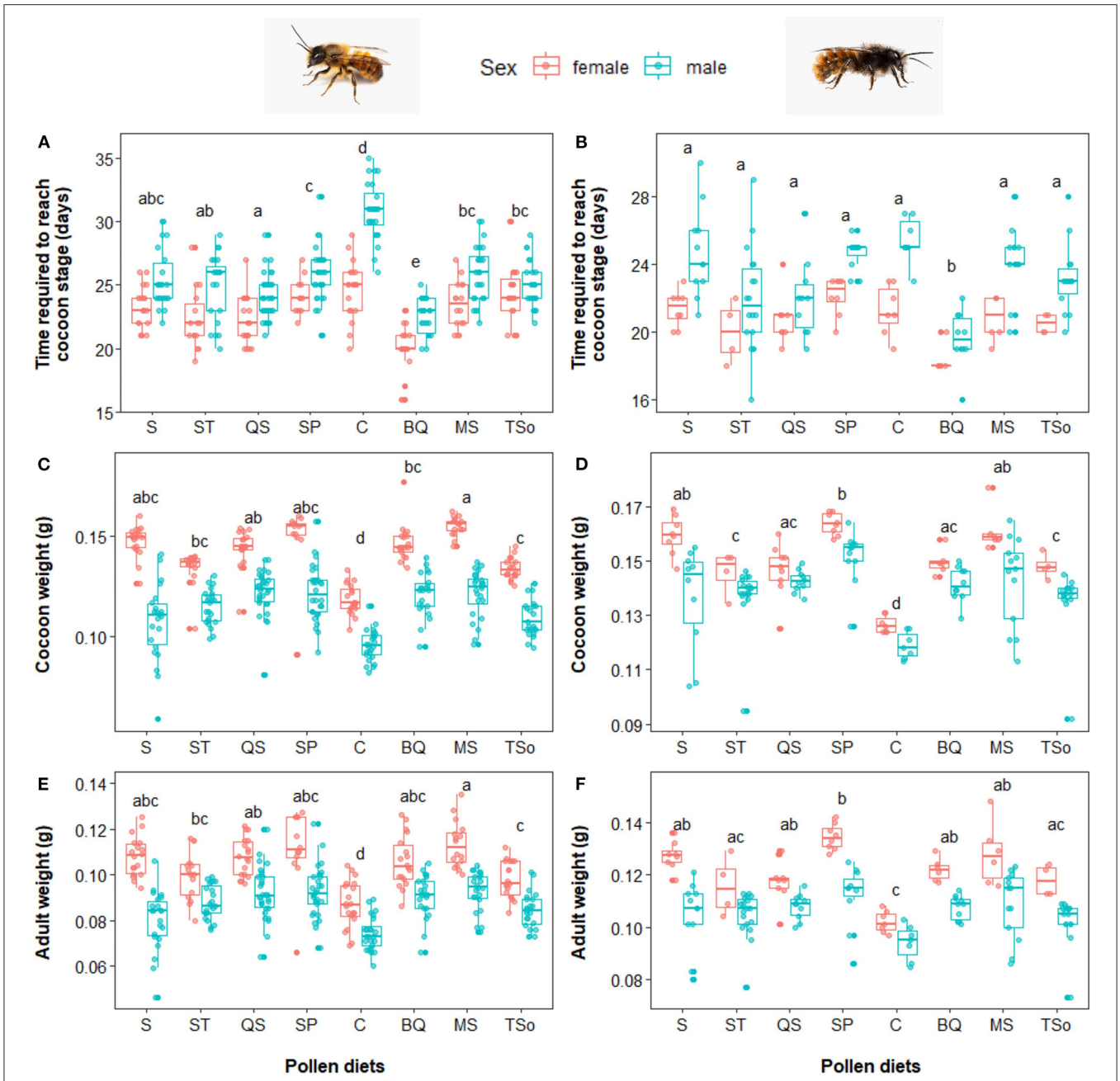

FIGURE 4 | Influence of pollen diets on (left) *Osmia bicornis* and (right) *Osmia cornuta*. **(A,B)** Time required for larvae to reach the cocoon stage (days), **(C,D)** cocoon weight (g) and **(E,F)** adult weight (g). Boxes indicate the 1st and 3rd interquartile range with line denoting median ($n = 40$ per pollen diet). Different letters indicate significant differences between pollen diets (ANOVA tests followed by Tukey's multiple comparison tests: $p < 0.05$).

(TSo diet). With more than 60 plant genera containing more than 40% of protein in pollen (Roulston and Cane, 2002), our diets are in the range of what can be find *in natura*, but no pollen with a particularly low or high protein content was tested here. As we used pollen blends and not monofloral diets, it was expected that no extreme values would be observed (Roger et al., 2017b). Lipids analyses also showed differences between the diets. Previous analyses showed that lipids can range from ∼1 to 20% in total content (Roulston and Cane, 2000; Roulston et al., 2000). With a variation from 4.6 to 9% of total lipid content, our diets are again in range with no extreme values. Note that the lipid analysis method may not completely lyse the pollen grains. There may therefore be a slight difference between the results of the analysis and what can be assimilated by the bee.

Amino acids analyses showed similar amino acids profiles were similar between pollen blends. These results are in line

with previous studies, suggesting that amino acid profiles are conserved among plants (Roulston and Cane, 2002; Weiner et al., 2010; Vanderplanck et al., 2014). However, differences between our diets have been observed in term of total and essential amino acids, and the amount of specific amino acids. In this regard, we have observed that C diet contains 54–72% less amino acids and 48–61% less total and essential amino acids, compared to other diets.

Finally, among our different diets, total sterol concentration and sterolic profiles were different with a lower total sterol content in the C diet. Those results corroborate with previous studies that showed a high variability in sterolic compounds concentrations across plant species (Vanderplanck et al., 2014, 2018).

Relationship Between Bee Performance Traits and Pollen Chemical Composition

B.terrestris, *O.bicornis* and *O.cornuta* worst performances were observed when fed with C diet, whereas a different result was recorded for *A.mellifera*, for which worst performance was observed when fed with TSo diet. *A.mellifera* performances fed with C diet were average, while TSo diet was good and average for *B.terrestris* and *Osmia* species, respectively. Diet effects were similar between *O.bicornis* and *O.cornuta*. As both species are included in the same genus, it can be expected that nutritional preferences are conserved.

Several studies pointed out protein and/or protein:lipid ratio as an indicator for pollen quality regarding developmental performances (Roulston and Cane, 2002; Smeets and Duchateau, 2003; Alaux et al., 2010; Nicolson, 2011). However, observed results and RSM analyses on *Bombus terrestris* showed that performance cannot be explained by protein, lipid or P:L ratio, while adult female mass of *Osmia* species is slightly affected by protein content. Some good diets (see S pollen mix) and bad diets (see C pollen mix) share the same protein, lipid, content and P:L ratio. Those results indicate a difference in nutritional abilities compared to *A. mellifera* for which RSM analyses highlighted an importance of protein and lipids for their survivability. Despite the common assumption that proteins are positively related to performances (Regali and Rasmont, 1995; Génissel et al., 2002; Roulston and Cane, 2002; Smeets and Duchateau, 2003; Alaux et al., 2010; Nicolson, 2011; Stabler et al., 2015), in our case, the only observed effects that can be related to protein content were a negative one on *Apis mellifera* when fed with a protein-rich diet (39.8%, TSo pollen mix) and a slight one on *Osmia* species that is difficult to identify as positive or negative, as both pollen with the highest (TSo) and lowest (C) protein content resulted in lighter adults compared to other diets. Interestingly, Roulston and Cane (2002) observed a higher mortality rate when *Lasioglossum zephyrum* individuals were fed with a diet containing more than 39% of total protein content. Moreover, it has already been shown that in laboratory conditions, mortality and ovary development in *A.mellifera* workers were negatively impacted when fed with pollen containing 32% of proteins compared to bees fed with 15% of proteins (Human et al., 2007). Other authors observed similar mortality results with protein-rich diet (Standifer, 1967; Herbert et al., 1977), suggesting that high levels of proteins can lead to deleterious effect.

Differences in amino acids content between the different diets did not impact *A. mellifera*, but seems to explain results observed with *B. terrestis* and *Osmia* species, with more than 3 times less pollen efficacy for *B. terrestris*, and up to −22 and −26% adult weight for *O.bicornis* and *O.cornuta,* respectively, when fed with C diet, which had the lowest amount of total and essential amino acids. These results are similar to those of Archer et al. (2021), who concluded that amino acid intake was positively correlated with bumblebee body mass, and underlined that the effects of total amino acids intake may depend on the blend of individual amino acids. Interestingly, bumblebees can perceive some amino acids via chemotactile antennal stimulation and distinguish different concentrations without being able to differentiate each amino acid (Ruedenauer et al., 2019). This way, bumblebees could therefore assess the overall quantity of some amino acids and adapt their foraging decisions. However, such strategy could also lead wrong decisions, i.e., a rich pollen in non-favorable amino acids proportions.

Finally, sterols analyses could explain observed results regarding *B. terrestris* and *O. cornuta* measured performance traits. Some sterolic compounds have already been reported to be positively correlated with larval growth (Vanderplanck et al., 2014). 24-methylenecholesterol is known to influence molting and ovary development (Svoboda et al., 1978, 1983; Human et al., 2007), while β-sitosterol and δ5-avenasterol are supposedly involved in metabolic pathways of *B. terrestris* or have a phagostimulant effect (Regali, 1996). Sterols in the C diet did not affect *A. mellifera* performances. However, these results could be explained by the performance traits tested in our experiment, which did not take into account reproductive performances. In this regard, it could be interesting to test the effect of diet on larval development and not only on adults, to better evaluate the sterols effect, known to have a positive effect on the larval development on other bee species. Nevertheless, TSo diet, which caused the lowest survival rate in *A. mellifera*, contained relatively high concentrations of cholesterol and δ7-stigmasterol. If no negative effect has been reported for cholesterol in the literature, maybe this compound was in excess for *A. mellifera*. On the other hand, δ7-stigmasterol have already been reported as not beneficial for bumblebees (Vanderplanck et al., 2018). *A. mellifera* could therefore be more sensitive to the presence of this compound compared to other species. Finally, *O. bicornis* reached cocoon stage quicker when fed with BQ pollen mix. This diet has a relatively high concentration of stigmasterol, suggesting a potential role in the larval development of this species. It has recently been reported by Ruedenauer et al. (2021) that bees can't taste sterols, limiting the bee ability to forage on plants with beneficial sterol composition or to avoid detrimental ones. Moreover, this work and previous studies showed that pollen consumption is not adjusted to compensate low nutritional quality of a diet (see Vanderplanck et al., 2014 for *B. terrestris* and Corby-Harris et al., 2018 for *A. mellifera*), suggesting that a diet with a detrimental sterol composition could be regularly consumed by the bees without them perceiving it and being able to adapt their consumption.

Good Diets for Everyone

Our results show that, if the 8 diet effects are different among species, similarities can be noted. Because the best performances of each species were shared across different diets, we could not define only one specific favorable diet. However, a global profile could be defined by comparing the nutritional factors of these diets. Based on this and previous studies, a generally good diet would require a high concentration of amino acids, and of 24-methylenecholesterol, β-sitosterol and δ5-avenasterol (Vanderplanck et al., 2014). P:L ratio did not impact measured parameters of this study. While Vaudo et al. (2016) have shown an impact of the P:L ratio on foraging behavior, the effects of this ratio on colony development remain relatively unclear. More studies should be done to see if the foraging strategy in favor of the P:L ratio results in effects on colony development, by artificially modifying the protein and lipid content of the same pollen without affecting the other compounds. Low protein content did not affect bees in this study. However, as all diets contained more than 20% of protein, we cannot conclude that proteins are not playing a role in diet quality. Regarding the literature, we hypothesize that protein content is important up to a certain threshold that can be close to 20% depending on the species (Regali and Rasmont, 1995; Génissel et al., 2002; Taseï and Aupinel, 2008b; Alaux et al., 2010), and can become detrimental if in excess (Standifer, 1967; Herbert et al., 1977; Roulston and Cane, 2002; Human et al., 2007). Once the minimum protein requirements are met, this work suggest that amino acid and sterol compositions are playing a key role in reproductive performances.

This study does did consider every element that can alter the quality of a pollen, such as pollenkit digestibility or secondary metabolites (alkaloids, lactones, diterpenes, or cyanogenic glycosides) (Peng et al., 1985; Detzel and Wink, 1993; London-Shafir et al., 2003; Williams, 2003; Gunduz et al., 2008; Kempf et al., 2010; Sedivy et al., 2012; Gosselin et al., 2013), and did not evaluate directly the sterols effect on *A. mellifera* due to measured parameters (only adults evaluated). Further research is therefore needed to fully comprehend the importance of each pollen-quality driver and interspecific differences. However, our study overall supports the need of conserving/introducing plant diversity into agro-ecosystems to meet the nutritional preferences of different bee species.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

AB and LB wrote the manuscript and conducted experiments on the bees. VL did all nutrient analyses. DS was involved in *Apis* experiments. YL and CA supervised *Apis* experiments and redaction. F-VG, FC, GS, and CC perfomed the palynological/pesticide residues analyses and wrote results about it. MV and DM supervised the whole work. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2022.824750/full#supplementary-material

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RESUME

Les abeilles mellifères (*Apis mellifera*), en tant que pollinisateurs majeurs des cultures, sont souvent exposées aux pesticides. Par conséquent, dans le cadre des procédures d'évaluation des risques liés aux pesticides, les tests réglementaires exigent des données toxicologiques sur les abeilles mellifères.

Dans ce contexte, le premier niveau d'évaluation repose sur des tests de toxicité aiguë et chronique mesurant le taux de survie des abeilles en conditions de laboratoire. Cependant, la mortalité liée à une dose ou concentration de pesticide peut varier en fonction de l'état physiologique des abeilles. Ainsi, mon premier objectif a été d'étudier cette variabilité intraspécifique afin de mieux évaluer les risques posés par les pesticides aux abeilles mellifères. J'ai d'abord mis en évidence que la qualité du pollen peut influencer la capacité des abeilles à métaboliser les pesticides ainsi que leur sensibilité à ces derniers, ce qui indique que la disponibilité des ressources polliniques peut entraîner une grande variation des réponses des abeilles mellifères aux pesticides. J'ai également démontré que les butineuses sont plus sensibles à un insecticide (sulfoxaflor) que les nourrices lors d'expositions aiguës et chroniques. Par conséquent, pour éviter une sous-estimation du risque posé par les insecticides aux abeilles mellifères, les abeilles butineuses devraient être incluses dans les tests réglementaires de toxicité des pesticides.

Ensuite, si un risque est identifié lors de l'évaluation de premier niveau, une évaluation de deuxième ou troisième niveau en conditions de terrain est nécessaire. Dans ce contexte, j'ai rédigé une synthèse montrant en particulier que la mesure de l'activité de vol des abeilles, de par sa pertinence écologique et des données qu'elle procure sur les taux de survie, représente une mesure particulièrement pertinente pour évaluer la toxicité des pesticides dans le cadre de tests réglementaires. Mon deuxième objectif a donc été de déterminer comment le suivi de l'activité de vol des abeilles à l'échelle individuelle et coloniale pourrait aider à améliorer l'évaluation de la toxicité des pesticides. J'ai ainsi démontré que l'exposition à une dose unique d'insecticide (sulfoxaflor) peut générer des effets différés jusqu'à l'activité de butinage. Ce résultat constitue un argument en faveur de tests de toxicité à plus long terme (> 48h) lors d'une exposition aiguë à un pesticide. Enfin, la quantification, à l'aide de compteur d'abeille, de l'activité de vol à l'échelle des colonies s'avère être très prometteuse pour identifier les taux de pertes populationnelles suite à une exposition à un pesticide (sulfoxaflor ici), et ainsi déterminer les risques consécutifs d'affaiblissement des colonies.

En conclusion, j'ai montré que les procédures d'évaluation des risques liés aux pesticides peuvent être améliorées en prenant en compte les facteurs physiologiques dans les tests de niveau 1 et en intégrant dans les tests de niveau 2 la surveillance de l'activité de vol des abeilles, en particulier à l'échelle coloniale.

Mots clés : *Apis mellifera*, évaluation des risques, pesticides, sulfoxaflor, azoxystrobine, variabilité intraspécifique, caste comportementale, pollen, activité de vol, taux de mortalité

ABSTRACT

Honeybees (*Apis mellifera*), as a major pollinator of crops are often exposed to pesticides. As a consequence, within the framework of pesticide risk assessment procedures, test guidelines require toxicological data on honeybees.

In this context, the first-tier assessment is based on acute and chronic toxicity tests measuring the survival rate of bees in laboratory conditions. However, the mortality related to a dose or a concentration of pesticide can vary depending on the physiological state of bees. Thus, my first objective was to study this intra-specific variability in order to better assess the risks posed by pesticides to honey bees. I showed that pollen quality can influence the ability of bees to eliminate pesticides as well as their sensitivity to them, indicating that the availability of pollen resources can lead to a large variation in honeybee responses to pesticides. I also showed that foragers are more sensitive to an insecticide (sulfoxaflor) than nurse bees upon acute and chronic exposures. Therefore, to avoid an underestimation of the risk posed by insecticides to honeybees, forager bees should be included in regulatory toxicity tests.

Then, if a risk is identified for honeybees in the first-tier assessment, a second or third-tier assessment (semi-field or field conditions) is required. In this context, I wrote a synthesis showing that the measure of bee flight activity, because of its ecological relevance and the data it provides on survival rates, is particularly a relevant endpoint to assess pesticides toxicity within the frame of regulatory tests. Then, my second objective was to determine how the monitoring of bee flight activity at the individual and colony levels could help to improve the assessment of pesticide toxicity. I found that exposure to a single dose of insecticide (sulfoxaflor) can generate delayed effects until foraging activity. This result provides an argument for more long-term toxicity tests (> 48h) upon acute exposure to a pesticide. Finally, the quantification of flight activity at the colony level, with the use of bee counters, proved to be promising to identify population loss rates following exposure to a pesticide (sulfoxaflor here), and consequently determine the risks of colony size reduction.

In conclusion, I showed that pesticide risk assessment procedures can be improved by considering physiological factors in Tier 1 tests and by integrating the monitoring of bee flight activity, especially at the colony level, in Tier 2 tests.

Keywords: *Apis mellifera*, risk assessment, pesticides, sulfoxaflor, azoxystrobin, intraspecific variability, behavioural caste, pollen, flight activity, mortality rate