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(TOOLS FOR) UNDERSTANDING AND IMPROVING HONEY BEE HEALTH

(OUTILS POUR) COMPRENDRE ET AMÉLIORER LA SANTÉ DES ABEILLES DOMESTIQUES

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“ *It is not the mountain we conquer but ourselves.”*

Sir Edmund Hillary

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Abbreviations

ABP	Animal by-products
ADI	Acceptable Daily Intake in mg kg^{-1} body weight day^{-1}
AESA	Association of Epidemiology and Animal Health
AHAW	Animal Health And Welfare
AMPA	Aminomethylphosphonic acid
ARfD	Acute Reference Dose in mg kg^{-1} body weight day^{-1}
AUC	Area under the curve
AVI	Algemene Vlaamse Imkervereniging
AVIB	Antwerpse Vereniging voor Imkersbelangen
b.w.	body weight
BIP	Bee Informed Partnership
BMP	Beekeeping Management practices
CARI	Research and Information Center on beekeeping
CART	Classification and regression tree
COE	Carboxylesterases
CTA	Classification tree analysis
CYP	Cyclochrome P450
DEET	N,N-Diéthyl-3-méthylbenzamide
df	degree of freedom
DT50/ DT90	Degradation time (in days) of 50/90% of the substance
DWV	Deformed wing virus
EDC	Estimated Daily Contribution
EDI	Estimated Daily Intake
EFSA	European Food Safety Authority
EMA	European Medicines Agency
EPA	Unites States Environmental protection Agency
EPILOBEE	Pan-European Epidemiological surveillance program on honey bee mortality
EU	European Union
EURL	European Union Reference Laboratoy for the bee health
FABW	Fédération des Apiculteurs du Brabant Wallon
FARAH	Fundamental and Applied Research for Animal and Health
FASFC	Federal Agency for the Safety of the Food Chain
FPAL	Fédération Provinciale d'Apiculture de Luxembourg
FPUPN	Fédération Provinciale des Unions Professionnelles de Namur
FRPLA	Fédération Royale Provinciale Liégeoise d'Apiculture
FRUPAH	Fédération des unions professionnelles apicoles du Hainaut
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GBH	Glyphosate-based herbicide
GC/MS-MS	Gas chromatography/tandem mass spectroscopy
GC-MSD	Das chromatography-mass selective detector
GST	Glutathione transferases
GST3	Glutathione S-transferase 3
HBM	Health Belief Model
HQ	Hazard quotient
INRA	Agriculture Research Institute
ITSAP	Technical and scientific research Institute for bees
IUCN	The International Union for Conservation of Nature
KOIV	Koninklijke Oost-Vlaamse Imkersbond
KWVIB	Koninklijke West-Vlaamse Imkersbond
LC/MS-MS	Liquid chromatography/tandem mass spectroscopy
LD_{50}	Acute lethal dose after 48h of exposition

LIB	Limburgse Imkersbond
LOD	Limit of detection
LOQ	Limit of quantification
MGST	Microsomal glutathione S-transferase 1
MRL	Maximum residue level
P450	cytochrome P450 monooxygenases
PPDB	Pesticides properties DataBase
QuEChERS	Quick Easy Cheap Effective Rugged Safe
RLP8	Receptor-like protein
ROC	Receiver operating characteristic
ROS	Reactive Oxygen Species
Sciensano	Institution issue de la fusion entre l'ancien Centre d'Étude et de Recherches Vétérinaires et Agrochimiques (CERVA) et l'ex-Institut scientifique de Santé publique (ISP)
SD	Standard deviation
SPM	Salford Predictive Modeler
SRABE	Société royale d'apiculture de Bruxelles et environs
TMDI	Theoretical Maximum Daily Intake,
UFAWB	Union des fédérations apicoles de Wallonie et de Bruxelles
UGent	University of Gent
ULiège	University of Liège
UREAR	Research Unit of Epidemiology and Risk Analysis applied to veterinary sciences
URRW	Union royale des ruchers wallons
VBVI	Verbond van Vlaams-Brabantse Imkersverenigingen
VNIF	Vlaams-Nederlandse Imkerfederatie
VSDB	Veterinary Substances DataBase

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Summary

Background: Bees are important pollinators of many crops and plants, contributing to about one-third of the human diet and providing immeasurable ecosystem services. Honey bee health has been declining since the end of the 1980s in Belgium, the rest of Europe and the USA. In 2012–2013, the Belgian winter colony loss rate was estimated at 34.6%, the highest percentage among 17 participating European countries in the European EPILOBEE study. There exist a variety of factors that negatively impact the health and survival of managed honey bee colonies, including management practices, the spread of parasites and pathogens, the loss of habitat, the reduced availability or quality of food resources, climate change, poor queen quality, as well as exposure to agricultural and apicultural pesticides both in the field and in the hive. These factors are often closely intertwined, and it is unlikely that a single stressor is driving colony losses. A clear and comprehensive overview of the impact of some potential risk factors on honey bee health was lacking in Belgium.

Methodology: This study consists of five parts. The first part aimed at **gathering data allowing us to create a comprehensive register of beekeeping management practices (BMP) and to correlate the data** of both parts of the country **with the registered colony loss rates to detect potential inappropriate BMP** and have a better understanding of risk factors in Belgium (**study 1)**. This was achieved by 186 face-to-face interviews and visits to apiaries throughout the country. The second part of our work aimed at assessing **the occurrence and the concentration of pesticide residues in beeswax, and at estimating their potential toxicity risk to bees.** The toxicity risk to bees expressed as the Hazard Quotient was calculated for each of the 186 extracted wax samples during the visits to the apiaries. A multivariate logistic regression model and a risk-based model were used to predict colony bee mortality (**study 2**). More specifically, for the acaricide flumethrin and the glyphosate-based herbicide, two specific studies to assess the health risk posed by these pesticide residues to honey bees and honey consumers were additionally implemented (**studies 3 and 4**). The third part of our work aimed **at initiating a change in BMP**. In this perspective, an unpreceded sociological online survey was designed using a grounded theory from health psychology that we adapted to the beekeepers (**study 5**). Meanwhile, beeswax adulteration emerged as an additive prominent problem for beekeepers. In the fourth phase of our work, we aimed at assessing **the current situation of beeswax adulteration in beekeepers and commercial wax in Belgium** through a nationwide survey (**study 6**). To date, no maximum residues limit has been set for adulterants or pesticide residues in beeswax, therefore in the fifth part of our study, we designed a **novel field realistic methodology to rear honey bees pupae** in newly formed colonies to reduce the influence of external factors such as *Varroa* infestation. **The impact of beeswax contaminations and adulteration on honey bees gene expression was examined in this last study** (**study 7**).

Results: From **study 1**, the results obtained confirmed the hypothesis of interactions between some BMP as factors in amplifying the risk of mortality to honey bees, the classification tree analysis allowed us to determine the relative importance and inter-relation among the different risk indicators of colony losses. Based on these results, an innovative BeeBestCheck tool was designed as inventory to improve and advise beekeepers on their BMP. In the three studies dealing with pesticide residue contaminations and their risk (**studies 2, 3 and 4**), we found 54 different pesticides and veterinary drug residues in beeswax in addition to acaricide flumethrin and herbicide glyphosate. The multivariate logistic regression model (**study 2**) showed a statistically significant influence of chlorfenvinphos on honey bee mortality. This national study provided guidelines on the re-use of beeswax by beekeepers and showed the necessity to introduce maximum residue levels for the global beeswax trade. An online tool (BeeToxWax) was developed to enable beekeepers and wax traders to estimate the potential risk to honey bee health associated with contaminated wax. The detected concentrations of flumethrin (**study 3**) did not represent a risk for human health, or honey bee health. In the glyphosate study (**study 4**) the maximum concentration found in beebread led to sub-lethal exposure to bees. Both studies concluded that clarifications and further research are needed to estimate the risk of active ingredients alone and in formulations, especially at levels below the regulatory safe limits and over longer durations. More studies are needed to assess synergies with other pesticides, and longer-term exposures at sub-lethal doses. The perception study (**study 5**) implemented to better understand amateur beekeepers' perception of risks affecting bee health to initiate change in BMP showed that beekeepers with a greater level of perceived risk combined with strong perceptions of the benefits of action have increased motivation to act in better and have acceptable loss rates. Despite a good general estimate of risks to bee colonies, the pesticide issue appears to be a source of confusion and poor understanding. Clear and harmonised information should be integrated into risk management recommendations. The results of this survey highlight the importance of looking beyond socio-economic determinants in any risk mitigation strategy associated with bee mortality when dealing with amateur beekeepers. The frequent presence of the adulterants such as stearin and paraffin in beeswax was highlighted by **study 6**, and the levels of stearin found are compatible with detrimental effects on bee brood. Again, the regulatory framework that defines beeswax purity criteria, to prevent beeswax adulteration and ensure beeswax safety is needed. Finally, in **study 7**, the gene expression profile of four genes involved in the major immune response to pathogens and environmental stress factors (Imd, dorsal, domeless and defensin), and two genes involved in detoxifications mechanisms (CYP6AS14 and CYP9Q3) were analysed on pupae raised in contaminated or adulterated beeswax. The immune system of pupae raised in acrinathrin-contaminated beeswax was triggered and the expression of CYP6AS14 was significantly upregulated at sublethal doses of the pesticide (exposure to 0.0125 and 0.025 mg/kg). Almost all expression levels of the tested immune and detoxification genes were down-regulated when pupae were exposed to sublethal concentrations of chlorpyrifos-contaminated wax, at higher concentrations, pupae seemed to have a

suppressed immunity. The exposure to stearin at higher percentages than 4%, triggered both the immune system and detoxification system.

Conclusion: At the start of our research, considerable data collection work implemented in the field was necessary to assess the beekeeping state of the art, gather beekeepers' concerns and examine some risk factors specifically impacting honey bee health in the Belgian context of amateur/hobbyist beekeeping. We have been able to point some of the risk factors of BMP and chemical contaminations, and have deepened our research on the chemical contaminations to estimate their impact on honey bee and pupae health, and gene expression. Our work has been shared with the beekeepers and the close collaboration with the sector has enabled us to develop tools that best meet the sector's expectations. Research in beekeeping faces a lot of difficulties due to the lack of epidemiological data and data such as pesticide lethal dose for larvae, it is partly those gaps that we tried to fill. The application of social sciences and looking into the behavioural mechanisms of the beekeepers have allowed us to point out levers that are conditions for a change in the beekeeping sector. We hope the change that has been initiated will run its course.

Résumé

Introduction: Les abeilles sont d'importants pollinisateurs de nombreuses cultures et plantes, contribuant à environ un tiers de l'alimentation humaine et fournissant des services écosystémiques incommensurables. La santé de l'abeille domestique est en déclin depuis la fin des années 1980 en Belgique, dans le reste de l'Europe et aux USA. En 2012-2013, le taux de mortalité hivernale belge des colonies d'abeilles a été estimé à 34,6%, pourcentage le plus élevé parmi les 17 pays européens participants à l'étude européenne EPILOBEE. Un nombre important de facteurs incluant la pratique apicole, la propagation de parasites et agents pathogènes, la dégradation de l'habitat, la réduction de la disponibilité ou de la qualité des ressources alimentaires, le changement climatique, la mauvaise qualité des reines, ainsi que l'exposition aux pesticides agricoles et apicoles, tant sur le terrain que dans la ruche, peuvent avoir un impact négatif sur la santé et la survie des colonies d'abeilles domestiques. Ces facteurs sont souvent étroitement liés et il est peu probable qu'un seul de ces facteurs de risque soit à l'origine des pertes de colonies. Un aperçu clair et complet de l'impact des facteurs de risque potentiels sur la santé des abeilles mellifères faisait défaut en Belgique.

Méthodologie: Ce travail est composé de cinq parties. La première étude visait à **recueillir des données nous permettant de créer un registre complet des pratiques apicoles en Belgique et de corréler les données de deux régions (Flandre et Wallonie) du pays avec les taux de mortalité de colonies enregistrés et ce dans le but de détecter les pratiques potentiellement inappropriées** et d'avoir une meilleure compréhension des facteurs de risque encourus en Belgique (**étude 1**). Pour ce faire, 186 entretiens en face à face couplés à des visites de ruchers ont été réalisés à travers tout le pays. La deuxième partie de notre travail visait à **évaluer la présence et la concentration de résidus de pesticides dans la cire d'abeille, et à estimer leur risque de toxicité potentiel pour les abeilles**. Le risque de toxicité pour les abeilles, exprimé par le quotient de risque, a été calculé pour chacun des 186 échantillons de cire extraits lors des visites de ruchers. Un modèle de régression logistique multivariée et un modèle basé sur le risque ont été utilisés pour prédire la mortalité des colonies (**étude 2**). Plus spécifiquement, pour l'acaricide fluméthrine et l'herbicide à base de glyphosate, deux études spécifiques visant à évaluer le risque sanitaire que représentent ces résidus de pesticides pour les abeilles et les consommateurs de miel ont été mises en œuvre en complément (**études 3 et 4**). Le troisième volet de notre travail visait à **initier un changement de pratique apicole**. Pour ce volet, une enquête sociologique inédite (diffusée en ligne) a été conçue en utilisant une théorie issue de la psychologie de la santé humaine que nous avons adaptée à la santé animale (**étude 5**). Parallèlement, la question de l'adultération de la cire d'abeille a émergé comme problématique préoccupante pour les apiculteurs. Dans le quatrième volet de notre travail, nous avons **évalué l'état des lieux de l'adultération de la cire d'abeille chez les apiculteurs et dans les cires issues du commerce en Belgique** par le biais d'une enquête nationale (**étude 6**). À ce jour, aucune limite maximale de résidus n'a été fixée pour les

adultérants ou les résidus de pesticides dans la cire d'abeille. Par conséquent, dans la cinquième partie de notre étude, nous avons conçu **une nouvelle méthode de production de couvain, en condition réelle dans des cires contaminées ou adultérées avec des concentrations de pesticides/adultérants proches des concentrations retrouvées dans les ruches**. Les cadres de cires contaminés ont été introduits dans des colonies nouvellement formées afin de réduire l'influence de facteurs externes tels que l'infestation par *Varroa destructor*. **L'impact des contaminations et de l'adultération de la cire d'abeille sur l'expression génique des abeilles a été examiné dans cette dernière étude** (**étude 7**).

Résultats: A partir de l'étude 1, les résultats obtenus ont confirmé l'hypothèse d'interactions entre certaines pratiques apicoles comme facteurs d'amplification du risque de mortalité des abeilles domestiques, l'analyse de l'arbre de classification nous a permis de déterminer l'importance relative et l'interrelation entre les différents indicateurs de risque de pertes de colonies. Sur la base de ces résultats, l'outil didactique BeeBestCheck a été conçu afin de conseiller les apiculteurs sur les améliorations possibles de leur pratique apicole. Dans les trois études traitant des contaminations par des résidus de pesticides et de leur risque (**études 2, 3 et 4**), 54 pesticides différents et résidus de traitements vétérinaires ont été trouvés dans la cire d'abeille, en plus de l'acaricide fluméthrine et de l'herbicide glyphosate. Le modèle de régression logistique multivariée (**étude 2**) a montré une influence statistiquement significative du chlorfenvinphos sur la mortalité des abeilles domestiques. Cette étude nationale a fourni des directives sur la réutilisation/le recyclage de la cire d'abeille par les apiculteurs et a montré la nécessité d'introduire des limites maximales de résidus pour le commerce mondial de la cire d'abeille. Un outil en ligne (BeeToxWax) a été développé pour permettre aux apiculteurs et aux transformateurs de cire d'estimer le risque de toxicité potentiel de cette dernière pour la santé des abeilles mellifères. Les concentrations détectées de fluméthrine (**étude 3**) ne représentaient pas un risque pour la santé humaine, ni pour celle des abeilles domestiques. Dans l'étude sur le glyphosate (**étude 4**), la concentration maximale trouvée dans le pain d'abeille a entraîné une exposition sublétale pour les abeilles. Les deux études ont conclu que des clarifications et des recherches supplémentaires sont nécessaires pour estimer le risque des matières actives, seules et en formulations, en particulier à des concentrations inférieures aux limites réglementaires de sécurité et prolongées dans le temps (effets chroniques). D'autres études sont nécessaires pour évaluer l'effet des synergies avec d'autres pesticides et les expositions à plus long terme à des doses sublétales. L'étude de perception (**étude 5**) mise en œuvre pour mieux comprendre la perception par les apiculteurs amateur/de loisir des risques affectant la santé des abeilles a montré que les apiculteurs ayant un niveau de perception élevé du risque combiné à une perception accrue des bénéfices de l'action contre ce risque ont une motivation augmentée pour mieux agir et ont des taux de perte acceptables. Malgré une bonne estimation générale des risques, la perception liée aux risques des pesticides n'est pas bien comprise. Des informations claires et harmonisées devraient être intégrées dans les recommandations de gestion des risques. Les résultats de cette enquête soulignent l'importance de s'intéresser aux déterminants socio-économiques dans toute

stratégie d'atténuation des risques associés à la mortalité des abeilles lorsqu'il s'agit d'apiculteurs amateurs (de loisir). La présence fréquente d'adultérants tels que la stéarine et la paraffine dans la cire d'abeille a été mise en évidence dans l'**étude 6**, et les niveaux de stéarine retrouvés sont compatibles avec des effets néfastes sur le couvain. Encore une fois, le cadre réglementaire qui définit les critères de pureté de la cire d'abeille, pour empêcher l'adultération de celle-ci et assurer la qualité toxicologique de la cire d'abeille, est nécessaire. Enfin, dans l'**étude 7**, le profil d'expression génique de quatre gènes majeurs impliqués dans la réponse immunitaire aux pathogènes et aux facteurs de stress environnementaux (Imd, dorsal, domeless et defensin), et deux gènes impliqués dans les mécanismes de détoxification (CYP6AS14 et CYP9Q3), ont été analysés sur des pupes élevées dans de la cire contaminée ou adultérée. Le système immunitaire des pupes d'abeilles élevées dans de la cire contaminée par de l'acrinathrine, a été déclenché et l'expression du gène CYP6AS14 impliqué dans les mécanismes de détoxication a été régulée à la hausse de manière significative à des doses sublétales du pesticide (exposition à 0,0125 et 0,025 mg/kg). Presque tous les niveaux d'expression des gènes immunitaires et de détoxification testés étaient régulés à la baisse lorsque les pupes étaient exposées à des concentrations sublétales de cire contaminée par le chlorpyrifos. A des concentrations plus élevées, l'immunité des pupes semble avoir été supprimée. L'exposition des pupes à la stéarine à des pourcentages supérieurs à 4 % a déclenché à la fois le système immunitaire et le système de détoxification.

Conclusion: Au début de notre travail de recherche, un travail considérable de collecte de données mis en œuvre sur le terrain a été nécessaire pour évaluer l'état des lieux de la pratique apicole, recueillir les préoccupations des apiculteurs et examiner certains facteurs de risque ayant spécifiquement un impact sur la santé des abeilles domestiques, dans le contexte belge d'une apiculture de loisir. Nous avons pu mettre en évidence certains des facteurs de risque liés aux pratiques apicoles et aux contaminations chimiques. L'étude de l'impact de contaminants sur la santé de l'abeille et sur l'expression génique des pupes a été réalisée. Les résultats et conclusions de nos travaux ont été partagés avec les apiculteurs et l'étroite collaboration avec le secteur nous a permis de développer des outils répondant au mieux aux attentes des apiculteurs. La recherche en apiculture rencontre beaucoup de difficultés en raison du manque de données épidémiologiques et de données telles que les doses létales des pesticides pour les larves, c'est en partie ces lacunes que nous avons essayé de combler. L'application des sciences sociales et l'étude des mécanismes comportementaux des apiculteurs nous ont permis de déterminer les leviers nécessaires à un changement de pratique apicole dans le secteur. Nous espérons que le changement amorcé suivra son cours.

Samenvatting

Introductie: Bijen zijn belangrijke bestuivers van vele gewassen en planten. Zij leveren een bijdrage aan ongeveer een derde van de menselijke voeding en leveren onmeetbare ecosysteemdiensten. In België,de rest van Europa en de VS gaat de gezondheid van honingbijen sinds het einde van de jaren tachtig achteruit. In 2012-2013 werd het Belgische winterverlies geschat op 34,6%, het hoogste percentage van de 17 deelnemende Europese landen aan de Europese EPILOBEE-studie. Er zijn verschillende factoren die de gezondheid en overleving van beheerde honingbijenkolonies negatief beïnvloeden, waaronder bedrijfsmethoden, de verspreiding van parasieten en ziekteverwekkers, het verlies van habitats, de verminderde beschikbaarheid of kwaliteit van voedselbronnen, klimaatverandering, slechte koninginnenkwaliteit en blootstelling aan landbouw en bijenteeltpesticiden, zowel in het veld als in de kast. Deze factoren zijn vaak nauw met elkaar verweven en het is onwaarschijnlijk dat één enkele stressfactor de oorzaak is van het verlies van kolonies. In België ontbrak een duidelijk en volledig overzicht van het effect van een aantal potentiële risicofactoren op de gezondheid van honingbijen.

Methodologie: Deze studie bestaat uit vijf delen. Het eerste deel had tot doel gegevens te verzamelen om een uitgebreid register van bijenteeltpraktijken op te stellen en de gegevens van beide landsdelen te correleren met de geregistreerde percentages van het kolonieverlies om mogelijke ongeschikte bijenteeltpraktijken op te sporen en een beter inzicht te krijgen in de risicofactoren in België (**studie 1**). Dit werd bereikt door 186 persoonlijke interviews en bezoeken aan bijenstanden in het hele land. Het tweede deel van ons werk had tot doel het voorkomen en de concentratie van residuen van bestrijdingsmiddelen in bijenwas te bepalen en het potentiële toxiciteitsrisico voor bijen in te schatten. Het toxiciteitsrisico voor bijen, uitgedrukt als de risicoquotiënt, werd berekend voor elk van de 186 geëxtraheerde was monsters tijdens de bezoeken aan de bijenstallen. Een multivariaat logistisch regressiemodel en een risico gebaseerd model werden gebruikt om de bijensterfte in kolonies te voorspellen (**studie 2**). Meer specifiek werden voor het acaricide flumethrin en het herbicide op basis van glyfosaat twee specifieke studies uitgevoerd om het gezondheidsrisico van deze bestrijdingsmiddelenresiduen voor honingbijen en honingconsumenten te bepalen (**studie 3 en 4**). Het derde deel van ons werk was erop gericht een verandering van de bijenteeltpraktijken op gang te brengen. In dit perspectief werd een niet eerder uitgevoerde sociologische online enquête ontworpen op basis van een theorie uit de gezondheidspsychologie die we aanpasten aan de imkers (**studie 5**). Ondertussen kwam bijenwasvervalsing naar voren als een aanvullend prominent probleem voor imkers. In de **vierde fase** van ons werk wilden we de huidige situatie van bijenwasvervalsing bij imkers en commerciële was in België bepalen door middel van een nationale enquête (**studie 6**). Tot op heden is er geen maximumlimiet vastgesteld voor vervalsingsmiddelen of residuen van bestrijdingsmiddelen in bijenwas. Daarom hebben we in het vijfde deel van onze studie een nieuwe veld-realistische methode

ontworpen om honingbijenpoppen op te kweken in pas gevormde kolonies om de invloed van externe factoren zoals Varroa-aantasting te verminderen. In deze laatste studie (**studie 7**) werd het effect van bijenwasverontreiniging en vervalsing op de genexpressie van honingbijen onderzocht.

Resultaten: Van **studie 1** bevestigden de verkregen resultaten de hypothese van interacties tussen sommige bijenteeltpraktijken als factoren in het versterken van het risico van sterfte bij honingbijen.De classificatieboomanalyse liet toe het relatieve belang en de onderlinge relatie tussen de verschillende risico-indicatoren van kolonieverlies te bepalen. Op basis van deze resultaten werd een innovatieve BeeBestCheck-tool ontworpen als inventaris om imkers te adviseren over hun bijenteeltpraktijken. In de drie studies naar de verontreiniging met residuen van pesticiden en hun risico (**studies 2, 3 en 4**) vonden wij 54 verschillende residuen van pesticiden en diergeneesmiddelen in bijenwas, naast het acaricide flumethrin en het herbicide glyfosaat. Het multivariate logistische regressiemodel (**studie 2**) toonde een statistisch significante invloed van chloorfenvinfos op de honingbijensterfte. Deze nationale studie leverde aanbevelingen op voor het hergebruik van bijenwas door bijenhouders en toonde de noodzaak om maximum residugehalten in te voeren voor de wereldwijde handel in bijenwas. Er is een onlinetool (BeeToxWax) ontwikkeld waarmee imkers en washandelaren het potentiële risico voor de gezondheid van honingbijen in verband met verontreinigde was kunnen inschatten. De vastgestelde concentraties flumethrin (**studie 3**) vormden geen risico voor de menselijke gezondheid of de gezondheid van honingbijen. In de glyfosaatstudie (**studie 4**) leidde de in bijenbrood aangetroffen maximumconcentratie tot subletale blootstelling van bijen. In beide studies werd geconcludeerd dat verduidelijkingen en verder onderzoek nodig zijn om het risico van werkzame stoffen alleen en in formuleringen in te schatten, met name bij niveaus onder de wettelijke veilige grenswaarden en gedurende langere perioden. Er zijn meer studies nodig om synergiën met andere pesticiden en blootstellingen op langere termijn bij subletale doses te bepalen. Uit de perceptiestudie (**studie 5**) die is uitgevoerd om meer inzicht te krijgen in de perceptie van de risico's voor de bijengezondheid door hobbyimkers om de aanzet te geven tot een verandering van de bijenteeltpraktijken bleek dat imkers met een betere perceptie van het risico in combinatie met een sterke perceptie van de voordelen van maatregelen meer gemotiveerd zijn om beter te handelen en aanvaardbare verliescijfers hebben. Ondanks een goede algemene inschatting van de risico's voor bijenvolken blijkt de pesticiden kwestie een bron van verwarring en onbegrip. In de aanbevelingen voor risicobeheer moet duidelijke en geharmoniseerde informatie worden opgenomen. Uit de resultaten van dit onderzoek blijkt hoe belangrijk het is om bij elke strategie voor risicobeperking in verband met bijensterfte verder te kijken dan alleen naar sociaal economische determinanten wanneer het gaat om hobby imkers. In **studie 6** werd gewezen op de frequente aanwezigheid van versnijdingsmiddelen als stearine en paraffine in bijenwas, en de aangetroffen hoeveelheden stearine zijn gepaard met schadelijke effecten op het bijenbroed. Ook hier is een regelgevend kader nodig dat zuiverheidscriteria voor bijenwas vaststelt om vervalsing van bijenwas te voorkomen en de veiligheid van bijenwas te waarborgen. In **studie 7** ten slotte werd het

genexpressieprofiel van vier genen die betrokken zijn bij de belangrijkste immuunreactie op pathogenen en omgevingsstressfactoren (Imd, dorsal, domeless en defensin), en twee genen die betrokken zijn bij ontgifting mechanismen (CYP6AS14 en CYP9Q3) geanalyseerd bij poppen die waren grootgebracht in besmette of versneden bijenwas. Het immuunsysteem van poppen opgekweekt in met acrinathrin besmette bijenwas werd geactiveerd en de expressie van CYP6AS14 was significant verhoogd bij subletale doses van het pesticide (blootstelling aan 0,0125 en 0,025 mg/kg). Bijna alle expressieniveaus van de geteste immuun en ontgifting genen waren neergereguleerd wanneer de poppen werden blootgesteld aan subletale concentraties van met chloorpyrifos verontreinigde was; bij hogere concentraties leken de poppen een onderdrukte immuniteit te hebben. De blootstelling aan stearine in hogere percentages dan 4% activeerde zowel het immuunsysteem als het ontgifting systeem.

Conclusie: Aan het begin van ons onderzoek was een aanzienlijke hoeveelheid gegevensverzameling op het terrein nodig om de situatie in de bijenteelt te bepalen, de bezorgdheid van de imkers te documenteren en een aantal risicofactoren te onderzoeken die specifiek van invloed zijn op de gezondheid van honingbijen in de Belgische context van hobby bijenteelt. We hebben een aantal risicofactoren uit het bijenteeltpraktijk en chemische verontreinigingen kunnen distilleren en ons onderzoek verdiept naar de chemische verontreiniging om de impact ervan op de gezondheid van honingbijen en poppen en op de genexpressie in te schatten. Ons werk werd gedeeld met de bijentelers en de nauwe samenwerking met de sector heeft ons in staat gesteld instrumenten te ontwikkelen die optimaal aan de verwachtingen van de sector beantwoorden. Het onderzoek in de bijenteelt ondervindt veel moeilijkheden door het gebrek aan epidemiologische gegevens en gegevens zoals de waarden van dodelijke doses pesticiden voor larven; het zijn gedeeltelijk die leemten die wij hebben proberen op te vullen. Dankzij de toepassing van de sociale wetenschappen en de bestudering van de gedragsmechanismen van de bijenhouders hebben wij hefbomen kunnen identificeren die voorwaarden zijn voor een verandering in de bijenteeltsector. Wij hopen dat de ingezette verandering haar beloop zal hebben.

General preamble

Since the end of the year 1990, beekeepers are facing severe and persistent mortalities in Belgium as well as in other world parts. Sound colony losses were reported by beekeepers and scientists but no official data were available.

In 2009, the European Food Safety Authorities (EFSA) launched a project on the description of bee surveillance programmes existing in Europe. The report highlighted the lack of comparable data and common operating systems to assess the mortality of bee colonies on the European scale, and a first harmonised active epidemiological surveillance programme on honey bee colony mortality (EPILOBEE) was set up. This first pan-European EPILOBEE study highlighted the important winter mortality rates within the Belgian colonies (respectively 32.4 % and 14.8 % for winter 2012-2013 and winter 2013-2014; corresponding to the 1st and 3rd highest mortality rates at the European level).

Other national and European monitoring projects such as HealthyBee (FASFC 2016-2018), APENET (Porrini et al., 2016), the German Bee Monitoring Project (Genersch et al., 2010b), EPILOBEE (Chauzat et al., 2016; Laurent et al., 2015) and COLOSS (Gray et al., 2019) highlighted the direct and/or indirect role of the beekeeper in ensuring the health and the productivity of honey bee colonies, nevertheless, the impacts of beekeeper knowledge and beekeeping management practices (BMP) have often been overlooked, despite honey bees being a managed pollinator. At the time, no comprehensive register for BMP with representative and comparable data across the different regions of Belgium and at the European level was available.

In the first instance, a field study aiming at gathering data allowing us to create this register was implemented. The collected data allowed us to have a better view of the BMP in Belgium and to correlate the data of both parts of the country with the registered colony loss rates to detect potentially inappropriate BMP and have a better understanding of mortality factors in Belgium. Better BMP can be implemented from a short-term perspective by individual beekeepers and have the potential to reduce colony losses. Best management practices were highlighted and an online tool for beekeepers was designed allowing them to evaluate the effect of their management practices on colony health (**study 1**) and giving them recommendations to improve it. During this field study, a concern was raised by most beekeepers: the impact of beeswax quality on brood development. In 2016, beekeepers massively reported poor brood development and excessive mortality after the use of commercial beeswax foundation in their hives.

The second phase of our work aimed at assessing the occurrence and the concentrations of pesticide residues in beeswax, and at estimating their potential toxicity risk to bees. The toxicity risk to bees expressed as the Hazard Quotient (HQ) was calculated for each of the 186 extracted wax samples. A multivariate logistic regression model and a risk-based model were used to predict colony bee mortality (**study 2**). More specifically, for the acaricide flumethrin and the glyphosate-based herbicide,

two specific studies to assess the health risk posed by these pesticide residues to honey bees and honey consumers were additionally implemented (**studies 3 and 4**). This national survey on beeswax contaminations provided guidelines on the use and recycling of beeswax by beekeepers and showed the necessity to introduce maximum residue levels for global trade in beeswax. An online tool was developed to enable beekeepers and wax traders to estimate the cumulative potential risk of pesticide residues in beeswax to honey bees.

The third phase of our work aimed at initiating a change in BMP. In this perspective, it was crucial to understand the factors determining this change we aimed at. Before applying adequate risk management, beekeepers need to perceive the impact of risks on the colony, as well as the benefits of the actions to undertake. An unpreceded sociological survey designed with a grounded theory from health psychology was used to build a framework adapted to the beekeepers (**study 5**).

Meanwhile, beeswax adulteration emerged as an additive prominent problem for beekeepers. In the fourth phase of our work, we aimed at assessing the current situation of beeswax adulteration in beekeepers and commercial wax in Belgium through a nationwide survey (**study 6**). To date, no maximum residues limit has been set for adulterants, therefore a novel field realistic methodology to produce honey bees pupae in contact with adulterants in beeswax was designed. The impact of beeswax contaminations and adulteration on honey bee gene expression was also examined in this last study (**study 7**).

This work provides an overview of the current BMP in Belgium, and their impact on honey bee health and exposes the determining factors for a behavioural change of the beekeepers. An assessment of pesticide residue contaminations and adulterations in beeswax and their related risk to honey bees and consumers' health are also provided. Tools, guidelines and recommendations for the beekeepers, veterinarians, beekeeping sector in general as well as the authorities are available to improve BMP, beeswax quality and recycling, to sanitize the beeswax commercial trade stream, and to set maximum residue levels (MRL) for some pesticide residues and adulterants.

Chapter 1 - Introduction

1. No bees, no food

We have all heard this sentence or similar ones. The majority of plant species (84%) benefit from insect pollination and 78% of temperate wildflowers need biotic pollination. Biotic pollination relies on living pollinators to move the pollen from one flower to another. The majority of European food production (76%) is dependent on pollination by both wild and domestic bees as well as other pollinators (**Figure 1**). It has been estimated that approximately 10% of the total economic value of the European agricultural output for human food production, which amounted to €22 billion in 2015 (€14.2 billion per year for the EU) (The European Greens, 2018), is due to insect pollination. The honey bee (*Apis mellifera* L.), a managed eusocial organism, provides highly valued pollination services for a wide variety of natural ecosystems and agricultural crops (Calderone, 2012). They rank as the most frequent single species of pollinator for crops worldwide (Garibaldi et al., 2014).

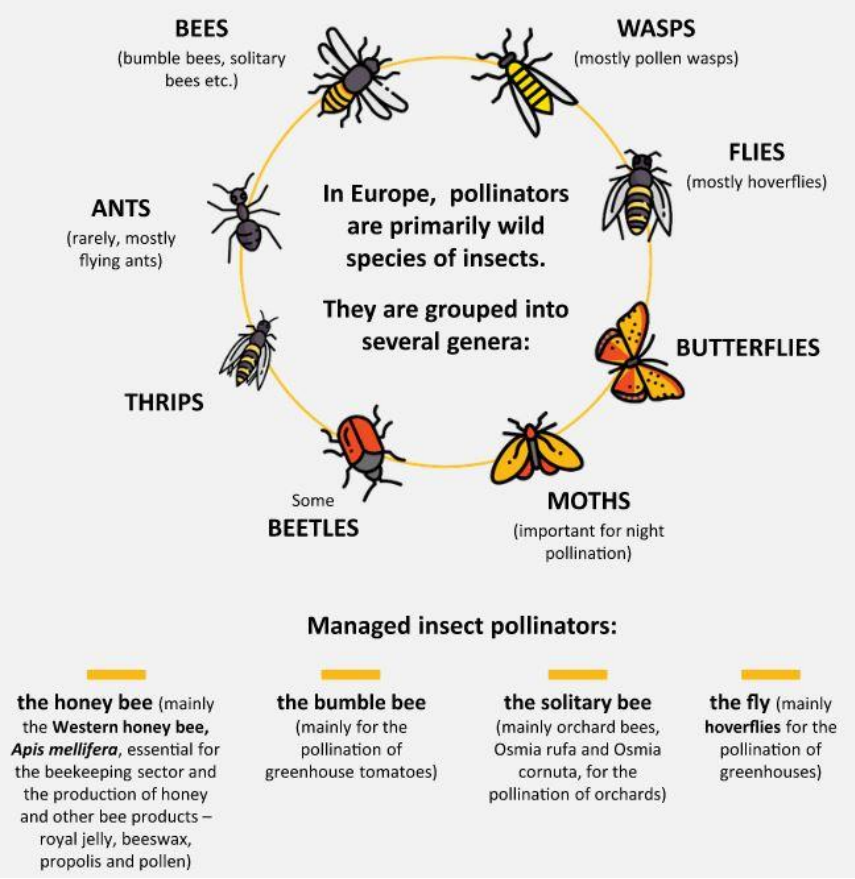

Figure 1: The different pollinators (ECA, 2020)

Pollination is thus an essential step in the reproductive process of the world's nearly 300,000 species of flowering plants because it is usually required for the production of seeds (Ollerton et al., 2011). Nevertheless, the diversity and abundance of wild and domestic insect pollinators have declined in many agricultural landscapes in Europe and the rest of the world (Ellis et al., 2010; Potts et al., 2010a; VanEngelsdorp et al., 2009).

Among the declining species, bees have attracted public attention. Fewer pollinators mean many plant species could decline or even disappear along with the organisms that directly or indirectly depend on them. In addition, the decline in numbers and diversity of pollinator populations affects food security with potential losses in agricultural yields. To tackle the issue and complement efforts at the EU and national levels, the European Commission presented in 2018 the EU Pollinators Initiative, the first comprehensive initiative at the EU level, focusing on wild pollinating insects. It aimed at improving knowledge about the decline, tackling the causes and raising awareness of the issue.

It is a fact that Belgium is no exception to the decline in bees observed worldwide. The honey bee, *Apis mellifera* L., is also threatened here. The excess mortality rate recorded in Belgian hives during winter 2012-2013 was ±33 %. Of the 370 known species of wild bees, over half are rare or in sharp decline and some have even completely disappeared from the country (Vereecken, 2012).

In Europe, 24% of bumblebee species are threatened by extinction and appear on the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (IUCN, 2014). It is however crucial to protect both honey bees and wild bees: wild bees are more effective pollinators than honey bees (Garibaldi et al., 2014), but honey bees are more "industrious". Furthermore, the data indicate that pollination, particularly that of crops, and therefore their productivity is significantly improved by the simultaneous presence of honey bees and wild bees (Brittain et al., 2013). Humans depend on bees for ecosystem services, and bees depend on humans for their survival (Jacobs et al., 2014). In this context, apiculture represents the close connection between these systems.

The different studies exposed in this manuscript work are exclusively directed toward domestic honey bees.

1.1 Honey bees (*Apis mellifera* L.)

Honey bees are native to Eurasia and Africa but have been spread to four continents by human beings. They are known for the construction of perennial colonial nests from wax, and the large size of their colonies. The first *Apis* bees appear in the fossil record at the Eocene-Oligocene boundary (34 million years ago). Twelve species of honey bee are currently recognised, with many subspecies (FAO, 2020). In contrast to all other bees in Europe, honey bees have both a highly sophisticated system of recruitment and large perennial colonies where they store honey reserves for periods with reduced nectar supply. These reserves are the main reason for a long shared sweet history between honey bees and humans (Crane, 1999; Roffet-Salque et al., 2015)

Honey bee hive number is declining in some parts of the world, but the global total is increasing, contrary to popular concern about the species extinction.

Managed Honey bees (*A. mellifera* L.) represent a unique opportunity to investigate complex health issues affecting a social species. The honey bee is a managed eusocial organism. Its health is mainly assessed at the colony level rather than at the individual level (Afssa, 2008). While honey bees enable us to investigate complex health issues affecting social organisms, defining precise risk or protective indicators remains challenging as some stressors are buffered at the colony level (Straub et al., 2015).

1.2 Honey bees, bioindicator environmental quality

Together with other bioindicators like lichens, mosses or snails, honey bees can be considered as living organisms able to contribute to the assessment of the quality of the environment under the influence of anthropization (Holt and Miller, 2011). The foragers of a single honey bee colony visit and samples numerous plants within a wide area surrounding the apiary. Techniques like waggle dance decoding have been used to determine the direction and distance flown by bees to find food resources (Kohl and Rutschmann, 2021; Nürnberger et al., 2019; Wario et al., 2017, von Frisch, 1946). The waggle dance is one of the tools, together with olfactory cues (Chaffiol et al., 2005), that aims at recruiting foragers to exploit the found sources used by bees to communicate with their sister bees where good sources of nutrition lay.

From several studies found in literature describing foraging distances we know nowadays that bees visit flowers either close to their colonies up to 15 km away (Couvillon et al., 2014; Danner et al., 2017). These distances depend on characteristics like the genetic and physiological status of the colony, the sugar content of nectar, the weather, or the time of the year (Couvillon et al., 2014) with bees flying over an average of 2 km in the summer in temperate regions, while they fly 500 m on average in spring or around 1.3 km on average in autumn. While foraging around, they also unintentionally collect airborne particles or substances diluted in the air (Girolami et al., 2012). Analyses of their products or bees themselves can therefore reveal the pollutants present in a wide area. Colony mortality or morbidity has been correlated with landscapes containing many pollutants (Smart et al., 2016), little nutritional resource diversity (Sgolastra et al., 2017; Woodcock et al., 2017) or a combination of one of these with other stress factors. Therefore, colony status can be used as a bioindicator of the quality of the environment surrounding the hive. This has led to using honey bees as a model for pollinators, and beekeeping products as biological indicators for environmental monitoring (Al Naggar et al., 2015; Balayiannis and Balayiannis, 2008; Bargańska et al., 2016; Celli and Maccagnani, 2003; Chauzat et al., 2011; Conti and Botrè, 2001; Durazzo et al., 2021; Perugini et al., 2018; Porrini et al., 2021). Monitoring of exposure to various environmental contaminants has been carried out in different studies, these contaminants include notably heavy metals (Celli and Maccagnani, 2003), plant protection products (Benuszak et al., 2017; Kiljanek et al., 2017; Mullin et al., 2016), polycyclic aromatic hydrocarbons

(Ciemniak et al., 2013; Kargar et al., 2017; Lambert et al., 2012; Perugini et al., 2009) and radioactive substances (Bargańska et al., 2016). Unfortunately, a specific source of contamination is often difficult to determine.

2. Why are pollinators declining?

Currently, there is no scientific data giving the full picture, but there is evidence of a considerable decline in pollinators, due primarily to human activities. A notable increase in failure of managed European honey bee colonies has been reported in the US, Europe and other areas of the Northern Hemisphere in recent years (Moritz et al., 2010; Potts et al., 2010b), but the underlying causes remain complex and may vary according to the region (Neumann and Carreck, 2010). Scientists and experts involved in researching the problem consider two forms of honey bee losses:

1. Annual (most frequent)—as a result of unsuccessful wintering caused by biotic factors (such as infections and parasites), acute intoxication, and several other causes, which are the subject of the discussion further in this work (van der Zee et al., 2012).
2. Multi-annual—permanent reduction in the number of bee colonies in separate, specific regions.

There is a broad consensus within the scientific community that a combination of several stressors is acting together to cause colony failure (Potts et al., 2010a; VanEngelsdorp et al., 2010). Nevertheless, very little is known about the synergies and interactions between these various factors, and not forgetting the additional influence of climate change.

There are five stressors of global importance that are thought to be relevant to the reducing number of bee colonies in different parts of the world. These are the anthropogenic-driven worldwide spread of pathogenic organisms and invasion of new alien species (e.g. Garigliany et al., 2017 and 2019), climate change and adverse weather/climatic conditions, habitat fragmentation and erosion of biodiversity and the subsequent decrease in food supplies (Durant and Otto, 2019), intensification of agricultural production including the use of fertilizers and pesticides that are increasingly cited by the scientific community as one of the major threats to bees (El Agrebi et al., 2020; Johnson et al., 2013; Perugini et al., 2018) and last but not least beekeeping management practices (BMP) and the loss of immunity due to poor genetic diversity within the *Apis mellifera* L. species (Espregueira Themudo et al., 2020) (**Figure 2**). This poor genetic diversity is primarily explained by the repeated selection of bees with favourable characteristics for beekeepers, such as honey productivity or non-aggressiveness. In section 4, the five stressors of global importance will be presented and explained.

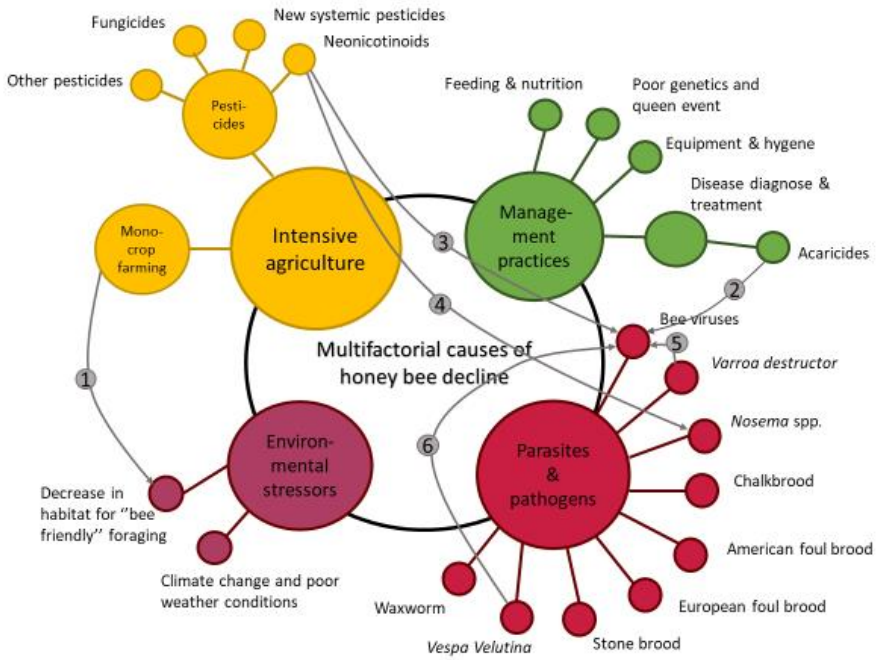

Figure 2: Multifactorial causes of honey bee decline, figure constructed as a result of the literature review of risk factors affecting honey bee health.

Legend: 1-Moncrop farming ► decrease in habitat: reduced growth or higher mortality with increasing urban or agricultural land use (Clermont et al., 2015; Dolezal et al., 2019; Sponsler and Johnson, 2015). 2-Acaricides ► bee viruses: May increase honey bees' susceptibility to viruses (Locke et al., 2014). 3-Neonicotinoids ► bee Viruses: Can increase honey bees' susceptibility to viruses by weakening their immune system (Di Prisco et al., 2013; Doublet et al., 2015). 4-Neonicotinoids ► Nosema spp: can increase the severity of the infection (Doublet et al., 2015). 5-*Varroa destructor* ► bee viruses: transmission and spread of viruses (Martin et al., 2012). 6-*Vespa velutina* ► Bee viruses: transmission and spread of new bee viruses (e.g. Garigliany et al., 2019 and 2017).

- ***The consequences of pollinators' decline***

Health means more than just the absence of disease. Food security is a huge aspect of health, for humans, livestock and bees. Honey bees are pollinators and by this, together with other insects, contribute substantially to food security. Without pollinators, our diets would be short of, or even completely lacking, many of the micronutrients essential for health, including vitamins A and C, calcium and fluoride. Smith et al. (2015) calculate that a complete loss of pollinators, a worst-case scenario, could reduce global supplies of fruit by 22.9%, vegetables by 16.3%, and nuts and seeds by 22.1%. The health impacts of the resulting dietary change would be substantial, increasing global human deaths yearly from non-communicable and malnutrition-related diseases by 1.42 million (an increase of 2.7%). A 50% loss of pollination services would be associated with 700 000 additional annual deaths (Smith et al., 2015).

Bees also produce, honey, beeswax, royal jelly and many other products used for human consumption. Honey bees and human health are linked to their environment. Bees represent a significant link in the food chain and ecosystem, and therefore healthy stocks of bees must be protected to enable the production of goods such as honey, pollen, propolis, royal jelly and beeswax (for honey bees), and the sustainable maintenance of the services that bees deliver (i.e. biodiversity and pollination).

3. Epidemiology applied to honey bee health

For researchers and beekeepers, a large number of questions remain unanswered: what is the relative share of these factors in the deaths identified? What is the distribution of these factors in time and space? What combinations of factors have an impact on bee health? What is the real level of health indicators in populations (mortality, morbidity in particular) and how do they change over time?

These are the questions that epidemiology seeks to answer through surveillance programmes or by setting up analytical studies designed to explain the epidemiological mechanisms affecting bee health. However, applying epidemiology to bee health requires a certain number of adaptations to the specificities of the beekeeping sector.

Epidemiology is the study of the distribution and determinants of states of health, including diseases, within a population (Woodward, 2014). Animal health epidemiology is a recent discipline, which has long been directly inspired by human health epidemiology methods. The multifactorial origin of the health problems observed in honey bees has been a matter of scientific concern for the past twenty years. Assuming that the origin of health problems is multifactorial is one thing, but explaining the mechanisms and, at the very least, determining the influence of each of the factors potentially involved is another (More et al., 2021).

Epidemiology is based on several fields of activity. Our research is based on the specificities of two of these fields: descriptive epidemiology completed by analytical epidemiology. **Descriptive epidemiology** (describes health characteristics of the population in space and time) permits the evaluation of trends in health and diseases, provides valuable information for the prevention of these diseases, allows the design of interventions, the conduct of additional research and permits also to identify problems to be studied by analytic methods and suggest areas that may be fruitful for investigation. Descriptive epidemiology can thus generate hypotheses of etiologic research (VanEngelsdorp et al., 2013).

Once hypotheses are generated, **analytical epidemiology** seeks to explain the causes of a health phenomenon and is employed to test the generated hypotheses by drawing samples and comparing

groups to determine whether health outcomes differ based on exposure status (Steinhauer and VanEngelsdorp, 2017; VanEngelsdorp et al., 2013).

4. Stress factors affecting honey bee health

4.1 Biotic factors

4.1.1 Genetic diversity

Honey bees (*Apis* spp.) have among the highest levels of multiple mating among social insects, the honey bee queens mate with 12 drones (males) on average (but up to 40), which creates a high level of intracolonic genetic diversity (Tarpy and Nielsen, 2002). Genetic diversity reached by multiple mating of the queen, influences a wide range of phenotypes or observable traits in honey bee colonies, from the expression of antimicrobial compounds, resistance to pathogens, thermoregulation, foraging behaviour, and nutritional status (Eckholm et al., 2015) and colony defence (Oxley and Oldroyd, 2010). An important hypothesis is that multiple mating is adaptive because it increases intracolonial genetic diversity and thereby reduces the likelihood that parasites or pathogens will catastrophically infect a colony (Palmer and Oldroyd, 2003). Thus a high genetic diversity increases the diversity of worker genotypes within a colony, which has been shown to confer significant adaptive advantages that result in higher colony productivity and survival (Tarpy and Pettis, 2013).

Besides natural selection pressures, human has contributed, deliberately or not, to shape the current diversity of honey bees worldwide. As exposed in the publication of Leclercq et al. 2018, four main factors are considered in reshaping the diversity of honey bees: honey bees' international trade, domestication and selection, the decline/development of wild and feral populations, and the socio-political, economic and cultural factors (Leclercq et al., 2018).

Domestication, professional breeding and commercial mass rearing of queens aim at selecting individuals with specific traits favourable to beekeepers, such as docility, lack of propensity to swarming, honey yield, and others may be selected for, but as it is difficult to have controlled mating, this is usually done through the import of stock from other areas, where these traits are more frequent. These practices consciously or unconsciously narrow the genetic diversity of domestic bees, increasing their susceptibility and the transmission rate of diseases between wild bee species.

4.1.2 Parasites and pathogens

Among the causative agents and pests in the honey bee colonies are *Varroa* mite (*Varroa destructor*), microsporidia (*Nosema apis*; *N. ceranae*, the more virulent one); fungi such as *Ascophaera*

apis; bacteria (*Paenibacillus larvae*, *Melissococcus plutonius*), small hive beetles (*Aethina tumida*), beeswax moths (*Pyralidae*), and others. A full list of all pests and pathogens associated with honey bees is out of the purview of this manuscript, and their prevalence and potential impact are variable in time and across regions. In any case, parasites and pathogens are definitively involved in the decline of honey bee colonies (Muli et al., 2014). ***Varroa destructor*** is presented as the most economically damaging threat to beekeeping (Genersch et al., 2010a; Maggi et al., 2016; Rosenkranz et al., 2010) (**Figure 3**). *Varroa destructor* is detrimental both because of its widespread prevalence and highly damaging effects, mostly from its associated viruses (Dainat et al., 2012).

Due to a short history of coevolution, the host-parasite relationship between *A. mellifera* and *V. destructor* is unbalanced, with honey bees suffering infestation effects at the individual, colony and population levels. *Varroa destructor* affects not only adult honey bees but also bee brood (Floris et al., 2020).

Figure 3: *Varroa destructor* mite inside a honey bee cell (Véto-pharma, n.d.)

Moreover, the arrival of *V. destructor* provided a new route of transmission for viruses, thereby modifying the viral community structure associated with bees. The relative abundance of viral species changed, and the prevalence of a select few, such as Deformed Wing Virus (DWV) increased (Martin et al., 2012, Matthijs et al., 2020). Among viruses that increased, particular strains were favoured resulting in a massive reduction in the genetic diversity of the remaining predominant strains. We need to keep in mind that pathogens recent virulence is more likely to have been fostered by the exposure of bees to pesticide-contaminated pollen and nectar (Long and Krupke, 2016) which weakens their immune system (Sánchez-Bayo et al., 2016; Tesovnik et al., 2017). The effects of *V. destructor* on honey bee health are still under review but the most documented ones are that the parasitism of honey bees by *V. destructor* decreases the body weight and water content of young emerging bees (Bowen-Walker and Gunn, 2001), the resulting reduction in weight of adult drones was related to the number of foundresses female mites (Duay et al., 2003; Strauss et al., 2016) and as the number of spermatozoids is correlated

with drone body size (Schlüns et al., 2003), the decrease in drone size induces a deficit in sperm production, and thus, the reproductive fitness (Duay et al., 2002). *Varroa destructor* also alters flying, homing and orientation abilities in foragers (Kralj and Fuchs, 2006; Peck et al., 2016), which in turn, limits efficiency in their ability to collect resources needed for colony development.

4.2 Abiotic factors

4.2.1 Habitat fragmentation, erosion of biodiversity and the decrease in food supplies

Bees are often described as the 'canaries in the coal mine' when it comes to the health of the environment. Biodiversity strengthens the productivity of any ecosystem (e.g. agricultural land, forest, and lake). The loss of biodiversity contributes to food and energy insecurity; increases vulnerability to natural disasters, such as floods or storms; and decreases the quality of both life and health. Wild and managed bees play a key role in maintaining biodiversity and in the recovery and restoration of degraded habitats. Intensively farmed land, as we have in Europe, is a hostile environment for bees: habitat loss due to destruction, fragmentation, or degradation of habitat by pollution, is the main threat to the survival of pollinators. When an ecosystem has been dramatically changed by human activities such as agriculture, commercial development, or water diversion, it may no longer be able to provide food, water and cover.

Land use and intensive agriculture can be a determinant of colony growth, honey production and survival correlates with particular land use practices, such as the percentage of agricultural land or the percentage of certain crops in the area surrounding the hive (Clermont et al., 2015; Dolezal et al., 2019). Several studies have indicated that honey bees show reduced growth or higher mortality with increasing urban or agricultural land use (Clermont et al., 2015; Dolezal et al., 2019; Sponsler and Johnson, 2015).

An urban setting or area can refer to towns, cities, and suburbs. Agricultural or rural settings are the opposite of urban areas. Rural areas, have low population density and large amounts of undeveloped or farmland.

We tend to accept that such practices are necessary to feed the growing human population, but we should challenge that assumption. Habitat loss is considered the most important driver of general species decline, both in abundance and diversity (Brown et al., 2016; Goulson et al., 2015).

4.2.2 Nutrition

All animals, even invertebrates, have an optimal diet that maximizes their fitness (or health), known as the "intake target" (Simpson and Raubenheimer, 2011). Bees obtain nutrients from nectar and pollen. When given a choice of what to eat, insects can adapt what they eat depending on their state of health (Lee et al., 2006). Honey bees are specially adapted to forage over a huge range (up to 15 km on a single flight), selecting what they bring back to the hive based on the colony's fitness. However, if this range is saturated with a single floral species, as can be the case in some agricultural land, then this choice may be taken away from the foragers, hindering their ability to reach their preferred intake target (Alaux et al., 2010). The link between nutritional diversity, richness and pollinator fitness is well established; high-quality diets have benefits for immune responses (Vaudo et al., 2015), reproduction and adult survival (Ruedenauer et al., 2020). Similarly, bees-fed honey, which consists of 30–45% fructose, 24–40% glucose, 0.1–4.8% disaccharides including sucrose, and minute amounts of micronutrients and amino acids exhibited increased expression in more genes involved in detoxification immunity, aromatic amino acid metabolism, oxidation and reduction, as compared to bees fed either sucrose or high fructose corn syrup (Mao et al., 2013). Although there is a lack of direct experimental data supporting dietary adaptation in honey bees, our current understanding is that honey bees that are under stress from pests or pathogens will consume a higher protein diet, whereas healthy bees may eat higher carbohydrate diets to enable greater exploitation of their environment.

Though there have been few quantitative assessments of the relationship between nutritional status and pathogen burden (Alaux et al., 2010), several studies suggest that insufficient protein and low-diversity diets negatively impact bees' ability to defend against pathogens (Wheeler and Robinson, 2014).

Figure 4: longitudinal section of a beeswax comb containing bee bread (pollen)

4.2.3 Climate change

There have been three cooling periods and three warming periods over the last 3000 years, during these alternating periods, bees did not disappear completely, even though they had undergone some population fluctuation (Mann et al., 2009; Neov et al., 2019). Climate change can impact honey

bees at different levels: it could have a direct influence on honey bee behaviour and physiology, it could alter the quality of the floral environment and increase or reduce colony harvesting capacity and development, and it could define new honey bee distribution ranges and give rise to new competitive relationships among species and races, as well as among their parasites and pathogens (Conte and Navajas, 2008). Climate change could increase species' extinction risk as temperatures and precipitation begin to exceed species' historically observed tolerances (Soroye et al., 2020). In concrete terms, for honey bees, temperature increase induces longer periods of brood rearing and foraging because of longer warm seasons. A longer brood period means more *V. destructor* reproduction cycles that may lead to an increase in mite populations (Le Conte et al., 2010). The increase in temperature also results in longer foraging periods. Foraging alters the lifespan of winter bees as their energy is limited. The reduction of the lifespan of winter bees can contribute to colony mortality. Colonies are not dormant during the winter: they remain active and maintain the hive temperature between 24 and 34 °C by forming a thermoregulating cluster (Heinrich, 1981). This enables them to survive long periods of cold temperatures (Döke et al., 2015). Due to global warming, an alien species may migrate to a new geographical area, in many cases, this migration is unfavourable for the local fauna due to competition for food resources or the transfer of various pests and diseases (Kerr et al., 2015).

4.2.4 Weather conditions

Unfavourable weather conditions and dramatic weather changes play an undeniable role in the reduction of bee colonies. Long periods of cold and rainy or hot and dry weather are associated with a lack of nectar and pollen (foraging), which inevitably leads to starvation and eventually the death of bee colonies. Although colonies can adapt to temperature changes due to thermoregulation (Stabentheiner et al., 2010), the weather has multiple effects on honey bees' foraging and behaviour (Riessberger and Crailsheim, 1997), wintering ability and *Varroa* control (Underwood and Currie, 2003). Two recent studies modelling the seasonal effects of temperature and precipitation on honey bee winter mortality in Austria (Switanek et al., 2017) and Luxembourg (Beyer et al., 2018) reported positive relationships between air temperature and colony losses throughout the year, except for some months of the year. Both studies indicated that warm winter temperatures in December, January and March were linked with high honey bee colony losses, while high temperatures in July were linked with low colony losses. High amounts of precipitation in January and October were linked with high honey bee colony losses in the Austrian study (Switanek et al., 2017). The same effect was observed in January in the Luxembourgish study (Beyer et al., 2018). Both studies agree that high precipitation values in February, March, May, September and November were coupled with low honey bee colony losses.

In summary, these two studies agree that high honey bee colony losses were linked with warm winters, low temperatures in July as well as low precipitation values in February, March, April,

September and November. The numerous relationships between weather variables and colony losses indicate the importance of considering weather effects when assessing the success or failure of *Varroa* control regimes to avoid confounding effects of weather and treatments (Beyer et al., 2018).

4.2.5 Wintering

During the winter, the colony ceases foraging for nectar and pollen and relies on its existing stores, collected during the plant blooming season. Furthermore, brood rearing ceases, and the colony is dependent on the survival of a long-lived cohort of bees that are produced in the autumn. These bees will live for several months, while worker bees produced in the summer only live for a few weeks. Thus, factors which undermine the ability of the bees to collect and store adequate amounts of food during the summer and fall, to thermoregulate effectively during the winter, or reduce the lifespan of winter bees, can contribute to colony mortality (Calovi et al., 2021). These factors include BMP that affect parasite and pathogen loads, particularly control of *Varroa* mites (Genersch et al., 2010b; van Dooremalen et al., 2012); forage quality and pesticide exposure due to the surrounding land use; and weather factors which influence the availability of forage, the thermoregulatory ability of the bees in the winter, and the amount of time before bees can initiate brood rearing in the spring (Switanek et al., 2017). Modelling and predicting honey bee winter survival requires consideration of all of these factors.

4.2.6 Beekeeping and management practices

Beekeeping, also called apiculture, refers to the totality of the actions implemented by a beekeeper to maintain the health of social bee species and to achieve its production objectives (EFSA AHAW panel, 2016; Formato and Smulders, 2011; Ritter and Pongthep, 2006; Rivera-Gomis et al., 2019a) (Figure 5). Beekeeping is different from honey-hunting, which involves “plundering wild nests of honey bees to obtain crops of honey and beeswax”. For thousands of years, we have known that honey can be obtained much more easily and conveniently if bees are encouraged to nest inside a man-made hive (FAO, 2009). Depending on the type of hive and the species and subspecies of bee, it is also possible to manage the colony to some extent. In many rural areas of the world, beekeeping is a widespread activity, with thousands of small-scale beekeepers depending on honey bees for their livelihoods. Social bees can provide humans with valuable hive products (honey, beeswax, propolis, pollen, royal jelly, queen bees and swarms) and services (pollination, apitherapy, apitourism and environmental monitoring) and play other important economic, cultural and social roles.

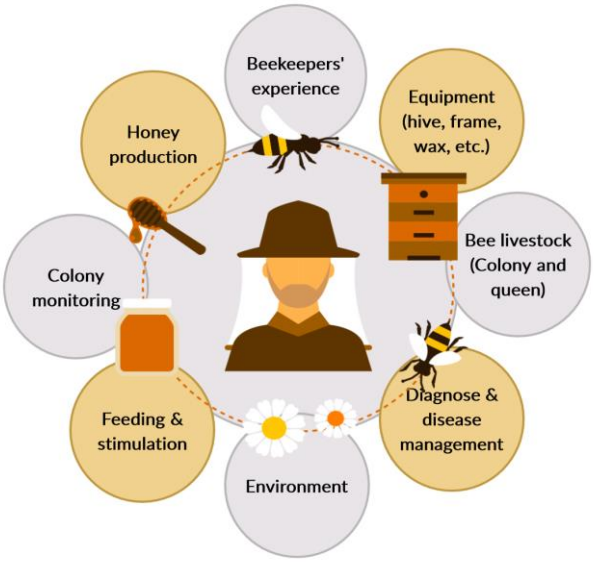

Figure 5: Beekeeping management practices can impact bee health and survival by themselves or in synergy with other factors (El Agrebi et al., 2021)

Many of the factors causing bee decline occur within the hive. To lift the stress factors exposed above, beekeepers can adapt their management practices. For example, when facing high pest pressure, beekeepers can reduce hazards through physical or chemical interventions (Giacobino et al., 2016; Jacques et al., 2017). While beekeepers are responsible for the immediate health of their bees, some beekeeping practices may be ultimately detrimental to colony health in the short and long term. Characteristics of bees that may be attractive to beekeepers, such as high productivity, a low tendency to swarm, and being easy to handle and manipulate, could be ultimately counter to honey bees' welfare (Lehébel-Péron et al., 2016). Over the past decade, concern about the negative effects of various in-hive treatments for *Varroa* has led to an increase in beekeepers practising treatment-free beekeeping (Loftus et al., 2016; Neumann and Blacquière, 2017). This approach can be highly problematic for the wider beekeeping community, as *Varroa* infestation has been widely associated with colony death (Thoms et al., 2018), and the spread of infection between neighbouring colonies through robbing and drifting (Peck and Seeley, 2019). Beekeepers' knowledge and practices are central to any efforts to reverse honey bee decline. The role of beekeepers in monitoring and ensuring honey bee health serves as a linkage between hive-based factors, and wider landscape scale factors affecting bees. Good management practices must be developed with proper training and experience (Steinhauer et al., 2018).

Over the last decade, considerable attention has been given to understanding stress factors impacting honey bee colony health and losses, but a clear overview of the main actions carried out by beekeepers and their role in the successful management of honey bees is only partially addressed (Sperandio et al., 2019) and the management practices' impact has often been overlooked. In the literature, very few publications about management practices are available (Sperandio et al., 2019;

Steinhauer et al., 2021; Underwood et al., 2019). So far, studies have not achieved to deliver a global picture of the exposure to these stressors and their interactions remain fairly unknown. The long-term success of beekeeping and honey bees relies upon continued exploration and monitoring of the ever-changing factors impacting bee health (Steinhauer, 2017).

- ***Beekeeping in Belgium***

Although integrated in the agricultural context, beekeeping is often left out for various reasons. Beekeeping is a specific form of animal husbandry with specific risks and needs that are not comparable to traditional animal husbandry. This is rarely understood and integrated into agricultural policies. Moreover, the status of the beekeeper is particular: unlike farmers, beekeepers rarely have land for their apiaries. Contrary to appearances, this makes them very dependent as they are also on the quality of the environment and the floral resources around the apiary. However, they do not master these two criteria which are crucial for the success of their breeding.

The monitoring network of the European Honey Programme estimates the number of Belgian beekeepers at 9180 in 2021, with a total number of hives around 100,000. Two third of the Belgian beekeeping sector is made up of amateur/hobbyist (non-professional) beekeepers, with less than 24 hives per apiary, whose source of income lay outside beekeeping, nevertheless, the activity can generate substantial incomes and profitability is more or less admitted through the sale of queens, honey or transhumance. Bees are kept as a pleasant pastime and for the intrinsic values of beekeeping (El Agrebi et al., 2021). Apiaries are thus relatively small operations. Beekeepers often have knowledge based on observation and self-experimentation. One-third of the sector is made of semi-professional beekeepers (24–150 colonies) and only eight beekeepers are professional (with more than 150 colonies). These categories based on the number of hives do not exhaust the multiplicity of profiles. Thus, the category of "hobbyist" alone covers very diverse realities, both in terms of conceptions and motivations: ensuring a small family consumption, "helping the bees" and acting for e.g. the environment, promoting beekeeping through educational activities, and collecting swarms.

Beekeepers are mostly represented at the regional or local level by a variety of institutions: federations, associations, apiaries and beekeeping groups. For example, ten federations are listed for Wallonia only and almost as many in Flanders. The history of the federations, internal conflicts, and regional specificities partly explain such diversity. This wide variety of profiles and institutions is an integral part of a complex system that is subject to many issues (**Figure 8**).

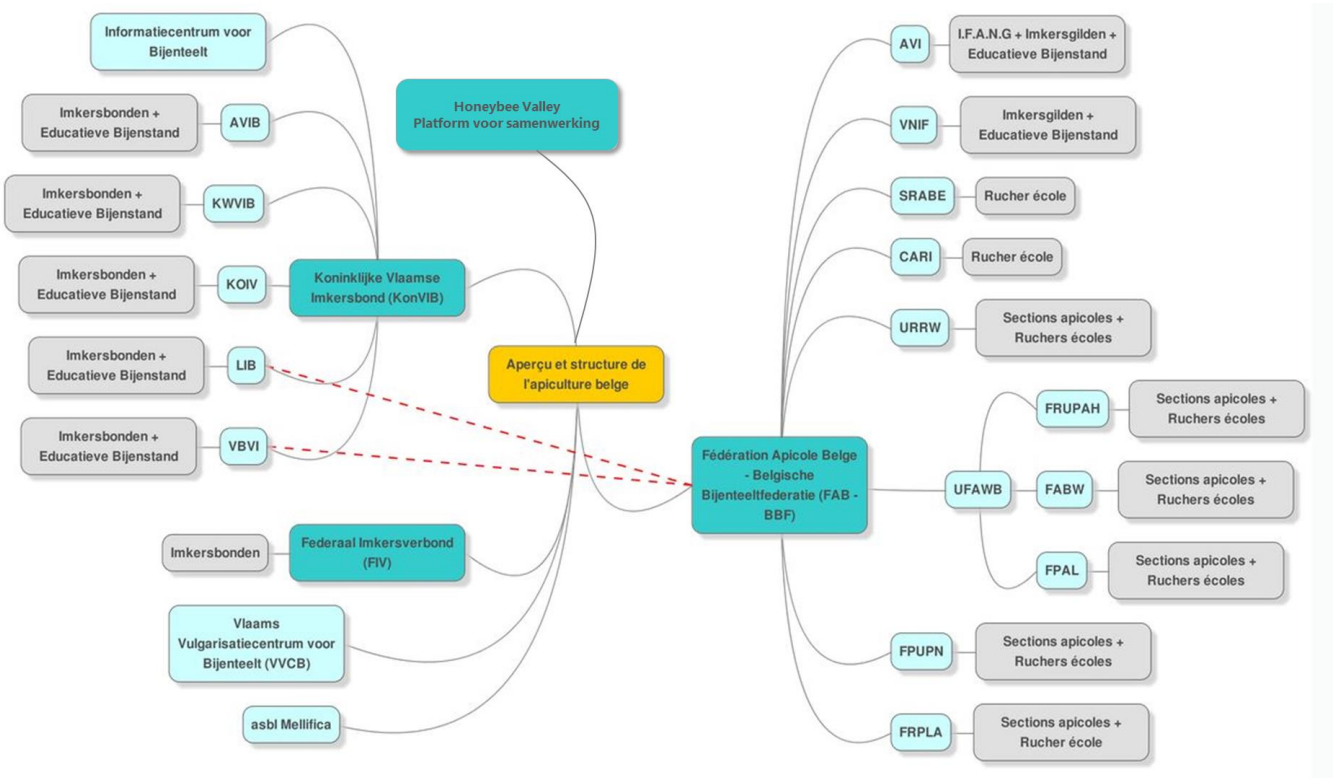

Figure 6: Overview of the multiple beekeeping institutions in Belgium ("Vive les abeilles," 2022)**.**

Legend: AVIB=Antwerpse Vereniging voor Imkersbelangen; KOIV=Koninklijke Oost-Vlaamse Imkersbond; KWVIB=Koninklijke West-Vlaamse Imkersbond; LIB=Limburgse Imkersbond; VBVI=Verbond van Vlaams-Brabantse Imkersverenigingen; AVI=Algemene Vlaamse Imkervereniging; VNIF=Vlaams-Nederlandse Imkerfederatie; SRABE=Société royale d'apiculture de Bruxelles et environs ; CARI=Centre Apicole de Recherche et d'Information; UFAWB=Union des fédérations apicoles de Wallonie et de Bruxelles; FRUPAH=Fédération des unions professionnelles apicoles du Hainaut; FABW=Fédération des Apiculteurs du Brabant Wallon; FPAL=Fédération Provinciale d'Apiculture de Luxembourg; URRW=Union royale des ruchers wallons; FRPLA=Fédération Royale Provinciale Liégeoise d'Apiculture; FPUPN=Fédération Provinciale des Unions Professionnelles de Namur)

The annual honey production was estimated in 2021 at 1 840 000 kg and an average yield of honey per hive and year of 25 kg. The average price for multi-floral honey at the site of production is ±13€/kg. The honey trade (and other hive products) is not organised since there is no link between producers, processors and distributors of honey. This micro-supply chain functions essentially on direct sale to consumers. Some beekeepers sell through small shops. Large-scale distribution is showing more and more interest in the sale of local products from small producers but no financial margin is taken on the sale of the product, the objective being to meet the expectations of consumers attracted by local products. The advantage of this micro-supply chain is that the beekeepers are protected from the

fluctuations of the international honey market. However, this does not protect them from the great perils that weigh on this same international market: the problem of beeswax traceability and the adulteration and contaminations of this product.

4.2.7 Pesticides

Pesticides are toxic chemicals used to control pests, weeds and pathogens. As with agriculture, the story of pesticides started in the Middle East where an extract of certain chrysanthemum flowers (known as pyrethrum) was very effective in killing flies and other insects, so they used it to control agricultural pests. It is only with the so-called 'Green Revolution' that was based on the use of chemical pesticides and fertilizers together with increased irrigation and genetic improvement for agricultural production that the chemical industry started to mass-produce synthetic toxic substances not only in killing insects (insecticides) and other animal pests (rodenticides) but also weeds (herbicides) and fungal diseases (fungicides). Hailed as the saviour of human starvation, the Green Revolution practices were quickly adopted worldwide and became the 'conventional' agriculture. Soon after, the side effects of pesticides were reported and it was realised that all pesticides are toxic to a greater or lesser degree, so their release could not be without risks to some kind or other organisms (Sanchez-Bayo, 2011).

4.2.7.1 Pesticide for agricultural use

Within the European Union, there are 484 active substances approved for use as pesticides according to Regulation (EC) No 1107/2009 of which 31% are herbicides, 21% insecticides, 17% fungicides, 9% acaricides and 2% rodenticides; the remaining 20% of products include a plethora molluscicides, algicides and nematicides, as well as plant growth regulators (6%) and natural or artificial pheromones (5%). In addition, 793 substances are no longer approved for use as pesticides, including most of the obsolete organochlorine insecticides, these have been banned for safety and environmental reasons or because they were no longer efficient due to resistance. Organochlorine pesticides have been increasingly replaced with more effective and 'safer' alternatives with faster biodegradation rates such as organophosphorus pesticides and neonicotinoids. However, most pesticides are not selective and still affect non-target species via water, soil, and contaminated plant (Gonalons and Farina, 2018). Modern formulations are invented to 'avoid persistent, bioaccumulative, and toxic properties.

Agricultural pesticides are typically applied directly to crop plants or fruit trees by spraying them. Some pesticides are applied as granules buried in the soil, or as seed dressings to protect the growing seedlings. Pesticides include a wide variety of chemicals, they can be divided into two main categories of interest: systemic pesticides and non-systemic pesticides and are increasingly being used all over the world. Systemic pesticides can penetrate plant organs and pass into the interior of plant tissue, leading to a better insecticidal effect. Furthermore, systemic pesticides are much more insensitive

to degradation by environmental factors and increase the difficulty of safety testing, especially in the pretreatment process. Non-systemic pesticides stay on the surface of leaves or other plant organs and are easily affected by environmental factors, such as rain, and sunlight (**Figure 7**). Therefore, from the point of safety detection, non-systemic pesticides can be simply cleared away by washing and extraction (Hou et al., 2016). The toxicity and specificity of pesticides depend on the mode of action of the active ingredients, while the effects on organisms depend on the dose they are exposed to.

The persistence of pesticides is evaluated by their half-life (t1/2), which is defined as the time required for half the amount of a chemical to disappear from a medium, that is, water, soil, air or biological tissues. Half-lives longer than 90 days indicate that the pesticide may accumulate since more than 5% of the amount applied will remain in the environment after 1 year (Sanchez-Bayo, 2011).

Pesticides are applied via different pathways (sprays, seed coatings etc.) in conventional agriculture as plant protection products, but not only, they are used in many professional non-agricultural settings in Belgium, including gardens, parks, and public spaces, sports fields and outdoor leisure areas. They also help the functioning of transportation corridors such as road shoulders, airport runways and railway tracks, as well as industrial sites and drainage infrastructure. Apart from their use as plant protection products they are also used as veterinary drugs in animal production and to protect domestic animals against ticks and fleas among other.

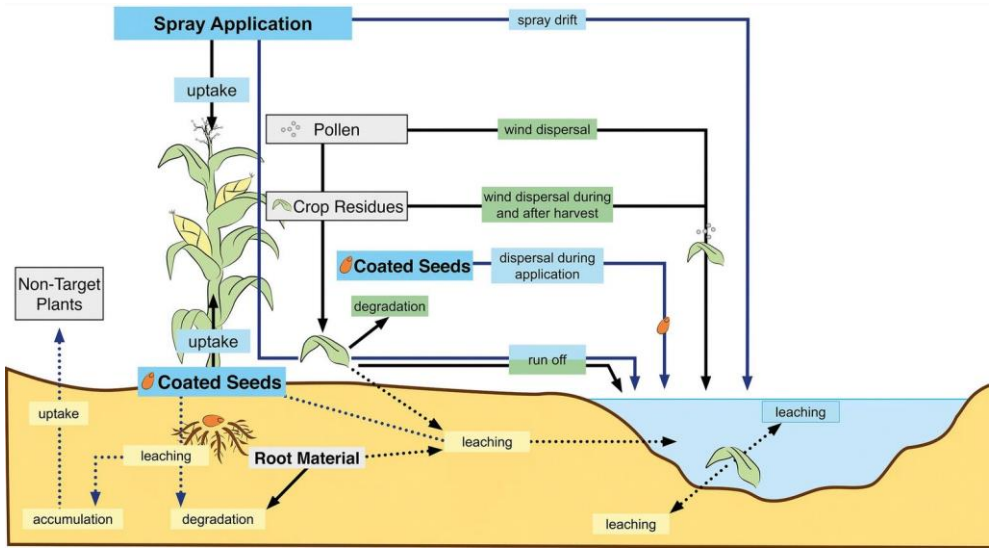

Figure 7: A schematic representation of pesticides' fate in the environment (Bundschuh et al., 2019)**.**

- ***Exposure to pesticides***

The exposure of bees to pesticides can occur through ingestion (oral) of residues found in the pollen and nectar of contaminated plants (crop plants or the weeds around the fields), and via direct contact with contaminated plants, pollen and beeswax (El Agrebi et al., 2020). We do not currently have

an accurate picture of what pesticides are used, where and in what amounts, nor do we have accurate measures of just what the maximum exposure is in agricultural or urban settings on blooming plants. Once contaminated pollen is collected, the potential transformations of pesticides in bee bread and royal jelly are also currently unknown. Clearly, the potential for pesticide involvement in declining honey bee health is far from being understood, and it is too early to discount them as key factors associated with bee mortality.

- ***Toxicological effects of pesticides***

Toxicological effects depend on the doses exposed to, and such effects may occur at individual, population and community levels (Sanchez-Bayo, 2011). When a pesticide is applied once, all non-target animals and plants that are directly exposed to it may experience short-term, acute toxic effects. In ecotoxicology, this is called pulse exposure to distinguish it from decreasing exposure to pesticides in a given environment. Pulse exposure can result in poisoning. The fundamental symptom indicating that poisoning occurs is the high number of dead bees, with fluctuating critical mortality thresholds depending on the national authority (Cutler et al., 2014; EFSA, 2013a) of research (Porrini et al., 2003). Typical clinical symptoms of acute insecticide poisoning include cramping, disoriented behaviour of bees, abnormal wing movements, and abdominal spasms of dying bees. The number of reported cases of poisoning incidents observed between 1994 and 2003 has decreased in the last years (Barnett et al., 2007). Pulse exposure and poisoning are not the subjects of this work.

What interests us most in the work, are the effects of decreasing exposure as the pesticide disappears progressively by natural decay, microbial degradation, and other dissipation routes (Sanchez-Bayo, 2011) or low doses effects named sublethal effects. Pesticide residues remaining in the plants, on the pollen; in the soil and water of the agricultural fields and surroundings can be taken up by honey bees and brought to the hive. For non-persistent and biodegradable pesticides, those residual amounts are sufficiently low to ensure the lethal dose (LD50s) for most species are not reached, although there is no guarantee they won't have any impact whatsoever – sublethal effects on some individuals may still take place. When pesticides persist in the environment for longer than one season (which occurs whenever half-lives are over 3 months) residues are expected to build up between consecutive annual applications. In such circumstances, all organisms chronically exposed are at risk of accumulating the toxicant in their tissues, and with time the internal doses may be sufficient to cause either sublethal or lethal.

4.2.7.2 Pesticide for beekeeping use

Beekeeping is no exception to this practice. If unmanaged, *Varroa* mite infestations can increase the mortality of bees in the colony within one season (Carreck et al., 2010). The lack of, or improper,

Varroa mite management is a significant driver for losses among beginning and amateur/hobbyist beekeepers (Traynor et al., 2016b). *Varroa* mites keeps on being the major reason for the use of beekeeper-applied miticides or varroacides since their introduction (**Figure 10**). Many recent studies have focused on the direct links between the decline in bees and pesticides. Nevertheless, we should keep in mind the indirect effects of pesticide presence in the environment: herbicides, often considered bee safe, can reduce the plant diversity and availability of food sources, which links back to the issue of resource quantity and diversity (Bretagnolle and Gaba, 2015).

➢ *Varroa control and the use of veterinary treatments*

Since its spread in 1984, *V. destructor* became the main honey bee disease in Belgium. The *Varroa* control strategies currently in use can be divided into four broad categories, the first two being the most widely used:

- the "conventional" treatments based on synthetic acaricides (**Table 1**);
- the "biological" treatments based on organic acids or essential oils (**Table 1**);
- the mechanical or population-based methods; and
- the selection of naturally varroa-resistant bees as a sustainable solution to fight the mite.

In Belgium, every year, recommendations for effective control of varroosis are edited by the Federal Agency for the Safety of the Food Chain (FASFC) for a uniform approach at the national level. Registered formulations are available in pharmacies, without a prescription, or can be supplied directly by the veterinarian. Other unregistered formulations in Belgium, based on other active substances, can also be obtained, necessarily via a veterinarian, by using the cascade system.

Figure 8: Substances used by Belgian beekeepers for *Varroa* control, left picture: Oxalic acid, right picture: tau-fluvalinate base formulation meant for agricultural use and not for *Varroa* control

➢ The cascade system

The cascade system was introduced to solve the general problem of the availability of veterinary medicinal products for minor species and minor uses. It provides the veterinarian with the opportunity to depart from the strict use of registered medicinal products in Belgium. Indeed, it is possible to use a medicinal product for animals of another species or animals of the same species but with another disease. On the other hand, the veterinarian may also prescribe a medicinal product for veterinary use, which is authorised in another member state of the European Union, a medicinal product for human use and even a magistral preparation.

Table 1: *Varroa destructor* treatment type and authorisation in Belgium in 2021

Treatment type	Active substance	Commercial product	Authorization in Belgium
Conventional	Bromopropylate	Folbex VA®	Not allowed
	Tau-Fluvalinate	Apistan®	Allowed under cascade
	Coumaphos	Perizin®	Allowed under cascade
		CheckMite+®	Allowed under cascade
	Amitraz	Apivar®	Allowed
	Flumethrin	Bayvarol®	Allowed under cascade
		PolyVar®Yellow	Allowed
Biological	Thymol	Api Life Var®	Allowed
		Thymovar®	Allowed
		Apiguard®	Allowed
	Oxalic and formic acid	VarroMed®	Allowed
	formic acid	MAQS®	Allowed under cascade
	Oxalic acid	Oxuvar®	Allowed
		Oxybee®	Allowed
		Dany's bienenwohl®	Allowed

➢ *Pesticide lethal and sublethal effects*

When bees are exposed to a toxic dose of pesticides (poisoning incident), dead bees surrounding the hive entrance are an obvious result. What is not so obvious, are the consequences of lower doses of one or more pesticides that may be encountered while foraging, or from collected pollen and nectar brought back to the hive. It is these sub-lethal impacts that have become the focus of much of the current research on pesticides. Many studies have documented the impacts of low levels of pesticide exposure that when ingested or put in contact with bees for longer periods resulting in chronic impacts.

The potency of a pesticide to any species is defined by the dose of a toxic chemical that is lethal to 50% of individuals of that species (LD50), and such dose varies from species to species. Doses lower than the LD50 are considered 'sublethal', but they can also cause mortality in a certain proportion of the

species population. In general, sublethal doses cause toxic effects that do not kill the organisms but still affect their normal functioning and health.

Adult worker honey bees are repeatedly exposed to pesticides during the collection of pollen and nectar (phytosanitary products) and bee larvae, as well as adult worker honey bees, are exposed to acaricides (applied in-hive for *V. destructor* control). By breading honey bee brood in contaminated beeswax and feeding developing honey bees with contaminated food, entire colonies can be exposed to multiple pesticides (Calatayud-Vernich et al., 2018; Fisher and Rangel, 2018). Low levels of pesticides have been shown to reduce associative learning of individual bees in laboratory studies using the probosci's extension response (Decourtye et al., 2004), altering maze learning performance in free-flying bees (Decourtye et al., 2010, Henry et al., 2012) and the loss of foraging efficiency in radio-tagged bees (Decourtye et al., 2011), and can cause changes in immune response and detoxification mechanisms (Boncristiani et al., 2012; Cizelj et al., 2016). The Proboscis Extension Response in honey bees is a natural behavioral reflex in which the honey bee extends its proboscis in response to antennal stimulation with a sugar solution. Honey bee larvae reared in cells contaminated with the miticides fluvalinate or coumaphos show a reduced developmental rate and delayed adult emergence along with reduced adult longevity (Wu et al., 2011). These changes in learning and behaviour can potentially alter normal colony-level functions, yet colony-level impacts remain to be verified. Overwintered, old, and poorly fed bees are more vulnerable to pesticides than young ones. This is most likely true because such bees have a decrease in vitellogenin – a hemolymph protein with antioxidant properties (Johnson, 2015). Compared to other insects, honeybees are extremely sensitive to pesticides, due to a deficiency in the number of genes encoding detoxification enzymes (Claudianos et al., 2006).

- ***Pesticide synergetic effects***

Synergistic effects occur when combined exposure to two factors results in an effect that is significantly greater than the sum of individual effects. Real-life exposure occurs to complex chemical mixtures, pesticides can affect each other according to the additivity (most commonly reported pattern of mixture response, approximately 80%) (Belden, 2022; Woodcock et al., 2017) and according to interaction concepts; **antagonism**, when joint toxicity is lower than expected (based on the default assumption of additivity) and **synergism**, when the joint toxicity is higher than expected (concerning assumptions of additivity) (Desneux et al., 2007; Potts et al., 2010a; Silins and Högberg, 2011). In all cases, the underlying mechanism of the interaction was associated with inhibition by one compound of the active sites of detoxifying cytochrome P450 enzymes thereby inhibiting the metabolism of the second compound (Mao et al., 2011).

Almost all studies to date have focused on the action of a single pesticide so very few combinations have been studied. We feel that this is a major limitation to our current level of understanding of pesticide impacts on bees.

- ***Adjuvants, formulants or co-formulants***

Another important mixture effect is potentiation: when a non-toxic substance (e.g. adjuvants) enhances the effect of a pesticide. The general terms adjuvants, formulants or co-formulants refer to the inert ingredients that are usually added to maintain long-term physical stability, but also to enhance the biological performance of the agrochemical, increasing the foliar uptake of herbicides, growth promoters and defoliants (Castro et al., 2014; Vandenberg et al., 2017). This could also mean that the contact toxicity of lipophobic pesticides could be underestimated and uncertain, and surfactants, penetrant enhancers, spreaders, and stickers, have a huge impact on the toxicity of active ingredients but are never included in the risk assessment.

The standardised method for studying acute contact pesticide toxicity towards *Apis mellifera* L. presupposes that the test substance is preferably applied as a solution in acetone or as a water solution with a wetting agent (Medrzycki et al., 2013). Other organic solvents of low toxicity to bees may be used but they must be administered in the negative reference. Acetone or water is only one of the pluralities of organic solvents used in pesticide formulations and the light of the other above arguments, they do not reflect reality.

More specifically, there is mounting evidence that organosilicon spray adjuvants used in various pesticide formulations may pose a more serious threat than previously realized as they have been demonstrated to both impair olfactory learning (Ciarlo et al., 2012) and increase viral pathogenicity in bees (Fine et al., 2016). According to a report from the European Chemicals Agency, there is strong evidence that some formulants are capable of independently exhibiting toxic properties, resulting in higher toxicity in the final pesticide product (BAuA, 2016).

4.3 Interactive and cumulative effects: the action of biotic and abiotic stressors

Honey bees in their natural settings rarely experience optimal conditions. On the contrary, during most of their life, they are forced to cope with sub-optimal conditions and occasionally with severe environmental stress. Recently, it has become increasingly accepted that the combined action of two or more adverse factors of different natures increases the risk of colony mortality.

Biotic and abiotic stressors can add up and interact. Bees are subjected to different stress factors at the same time and with an accumulating effect over time. In doing so, each factor reduces the ability of bees to overcome the negative effects of the action of other stressors. The mortality of bees and bee colonies is likely to be lower if, for example, the parasite-infested hive is not further exposed to sublethal doses of toxic substances, incl. antibiotics and acaricides used in beekeeping (Goulson et al., 2015). Interactions between the effects of a natural stressor and chemical substances can sometimes result in greater effects than expected from either of the stress types alone (Holmstrup et al., 2010).

Recent studies have shown increased larval or worker honey bee mortality due to the additive or synergistic interactions between sub-lethal doses of neonicotinoid, and infection by the *Nosema ceranae* (Aufauvre et al., 2012; Retschnig et al., 2014). Moreover, the combination of neonicotinoid pesticides and *V. destructor* contributes to the decrease in the winter honey bee population in the colony (Straub et al., 2019; Van der Zee et al., 2015), the decreases in the bee's flying ability (Blanken et al., 2015) and impacts the honey bee homing behaviour (Monchanin et al., 2019).

Other studies showed that honey bee colonies need proper and balanced nutrition to maintain their development and reproduction (Paoli et al., 2014). A large number of direct anthropogenic drivers produce alterations in diversity and may even lead to the extinction of many flowering plants which are the main food sources for honey bees (Barber and Gorden, 2013). These anthropogenic interventions may lead to malnutrition, i.e., reducing the activity of the immune system and potentially the function of some important detoxification enzymes; there is an elevated risk of the individual and combined impact of pesticides and pathogens on honey bees (Goulson et al., 2015; Vanbergen et al., 2013). From what has been said so far, it is clear that the interaction between anthropogenic direct and natural direct drivers may represent a serious threat to honey bee health and survival.

5. Beeswax as a risk factor for honey bees

5.1 The matrix beeswax

Beeswax is a fundamental material for the colony. It is worthwhile acknowledging that beeswax is produced endogenously by specialized glands in adult bees, then manipulated in the mandibles where salivary secretions are added and finally placed in the growing comb structure. Beeswax does experience some ageing effects (Fröhlich et al., 2000a), but its composition is relatively stable over time. The thermal properties of beeswax contribute to heat retention and thermoregulation in the hive. Beeswax also has interesting structural properties that vary among species. Beeswax is also important as a source of nestmate recognition cues (Buchwald et al., 2009). The chemical composition of beeswax consists of a blend of more than 300 compounds including hydrocarbons (14%), monoesters (35%), diesters (14%), hydroxy polyesters (8%) and free acids (12%) (Callow 1963; Tulloch 1980). As a result, beeswax is a

fatty matrix where lipophilic compounds (with high partition coefficient octanol/water (log P or Kow)) will tend to accumulate and stabilise. The temperatures at which beeswax is handled for beekeeping purposes like the production of the beeswax foundation are about 66°C, this range of temperatures may not degrade the pollutants present there.

While the chemical composition of beeswax is relatively well known, the contribution of individual compounds, or classes of compound, to the functions of the beeswax are less well understood (Buchwald et al., 2009).

5.1.1 Beeswax European legislation

Currently, there are no specific standards in the legislation for the composition and chemical contamination of beeswax specifically aimed at the protection of bee health ((CE) n° 1069/2009 and (UE) n° 142/2011), however, beeswax for use in beekeeping is regulated indirectly. The European legislation on animal by-products (ABPs) defines beeswax as an "apiculture product" used in beekeeping (Regulation (EC) No 1774/2002) and categorises beeswax as an ABP Category 3 material, i.e. not intended for human consumption. Thus, this categorisation does not prevent the presence of contaminants and/or adulterants.

The lack of standards for the composition and chemical contamination of beeswax specifically aimed at the protection of bee health is problematic. There are no legal standards set to define the toxicological quality of waxes or to set limits on the import of poor-quality waxes. Nevertheless, substantial work has been carried out in Belgium by the scientific committee of the Federal Agency for the Safety of the Food Chain (FASFC) where limits for 9 pesticide and veterinary drug residues were set and where limiting the sale of re-melted beeswax that exceeds these limits is recommended (Scientific Committee of the FASFC, 2018).

5.1.2 Beeswax contamination routes

As exposed above, beeswax is produced by honey bees or recycled and added by the beekeeper to the hive. Though beeswax guarantees the stability of the hive, it has often a low replacement rate and can remain in the hive for many years, leading to an accumulation of lipophilic substances applied in beekeeping and/or agriculture (Chauzat and Faucon, 2007; Lambert, 2013; Mullin et al., 2010). Contaminations in the hive can occur through different pathways: (i) forager honey bees can bring environmental contaminants from crops and plants, water and soil to the hive (ii) Beekeepers can provide honey bee colonies with contaminated manufactured beeswax sheets, which the bees draw out into the full depth comb. The raw materials for beeswax manufacture are recycled from old brood combs, honeycombs and beeswax cappings, as recycled materials, they can also contain chemical substances.

(iii) The use of conventional veterinary treatments based on synthetic acaricides to control *Varroa* infestation can lead to contaminations and can lead to accumulations (**Figure 9**).

Figure 9: Pesticide residues contamination routes of beeswax

Once inside the hive, these residues of pollutants may migrate within beeswax in a time frame of weeks or months (Wu et al., 2011) and bees can help their transfer by moving food stores around (Zeggane et al., 2005). Given that beebread and honey are stored in beeswax cells, a transfer of pollutants among in-hive matrices is possible. Studies done with antibiotics showed that the larger the number of residues in beeswax, the larger the number of residues transferred to honey, although the transfer rate varies depending on the concentration studied and ranged between 15.6% and 56.9% (Reybroeck et al., 2010). The level of transfer seems to be linked to the n-Octanol/Water Partition Coefficient (Kow) (ratio of the concentration of a chemical in n-octanol and water at equilibrium at a specified temperature) which determines the affinity to water. The transfer from nectar to beeswax was very little (3%). With a similar approach, Jan and Černe (1993) fed colonies with various organochlorine compounds and followed their transfer to beeswax and honey stored by bees. As in Reybroeck et al., 2010, larger concentrations of pollutants resulted in larger concentrations in both beeswax and honey. Recovery of the different compounds ranged between 1% and 53% in beeswax and between 0.2% and 3% in honey.

5.1.3 Assessing environmental contaminations with beeswax

Environmental monitoring can be conducted using biotic or abiotic systems, and a combination of physical, chemical, and genomic analyses (Cordier et al., 2021). As the main managed pollinator of

agricultural systems, *A. mellifera* L. has diverse interactions with the environment and is a ‘micro livestock of global distribution’(Cunningham et al., 2022).

As exposed earlier, beeswax is a complex mixture consisting mainly of esters of higher fatty acids (Aichholz and Lorbeer, 1999; Tulloch, 1980). Due to its high composition in fatty acids, and as most acaricides are fat-soluble and non-volatile (Wallner, 1999), beeswax is a relevant matrix to assess in-hive chemical exposure history for lipophilic compounds (Lozano et al., 2019; Ravoet et al., 2015). Of all beehive products, it has the lowest replacement rate, can remain in the hive for many years and is recycled by the beekeepers into new beeswax foundations for comb building, thus leading to a greater accumulation of different pesticide residues used in beekeeping and agriculture (Chauzat and Faucon, 2007; Johnson et al., 2010). Beeswax can be considered a contaminant reservoir or a final sink for pesticides (Bogdanov, 2004; Bommuraj et al., 2019). It is for all the reasons listed above combined with beeswax properties that it remains extremely difficult to differentiate between old and new contaminations and to determine with certainty their origin.

5.1.4 Beeswax contamination risk to bees and consumers

Pesticide accumulation through the years and beeswax recycling may lead to residue levels that exceed the maximal limits and thus pose a health risk for honey bees and consumers. Even though most residues remain in the beeswax because of their liposolubility, residues' migration from the beeswax to beebread, and larvae is a crucial factor that could affect the evolution of the colony (Murcia Morales et al., 2020). Few studies have shown that residue accumulation can affect worker honey bee and queen development (Haarmann et al., 2002), bee longevity (Wu et al., 2011), and colony performance (Desneux et al., 2007). We should also not forget that even though beeswax is primarily used in beekeeping it is also used in the chemical, cosmetic, pharmaceutical and food industries. The contamination of this matrix can thus represent a health risk for the consumer.

Considering the human dimension of honey bee products, pesticide contamination becomes not only important for bees, but also for us. Honey can be eaten in Belgium at a rate of 15 Kg head/year (heavy consumer) and pollen has become a food complement for many (Bruneau, pers. Comm.). Propolis or royal jelly are often used for pharmacological purposes, while beeswax can be consumed as “comb honey” or as a food additive (E901). Maximum Residue Levels (MRLs) of pesticides are set up to ensure the safety of consumers and are ruled by Regulation 396/2005/EC (Commission regulation, 2006). However, they are described only for honey, and the consumption of other beekeeping products possibly posing a risk to human health (Wilmart et al., 2016).

5.1.5 Beeswax adulteration

Beeswax adulteration is the addition of cheap hydrocarbons of foreign origin, e.g., paraffin, microcrystalline wax, stearin or beef tallow. It is one of the main quality issues of beeswax production together with beeswax pesticide residue contamination and represents a long-term and increasing problem worldwide. The high price of beeswax when compared with other solid fatty products makes beeswax an attractive target for adulteration. These adulterants may pose health concerns to honey bees which might be in contact (i.e. from larvae developing in beeswax and from adults manipulating the beeswax when building combs) (**Figure 12**) or consume contaminated food (stored in beeswax) (Chęć et al., 2021; Semkiw and Skubida, 2013).

Figure 10: Bees refusing to build the adulterate beeswax and building with their beeswax instead - by André Jusseret, 2016.

Recent studies demonstrated that beeswax comb foundation adulterated with stearic and palmitic acids affected brood development (Reybroeck, 2017), where mortality rates above 45% were observed with a minimum of 5% and 7.5% of stearic and palmitic acids, respectively. Around 80% mortality rates were found with beeswax comb foundation containing 10% of mixtures of added fatty acids. Therefore, it was concluded that the beeswax comb foundation made with stearic and palmitic acids was inappropriate for use in apiculture (Scientific Committee of the FASFC, 2018). Except for the study of Reybroeck et al. (2017), the effects on bees are understudied and still debated.

6. Impact of pesticide exposure on the bee gene expression

Gene expression is a fundamental life process providing a bridge between information encoded within a gene and a final functional gene product, such as a protein or non-coding RNA (ncRNA), it is also the physiological response to chemical and biological threats. It is vital for maintaining normal cellular structure and function and is also the basis for developmental changes, such as differentiation and morphogenesis (Parker, 2013).

The ability to regulate gene expression allows cells to deliver a functional protein whenever it is needed for their normal functioning or survival. This mechanism underlies various physiological and pathological processes, including cellular adaptations to novel environments, maintenance of homeostasis, and recovery from damages (Parker, 2013).

6.1 Immune system of honey bees

Although honey bees lack the complex adaptive immune system of vertebrates, they have an innate immune system (Larsen et al., 2019). The innate immune system of bees is based on cellular defences with circulating haemocytes in the haemolymph, and humoral defences are based on the recognition of pathogen-associated molecular patterns through host proteins called pattern recognition receptors (Antúnez et al., 2009). After recognition, the Toll, Imd, Janus kinase (JAK)/STAT and/or JNK pathways transmit a signal to the nucleus of the cell to trigger the expression of defence-related genes that regulate the production of antimicrobial peptides, such as hymenoptaecin, defensin1 and defensin2, and stress-related genes, such as blue cheese, involved in autophagasome trafficking to lysosome (Evans, 2006; Hamiduzzaman et al., 2012). However, pathogens can also suppress host defences. For example, *N. ceranae* can cause widespread immunosuppression in honey bees, such as reduced expression of genes for Gram-negative binding protein, peptidoglycan recognition protein and various antimicrobial peptides (Li et al., 2018). It is also interesting to mention the study of De Smet et al. that highlighted the different context-dependent effects of. pesticide exposure on the honey bee response. When exposed in field conditions, honey bees were able to set up an immune reaction while bees housed in artificial cages suppress this reaction (De Smet et al., 2017).

6.2 Detoxification mechanism of honey bees

One of the principal mechanisms used by insects to escape the adverse effects of both natural and synthetic toxins, such as natural pyrethroids and pesticides, is metabolic resistance (Rand et al., 2015). The major enzyme superfamilies responsible for the metabolism or detoxification of toxins are the cytochrome P450 monooxygenases (P450s), glutathione transferases (GSTs) and carboxylesterases (COEs) (Feyereisen, 2006; Li et al., 2007). The sequencing and annotation of the honey bee genome

revealed a 50% or greater reduction in the number of genes encoding for these enzyme families relative to other insect genomes (Claudianos et al., 2006). The smaller number of detoxification genes may limit the capacity of honey bees to metabolize multiple toxins simultaneously, causing bees to be more sensitive to synergistic interactions of pesticides e.g. competitive inhibition of P450s (Johnson et al., 2009, 2006). It has been shown in different studies that the detoxification reaction in honey bees depended on the housing condition (De Smet et al. 2017; Pettis et al. 2012).

7. Perception of the risks affecting colony loss

Colony management by beekeepers is of utmost importance for the health and survival of honey bee colonies. Beekeeping management practices vary from low to high intervention regarding the use of chemicals, hive manipulations, and supplemental feeding. Before applying adequate BMP, beekeepers need to perceive the impact of stress factors representing a risk for the colony. They also need to perceive the benefits of certain BMP for implementing them. Risk perception consists of the importance that individuals give to an at-risk situation (Lamarque et al., 2011; Shackleton et al., 2019).

Understanding beekeepers' perception of these risks is essential to analyse the reasons for adopting or rejecting some BMP. Identifying and preventing risks associated with BMP may help avoid exacerbating colony loss rate (Giacobino et al., 2014). It is known that risk perception is determined by different social and environmental factors affecting individuals, such as the degree of knowledge they have and/or the environment in which they live (Martín-López et al., 2012).

Risk perception is usually divided into two components: the probability for the risk to occur and its likely impact on the apiary. Independently of the risk perceptions, the beekeeper's general risk attitude is a key psychological factor which influences the risk-related behaviours and therefore the adoption of BMP by the beekeepers (van Winsen et al., 2016). A beekeeper who has a high-risk attitude (personal tendency to take risks) will more likely have a lower perception of risks and will be less likely to adopt any risk management strategy.

Therefore, a grounded theory from health psychology was used to build a framework adapted to the beekeepers (**Study 5**): the Health Belief Model (HBM) (Janz and Becker, 1984; Rosenstock, 1974). The HBM was specifically developed for the understanding of health-related behaviour

8. Future perspectives and hope for bees

On 1 June 2018, the European Commission adopted a Communication on the first-ever EU initiative on wild pollinators. The initiative sets strategic objectives and a set of actions to be taken by the EU and its Member States to address the decline of pollinators in the EU and contribute to global conservation efforts. It sets the framework for an integrated approach to the problem and more effective

use of existing tools and policies. The initiative sets long-term objectives (towards 2030), and short-term actions under three priorities:

1. Improving knowledge of pollinator decline, its causes and consequences;
2. Tackling the causes of pollinator decline; and
3. Raising awareness, engaging society at large and promoting collaboration.

On 20 May 2020, the commission adopted the EU biodiversity strategy for 2030 and the farm-to-fork strategy, both of which are flagship initiatives under the European Green Deal. These strategies will boost actions to reverse the decline of pollinators through commitments and targets for nature protection and the EU nature-restoration plan. Together with the new EU strategy on adaptation to climate change, and strengthened ambition on both climate neutrality and zero pollution, these strategies will help to tackle the main threats to wild pollinators such as land-use change, intensive agricultural management and pesticide use, environmental pollution, invasive alien species and climate change (European Commission, 2021). Similarly, on national level, a national strategy in favour of pollinators has been defined aswell (Federal Public Service, 2021).

Reversing honey bee declines will require the integration of hive-specific solutions, a reassessment of engagement with the many stakeholders whose actions affect honey bee health, and recontextualising both of these within landscape scale efforts. Also, veterinary research needs to adopt a “One-Health” approach to address the scope of crises that pollinators face.

- **Towards a One Health approach**

One Health is described as an approach to global health that emphasises the interconnectedness of human, animal and environmental health, noting in particular that human health depends on a healthy and functioning ecosystem. This approach focuses on linkage between the health of humans, animals, and the environment by improving intersectional communication and collaboration through research and policy (WHO, 2017). The One-Health approach emphasises a holistic understanding to tackle challenges (Destoumieux-Garzón et al., 2018). Human and animal health are threatened by antimicrobial resistance, environmental pollution, and the development of multifactorial diseases. This highlights the increasing globalization of health risks and the importance of the human-animal-ecosystem interface in the evolution and emergence of pathogens.

As exposed previously, honey bee health is depending on various stressors and their complex interactions. Thus, honey bee health must be understood on a global scale and from a global and crosscutting perspective, integrating human health, animal health, plant health, ecosystems health, and biodiversity (Destoumieux-Garzón et al., 2018).

Honeybees can serve as a One Health model organism to study these interactions between environmental change and diseases because of their inseparable symbiosis with environmental health determinants (Conti and Botrè, 2001; Porrini et al., 2021) (**Figure 14**). For example, environmental pollutants in water, soil, and air can negatively impact bee and hive health by seeping into pollen and honey foods (Mullin et al., 2010; Smart et al., 2016). In addition, warming temperatures and other climatic factors related to climate change may increase the prevalence and spread of bee diseases (Runckel et al., 2011) and reduce the effectiveness of antimicrobials in treating some pathogens (MacFadden et al., 2018). In addition, the efficacy of Varroa treatment is challenged by years of use, which contributes to the increase in drug resistance (Thompson et al., 2002).

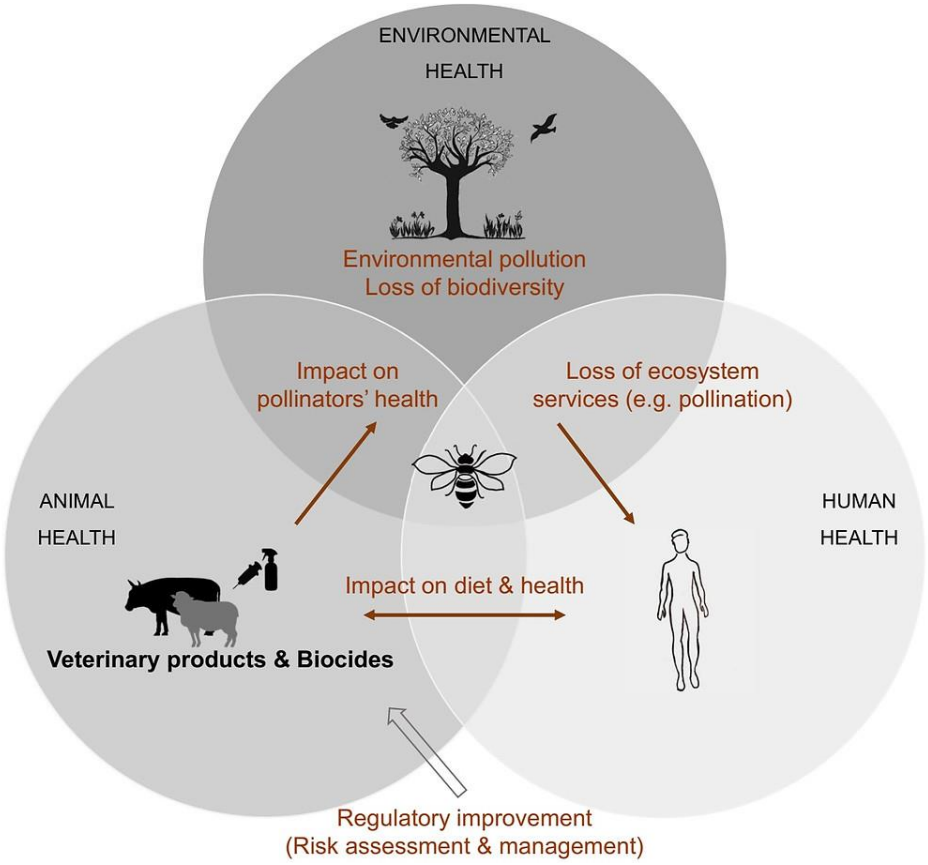

Figure 11: Honey bees and the one health approach (Mahefarisoa et al., 2021)

Bee research efforts committed to studying biotic risk factors of honey bees (mainly *Varroa* sp.) were predominant over the past three decades, but research on threats to bees has now reached the shifting point from biotic risk factors to global change as the prime concern of scientists (Decourtye et al., 2019). This rise of global change science reveals prevailing topics, for current and future years: climate change, landscape alteration, agricultural intensification and invasive species. Research on floral resources and habitat loss is one of the rapidly expanding topics and the overall research on pesticide effects, exposure assessment and other toxicological topics also developed substantially. In addition to broad pesticide categories, a diversified range of polluting substances are nowadays considered

(Decourtye et al., 2019). Wild bees represent the main focus of studies on resource and habitat loss, nevertheless much more efforts should be devoted to these native species.

Chapter 2 - Objectives

1. General objective

The overall objective of this thesis is **to better understand the risk factors affecting honey bee health** in the Belgian beekeeping context and **to provide tools as well as guidance and recommendations** to the beekeeping sector **to alleviate these risks**.

Specific objectives

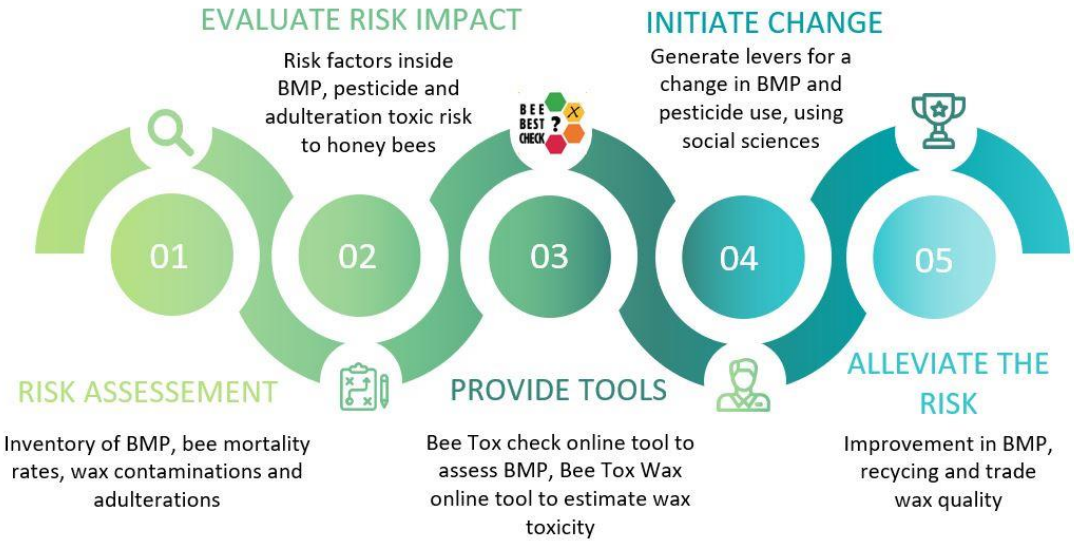

Figure 12: Specific objectives of the thesis

Although indications of the role played by BMP on honey bee health exist, very little information was available in Belgium. Our study aimed **to characterize BMP** carried out in Belgium**, determine the possible correlation between BMP and colony losses** and **provide a tool** for beekeepers to evaluate the effect of BMP on colony health (**study 1**).

The second specific objective of our work was **to assess the occurrence and the concentrations of pesticide residues in beeswax** on the national level and **estimate the potential toxic risk of beeswax to honey bees.** More specifically, the risk to bees and consumers of two pesticides (flumethrin and glyphosate) was examined. This work resulted in the creation of a pesticide toxicity estimation tool. In a related study, we also assessed **the current situation of beeswax adulteration** in beekeepers' and commercial beeswax in Belgium through a nationwide survey (**studies 2, 3, 4 and 6**).

The third objective of our work aimed at **initiating a change in BMP**, therefore an unpreceded sociological survey designed with a grounded theory from health psychology was used (**study 5**).

Last but not least, the impact of these contaminations and adulterations in beeswax on honey bee development has been investigated. Therefore, a novel field realistic methodology to rear honey bees pupae in contact with adulterants and contaminants in beeswax has been tested. Rearing honey bees pupae allowed us to characterise the impact of contaminated and adulterated beeswax on brood mortality and honey bee gene expression (**study 7**).

This work gives an overview of the current BMP, beeswax pesticide residues contaminations and adulteration but also the related risk to honey bees and consumers' health. This work also provides guidelines for the beekeepers in terms of BMP and beeswax recycling, guidelines and recommendations for authorities in terms of MRL for some pesticides residues and adulterants and in terms of determining factors for best BMP behaviour change as well as tools for the beeswax manufacturers to estimate the risk associated with contaminated beeswax to sanitize beeswax commercial trade stream.

Chapter 3 - Experimental section

Experimental section

Study 1

Risk and protectives indicators of beekeeping management practices

Science of the Total Environment 2021,799:1-10

Noëmie El Agrebi, Nathalie Steinhauer, Simone Tosi, Laurent Leinartz, Dirk C. de Graaf and Claude Saegerman

Preamble

Honey bees are social and domesticated insects that live in a colony. The beekeeper plays thus a key role in maintaining the health status of managed honey bee colonies. While good management can alleviate stress, poor management can accentuate it. However, a clear overview of the main actions carried out by beekeepers and their role in the successful management of honey bees was never addressed in Belgium. A field study aiming at gathering data allowing us to create a management register was implemented. The collected data allowed us to have a better view of the bee management practices (BMP) in the two Belgian regions and to correlate the data of both parts of the country with the registered colony loss rates to detect potentially inappropriate BMP and have a better understanding of mortality factors in Belgium.

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Risk and protective indicators of beekeeping management practices

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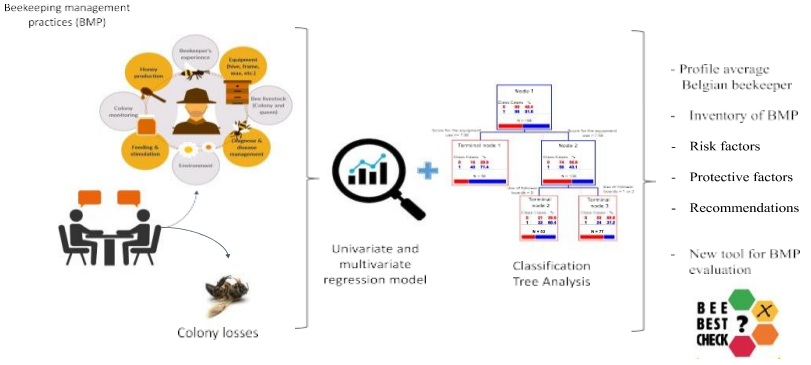
HIGHLIGHTS

- Cross sectional study with face-to-face interviews (n = 186 beekeepers)
- Characterization of beekeeping management practices carried out in Belgium
- Evidences of a relationship between beekeeping management practices and colony losses
- BeeBestCheck online tool freely available on smartphone and computer

GRAPHICAL ABSTRACT



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ABSTRACT

Explaining the reasons for the high honey bee (*Apis mellifera*) colony loss rate in recent years has become a top global research priority in apicultural and agricultural sciences. Although there are indications of the role played by beekeeping management practices on honey bee health, very little information is currently available. Our study aimed to characterize the beekeeping management practices carried out in Belgium, and to determine the relationship between beekeeping management practices and colony losses. Variables obtained from face-to-face questioning of a representative randomized and stratified sample of Belgian beekeepers (n = 186) were integrated into a logistic regression model (univariate and multivariate) and correlated to the declared colony loss rates to identify risk and protective indicators. We used a classification tree analysis to validate the results. We present evidence of a relationship between poor beekeeping management practices and colony losses. The main factors protecting honey bee colonies are the aptitude of the beekeeper to change his management practices, the hive type, the equipment origin and hygiene, wintering in proper conditions (the use of divider boards, i.e. board blocks or space fillers off part of the hive body), the colony strength estimation before wintering, winter monitoring, and last but not least, appropriate integrated pest management. Proper estimation of the Varroa infestation level should be performed prior to treatment. The consequences of poor beekeeping practices on honey bee health can be addressed by proper training of beekeepers. An online tool was developed and published for beekeepers allowing them to evaluate the effect of their management practices on colony health.

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1. Introduction

Honey bees (*Apis mellifera* L.) generate a wide range of products for human consumption but more importantly provide irreplaceable pollination services to agricultural and natural ecosystems. The honey bee is a managed eusocial organism. Its health is mainly assessed at the colony level rather than at the individual level (Afssa, 2008). While honey bees enable us to investigate complex health issues affecting social organisms, defining precise risk or protective indicators remains challenging as some stressors are buffered at the colony level (Straub et al., 2015).

Beekeeping management practices (BMP) represent the totality of the actions implemented by a beekeeper to maintain healthy honey bee colonies and to achieve its production objectives (EFSA AHAW panel, 2016; Formato and Smulders, 2011; Ritter and Pongthep, 2006; Rivera-Gomis et al., 2019) (Fig. 1). For example, when facing high pest pressure, beekeepers can reduce hazards through physical or chemical interventions (Giacobino et al., 2016; Jacques et al., 2017). While good management can alleviate stress, poor management can accentuate it. Good management practices must be developed with proper training and experience (Steinhauer et al., 2018). The beekeeper plays thus a key role in maintaining the health status of managed honey bee colonies. However, a clear overview of the main actions carried out by beekeepers and their role in the successful management of honey bees is only partially addressed (Sperandio et al., 2019).

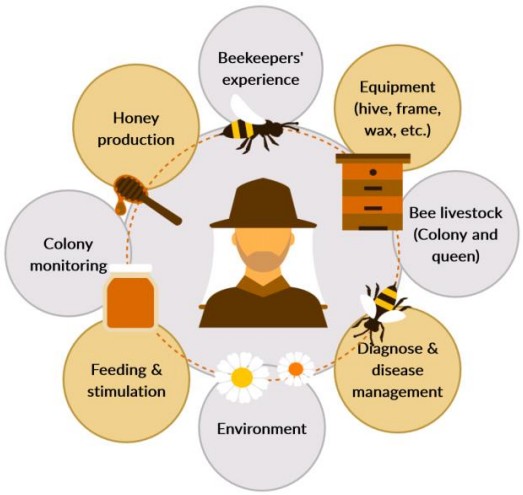

Fig. 1 Beekeeping management practices can affect bee health alone or in combination with other factors

Over the last decade, considerable attention has been given to understand stress factors impacting honey bee colony health and losses, but the management practices' impact has often been overlooked. In the literature, very few publications about management practices are available (Sperandio et al., 2019; Steinhauer et al., 2021; Underwood et al., 2019). Nevertheless, national and European monitoring projects such as HealthyBee (Federal Agency for the Safety of the Food Chain, Belgium, 2016-2018), APENET (Porrini et al., 2016) and COLOSS (Gray et al., 2019) highlighted the direct and/or indirect role of the beekeeper in ensuring health and performance of honey bee colonies. Better BMP can be implemented from a short-term perspective by individual beekeepers and may have the potential to reduce colony losses (Clermont et al., 2014). In Belgium, information on the correlation between beekeeping management practices and the registered colony loss rates is still lacking. To date, there is no comprehensive register of beekeeping practices in Belgium. A register with representative and comparable information across the different regions could help target inappropriate BMP.

Honey bee health has been declining since the end of the 1980s in Belgium as well as in the rest of Europe (Ellis et al., 2010; Potts et al., 2010; VanEngelsdorp et al., 2009). Epidemiological standardized methods to collect comparable and robust data were set up with the pan-European surveillance program on honey bee colony losses (Laurent et al., 2015). In 2012–2013, the Belgian winter loss rate was estimated at 34.6%, the highest percentage among 17 participating European countries in the European EPILOBEE study of the same year (loss of 32.8% overall) (Fig. 2). Before the emergence of the Varroa mite, no historical data regarding the acceptable (winter) mortality levels of colony losses in Europe were set, and these levels may vary between countries (Chauzat et al., 2016; Steinhauer et al., 2014).

We hypothesize that some implemented BMP can have an impact on honey bee health and consequently on colony losses. Our study aimed to characterize the beekeeping management practices carried out in Belgium, in order to determine the relationship between beekeeping management practices and colony losses.

2. Materials and methods

2.1. The Belgian beekeeping

The monitoring network of the European Honey Programme estimates that 2/3 of the Belgian beekeeping sector is made up of hobbyist beekeepers, who's source of income lay outside beekeeping. They keep bees as a pleasant pastime and for the intrinsic values of beekeeping (El Agrebi et al., 2021). Honey bees are largely maintained in stationary apiaries, for honey production, by hobby beekeepers (1–15 colonies) or experienced hobby beekeepers (16–50 colonies). Apiaries are thus relatively small operations. Beekeepers often have a knowledge based on observation and self-experimentation. One-third of the sector is made of semi-professional beekeepers (50–150 colonies) and only seven beekeepers are professional (with more than 150 colonies). The European Union co-finances aid programs for beekeeping. In Belgium, they are developed at regional levels (Flanders and

Wallonia), in consultation with representatives of the sector.

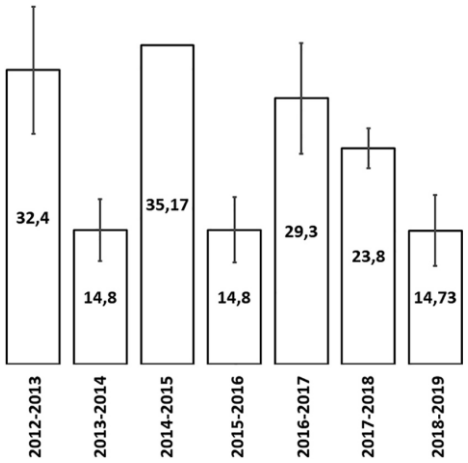

Fig. 2. Winter loss rate in percent from 2012 to 2019 in Belgium. Legend: Data collected from the EPILOBEE consortium and the Belgian Federal Agency for the Safety of the Food Chain (FASFC) for the years 2012 to 2015, from the Belgian beekeeping federation for the years 2014–2015 (only global data available), from the ULiège Faculty of veterinary medicine for the years 2015–2016, and from the Belgian institute of health (Sciensano) for the years 2016 to 2019.

2.2.Study design and sample size

A cross-sectional study was carried out from May to November 2016 in Belgium. There is no precise, comprehensive figures for the entire beekeeping sector in Belgium since 2015, as an undefined number of beekeepers are reluctant to the register of competent authority (Federal Agency for the Safety of the Food Chain) or to beekeepers associations. In this study, we started from the list of beekeepers officially registered (FASFC) in 2015 (n = 4949). Out of this list, 20 beekeepers were randomly selected per province (n = 10 provinces) following stratified randomization procedures (computerized random numbers) (Moher et al., 2010).

Potential explanatory variables were obtained from structured face-to-face interviews, with predetermined questions designed in advance and directed towards BMP. To facilitate data processing, most questions were close-ended (N = 140) (dichotomous or multiple choice). Open-ended (N = 3) questions were designed and asked in a simple, neutral, and comprehensive way (van der Zee et al., 2013) and were used to assess beekeepers' concerns. The loss rate of each apiary was assessed. A detailed list of the survey questions and results is available in Appendix 1.

2.3.Data collection

2.4.Data on colony losses

The loss rate was based on beekeepers declarations. The overall loss rate is the proportion calculated as the total number of lost colonies (at the end of the winter or end of the season) divided by the total number of colonies before winter. The following definitions are provided to understand the part of methodology carried out in this study. Winter is defined as the period between the end of pre-winter colony preparations and the start of the new foraging season (van der Zee et al., 2013). Seasonal losses occur during the beekeeping season. The year losses are the sum of winter and seasonal losses. The colony loss metric is subject to discussion as BMP vary between regions and between professional and hobby beekeepers. Merging weak colonies into stronger ones decreases the number of colonies in an apiary, but to define those as dead would be inaccurate, so they are considered lost. We have set the acceptable winter mortality level at 10% according to earlier work (Haubruge et al., 2006; El Agrebi et al., 2020; El Agrebi et al., 2021), this rate is generally considered acceptable.

2.5. Varroa control classification

In Belgium, the strategies used to control Varroa are diverse (active substance, formulation, biotechnical control methods, time of treatment, and the treatment frequency), and most beekeepers apply a combination of various Varroa control methods. Thus, because statistical methods require a sufficient number of replicates, Varroa control methods were classified in models according to most frequent combinations (Table 1). The Varroa mite control notice issued yearly by the FASFC (2015–2016) recommended to accurately estimate the Varroa mite infestation in the colonies, then to apply two treatments a year: the first after the last honey harvest and the second in winter in the absence of brood when all/nearly all mites are phoretic.

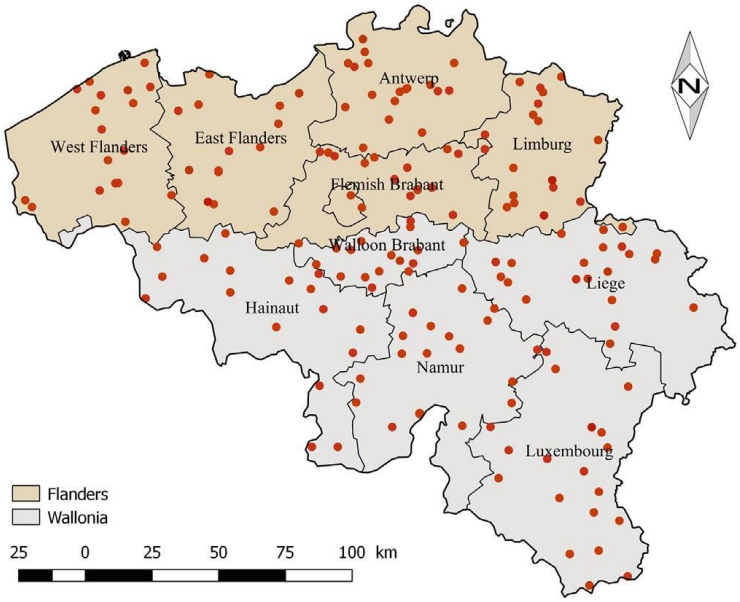

Fig. 3. Surveyed beekeepers' geographic locations in each province (n = 20), in Flanders (n = 100), and Wallonia (n = 100) in 2015–2016.

2.6.Equipment scoring

A score was assigned to the origin of the equipment (new, self-made, second hand) and its reuse after colony losses (yes, after disinfection, or no), as well as to the origin of the beeswax (recycled from own beeswax, recycled from commercial beeswax or commercially purchased beeswax). For these three variables, an overall score was calculated for each beekeeper as statistical methods require a sufficient number of replicates and most beekeepers have different combinations of practices regarding the origin of the beeswax, the origin of the equipment and its reuse after colony losses.

2.7.Statistical analysis

2.7.1. Identification of risk and protective indicators using logistic regression

Logistic regression models were performed in Stata SE 14.1® (StataCorp LP, College Station, TX, USA), to evaluate the effect of the selected explanatory variables on the binary outcome loss rate (threshold 10% according to (El Agrebi et al., 2021)). First, a univariate analysis was conducted and odds ratios (OR) with 95% confidence intervals (95% CI) were calculated for each variable. Then, a multivariate logistic regression analysis was performed using the variables with a p-value <0.10 in the univariate analysis (in order to be conservative) (Renault et al., 2020). The model was progressively simplified by removing the least significant variable with a $p > 0.05$. The model was considered complete, either when all variables had a significant p-value (<0.05), or when it could not be further simplified without having a significant difference between the most complex and the simpler model (likelihood ratio test with a p-value < 0.05) (Renault et al., 2020). The goodness of fit was assessed using the Hosmer–Lemeshow goodness-of-fit test (Petrie and Watson, 2014). The limit of statistical significance of the tests performed was defined at p-value ≤ 0.05.

Table 1: Model of the *Varroa* treatment combinations in Belgium.

Model treatments combination	
A.	No *Varroa* control
B.	Two treatments: thymol after harvest + organic acids (formic, oxalic) in winter
C.	Two treatments: EU-authorized veterinary medicinal products (VMP) after Harvest (Amitraz and Tau-fluvalinate) + organic acids in winter
D.	One treatment with organic acids (summer or winter)
E.	Two treatments or more; summer and winter with organic acids + other substances (essential oils other than thymol)
F.	One treatment with EU-authorized veterinary medicinal products (VMP) (Amitraz and Tau-fluvalinate)

2.7.2 Classification tree analysis

We performed a classification tree analysis (CTA) in an attempt to better understand the relative importance and inter-relations among different risk variables in explaining colony losses using the acceptable level of 10% (El Agrebi et al., 2021). The CTA is a non-linear and non-parametric model that is fitted by binary recursive partitioning of multidimensional covariate space (Breiman et al., 1984). Using Salford Predictive Modeler (SPM) software (Salford Systems, San Diego, CA, USA), the analysis successively splits the data set into increasingly homogeneous subsets until it is stratified to meet specified criteria. The Gini index was used as the splitting method, and 10-fold cross-validation was used to test the predictive capacity of the obtained trees. SPM performs cross validation by growing maximal classification trees on subsets of data then calculating error rates based on unused

portions of the data set (Chaber and Saegerman, 2017). To accomplish this, SPM divides the data set into 10 randomly selected and roughly equal parts, with each 'part' containing a similar distribution of data from the populations of interest (i.e. colony strength estimation). SPM then uses the first nine parts of the data, constructs the largest possible tree and uses the remaining 1/10 of the data to obtain initial estimates of the error rate of the selected subtree. The process is repeated using different combinations of the remaining nine subsets of data and a different 1/10 data subset to test the resulting tree. This process is repeated until each 1/10 subset of the data has been used as to test a tree that was grown using a 9/10 data subset. The results of the 10 mini-tests are then combined to calculate error rates for trees of each possible size; these error rates are applied to prune the tree grown using the entire data set. The consequence of this process is a set of fairly reliable estimates of the independent predictive accuracy of the tree, even when some of the data for independent variables are incomplete and/or comparatively small. For each node in a classification generated tree, the 'primary splitter' is the variable that best splits the node, maximizing the purity of the resulting nodes.

3. Results

The completion rate during the face-to-face questionnaire interview was 99.71%. The few absence of answers was due to an alternative BMP (minimal intervention) or a reluctance to talk about the quantity of produced honey.

Beekeepers' age distribution was not normally distributed (ShapiroWilk W test for normal data; p-value = 0.0001). Median age was 60 years old (min–max = 20–90, mean = 57, SD = 15, n = 186), 87.2% had followed a beekeeping training, 91.5% were members of a beekeepers association and 59.6% of them used a logbook or took quick notes (23.4%). Beekeepers with 10 years or more of experience represented 54.8% of the subset. The median number of colonies in the apiaries was 8.5 (min–max, 1–60, mean 11.4, SD ± 9.9), i.e. these were exclusively hobby/non-professional beekeepers. The vast majority of the apiaries were located in a rural environment (72.2%) surrounded by agricultural environment and/or gardens with immediate crop proximity (<3000 m) (92%) and estimated abundant vegetation (52.9%). The motivations for beekeeping were various and included interest for honey bees (58.8%), ecological concerns (47.6%), continuing a family activity (21.9%) and honey production (23.5%). The number of colonies and loss rates per season are shown in Table 2.

Table 2: Loss rates for the year 2015–2016 (n = 186 beekeepers).

Year 2015–2016	Average loss rate in per cent (95% confidence interval)
Winter	11.8 (9.1–14.5)
Seasonal	3.0 (0.3–5.7)
Yearly	14.8 (11.2–18.3)

The losses due to a lack of hazard prevention, thus a lack of good management practices (GMP) in the colonies, were also assessed. Beekeepers estimated that the lack of GMP and hazards encountered in BMP might have been the cause of 41.7% of the year's losses. The most encountered not prevented hazard was colony weakness (Fig. 4).

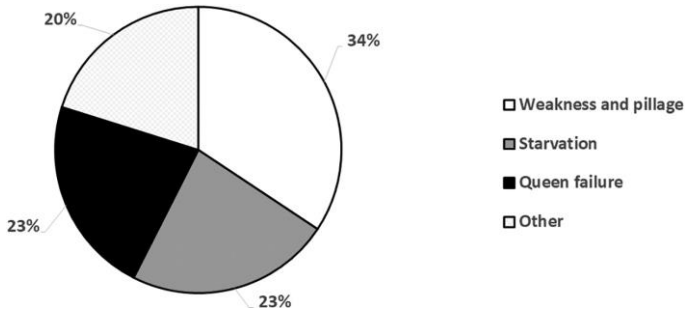

Fig. 4. Distribution of the most encountered (not prevented) hazard leading to colony losses (n = 2175 colonies).

The Buckfast was the dominant breed of honey bees kept (40.4%), followed by *Apis mellifera carnica* (38.3%) and the dark honey bee (*Apis mellifera mellifera*) (17.7%). Stationary apiaries prevailed; transhumance was practiced by 19.7% of the beekeepers, almost exclusively by Flemish beekeepers (94.4%). The hive type "Dadant Blatt" (10–12 frames) was the most frequent type used in Belgium (46.5%), with 88.5% of use in the Walloon region.

In Flanders, the tendency was different, with 53.8% of the beekeepers using the "simplex" hive type. Queen rearing was practiced by 58.8% of the beekeepers; with a median value of 3 (min–max; 0–72) queens produced per year. Of the selfproduced queens, 71% were marked, 56.7% were for personal use. On average, beekeepers bought 1.2 queens a year, 91% of them were reared nationally, and 10.7% came from the EU. In-hive, 51% of the queens were younger than a year, 31.3% were between 1 and 2 years old and 17.7% were older than 2 years. Most new queens were introduced in spring (60%) versus in autumn (40%). One of the most commonly used reproduction methods was the division of colonies with 48.8%, followed by the introduction of mated queens (42.8%).

The average of newly started colonies per year and per beekeeper was 4.6 (min–max; 0–30). About half of these newly started colonies (2.02; min–max: 0–25) were handed off to other beekeepers. The number of introduced swarms was on average 1.28 (min–max: 0–14) per beekeepers a year, 34.2% of these swarms were collected (wild swarms), 33.3% were received from another beekeeper, 25.6% were own swarms recovered, and 9.4 were bought from EU origin.

The most prevalent breeding criterion was honey bee stock gentleness (75.9%), followed equally by the stock productivity and queen laying rate (28.9%). Hygienic behavior and Varroa tolerance were only mentioned respectively by 10.7% and 11.8% of the beekeepers.

Winter preparation usually begins after the last honey harvest, starting with an anti-Varroa treatment. Adapting the hive space to the colony size by using divider boards[1] was a practice used by 38.5% of beekeepers. Reducing the flight entrance was a common practice (71.1%), as well as the control of the presence/laying activity of the queen (74.3%). Colony strength estimation before wintering was performed by 82.5% of beekeepers, 63.1% of their colonies were estimated as strong, 14.7% as acceptable, and 20.3% as weak. Winter monitoring was implemented by 77.5% of the beekeepers, mostly by controlling the bottom board (68.4%), less than once a month (48.3%). The most practiced airing mode was removing the hive bottom board (60%). A grid was largely used (92%) in the hive as the bottom, and hives were generally 40 cm above the ground.

After winter, beekeepers performed the first hive check-up before April (54.8%). During spring monitoring, 45.3% of the beekeepers used a divider board to reduce the hive space, 88.3% checked the brood quantity, and the pollen entries (90.7%). After winter, 23.4% of the beekeepers gathered weak colonies with stronger ones. Swarming control was implemented by 80.6% of beekeepers; the most common control techniques used were royal cell destruction (54.9%), and artificial swarming (33.5%). In summer, brood quality and uniformity control were done by 85.2% of beekeepers as well as the food quantity, and position (78.7%). Hive pillage by wasps (Vespula germanica) was experienced by 36.4% of beekeepers in 2015.

Concerning the equipment and its hygiene, 66.8% of the beekeepers disinfected their equipment after mortality before re-use. The most common disinfection technique used was scraping (53.5%), and using a blowtorch (62%). Reagents as hot water with washing soda or chlorine bleach are also used as disinfectant. Most beekeepers (58.3%) renewed 25 to 50% of the beeswax frames per year. Beeswax was recycled by 32.6% of the beekeepers, or bought by 57.2% (commercial beeswax). Most beekeepers (63.2%) were confronted with the presence of wax moth in the beeswax frames.

Right after harvest, 36% of the beekeepers fed their colonies, 71.6% of them using homemade sugar syrup for this purpose. Winter feeding was done by 80.2% of beekeepers, the use of commercial products, in this case, was preferred by 63% of the beekeepers, and the average quantity that was given to the bees for feeding was 13.3 ± 4.5 kg. Feeding after winter was done by 44.4% of the beekeepers, and 65.4% of them used a commercial sugar paste.

The actions implemented to control Varroa and honey bee diseases in Belgian apiaries are shown in Table 3. The majority of the beekeepers (29.5%) used organic acids (oxalic/formic/lactic) as treatment substances in summer and winter. Thymol in combination with organic acids was applied by 27.3% of beekeepers. Organic acids in a single-use were applied by 10.4% of the beekeepers. Summer treatment with EU-authorized veterinary medicinal products combined with organic acids was used by 8.2% of the beekeepers.

Table 3: Implemented actions to control Varroa and bee diseases in Belgian apiaries (n=186).

Category	Sub-category	Variable	Percent
Varroa control	Veterinary advice	For prescription	88
	Varroa management	Automatical treatment without diagnose	80
		Infestation rate determination	19.3
		Counting natural *Varroa* fall	42.2
Diseases	*Varroa* reported infestation rates	Lack of knowledge	82.9
	Biotechnical methods/drone brood removal	Implemented	36.4
	Treatment efficacy check	Implemented	74.3
	Nosema	Detected	6.95
	Deformed wing virus (DWV)	Detected	39.6
		No significant detection in honey bees	80

Only 36.4% of the beekeepers used biotechnical means (drone brood removal, bottom boards screening and powder sugar dusting) in addition to treatments for Varroa control. The summer treatment was applied by 62.9% of the beekeepers in August, after the harvest. The winter treatment was mostly applied in December, between Christmas and New Year (57.8% of beekeepers).

The honey yield question was not answered by 3.8% of the beekeepers. Of the respondents, 76.9% of the respondents harvested honey twice a year. The average ± standard error production on a yearly base per colony considering all colonies in the apiary was 27.5 ± 16.9 kg per colony per year with a median of 25.0 kg per colony per year (not normally distributed; Shapiro-Wilk W test for normal data, p-value < 0.00001). The average honey yield considering only colonies in full production capacity was 31.03 ± 18.8 kg per colony per year with a median of 27.4 kg per colony per year (not normally distributed; Shapiro-Wilk W test for normal

[1] The divider boards are board blocks or space fillers off part of the hive body; so the honey bees are not overwhelmed with space when starting a smaller colony. Yet is easy to move as they grow.

data, p-value < 0.00001). No significant difference was found between Flanders and Wallonia in terms of yearly yield (Wilcoxon rank-sum test) for all colonies in the apiary (p-value = 0.22) and for colonies in full production capacity (p-value = 0.38).

The open-ended questions from the interview allowed the beekeepers to express their concerns about colony losses. Most common concerns were the following: among the colonies that died, a high number had an apparent queen issue on the previous inspection (queenless colonies, drone-laying queens, unfertilized queens), the lack of clear guidelines concerning efficient and alternative varroosis veterinary treatments, trade beeswax quality, and in-hive contaminations.

3.1. Identification of risk and protective indicators of colony losses using logistic regression

3.1.1. Univariate logistic regression analysis

We tested 98 explanatory variables compared to the dependent variable yearly loss rate. We found a significant association between colony losses and the overall global score given to the equipment used (OR = 0.88; 90% CI: 0.79–0.99; p-value = 0.03). The higher the beekeeper scored with the equipment, the more it was considered as a protective indicator (Table 4). The use of divider board(s) also appeared to be a protective indicator, since with beekeepers using a divider board (OR = 0.39; 90% CI: 0.19–0.78; p-value = 0,008) being less likely to have losses. Beekeepers who estimated their colony strength in the fall were also less likely to have losses (OR = 0.37; 90% CI: 0.15–0.89; p-value= 0.03). Beekeepers with the highest number of strong colonies before wintering (76–100%) faced a higher losses risk (OR = 2.33; 90% CI: 1.09–5; p-value=0.03). The beekeepers who checked their colonies once a month during winter were less at risk for losses (OR = 0.25; 90% CI: 0.12–0.54; p-value<0,001). Finally checking the efficiency of the treatment against Varroa mite appeared to be a protective indicator (OR = 0.44; 90% CI: 0.20–0.95; p-value = 0.04).

Table 4: Most relevant explanatory variables evaluated for potential association with yearly colony losses in 186 apiaries, using a univariate logistic regression analysis.

Variable	Variable type	Modalities	Odds ratio	p-Value
Practice improvement	Categorical	Absolutely	Reference	–
		Why not	0.53 (0.24–1.13)	0.10
		No	0.93 (0.42–2.07)	0.87
		Don't know	4.11 (0.19–87.48)	0.37
Hive type	Categorical	Dadant 10–12 frames	Reference	–
		Simplex	1.12 (0.52–2.40)	0.77
		Other hive types	2.05 (0.95–4.41)	0.07
Score given to the equipment origin and hygiene	Continuous	Number	0.88 (0.79–0.99)	0.03
Apis mellifera carnica	Binary	No	Reference	–
		Yes	2.37 (0.93–6.07)	0.07
Use of divider board(s)	Categorical	No	Reference	–
		Yes	0.39 (0.19–0.78)	0.008
		Sometimes	0.34 (0.11–0.06)	0.063
Colony strength estimation	Binary	No	Reference	–
		Yes	0.37 (0.15–0.89)	0.03
Adjust the hive space to the colony size before winter feeding	Categorical	No	Reference	–
		Yes	0.56 (0.29–1.08)	0.08
		Sometimes	0.58 (0.19–1.76)	0.33
Winter monitoring	Categorical	No	Reference	–
		Yes	0.25 (0.12–0.54)	<0.001
Colony strength	Categorical	A	Reference	–
		B	2.38 (0.87–6.50)	0.09
		C	1.11 (0.36–3.48)	0.86
		D	2.33 (1.09–5.00)	0.03
Disease declaration to authorities	Binary	No	Reference	–
		Yes	0.50 (0.24–1.04)	0.06
Infestation rate determination	Binary	No	Reference	–
		Yes	0.50 (0.24–1.04)	0.06
Varroa management with biotechnical methods	Binary	No	Reference	–
		Yes	0.57 (0.31–1.03)	0.06
Treatment efficacy check	Categorical	No	Reference	–
		Yes	0.44 (0.20–0.95)	0.04
		Sometimes	0.88 (0.21–3.59)	0.85
Model of the *Varroa* treatments combinations	Categorical	A	Reference	–
		B	0.52 (0.18–1.50)	0.23
		C	0.47 (0.0–2.39)	0.36
		D	0.63 (0.29–1.35)	0.23
		E	0.38 (0.13–1.15)	0.09
		F	1.87 (0.18–19.73)	0.60

Legend: Statistical significance when p-value < 0.05. Colony strength: A (0—25%), B (26—50%), C (51—75%), and D (76—100%). Treatments model: see Table 1 for definition of models A, B, C, D, E and F.

3.1.2. Multivariate logistic regression analysis

The multivariate logistic regression analysis (Table 5) confirmed the significant positive association between colony losses and the equipment score (OR = 0.75; 95% CI: 0.59–0.96; p-value = 0.025) as well as the use of divider boards as protective indicators (OR = 0.094; 95% CI: 0.026–0.32; p-value = 0.00). Supplementary protective indicators of losses were found with the use biotechnical methods to control Varroa infestation (OR = 0.22; 95% CI: 0.051–0.96; p-value = 0.04), treatment model E

corresponding to two treatments or more; summer and winter with organic acids + other substances (e.g. essential oils other than thymol) (OR = 0.131; 95% CI: 0.017–0.99; p-value = 0.049) compared to model A (no Varroa control). The model showed additional risk indicators: beekeepers that were not open to change in their beekeeping practices were at risk of higher colony losses (OR = 8.89; 95% CI: 1.15–68.1; p-value = 0.035) compared to the beekeepers who were willing to improve their BMP, the use of other types of hives other than Dadant-Blatt (OR = 8.62; 95% CI: 1.66–44.61; p-value = 0.01) or combining Dadant-Blatt with another hive types (OR = 8.81; 95% CI: 1.21–55.27; p-value = 0.031) increased the risk of colony losses. Beekeepers who declared overwintering a majority of strong colonies (>75%) were also more at risk of colony losses (OR = 2.24; 95% CI: 0.22–0.88; p-value = 0.437). The Hosmer–Lemeshow test showed that the model fit the data correctly (Ch2 = 6.26, df = 8, p = 0.62).

Table 5: Results of the final multivariate logistic regression analysis testing the association between the 15 most significant beekeeping management practices out of the univariate model with a p-value < 0.10 and colony losses in n = 186 apiaries.

Variable	Variable type		Odds ratio	p-value
Practice improvement	Categorical	A	Reference	–
		B	0.31 (0.067–1.51)	0.15
		C	8.89 (1.15–68.1)	0.035
Hive type	Categorical	A	Reference	–
		B	4.05 (0.68–24.1)	0.124
		C	8.62 (1.66–44.61)	0.01
		D	8.18 (1.21–55.27)	0.031
		E	0.816 (0.012–51.64)	0.92
Score given to the equipment origin and hygiene	Continuous	Number	0.75 (0.59–0.96)	0.025
Use divider board	Categorical	0	Reference	–
		1	0.094 (0.026–0.32)	<0.001
		2	0.33 (0.04–2.46)	0.028
Varroa management with biotechnical methods/drone brood removal	Binary	No	Reference	–
		Yes	0.22 (0.051–0.96)	0.04
Treatment models	Categorical	A	Reference	–
		B	0.498 (0.086–2.86)	0.436
		C	0.208 (0.010–4.12)	0.303
		D	0.681 (0.18–2.53)	0.568
		E	0.131 (0.017–0.99)	0.049
		F	0.915 (0.038–21.93)	0.956

Legend: Statistical significance when p-value < 0.05. Practice improvement: A (absolutely), B (why not), and C (no). Hive type: A (Dadant 10–12 frames), B (Simplex hive), C (Other types); D (Dadant + other types), and E (Simplex + other types). Treatments model: see Table 1 for definition of models A, B, C, D, E and F.

3.2 Classification tree analysis

The classification tree analysis (CTA) allowed to determine the relative importance and inter-relation among the different risk indicators of colony losses. We conducted the CTA with variables having a p-value <0.10 from univariate logistic regression analysis. The CTA showed that the score of the equipment (variable importance [VI]: 100) and the use of divider boards (VI: 80.2) were the two predictor variables with the strongest overall discriminating power (Table 6; Fig. 5). Eight additional variables, i.e., variables that did not act as nodes on the selected CTA (Fig. 5), also had significant discriminating power (DP), in decreasing order: the bee breed Carnica (DP: 27.0), tightening colonies before feeding (DP: 13.8), check of treatment efficiency (DP: 11.8), winter check (DP: 11.5), and estimation of the colony strength (DP: 9.9) (Table 6). The root node was first split based on the score of the equipment, clearly indicating that the score of the equipment was the strongest protective indicator. In the first node, when the overall global score of equipment was ≤7.5, 71.4% of the beekeepers (n = 40/56) had mortality rates higher than 10%. In the second node when the overall global score of the equipment was >7.5, 56.9% of beekeepers (n = 130/186) had a mortality rate lower than 10%. For the third node, 31.2% of the beekeepers (n = 77/130) who used one or two divider boards had mortality rates under 10% (Fig. 5). The sensitivity of the tree was 75% (95% CI: 65.1–83.3) and the specificity was 85.6% (95% CI: 76.6–92.1).

4. Discussion

Honey bees (*Apis mellifera* L.) generate a wide range of products for human consumption but more importantly provide irreplaceable pollination services to agricultural and natural ecosystems. To contribute to the maintenance of the population of honey bees, we characterize bee management practices (BMP) carried out in Belgium and present evidence of a relationship between poor beekeeping management practices and colony losses. In general, no significant differences between the two Belgian regions in terms of BMP were found. Our study allowed the identification of risk and protective indicators of BMP and ranked them according to their relative importance and inter-relations among different indicators in explaining colony losses.

According to this study, the winter loss rate reported by the Belgian beekeepers in spring 2016 was 11.8% (±3.6%), which is in line with the winter loss rate of 12.2% published by the COLOSS monitoring group for the same year (Brodschneider et al., 2017). This rate is not particularly alarming given the acceptable losses rate of 10% (El Agrebi et al., 2021). Varroa control is known to

have a tremendous influence on colony losses (Flores et al., 2021; Francis et al., 2013; Noël et al., 2020; van Dooremalen et al., 2012). For Varroa control (2015–2016), only four veterinary medicinal products were authorized in Belgium to treat Varroa: three based on thymol extract to which Varroa have shown resistance for several years (Bonafos et al., 2011) (Thymovar 15 g bee-hive strip, Apiguard 12,5 g gel, and Apilife Var 8 g bee-hive strip (FASFC, 2015)), and one based on flumethrin (PolyVar Yellow 275 mg bee-hive strip) comparable to the fluvalinate molecule, the active ingredient in Apistan (10,3% w/w bee-hive strip), abandoned a few years ago due to Varroa resistance (Elzen et al., 2000; Rodríguez-Dehaibes et al., 2005) but still authorized in other EU countries so applicable by cascade[2] in Belgium. A small percentage of beekeepers (8.2%) did not use any Varroa control, relying on a Varroa-resistant honey bee selection or a non-interventionist approach.

Organic acids and thymol are the most widely used control method for Varroa. Nevertheless, the beeswax contamination studies related to this same beekeepers sample (n = 186 for multi-residue analysis and n= 124 for flumethrin analysis) (El Agrebi et al., 2020, 2019) revealed the presence of typical residues of beekeeper-applied veterinary medicinal products such as tau-fluvalinate and coumaphos, in 97.3% of the samples, and the presence of flumethrin in 21.8% of the samples. The presence of these veterinary medicinal products is in contradiction with the beekeepers' declaration. These contaminations could come from (e.g.) the recycling of beeswax from varied origins.

Table 6: Ranking of management predictor variables by overall discriminatory power, using classification regression tree.

Variable	Relative importance
Score given to the equipment origin and hygiene	100
Use of divider boards	80.2
Apis mellifera carnica	27.0
Adjust the space to the colony size	13.8
Treatment efficacy check	11.8
Winter check	11.5
Estimation of colony strength	9.9

Biotechnical methods including drone brood removal (Calderone, 2005), bottom boards screening (Delaplane et al., 2005), powder sugar dusting (Berry et al., 2012) in combination with other Varroa control was used by 36.4% of the beekeepers. The use of biotechnical methods to control Varroa infestation levels in combination with classical treatments was confirmed to be a protective indicator. This is in line with the study of Giacobino et al., 2015, that showed an increased treatment failure risk when the percentage of Varroa infestation prior to treatment was >3% (Giacobino et al., 2015). Sustainable Varroa control is a labor-intensive process requiring a combination of different measures, e.g. monitoring of mite fall, drone brood removal trapping (Calderone, 2005; Charriére et al., 2003), and application of miticides in rotation. Such “integrated pest management” needs to consider the population dynamics of Varroa as well as the honey bee colony so that measures can be applied at appropriate times of the year (Rosenkranz et al., 2010).

In our study, Varroa control model in at least two treatments (one in summer and one in winter), one with organic acids and one with alternative substances (mostly essential oils, other than thymol) offered the most protection against colony losses. Varroa infestation level was rarely estimated prior to treatment. Nevertheless, half of the beekeepers followed up the natural Varroa fall (counting Varroa natural mortality). Various studies gave contradictory conclusions regarding the accuracy of the natural fall method to determine total infestation rate since natural mite fall is largely determined by the amount of emerging infested brood, but it is in general considered as a good indicator of colony infestation (Branco et al., 2006). The majority of the beekeepers (82.9%) lacked proper knowledge of the Varroa infestation rates in their apiaries. These results are worrying as we know that treatment efficiency is highly associated with mite infestation before treatment (Giacobino et al., 2015).

2 The cascade system provides the veterinarian the opportunity to depart from the strict use of registered medicinal products in Belgium. Indeed, it is possible to use a medicinal product for animals of another species or animals of the same species but for another disease. On the other hand, the veterinarian may also prescribe a medicinal product for veterinary use, which is authorized in another Member state of the European Union, a medicinal product for human use and even a magisterial preparation.

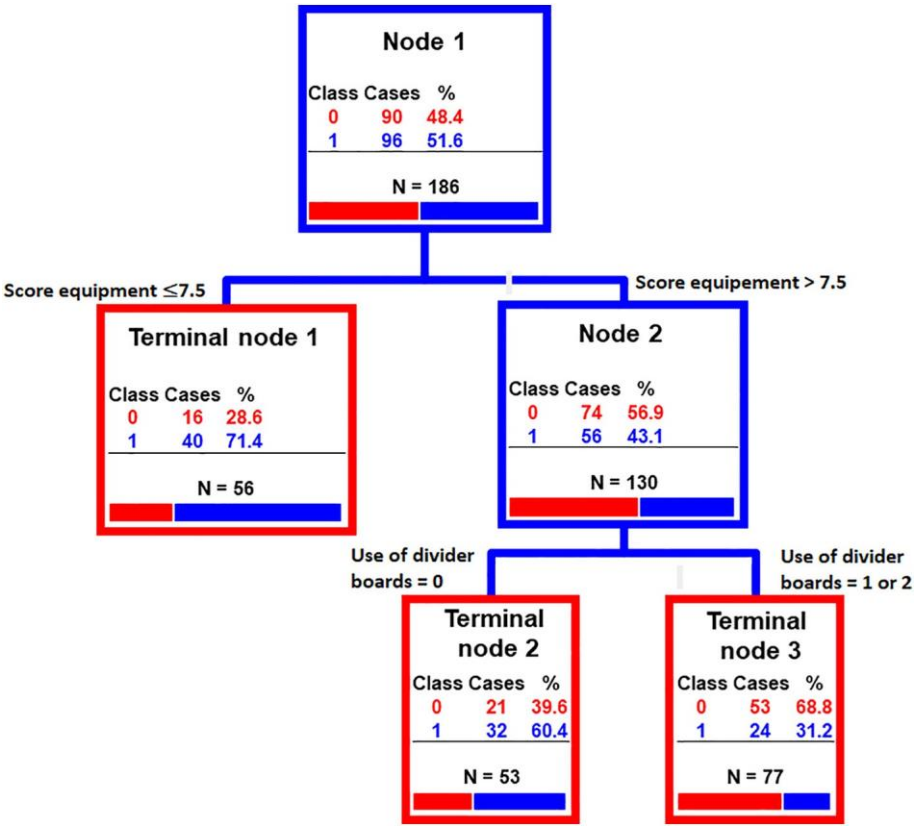

Fig. 5. Classification tree analysis for studying the relative importance and interrelation among the different risk indicators and the colony losses. Legend: Class: colony losses above (1) or below 10% (0). The blue-bordered boxes are the nodes that can be further divided into other nodes or terminal nodes. The red-bordered boxes are the terminal nodes that cannot be divided anymore. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Checking the efficiency of the treatment after its application (applied by 74,3% of the beekeepers) was confirmed to be a protective factor for colony survival. This result is in line with the results of Giacobino et al. (2014) who found that beekeepers who indicated that they did not monitor colonies after mite treatment, were associated with an increased risk of presenting high-intensity infestation and thus colony losses (Giacobino et al., 2014).

The hive type 'Dadant Blatt' used by 46.5% of the beekeepers decreased losses risk compared to all other hive types, the use of other hive types in combination with 'Dadant Blatt' even appeared to increase losses risk. In small apiaries, the use of different types of hives can lead to incompatibility of equipment to remedy problems faced by colonies. The hive type could affect honey bee colony losses by their size, shape, segmentation, building materials, management strategy, or suitability for honey bee parasites (Clermont et al., 2014). The frame of the 'Dadant Blatt' hive is bigger than any other type, this size allows the simultaneous presence of brood and food source in immediate proximity, which might ease colony survival through the winter. A significant relation between loss rate and the global equipment score was found. The overall global score of equipment was calculated as the sum of the scores given to the origin of the equipment (new, self-made, second hand), its re-use after colony losses (yes, after disinfection, or no), and the origin of the beeswax (recycled from own beeswax, recycled from commercial beeswax or commercially purchased beeswax). The higher the beekeeper scored with the equipment global score, the more the factor was protective. Monitoring and keeping the woodenware of hives in good conditions is recommended among best management practices (Heintz et al., 2011), practice good hygiene when dealing with dead colonies (combs, food stores, boxes, etc.) has been ranked and validated as most relevance BMP with a 3.8/4 (Rivera-Gomis et al., 2019). Using own beeswax (preferably capping) is also recommended (El Agrebi et al., 2020; ITSAP, 2017; Vergaert, 2017).

Interestingly, confining the colony to match its need in space and temperature while the colony fluctuates in volume with the use of divider board(s) appeared to be a protective indicator. To date, no other study has looked at this as a potential factor that could influence colony losses. Nevertheless, it has been ranked as a moderately relevant BMP (2.3/4) (Rivera-Gomis et al., 2019).

Beekeepers that estimated colony strength during the beekeeping season and before wintering were less likely to have losses. Moreover, beekeepers that declared the highest number of overwintered strong colonies in fall were those at greater risk of losses. Indeed, the mite population increase is related to colony growth and total incoming and outcoming foragers (DeGrandi-Hoffman et al., 2016). The biggest the colony is, the higher the infestation. Wintering colonies in good conditions and monitoring them through the winter also appeared to be a protective indicator of colony losses. This is rather an indicative of the duality of the BMP that is associated with the success of colony overwintering (Steinhauer et al., 2021).

5. Conclusion

The results of our study indicate that certain BMP are associated to lower colony loss rate. Beekeepers who are not open to improve their BMP are at risk of higher mortality rates. Evolution in management practices is needed as honey bees are exposed to frequent changes in land use, pesticide use, climate, emerging predators, diseases. Adapting BMP to these changes and

monitoring the needs of evolving colonies is of crucial importance for their survival. Improving BMP will not prevent all losses, but few behavioural changes including a proper comb management, equipment hygiene, and Varroa management, can lead to a non-negligible reduction of the risk of colony losses. We, therefore, recommend the development of a best beekeeping management practices guide, focused on honey bee health rather than on honey production. Having a colony monitoring system in place is also recommended even if it is difficult to conclusively establish the temporal cause and effect relationship. Based on the results of this survey, to improve BMP, an innovative BeeBestCheck tool was designed as inventory to improve BMP and advice beekeepers on their BMP (Appendix 2).

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Abbreviations

BMP	Beekeeping management practices
CTA	Classification tree analysis
FASFC	Federal Agency for the Safety of the Food Chain
ITSAP	Technical and Scientific Institute of Beekeeping and Pollination
Sciensano	Belgian Institute of Health
SPM	Salford Predictive Modeler
ULiège	University of Liège

Credit authorship contribution statement

Noëmie El Agrebi: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization, Project administration. **Nathalie Steinhauer**: Writing – review & editing. **Simone Tosi**: Writing review & editing. **Laurent Leinartz**: Software, Writing – review & editing. **Dirk C. de Graaf**: Conceptualization, Writing – review & editing. **Claude Saegerman**: Conceptualization, Methodology, Validation, Formal analysis, Resources, Data curation, Writing – original draft, Writing review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

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

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Experimental section

Study 2

Pesticide and veterinary drug residues in Belgian beeswax: Occurrence, toxicity, and risk to honey bees

Science of the Total Environment 745 (2020) 141036

Noëmie El Agrebi, Kirsten Traynor, Olivier Wilmart ,Simone Tosi ,Laurent Leinartz , Ellen Danneels, Dirk C. de Graaf and Claude Saegerman

Preamble

The contribution of pesticide residues to the global decline of honey bees and other pollinators has received much attention from the scientific community. At the national level, no assessment of pesticide residues in interaction with honey bees was available. To overcome this lack and improve our understanding of pesticide residue occurrence and concentrations in the hive, beeswax was sampled from 182 beekeepers throught Belgium and screened for the presence of 294 different residues. Beeswax exposure risk to honey bees was assessed using a cumulative Hazard Quotient (HQ) risk formula, in addition, we split the beeswax in four types (capping wax, recycled wax, comb wax and wax from the honey super) and compared their toxicity risk and discussed the potential implications for beekeeping management practices. As a result of this study, an online tool (BeeToxWax) to estimate beeswax's potential toxicity to bees was designed and made available for the beekeepers and wax manufacturers. This tool is now largely used in Belgium and outside.

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Pesticide and veterinary drug residues in Belgian beeswax: Occurrence, toxicity, and risk to honey bees

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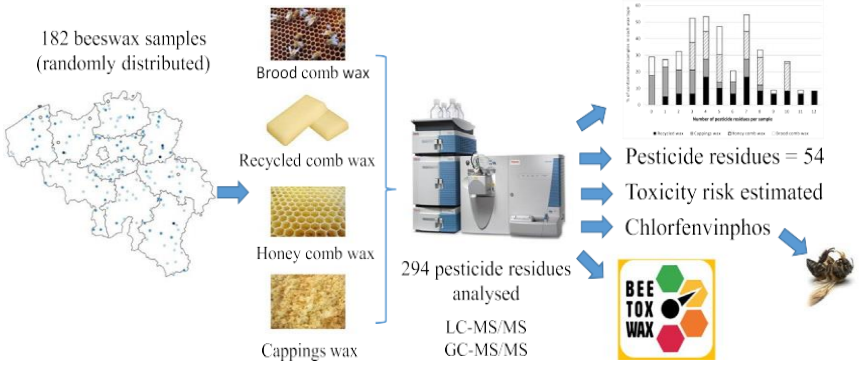
HIGHLIGHTS

- Pesticide levels in brood comb, recycled comb, honey comb, and cappings wax were compared.
- 54 different pesticide and veterinary drug residues were found in the four types of beeswax.
- In-hive applied or high lipophilic residues are more likely to be found in beeswax.
- A statistically significant influence of chlorfenvinphos on bee mortality was found.
- Cappings wax was substantially less contaminated

GRAPHICAL ABSTRACT



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abstract

Pesticide and veterinary drug residues are one of the stress factors affecting bee health and mortality. To investigate the occurrence, the concentration and the toxicity risk to bees of pesticide residues in four different types of beeswax (brood comb wax, recycled comb wax, honey comb wax, and cappings wax), 182 samples were collected from apiaries located all over the Belgian territories, during spring 2016 and analysed by LCMS/MS and GC–MS/MS for the presence of 294 chemical residues. The toxicity risk to bees expressed as the Hazard Quotient (HQ) was calculated for each wax sample, according to two scenarios with different tau-fluvalinate LD_{50} values. Residues showing the highest prevalence were correlated to bee mortality in a multivariate logisticregression model and a risk-based model was used to predict colony bee mortality. Altogether, 54 different pesticide and veterinary drug residues were found in the four types of beeswax. The residues with a higher likelihood to be retained in beeswax are applied in-hive or with a high lipophilic nature. The multivariate logistic regression model showed a statistically significant influence of chlorfenvinphos on bee mortality. All our results indicatedthat cappings wax was substantially less contaminated. This national survey on beeswax contamination provides guidelines on the re-use of beeswax by beekeepers and shows the necessity to introduce maximum residue levels for global trade in beeswax. An online tool was developed to enable beekeepers and wax traders to estimate therisk to honey bee health associated with contaminated wax.

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1. Introduction

The presence of residues in apicultural matrices reflects anthropogenic activities whether they come from agricultural use or veterinary treatments (Balayiannis and Balayiannis, 2008; Berthoud et al., 2010). Honey bees and other pollinators are at risk from multiple stress factors (Berthoud et al., 2010; Dainat et al., 2012; Goulson et al., 2015; Le Conte et al., 2010; VanEngelsdorp et al., 2008) and pesticide residues play an undeniable role. The contribution of pesticide residues to the global decline of honey bees and other pollinators has lately received much attention from the scientific community (Mitchell et al., 2017; Tsvetkov et al., 2017; Woodcock et al., 2017). Since the arrival of the parasitic mite Varroa destructor in Belgium in 1984, the most common means of controlling Varroa has been through the use of synthetic acaricides (Bogdanov et al., 1998; Mullin et al., 2010). Treatments are placed in-hive, thus exposing not only the mites to the compound but honey bee eggs, larvae, adults, and beehive products. Residues acute toxicity to honey bees is characterised by the determination of the acute median lethal dose (LD50) values, which is the residue dose that is required to kill half of the tested animals. LD50 values may differ based on the route of exposure and species exposed (Haschek et al., 2013).

Regarding adult honey bees, residues associated with acute contact LD50 values inferior to 2 µg bee-1 are considered as highly toxic, moderately toxic with acute contact LD50 values between 2 and 10.99 µg bee-1, slightly toxic with acute contact LD50 values between 11 and 100 µg bee-1, and essentially non-toxic with acute contact LD50 values higher than 100 µg bee-1 (Washington State Department of Agriculture, 2010). The persistence of a residue depends on its physical and chemical properties (partition coefficients, degradation rates, deposition rates) and the characteristics of the contaminated matrix. Honey bees are typically exposed to a cocktail of residues; in-hive (beekeeper applied) acaricides and other veterinary drugs applied over long time periods and out-of-hive (farmer applied) pesticides encountered in pollen, nec tar, and water during their foraging activity (Bogdanov, 2006; Chauzat et al., 2011; Rortais et al., 2005). These pesticide residue mixtures may act alone or in interaction (Carnesecchi et al., 2019), in ways currently difficult to predict, potentially creating a toxic environment for honey bee growth and development (Tomé et al., 2020; Yao et al., 2018; Zhu et al., 2014).

Beeswax is a natural honey bee product. It is secreted in liquid form by specialized wax glands in the abdomen of younger worker bees (aged between 12 and 18 days) (Bogdanov, 2016) and solidifies into translucent white scales when in contact with air. Wax combs are constructed from these wax scales, molded into shape by honey bee mandibles. In Europe, as wax production is not the aim in beekeeping, beekeepers provide bee colonies with manufactured wax sheets of foundation, which the bees draw out into the full depth comb. The raw materials for wax manufacture are recycled from old brood combs, honey combs and cappings wax. Cappings wax contains almost exclusively pure wax. Beeswax is a complex mixture consisting mainly of esters of higher fatty acids (Aichholz and Lorbeer, 1999; Tulloch, 1980). Due to its high composition in fatty acids, and as most acaricides are fat-soluble and nonvolatile (Wallner, 1999), beeswax is a relevant matrix to assess in-hive chemical exposure history for lipophilic compounds (Lozano et al., 2019; Ravoet et al., 2015). Of all beehive products, it has the lowest replacement rate, can remain in the hive for many years and is recycled by the beekeepers into new wax foundations for comb building, thus leading to a greater accumulation of different pesticide residues used in beekeeping and agriculture (Chauzat and Faucon, 2007; Mullin et al., 2010). Beeswax can be considered as a contaminant reservoir (Yáñez et al., 2013) ora final sink (Bommuraj et al., 2019). Even though most residues remain in the wax, residues migration from the wax to beebread, and larvae is a crucial factor that could affect the evolution of the colony (Murcia Morales et al., 2020). A residue accumulation can affect worker honey bee and queen development (Haarmann et al., 2002), bee longevity (Wu et al., 2011), and colony performance (Desneux et al., 2007).

Assessment/registration authorities like e.g. World Health Organisation (WHO), United States Environmental Agency (EPA), European Food Safety Authority (EFSA), and European Medicines Agency (EMA) ensure that each registered pesticide/veterinary drug continues to meet the highest standards of safety to protect human health and the environment. Within this context, older pesticides are being reviewed to ensure that they meet current scientific and regulatory standards. As an example, EPA screening level assessors re-evaluated in 2005 tau-fluvalinate, one of the acaricides frequently used for Varroa control and reset its median acute contact lethal dose (LD50) at 0.2 µg bee 1 (EPA, 2005). This classifies tau-fluvalinate as highly toxic to honey bees. Tau-fluvalinate is expected to pose an acute health risk to non-target insects. Nevertheless, in Europe, the acute LD50 of tau-fluvalinate is still set at 12 µg bee-1 (worst case from 24, 48 and 72-hour values) reported by the University of Hertfordshire Pesticide Properties DataBase (PPDB) (Lewis et al., 2016).

The European legislation on animal by-products (ABPs) defines beeswax as an “apiculture product” used in beekeeping (Regulation (EC) No 1774/2002) and categorises beeswax as an ABP Category 3 material, i.e. not intended for human consumption (Regulation EC No 1069/2009). This categorisation does not prevent the presence of contaminants and/or adulterants. Moreover, it allows the commercialisation of beeswax used in apiculture without previous quality (authenticity) control. In Belgium, the guidelines contained in the advice 18–2018 (Scientific Committee of the FASFC, 2018) set the limits for pesticide and veterinary drug residues at 9 different products and proposed limiting the sale of re-melted beeswax that exceeds these limits.

This first national pilot survey aimed to improve our understanding of the pesticide residues currently present, their rate of occurrence, and their concentration in four types of beeswax. The survey also aimed to assess the exposure risk to honey bees, comparing the toxicity of pesticide residues in the four beeswax types and the potential implications for beekeeping management practices.

The results obtained led us to develop an online tool (BeeToxWax) to empower beekeepers and wax traders to estimate the risk to honey bees associated with contaminated wax based on the residue concentrations reported in a laboratory analysis report and the pesticide residues acute LD50. The tool gives automated real-time recommendations on whether the tested sample can be reused in a colony or should be discarded based on the current scientific literature: contact Hazard Quotient (HQ) value over 250 are considered to have significant toxicity and elevated toxicity is associated with HQ values over 5000 (Traynor et al., 2016). The tool is a web-based calculator of risk associated with contaminated wax; its use could be an important strategy to sanitize beeswax available in the commercial trade stream (https://www.beetools. uliege.be).

2. Materials and methods

3.3 *Beeswax and residues*

3.3.1 Origin and characterisation of the wax samples

A total of 200 beekeepers were randomly selected from the Federal Agency for the Safety of the Food Chain (FASFC) beekeepers database including 4949 registered beekeepers in 2015. Beeswax wax collected from a single hive out of one apiary per beekeeper during spring 2016. The number of beekeepers was stratified by province. Out of the selected beekeepers (N = 200), 91.5% of the beekeepers provided a wax sample of sufficient amount (100 g) for analysis (182 samples). Wax samples were differentiated into four types: brood comb[3] wax (N = 89), recycled comb[4] wax (N = 59), honey comb[5] wax (N = 6), and cappings[6] wax (N = 28). The different types of waxes are easily identifiable by colour, shape, and consistency. Brood combs are dark, honey combs are light with no pupal cocoons, cappings wax is cut off comb when extracting honey and melted wax is received as a block or pressed into sheets of foundation. Beekeepers donated less honey comb wax as they reuse these light coloured frames for honey production. The samples were free of beebread, honey or brood, they were kept in hermetic plastic bags and stored at −20 °C until analysis.

3.3.2 Multi-residue analysis

Analysis of beeswax was carried out at an independent laboratory in Germany (Intertek Food Services GmbH) according to the European EN 15662 method (CEN 2008), between October 2016 and January 2017, using a common analytical protocol (QuEChERS) designed for the analysis of food materials and suitably adapted. All residues were analysed using multi-residue GC–MS/MS and LC-MS/MS methods covering 294 different substances with detection limits (LOD) of 0.003 mg/kg and limits of quantification (LOQ) of 0.01 mg/kg in most cases and with recoveries between 70% and 120%. The quality control is done using quality control samples and spiking experiments.

Generally, 10 ml of deionized water (BarnsteadTM, Nanopure DiamondTM, Thermo Scientific) was added to approximately 5 g of beeswax accurately weighed into a 50 ml-Teflon centrifuge tube. 10 ml of acetonitrile (HPLC Gradient Grade, VWR) was added together with an internal standard solution containing isoproturon-d6 for LCMS/MS analysis, anthracene-d10 for GC–MS/MS analysis and octachlorostyrene for negative chemical ionization GC–MSD analysis.

The whole preparation was mixed using a horizontal shaker for 20–30 min. Then 6.5 g QuEChERS salt mixture was added, consisting of 4 g of anhydrous magnesium sulfate, 1 g of sodium chloride, 1 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogen citrate sesquihydrate, and the whole was mixed by hand for approximately 1 min, then centrifuged for 13 min at 10,000 Relative Centrifugal Force (RCF; refrigerated centrifuge Rotina 380 R). 7 ml of the supernatant was transferred to a tube containing 1 g of anhydrous calcium chloride and 300 mg of PSA as a sorbent. After briefly shaking by hand, this mixture was centrifuged again for 13 min at 10,000 RCF. 1 mL of the supernatant was then removed for LC-MS/MS analysis. Further two aliquots of 1 ml each were filled into vials and 8 µl of 5% formic acid solution in acetonitrile were added as analyte protectant for GC–MS/MS and GC–MSD analyses, respectively. The addition of 5% formic acid solution in acetonitrile is done to stabilize the analytes in the solution. This is not meant as a classical Analyte Protectants (AP) for GC–MS analysis. An AP-Mix (mixture of 3-Ethoxypropandiol, Shikimic acid, Glucuronolactone and Sorbitol) for GC–MS analysis was used to block free active spaces on the liner to prevent interactions between the liner and the analytes.

LC-MS/MS was performed on a Thermo Scientific system consisting of an Accela 1250 pump and a TSQ Quantum Access mass spectrometerwith a Hypersil Gold C8 (150 × 2.1 mm, 5 µm) column. The GC-MS/MSsystem was a GC 7890 equipped with a HP-

3 Wax comb in which the brood was reared

4 Melted old brood and/or honey wax comb to be reused.

5 Wax comb in which honey was stored.

6 Virgin wax covering on sealed honey combs rendered by beekeepers.

5 ms column (30 m × 0.25 mm × 0.25 µm, Varian) combined to a 7000 Triple Quadrupole mass spectrometer (Agilent Technologies). The GC-MSD system consisted of a GC 6890 N with a VF-5 ms column (30 m × 0.25 mm × 0.25 µm, Varian) combined to a 5975 XL inert MS (Agilent Technologies).

3.3.3 Regression modelling of residue per wax type

In a first step, a descriptive analysis was performed to examine data for completeness and validity and to identify the wax type with the least residues. After this validation, a univariate logistic regression model was performed for each residue (N = 54) to examine associations between a range of independent variables (i.e. the four wax types, with cappings wax considered as the purest reference wax) and the outcome of interest (each residue). The level of statistical significance was set to P = 0.05.

3.3.4 Hazard Quotient and toxicity to bees

To estimate contaminated wax contact toxicity to bees, a mean Hazard Quotient (HQ) was calculated for each of the four wax types. Until now, toxicity for larvae has not been well studied. As chronic median lethal dose data for bees are extremely rare (EFSA, 2012), the acute contact median lethal dose (LD50 48 h for adult bees) was used in the HQ calculation. Per sample then gathered by wax type, HQ was calculated as the sum of the concentration of the residue (mg kg-1) divided by its respective acute contact LD50 (µg bee-1). The HQ provides an estimate based on percentages of LD50 equivalents present in the wax. For oral contact in pollen instead of beeswax, HQ is considered notable when it is N50 and is considered as elevated when it is N500 (Stoner et al., 2013). In beeswax, pesticide residues are embedded in the matrix and not all residues are in contact with honey bees. Only a fraction of the pesticide load is exposed to the individuals of the colony, so HQ in beeswax samples was considered as notable when N250 (Calatayud-Vernich et al., 2019). Samples with contact HQ beeswax N5000 were considered to have an elevated pesticide load (Traynor et al., 2016).

Acute contact LD50 values were retrieved from the Pesticide Properties DataBase (PPDB) and the Veterinary Substances DataBase (VSDB) reported by the University of Hertfordshire (Lewis et al., 2016) or from some additional primary literature (Sanchez-Bayo and Goka, 2014; Stoner et al., 2013) (Table 1). For substances with multiple LD50, the lowest value was considered according to a conservative scenario. For unknown contact LD50, when possible, the LD50 of the respective parent compound was used in the HQ calculation acknowledging that some metabolites may have either lower or higher toxicity than the par ent compound (Suchail et al., 2001). When the substance was not assimilated to a pesticide (e.g. solvent), a low toxicity value of 200 µg bee -1 was assigned. In the case of tau-fluvalinate, both values proposed by the EPA (0.2 µg bee 1) and PPDB (12 µg bee 1) were considered in two toxicity scenarios as an important 60-fold disparity appeared with its toxicity.

Cumulative risk by contact exposure estimate.

To assess the risk to larvae in contact with contaminated wax topical contact during their development, it is necessary to consider the frequency of detection of each pesticide residue in this matrix, because prevalence indicates the probability of exposure to the contaminants. We used the method suggested by Sanchez-Bayo and Goka (Sanchez-Bayo and Goka, 2014) that takes into consideration the cell weight (0.0232 g) (El Agrebi et al., 2019) and the development time (21 days) of bee larvae (Eq. (1))

$$\text{Risk (\%)} = \left(\frac{\text{Frequency \% x Residue concentration [µg/g]}}{\text{LD}_{50}\text{ acute contact [µg bee}^{-1}\text{]}}\right) \text{x } 0{,}023 \text{ [g] x } 21 \text{ [days]} \qquad \text{(Eq. 1)}$$

$$\text{Cumulative risk}_{P1-P54}\text{ (\%)} = \sum_{P=1}^{54}\left(\frac{\text{Frequency \% x Residue concentration [µg/g]}}{\text{LD}_{50}\text{ acute contact [µg bee}^{-1}\text{]}}\right) \text{x } 0{,}023 \text{ [g] x } 21 \text{ [days]} \qquad \text{(Eq. 2)}$$

Eq. (1) indicates the percentage of risk (i.e. likelihood of causing 50% mortality) caused by a given pesticide residue on honey bee larvae that come into contact with contaminated wax during their development. For each wax type, a cumulative risk by contact exposure was calculated as the summation of the risk caused by each pesticide in the sample (Eq. (2)). The cumulative risk expresses the risk that larvae would be exposed to during their development to a higher pesticide dose than the contact lethal dose (LD50).

2.2 Pesticide and veterinary drug residues and honey bee mortality

2.2.1 Data on bee mortality

Sampling was conducted jointly with a questionnaire to record colony losses and management practices. The total loss rate was calculated by dividing the total number of colonies lost between September 2015 and April 2016 (winter and seasonal) by the number of colonies in September 2015 multiplied by 100 (Clermont et al., 2014) excluding removed, sold and purchased colonies. Bee mortality rate in function of the presence or the absence of a specific pesticide residue was tested with a two-sample Wilcoxon rank-sum (Mann-Whitney) test for significance. The limit of statistical significance of the test was defined as 0.05.

2.2.2 Logistic regression model

A univariate logistic regression model was used to explain colony mortality expressed as a binary dependent variable, taking into account the acceptable level of mortality (0 for colony mortality rates ≤10%; 1 for colony mortality rates N10% (Morgenthaler, 1968)) associated with residues. Then, a multivariate logistic regression was performed using the most significant variables (P b 0.1) out of the univariate model. Finally, in a backward stepwise multivariate model, the least significant variable (with the highest P value) were eliminated in a step-by-step approach. At each stage, a likelihood ratio test was used to compare the complex and simplified models. When there was no significant difference between them (using value of P N 0.10), the simplified model was used. The interaction between variables in the multivariate final retained model was tested. All models and tests were performed using Stata SE 14.1® (StataCorp LP, College Station, TX, USA) and the limit of statistical significance of performed tests was defined as 0.05.

2.2.3 Development of a risk-based model

To predict the colony mortality expressed as a binary variable (0 for colony mortality rates ≤10%; 1 for colony mortality rates N10%) in function of the different combinations of pesticide residues present in each beeswax sample, a Receiver Operating Characteristic (ROC) curve was established. For this, only the 10 residues with a P-value b0.20 in the previous univariate logistic regression analysis were retained. Next, ten different receivers operating characteristic (ROC) curves were established (i.e. with the first, the two first, the three first, until the ten-first pesticide residues retained). The ROC is a probability curve that plotted with true-positive results (Y-Axis) against the false-positive results (X-Axis). Each point of the curve is determined by a specific threshold = cut-off (i.e. a certain combination of pesticide residues). The area under the ROC curve (AUC) is the performance measurement for the classification test at various threshold settings. over-dispersion of the variable outcome. Possible residue synergies were looked for in residue combinations.

3 Results

3.1 Beeswax, pesticides and veterinary drug residues

Descriptive data of the residues found in Belgian beeswax are presented in Table 1. The analysed samples revealed a contamination prevalence of 97.3% and the presence of 54 different compounds for all wax types jointly. Per sample, the number of different residues ranged from 1 to 12 with a median value of 5. Ten different residues were commonly found in the four wax types. Acaricides (i.e. tau-fluvalinate and coumaphos) have the highest prevalence in all wax types (respectively 89.6% and 78.6%), followed by propargite, chlorfenvinphos, bromopropylate (including metabolite 4,4′-Dibromo-benzophenone). Also, the insecticide permethrin, the repellent DEET (diethyltoluamide), the fungicide pentachloroanisole, and its metabolite pentachlorophenol, as well as the performance enhancer substance piperonyl butoxide were frequently found in the wax samples. The frequency of occurrence of each residue per wax type is shown in Table 1. The percentage of contaminated samples as a function of the number of residues per wax type is shown in Fig. 1. This percentage is significantly higher in cappings wax for lower residue numbers than in the other wax types (Negative binomial regression; P b 0.001).

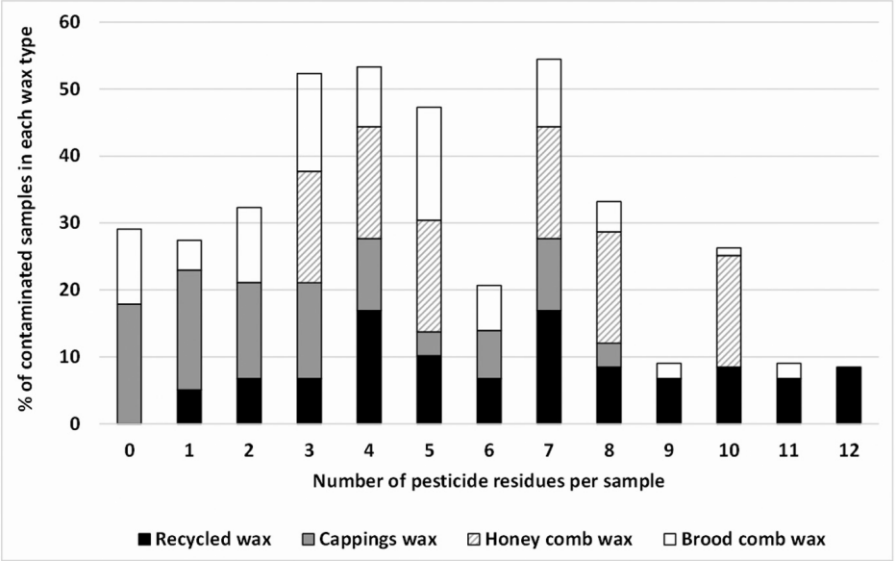

Fig. 1. Percentage of contaminated samples with 0 to 12 pesticides in the four wax types, brood comb wax (N = 89), recycled comb wax (N = 59), honey comb wax (N = 6) and cappings wax (N = 28).

3.2 Frequency of pesticide and veterinary drug residues per wax type

3.2.1 Brood comb wax

A total of 41 different residues were found in brood comb wax (N = 89). The median number of different residues per sample was 5 (min-max, 1–12). Residues with the highest prevalence were tau-fluvalinate (92.1%), coumaphos (80.9%), propargite (57.3%), amitraz (28.1%), and DEET (25.8%). The highest maximum concentrations were observed for amitraz (including the metabolites containing the 2,4 dimethylaniline expressed as amitraz) with 16.7 mg kg^{-1} followed by cypermethrin (9.3 mg kg^{-1}), and by tau-fluvalinate (6.46 mg kg^{-1}). Seven highly toxic residues to bees (with DL50b2 µg bee^{-1}) were found: chlorpyrifos (-ethyl) and permethrin, both in 13.5% of the samples, cypermethrin in 4.5% of the samples, acrinathrin, lindane, p,p′DDT, and pyridaben in 1.1% of the samples. The neonicotinoid thiacloprid was detected in 2.2% of the sample with a maximum concentration of 0.046 mg kg^{-1} (Table 1).

Increased odds of tau-fluvalinate were observed in brood comb wax compared with reference cappings wax (OR = 5.36 with 95% CI: 1.82–15.73; P = 0.002) (Table 2).

3.2.2 Recycled comb wax

In recycled comb wax (N = 59), 42 different residues were quantified. The median number of different residues per sample was 7 (min-max: 1–12). Residues with the highest prevalence were taufluvalinate (94.4%), coumaphos (89.8%), propargite (57.6%), DEET (52.5%), Piperonyl butoxide (40.7%), bromopropylate (39%), chlorfenvinphos (32.2%), permethrin (27.1%), chlorpropham (25.4%) and pentachloroanisole (23.7%). Tau-fluvalinate had the highest concentration with 8.68 mg kg 1 followed by coumaphos with 7.41 mg kg^{-1} and chlorpyrifos (-ethyl) with 4.38 mg kg^{-1} (Table 1). Highly toxic. residues to bees (with DL50b2 µg bee 1) were found: permethrin in 27.1% of the samples, chlorpyrifos (-ethyl) in 11.9%, p,p′-DDT and lindane in 8.5%, dianizon in 3.4%, cypermethrin, acrinathrin, deltamethrin, DDT and tetramethrin in 1.7% of the samples. The neonicotinoid thiacloprid was detected in one sample with a maximum concentration of 0.014 mg kg^{-1}.

Increased odds of coumaphos were observed in recycled comb wax compared with reference cappings wax (OR = 10.19 with 95% CI: 3.31–31.37; p = 0.000) (Table 2).

Table 2. Univariate logistic regression model outputs for residues detection rate in brood comb wax, in recycled comb wax and in honey comb wax with cappings wax as reference.

Residue	Wax type	Odds ratio	95% confidence interval	P-value
Brompropylat*	Recycled	3.51	(1.13–10.86)	0.03
Chlorpyrifos (-ethyl)	Honey comb	31.67	(1.29–772.98)	0.034
Coumaphos	Brood comb	4.89	(1.96–12.15)	0.001
	Recycled	10.19	(3.31–31.37)	0.000
Fenpyroximate	Honey comb	57.00	(2.40–1349.32)	0.012
Pentachloranisole	Recycled	8.40	(1.04–67.51)	0.045
Piperonylbutoxide	Recycled	2.95	(1.02–8.52)	0.046
	Honey comb	7.69	(1.26–46.68)	0.027
Propargite	Brood comb	4.03	(1.55–10.44)	0.004
	Recycled	4.08	(1.50–11.08)	0.006
	Honey comb	15.00	(1.48–151.28)	0.022
tau-Fluvalinate	Brood comb	5.36	(1.82–15.73)	0.002
	Recycled	7.86	(2.08–29.71)	0.002

Legend: *An example of interpretation is presented: significant more detection of Brompropylat was found in recycled comb in comparison with the cappings wax as reference. Other beeswax types are not different from the reference.

3.2.3 Honey comb wax

The results interpretations for this wax type are only indicative as they are derived from a comparatively smaller sample size. Honey comb wax (N = 6) contained 13 different pesticide residues, the median number of different residues per sample was 6 (min-max: 3–10). Tau-fluvalinate was detected in 100% of the samples, coumaphos, and propargite in 83.3% of the samples, piperonyl butoxide in 66.7%, and fenpyroximate in 50% of the samples. Six molecules were found in 33.3% of the analysed samples, i.e. bromopropylate (and its metabolite 4,4′-Dibromo-benzophenone), chlorfenvinphos, chlorpyrifos (-ethyl), pentachloranisole, and permethrin (Table 1). In honey comb, two insecticides considered as highly toxic to bees (b2 µg bee^{-1}) were detected: permethrin and chlorpyrifos (-ethyl).

Table 1. Residue levels of pesticides found in the four types of beeswax in Belgian apiaries. The type of each active substance, the contact acute median lethal dose and number of positive samples found are reported.

Active ingredient	Active ingredient type				Contact acute 48 h LD50 (µg bee-1)			Brood comb wax (n = 89)					Recycled comb wax (n = 59)
	Insecticide	Fungicide	Acaricide	Other	PPDB/VSDB	Stoner et al., 2013	Sanchez-Bayo and Goka, 2014	# positive samples	Frequency (%)	Mean (mg/kg)	Min (mg/kg)	Max (mg/kg)	Frequency (%)
Acrinathrin	X		X		0.084		0.17	1	1.1%	0.014	0.014	0.014	1.7
Amitraz (incl. Metabolites)	X		X	Antiparasite	50			25	28.1	0.740	0.010	16.7	16.9
Azoxystrobin		X			200	200		3	3.4	0.047	0.011	0.117	1.7
Biphenyl	X	X	X		/								1.7
Boscalid		X			200	200		5	5.6	0.121	0.038	0.310	11.9
Bromopropylate			X		/			22	24.7	0.024	0.010	0.058	39.0
Captan		X		Bactericide	200			3	3.4	0.646	0.014	1.837	8.5
Carbendazim		X		Metabolite	50	50		6	6.7	0.040	0.014	0.098	
Chlorfenvinphos	X		X	Sheep dip	/		4.1	20	22.5	0.036	0.012	0.084	32.2
Chloropropylate	X		X		/			2	2.2	0.024	0.011	0.036	5.1
Chlorothalonil		X			101		135.32	1	1.1	0.066	0.066	0.066	
Chlorpropham				Herbicide	86			3	3.4	0.034	0.025	0.053	25.4
Chlorpyrifos (-ethyl)	X				0.059	0.01	0.07	12	13.5	0.025	0.011	0.041	11.9
Coumaphos	X		X	Antiparasite	/	24	20.29	72	80.9	0.150	0.010	2.257	89.8
Cypermethrin	X			Sheep dip	0.02		0.03	4	4.5	2.34	0.023	9.300	
Cyprodinil		X			784	784		2	2.2	0.063	0.062	0.063	10.2
p,p′-DDE (Dichlorodiphenyldichloroethylene)				Metabolite	/								1.7
DDT (Sum, expressed as DDT)	X				0.54								1.7
o,p′-DDT				Isomer	0.54								3.4
p,p′-DDT (Chlorophenothane)	X				0.54			1	1.1	0.010	0.010	0.010	8.5
DEET (diethyltoluamid)	X			Repellent	/			23	25.8	0.102	0.010	0.707	52.5
Deltamethrin	X			Metabolite	0.0015		0.02						1.7
Diazinon	X	X		Repellent	0.13	0.22	0.38						3.4

Dibromobenzophenone				Metabolite	Not listed			3	3.4	0.013	0.010	0.015	1.7
Dichlofluanid		X			16			3	3.4	0.174	0.012	0.494	11.9
Dichlorobenzophenone				Metabolite	Not listed								
Dimethomorph		X			102	10		2	2.2	0.285	0.046	0.523	
Dimoxystrobin		X			100			1	1.1	0.022	0.022	0.022	
Etridiazole		X			/								1.7
Fenpyroximate			X		15.8			8	9.0	0.029	0.010	0.064	6.8
tau-Fluvalinate	X		X		12	0.2	8.66	82	92.1	0.530	0.010	6.460	94.9
Hexythiazox			X		200			9	10.1	0.015	0.010	0.030	3.4
Iprodione		X			200			10	11.2	0.058	0.010	0.130	22.0
Lindane (γ-HCH)	X		X		0.23			1	1.1	0.023	0.023	0.023	8.5
Metalaxyl		X			200	100		1	1.1	0.015	0.015	0.015	
Methoxychlor	X				23.6								3.4
Parathion	X		X		/			1	1.1	0.016	0.016	0.016	
Pendimethalin				Herbicide	100	49.8		5	5.6	0.017	0.012	0.030	
Pentachloroanisole				Metabolite Pentachlorophenol	48			6	6.7	0.026	0.010	0.065	23.7
Permethrin (Sum all Isomere)	X			Antiparasite	0.29		0.06	12	13.5	0.077	0.011	0.311	27.1
2-phenylphenol		X		Other substance	/			17	19.1	0.022	0.010	0.074	8.5
Piperonyl butoxide				Performance enhancer	294			20	22.5	0.055	0.010	0.376	40.7
Pirimicarb	X				53.1	12.56		2	2.2	0.014	0.011	0.016	
Propamocarb		X			100			3	3.4	0.018	0.010	0.027	1.7
Propargite			X		47.9			51	57.3	0.124	0.011	0.375	57.6
Propiconazole		X			100	25		1	1.1	0.378	0.378	0.378	3.4
Pyridaben	X		X		0.024		0.05	1	1.1	0.010	0.010	0.010	
Pyrimethanil		X			100	100		3	3.4	0.048	0.012	0.080	11.9
Tebuconazole		X		Plant growth regulator	200								1.7
Tetradifon	X		X		11								5.1
Tetramethrin	X				/								1.7
Thiacloprid	X			Molluscicide	38.82	37.83		2	2.2	0.030	0.014	0.046	1.7
Trifloxystrobin		X			100	200		1	1.1	0.025	0.025	0.025	
Vinclozolin		X			/								1.7

Legend: Amitraz, including the metabolites DMPF, DMF and the 2,4 -dimethylaniline moiety; PPDB/VSDB, data was retrieved from Pesticide Properties DataBase and Veterinary Substances DataBase (Lewis et al., 2016).

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The highest maximum concentrations were observed for taufluvalinate with 0.91 mg kg¯1 followed by DEET with 0.78 mg¯kg 1 and coumaphos with 0.45 mg kg¯1. Two highly toxic residues to bees (with DL50 b 2 μg bee 1) were detected: permethrin and chlorpyrifos (ethyl). No trace of thiacloprid (neonicotinoids) was detected in honey comb wax.

Increased odds of fenpyroximate were observed in honey comb wax compared with reference cappings wax (OR = 57 with 95% CI: 2.40–1349.32 [wide range due to small sample size]; P = 0.012) (Table 2).

3.2.4 Cappings wax

In cappings wax (N = 28), 18 different residues were detected. The median number of different residues per sample was 3 (min-max: 0–8) (Table 1). Tau-fluvalinate (65.5%), coumaphos (44.48%), DEET (37.93%), propargite (24.1%) and piperonyl butoxide (17.2%) were the most frequently detected residues in cappings wax. The highest maximum concentrations were observed for coumaphos with 0.93 mg kg¯1 followed by tau-fluvalinate with 0.53 mg kg¯1 and propargite with 0.45 mg kg¯1. Permethrin (13.8%) was the only substance found with high toxicity to bees. No trace of thiacloprid (neonicotinoids) was detected in cappings wax.

3.3 Wax Hazard Quotient and toxicity to bees

Overall, in the first scenario (tau-fluvalinate DL50 = 12 μg bee 1), out of N = 182, 123 samples of samples (67.5%) had a low HQ value (b250), 55 samples (30.2%) had significant toxicity (250 b HQ b 5000) and 4 samples (2.2%) of the total number of samples had elevated toxicity to bees (HQ N 5000) (Fig. 2). At the territorial level, the samples with the highest HQ (N = 4) were reported in the province of Luxembourg (max = 466,246), in Limburg (max = 5242 and 74,208) and East Flanders (max = 17,536) (Table 3). Detailed results per wax type are shown in Table 4. With the second toxicity scenario (taufluvalinate DL50 = 0.2 μg bee¯1), the HQ levels approach alarming levels and the number of samples exceeding the threshold values increases (Table 4).

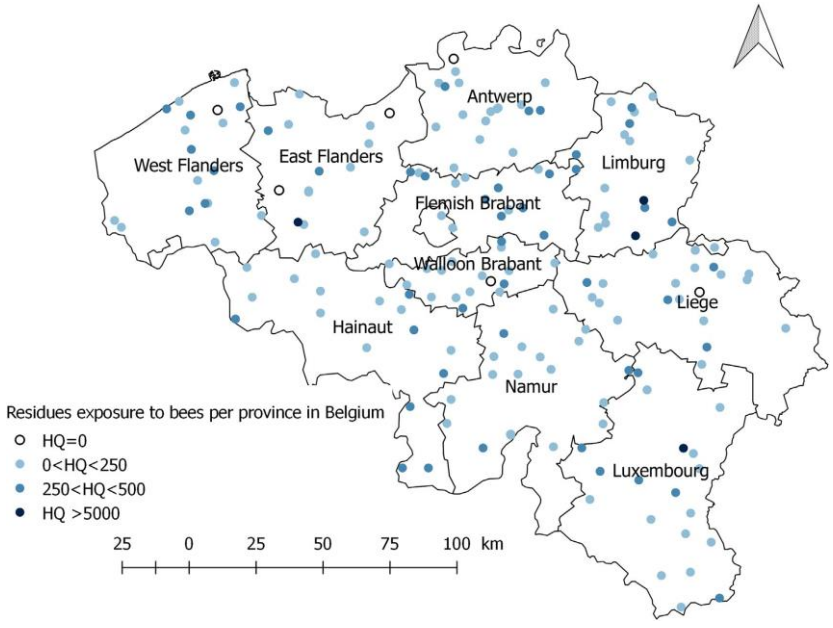

Fig. 2. Residues exposure risk to bees per province in Belgium (*N* = 182).

Table 3. Hazard quotient values per province in Belgium.

Provinces	Mean HQ	S.D.	Median HQ	Min HQ	Max HQ
Antwerp	79	150	14	0,0	533
Eastern Flanders	1494	4515	10	0,0	17,536
Flemish Brabant	369	273	2	0,6	837
Hainaut	482	769	189	3,0	2408
Liège	251	545	28	1,4	2295
Limburg	4996	17,896	131	4,1	74,208
Luxemburg	24,738	106,917	36	1,9	466,249
Namur	374	1085	22	0,1	4561
Walloon Brabant	108	157	44	0,2	501
Western Flanders	266	337	86	0,0	1081

3.4 Cumulative risk by contact exposure to bee larvae

In the scenario where tau-fluvalinate mean DL50 = 12 µg bee 1 and considering the Eq. (2), the risk posed to bee larvae by the presence of residues in brood comb, recycled comb, honey comb, and cappings waxes is respectively of 15.12%, 12.3%, 4.79%, and 0.73%. With the second scenario (tau-fluvalinate DL50= 0.2 µg bee^{-}1) the cumulative risk for bee larvae in the four wax types are respectively of 122%, 119%, 104%, and 16% (Table 4).

Table 4. Risk to bees expressed in percentage, Hazard Quotient (HQ) and HQ values exceeding threshold toxicity in beeswax for the four wax types (brood comb wax, recycled comb wax, cappings wax and honey comb wax) for two different tau-fluvalinate LD50 values

Tau-fluvalinate DL_{50} (µg bee^{-1})		HQ value	Brood comb wax (N=89)	Recycled wax (N=59)	Cappings wax (N=28)	Honey comb wax (N=6)
12 (Lewis et al.,2016)	HQ_1	Mean	5,562	1,901	54	213
		SD	49,395	9,855	116	193
		Median	27	136	4	169
		Min	0	0	0	6
		Max	466,249	74,208	507	452
		250 > value > 5000	24	26	2	3
		Value > 5000	2	2	0	0
	Risk %	Mean	0.151	0.123	0.007	0.048
		SD	0.010	2.399	0.011	0.044
		Median	1.079	0.553	0.001	0.034
		Min	0	0	0	0
		Max	10.193	4.264	0.039	0.090
0.2 (EPA, 2008)	HQ_2	Mean	7,961	4,238	533	2,262
		SD	49,745	11,341	744	1,581
		Median	753	1,33	184	2,466
		Min	0	0	0	75
		Max	468,324	75,476	2,677	4,584
		250 > value > 5000	54	42	11	5
		Value > 5000	10	9	0	0
	Risk %	Mean	1.219	1.194	0.160	1.037
		SD	2.924	2.706	0.053	1.064
		Median	0.283	0.507	0.240	0.771
		Min	0	0	0	0
		Max	14.376	19.958	0.871	2.213

Legend: LD50, acute median lethal dose; HQ1 and HQ2, Hazard Quotient calculated 2 different tau-fluvalinate LD50 values.

3.5 Pesticide and veterinary drug residues and honey bee mortality

3.5.1 Logistic regression model

An individual residue's possible correlation with mortality rates was tested using a univariate logistic regression model (Table 5). After multivariate logistic regression analysis, only chlorfenvinphos exhibited a significant correlation with bee mortality (OR = 2.15; 95% CI: 1.04–4.44; P = 0.038). Moreover, no interaction between chlorfenvinphos and permethrin was found in the final multivariate logistic regression model. In addition, bee mortality rate was significantly higher in samples contained Chlorenvinfos (two-sample Wilcoxon rank-sum test; P = 0.026).

3.5.2 Development of a risk-based model

The area under the ROC curve (AUC) was estimated for 10 different ROC curves, i.e. with the first, the first two, the first three, until it incorporated the first ten most commonly found residues presented in Table 5. The two ROC curves with close higher AUC were retained for future fitting of the binomial model (Fig. 3). The final model retained and presented in Fig. 4 corresponds to the ROC curve fitted with the higher AUC, i.e. the ROC curve fitted with the three first pesticide residues related to the colony bee mortality (i.e. bromopropylate, chlorfenvinphos and chlorpyrifos-(ethyl)). For this final ROC curve, the AUC = 0.6128 (Fig. 4). Considering this final ROC curve, the best cut-off related to the prediction of the colony bee mortality corresponds to at least one of these three residues (Fig. 4).

Table 5**.** Univariate logistic regression model outcome, pesticides with possible correlation to bee mortality (only pesticides with P value <0.2 are presented).

Pesticides with *P* value <0.20	Odds ratio	95% CI	*P* value
Bromopropylate	1.68	0.86–3.27	0.124
Chlorfenvinphos	2.24	1.09–4.58	0.028*
Chlorpyrifos (-ethyl)	2.38	0.88–6.44	0.088
Diclofluanid	2.43	0.62–9.46	0.2
Pendimethalin	0.21	0.02–1.90	0.16
Permethrin	2.06	0.93–4.53	0.072
Piperonylbutoxid	1.58	0.82–3.04	0.175
p,p′-DDT	4.51	0.52–39.4	0.17
Propargite	1.80	0.99–3.25	0.051
Thiacloprid	0.12	0.007–2.34	0.16

Legend: CI, confidence interval; * P value <0.05.

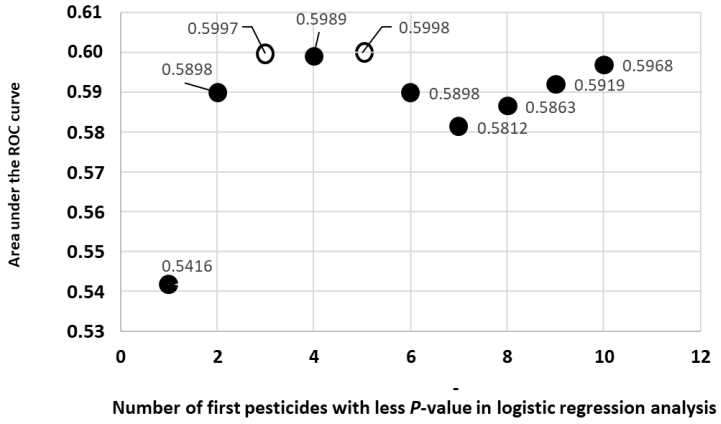

Fig. 3. Area under the curve estimated for each of the ten receiver operating characteristic(ROC) curves tested. Legend: Circle, area under the curve for each of the ten different receiver operating characteristic (ROC) curves tested; black circle, ROC curve not retained; circle with white centre, two best ROC curves retained

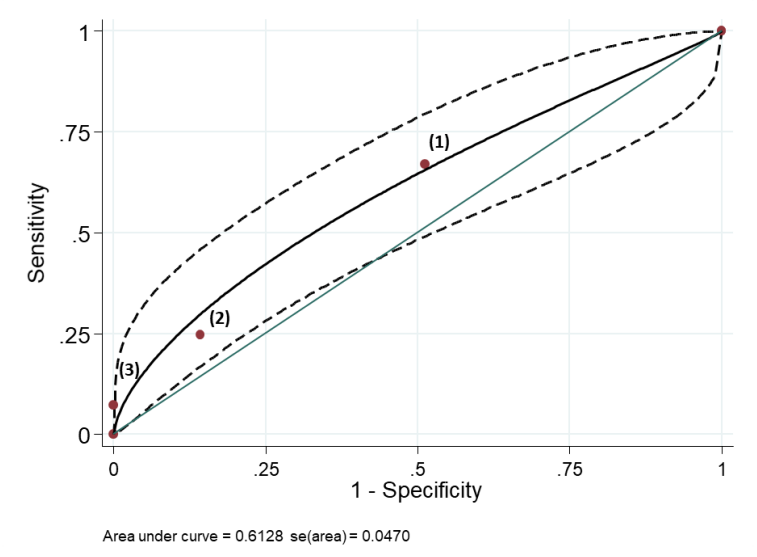

Fig. 4. Best predicted receiver operating characteristic (ROC) curve. Legend: ROC, ReceiverOperating Characteristic; solid line, fitted ROC curve (fitting binormal model); dashedlines, 95% confidence interval of the fitted ROC curve; cut-off (= number of pesticides in the combination of the three pesticide residues considered) was noted as a number inbrackets. The best cut-off corresponds to at least one pesticide residue(s) of the threeconsidered.

3.6 Potential interactions of residues

We looked for the most prevalent pesticide combination in all wax types combined (N = 182). The most frequent combination was taufluvalinate together with coumaphos (N = 142), tau-fluvalinate together with coumaphos, and propargite (N = 94), tau-fluvalinate together with DEET and coumaphos (N = 56), coumaphos together with propargite and tau-fluvalinate (N = 48), coumaphos together with chlorfenvinphos (N = 44). Other relevant combinations with proven synergies we detected were; amitraz together with taufluvalinate (N = 37), piperonyl butoxide together with fenpyroximate (N = 9), chlorothalonil together with coumaphos (N = 1), and taufluvalinate (N = 1).

4. Discussion

4.1. Validation of analytical method

The QuEChERS extraction method followed by LC-MS/MS is well established to assess pesticide residues in beeswax (Herrera López et al., 2016; Niell et al., 2014; Svečnjak et al., 2019). From an analytical point of view, sample preparation should guarantee the representativeness and complete extraction of the residues for a high recovery (Niell et al., 2014). As pesticide residues in beeswax samples are not evenly distributed, beeswax wax was grounded and homogenised using liquid nitrogen. This method allows limits of quantification (LOQs) of 0.01 mg/kg and limits of detection (LODs) of 0.003 mg/kg for most residues, these limits were considered as the lowest successfully validated levels, that is, the levels at which acceptable recoveries (70–120%) were achieved.

Pesticides and veterinary drug residues in beeswax. The results confirmed our first hypothesis; residues of pesticides applied in agriculture and as veterinary drugs in-hive are ubiquitous contaminants in beeswax. In 2012, Ravoet et al. (2015) already reported the presence of 18 pesticide residues in a restricted area in Flanders with a similar median number of residues per wax sample. Simon-Delso et al. (2014) analysed 54 wax samples for 99 different residues, detecting 15 different active ingredients overall. Worldwide, numerous studies (Boi et al., 2016; Calatayud-Vernich et al., 2017; Chauzat and Faucon, 2007; Fulton et al., 2019; Harriet et al., 2017; Lozano et al., 2019; Serra-Bonvehí and Orantes-Bermejo, 2010; Shimshoni et al., 2019; Zawislak et al., 2019) acknowledge that beeswax is a major contamination sink for pesticide residues, thereby constituting hazardous health implications for bees and potentially for humans. Overall in our study, typical residues of beekeeper-applied veterinary treatments such as tau-fluvalinate (Apistan®) and coumaphos (Checkmite®) had the highest contamination prevalence and concentrations. These products, by design, have low toxicity relative to the dose required for adverse effects. Pesticide residues from agricultural were found with lower prevalence and concentrations, nevertheless, these products have higher toxicity to bees and are known to have synergistic effects with other pesticides, which increase the toxicity of one or more of the compounds (Johnson et al., 2013; Thompson and Wilkins, 2003).

Tau-fluvalinate and coumaphos are currently not approved in Belgium but are permitted in at least one of the other Member States of the European Union. Through the "cascade system[7]" (El Agrebi et al., 2019), they can, therefore, be used in Belgium, under certain conditions and the responsibility of a veterinarian. Their frequent use over the past few years resulted in substantial residue levels in beeswax. These Varroa-treatments are well known and have previously been reported worldwide as prevalent contaminants in honey bee colonies (Bommuraj et al., 2019; Chauzat and Faucon, 2007; Harriet et al., 2017; Herrera López et al., 2016; Johnson et al., 2010; Mullin et al., 2010; Perugini et al., 2018; VanEngelsdorp et al., 2010). The high chemical stability and the low migration rate of these highly lipophilic acaricides drive them to accumulate in wax to concentrations up to the mg $kg^{-}1$(Lozano et al., 2019). This phenomenon seems to occur especially with coumaphos, whose concentration levels vary significantly from 0.01 mg $kg^{-}1$ up to 7.41 mg $kg^{-}1$, probably due to different application events, but also to its high beeswax persistence (half-life of 115–356 days) (Martel et al., 2007; Zhu et al., 2014) and the extensive recycling of old beeswax into new foundations.In contrast amitraz (Apivar®), an approved acaricide that is frequently used in Belgium is rarely detected in beeswax samples, due to its short half-life, requiring its quantification indirectly through its metabolites (Shimshoni et al., 2019). Amitraz is reported to degrade within 1 day in beeswax and within 10 days in honey (Korta et al., 2001). In our study, one very high amitraz detection (16.7 mg kg 1) was registered in a comb wax; probably due to a massive recent application. No other value exceeded 0.54 mg $kg^{-}1$.

Other acaricides were also found with a high prevalence (28%) such as bromopropylate (and its metabolite dibromo-benzophenone). This acaricide was used in the early years of Varroa-treatments (e.g. Folbex VA®), in addition to its agricultural use against other mites. Bromopropylate shows high lipophilic properties (log P = 5.4) and high persistence, therefore its use in agriculture was banned in Europe in 2003 and Belgium in 2007 (Commission Regulation (EC) No 2076/ 2002). Nowadays, its use in beekeeping is no longer approved. The acaricide propargite was detected with a high prevalence (53.3%) as well. This residue comes from agricultural applications and not from Varroa control. Its accumulation in beeswax came from external contaminants

[7] The cascade system was introduced to solve the general problem of availability of veterinary medicinal products for minor species and for minor uses.

brought back to the hive via foraging. Its use is no longer authorized by the European regulation (EC) 1107/2009.

Agricultural pesticides such as piperonyl butoxide were found with a prevalence of 29.1%, it is a classic P450 inhibitor that has been reported to increase the toxicity of thiacloprid to honey bee (Iwasa et al., 2004) and to affect the bee's ability to detoxify, contributing significantly to honey bee intolerance of pyrethroid insecticides (Johnson et al., 2006). Diethyltoluamide (DEET) an insect repellent, was found with a prevalence of 36.3%, confirming its presence in Belgian beeswax (Ravoet et al., 2015). DEET has relatively high lipophilic properties (Log P = 2.1), which could explain its accumulation. Nevertheless, DEET contamination source could not be determined.

We analysed the samples for six neonicotinoids insecticides. Similarly to the study of Simon-Delso et al., (2014), only residues of thiacloprid were detected in 3 wax samples. In the past years, neonicotinoids have been under particular surveillance for their implication in honey bee losses, and their use as seed treatments has been partially restricted in the European Union (European Commission, 2013).

More alarming was the detection of highly toxic to bees and EU banned molecules such as lindane (gamma-HCH) (prevalence of 3.29%) and dichlorodiphenyltrichloroethane (DDT) including its breakdown product dichlorodiphenyldichloroethylene (prevalence of 0.54%). Since 2008, all uses of lindane are banned in the EU. In 2009, lindane and two other HCH-isomers were included in the Stockholm Convention (ECE/EB.AIR/104) on Persistent Organic Pollutants (POPs) to achieve the global elimination of these substances (Vega et al., 2016). DDT's use has also been banned in Europe since 2009 (Regulation (EC) No 1107/2009). Our results confirm previous ones showing that pesticides can continue to contaminate the environment long after their ban (Tosi et al., 2018).

4.2 Pesticide and veterinary drug residues and honey bee mortality

In the multivariate logistic regression analysis, only chlorfenvinphos appeared to have a significant correlation with bee mortality (OR = 2.15; 95% CI: 1.04–4.44; P = 0.038), in the risk-based model, this compound was also targeted. The Honey bee mortality data used should be interpreted with caution as the underlying factors responsible for bee mortality are generally multi-factorial (Potts et al., 2010). Chlorfenvinphos use is no longer authorized for agricultural use in the EU (Commission Regulation (EC) No 2076/2002) and is not approved as veterinary treatment for controlling this, the molecule was found in 24.7% of all wax samples. As no maximum residue level (MRL) was defined for the substance, a default value of 0.01 mg kg^{-1} is applied as MRL for honey following Reg. (EC) No 396/2005. The mean concentration in beeswax of the positive samples to chlorfenvinphos (all wax types together) is 0.033 mg kg^{-1} (min-max: 0.01–0.15 mg kg^{-1}), thus exceeding the MRL set for the honey of 0.01 mg kg^{-1}. Chlorfenvinphos presence has already been reported in a previous Belgian survey, with 50% occurrence (N = 10) and a concentration fluctuating between 0.008 and 0.015 mg kg^{-1} (Ravoet et al., 2015) as well as in a German study in 8.6% of the analysed samples (N = 288), with concentrations ranging from 0.001 to 6.4 mg kg^{-1} (Shimshoni et al., 2019). In Italy, 34.5% of the analysed wax samples (N = 178) were positive to chlorfenvinphos with concentrations reported of 0.01 to 0.63 mg kg^{-1} (Perugini et al., 2018). Pollen was as well continuously contaminated over months and years (Tosi et al., 2018). In Spain, 88.5% of the samples were found positive for chlorfenvinphos, with concentrations up to 10.64 mg kg^{-1} during a survey between 1996 and 2006 (Orantes-Bermejo et al., 2010). In another Spanish study, differentiating wax types, Calatayud-Vernich et al. (2019) reported a 100% prevalence and concentrations ranging from 0.21 to 0.79 mg kg^{-1} in old comb wax, 33.3% prevalence and concentrations ranging from 0.005 to 0.05 mg kg^{-1} in cappings wax.

Studies on the effects of chlorfenvinphos on honey bee larvae health are not yet available. However, like coumaphos, it is an organophosphorus insecticide, whose adverse effects on adult worker bees have been studied at different levels (Fell and Tignor, 2001; Haarmann et al., 2002; Pettis et al., 2004). The origin of chlorfenvinphos in Belgian waxes is uncertain. Chlorfenvinphos illegal use as acaricide has been suspected in Spain, Portugal, France, and Italy (Orantes-Bermejo et al., 2010), where unauthorized chemicals are used as an alternative to the limited efficacy of some authorized treatments. The residues may also have been taken up by honey bees during the collection of nectar and/or pollen in the environment around the hive when the pesticide was illegally applied on flowering crops (Lozano et al., 2019). Chlorfenvinphos could also originate from river pollution, the substance has been identified among 45 other as a priority substances to be monitored in the European Union (Directive 2013/39/EU) (Pistocchi et al., 2019). Another possible route of wax contamination is the use of legally traded wax from non-EU countries. Chlorfenvinphos concentrations in Belgian beeswax appear to be low and therefore does not seem to be the result of illegal use in-hive as veterinary treatment.

4.4 Wax Hazard Quotient and toxicity to honey bees

Overall, in the first scenario (tau-fluvalinate DL50 = 12 μg bee 1), the majority of the analysed samples (68%) had a low HQ value (b250) and should not represent a danger for honey bees, nevertheless, 30.2% have significant HQ.

The mean HQ value for brood comb (N = 89) showed the highest tox icity to bees (μ = 5562; σ = 49,395: min-max: 0–466,249), this value is due to one sample with an extremely high HQ value (466,249) elevating the HQ mean significantly from 326.7 to 5562. The sample contaminated with a high concentration of cypermethrin (9.3 mg kg^{-1}) was recorded in the province of Luxembourg, where agricultural land is essentially devoted to dairy and, above all, meat cattle farming. Cypermethrin is used massively in livestock worldwide for topical administration, either as concentrates for dipping or spraying or in ready-to-use products such as pour-on, dressings, and ear-tags. In recycled wax (N = 59) (μ = 1901; σ = 9855; min-max: 0–74,208) mean HQ value is significantly high (250 b HQ b 5000) but again, was due to 2 samples with extremely elevated toxicity values (17,536 and 74,208). These values elevated the mean HQ value from 358.2 to 1901. Two contaminants (HQ = 74,208 and 5242) were located in the province of Limburg, in a region devoted to horticulture, the other in East Flanders (HQ = 17,536). The contaminations in

Limburg are due to permethrin (0.31 mg kg^{-1}) and chlorpyrifos (ethyl) (4.38 mg kg^{-1}) both used over a long period respectively to control Lepidoptera and Coleoptera in ornamental, fruit and vegetable crops and a wide range of foliar pests. In East Flanders (cattle farming), the contamination was due to the presence of deltamethrin (0.026 mg kg^{-1}) and lindane (0.021 mg kg^{-1}). Deltamethrin is a pyrethroid insecticide used to eradicate external parasites on animal farms, lindane an obsolete topical substance that was used to treat parasites. Honey comb (N = 6) (μ = 213; σ = 193: min-max: 6–452), had three samples with significant tox icity. The limited number of honey comb wax samples does not allow us to draw clear conclusions about this wax type.

Cappings wax (N = 28) had the lowest mean (μ = 53; σ = 114; min-max: 0–507), this maximum value (507) is due to permethrin contamination in a single sample. Two samples were found with significant toxicity (250 b HQ b 5000). Cappings wax and honey comb wax toxicity can be considered as low or non-toxic to bees compared to recycled and brood comb wax.

The results of our study are very much in line with the findings of Calatayud-Vernich et al., 2017; where pyrethroids together with organophosphate chlorpyrifos were the main contributors to the HQ scores. This is due to their great toxicity through contact for honey bees and/or significant concentrations in the samples. Furthermore, cappings wax were also substantially less contaminated than foundations (made out of recycled wax) (Calatayud- Vernich et al., 2017; Harriet et al., 2017) and old combs beeswax.

With the second toxicity scenario (tau-fluvalinate DL50 = 0.2 µg bee 1), the HQ levels near alarming levels, and the number of samples exceeding threshold values increases. A revision is needed to clarify Tau-fluvalinate DL50 value.

The HQ model used in this study is simplistic as it considers toxic effects as cumulative and additive but does not take into account any synergistic or antagonistic effects, as these are not yet well documented and thus not yet integrated into the used equation. Better models for estimation of potential adverse effects of residue cocktails with greater reliability than those already existing are needed to assess more properly the potential risks of residues.

Cumulative risk by contact exposure to honey bee larvae.

The highest risk was posed by brood comb wax where 15% of larvae were exposed to pesticide doses higher than the lethal dose, followed by the recycled comb, and honey comb wax. Our results point out that cappings wax was substantially less contaminated than the 3 other wax types and presented a very limited risk (0.7%). In the scenario where tau-fluvalinate DL50= 0.2 µg bee 1, the cumulative by contact exposure risk increased considerably to exceed 100% except for cappings wax (16%) but is still high. However, it may not be appropriate to assess risk by acute DL50 values for adult honey bees when it is the larvae that develop in wax for a specific time, hence the cumulative risk value estimated using the current calculation represents an inaccurate scenario, but to date, the necessary toxicity values for larvae is not sufficiently documented. Other studies already estimated contact exposure risk to worker bees or bee larvae of single pesticides (Harriet et al., 2017; Sanchez-Bayo and Goka, 2014). Using a slightly different equation, Harriet et al., 2017, found Chlorpyrifos-ethyl (198%) and coumaphos (21%) to have the highest risk to bee larvae.

4.5 Potential synergies and interactions of residues

With up to 12 different residues detected in a single wax sample, it is very difficult to elucidate the potential interactions of products. The risk assessments may thus underestimate the true risk to bees, as the more residues in a given sample, the greater potential for unexpected synergistic interactions. The most prevalent combinations included acaricides for Varroa treatments as they are directly applied in the hive. Pesticide residues synergies have scarcely been evaluated, nevertheless, the current pesticide combinations would probably damage colony health, because synergistic effects have been identified for combinations such as piperonyl butoxide that seems to increases the toxicity of fenpyroximate, while amitraz seems to increase the toxicity of taufluvalinate (Johnson et al., 2013).

4 Conclusion

Bees are at risk from many stress factors, which occur individually but most commonly in combinations, affecting bee health and mortality. Pesticides are one of the factors impacting colony health. Our study highlights the ubiquitous presence of pesticides in all wax types, besides veterinary drug residues have the highest concentration and prevalence but the lowest toxicity compared to agricultural pesticides that have a lower prevalence but higher toxicity to bees and can have synergistic effects with other pesticides. Significantly lower residue diversity and concentrations were found in cappings wax compared to the other three types. Brood comb wax exhibited the highest rates of contamination. In light of these results, beekeepers should replace brood comb wax more frequently than recommended (1/4 to 1/3 of than old brood frames (ITSAP, 2017)) rather than recycling them back into the wax stream, where they will continue to potentially impact colony health. We highly recommend the use of greater amounts of cappings wax in the manufacturing process of foundation, the substrate beekeepers purchase to aid their bees in building comb, as well as using organic or natural wax sources to gradually decrease residues in the colony matrix. Furthermore, the marketing and the recommendation regarding the use of plant protection products and as well as veterinary treatments should take into account that compounds with highly lipophilic properties accumulate in wax. Given the large number of residues found in beeswax and the amount of potential synergistic effects among the different residues detected, we recommend testing commonly found combinations in field experiments to determine the potential synergetic effects on colony health. The use of alternative veterinary substances (e.g. acids) should be encouraged. An educational campaign for users of pesticides or veterinary drugs is needed to

increase awareness and good practices. The use of the BeeToxWax tool designed to estimate the risk associated with contaminated beeswax is recommended when pesticide analyses are available (Appendix 2). It is crucial to introduce maximum residue limits for beeswax trade, taking into account residue toxicity for bees and, ideally, for their larvae. Furthermore, EPA and PPDB toxicity values for tau-fluvalinate should be scientifically re-examined in depth.

Abbreviations used

ABP animal by-products
AUC Area under the curve
DEET N,N-Diéthyl-3-méthylbenzamide
EFSA European Food Safety Authority EMA European Medicines Agency
EPA Unites States Environmental protection Agency FASFC Federal Agency for the Safety of the Food Chain HQ Hazard Quotient
LC/MS-MS liquid chromatography/tandem mass spectroscopy
GC/MS-MS gas chromatography/tandem mass spectroscopy
GC-MSD gas chromatography-mass selective detector
LD50 Acute median lethal dose after 48 h of exposition = is a statistically derived single dose of a substance that can cause death in 50% of animals when administered by the oral route/by contact. The LD50 value is expressed in µg of test substance per bee. For pesticides, the test substance may be either an active ingredient (a.i.) or a formulated product containing one or more than one active ingredient (OECD, 2017)
LOD Limit of detection
LOQ Limit of quantification
MRL Maximum Residue Limit
PPDB Pesticides properties DataBase
QuEChERS Quick Easy Cheap Effective Rugged Safe
ROC Receiver operating characteristic
VSDB Veterinary Substances DataBase

CRediT authorship contribution statement

Noëmie El Agrebi: Conceptualization, Methodology, Software, Data curation, Writing original draft, Visualization, Investigation. **Kirsten Traynor**: Writing review & editing. **Olivier Wilmart**: Writing review & editing, Data curation, Validation. **Simone Tosi**: Writing review & editing. **Laurent Leinartz**: Software, Visualization. **Ellen Danneels**: Writing review & editing. **Dirk C. de Graaf**: Writing review & editing. **Claude Saegerman**: Conceptualization, Methodology, Software, Data curation, Visualization, Investigation, Writing review & editing.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi. org/10.1016/j.scitotenv.2020.141036.

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Experimental section

Study 3

Belgian case study on flumethrin residues in beeswax: possible impact on honey bee and prediction of the maximum daily intake for consumers

Science of the Total Environment 687 (2019) 712–719

Noëmie El Agrebi, OlivierWilmart, Bruno Urbain, Ellen L. Danneels, Dirk C. de Graaf and Claude Saegerman

Preamble

Flumethrin is a synthetic pyrethroid ectoparasiticide commonly used in veterinary medicine and one of the varroacides used for the control and treatment of *Varroa* mites in beekeeping. In Belgium, until February 2017, flumethrin was only authorised under veterinary prescription using the "cascade system". Until today, no maximum residue limit (MRL) due to the veterinary use of flumethrin is required in honey according to the European Commission Regulation, because the residue levels in honey were generally lower than the limit of detection (LOD) of the analytical method (1–2 µg/kg), and this while, at the same time, the concentration of flumethrin in the beeswax from the same treated hives amounted to 130 µg/kg. The objective of the study was to perform a flumethrin nationwide monitoring of comb wax to determine the prevalence rates and contamination levels in Belgian apiaries. The novelty of this study was testing the possible relation between flumethrin residue concentrations and honey bee mortality as well as the assessment of the risk posed by flumethrin residues in beeswax to honey bees through contact or oral exposure (mastication).

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Belgian case study on flumethrin residues in beeswax: Possible impact on honey bee and prediction of the maximum daily intake for consumers

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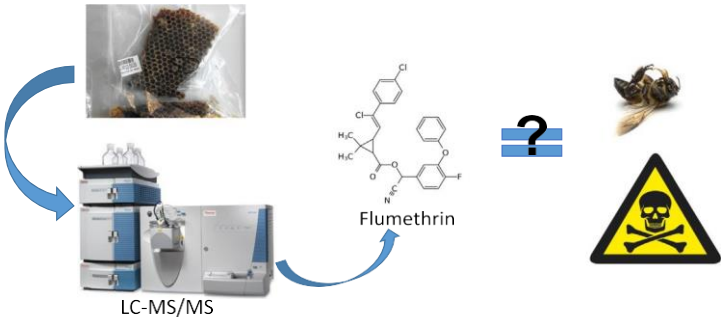
HIGHLIGHTS

- Study of flumethrin presence in beeswax and its risk to bees and human health
- Mean Flumethrin prevalence rate in beeswax was of 21.77% (95% CI: 14.87–30.08).
- Oral hazard quotient is above recommended threshold representing a risk to bees.
- No relation was established between flumethrin in beeswax and bee mortality.
- Flumethrin residues in beeswax and in honey do not pose a risk to human health.

GRAPHICAL ABSTRACT



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abstract

To assess the health risk posed by flumethrin residues in beeswax to honey bees and honey consumers, 124 wax samples randomly distributed in Belgium were analysed for flumethrin residues using liquid chromatography/tandem mass spectrometry. The risk posed by flumethrin residues in beeswax to honey bee health was assessed through the calculation of a non-pondered and a pondered Hazard Quotient by the prevalence rate of flumethrin considering an oral or topical exposure. No statistical difference was found when comparing both the average flumethrin residues concentrations and contact and oral pondered hazard quotients between apiaries with lower and equal or higher than 10% of colony loss. Flumethrin residues estimated daily intake by Belgian consumers through honey and wax ingestion was estimated via a deterministic (worst-case scenario) and a probabilistic approach. The probabilistic approach was not possible for beeswax consumption due to the lack of individual consumption data. The highest estimated exposure was 0.1% of the theoretical maximum daily intake for both approaches, meaning no risk for human health.

Abbreviations:ADI, acceptable daily intake; bw, body weight; df, degree of freedom; EDC, estimated daily contribution; EDI, estimated daily intake; FASFC, Federal Agency for the Safety of the Food Chain; HQ, hazard quotient; LC-MS/MS, liquid chromatography/tandem mass spectrometry; LD50, median lethal dose; LOQ, limit of quantification; MRL, maximum residue limit; QuEChERS, quick, easy, cheap, effective, rugged, and safe; S.D., standard deviation; TMDI, theoretical maximum daily intake; VSDB, Veterinary Substances Data Base.

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1. Introduction

Honey bee health and mortality are of concern (Aizen et al., 2009; Fontaine et al., 2005; Garibaldi et al., 2014; Klein et al., 2007; Pettis et al., 2012; Potts et al., 2010; Sanchez-Bayo and Goka, 2014) in North America as well as in Europe, and particularly in Belgium. Honey bee colony development success depends partly on the management of the ectoparasitic bee mite *Varroa destructor*, which has historically been treated using varroacides that may also impact honey bee health (Johnson et al., 2013). Many studies have pointed out pesticides as one of the main stressors affecting colony development/survival (e.g. Balbuena et al., 2015; Johnson et al., 2013; Rumkee et al., 2015). Honey bee exposure to pesticides may result in adverse health impacts such as acute and chronic mortality or sub-lethal effects (Chauzat et al., 2009; EFSA, 2012; Hardstone and Scott, 2010). Understanding and quantifying the risks of pesticides entering the hive is challenging as pesticide risk is currently determined via short-term acute contact and oral toxicity tests on adult bees (i.e., LD_{50}), which avoid synergistic, cumulative, sublethal effects on the colony (Traynor et al., 2016) and which do not take the possible toxicokinetic profile of bees into account (Hesketh et al., 2016). Honey bee chronic toxicity tests over 10 days are suggested by the OECD (OECD, 2018), as well as the standardized chronic toxicity tests for larvae (OECD, 2013).

Flumethrin is a synthetic pyrethroid ectoparasiticide commonly used in veterinary medicine and one of the varroacides used for the control and treatment of *Varroa* mites in beekeeping (Johnson, 2014; Oruc et al., 2012). In beekeeping, strips impregnated with 3.6 mg of the active substance flumethrin are suspended into the space between the combs in the central brood rearing area for several weeks; normally developed colonies receive four strips per brood chamber (EMEA, 1998). Flumethrin belongs to group 1, highly toxic to honey bees pesticides, from the pyrethroids class of synthetic insecticides, based on the structure and insecticidal activity of the pyrethrins, with a broad range of toxicity to adult bees (Oruc et al., 2012). Flumethrin acute oral DL_{50} is 0.178 µg/bee (Oruc et al., 2012) and its contact DL_{50} is 0.05 µg/bee (Perez Santiago et al., 2000). Flumethrin affects the insect nervous system by causing multiple action potentials in the nerve cells, by delaying the closing of ion channels (Oruc et al., 2012). In addition of being highly toxic to adult bees (Oruc et al., 2012), applying varroacides in honey bee colonies leaves residues in bee products, especially in beeswax. Varroacides accumulate in beeswax with years of treatments, reaching such high concentration levels up to the mg kg^{-1} (Lozano et al., 2019), given that they are mostly fat-soluble, non-volatile (Wilmart et al., 2016) and given that old comb beeswax is recycled continuously into new foundations (Ravoet et al., 2015; Tlak Gajger et al., 2016). Beeswax is primarily used in beekeeping to produce comb foundations but also in the chemical, cosmetic, pharmaceutical and food industries. Beeswax is a natural wax produced by the worker bees in their wax-producing mirror glands on the inner sides of the sternites on abdominal segments (Reybroeck et al., 2010). The new wax scales are masticated by the worker bees and used to build honeycomb cells in which brood is raised, and nectar and pollen are stored (Ravoet et al., 2015; Thompson, 2012). Ripened honey is also capped with wax (EFSA, 2007). Contact between beeswax and honey enables chemical transfer between these two matrices (Tremolada and Vighi, 2014). This carry-over could lead to an exceeding maximum residue limits, which could pose a health risk to consumers and honey bee health (Benuszak et al., 2017; Wilmart et al., 2016). Nevertheless, transfer of flumethrin from beeswax to honey has been estimated as negligible (EMEA, 1998; Karazafiris et al., 2012; Wallner, 1999) as its octanol-water partition coefficient at pH 7 and 20 °C (i.e. Log P) is 6.2 (Veterinary Substances Data Base, Pesticide Properties DataBase: http://sitem.herts.ac.uk/aeru/ppdb/en/atoz.htm), which corresponds to a highly lipophilic substance. At European level, the EU commission has set the average consumption of honey at 5 g/ capita/day representing a very small part of the total diet (EU Commission, 2018). For Belgium, the value of 50 g honey per day and per person is recorded as the 95th percentile of the chronic daily consumption (consumers only) for an adult according to the EFSA Comprehensive European Food Consumption Database (Wilmart et al., 2016). In addition, in Belgium, the average consumption of 16.41 g honey per day and per person (honey consumers only) was recorded in consumers older than 14 years of both sex (De Vriese et al., 2005). Concerning beeswax, a consumption of 1.29 g beeswax per day and per person was calculated by the EFSA (EFSA, 2007). This conservative assumption is based on the 95th percentile of consumption of foodstuffs containing beeswax as they increase human exposure through secondary routes such as consumption of food additives, coating agents in pastry preparation, capsules and tablets, surface treatment of certain fruits (EFSA, 2007). The consumption of honey and beeswax only as foodstuffs were taken into account, not as cosmetics or pharmaceuticals. We considered the consumer as an adult of 60 kg body weight (bw) (Wilmart et al., 2016). Pyrethroids, including flumethrin, show almost negligible acute toxicity to humans but are highly toxic to target organism. The main effects of pyrethroids are neurotoxicity at high doses and liver hypertrophy, which are reversible if death does not occur. Symptoms of chronic toxicity of pyrethroids include memory loss, change in immunity system, behavioral problems, thyroid problem etc. (Patel and Patil, 2016).

In Belgium, until February 2017, flumethrin was only authorised under veterinary prescription using the "cascade system". The cascade system was introduced to solve the general problem of availability of veterinary medicinal products for minor species and for minor uses (Reybroeck et al., 2010). Until today, no maximum residue limit (MRL) due to the veterinary use of flumethrin is required in honey according to European Commission Regulation (EU, 2002), because the residue levels in honey were generally lower than the limit of detection (LOD) of the analytical method (1–2 µg/kg), and this while, at the same time, the concentration of flumethrin in the beeswax from the same treated hives amounted to 130 µg/kg (EMEA, 1998).

In February 2017, flumethrin veterinary medicine product obtained a European Marketing Authorisation (MA) in several EU member states, including Belgium. This product is commercialised under the name of PolyVar Yellow® (275 mg bee hive strip containing holes). The strips should be fitted at the entrance in a way that the bees are forced to enter or leave the hive only through the holes of the strip.

The present unprecedented study was motivated by the high losses of honey bee colonies observed in Belgium last years. The objective of the study was to perform a flumethrin nationwide monitoring of comb wax in order to determine the prevalence rates and the contamination levels in Belgian apiaries. During the survey (beekeeping season 2016), beeswax samples were collected and honey bee mortality rates were registered (from May to October 2016). The novelty of this study was testing the possible relation between flumethrin residues concentrations and honey bee mortality as well as the assessment of the risk posed by flumethrin residues in beeswax to honey bees through contact or oral exposure (mastication). For this last purpose, the masticated wax quantity had to be beforehand estimated, as these quantities were not known. In addition, using both deterministic and probabilistic approaches, we estimated according to different scenarios the daily intake of flumethrin residues through consumption of beeswax and honey per day and per person and we compared it to the theoretical maximum daily intake (TMDI) estimated by EMEA (1998) as equal to 108 µg/day.

2. Materials and methods (including safety information)

a. *Epidemiological unit of interest*

When applied to beekeeping, it is important to define the "epidemiological unit" for which the case definition is being applied. Epidemiological units are the groups which make up the population of interest, and can range from individual bees, colonies, and apiaries (VanEngelsdorp et al., 2013). For this study, the epidemiological unit used to assess the risk for honey bee health is the individual adult honey bee, Nevertheless, larvae reared in cells are in closer contact with residues contained in the wax (Chauzat and Faucon, 2007) and are thus more at risk than bees. Unfortunately, the risk to larvae could not be assessed as we still lack acute and chronic, contact and oral toxicity tests (i.e., LD_{50}) for larvae. To characterise honey bee mortality, the colony was considered as the unit of interest.

b. *Beeswax sampling*

One sample of 20 g of comb wax was withdrawn from 1 hive per apiary out of 124 apiaries, randomly selected and uniformly spread in each of the ten Belgian provinces (Fig. 1). Whenever possible, samples were collected from an area of used brood comb, out of the hive body, not containing any beebread, honey or brood (Traynor et al., 2016). The sampled bee colonies seemed healthy, with no clinical signs of infectious diseases or acute intoxication (Ravoet et al., 2015). Potential variations in climatic factors between different sampling locations were minimised by collecting beeswax matrices during the same beekeeping season, from May to October 2016. In Belgium, veterinary treatments against *Varroa* mite are applied typically two times a year: first around New Year (oxalic acid) in the absence of brood, then right after honey harvest (varroacide), meaning between the 15th of July and 1st of August.

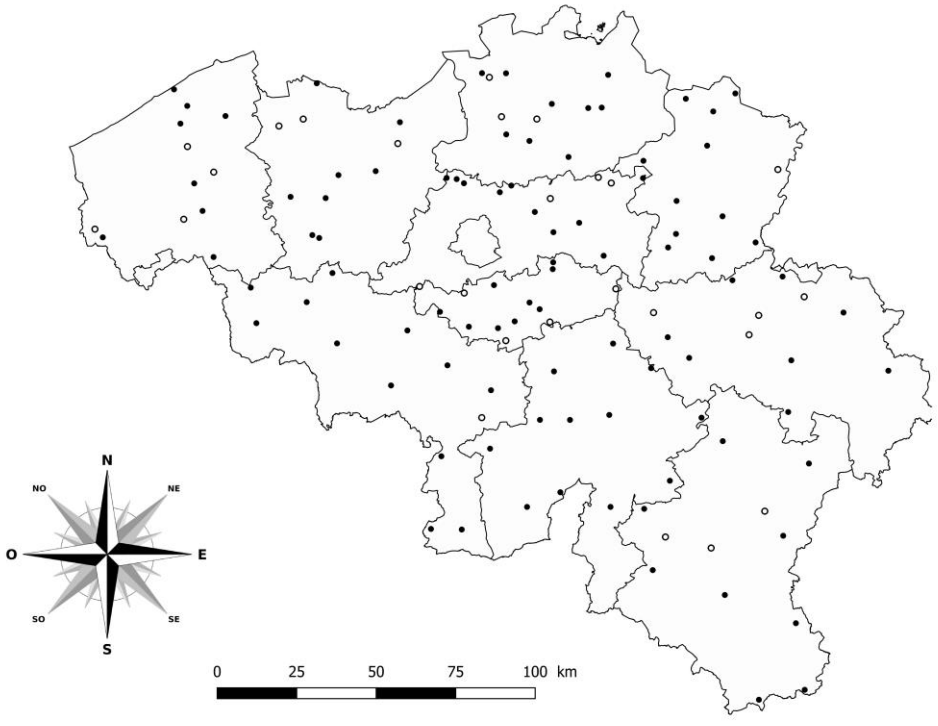

Fig. 1. ● negative sample for flumethrin; ○ positive sample for flumethrin.

c. Flumethrin detection and residue concentration

Beeswax was analysed for the presence of flumethrin residues by GRIPA test laboratory (Beaucouzé, France) according to the European NF EN 15662 method (CEN, 2008). Solubilisation was made with acetonitrile (ACN) before the addition of water and citrate salts (sodium chloride, magnesium sulfate, sodium citrate and sodium hydrogenocitrate sesquihydrate) in order to separate ACN from water. The ACN extract was shaken and centrifuged to purify it with dispersive QuEChERS's salt (mix of $MgSO_4$ and Primary and Secondary Amine). The final extract was directly used for LC-MS/MS. Quantification was performed using addition of flumethrin in blank beeswax extract before injection (calibration curve from 1 µg/l to 50 µg/l). Samples with higher contents are diluted with ACN to integrate the linearity range. Flumethrin was also added before extraction (different level between 20 µg/kg and 100 µg/kg) to another blank beeswax sample (each analysis batch) to obtain recovery rate and the validation of the capacity to detect and quantitate with good accuracy the limit of quantification (LOQ) set to 20 µg/kg. Mean recovery is 95% with a relative standard deviation of 13.

Analysis in LC/MS/MS has been done on Sciex 5500 Qtrap with Shimadzu HPLC pump (LC 20XAD) and Synergi Hydro RP® column from Phenomenex. Two transitions were followed to ensure the specificity of the method in negative electrospray mode (508 give 481 and 510 give 483). The Multiple Reaction Monitoring ratio (MRM ratio) criteria was fixed at 30% to discriminate false positive. A signal to noise ratio under 10 is not acceptable for both transitions to measure calibration point.

d. Data collection on colony loss

A questionnaire was filled out together with the sampling in order to record the general colony losses. The percentage of losses per apiary is the difference between the number of colonies in April 2016 and in September 2015 divided by the number of colonies (including splits) in September 2016 and multiplied by one hundred (Clermont et al., 2014).

We took in consideration, within the population of interest, the apiary size. Apiaries with large numbers of colonies will have a greater influence on the total colony loss metric than the apiaries with only few colonies (Kulhanek et al., 2017).

General colony loss is the most accurate snapshot of losses in Belgian apiaries over a fixed period of time (end beekeeping season) giving us a precise figure of the proportion of all colonies that died in Belgium.

e. Non-pondered and pondered wax hazard quotient

The acute risk of flumethrin residues in beeswax to bees was assessed separately considering an oral or a contact exposure. Since residue concentrations are significantly higher in wax, and migration poorly understood in this matrix, only samples with a HQ_{wax} ≥ 5000 are associated with an elevated risk to honey bees (Traynor et al., 2016). As no information was found on the amount of masticated beeswax by honey bees, the following scenario was used. The cell weight was estimated by the average weight of 4 wax samples (1 dm^2 each) from a body wax frame divided by the number of cells which were counted recto and verso (n = 800). In this condition, the estimated cell weight was in average 0.0232 g (S.D. = 0.0015 g).

For a colony including ±50,000 bees and considering that 50% (or 25,000) of them are foragers, 20% (or 5000) of them develop the ability to produce wax during 7 days (Winston, 1987). Worker honey bees build 3 sheets (34.6 cm × 19.9 cm = 6.88 dm^2 each) of wax (initially 65 g per sheet) within a Simplex body in 2 days by stretching and incorporating newly produced wax (Winston, 1987). Once built, these 3 sheets consisting of 800 cells per dm^2, each weighing 0.0232 g will bring the weight of the 3 build wax sheet to 383 g (6.88dm^2 × 800 × 0.0232 g × 3 = 383 g). This amount of beeswax corresponds to 0.0383 g of masticated wax per bee and by day (= 383 g/(5000 bees × 2 days)).

The acute toxicity determines the inherent toxicity of flumethrin to bees in experimental conditions. Currently, typical risk assessments consider only acute toxicity of chemicals either by topical or oral exposure, measured 24 or 48 h after exposure (Sanchez-Bayo and Goka, 2014).

The result as a certain dose expressed in µg/bee is a parameter and does not express the hazard of the product in the field. For this reason, we calculated the Hazard Quotient (HQ) per bee and sample for the specific matrix beeswax using a similar method described by (Stoner et al., 2013) and for which the equations are the following:

$$HQ_{contact\ wax}\ per\ bee = \frac{Residue\ concentration\ in\ \mu g/kg\ beeswax}{LD_{50}\text{contact in } \mu g\ bee^{-1}} \quad (1)$$

$$HQ_{Oral\ wax}\ per\ bee = \frac{Residue\ concentration\ in\ \mu g/kg\ beeswax}{LD_{50}\text{oral in } \mu g\ bee^{-1}} \quad (2)$$

For contact and oral routes, this standard calculation per sample is not fully a measure of the risk of honey bees being exposed to flumethrin residues through the beeswax, because it does not indicate the probability of a hazard to occur. To estimate the risk of honey bees being affected by flumethrin residues contaminated beeswax, it is necessary to consider also the frequency of detection of these residues in this matrix in Belgium, because prevalence indicates the probability of exposure to the contaminant (Sanchez-Bayo and Goka, 2014). Prevalence rate is the percentage of positive samples per province or par region.

Therefore, a pondered HQ (PHQ) should incorporate this probability as follow:

$$PHQ = HQ_{contact\ wax} \times prevalence\ rate\ [\%] \quad (3)$$

$$PHQ = HQ_{oral\ wax} \times prevalence\ rate\ [\%] \quad (4)$$

2.6. Flumethrin residues estimated daily intake for Belgian consumers of honey and beeswax

The estimated daily intake (EDI) of flumethrin residues by consumers through the consumption of honey and beeswax was assessed using both deterministic and probabilistic approaches. In the determistic approach, the EDI was based on a worst-case scenario (EDI_{WCS}). The EDI_{WCS} was calculated on the basis of the percentile 95 (P95) of flumethrin residues concentrations found in beeswax of Belgian hives multiplied respectively by the P95 of honey consumption (i.e. 50 g of honey per day and per person) and the P95 of the beeswax consumption (i.e. 1.29 g beeswax per day and per person) (Wilmart et al., 2016). In the probabilistic approach, both distributions of flumethrin residues concentrations and honey consumption were considered. For this approach, honey consumption data were extracted from the national human consumption survey performed in 2004 in Belgium (De Vriese et al., 2005). This consumption survey concerns adults older than 14 years of both sex (n = 3083 persons involved). In this approach, individual consumption and contamination data are converted into a distribution function (Table 1) and computed using @Risk software (version 7.5; Palisade Corporation, New York, NY, USA). Afterward, distribution functions are combined using a Monte Carlo simulation with 100,000 iterations to obtain a function of flumethrin EDI.

Table 1. List of the distributions used for the probabilistic risk assessment (only "consuming" people are presented) according to the @Risk software notations

Parameter	Distribution function in @Risk
Flumethrin contamination	
Lower bound approach	RiskExpon(0,014919;RiskShift(0))
Middel bound approach	RiskPareto(2,7117;0,01)
Upper bound approach	RiskPareto(4,5905;0,02)
Honey consumption	RiskLognorm(15,277;19,221;RiskShift(0,77739))

Legend: P95, percentile 95.

To our knowledge, very few studies exist about contaminations of honey by contaminated beeswax. The percentage of transfer depends on the lipophilicity of the active substance. The Log *P* values or the logarithm of the ratio of the concentrations of flumethrin in the solvents octanol and water is of 6.2 (http://sitem.herts.ac.uk/aeru/vsdb/ Reports/1480.htm). Chemicals with low Log P values (e.g., b1) may be considered relatively hydrophilic; conversely, chemicals with high Log P values (e.g., N4) are very hydrophobic, in other words, highly lipophilic. Flumethrin is highly lipophilic (Log P of 6.2). This induces a very low transfer from beeswax to honey. A previous study that aimed to determine the limit after which the concentration of active varroacide constituents in the frame wax move and become quantitatively detectable in honey, with a detection threshold for flumethrin residues of 5 µg/kg, showed that there was no detectable transfer of flumethrin residues from wax into honey, in experimental conditions (Wallner, 1992).

For this reason, we consider only 1% of flumethrin residues migrating to honey. Considering that a frame completely filled with honey contained approximately 1.84 kg of honey (Simplex standard frame), the wax/honey ratio is 128/1840 g = 0.069 (Reybroeck et al., 2010).

The EDI of flumethrin residues by the consumer is:

$$EDI_{wax} = BDC\ per\ person\ X\ beeswax\ contamination \quad (5)$$

which, BDC is the beeswax daily consumption.

$$EDC_{honey} = HDC\ per\ person\ X\ beeswax\ contamination X\ 0.069 X\ 0.01 \quad (6)$$

which, HDC is the honey daily consumption, 0.069 the wax/honey ratio and 0.01 the maximum transfer from beeswax to honey.

2.7. Statistical analysis

Comparison between prevalence rates and flumethrin residues concentrations between regions were respectively assessed using a Chi2 and a Mann-Whitney *U* test. The relation between the average flumethrin residues concentrations and the colony losses was tested using both the Pearson correlation coefficient and Spearman rank correlation coefficient. The average of flumethrin residues concentrations between groups of colonies with loss lower and equal or higher than 10% was tested using Two-sample *t*-test with unequal variances. In addition, a negative binomial regression was used to investigate the relation between both contact and oral pondered hazard quotient. In this analysis, the number of colony losses was weighted by the size of apiary as exposure. All statistical analyses were carried out in STATA/SE 14.2 (StataCorp, College Station, TX, USA). The limit of statistical significance of the tests performed was defined as 0.05.

9. Results & discussion

a. *Flumethrin detection and concentration in positive beeswax samples*

Out of the 124 analysed beeswax samples (61 from Flanders and 63 from Wallonia), 27 samples were found with flumethrin residues (14 from Flanders and 13 from Wallonia) (Table 2). Unexpectedly, the Belgian mean prevalence rate was of 21.77% (95% CI: 14.87–30.08) with no significant difference in prevalence rates between Flanders and Wallonia ($Chi2_{(\alpha=0.05;\ 1\ d.d.l.)} = 0.10$; $P = 0.76$), showing that flumethrin use in Belgium is quite widespread at the national level even if the substance was only authorised under veterinary prescription before February 2017, in the case of the "cascade system". The Belgian average flumethrin residues concentration for contaminated beeswax samples was 68.52 µg/kg with a standard deviation of 58.2 µg/kg (median = 48 µg/kg; min = 21 µg/kg and max = 280 µg/kg) with no significant difference in the flumethrin residues concentration between regions (Mann-Whitney *U* test; $P = 0.08$). The highest concentrations were observed for samples from provinces of Antwerp (280 µg/kg) and of Walloon Brabant (190 µg/kg) (Table 2). As for prevalence rates, no significant difference in flumethrin residues concentrations was found between both regions.

Surprisingly, no beekeeper indicated using this substance for varroosis treatment in the associated face-to-face questionnaire (El Agrebi, personal communication). The origin of the contamination could provide from historic use of flumethrin by the beekeepers (previous years) or from flumethrin residues contaminated trade beeswax before it use by the beekeepers as flumethrin shows high lipophilic properties (Log $P = 6.2$) (Lewis et al., 2016) and remains in beeswax.

In a study on the prevalence of pesticides residues in beeswax in Spain (Calatayud-Vernich et al., 2017), the flumethrin residues mean concentration found was of 90.5 µg/kg (min-max; 48–170.1 µg/kg). Nevertheless, the limit of quantification (LOQ) for flumethrin in the Spanish study was lower (12.5 µg/kg) than in the present study (20 µg/kg). The prevalence rate in Spain was of 81.8% (Calatayud-Vernich et al., 2017).

b. *Non-pondered and pondered wax hazard quotient*

The exposure of honey bees to pesticides residues involves both contact and oral routes (Alix and Vergnet, 2007). For both exposure routes, the non-pondered HQ_{wax} is compared with a trigger value of 50 (Alix and Vergnet, 2007) considered as a risk to adult worker honey bees. Nevertheless, since residues concentrations can be significantly higher in wax, and transmission routes poorly understood in this matrix, only values of *contact* HQ_{wax} N 5000 correspond to an elevated risk to honey bees (Traynor et al., 2016). Contact non-pondered hazard quotient ranged from 420 to 5600 with a mean value of 1370 (S.D. = 1164) and 1 out of 27 beeswax samples was associated with an elevated risk to honey bee health with a value of 5600. Oral non-pondered hazard quotient ranged from 118 to 1573 with a mean value of 385 (S.D. = 327). When the flumethrin residues prevalence rates were considered (PHQ) the mean contact PHQ decrease up to 384 (S.D. = 333; min-max = 51–1292) and the mean oral PHQ decrease up to 108 (S.D. = 94; min-max = 14–363) (Table 2) but remained above the trigger value of 50 and represents thus a risk to bees. At best, the HQ provides an underestimate of total exposure and does not take into account flumethrin cumulative and sub-lethal effects on the colony. Because of the specific toxicokinetic profile of honey bees compared with other insects, it is recognised that toxicokinetic data can provide useful information on the potential biological persistence of a pesticide residue which, in some cases, could have effects after continuous exposure that maybe more marked compared with their short-term effects (EFSA, 2013). Unfortunately, the current state of knowledge does not permit the development of more robust models that include these factors, and thus we used this more simplistic model as a point of departure to help understand the risk posed by the real world exposure experienced by honey bee colonies (Traynor et al., 2016). More precise calculation would use the LD_{10} instead of LD_{50}'s or the same but for chronic toxicity, however this toxicological reference dose is currently not available for flumethrin (Traynor et al., 2016).

Table 2: Prevalence rate and concentration of flumethrin residues (µg/kg) in beeswax samples from the two Belgian regions and calculated oral/contact HQ/PHQ for each province.

				Concentration (µg/kg) in Np				HQ		PHQ	
Area	Np	Nn	Prevalence rate (%)	Min	Max	Average	Median	Contact	Oral	Contact	Oral
Antwerp	3	10	23.08%	29	280	143	120	2860	803	660	185
Limburg	1	11	8.33%	31	31	31	NA	620	174	51	14
East Flanders	3	9	25%	26	40	35	40	707	199	177	50
Flemish Brabant	3	9	25%	54	59	57	58	1140	320	285	81
West Flanders	4	8	33.33%	21	50	32	29	645	181	215	60
Flanders	14	47	22.95%	21	280	62	40	1237	348	348	86
Wallon Brabant	5	9	35.71%	54	190	104	94	2084	585	694	195
Hainaut	1	12	7.69%	46	46	46	NA	920	258	64	18
Liège	4	8	33.33%	32	140	72	58	1435	403	478	134
Luxemburg	3	9	25%	41	48	43	41	867	243	217	61
Namur	0	12	0%	NA	NA	NA	NA	NA	NA	NA	NA
Wallonia	13	50	20.63%	32	190	76	54	1514	425	425	132
Belgium	27	97	21.77%	21	280	69	48	1370	385	385	108

Legend: Np, number of positive; Nn, number of negative; HQ, hazard quotient; PHQ, pondered hazard quotient; NA, non-applicable

c. Flumethrin residues concentrations and pondered hazard quotients compared to colony loss

We found no linear (Pearson correlation coefficient = −0,22; $P = 0.55$) and no non-parametric (Spearman rank correlation coefficient
= 0.13; $P = 0.73$) relation between average flumethrin residues concentrations expressed in μg/kg compared to average mortalities expressed in percent per province in Belgium (Fig. 2). There is no significant difference concerning the average flumethrin residues concentrations between apiaries with lower and equal or higher than 10% of colony loss (Two-sample *t*-test with unequal variances; $P = 0.60$).

There is no significant difference concerning the contact (negative binomial regression; $P = 0.537$) and oral (negative binomial regression; $P = 0.535$) pondered hazard quotients between apiaries with less and equal or higher than 10% of colony loss.

In this study, we only focused on flumethrin residues contaminations and their possible direct impact on honey bee mortality. Till today, no specific causal agent has yet been identified, but there is a wide consensus on the multifactorial origin of colony losses that are often associated with high infection levels of parasites and/or pathogens (Neumann and Carreck, 2015; Ratnieks and Carreck, 2010). There is no consensus either, regarding the relative importance of these factors, singly or in combination (VanEngelsdorp and Meixner, 2010). We can't thus identify flumethrin residues in beeswax as a risk factor of bee mortality alone, but it could be one in combination with other pesticides like fungicides (Thompson, 2012).

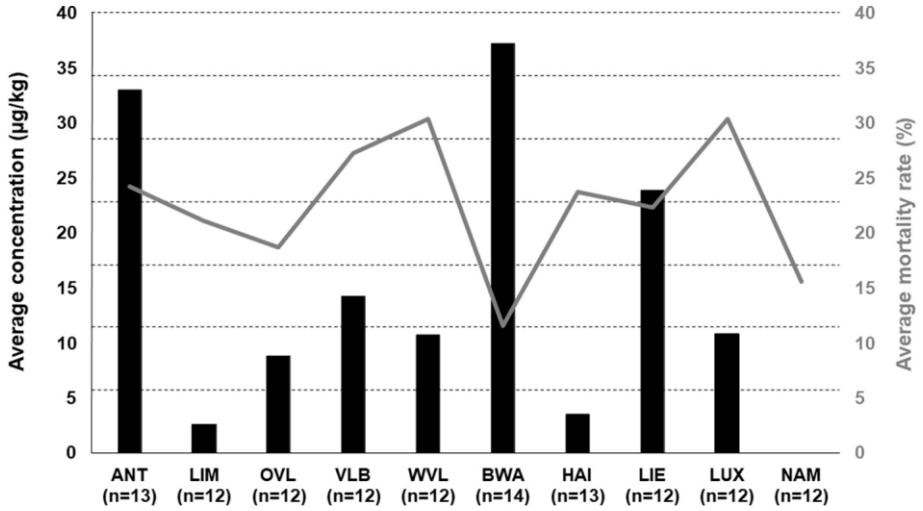

Fig. 2. Provinces as ANT (Antwerp), LIM (Limburg), OVL (East Flanders), VLB (Flemish Brabant), WVL (West Flanders), BWA (Walloon Brabant), HAI (Hainaut), LIE (Liège), LUX (Luxembourg), NAM (Namur).

d. Belgian honey and/or beeswax consumer's flumethrin residues estimated daily intake

The EDI of flumethrin residues by consumers through the consumption of honey and beeswax was estimated with both determinist and probabilistic approaches. In the determinist approach, only the EDI in a worst-case scenario (P95 for wax flumethrin residues concentration and for consumption data) (EDI_{wcs}) was considered. For the flumethrin concentration conversion from wax to honey, we have considered a wax/honey ratio of 0.069 and only 1% of flumethrin residues migrating from wax to honey due to the high lipophilicity (high Log P) of flumethrin. In the probabilistic approach, due to the absence of information on individual consumption of beeswax, the exposure assessment could only be performed for honey.

i. Deterministic approach

In the worst-case scenario, the flumethrin residues estimated daily intake through beeswax consumption ($EDI_{wcs-wax}$) is of 0.0955 μg per day and per person (Eq. (5)). Flumethrin residues EDI in honey ($EDI_{wcs-honey}$) is of 0.00256 μg per day and per person (Eq. (6)) (Table 3). Both values represent b0.1% of the TMDI.

As no randomness is involved in this model, the result of it can be validated nevertheless the multiplication of prudent assessment factors which may result in an overestimated overall result and therefore unrealistic reference values.

ii. Probabilistic approach

Considering the lower (for each value below the LOQ, 0 was attributed), the middle (for each value below the LOQ, value of LOQ/2 was attributed) and the upper (for each value below the LOQ, value of LOQ was attributed) bound approaches, the mean EDI_{honey} was respectively 0.00013, 0.00014 and 0.00072 μg flumethrin per day and per person. In the same way, the P95 EDI_{honey} was respectively 0.0004, 0.0004 and 0.00069 μg flumethrin per day and per person. In all the previous cases, the mean EDI_{honey} represents b0.0002% of the TMDI. When we consider the maximum values of the EDI_{honey} in each approach, the maximum percentage of the TMDI was b0.007%. The probabilistic approach was not possible for beeswax consumption due to

the lack of individual consumption data.

This approach intends to describe more clearly variability and/or uncertainties in yielding quantitative insight into both the possible range and the relative likelihood of values for model outputs. According to both approaches, flumethrin residues in beeswax and in honey do not pose a risk to human health (= calculated EDI values very much lower than the ADI). This is mainly due to the low level of beeswax consumption and to the low level of flumethrin residues in honey. Nevertheless, other foodstuffs can contribute to the consumers' exposure to flumethrin residues (EMEA, 1998; Wilmart et al., 2016).

Table 3: Deterministic calculation of the estimated daily intake in the worst-case scenario* for honey and/or beeswax consumers only expressed in percentage of the TMDI.

Parameter	Consumption	
	Honey	Wax
Flumethrin residue concentration in wax (mg/kg)	–	0.07405
Flumethrin residue concentration in wax (µg/kg)	–	74.05
Flumethrin residue concentration in honey (µg/kg) considering the wax/honey ratio (0,069) and the coefficient of migration (0.01)	0.05109	–
Consumed quantity (g per day and person)	50	1.29
Consumed quantity (kg per day and person)	0.05	0.00129
Body weight (kg)	60	60
EDI (µg per day and per kg of body weight)	0.00004258	0.00159208
EDI (µg per day and per person of 60 kg)	0.;002555	0.0955245
TMDI (µg per day and per person of 60 kg)	108	108
% of TMDI	0.0024	0.0885

Legend: *Percentile 95 (P95) of the flumethrin residue concentration and P95 of the honey consumption.

10. Conclusions

The results of this study highlight the importance of considering the risk of pesticides both for honey bee health and for human health perspectives. Flumethrin residues in beeswax and in honey do not appear to pose a risk to human health but represent a risk to honey bee health. The benefit of the flumethrin use should be considered in regard to its toxic effects on bees. In order to decrease the level of pesticide residues in beeswax, we recommend to (i) inform beekeepers about flumethrin risks (HQ and PHQ) to honey bee health and to its correct use, (ii) replace the old frames from the brood chamber by low residue beeswax foundation in order to ensure a complete frame turnover in the hive after 2 to 3 years. The exclusion of honey and beeswax frames that are in contact with the strips could also lead to a drastic reduction of residual flumethrin concentrations in the final product.

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Experimental section

Study 4

Honey bee and consumer's exposure and risk characterisation to glyphosate-based herbicide (GBH) and its degradation product (AMPA): Residues in beebread, wax, and honey

Science of the Total Environment 704 (2020) 135312

Noëmie El Agrebi, Simone Tosi , Olivier Wilmart, Marie-Louise Scippo, Dirk C. de Graaf and Claude Saegerman

Preamble

Glyphosate is a controversial weed killer for its potential effects on human health. This study is the first Belgium-wide monitoring investigating the extent of the contamination of the apicultural products by glyphosate residues and their main degradation product. Two bee matrices were investigated: beebread and innovatively beeswax from the brood chamber. Additionally, to detect a possible transfer of glyphosate residues from beeswax to honey, we analysed wax from the honey super together with corresponding extracted honey samples (pairwise samples). Based on glyphosate residues and AMPA exposure and toxicity, we assessed their risk to the honey bee (*Apis mellifera* L.) and human health using published data (i.e. international standards).

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Honey bee and consumer's exposure and risk characterisation to glyphosate-based herbicide (GBH) and its degradation product (AMPA): Residues in beebread, wax, and honey

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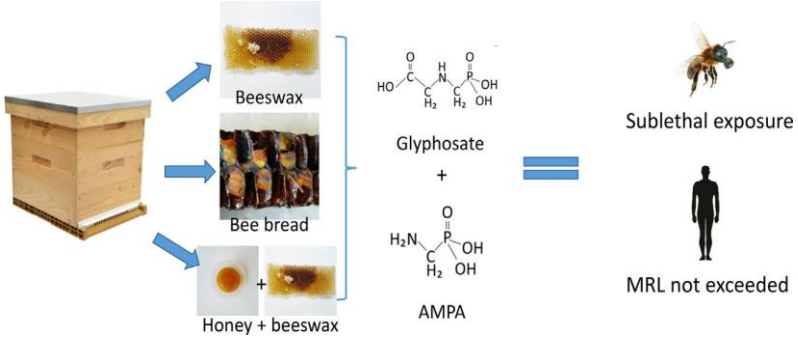
HIGHLIGHTS

- Assessement of GBH and AMPA in beebread, beeswax and honey.
- Estimation of bee and human exposures to GBH residues and AMPA.
- GBH residues were found in beeswax samples while glyphosate is non-lypophilic.
- Maximum GBH residues can cause sub-lethal effects on honey bees.
- Risk assessment of glyphosate (active ingredient) may underestimate the risks caused by its formulation.

GRAPHICAL ABSTRACT



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glyphosate-based herbicide (GBH)

ABSTRACT

In order to assess bee and human exposure to residues of glyphosate-based herbicide (GBH) and its main degradation products aminomethylphosphonic acid (AMPA) and to characterise the risk posed by these substances, we analysed 3 different bee matrices; beebread (N = 81), wax (N = 100) and 10-paired samples of wax/honey collected in 2016/2017 from 379 Belgian apiaries. A high-performance liquid chromatography-electrospray ionisation tandem mass spectrometry (HPLC-ESI-MS-MS) was used as analytical method. Limit of quantification and detection (LOQ and LOD) for GBH residues and AMPA in the 3 matrices was respectively of 10 ng g—1 and 1 ng g—1. In beebread, 81.5% of the samples showed a residue concentration > LOQ and 9.9% of the samples a residue concentration < LOQ (detection without quantification); no significant difference in detection rate was found between the north and the south of the country. Glyphosate was detected in beeswax less frequently than in beebread (i.e. 26% >LOQ versus 81.5% >LOQ). The maximum GBH residues and AMPA concentration found in beebread (respectively 700 ng g—1 and 250 ng g—1) led to sub-lethal exposure to bees. The Hazard Quotient (HQ) for beebread and beeswax (7 and 3.2, respectively) were far below the "safety" oral and contact thresholds for bees. For human health, the highest exposure to

GBH residues in pollen corresponded to 0.312% and 0.187% of the ADI and of the ARfD respectively and, to 0.002% and to 0.001% for beeswax. No transfer of glyphosate from wax to honey was detected. Considering our results and the available regulatory data on the glyphosate molecule considered solely, not including the adjuvants in GBH formulation, the consumption of these three contaminated matrices would not be a food safety issue. Nonetheless, caution should be taken in the interpretation of the results as new studies indicate possible glyphosate/GBH residues toxicity below regulatory limits and at chronic sub-lethal doses.

Abbreviations: ADI, Acceptable Daily Intake in mg kg^{-1} body weight day^{-1}; AMPA, Aminomethylphosphonic acid; ARfD, Acute Reference Dose in mg kg^{-1} body weight day^{-1}; b.w., body weight; DT_{50}/ DT_{90}, Degradation time (in days) of 50/90% of the substance; FASFC, Federal Agency for the Safety of the Food Chain; GHB, Glyphosate-based herbicide; LD_{50}, median lethal dose = is a statistically derived single dose of a substance that can cause death in 50 per cent of animals when administered by the oral route/by contact. The LD_{50} value is expressed in lg of test substance per bee. For pesticides, the test substance may be either an active ingredient (a.i.) or a formulated product containing one or more than one active ingredient; LOD, Limit of detection; LOQ, Limit of quantification; MRL, Maximum Residue Limit; N, Number of samples; ppb, part per billion (ex. ng g^{-1}).

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1. Introduction

Honey bees (*Apis mellifera* L.) are the main pollinators in agricultural ecosystems (Aizen et al., 2009). By gathering nectar and pollen from blooming plants several kilometers away, they take any contaminants present in those resources back to the hive. The glyphosate-based herbicide (GBH), N-(phosphonomethyl) glycine, was introduced in 1974 (Benbrook, 2016) as a broad-spectrum herbicide, used for weed control under the common trade name of "Roundup®" (Monsanto). The use of GBH has spread rapidly in the last few decades (Goldsborough and Brown, 1988) to become one of the most commonly used agrochemicals worldwide (Dill et al., 2010; Duke and Powles, 2008). GBH use is still increasing every year (Benbrook, 2016). In Belgium, 471.19 tons of GBH were sold in 2015 (Service Public de Wallonie, 2017). GBH is typically applied via direct spray onto foliage (Giesy et al., 2000). For honey bees, beside pesticide-treated crops (Aktar et al., 2009), the other contamination routes are typically a consequence of drift, as residues of the herbicide can be found on the non-target plants (Krupke et al., 2012; Simon-Delso et al., 2017). Another possible contamination route is the wide use of GBH in urban areas, for domestic and for minor non-agricultural applications including weed control in railway lines, parks, and home gardens (Di Pasquale et al., 2013; Silva et al., 2018), where honey bees can potentially collect resources.

The commercial pesticide formulations are not single molecules, but mixtures (Mesnage et al., 2019). Most studies documenting pesticide effects on honey bees are performed without the formulation or other relevant spray adjuvant components used when applying the active ingredient, most often due to lack of such required tests for product registration (Mullin et al., 2015). Some of the compounds used in the formulation of end-use pesticide products are regulated as 'active', such as glyphosate in Roundup®, while other compounds are categorized as 'inert'. Such inert ingredients are variously called "co-formulants", "adjuvants", or "other ingredients" (EPA, 1997). They are specifically added to increase the efficacy of the active ingredient and can be more toxic to bees than the 'active' substance alone (Mesnage et al., 2013; Tsui and Chu, 2003). While technical glyphosate do not show high toxicity for honey bees, common formulations such as WeatherMAX® do (Boily et al., 2013). Severe impacts of agrochemical formulants on bee toxicity of pesticide active ingredients have been documented (Mullin, 2015).

Glyphosate molecule has a direct carbon-phosphorus (C–P) bond resistant to physicochemical impacts (Shushkova et al., 2010). Although this C-P bond is chemically very stable, it is broken down in living (Arregui et al., 2004) and dead plant material, and in soil by various microorganisms (Mamy et al., 2016) resulting in the degradation product aminomethyl phosphonic acid (AMPA) (Bruggen and Jr, 2017). The AMPA is the most abundant degradation product of glyphosate (Blot et al., 2019; Shushkova et al., 2010; Singh and Singh, 2016; Zhang et al., 2015). The AMPA enters into the environment, contaminating water, soil and indirectly, bee products (Manning, 2018). The AMPA has greater environmental persistence than glyphosate (Grunewald et al., 2001; Mamy et al., 2016). Glyphosate degradation time (DT_{50}) in soil (aerobic) ranges between 1.0 and 67.7 days, DT_{90} ranges between 9.3 and 1661 days (lab studies at 20 °C) (European Commission, 2016) but could be longer in water and soil than previously recognized (Myers et al., 2016). In soil, AMPA degradation time (DT_{50}) ranges between 39.0 and 330.7 days, DT_{90} ranges between 129.5 and 998.9 days (lab studies at 20 °C) (European Commission, 2016). Although the glyphosate exhibits low toxicity to adult honey bees (median lethal dose 48 h after exposition (LD_{50}) 100 lg bee^{-1} (Pesticide Properties DataBase (PPDB) (Lewis et al., 2016)), several recent research (Boily et al., 2013; Dai et al., 2017; Faita et al., 2018; Helmer et al., 2015; Herbert et al., 2014; Jumarie et al., 2017; Mengoni Goñalons and Farina, 2018; Motta et al., 2018) raise the possibility of health effects on bees associated with chronic, sub-lethal doses related to the accumulation of this compound in the hive. Furthermore, Liao et al. (2017) reported that bees display a contradictory preference for flowers that contain glyphosate in sugar water at 10 ppb. Therefore, glyphosate is not an obstacle for bees to visit floral nectar that contains it. The impact of sublethal chronic effects is particularly important for social insects since they could affect the entire bee colony. Furthermore, bee tolerance to glyphosate does not imply its harmlessness, contributing to increase the allostatic load of a colony (Vázquez et al., 2018).

Beebread has a slightly different composition than pollen, as the bees add honey and bee secretions to the pollen to make a nutritional protein source for adult and developing bees, large amounts being consumed by nurse bees, and to a lesser extent by larvae (Rortais et al., 2005). Beebread is mainly composed of pollen, which is consistently contaminated by pesticides over seasons and years (Tosi et al., 2018). Pollen is a valuable dietary supplement for humans and the contamination of this food matrix might represent a health risk.

Beeswax is a fundamental material for the colony. It is produced by bees or added by the beekeeper in the hive. Though beeswax guarantees the stability of the hive, it has often a low replacement rate and can remain in the hive for many years, leading to an accumulation of chemical substances applied in beekeeping and/or in agriculture (Chauzat and Faucon, 2007; Lambert et al., 2013; Mullin et al., 2010). Beeswax is primarily used in beekeeping but also in the chemical, cosmetic, pharmaceutical and food industries. The contamination of this matrix can thus represent a health risk for the consumer. GBH and other pesticides are introduced into the wax by contact with bees, by contaminated food resources which are stored in the hive or by newly secreted wax that is already contaminated with the pesticide (Bonzini et al., 2011). Regarding human health, tests about the safety of glyphosate and GBH residues conducted by several regulatory agencies and scientific institutions worldwide have concluded that there is no indication of any human health concern (EPA, 1993; European Commission, 2002). Nevertheless, questions regarding their safety raised progressively (Mesnage et al., 2015; Myers et al., 2016; Williams et al., 2000). Mesnage et al. (2015) revealed in their study that GBH residues could be toxic to humans below the regulatory Maximum Residue Level (MRL). It includes teratogenic, tumorigenic and hepatorenal effects. These effects could be explained by endocrine disruption and oxidative stress, causing metabolic alterations, depending on dose and exposure time. Some effects were detected in the range of the acceptable daily intake. Toxic effects of commercial formulations can also be explained by GBH adjuvants, which have their own toxicity but also enhance glyphosate toxicity (Mesnage et al., 2015). Similar concerns about AMPA toxicity to human have been raised, as its genotoxicity was proven (Mañas et al., 2009).

This study is the first Belgium-wide monitoring investigating the extent of the contamination of the apicultural products by GBH residues and AMPA. Two bee matrices were first investigated: beebread and innovatively beeswax from the brood chamber. Additionally, in order to detect a possible transfer of GBH residues from beeswax to honey, we analysed wax from the honey super together with corresponding extracted honey samples (pairwise samples). Based on GBH residues and AMPA exposure and toxicity, we assessed their risk to the honey bee (*Apis mellifera L.*) and human health using published data (i.e. international standards).

2. Materials and methods

Three different bee matrices were sampled for the analysis of GBH residues and AMPA: (i) beebread (N = 179), (ii) wax from the brood chamber (N = 100) and additionally (iii) a combination of wax from the honey super and corresponding extracted honey (N = 10). We used 379 non-professional apiary sites located in Belgium, including 2,997 colonies of *Apis mellifera*. For beebread and wax sampling, apiaries were selected (193 for beebread, 186 for wax and honey) from the Federal Agency for the Safety of the Food Chain (FASFC) apiaries database that included 4,949 registered beekeepers in 2015. The apiaries were stratified by province (N = 20/province and 10 provinces in Belgium) and randomly distributed in Flanders (northern Belgium) and Wallonia (southern Belgium). All sampled bee colonies seemed healthy, with no clinical signs of infectious diseases or acute intoxication (Ravoet et al., 2015). Quantum GIS (QGIS Development Team, 2009; http://qgis. osgeo.org) was used to create the maps in Fig. 1 and Fig. 2.

The risks posed by formulated products in the present study are restricted to the active ingredient glyphosate plus AMPA and the total risk of commercial products utilized by farmers is not the subject of this study.

2.1. Beebread collection

Beebread sampling (N = 179) was carried out by FASFC beekeepers and apiary technicians (Healthy Bee national monitoring program) between September and October 2016 from 193 apiaries including 865 colonies, out of 75 municipalities covering the entire Belgian territory (Fig. 1). The samples were provided with a protocol defining sampling collection details and were personally instructed by expert beekeepers to improve the harmonization of the procedure across apiaries. At each apiary, one hive was sampled randomly by cutting a comb portion of 8 by 8 cm filled with beebread. The coded samples were kept in hermetic plastic bags and stored at 20 °C the same day in order to be processed. A cool-box was used for shipment of samples from FASFC to Liège University to ensure that samples were maintained frozen (Tosi et al., 2018) until processing.

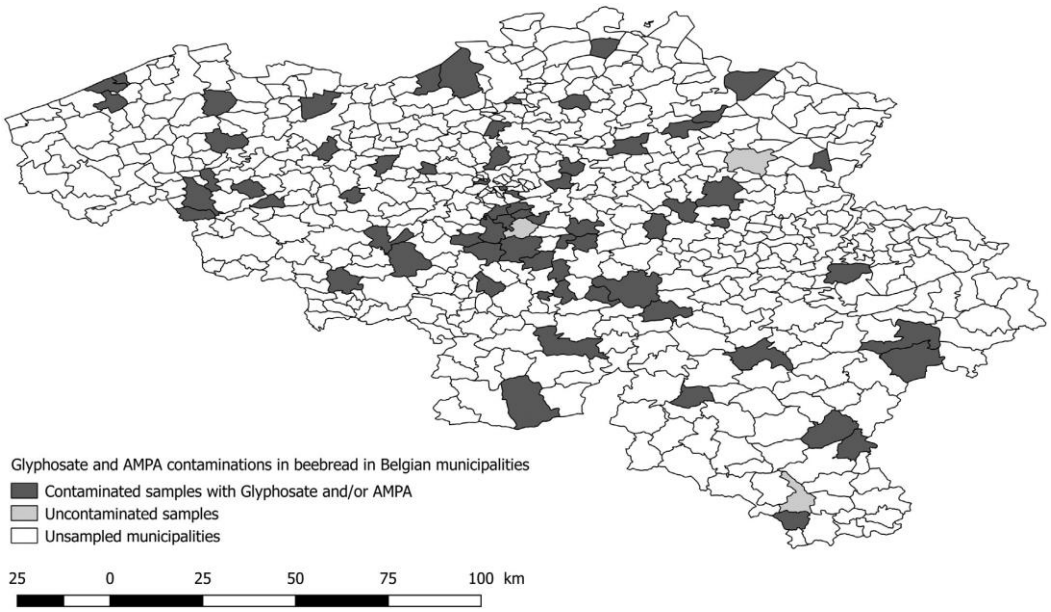

Fig. 1. Glyphosate residues and AMPA contaminations in beebread across Belgium, in 2016.

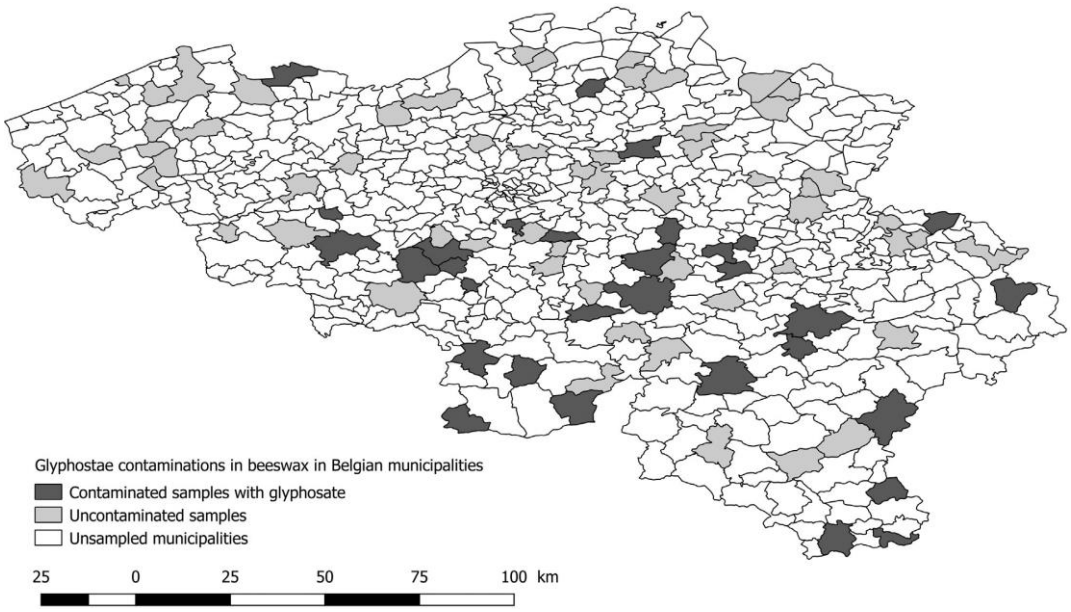

Fig. 2. Glyphosate residues and AMPA contaminations in beeswax across Belgium, in 2016.

2.2. Beebread extraction

For analyse purpose, 20 g of beebread were extracted manually from each comb sample using a disposable surgical blade (1 blade per sample). Cleaned beebread samples were stored in a 60 mL marked sterile polycarbonate containers with screw cap. Only 81 samples of beebread could be extracted from the 179 comb samples in adequate amounts for analysis.

5.1 Wax collection

Twenty grams (20 g) of wax from the brood chamber were sampled during spring 2016. Together with sampling, wax renewal rates were registered in a questionnaire (<50% and 50%). The coded samples were kept in hermetic plastic bags and stored the same day at -20°C until analysis. Financial limitations allowed us to randomly select only 100 wax samples out of the 186 original samples (2,132 hives). These 100 samples were equally distributed between Flanders and Wallonia in 89 municipalities (Fig. 2).

5.2 Honey/wax sampling

After wax analysis, out of the 32 beekeepers with the highest GBH residues contaminations in wax from the brood chamber, 10 beekeepers were randomly selected. Among these beekeepers, samples of 20 g of wax and of 50 g of honey harvested in summer 2017 were extracted both from the honey super (pairwise samples). The coded wax samples were kept in hermetic plastic bags, honey in polypropylene disposable containers and shipped the same day to the laboratory. Sampling and analysis of honey for GBH residues and AMPA were performed in September 2017 in the same laboratory and according to a similar method as for beebread and beeswax. Concentrations of GBH residues measured in honey were compared to the Maximum Residue Level (MRL) for human consumption (50 ng g^{-1}) (Regulation (EC) No 396/2005).

5.3 Glyphosate-based herbicide residues and AMPA detection

The GBH residues and AMPA analyses were carried out between May and June 2017 (September 2017 for the 10-paired samples of wax/honey) by the Phytocontrol laboratory (France) ISO 17,025 accredited under the number N° 1–1904 for the analysis of bee products by the French competent authority. The analysis method used for the targeted matrices (beebread, beeswax, and honey) was a high-performance liquid chromatography-electrospray ionisation tandem mass spectrometry (HPLC-ESI-MS-MS). The analytes were extracted using an aqueous solution followed by a simple clean up with a C18 solid-phase extraction (SPE) cartridge, and then glyphosate and AMPA were derivatized using 9fluorenylmethoxycarbonyl (FMOC-Cl) in borate buffer. For beeswax, an additional hexane treatment was used in order to defat the extract. The derivatives of glyphosate and AMPA were separated on a C18 column (105 4.6 mm; 5 mm) with gradient elution with the mobile phase of acetonitrile and 5 mmol/L ammonium acetate (pH 9), and finally detected with negative ion electrospray ionization-mass spectrometry (ESI-MS) in multiple reaction monitoring (MRM) mode (drying gas flow at 15 mL/min, nebulizing gas flow at 3 L/min). Limits of quantification (LOQ) for both glyphosate and AMPA in the 3 matrices were 10 ng g—1, while limits of detection (LOD) were 1 ng g—1. Matrix effects were compensated by the addition of 13C labeled glyphosate (used as internal standard) to the sample prior extraction, as well as in spiked samples used to set up the calibration curve. Three levels of spiking, including the LOQ, were performed on several matrices of different categories, which were analysed in condition of repeatability and intermediate fidelity. The mean spiked recoveries of glyphosate and AMPA at 3 spiked levels ranged from 72.2% to 112.9% with the relative standard deviations (RSD, n = 5) of 0.1% 4.5%. The tolerance interval was plotted with a beta probability of 80%, which represents the proportion of future values that the routine method will produce over the entire field of application. This allows to ensure that the molecule of glyphosate is extracted correctly and to correct any matrix effects.

5.4 Exposure assessment and risk characterisation to honey bee health

We estimated the Hazard Quotient (HQ) for honey bees using the method described by (Stoner and Eitzer, 2013). The HQ is calculated as the exposure divided by the toxicity expressed, in this study, as the maximum residue concentration (ng g^{-1} or ppb) in beebread samples divided by the oral acute LD_{50} (mg/bee) and multiplied by 100. An adult bee that consumed 100 mg pollen with an HQ of 1000 would have consumed approximately 10% of the LD_{50} for the pesticide during this development stage (=10 days as nurse bee) (Calatayud-Vernich et al., 2018). Assuming that 10% of the LD_{50} should never be exceeded (Atkins et al., 1981), the HQ value of 1000 would correspond to the limit of concern for bee health (Stoner et al., 2013; Traynor et al., 2016). For beeswax, we used a contact HQ of 5000 as a threshold safety value, since residue concentrations are significantly higher in wax, and contact exposure routes are poorly understood in this matrix (Traynor et al., 2016).

Then, we also assessed the risk posed by GBH residues and AMPA in beebread to honey bee health through the assessment of the honey bee exposure to these compounds through beebread consumption. To estimate the beebread consumption, we used published pollen consumption values. A nurse bee consumes between 13 and 120 mg of pollen during its first 10 days of life (OECD, 1998; Rortais et al., 2005) with a mean value equal to 65 mg (Chauzat and Faucon, 2007). As a worst-case scenario, we took into account the maximum consumption level of 12 mg of pollen per day. Then, we multiplied this highest level of consumption with the highest GBH residues and AMPA concentrations. Finally, we compared the exposure levels with the oral acute LD_{50} of these compounds.

Until very recently, risk assessment procedures did not implement yet the side-effects of pesticides on developing brood and the chronic effects in general (OECD, 2017). We could only assess the acute risk for adult bees since the possible toxicity of GBH residues on bee larvae is currently not sufficiently characterized.

5.5 Risk to consumer's health

For human health, GBH residues toxicity has been redefined in 2015 (European Food Safety Authority, 2015); an acceptable daily intake (ADI) for consumers has been set to 0.3 mg kg^{-1} body weight day^{-1} and the acute reference dose (ARfD) at 0.5 mg kg^{-1} body weight day^{-1}. Concerning AMPA residues, only the ADI value is available (0.3 mg kg^{-1} body weight day^{-1}). ADI is the quantity of a chemical that can be ingested daily for a lifetime causing no harm (on the basis of all known facts) (Renwick, 2002). ARfD is the quantity of a chemical that can be ingested by a person at a single time causing no harm. MRL is the maximum concentration of pesticide residue legally permitted in or on food commodities or animal feeds (Food and Authority, 2017).

Then, we assessed the risk posed by GBH residues and AMPA in beebread and beeswax to consumer's health through the assessment of the consumer exposure to these compounds through pollen and beeswax consumption. Thus, we assumed that beebread contamination levels correspond to pollen contamination levels. To estimate the pollen and beeswax

consumption, we used published consumption data. According to EFSA (EFSA, 2007), the 95th percentile of the daily consumption of beeswax corresponds to 1.29 g/person, which is 0.022 g/kg b.w. for a 60 kg individual. Concerning the daily consumption of pollen, the highest 95th percentile value recorded in the EFSA Comprehensive European Food Consumption Database (EFSA, 2018) corresponds to 69.55 g/person, that is 1.35 g/kg b.w. for a 52 kg individual, in France (according to the second version of the FoodEx food classification system). Then, as a worst-case scenario, we multiplied these high levels of consumption with the highest GBH residues and AMPA concentrations. Finally, we compared the exposure levels with the reference toxicological values of these compounds (above mentioned) to characterize the risk.

5.6 Statistical analysis

Yearly wax renewal rates were divided into 2 categories: <50% and 50% of wax frames changed per year in the brood chamber. A Fisher's exact test was used to compare the annual renewal rate of wax frames between regions (Flanders *versus* Wallonia). A Fisher's exact test was used for each pairwise comparison of frequency of detection of GBH residues and AMPA depending on the region/country and the matrix for GBH residues only (beebread *versus* beeswax). A two-sample Wilcoxon rank-sum (MannWhitney) (i.e. non-parametric test) test was used for each pairwise comparison of concentration of GBH residues and AMPA depending on the region/country and the matrix for GBH residues only (beebread *versus* beeswax).

A logistic regression (odds ratio's (OR) with 95% confidence intervals (95% CI)) was used to test a possible risk factor of GBH residues detection in beeswax and regions (Stata SE 14.1®, StataCorp LP, College Station, TX, USA). For all tests, a level of significance of 5% was used and divided, if needed, by the number of comparisons performed for the Bonferroni correction.

6 Results

6.1 Glyphosate-based herbicide residues and AMPA in beebread

In beebread, a high detection of GBH residues was registered (91.4% of positive samples overall) and AMPA (25.9% positive samples) in both Belgian regions. Glyphosate LOQ value (10 ng g^{-1}) was lower than the glyphosate median lethal doses LD_{50} for bees (10^6 ng g^{-1}). No significant difference of contamination prevalence in beebread between regions was confirmed by a one-tailed Fisher's exact test $_{(1 \text{ degree of freedom; } a=0.05)}$ (N = 81; p > 0.20) (Table 1). GBH residues and AMPA were not detected in only 6 samples (7.4%), coming from 3 of the 75 sampled municipalities (Fig. 1). Only 2 samples contained AMPA without GBH residue.

6.2 Exposure assessment and risk characterisation of GBH residues in beebread for honey bees

Based on the honey bee oral acute LD_{50} (48 h) of glyphosate (100 mg bee^{-1} = moderate toxicity for adult bees) (Conclusion on the peer review of the pesticide risk assessment of the active substance glyphosate 2015; Lewis et al., 2016) and on the maximum concentration of GBH residues detected in beebread (700 ng g^{-1}), the estimated maximum HQ (oral) of GBH residues for beebread found in Belgium is equal to 7 (=700/100). Because the honey bee oral acute LD_{50} (48 h) of AMPA is currently unknown in published data, it was impossible to estimate its corresponding HQ.

Considering the maximum consumption level of 12 mg of pollen per day (Rortais et al., 2005) (worst-case) and the maximum concentration of GBH residues detected in beebread (700 ng g^{-1}), this would correspond to a dose of 84 ng of GBH residues ingested per nurse bee over 10 days (0.012 g 700 ng g^{-1} 10 days). This exposure level corresponds to about 0.08% of the oral glyphosate LD_{50}. As mentioned, in the open literature, no oral acute LD_{50} (48 h) for AMPA is available. To assess the risk of AMPA to bees, we used, therefore, the parent compound glyphosate LD_{50} (Traynor et al., 2016). AMPA detection in beebread (250 ng g^{-1}) would correspond to about 0.03% of oral glyphosate LD50. Cumulatively, GBH and AMPA maximal concentration would correspond to about 0.12% of oral glyphosate LD50.

Table 1: GBH and AMPA detection, residue levels and hazard risk to bees in beebread, beeswax and honey samples in Flanders (North Belgium), Wallonia (South Belgium) and Belgium

Matrix	Region	Sampling period	Nb. analysed samples	Nb. samples > LOQ	Nb. samples < LOQ	Nb. samples detected	% samples > LOQ	% samples < LOQ	% samples detected	Multi-test for detection	Average [] ng g-1	S.D. [] ng g-1	Multi-test for []	Max [] ng g-1	Median [] ng g-1	Max HQ
	GBH															
	Flanders		39	34	3	37	87.2%	7.7%	94.9%	aa	58.44	133.28	aa	700	23	7
	Wallonia	Fall 2016	42	32	5	37	76.2%	11.9%	88.1%	aa	52.41	39.70	aa	160	49.5	1.6
Beebread	Belgium		81	66	8	74	81.5%	9.9%	91.4%	aa	55.52	98.89	aa	700	26	7
	AMPA															
	Flanders		39	5	3	8	12.8%	7.69%	20.5%	a-	39.8	25.16	a-	77	38	0.8
	Wallonia	Fall 2016	42	10	3	13	23.8%	7.14%	30.9%	a-	80.8	78.09	a-	250	58.5	2.5
	Belgium		81	15	6	21	18.5%	7.4%	25.9%	a-	67.13	67.09	a-	250	44	2.5
	GBH															
	Flanders		48	3	1	4	6.3%	2.08%	8.3%	ab	28.33	22.90	aa	54	21	0.5
Beeswax	Wallonia	Spring 2016	52	23	5	28	44.2%	9.62%	53.8%	bb	66.43	84.01	aa	320	40	3.2
	Belgium		100	26	6	32	26%	6%	32%	cb	62.04	80.05	aa	320	36	3.2
	Flanders		2	0	1	1	0%	50%	50%	/	/	/	/	/	/	/
Honey	Wallonia	Summer 2017	8	1	0	1	12.5%	0%	13%	/	11	/	/	11	11	/
	Belgium		10	1	1	2	10%	10%	20%	/	11	/	/	11	11	/

Legend: GBH: Glyphosate based herbicide, Nb.: number; > LOQ: detection with quantification, <LOQ: detection without quantification, []: concentration; AMPA: aminomethylphosphonic acid; HR beebread (oral) threshold value = 1000; HR wax (contact) threshold value = 5000, + detection is the sum of samples >LOQ and <LOD; S.D.: standard deviation; /: not determined. Multi-testing: a Fisher's exact test and a two-sample Wilcoxon rank-sum (Mann-Whitney) test were respectively used for each pairwise comparison of frequency of detection and mean concentration of the compounds. Different letters were used for significant differences. The first position letter corresponds to the comparison of regions for a same matrix; the second position letter corresponds to the comparison of breebread and beeswax for the mean concentration of GBH. A level of significance of 5% was used, divided by the number of tests performed for the Bonferroni correction.

6.3 Glyphosate-based herbicide residues and AMPA in beeswax

GBH residues were found in 32% of Belgian beeswax positive samples (N=100, T1). A significantly higher GBH residues prevalence was found in Wallonia (52.8% positive sample), as compared to Flanders (8.3% positive samples, Chi-square=23.76; df=1; p<0.001; confirmed by a logistic regression comparing contaminations in both regions (with Flanders as a reference): OR=18.4, 95% CI=4.66-72.60, p<0.001). The two-sample t-test with unequal variances (Welch's t-test) showed that average GBH residues concentration observed in Wallonia is significantly higher than in Flanders (p=0.0491).

6.4 Exposure assessment of GBH in beeswax

No trace of AMPA has been detected in beeswax. HR (contact) of beeswax for the maximum GBH residues concentration in Belgium is equal to 3.2 (= 320/100).

6.5 Wax renewal rate in Flanders and Wallonia

Beekeepers should renew the wax foundation of their bee colonies periodically. This improves bee health reducing the disease and chemical load of beeswax and allowing bees to rear their brood in a freshly built environment. Flemish beekeepers had a significant higher wax renewal rate (≥50% per year) as compared to Walloon ones (N =100, Fisher's exact test, p=0.017) (data not shown).

6.6 Risk assessment for the consumer of contaminated beebread and beeswax

As shown in table 1, GBH residues contaminated more frequently beebread (87.2% >LOQ) than beeswax (26% >LOQ) but the average concentration found in beebread (55.5 ng g-1) and wax (62 ng g-1) were comparable. A high consumption level (95th percentile) of the most contaminated pollen and beeswax by GBH residues, according to our results, leads to an exposure of respectively 0.936 and 0.007 µg GBH residues kg-1 b.w. day-1 through beeswax and pollen consumption. Concerning AMPA, the highest exposure corresponds to 0.334 µg AMPA kg-1 b.w. day-1 through pollen consumption.

6.7 Transfer of GBH residues and AMPA from wax to honey

We wondered if a transfer of GBH residues and AMPA from beeswax to honey was possible. Thus, to further test this hypothesis, we concomitantly collected both wax and honey from the bee colony honey supers of 10 apiaries. We found 1 out of 10 wax samples (10%) contaminated with GBH residues (concentration: 48 ng g-1). In honey, 2 out of 10 samples were contaminated by GBH residues (20%; 11 ng g-1 for the first sample and a detection lower than the quantification limit [LOQ] <10 ng g-1 for the second sample). These 3 positive GBH samples came from different bee colonies. No trace of AMPA was detected in any of the matrices. The highest GBH residues concentration detected in honey was about 5 times lower than the MRL (50 ng g^{-1}).

7 Discussion

7.1 Beebread

Our study showed an extended presence of GBH residues in beebread (81.5% positive samples at the national level) in both Belgian regions. AMPA was found in 18.5% of beebread samples at the national level. Only 2 samples contained AMPA without GBH residue. The LOQ values for glyphosate and AMPA are of 10 ng g^{-1}, which makes the analysis method very sensitive. Simultaneous AMPA/GBH residues detection in beebread could be explained by the GBH residues

degradation in the matrix or by their simultaneous occurrence in the environment. In soil, the primary pathway degradation of glyphosate residues is microbial action, which yields AMPA and glyoxylic acid (Roberts et al., 1999). The maximum GBH residues concentration found (700 ng g^{-1}) led to sublethal exposure (not acutely toxic to bees), corresponding to a dose of 84 ng bee^{-1} (0.08% of its LD_{50}), ingested over the first 10 days of life of a nurse bee. AMPA dose in beebread also corresponded to a sub-lethal exposure (to about 0.03% of oral glyphosate LD_{50}) alone or cumulated with GBH residues (about 0.12% of oral glyphosate LD_{50}). However, while the LD_{50} is measured as a one-time dose, bees could be exposed to GBH residues contaminated beebread for a longer period, when re-contamination occurs, since glyphosate degradation time DT_{50} ranges between 1.0 and 67.7 days. Therefore, the use of the LD_{50} as a single benchmark could underestimate the exposure risk to bees.

Bee and bee colony health is significantly impaired by doses that are lower than those we found through sub-lethal effects. Helmer et al. (Helmer et al., 2015) orally exposed bees to sub-lethal field realistic doses of GBH residues (1.25, 2.50, and 5.00 ng bee^{-1}) and showed a significant decrease ($p < 0.05$; $n = 40$) of beta-carotene and protein levels in their bodies after 10 days. Our results confirm Helmer's field-realistic doses (lower than 700 ppb, corresponding to 84 ng bee^{-1}). Other studies (Herbert et al., 2014), showed that adult *A. mellifera* workers exposed orally to 2.5 and 5 mg l^{-1} of GBH residues (field-realistic doses equivalent) presented reduced sucrose sensitivity leading to loss and difficulty in establishing associative memories, which, in turn, could cause inefficient collection of nectar and pollen for the colony and, finally, compromise its survival. Oral exposure to GBH residues concentrations (2.5, 5.0, and 10.0 mg l^{-1}, corresponding to a dose of 0.125, 0.25, and0.5 lg bee-1) affects honey bee cognitive abilities, with potential long-term negative consequences for colony foraging success (Balbuena et al., 2015). Exposures to 5 and 10 mg l^{-1} of GBH residues (dose of 0.25 and 0.5 lg bee-1) perturb the gut microbiota of honey bees. Bee gut symbionts influence bee development, nutrition, and defence against natural enemies (Motta et al., 2018). Perturbations of these gut communities may affect bee susceptibility to environmental stressors, including poor nutrition (Tosi et al., 2017) and pathogens (Motta et al., 2018). Moreover, in evaluating the effect of Roundup® on the royal jelly-producing glands, Faita et al.. (2018) showed that exposure to GBH residues resulted in the alteration of these glands that can trigger damage to the development and survival of bee colonies.

Regarding AMPA, no trace was found in honey and beeswax. In beebread, the maximum AMPA concentration was 250 ng g^{-1}. Because no information on AMPA toxicity to bees is available yet in the open literature, we were not able to assess its risks to bees. Nevertheless, Blot et al.. (2019) confirmed that glyphosate have sub-lethal effects on the honey bee microbiota, while AMPA did not induce any significant change.

7.2 Beeswax

Measured GBH residues concentrations should not cause acute lethal effects since the estimated HQ for beebread and beeswax (7 and 3.2, respectively) were far below the "safety" oral and contact thresholds (1000 and 5000, respectively). Since beebread can be stored in the hive for months after collection in the field, glyphosate degradation have likely reduced its concentration over time. Furthermore, bees typically collect multiple chemicals simultaneously (Tosi et al., 2018). Because bees are bio-indicators of environmental health and pollution, residues found in bee products provide valuable information on environmental punctual contamination or accumulation which, nevertheless, might be underestimated (i.e. residue degradation, dilution of highly-concentrated samples, technical limitations such as LOD) or overestimated (i.e. accumulation of contaminated pollen) (Tosi et al., 2018).

Due to glyphosate high water solubility and a very low octanol/ water partition coefficient (Log P (=Log Kow) at pH 7 and at 20°C =3.2), GBH residues were expected to be found only in beebread but not in wax (a very hydrophobic matrix). Beeswax samples contamination rate was of 26% at the national level. The addition of surfactant in the formulation of end-use pesticide products is at the origin of the phenomenon allowing glyphosate, which is water-soluble, to penetrate lipid-based structures (Shokri et al., 2001). Nevertheless, the risk assessment for honey bees and the consumer has been evaluated for glyphosate molecule solely without the concomitant formulation ingredients and adjuvants, nor other possibly concurring pesticides (Tosi et al., 2018). The use of the glyphosate/AMPA molecule solely does not render the combined toxic effects of the formulation constituents nor the synergetic potential effects of pesticide combinations.

Wallonia had both a higher GBH residue detection rate (53.8%) and a significantly lower rate of wax foundation renewal rate, as compared to Flanders ($p = 0.017$). This supports our hypothesis that the beekeeping management practice of renewing wax foundation can protect bees from the accumulation of pesticide residues inside the hive. No trace of AMPA could be detected in beeswax, probably because the matrix is not suitable for microorganism growth due to its rich hydrophobic protective properties (Fratini et al., 2016), resulting in no degradation of glyphosate in AMPA. Beeswax's conservative properties for pesticide residues combined with the beekeeping practice of wax recycling (Perugini et al., 2018), may be at the origin of the unequal detection of GBH residues in Flanders and Wallonia. This result highlights the importance of replacing at least 50% of wax frames per year, the current recommendation being the yearly replacement of 25 to 33% of the wax from the brood chamber (ITSAP, 2017; Vergaert, 2017).

For human health, the highest exposure to GBH residues in pollen corresponds to 0.312% and 0.187% respectively of the ADI and of the ARfD, and this through the pollen consumption (69.55 g day^{-1} $person^{-1}$ of contaminated pollen with 700 ng of GBH residues g^{-1}). The exposure to GBH residues through the beeswax consumption (1.29 g day^{-1} $person^{-1}$ of contaminated beeswax with 320 ng of GBH residues g^{-1}) corresponds to only 0.002% and 0.001% respectively of the ADI and of the ARfD. Concerning AMPA, the highest exposure to this compound corresponds to 0.111% of the ADI, and this through the pollen consumption (69.55 g day^{-1} $person^{-1}$ of contaminated pollen with 250 ng of AMPA g^{-1}).

7.3 Honey

The honey analysis resulted in a maximum GBH residues concentration of 11 ng g^{-1}, not exceeding the EU MRL (50 ng g^{-1}) for honey and theoretically meaning no risk for the consumer. In a survey on GBH residues in honey

samples originating from different countries (Brazil, Canada, China, Germany, Greece, Hungary, India, Korea, Mexico, Uruguay, New Zealand, Spain, Taiwan, Ukraine, Vietnam and USA), GBH residues were found in fifty nine per cent (59%) of analysed samples, with concentrations ranging between 17 and 163 ng g^{-1} (mean = 64 ng g^{-1}) (Rubio et al., 2014). Our concomitant analyses of wax and honey in samples (N = 10) from honey supers resulted in one wax sample being contaminated (48 ng g^{-1}). The low contamination in honey supers suggests that GBH residues are mostly stored in the brood chamber, where pollen and nectar are stored and where most bee activity occurs. This preliminary study showed no transfer from wax to honey. Because our results on the concomitant honey/wax contamination are based on limited data (N = 10), they should be confirmed with further studies.

For human health, considering our results and the assumptions we made with the available regulatory data, the consumption of these three contaminated food matrices (pollen, beeswax, and honey) would not be a food safety issue, nonetheless, caution should be taken in the interpretation the results as new studies confirmed glyphosate toxicity below regulatory limits (Mesnage et al., 2015), and the genotoxicity of AMPA (Mañas et al., 2009).

Bees are major pollinators in agricultural systems. Beebread, beeswax, and honey pesticide residue contamination can impact the viability of a colony when larvae develop on highly contaminated beeswax and feed with contaminated food (Orantes- Bermejo et al., 2010). Even a low concentration of pesticide residues can have amplified toxic effects on animals, including bees, through interactions with other chemicals (Zhu et al., 2017) or environmental stressors. The pesticide risk to bees can synergistically amplify the adverse effect of non-chemical stressors too and conversely, nutritional stress can synergistically increase the toxicity of pesticides (Tosi et al., 2017).

At best, our study gives a glimpse of bees and human exposures to GBH residues. At this stage, glyphosate is analysed alone, even though it is never used in this form but only as part of a mixture with adjuvants in commercial formulations. Clarifications and further research are needed to estimate the risk of the herbicide alone and in formulations (i.e. with the adjuvants), especially at levels below the regulatory safe limits and over longer durations. More studies are needed to assess synergies with other pesticides, and longer-term exposures at sub-lethal doses. More transparency is needed regarding the commercial formulation products.

Declaration of Competing Interest

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

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Experimental section

Study 5

Beekeepers perception of risks affecting colony loss: A pilot survey

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Preamble

The conclusion of the findings of the four studies carried out led us to want to understand beekeepers' perception of risk factors, intending to initiate a change in bee management practices (BMP), identify and prevent risks associated with beekeeping management may help avoid exacerbating colony loss rate. Before applying adequate risk management, beekeepers need to perceive the impact of risks on the colony, as well as the benefits of the actions to undertake. An unpreceded sociological survey designed with a grounded theory from health psychology was used to build a framework adapted to the beekeepers.

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ORIGINAL ARTICLE

Beekeepers' perception of risks affecting colony loss: A pilot survey

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Abstract

Understanding amateur beekeepers' perception of risks affecting bee health and mortality is essential to analyse the reasons for adopting or rejecting good management practices. A perception survey on how beekeepers perceive and manage factors related to climate change, *Varroa* infestation, management practices, and pesticide exposure was designed and launched online. This unpreceded sociological survey involved 355 beekeepers spread all over Belgium. A two-sample *t* test with unequal variances comparing beekeepers with colony loss rates below or exceeding the acceptable level, that is <10% and ≥10%, indicates that beekeepers (*N* = 213) with colony loss rates <10% generally have greater average levels of perceived risks and the benefits of action that lead to increased motivation to act in better ways. The results of this survey highlight the importance of looking beyond socio-economic de-terminants in any risk mitigation strategy associated with bee mortality when dealing with amateur beekeepers.

KEYWORDS

barriers, benefits, colony loss, Perception, risk, severity

2 | INTRODUCTION

While wild bees are acknowledged to be extremely important pollinators for many plant species, honey bees (*Apis mellifera*) remain the most economically and easily managed pollinator of the main crop monocultures worldwide (Klein et al., 2007). In recent years, the decline in pollinators, both wild and managed, has gained much attention (Samson-Robert et al., 2017), and increasing research efforts (Lundin et al., 2015). In light of these studies, a suite of numerous and interacting factors have been highlighted as possible variables having an impact on bee decline and mortality. These factors include the loss of foraging resources due to habitat loss and its homogenization (Kennedy et al., 2013), the introduction of invasive species (Monceau et al., 2014), climate change (Dennis & Kemp, 2016; Murcia Morales et al., 2020; Neumann & Carreck, 2010; Switanek et al., 2017), parasites (Goulson et al., 2015; Muli et al., 2014), pathogens (Doublet et al., 2015; Mondet et al., 2014), loss of genetic diversity (Oldroyd, 2007), exposure to pesticides (Cresswell et al., 2012; James & Xu, 2012; Johnson et al., 2010; Nazzi et al., 2012) and beekeeping management practices (Giacobino et al., 2017; Steinhauer, 2017; Steinhauer et al., 2020; vanEngelsdorp et al., 2012). Honey bees are managed pollinators, their survival relies thus on the competence and experience of the beekeeper (Steinhauer et al., 2018). Nevertheless, the impacts of beekeepers knowledge and management practices have often been overlooked (Jacques et al., 2017). When facing (e.g.) high pest pressure, beekeepers can reduce risks through physical or chemical interventions (Giacobino et al., 2014). While good management can alleviate stress, poor management can accentuate it. Good management practices or good risk management must be developed with proper education and experience (Steinhauer et al., 2018).

The Belgian beekeeping context is unique since 2/3 of the sector is made up of leisure beekeepers. The monitoring network of the European Honey Programme estimates at 1/3 the Belgian beekeepers with an economic profile. The beekeepers' category is defined by the size of the apiary as follow: amateur beekeepers (1-15 colonies), experienced amateurs (16-50 colonies), backyard beekeepers (51-150 colonies) and professional beekeepers (151-500 colonies) (Clermont et al., 2014). Honey bees are largely kept in stationary apiaries, for honey production, by passionate amateur beekeepers with relatively small operations and often, with a knowledge based on observation and experimentation. Beekeepers main occupation and source of income lay outside beekeeping; they keep bees because of the activity satisfaction they derive and the intrinsic values attached to beekeeping.

Before applying adequate risk management, beekeepers need to perceive the impact of risks on the colony, as well as the benefits of certain beekeeping management practices. Understanding beekeepers' perception of risks affecting honey bee health and mortality is essential to analyse the reasons for adopting or rejecting some beekeeping management practices. Identifying and preventing risks associated with beekeeping management may help avoid exacerbating colony loss rate (Giacobino et al., 2014). Risk perception consists of the importance that individuals give to an at-risk situation (Dewitt et al., 2015; Lamarque et al., 2011; Shackleton et al., 2019). It is known that risk perception is determined by different social and environmental factors affecting individuals, such as the degree of knowledge they have and/or the environment in which they live (Martín-López et al., 2012).

In this study, a grounded theory from health psychology was used to build a framework adapted to the beekeepers: the Health Belief Model (HBM) (Janz & Becker, 1984; Rosenstock, 1974) (Figure 1). The HBM was specifically developed for the understanding of health-related behaviour (Vande Velde et al., 2015). It has four key concepts: (1) perceived susceptibility is an individual's belief that a risk can occur. The relationship of perceived susceptibility to taking a risk management action is modified by (2) perceived severity of the risk, (3) the perceived benefits of risk management to mitigate the risk and its consequences, and the (4) perceived barriers to taking action. Beyond these, actions or intentions, health responsibility, and influences can also modify the relationship of perceived susceptibility to action. Actions (or intentions) include recognized clinical signs, knowledge and education. It is expected that greater levels of perceived risk combined with strong perceptions of the benefits of action will lead to increased motivation to act in better ways. Other intangible elements of risk perception and other motivations for strategy adoption within animal health risk management often remain unidentified though research on these issues is beginning to emerge (Ellis-Iversen et al., 2010; Jansen et al., 2009; Valeeva et al., 2007). This may be one of the reasons why the adoption of risk management strategies is hard to predict and influence (Valeeva et al., 2011).

This cross-sectional survey aimed to estimate the current state of perception of risks related to bee health and mortality at the level of amateur beekeepers in Belgium and to assess a possible association between colony loss, the perceived

susceptibility, severity, benefits and barriers as well as the demography, the actions or intentions, the health responsibility, and the influences.

Conventional production economics suggests that producers decisions are essentially economic ones, driven by the desire to maximize household welfare, net income or profit (Garforth, 2015). Since 2/3 of the Belgian beekeeping sector is made up of leisure beekeepers, we need to look beyond economic drivers in the search for an understanding of beekeepers decision and behaviour

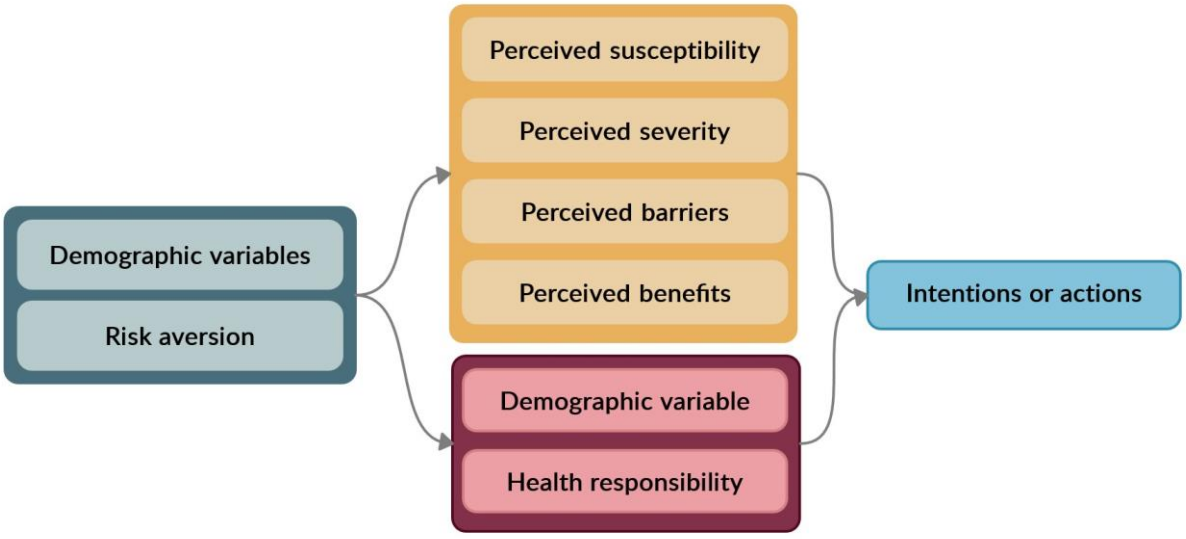

FIGURE 1 Basic elements of the health belief model applied for beekeepers in this survey

2 | MATERIALS AND METHODS

2.1 | Development of the perception survey

Compared to the number of registered beekeepers in the Federal Agency for the Safety of the Food Chain (FASFC) data-base was evaluated with a chi-squared test ($\alpha = 0.05$; df = 1). A two- sample t-test with unequal variances (Welch test) was performed to compare the means of each variable regarding the colony loss rate; that is under the 10% colonies loss threshold and above or equal the 10% (Morgenthaler, 1968). The cut-off level of 0.05 was considered as the p-value for significance. To check whether any clustering of responses indicative of a cultural influence would appear (i.e. northern versus southern parts of the country), a two-sample Wilcoxon rank-sum (Mann-Whitney) test was used.

3 | RESULTS

3.1 | Study population

The survey recorded 627 responses all over Belgium, from which 355 were considered complete and valid (i.e. 213 and 142 beekeepers with colony loss rate <10% and ≥10%, respectively). These valid answers represent 6.7% of the registered beekeepers number (N = 5,852) in Belgium in 2017. The number of French and Dutch-speaking beekeepers who participated in the survey is proportional to the number of Belgian beekeepers in each part of the country (Table 1). Indeed, the participation rate for the entire Belgian territory was 10.71% and was statistically proportional to the registered beekeepers on the FASFC database (Chi-squared test (1df; $\alpha = 0.05$) = 0.0007; p-value = 0.98). The completion rate (fully completed surveys) was 56.6%; the number of respondents per region was also statistically proportional to the number of participants to the survey per region (Chi-squared

test (1df; α = 0.05) = 0.14; p-value = 0.70) (Table 1). In total, the respondents managed 3,919 living colonies on 1 September 2016 with a median number of 7 colonies per beekeeper (min-max 1-150; mean 11.2; SD ± 14.8). Nationwide, beekeepers average experience was 16.9 (standard deviation = 15.6) years and the average loss rate for 2016 was 14.5% (standard deviation = 22.4). The average score (scale from 0 to 100) given by the beekeepers to their risk aversion was 60.4 with a standard deviation of 13.8 (Table 2). The 10% colony loss threshold was assessed with a kernel density estimation. This figure showed that the preliminary cut-off value of 10% is appropriate for Belgium.

TABLE 1 Participation and completion rates to the survey among Belgian beekeepers in 2017 (*N* = 627)

Region	No. FASCA Beekeepers 2017	Proportion beekeepers per region (%)	no. Total Participants	Participation rates	no. Complete survey 06/02/18	Completion rate among participants	Completion rate among registered beekeepers
Wallonia	1935	33.1%	207	10.7%	113	54.6%	5.8%
Male			*193*	*9.97%*	*102*		
Female			*14*	*0.72%*	*11*		
Flanders	3917	66.9%	420	10.72%	242	57.6%	6.2%
Male			387	*9.88%*	*225*		
Female			*33*	*0.84%*	*17*		
Belgium	5852	100.0%	627	10.71%	355	56.6%	6%
Male			*580*	*9.91%*	*327*		
Female			*47*	*0.80%*	*28*		

Legend: Participants are the beekeepers that participated in the survey with or without completing all the survey questions
.

3.2 | Descriptive analysis

3.2.1 | Health responsibility

For health responsibility, beekeepers cared the most about the quality of the honey they produce (average 90.9 and standard deviation 19.7). Nevertheless, bee health was as important as the honey production (average 80 and standard deviation 26.6), as well as bee health and environment protection (average 80.1 and standard deviation 25.7). For the beekeepers, a colony loss represented more than only an economical loss (average 91.7 and standard deviation 16.7) (Table 2).

3.2.2 | Perception of climate change

Beekeepers' opinions were divided on climate change impact on bee health (susceptibility) (average 65.3 and standard deviation 31.2) and climate change severity (average 59.8 and standard deviation 32.1). The scores given had important disparities and high standard deviation values compared to their respective average values. The perceived benefits of acting to mitigate climate change (average 60.3 and standard deviation 24.7) and the perception of barriers to mitigate it (average 72.2 and standard deviation 29.9) appeared though more uniform (Table 2). The two-sample Wilcoxon rank-sum (Mann-Whitney) test showed that the severity of climate change was perceived more significantly by the Walloon beekeepers (p =.0004).

3.2.3 | Perception of *Varroa* infestation

For Varroa perception, parasite susceptibility scored high (average 85.2 and standard deviation 24.1). Nevertheless, the perception of the severity of Varroa was less important (average 65.8 and standard deviation 22.3) (Table 2). The benefits of mitigating Varroa risk were positive and consistent (average 64.8 and standard deviation 20), and the barriers to reduce the Varroa risk did not seem challenging (average 63.1 and standard deviation 25.3).

.3.2.4 | Perception of pesticide exposure

For susceptibility and barriers, the distinction between pesticides coming out of agriculture and veterinary drugs was made. The susceptibility of high exposure to veterinary drugs scored low (average 19.5 and standard deviation 25.4), while susceptibility of agricultural pesticides scored higher (average 37.4 and standard deviation 31). Besides, a significant difference in the perceived susceptibility of exposure to pesticides; coming out of agriculture ($p = .014$) and due to veterinary use ($p = .046$) was found between the two Belgian regions since susceptibility was perceived as more severe in Wallonia (two-sample Wilcoxon rank-sum (Mann–-Whitney) test; $p < .05$). The scores concerning the severity of veterinary drug/agricultural pesticide (average 69 and standard deviation 21.6) and their benefits on colonies (average 58.9 and standard deviation 22.3) (agriculture and veterinary jointly) scored, respectively, high and moderately high; the answers to the questions were uniform as standard deviations were low. The barriers for reducing agricultural pesticides (average 34.5 and standard deviation 31.1) scored low similarly to the barrier for reducing pesticide-based veterinary drugs (average 39.2 and standard deviation 36.2) that were perceived significantly lower in Flanders ($p = .008$).

3.2.5 | Perception of management practices

Management practices stood out with the highest scores and with the most consistent opinions in terms of susceptibility (average 87.9 and standard deviation 20.3), severity (average 86.4 and standard deviation 20) and barriers (average 72.6 and standard deviation 16.4) compared to all other variables (Table 2). These concepts were well understood by the beekeepers. Nevertheless, they were not cohesive on the influence of the hive type on colony health, this lowered the benefits average score (average 67.4 and standard deviation 16) (Table 1)

3.2.6 | Intentions or implemented actions

The intentions or actions already implemented to mitigate the risks, scored generally high: for the equipment hygiene (average 78 and standard deviation 29.6), for the diagnosis and regular monitoring of Varroa infestations (average 77.6 and standard deviation 27.9), for beekeepers adaptation to environmental changes through their management practices (average 72.9 and standard deviation 28.8), and the complete replacement of old comb wax in the hive body every four years (average 83.1 and standard deviation 26.1). Combining Varroa treatments scored high (average 73.3 and standard deviation 34.1) but treating only the colonies affected by moderate to high infestations of Varroa was more controversial (average 39.3 and standard deviation 36.1). The more sensible use of varroacides to delay resistance development scored relatively high (average 65.8 and standard deviation 37.9). Avoiding to overwinter weak colonies scored high (average 73.1 and standard deviation 33), but avoiding to overwinter too strong colonies scored low (average 14.8 and standard deviation 23.5). The score awarded by the beekeepers to the use of a partition in the winter was moderate (average 54.6 and standard deviation 37.5). Nevertheless, the use of partitions was significantly higher in Wallonia ($p < .01$).

TA B L E 2 Average results (scale: between 0 and 100) and standard deviation for measures of demography, risk aversion, health responsibility, perception of climate change, Varroa infestation, management practices and exposure to pesticides), actions or intentions, and influences

	Variables		Mean	Standard deviation
	Beekeeping experience (years)		16.9	15.6
		Flanders	17.3	15.7
		Wallonia	16.1	15.6
Demography	Gender	Male %	92.5	-
		Female %	7.5	-
	No. hives 1 September 2016		11	14.8
	No. new colonies 2016-2017		5.2	7

	No. losses 1 September 2017		1.7	3.4
	No. colonies 1 September 2017		16.3	20.3
	Average Loss 2017 Belgium [%]		14.50	22.4
		Dutch speaking	13.7	22.4
		French speaking	16.2	22.5
Psychological characteristics	Relative risk aversion		60.4	13.8
Health responsibility	- Bee health is reflective of the health of the environment		79.9	25.8
	- I believe I am responsible for the health of the people consuming my honey		90.9	19.7
	- The loss of my colonies represents more than just a material loss		91.7	16.7
	- A colony is easily replaceable next year, without too much effort		43.6	32.1
	- Honey production is as important as the health of my colonies		79.9	26.6
Climate change	Susceptibility	- Long and mild winters are more and more frequent, and worrying for beekeeping	65.2	31.2
	Severity	- Long and mild winters have a significant impact on the strength/survivorship of my colonies over the winter	59.7	32.1
	Benefits	- Efficiency of Equalizing colony strength to limit the impact of climatic changes	60.3	24.7
	Barriers	- Climate change is an inevitability and its consequences on beekeeping are unavoidable	69.7	32.5
		- Overwintering only medium to strong colonies is not an important time investment	74.7	27.3
		Average score Barriers	72.2	29.9
***Varroa* infestation**	Susceptibility	- Every year, all of my colonies are affected by Varroa	85.2	24.1
	Severity	-Varroa has had a large economic impact on my operation in the last 3 years	44.3	34.3
		- An uncontrolled Varroa infestation would have a large negative impact on the health of the colony	87.8	21.6
		Average score severity	65.8	22.3
	Benefits	- Beekeepers should combine several methods (chemical/acid/biotechnical) to better control Varroa	70.1	35.1
		- Beekeepers should not systematically treat all colonies in an apiary without diagnosis of the infestation level (amitraz, apistan, apivar, polyvar...)	61.6	37.5
		- Beekeepers should adopt a more sensible use of varroacides to delay the development of resistance	62.4	37.9
		Average score benefits	64.8	20
	Barriers	- The prevention of Varroa infestation relies above all on measures put in place by authorities, still at my individual beekeepers level, there's much I can do about it.	72.0	32.4
		- Are the recommendations from FASFC regarding Varroa treatments efficient (the efficiency is assessed considering both the cost of the product, time invested in its application, and its capability to prevent the disease and/or colony losses)	54.2	29.8
		- The individual diagnosis of Varroa in each colony before selective application of Varroa treatment does not require too much time.	63.1	33.4
		Average score barriers	63.1	25.3
Pesticide exposure	Susceptibility	My colonies are affected by abnormally high exposure to agricultural-related pesticides	37.2	31
		My colonies are affected by abnormally high exposure to beekeeping-related pesticides (systematic or repetitive anti-Varroa chemical treatments)	19.5	25.4
	Severity	- Pesticide contaminations (both agricultural and beekeeping-related) and their accumulation in beeswax are responsible for the high colony losses	50	30
		- Pesticide use (in agriculture and beekeeping) leads to cancer in humans	67.2	30.6
		- Pesticide use (in agriculture and beekeeping) leads to environmental contaminations	83.5	24.4

		- Pesticide cocktails (used in agriculture and beekeepeing) have a higher impact on colonies than single pesticides	75.6	29.4
		Average score severity	69	21.6
	Benefits	- Beekeepers should limit the use of Varroacides by monitoring the infestation level of each colony before treatment	65.2	33.3
		- Beekeepers should alternate the use of synthetic (eg. apivar) and organic (eg. Oxalic acid) varroacides or other non chemical treatments	69	34.8
		- Beekeepers should only treat the colonies affected by moderate to severe Varroa infestation	42.3	35.7
		Average score benefits	58.9	22.3
	Barriers	- The reduction of pesticide use by farmers/authorities in agriculture is foreseeable soon	65	31.1
		- Systematically treating the colonies with conventionel Varroa treatment products (Apistan,Apivar...) is necessary for the survival of colonies.	39.2	36.1
Management practices	Susceptibility	- Not using good beekeeping practices can cause colonies to weaken	87.9	20.3
	Severity	-If you were not using good beekeeping management practices, what would be the probability of colony loss?	86.4	20
	Benefits	- Disinfecting beekeeping equipment before its re-use (blowtorch/disinfectant...)	74.3	30.7
		- Monitoring regularly for Varroa infestation levels	80.4	24.3
		- The choice of hive type	28.4	29.7
		- The adaptability of the beekeepers practices to environmental changes	70.6	29.6
		- The full replacement of wax from the brood chamber every 4 years	83.2	24.2
		Average score benefits	67.4	16
	Barriers	- I am constantly improving my beekeeping practices/knowledge	90.2	17.4
		- A change in my beekeeping practices would not require a considerable investment in time	64.6	29.3
		- A change in my beekeeping practices would be accepted by the other beekeepers/association/federation	62.9	28.9
		Average score barriers	72.6	16.4
Intention or actions	- Avoid overwintering too weak colonies		73.5	32.8
	- Avoid overwintering too strong colonies		14.8	23.6
	- Partition (with one or two partitions) my colonies to limit their width		54.6	37.5
	- Adapt my beekeeping practices according to environmental changes		72.9	28.8
	- Disinfect my beekeeping equipment before reusing it (blowtorch/disinfectant...)		78.0	29.6
	- Combine several methods (chemical/acid/biotechnical) to better control Varroa		73.3	34.1
	- Regularly monitor for Varroa infestation levels colony by colony		77.6	27.9
	- Only treat colonies affected by moderate to high infestations of Varroa		39.3	36.1
	- Use varroacides more sensibly (apivar, apistan …) in order to delay the development of resistance		65.8	37.9
	- Replace all wax from the brood chamber every 4 years		83.1	26.1
	- Avoid introducing swarms from unknown origin in my apiary without quarantine/treatment		70.9	35.8
Influences	- My colony losses		76.5	28.9
	- The beekeepers in my association/federation		56.5	31.3
	- Research and information centers		65.2	29.5
	- Universities		54.9	33.1
	- The news (newspapers, journal, magazines, internet, …)		54.7	30.2
	- The recommendations/mandatory actions from FASFC		53.4	32.1
	- My health		77.5	29.4

- The protection of the environment	83.0	23.3
- Honey production	52.6	30.8
- Queen production	54.2	35

3.2.7 | Influences

Beekeepers are most often guided in their risk management choices by the protection of the environment (average 83 and standard deviation 23.3), this more importantly in Wallonia (p <.01). The protection of their health (average 77.5 and standard deviation 29.4), as well as the loss of their colonies (average 76.5 and standard deviation 28.9), influenced their risk management. Research and information centres (average 64.9 and standard deviation 29.4) seemed to have more influence on their risk management than beekeeping federations and unions (average 56.8 and standard deviation 31.2), and universities (average 55 and standard deviation 33.2). The influence of universities seemed significantly more important in Flanders than in Wallonia (p =.027). Honey production seemed of secondary importance (average 54 and standard deviation 35) in the risk management choices and appeared significantly more important in Flanders (p =.004).

TA B L E 3 Welch test's significant variables (p-value <0.05) for loss rates above and under 10%

		<10% loss (*N* = 213)		≥10% loss (*N* = 142)		
Variable	**Item**	**Average**	**Standard deviation**	**Average**	**Standard deviation**	**Welch test**
Demographic variables	No. colonies September 1, 2016	12.67	17.25	8.58	9.41	<0.0001
	No. split/increased/bought colonies between 1 September 2016 and 1 April 2017	6.31	8.17	3.65	4.25	<0.0001
	No. lost colonies 1 September 2017	0.38	0.83	3.74	4.65	<0.0001
	No. living colonies on 1 April 2017	18.98	23.59	12.23	12.85	0.0003
Health responsibility	Bee health/environment awareness	78.26	27.25	82.83	22.95	0.04
	Splits and making up colonies, without much effort	49.29	33.51	35.49	27.46	<0.0001
Susceptibility	*Varroa* infestation	71.38	22.85	63.75	25.26	0.002
	Management practices	90.15	16.95	84.52	24.16	0.008
	Exposure to pesticide	34.46	29.52	41.69	32.63	0.02
Severity	Climate change	55.45	33.12	66.12	29.87	0.009
	Varroa infestation	63.80	21.56	68.90	23.16	0.02
	Management practices	88.31	18.34	83.40	21.90	0.014
Benefits	Management practices	68.96	15.11	65.10	17.12	0.015
Intentions or actions	Avoid overwintering too weak colonies	77.04	30.97	67.30	35.22	0.004
	Equipment disinfection before reuse	80.78	27.53	74.14	31.83	0.02
	Complete wax replacement every 4 years	85.28	25.01	80.33	27.04	0.04
	Avoid introducing swarms from unknown origin without quarantine/treatment	75.13	34.47	64.57	37.10	0.004
Influences	Colony losses	74.69	31.04	79.94	24.97	0.04
	Packages/queen production	57.34	34.59	48.85	35.22	0.013

3.3 | Statistical analysis

The Welch test was performed to compare loss values above and below the acceptable mortality threshold defined at 10%. Results indicate that beekeepers with loss rates lower than 10% had a higher average number of colonies (average 12.67 and standard deviation 17.25; *p*-value <0.0001) and a significantly higher ability to split and make up for losses (average 6.31 and standard deviation 8.17; *p*-value <0.0001). Their score in perceiving *Varroa* infestation occurrence was significantly higher (average 71.38 and standard deviation 22.85; *p*-value = 0.002), and they were well aware of management practices positive impact (average 90.15 and standard deviation 16.95; *p*-value = 0.008) and severity on their colonies (average 88.31 and standard deviation 18.34; *p*-value = 0.014). Nevertheless, these beekeepers scored significantly lower in the perception of climate change severity (average 55.45 and standard deviation 33.12; *p*-value = 0.009) and the perception of *Varroa* infestation severity (average

63.8 and standard deviation 21.56; *p*-value = 0.02). They better perceived the benefits of good management practices (average 68.96 and standard deviation 15.11; *p*-value = 0.015) and scored higher at all questions related to actions/intentions. Significant results of the two-sample *t* test with unequal variances are depicted in Table 3.

4 | DISCUSSION

This first nationwide cross-sectional survey focused on how beekeepers perceive and manage risks (climate change, Varroa mite, management practices and exposure to pesticides), in their colonies, in Belgium. Social science theories and disciplines offer tools that can help explore the rationality of beekeepers' behaviour concerning risk factors. These tools include several models from social psychology (the Theory of Reasoned Action, Theory of Planned Behaviour and Health Belief Model), which were originally used to analyse human health behaviour but have more recently been used to understand the factors that affect farmers' or beekeepers' animal health management. Understanding beekeepers' perception of risks affecting their colonies' health and mortality is crucial to better understanding beekeepers attitudes towards risks and potentially, to adopt adapted management practices.

Population representativeness was achieved when compared to the number of voluntarily registered beekeepers on the FASFC database. Representativeness was confirmed by a chi-squared test, and the respondents were the subset of the target population. Nevertheless, the real number of beekeepers in Belgium and per region was difficult to obtain, as most beekeepers are amateurs and reluctant to register themselves.

Beekeepers' general attitude towards risk was derived by measuring their degree of risk aversion using four statements on general issues. Beekeepers seemed to have a common understanding of general risk. Nevertheless, for most questions, beekeepers' perception seemed divergent, as important disparities appeared with standard deviation values that were high compared to their respective average values (i.e. Climate change perception, though it has been pointed out as one of the causes of colony loss by scientific research (Dennis & Kemp, 2016; Flores et al., 2019)). Risk perception varies according to the beekeepers' gender, the amount of knowledge obtained through experience and education (Lamarque et al., 2011; Martín-López et al., 2012; Shackleton et al., 2019), and their behaviour. Another possible explanation for this disparity is the lack of heterogeneity in beekeeping education. The results of the two-sample Wilcoxon rank-sum (Mann-Whitney) test, used to check the possible clustering of responses according to the region (Flanders and Wallonia) indicated a cultural or regional influence on the perception of some variables.

For *Varroa* perception, parasite susceptibility (the belief that an infestation can occur) was well perceived unlike the severity of the parasite (the perceived outcome of the infestation). The benefits of mitigating *Varroa* risk (expected effectiveness of the practices to modify the infestation's consequences) were understood, and the answers were uniform without being unanimous. The susceptibility of exposure to veterinary drugs was less important than the exposure to agricultural pesticides; additionally, the perceived risk of pesticide exposure did not seem to be alarming. A significant difference in this risk perception was found between the two Belgian regions as the risk was perceived as more severe by the Walloon beekeepers. Reducing the use of veterinary drugs and agricultural pesticides was perceived as difficult to accomplish. This is comprehensible as some beekeepers see veterinary drugs as the cost for low infested colonies. Agricultural pesticides are out of their preview. The scores awarded in response to the question dealing with reducing the use of veterinary drugs were not uniform and their mean had a high standard deviation value. As suggested in one of the few studies on beekeeper beliefs and stewardship, the lack of homogeneity in *Varroa* and pesticide risk perception could result from the coexistence of different beekeeping groups that tend to treat *Varroa* in different ways (Thoms et al., 2018; Underwood et al., 2019). Three trends in the management of *Varroa* seem to coexist among Belgian beekeepers: the first trend is the use of systematic drug treatments, without diagnosis of *Varroa* infestations. The second trend implements the monitoring of *Varroa* infestations and the use of acids (oxalic) to decrease *Varroa* pressure on honey bee colonies when required, corresponding to national *Varroa* control recommendations. The third trend is to start relying on the selection of *Varroa*-resistant honey bees instead of treating them.

Compared to the three previously assessed risks (climate change, *Varroa* infestation and pesticide exposure), the respondents seemed to perceive the importance of management practices more uniformly than any of the three other risks. This was of utmost importance for the health and survival of honey bee colonies, as management practices are crucial to compensate for the effects made

worse by the *Varroa* infestations, climate changes, and many other interacting stress factors for honey bees. Beekeepers felt the most responsible for the quality of the honey they produce and were mostly influenced by their health and environment protection as well as by the colony loss. These elements could be considered as a lever for adopting better management practices.

The parametric Welch test was performed to compare the perception of beekeepers with colony loss of acceptable and non- acceptable levels, assuming that beekeepers with acceptable loss rates have better risk management. There are no historical values regarding the acceptable levels of colony losses in Europe, numbers varying according to countries. Nevertheless, this question has been addressed and discussed in the first EPILOBEE program, where different colony losses were reported in European countries (Charrière & Neumann, 2010; Genersch et al., 2010) and outside Europe (Engelsdorp et al., 2008). The empirical threshold of 10% is considered acceptable by the European Union Reference Laboratory for Bee Health (EURL) for European winter honey bee colony mortality. In some areas of Europe and other parts of the world, higher or lower mortality rates can be considered bearable by beekeepers and scientists. The empirical threshold of 10% is considered acceptable in Belgium; this value was confirmed by the Kernel density of mortality.

The results indicate that beekeepers with acceptable loss rates had a higher average number of colonies, and had a better ability to split and make up for losses than the ones with non-acceptable loss rates. The size of the apiary and the age and experience of the beekeeper have already been reported as factors directly linked to the survival of the honey bee colony (Brodschneider et al., 2016; Jacques et al., 2017). We assume that these results express better capacities in risk management and thus in management practices, and a proactive approach of beekeeping. The scores of the benefits of reducing the risk of colony loss through better management practices confirm our assumption. These risks were significantly better perceived by the beekeepers with acceptable loss. This confirms the hypothesis that greater levels of perceived risk combined with strong perceptions of the benefits of action would lead to increased motivation to act in better ways. Nevertheless, those same beekeepers had a poor perception of climate change severity and *Varroa* infestation severity. We cannot state with certainty whether these perceptions were due to the beekeepers' resilience or the lack of the perception of the impact resulting from good management practices. No other studies allowing comparison are currently available.

5 | CONCLUSIONS

The overwhelming majority of Belgian beekeepers are amateur beekeepers. Understanding their perception of the risks affecting colony health and mortality is crucial to analyse the reasons for adopting or rejecting some beekeeping management practices. Beekeepers with a greater level of perceived risk combined with strong perceptions of the benefits of action have increased motivation to act in better ways and have acceptable loss rates. Despite a good general estimate of risks to bee colonies, the agricultural pesticides, and veterinary drug treatment issue appears to be a source of confusion and misunderstanding. Clear and harmonised information should be integrated into risk management recommendations. We need to take different approaches with the different beekeeper groups who are convinced of the efficacy of managing *Varroa* their way. The low consideration of the financial impact that the loss of a colony entails seems to be an obstacle to the implementation of measures to limit the risk. The results of this survey highlight the importance of looking beyond socio-economic determinants in any strategy aimed at mitigating the risks associated with colony loss. To successfully translate recommendations in such a way that the adoption of good management practices will be facilitated, more socio-psychological research is essential.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL APPROVAL

Due to the nature of the survey and the low risk posed to participants, formal approval from an Ethics Committee was not a requirement at the time of the survey.

DATA AVAILABILITY STATEMENT

The data that support the findings of this survey are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Experimental section

Study 6

Adulteration of beeswax: A first nationwide survey from Belgium

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Noëmie El Agrebi and Lidija Svečnjak, Jelena Horvatinec, Véronique Renault, Agnes Rortais, Jean-Pierre Cravedi and Claude Saegerman

Preamble

Around 2016, an increasing number of reports dealing with the effects of contaminated or adulterated foundations as the main cause of poor brood and colony development. Beekeepers reported that affected colonies were showing a holey brood pattern and a decline in population size. The bees accepted the comb material in the breeding area poorly, and young larvae died. The symptoms have been linked to various possible causes including diseases, poor quality queens, residues of pesticides in wax, and poor quality of the wax foundation. To investigate this issue and assess the adulteration in Belgian beeswax from the beekeepers and the trade we implemented a first nationwide survey.

PLOS ONE

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RESEARCH ARTICLE

Adulteration of beeswax: A first nationwide survey from Belgium

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Abstract

Beeswax is intended for use in the beekeeping sector but also in the agro-food, pharmaceutical or cosmetics sectors. The adulteration of beeswax is an emerging issue that was reported lately on several occasions in the scientific literature. This issue tends to become more frequent and global, but its exact extent is not accurately defined. The present study aims to assess the current situation in Belgium through a nationwide survey. Randomized beeswax samples originating from Belgian beekeepers (N = 98) and commercial suppliers (N = 9) were analysed with a Fourier transform infrared spectroscopy (FTIR) coupled with Attenuated Total Reflectance (ATR) accessory (FTIR-ATR spectroscopy) for adulteration. The survey revealed a frequency of 9.2% and 33.3% of adulteration in beekeepers' beeswax samples (9 samples out of 98: 2 with paraffin and 7 with stearin/stearic acid) and commercial beeswax samples (3 samples out of 9: all adulterated with stearin/stearic acid), respectively. The analysed samples were adulterated with various percentages of paraffin (12 to 78.8%) and stearin/stearic acid (1.2 to 20.8%). This survey indicates that in the beekeeper's samples, beeswax adulteration was more frequent in comb foundation and crude beeswax than in comb wax. With the example of this nationwide survey conducted in Belgium, this study shows the emergence of the issue and the urgent need for action to safeguard the health of both honey bees health and humans, in particular with the setting of a proper regulation legal framework and a specific routine analytical testing of commercial beeswax to ensure beeswax quality.

Introduction

Honey bees (*Apis mellifera* L.) are the main pollinators in agricultural ecosystems [1]. Beeswax is essential for the beekeeping sector (production of comb foundations) but also for agro-food, pharmaceutical and cosmetics sectors. In Europe, beeswax is considered as an animal by-product Category 3 material and, therefore, it is not intended for human consumption [2]. However, beeswax is an authorized food additive in the European Union [3] and is a food sub-stance considered as safe according to the U.S. Food & Drug Administration, FDA (21CFR184, 1973) [4]. In some cases, honey is sold with honeycombs to demonstrate its authenticity [5,6], resulting in a dietary exposure to beeswax for consumers eating both the honey and the comb and those exposed to honey comb debris present in honey. As reported by Hargrove et al. [7] consumption of beeswax may reach a few grams per day and per person in a small portion of the population [7], but such dietary exposure could be increased with this practice being more frequently advertised and promoted via internet (online sales of ready-to-eat honeycombs).Therefore, there is a concern that the adulterated beeswax might enter into the food chain (e.g. through the use of honeycombs) and present a risk to human health [8].

On a global scale, between 2016 and 2018, a yearly average production of 1.9 million tonnes of honey and 69,000 tonnes of beeswax were registered in the FAOSTAT database [9]. Indeed, managed honey bee colonies represent an important source of goods and income [10]. Despite a slow increase of managed honey bee colonies to face agricultural demand for pollination [11], several monitoring programs indicate a global decline in bee populations around the world (e.g. [12–14]). Multiple stress factors, or drivers [15] affecting honey bees, alone or in combination [16–20] are referred to as a possible explanation of this decline.

Besides, in the recent years, beeswax adulteration with paraffin and/or stearin (e.g. [21–26]) has become a growing and alarming concern. The practice of adulteration is emphasised by the fact that beeswax is often salvaged, re-melted, and reused within the beekeeping sector [27].

However, few representative (randomized survey) and published reports are available on the prevalence, the type and the level of adulteration of beeswax. At European level, the most recent study [28] using an advanced method of detection of adulteration (Fourier transform infrared spectroscopy (FTIR) coupled with Attenuated Total Reflectance (ATR) accessory, so-called FTIR-ATR spectroscopy) revealed that, among 137 samples of comb foundation or wax blocks originating from 15 different countries sampled between 2016 and 2018, 59.9% were adulterated by paraffin and/or stearin. Within these samples, levels of adulteration were comprised between 5–93.5% (for paraffin) and/or stearin (solely in Belgium and The Netherlands representing 7.3% of the samples with a level of adulteration between 18.75 and 31.25%). No trace of other adulterants (e.g. tallow, carnauba wax) were detected.

The effect of beeswax adulteration on honey bee health (especially on brood) and human health (through the consumption of bee products) are currently poorly studied [27,28]. However, in Belgium, adverse effects of adulterated beeswax foundations on bee brood development were recently identified [29–31]. This study showed that adulteration levels as low as 5% and 7.5% of stearic and palmitic acids, respectively led to brood mortality rates above 45%.

According to the EU Food Fraud Network (https://ec.europa.eu/food/safety/food-fraud/ ffn_en), a dedicated Network for cross-border non-compliances related to food and feed, adulteration of beeswax, that is intended for honey production, with paraffin and/or stearin is considered as a fraud, when meeting four criteria (violation of Law, intention, economic gain, and consumer deception) [32].

To clarify the situation, the European Commission requested the European Food Safety Authority (EFSA) to define purity criteria for beeswax and to assess the health risks for honey bees and humans [8,31,33].

The present study aims to assess the current situation of beeswax authenticity in Belgium through a nationwide cross-sectional survey. Randomized beeswax samples originating from Belgian beekeepers, and commercial suppliers were analysed for adulteration using FTIR-ATR spectroscopy.

Materials and methods

Sample selection

In Belgium, 200 beekeepers were randomly selected from the Federal Agency for the Safety of the Food Chain (FASFC) beekeepers database, which included 4,949 registered beekeepers in 2015. One apiary per beekeeper was sampled for beeswax between May and November 2016. The number of beekeepers was stratified by province. Out of the selected beekeepers (N = 200), 91.5% of them provided a beeswax sample for analysis (N = 182).

To be able to detect an expected minimum prevalence of 3% of adulteration with a confidence level of 95%, and considering a population size of 4,949 registered beekeepers, we estimated the sample size for this survey at 98 beekeepers. Indeed, a sub-sample of 98 samples was randomized, and further submitted to the laboratory for analysis of adulterants (i.e. paraffin, and stearin/stearic acid). All sampled bee colonies seemed healthy, with no clinical signs of infectious diseases or acute intoxication.

Eight beeswax (comb foundation) samples randomly collected from different commercial suppliers in Belgium and one additional, achieved by the FASFC, from a Chinese batch of beeswax (2015) where mosaic brood was reported, were analysed for adulteration. All samples were kept in hermetic plastic bags and stored at -20˚C until analysis.

Sample preparation

Comb wax samples collected from the beekeepers were melted by boiling water prior to further analysis in order to remove hive-originating impurities and homogenize the samples into crude beeswax. In case of significant contamination (e.g. significant amount of residues of cocoons in brood combs), samples were re-melted 2–3 times until they were completely purified. Crude beeswax samples and comb foundations were analysed as obtained.

Beeswax adulteration detection by FTIR-ATR spectroscopy

Preparation of in-house reference material (genuine/authentic beeswax, adulterants, and adulterant-beeswax mixtures containing different proportions of adulterants) for calibration purposes, was performed according to the procedure described in a chapter of the BEEBOOK manual on standard methods for A. mellifera beeswax research by Svečnjak et al. [34] (see section "6.2.5.1. Generating IR spectral database of reference samples") with a modification of preparing the adulterant-beeswax mixtures by following 5% increasing sequence of adulterant addition (instead of originally proposed 10%) to improve precision in detecting adulterants in beeswax. For this, in total 38 adulterant-beeswax mixtures were prepared: 18 paraffin-beeswax mixtures (containing 5 to 95% of paraffin; Paraffinum solidum, Ph.Eur. 7,8, Kemig, Croatia), and 18 stearic acid-beeswax mixtures (containing 5 to 95% of stearic acid; Acidum stearicum, Ph.Eur. 8.1, Kemig, Croatia). Mixtures were placed in a temperature chamber for 3h at 90˚C for melting and homogenization. Pure paraffin, pure stearic acid, as well as genuine (pure) beeswax, were subjected to the same temperature treatment in the same way as adulterant-beeswax mixtures.

Beeswax samples were analysed by Fourier transform infrared spectroscopy (FT-IR) using an Attenuated Total Reflectance (ATR) recording technique. Infrared (IR) spectra of investigated beeswax samples were acquired using Cary 660 Fourier transform mid-infrared spectrometer (Agilent Technologies, Palo Alto, CA, USA) with a DTGS (deuterated triglycine sulphate) detector and CsI (cesium iodide) optics, coupled with Golden Gate high temperature (up to 200˚C) heated single-reflection diamond ATR accessory (Specac).

FTIR-ATR spectra of prepared in house reference material and collected Belgian beeswax samples were recorded under the same conditions (in the liquid state at 75˚C; spectral range: 4000–400 cm-1; spectral resolution: 4 cm-1; 64 scans/spectrum) in accordance with the method described by Svečnjak et al. [34] in the BEEBOOK section "5.3.2. Analysis of beeswax by IR spectroscopy/5.3.2.1. FTIR-ATR recording technique".

Raw spectral data were stored and pre-analyzed using the software package Resolutions Pro version 5.3.0 (2015) (Agilent Technologies, Palo Alto, CA, USA). Further chemometric model-ling and statistical analyses were performed using the software package specialized for spectral data analysis—Origin version 8.1 (Origin Lab Corporation, Northampton, MA, USA). Prediction strength and prediction error of calibration model were estimated by the simple linear regression whereas prediction strength and prediction error in detecting the adulteration level were determined, i.e. coefficient of determination (R2) and standard error (SE). Quantification of adulterants in beeswax was carried out automatically using the instrument software (Resolutions Pro) after establishing and evaluating the calibration procedure.

Epidemiological analysis

Data on bee mortality

The sampling in beekeepers (N = 98) was conducted jointly with a questionnaire to record colony losses and management practices. The total loss rate (winter and seasonal) was calculated by dividing the total number of colonies lost between September 2015 and April 2016 by the number of colonies in September 2015 multiplied by 100 [35] excluding removed, sold, and purchased colonies.

Mapping

The map (Fig 1) was produced by a co-author (VR) with quantum-GIS. The GPS data for the country and regional boundaries originate from a copyright free website: DIVA-GIS | free, simple & effective (diva-gis.org). The coordinates of the sample points were collected during the survey and registered into an Excel file. They have been projected with quantum GIS on the country layer and the map. This is therefore an original map with no copyright issues.

Statistical analyses

The percentage and corresponding 95% confidence interval (95% CI) of paraffin and stearin/stearic acid adulteration was estimated using an exact binomial distribution [36].

Two logistic regressions were performed. The first one was done using both samples form beekeepers and commercial suppliers (N = 107) and for which the information on the type of beeswax was available (i.e. comb wax as a reference group, comb foundation and crude beeswax from beekeepers and another beeswax from the commercial suppliers). The second one was done using only samples from beekeepers (N = 98) for which more information was available. For the second one, a univariate logistic regression model was used to explain adulteration expressed as binary dependent variable ("1" as adulterated and "0" as non-adulterated beeswax samples). The following exploratory variables were considered: the type of beeswax (categorical variable, which includes comb wax as a reference group, comb foundation, and crude beeswax), the year of introduction of the beeswax in the hive (categorical variable), the province of origin of the beekeepers (categorical variable), and the colony loss rate (continuous variable).

For the type of beeswax, the following definition was used: (i) comb wax (beeswax from old combs from the brood chamber provided by some beekeepers), (ii) comb foundation (beeswax foundation present in beekeepers as a mixture of beeswax from different trade origins), (iii) crude beeswax (melted old brood, and/or honey wax combs, and or cappings to be reused), and (iv) beeswax form suppliers (foundation sold by suppliers).

For the colony loss rate, two binary levels were considered: "0" for colony mortality rates <10%, and "1" for colony mortality rates >10% [37].

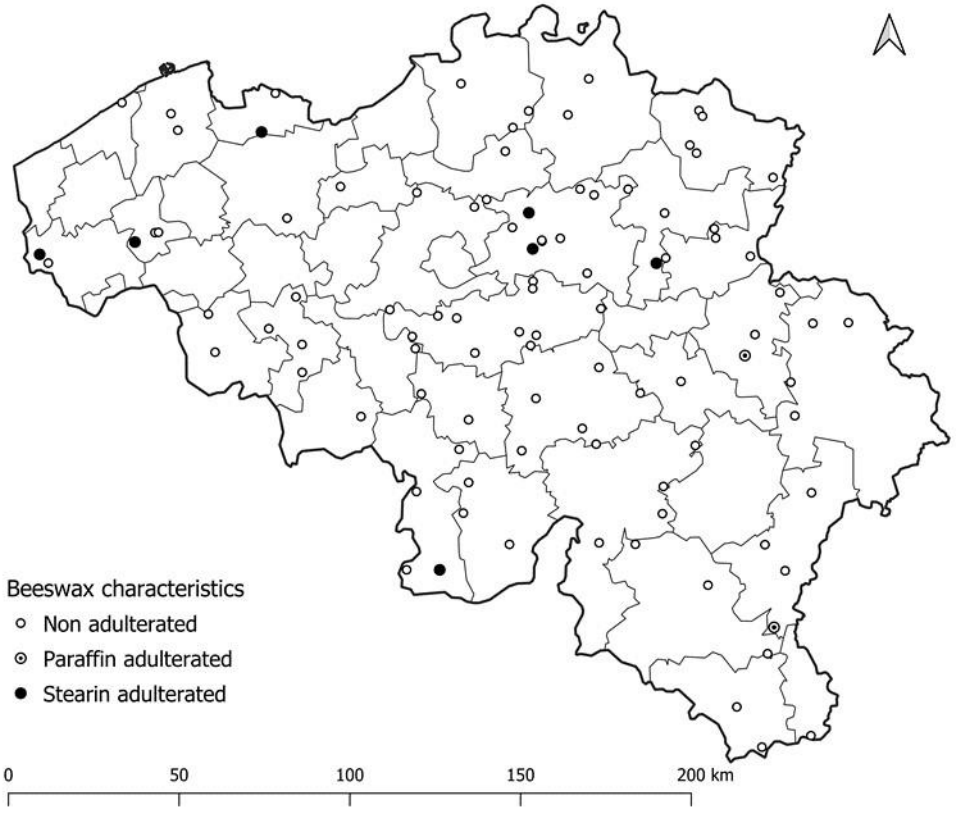

Fig 1. Location of the samples provided by the beekeepers (N = 98) in the Belgian administrative districts.

Then, a multivariate logistic regression was performed using the most significant variables (p-value < 0.2) out of the univariate model. The use of the Firth logit method allowed inference of odds ratios and 95% confidence interval (CI) when complete separation (zero-cells) occurred [38]. Finally, in a backward stepwise multivariate model, the least significant variable (with the highest p-value) were eliminated in a step-by-step approach. At each stage, a likelihood ratio test was used to compare the complex and simplified models. When there was no significant difference between them (using value of P > 0.05), the simplified model was used. The goodness of fit of the final multivariate model was assessed using the Hosmer-Lemeshow goodness-of-fit test [36]. All models and tests were performed using Stata SE 14.11 (StataCorp LP, College Station, TX, USA), and the limit of statistical significance of performed tests was defined as 0.05.

Results

Indirect validity of the randomization of beeswax samples provided by some beekeepers

For this cross-sectional survey, the sample size used was of 98 samples out of the 182 original beeswax samples (see materials and methods). For this reason, the representativeness of the sample subset was tested at the province level, related to the whole sample dataset after randomization. The 98 samples of beeswax represent accurately the whole sample dataset (Fisher's exact test (df = 9); p-value = 0.69).

Adulteration of randomized beeswax samples provided by some beekeepers

The samples (N = 98) were randomized, and collected from each Belgian province to be analysed for adulteration (**Fig 1**). The level of adulteration in analysed beeswax samples was determined based on the IR spectra of reference standards (genuine beeswax, adulterants, and adulterant-beeswax mixtures containing different proportions of adulterants) and calibration curves generated for prepared adulterant-beeswax mixtures. As presented in **Fig 2**, FTIR-ATR spectra of beeswax and different types of adulterants (an example of paraffin and stearic acid) exhibit specific spectral features with the most prominent and indicative absorption bands in the fingerprint region (1800-800cm-1). IR spectra of prepared adulterant-beeswax mixtures containing 5 to 95% (w/w) of adulterants, i.e. paraffin-beeswax mixtures (**Fig 3**), and stearic acid-beeswax mixtures (**Fig 4**), also revealed a specific trend of spectral alterations reflected in decreasing (following the addition of paraffin) and increasing (following the addition of stearic acid) intensities of absorption bands related to esters and free fatty acids. Two spectral regions with target peak areas showing the best correlation between the instrument response and known proportions of adulterant in the adulterant-beeswax reference standards were chosen for further calibration process and quantification of adulterants in analysed beeswax samples.

For paraffin, a target peak area 1750–1727 cm-1 (with an absorption maximum at 1738 cm-1) and 1198–1147 cm-1 (with an absorption maximum at 1171 cm-1) showed the best prediction performance (Pearson's r = 0.9994, R2 = 0.9987, SE = 0.00097—Figs 5 and 6, and Pearson's r = 0.9996, R2 = 0.9993, SE = 0.00017—Figs 7 and 8, respectively), and were therefore used for detecting the paraffin share in analysed beeswax samples. The amount of stearic acid in analysed beeswax samples was estimated based on 1721–1707 cm-1 (with an absorption maximum at 1710 cm-1) and 1308–1253 cm-1 target peak areas (with an absorption maximum at 1281 cm-1) that revealed the best prediction performance parameters, i.e. Pearson's r = 0.9994, R2 = 0.9987, SE = 0.00111—Figs 9 and 10, and Pearson's r = 0.9999, R2 = 0.9999, SE = 0.00005—Figs 11 and 12, respectively. The amount of adulterants (as %, w/w) in analysed beeswax samples was determined as an average value of instrument response for the above-mentioned reference peaks for each adulterant type, i.e. paraffin and stearic acid. Given that stearic acid and a widespread cheap substance called "stearin" (commercially available as a mixture of stearic and palmitic acid, or even as a pure stearic acid) exhibit almost the same spectral features (S1 Fig), the same calibration curve can be used for the detection of both substances. Therefore, the terminology stearin/stearic acid is used further in the text.

Beeswax samples were adulterated with paraffin (N = 2) and stearin/stearic acid (N = 7), but no multi-adulteration was observed. Also, no traces of other adulterants (such as tallow and carnauba wax) or other foreign substances were detected (S2A Fig). Indeed, the level of adulteration of beeswax samples provided by some beekeepers was calculated as 2.04% (95% confidence interval [CI]: 0.25–7.18), and 7.14% (95% CI: 2.92–14.16%) for paraffin and stearin/stearic acid, respectively. The level of beeswax adulteration with paraffin was 12% and 78.8% (Fig 13). The level of beeswax adulteration with stearin/stearic acid (N = 7; i.e. 1.2, 2.2, 2.3, 2.4, 7, 8.1 and 11.9%, respectively) (Fig 14).

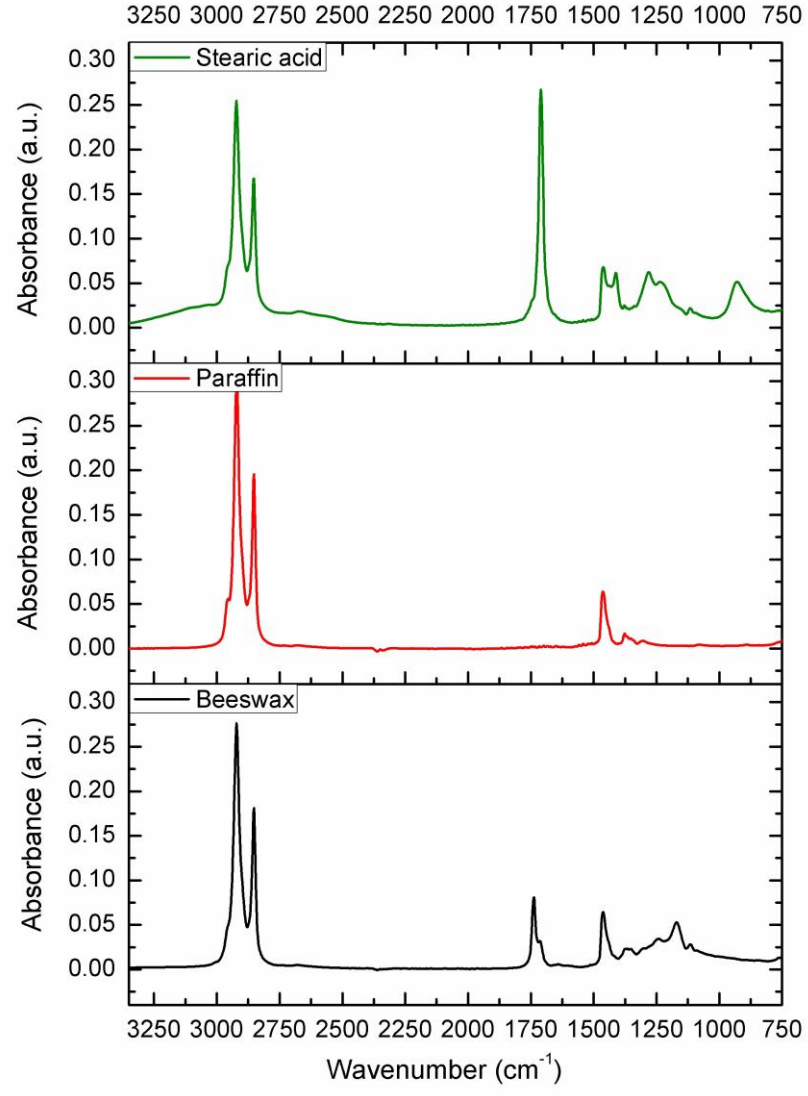

Figure 2. FTIR-ATR spectra of reference standards used for calibration - genuine (pure) beeswax, and adulterants (paraffin - Paraffinum solidum, stearic acid - Acidum stearicum)

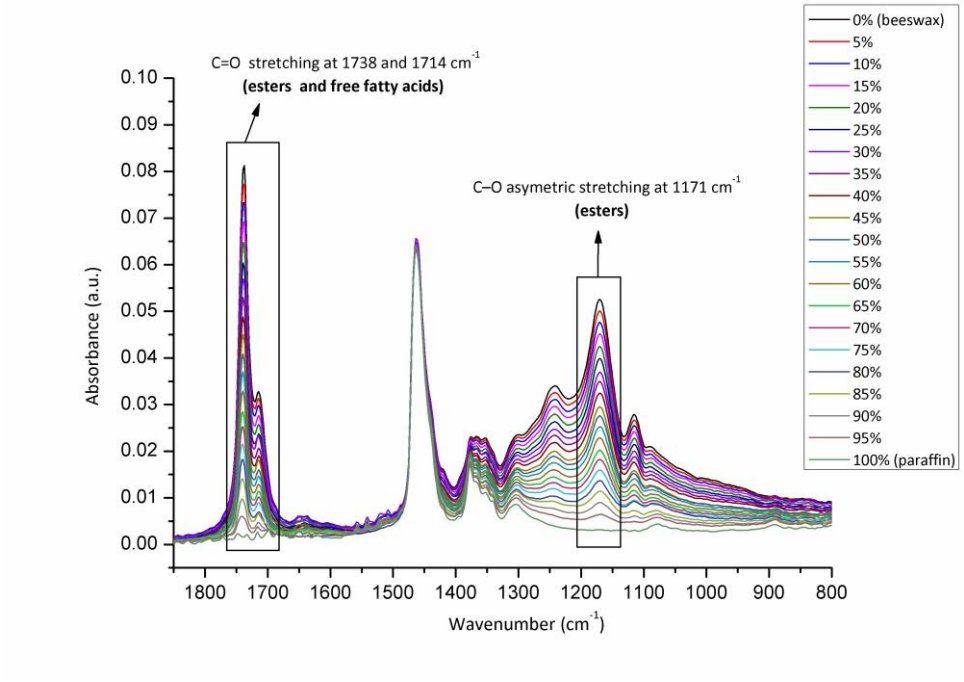

Figure 3. FTIR-ATR spectra of reference standards (paraffin-beeswax mixtures containing different proportions of paraffin) used for calibration. Wavenumber, the number of waves per unit distance; cm, centimetre; a.u. is for theabsorbance unit

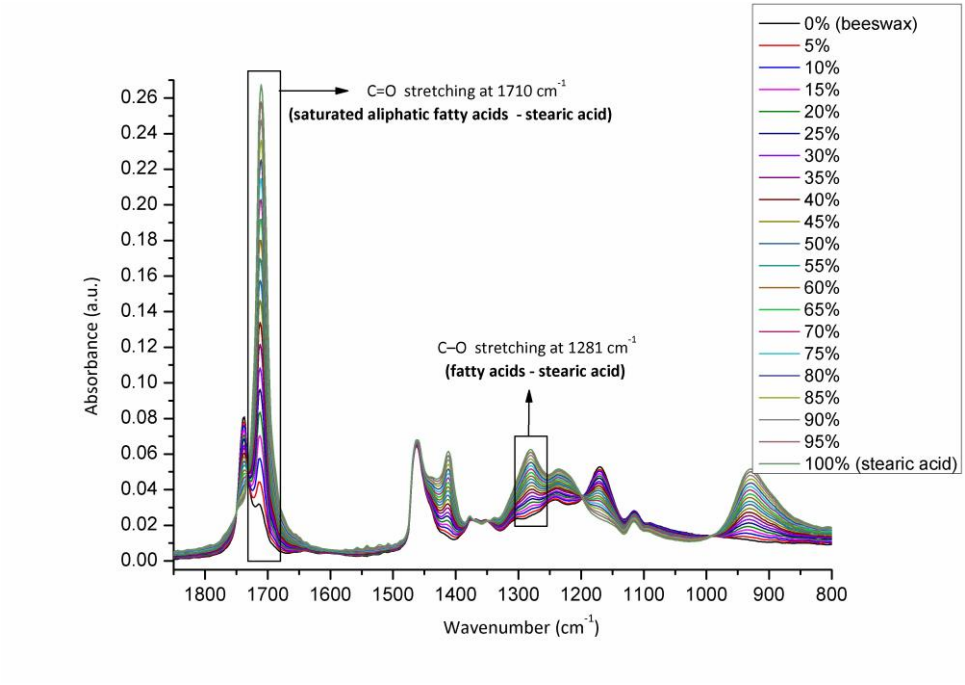

Figure 4. FTIR-ATR spectra of reference standards (stearic acid-beeswax mixtures containing different proportions of stearic acid) used for calibration. Wavenumber, the number of waves per unit distance; cm, centimetre; a.u. is for the absorbance unit

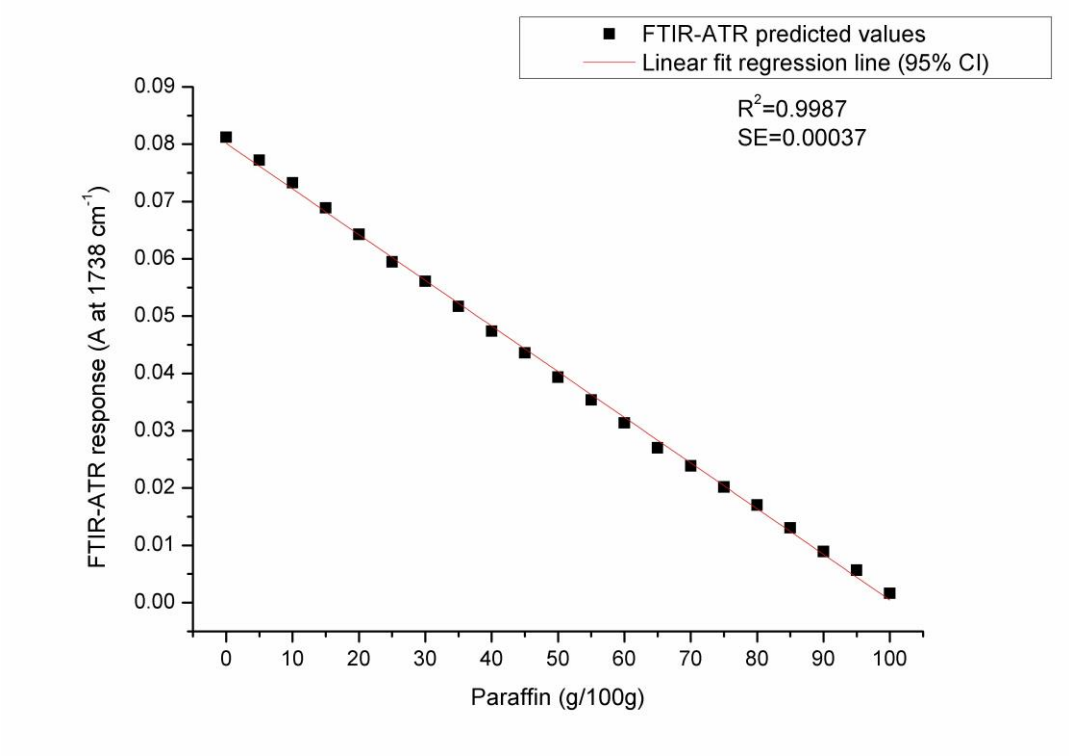

Figure 5. Prediction performance parameters of the calibration curve constructed for determination of the paraffin share in beeswax: A scatter plot of FTIR-ATR predicted values (instrument response) versus real (known) paraffin share values using the spectral region with an absorption maximum at 1738 cm-1

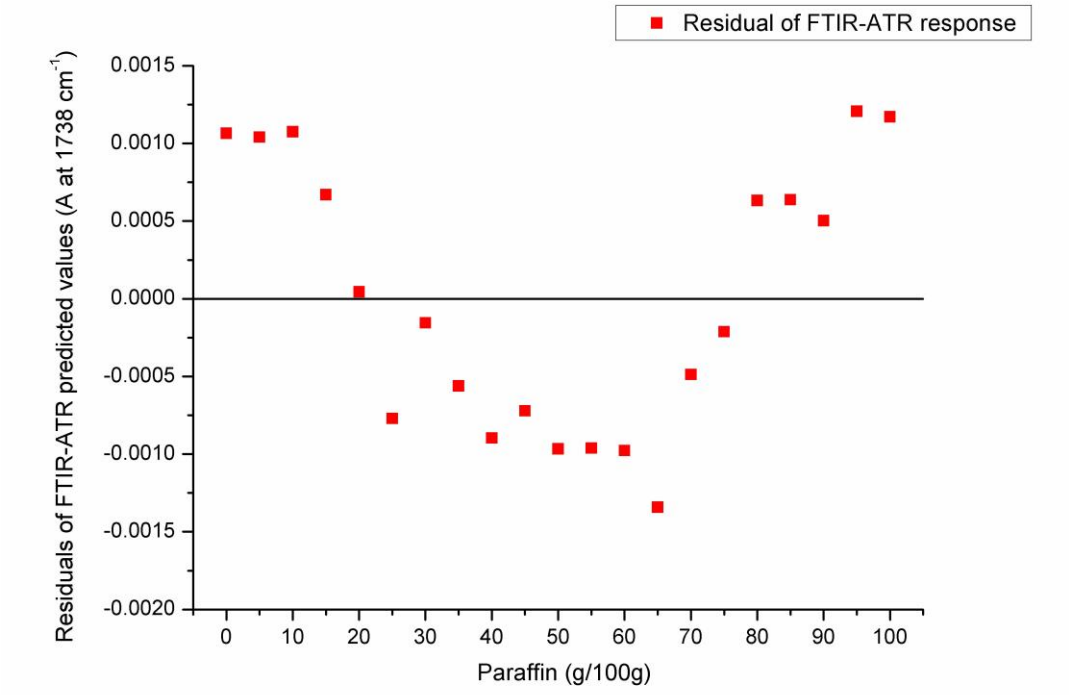

Figure 6. Residuals of FTIR-ATR prediction in the spectral region with an absorption maximum at 1738 cm-1.

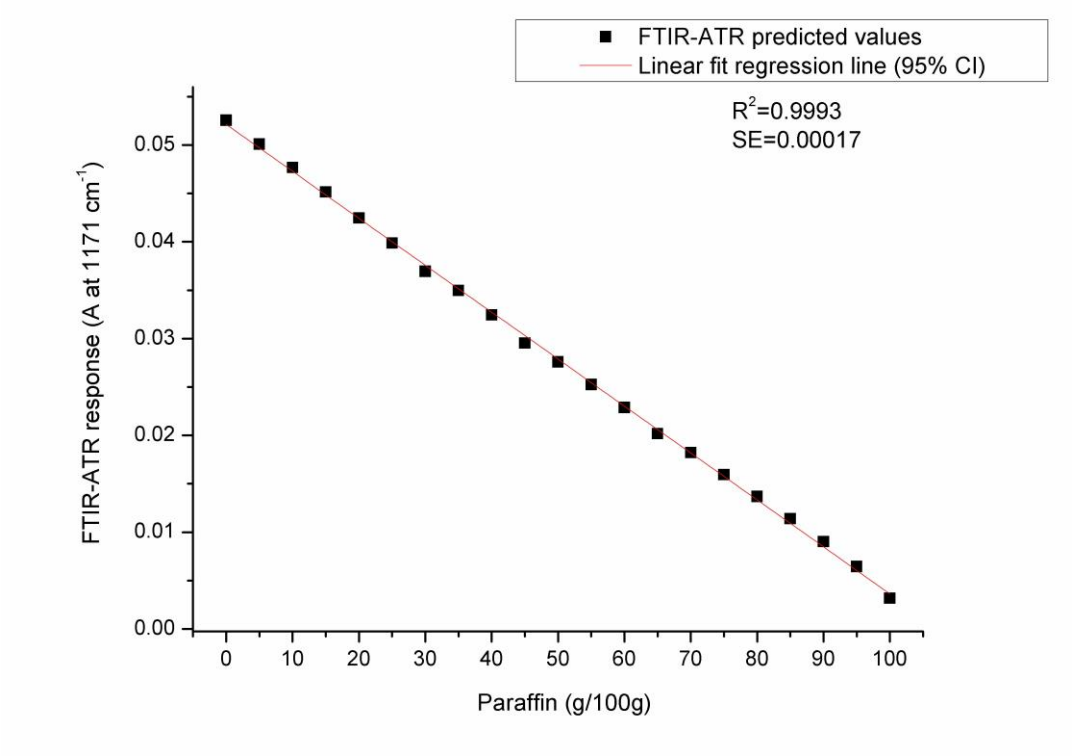

Figure 7. A scatter plot of FTIR-ATR predicted values (instrument response) versus real (known) paraffin share values using the spectral region with an absorption maximum at 1171 cm-1

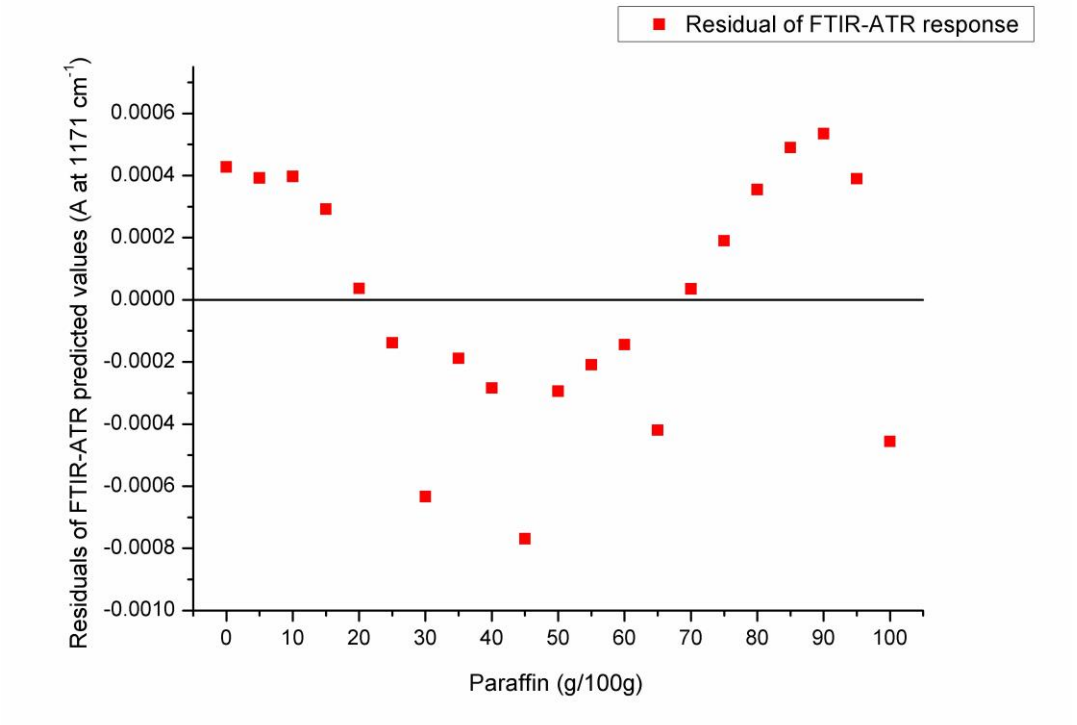

Figure 8. Residuals of FTIR-ATR prediction in the spectral region with an absorption maximum at 1171 cm-1

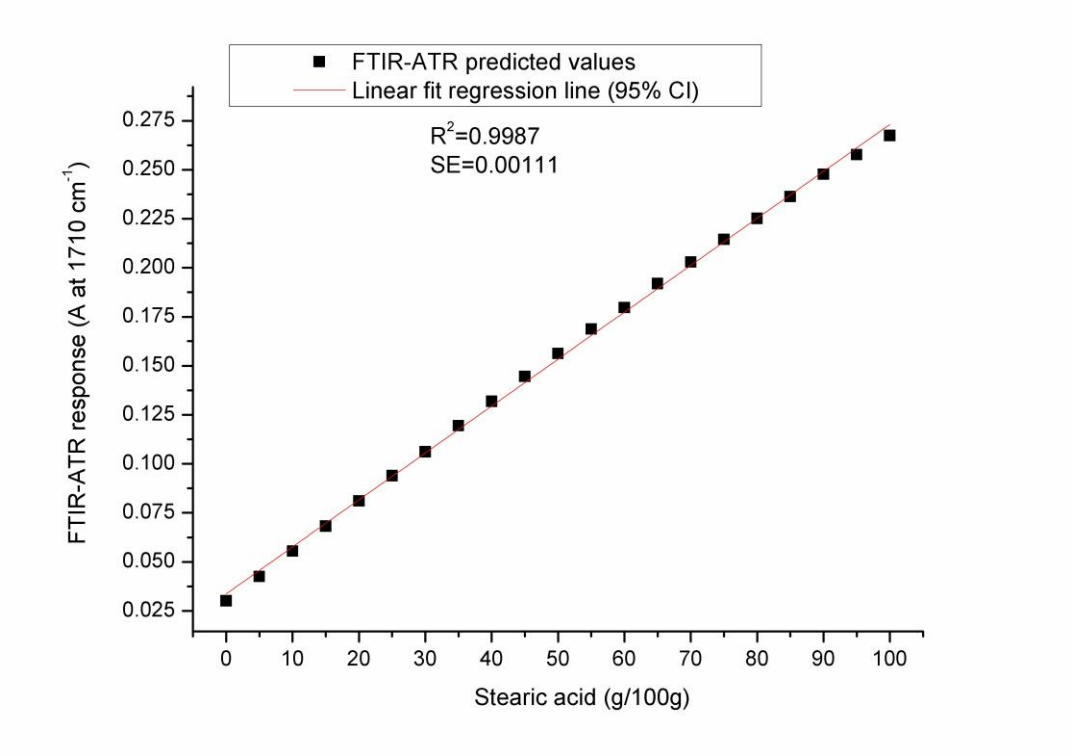

Figure 9. Prediction performance parameters of the calibration curve constructed for determination of the stearic acid share in beeswax: A scatter plot of FTIR-ATR predicted values (instrument response) versus real (known) stearic acid share values using the spectral region with an absorption maximum at 1710 cm-1

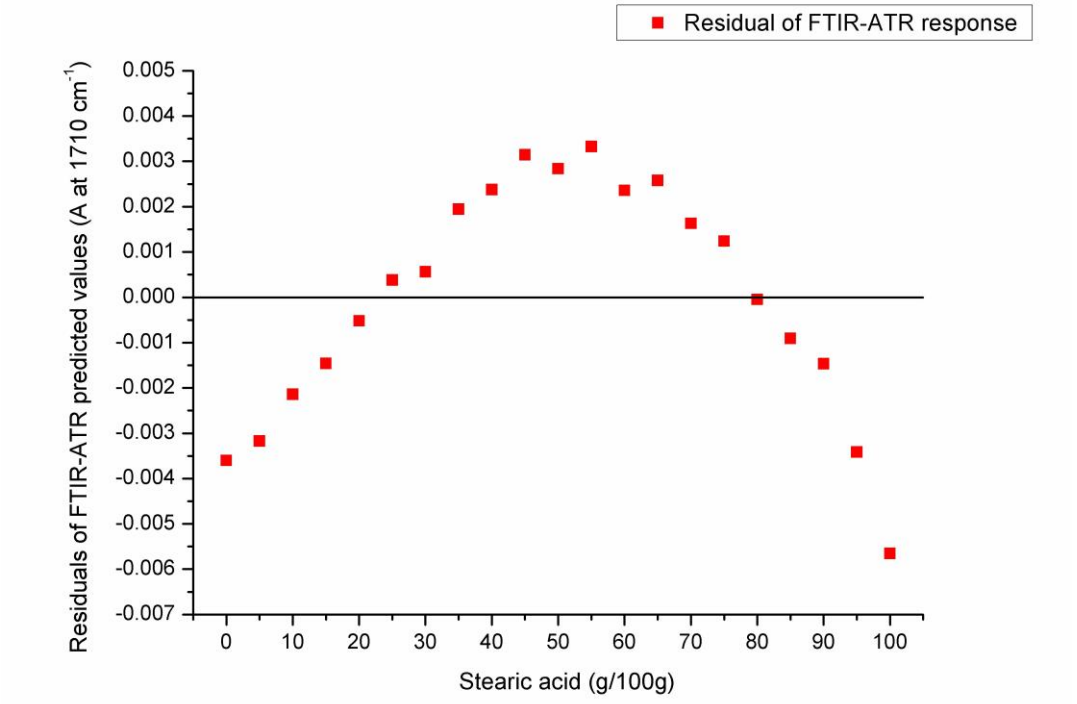

Figure 10. Residuals of FTIR-ATR prediction in the spectral region with an absorption maximum at 1710 cm-1

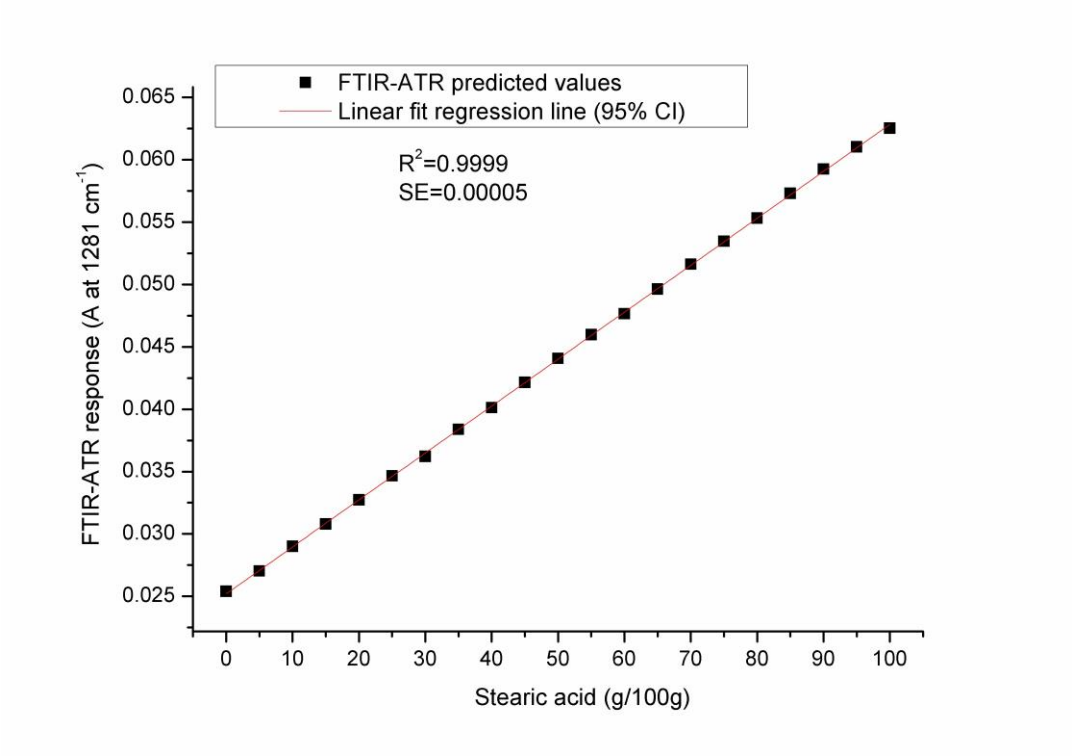

Figure 11. A scatter plot of FTIR-ATR predicted values (instrument response) versus real (known) stearic acid share values using the spectral region with an absorption maximum at 11281 cm-1.

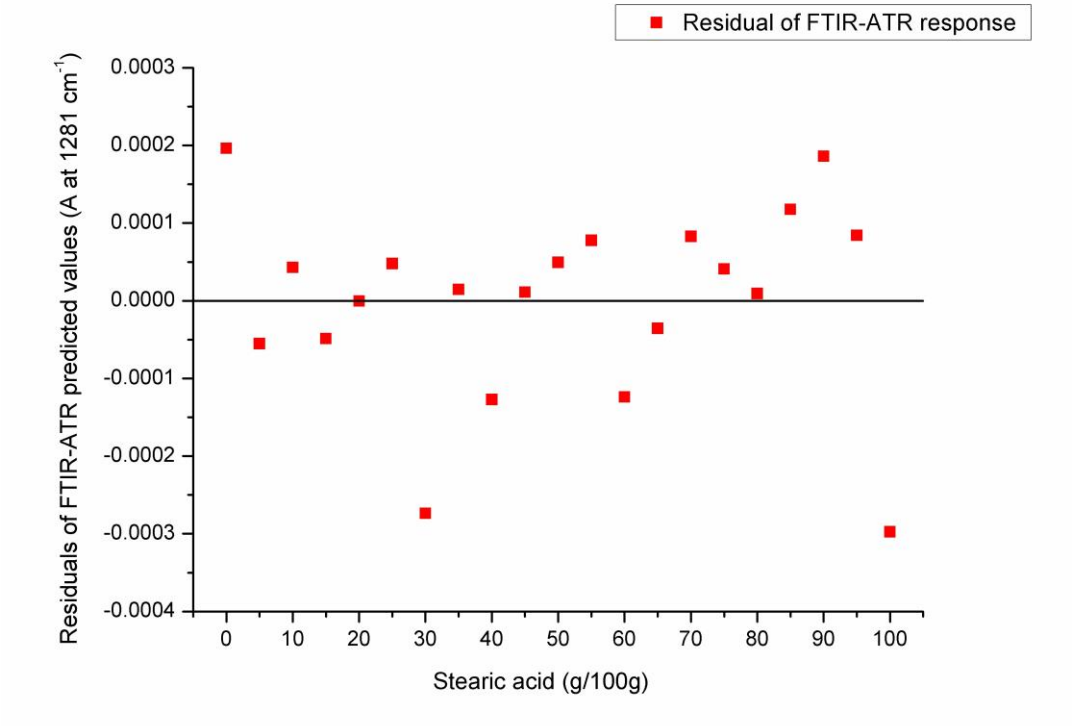

Figure 12. Residuals of FTIR-ATR prediction in the spectral region with an absorption maximum at 1281 cm-1

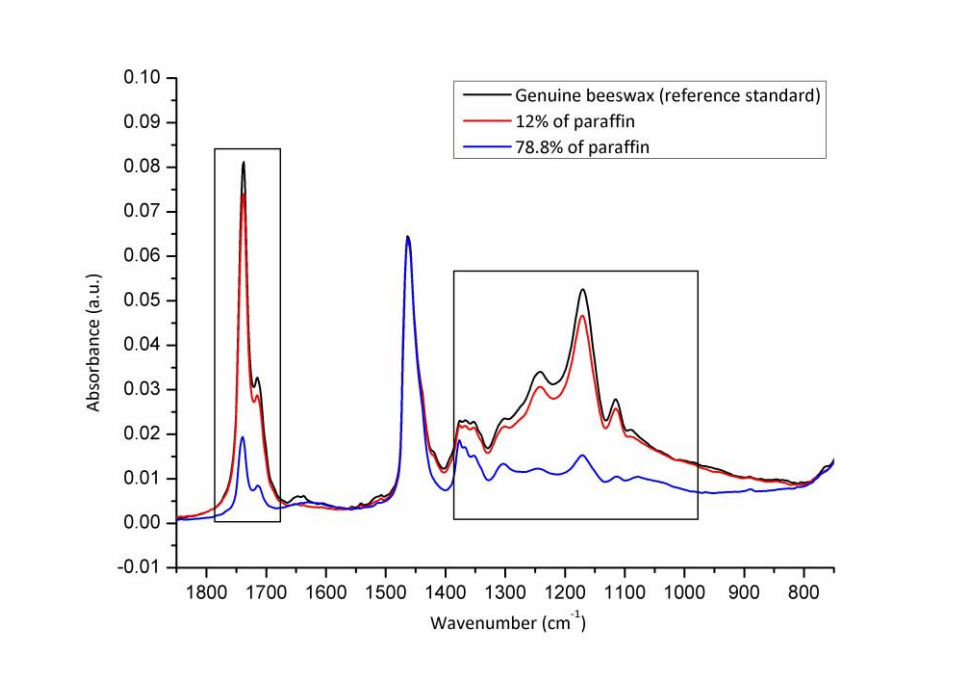

Figure 13. Adulterated beeswax samples versus genuine beeswax (reference standard) with an emphasis on spectral regions indicative for adulteration detection: Paraffin-adulterated beeswax samples. Due to some spectra overlaps (close share of spectra to 2%), only spectra with more than 2% of difference were presented in this figure. Wavenumber, the number of waves per unit distance; cm, centimetre; a.u. is for the absorbance unit.

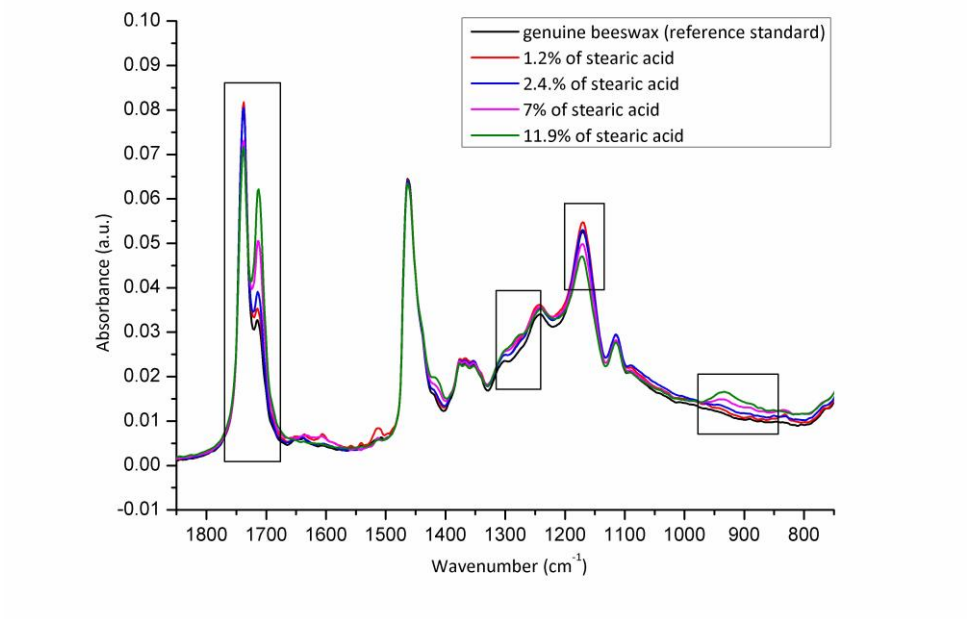

Figure 14. Adulterated beeswax samples versus genuine beeswax (reference standard) with an emphasis on spectral regions indicative for adulteration detection: Stearic acid—adulterated beeswax samples. Due to some spectra overlaps (close share of spectra to 2%), only spectra with more than 2% of difference were presented in this figure. Wavenumber, the number of waves per unit distance; cm, centimetre; a.u. is for the absorbance unit.

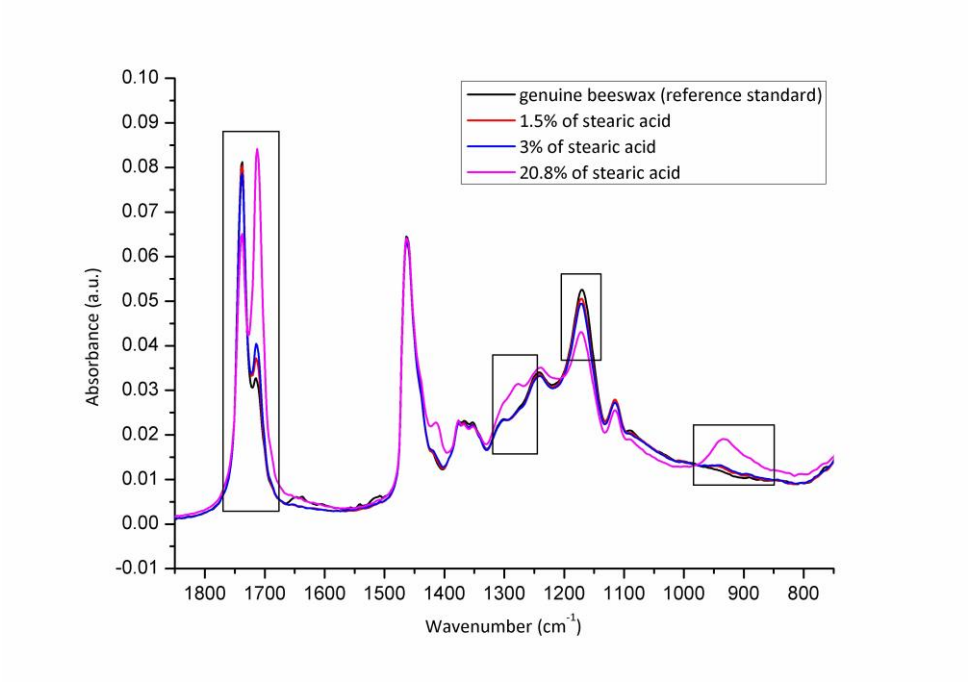

Figure 15. Comb foundation samples adulterated with stearic acid (N = 3) versus genuine beeswax (reference standard) with an emphasis on spectral regions indicative for adulteration detection. Wavenumber, the number of waves per unit distance; cm, centimetre; a.u. is for the absorbance unit.

Adulteration of beeswax samples from commercial suppliers (trade wax)

The eight wax samples collected from different commercial suppliers, and the one additional, achieved by the FASFC, were analysed for adulteration. None of the tested samples was adulterated with paraffin but 3 of them (33%) were adulterated with stearin/stearic acid. The adulteration percentages were 1.5, 3, and 20.8%, respectively (Fig 15). The most adulterated sample containing a level of 20.8% of stearin/stearic acid corresponds to the one where mosaic brood was reported. The IR spectra of other comb foundations analysed (N = 6) revealed no trace of other adulterants (S2B Fig).

Logistic regression analysis

Due to a low percentage of paraffin and stearin/stearic acid adulteration found in the beeswax samples coming from the beekeepers, the two types of adulterants were considered in the same logistic regression analysis.

Beeswax samples from both beekeepers and commercial suppliers.

Adulteration is more likely to occur in crude beeswax (OR = 7.70; 95% CI: 1.45–40.93; p-value = 0.017) and in comb foundation (OR = 14.75; 95% CI: 2.04–106.46: p-value = 0.008) than in comb wax as a reference group (Table 1).

Beeswax samples form beekeepers.

In both of the univariate (Table 2) and the multivariate analyses, only one exploratory variable was related to adulterated beeswax samples, i.e. the type of beeswax. Indeed, adulteration is more likely to occur in crude beeswax (OR = 7.70; 95% CI: 1.45–40.93; p-value = 0.017) compared to comb wax as a reference group (Table 2). The Hosmer–Lemeshow test showed that the final model fits the data well (Chi2 = 0.00, df = 1, p-value = 1).

Table 1. Contingency table of results for adulteration of beeswax.

Origin of samples	Type of beeswax	Adulterated	Non-adulterated	Total
Beekeepers	Comb wax	2	59	61
	Comb foundation	1	7	8
	Crude beeswax	6	23	29
Commercial suppliers	Comb foundation	3	6	9

Total	12	95	107

Table 2. Univariate logistic regression analysis for adulterated versus non-adulterated Belgian beeswax samples.

Variable	Modalities		Odds ratio	(95% CI)	*p*-value
Beeswax type	Comb wax		Reference	-	-
	Comb foundation		4.21	(0.34–52.64)	0.264
	Crude beeswax		7.70	(1.45–40.93)	0.017*
Year of introduction in the hive		2013	Reference	-	-
		2014	2.71	(0.10–74.55)	0.55
		2015	2.71	(0.14–51.60)	0.51
		2016	2.48	(0.09–68.14)	0.60
Location (province)	Antwerp		Reference	-	-
	Flemish Brabant		3.57	(0.15–85.68)	0.43
	Walloon Brabant		0.65	(0.01–36.56)	0.84
	Western Flanders		5.77	(0.23–143.37)	0.29
	Eastern Flanders		6.43	(0.21–201.07)	0.29
	Hainaut		1.67	(0.06–46.23)	0.76
	Liège		3.00	(0.10–86.09)	0.52
	Limburg		2.14	(0.08–60.17)	0.65
	Luxembourg		2.37	(0.08–66.88)	0.61
	Namur		0.56	(0.01–30.95)	0.77
Mortality rate (colony level)	Continuous variable		0.12	(0.002–9.68)	0.35

* *p*-value less than 0.05.

Discussion

The presence of adulteration of beeswax by paraffin or stearin/stearic acid from samples collected in Belgium was confirmed using a randomized cross-sectional nationwide survey. Based on a logistic regression analysis, using both paraffin and stearin/stearic acid (due to the relatively limited number of positive samples), significantly more adulteration was found in crude beeswax and comb foundation samples than in comb wax as a reference group.

This result demonstrates that beekeepers should preferentially use and recycle their own waxes (e.g. cappings wax) rather than using trade wax, following good management practices for wax recycling. In addition, it shows the need for more appropriate guidelines for beeswax production, trade and sale. Beeswax traceability and authentication should be conducted with regular surveillance beekeeping programs. To conduct such surveillance programs, the determination and use of purity criteria (using physico-chemical methods) for beeswax intended for use in beekeeping should be implemented [33]. The use of more advanced methods (e.g. FTIR-ATR spectroscopy) should be promoted, and risk-based survey (e.g. based on trade business of beeswax, and/or by identification, and tracking of emerging risks from beeswax adulteration in the media as recently suggested by Rortais et al. [8]) should be designed and performed. Despite the use of an advanced analytical method (i.e. FTIR-ATR spectroscopy) with a limit of detection in Spain by Serra Bonvehı´, and Orantes Bermejo [23]. However, in Spain, paraffin adulteration was mostly observed, while in Belgium, stearin/stearic acid adulteration appears to be predominant. This observation is confirmed by the study of Svečnjak et al. [28] which indicates the presence of stearin/stearic acid as adulterant only in Belgium, and The Netherlands amongst the 15 European countries tested. Despite the absence of evidence of a possible effect of the location on the adulteration of beeswax samples (both with paraffin and stearin/stearic acid), if we compare the location of the two different adulterants separately (Fig 1), stearin/stearic acid adulteration was exclusively observed in the northern part of the country, whereas, paraffin adulteration was restricted to the southern part. These observations should be in favour of different business networks of adulterated beeswax that need to be further investigated by ad hoc authorities to detect the fraud source.

When detecting beeswax adulteration, FTIR-ATR spectroscopy technique has the advantage to detect adulteration at a relatively low level (< 3%) for paraffin, beef tallow, stearin, stearic acid, palmitin, and carnauba wax [39], and its ability to detect mixtures of beeswax adulterants with the same accuracy as single substances [40].

Despite the limited number of beeswax samples from trade (commercial beeswax), 3 out of 9 samples were adulterated by stearin/stearic acid (33.3%). Two of them with a low level (>3%) but one with a high level (20.8%). This last trade beeswax sample was imported from China in 2015. In addition, mosaic brood was reported by several Belgian beekeepers who used wax from this batch when renewing hive foundations. Considering the results of a previous work [30], it is expected that the level of adulteration observed in this survey, could possibly reduce the brood survival rate to less than 55%, confirming the detrimental effect of beeswax adulteration by stearin on bee health.

Beeswax adulteration is an emerging issue and could be a challenge for bee health, as recently shown for stearin, and palmitin [30,41] and possibly for human health too, due to the potential presence of hazardous substances in unrefined paraffin of fossil origin that could be used as adulterant. Carcinogenic compounds such as polyaromatic hydrocarbons (PAHs) are known to be present at substantial concentrations (up to 1%) in unrefined waxes originating from various crude oils [42]. Consequently, the European Commission requested EFSA to define purity criteria for beeswax, and to assess risks for honey bees, and humans [31].

Conclusion

Beeswax adulteration is a fraud and an emerging issue. It brings the beekeeping sector into disruption. This survey shows that adulteration by paraffin or stearin/stearic acid in crude beeswax and comb foundation is more frequent than in comb wax. The level of stearin/stearic acid adulterant found is compatible with a detrimental effect on brood. The use of paraffins of petrogenic origin as adulterant must be considered of possible concern for human health, especially for unrefined paraffins that may contain carcinogenic substances such as PAHs, nevertheless, this needs to be properly assessed in the future. There is an urgent need for routine analytical testing of beeswax adulterants and their possible contaminants used in apiculture, in order to produce a regulatory framework that defines beeswax purity criteria, to prevent beeswax adulteration and to ensure the safety of crude, and trade beeswax.

Supporting information

S1 Fig. FTIR-ATR spectra of stearic acid versus 'stearin' (commercially available as "stearin for candles", a mixture of stearic and palmitic acid) showing the same spectral features. Wavenumber, the number of waves per unit distance; cm, centimetre; a.u. is for the absorbance unit.

(TIF)

S2 Fig. Comparative spectral features of: An average spectrum of non-adulterated beeswax samples (N = 88) versus genuine beeswax (reference standard) [A] an average spectrum of non-adulterated comb foundation samples (N = 6) versus genuine beeswax (reference standard) [B]. Wavenumber, the number of waves per unit distance; cm, centimetre; a.u. is for the absorbance unit.

(TIF)

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Experimental section

Study 7

A field realistic model to assess the effects of chlorpyriphos-ethyl, acrinathrin and stearin on honey bee gene expression

Science of the total environment (in review)

Noëmie El Agrebi and Lina De Smet , Caroline Douny, Marie-Louise Scippo, Lidija Svečnjak , Dirk C. de Graaf and Claude Saegerman

Preamble

To date, no maximum limit for pesticide residues or adulterants specifically aimed at the protection of bee health has been set. Previous research on the effects of pesticide residues by contact exposure on honey bee health has typically focused on adult honey bees, in in-vitro conditions, however, field experiments are essential to the risk assessment concerning pesticide impact on immature bees and brood development. To assess the risk of these contaminants in beeswax for honey bee and pupae development, a novel field realistic methodology to rear honey bee pupae in contact with adulterants and contaminants has been developed. The impact of beeswax contaminations and adulteration on honey bees' gene expression was also examined in this last study.

A field realistic model to assess the effects of chlorpyriphos-ethyl, acrinathrin and stearin on honey bee gene expression

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Abstract

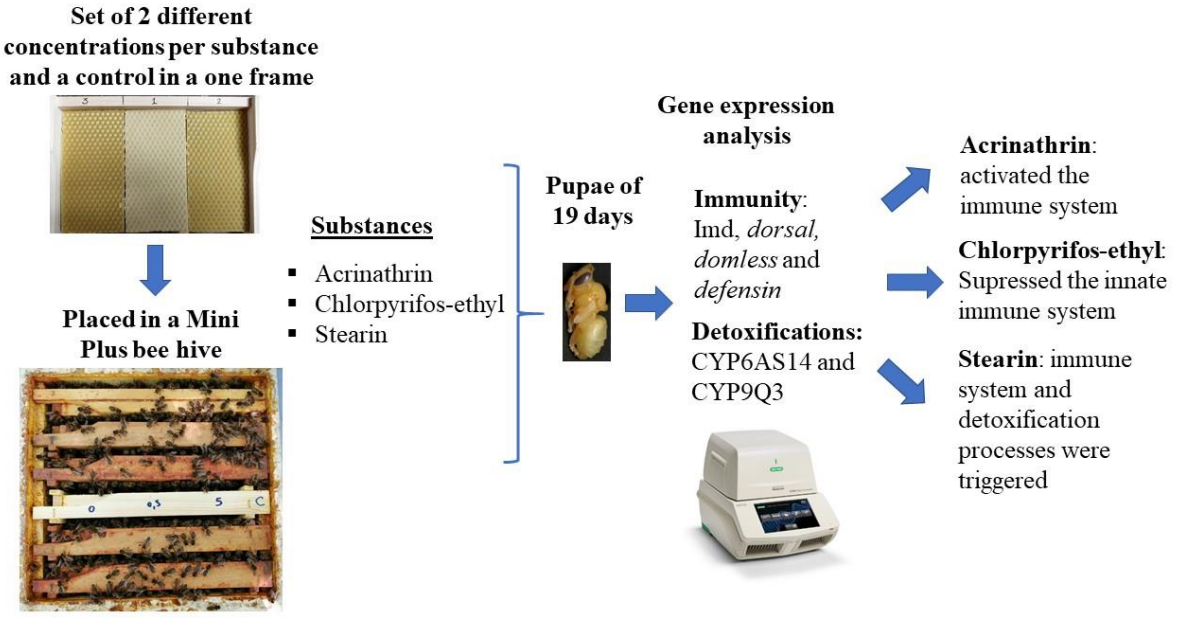
While the number of studies about the sublethal effects of chemical residues in beeswax on adult honey bees is increasing, the study protocols assessing the impacts on honey bee pupae in realistic conditions still need to be investigated. Moreover, little is known about the residue's effect on gene expression in honey bee pupae. This study reports the effects of chlorpyriphos-ethyl, acrinathrin and stearin (used as model substances) worker pupae exposure through contaminated or adulterated beeswax on their gene expression, using a novel *in vivo* realistic model. Larvae were reared in acrinathrin (0.0125, 0.025, 0.1 and 1 mg/kg) and chlorpyriphos-ethyl (0.005, 0.01, 0.5 and 5 mg/kg) contaminated or stearin adulterated beeswax (3, 4, 5, 6 and 9%) in newly formed colonies to reduce the influence of external factors such as *Varroa* infestation. Honey bee pupae were extracted from the comb after 19 days of rearing and were analysed for the gene expression profile of four genes involved in the major immune response to pathogens and environmental stress factors (*Imd*, *dorsal*, *domeless* and *defensin*), and two genes involved in detoxifications mechanisms (CYP6AS14 and CYP9Q3). We found that the immune system of pupae raised in acrinathrin-contaminated wax was triggered and the expression of CYP6AS14 was significantly upregulated (exposure to 0.0125 and 0.025 mg/kg). Almost all expression levels of the tested immune and detoxification genes were down-regulated when pupae were exposed to chlorpyrifos-contaminated wax. The exposure to stearin triggered the immune system and detoxification system of the pupae. For an economically and emblematic important species such as honey bees, the identification of substance-specific response factors might ultimately serve to identify molecules that are safer for bees and the ecosystem's health.

Keywords: Honey bees, beeswax, pesticide residues, adulteration, gene expression, acrinathrin, chlorpyriphos-ethyl, stearin, field conditions.

Highlights

- Field realistic model for pupae rearing in contaminated and adulterated beeswax
- The effect of pesticides and adulterants on the expression of key immune and detoxification enzymes coding genes was determined
- Chlorpyrifos exposure down-regulated all expression levels of immune and detoxification genes
- Acrinathrin exposure activated the pupae immune system
- Exposure to stearin triggered the immune and detoxification pupae system

GRAPHICAL ABSTRACT



INTRODUCTION

In modern beekeeping, removable frames are used to allow beekeepers to extract honey and inspect the hive without damaging the comb. The comb can then be relocated inside the hive and reused. This reduces the time and energy that honey bees spend on producing wax. In good beekeeping practice, brood combs are ideally replaced after three years (Al-Kahtani and A. Taha, 2021). The old comb wax recycled by melting the combs together with wax cell cappings in water vapour-producing blocks for the manufacturing of new comb foundations.

For many years, the use of pesticides was considered the main pest management strategy. Many pesticides from veterinary but also from agricultural use remain in the recycled wax and the newly produced comb foundations (Martel et al., 2007; Perugini et al., 2018; Sanchez-Bayo and Goka, 2014). When applied in the environment, in the hive or present in the wax foundation, apart from their immediate lethal effects, pesticides can generate insidious sublethal effects that impact the behaviour (Weick and Thorn, 2002; Aliouane et al., 2009; Yang et al., 2008), the reproduction, the development, and can generate resistance of the organisms chronically exposed to their active substances (Desneux et al., 2007).

Another emerging problem of beeswax for honey bees is its adulteration by the addition of natural or synthetic substances of wide availability and a low price. The most common sources of adulterations are hydrocarbons from paraffin and microcrystalline waxes, triglycerides from palmitic acid, fat and hardened beef tallow, industrially produced fatty acids (palmitic, stearic acid), long-chain alcohols (C16-C18), and C32-C36 synthetic esters (Bogdanov, 2016, 2009; Svečnjak et al., 2019; Waś et al., 2016).

The contamination and adulteration of beeswax is an issue that has been reported lately on several occasions in the scientific literature (Bernal et al., 2005; Bogdanov, 2004; El Agrebi et al., 2021; Noëmie El Agrebi et al., 2020; Maia et al., 2013; Špaldoňová et al., 2021; Svečnjak, 2018; Svečnjak et al., 2015; Tanner and Lichtenberg-Kraag, 2019; Tulloch, 1973; Wilmart et al., 2021, 2016). The effects of these contaminated or adulterated beeswax foundations seem to be the main cause of poor brood and colony development (Chęć et al., 2021; Reybroeck, 2018). The highly sensitive honey bee larvae and pupae are exposed to contaminants during their development when these substances migrate from the beeswax into the larval jelly or when larvae come into direct contact with the beeswax (Wilmart et al., 2021).

Among pesticide residues, the organophosphate insecticide chlorpyrifos, also known as chlorpyrifos ethyl (contact acute LD_{50} [worst case from 24, 48 and 72-hour values] = 0.068 µg/bee) is one of the most commonly found agrochemicals in beeswax (Payne et al., 2019). It was found in the

Belgian apiaries with a prevalence of 5.9% in 2021 and 2022, and 13.5% in 2016 (Noëmie El Agrebi et al., 2020). The high prevalence of chlorpyrifos has been confirmed in recent years by other studies (Al Naggar et al., 2015; Calatayud-Vernich et al., 2018; Tosi et al., 2018; Traynor et al., 2016a). Organophosphate insecticides, like chlorpyrifos and coumaphos, act on the insect nervous system by inhibiting acetylcholinesterase, the enzyme that inactivates the neurotransmitter acetylcholine in the synapses of the insect central nervous system (Casida and Durkin, 2013). Several effects such as an increase in apoptosis have been reported in larvae treated with chlorpyrifos orally compared to untreated larvae (Gregorc and Ellis, 2011). The contact exposure with field-relevant concentrations of chlorpyrifos in combination with chlorothalonil showed a decreased spermatozoa viability in sexually mature drones (Fisher and Rangel, 2018). Although not much is known about the effects of chlorpyrifos on honey bees and honey bee pupae, it can cause substantial synergistic effects when combined with other pesticides, leading to high larval mortality (Dai et al., 2019, 2017).

Another pesticide residue group to which honey bees are often exposed is pyrethroids. Acrinathrin (contact acute LD_{50} [worst case from 24, 48 and 72-hour values] = 0.084 µg/bee) is a pyrethroid insecticide and acaricide derived from hexafluoro-2-propanol. In beekeeping, it was used to control the mite *Varroa jacobsoni*, though its high toxicity and *Varroa* developed resistance. Acrinathrin was found in larvae after direct contact with contaminated beeswax (Murcia Morales et al., 2020). The standard Hazard Quotient (HQ) value of acrinathrin which expresses its potential toxicity to bees, exceeds the trigger value of 50, indicating the need for further refinement of the risk assessment of this substance (EFSA, 2013b). The results of semi-field and field tests confirmed that the application of acrinathrin leads to increased mortality of bees immediately after application and up to 3 days after application. No significant effects were observed on honey bee colony strength or bee brood. Risk mitigation was suggested to minimize exposure to honeybees immediately after application and up to 4 days after application of acrinathrin (EFSA, 2013b).

Recently, stearin, a mixture of stearic and palmitic acids, was reported as one of the main adulterants of beeswax (El Agrebi and Svečnjak, 2021). Moreover, preliminary studies conducted in Belgium (Reybroeck, 2018), Poland (Chęć et al., 2021) and Germany (Tanner and Lichtenberg-Kraag, 2019) confirmed an association between the presence of stearin at certain levels and detrimental effects on bee brood.

Previous research on the effects of pesticide residues by contact exposure on honey bee health has typically focused on adult honeybees, in *in vitro* conditions. However, field experiments are essential to the risk assessment of pesticide impact on immature bees and brood development. This study focuses on the response of pupae reared in contaminated beeswax with field-realistic concentrations of chlorpyrifos-ethyl, acrinathrin and stearin. The expression profile of some key immune and detoxification genes was followed. The three major immune response pathways were studied by

following the expression level of the genes *relish* (involved in the Imd pathway), *domeless* (involved in the Janus kinase-signal transducer and activator of transcription [Jak-STAT] pathway) and *dorsal* (involved in the Toll pathway) (Brutscher et al., 2015). *Defensin* was used as a marker for the production of antimicrobial peptides. Chemicals may trigger some detoxification pathways with CYP6AS14 and CYP9Q3 as key enzymes in the degradation process. Oxidative stress generated by exposure to chemicals can be mapped by following the expression of catalase and glutathione-S-transferase (GST) coding genes. The results of expression profiling will provide insight into how the pupae respond to and deal with exposure to chemicals.

MATERIALS AND METHODS

Virgin beeswax selection

Virgin beeswax was purchased from an organic beeswax producer and analysed using a multi-residue analysis by gas chromatography-tandem mass spectrometry (GC-MS/MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods covering 294 different substances with detection limits (LOD) of 0.003 mg/kg and limits of quantification (LOQ) of 0.01 mg/kg in most cases. The analysis was carried out in an independent laboratory in Germany (Intertek Food Services GmbH) according to the European EN 15662 method (CEN 2008), using a common analytical protocol (QuEChERS) designed for the analysis of food materials and suitably adapted (Noëmie El Agrebi et al., 2020). The virgin beeswax was also analysed by Fourier transform infrared spectroscopy (FTIR) coupled with a single-reflection diamond Attenuated Total Reflectance (ATR) system (FTIR-ATR spectroscopy) according to the methods for adulteration detection developed by Svečnjak et al. (2019). Both analyses confirmed the absence of any chemical contamination by pesticides or adulteration in the foundation wax.

Beeswax contamination and residue analysis

To obtain beeswax foundations with a chlorpyrifos-ethyl concentration of 0.005 and 0.01 mg/kg in the first year (2020) and of 0.5 and 5 mg/kg in the second year (2021), 9.2 mg of the substance (chlorpyrifos-ethyl, purity 99.49%, purchased from LGC) was added to 9.2 ml of dimethyl sulfoxide (DMSO) (99.9%), and then it was diluted 10 times. A certain volume of the diluted solution (25, 50, 2500 or 25 000 µl) was added to 500 g of the melted beeswax and homogenised using a magnetic stirring bar on a heating plate at 65°C for 5 minutes, to obtain the concentration of 0.005, 0.01, 0.5 and 5 mg/kg, respectively. The same procedure was applied to obtain beeswax foundations with an acrinathrin concentration of 0.0125 and 0.025 mg/kg in the first year and 0.1 and 1 mg/kg in the second year. The new beeswax foundations were formed with a foundation mould (**Figure 1**). The concentrations were chosen as similar to the respective concentrations of chlorpyrifos-ethyl (Calatayud-

Vernich et al., 2017; Noëmie El Agrebi et al., 2020; Serra-Bonvehí and Orantes-Bermejo, 2010) and acrinathrin (Calatayud-Vernich et al., 2017; Marti et al., 2022; Serra-Bonvehí and Orantes-Bermejo, 2010) found in commercial or beekeepers beeswax.

Beeswax adulteration

Stearin (Radiacid 0464 – CAS No. 67701-03-5) was obtained from Oleon, NV. Stearin used in this study was a solid mixture of stearic and palmitic acids. Its melting temperature depends on the ratio of components. In our case, the melting point was 55 °C. The stearin was composed of 58% of palmitic acid (C16) and 40% of stearic acid (C18). Stearin was added to the melted beeswax at the following percentages (w/w) the first year: 3, 6 and 9%, and 3, 4 and 5% the second year. The effective percentages of stearin in the beeswax after supplementation were evaluated by FTIR-ATR spectroscopy.

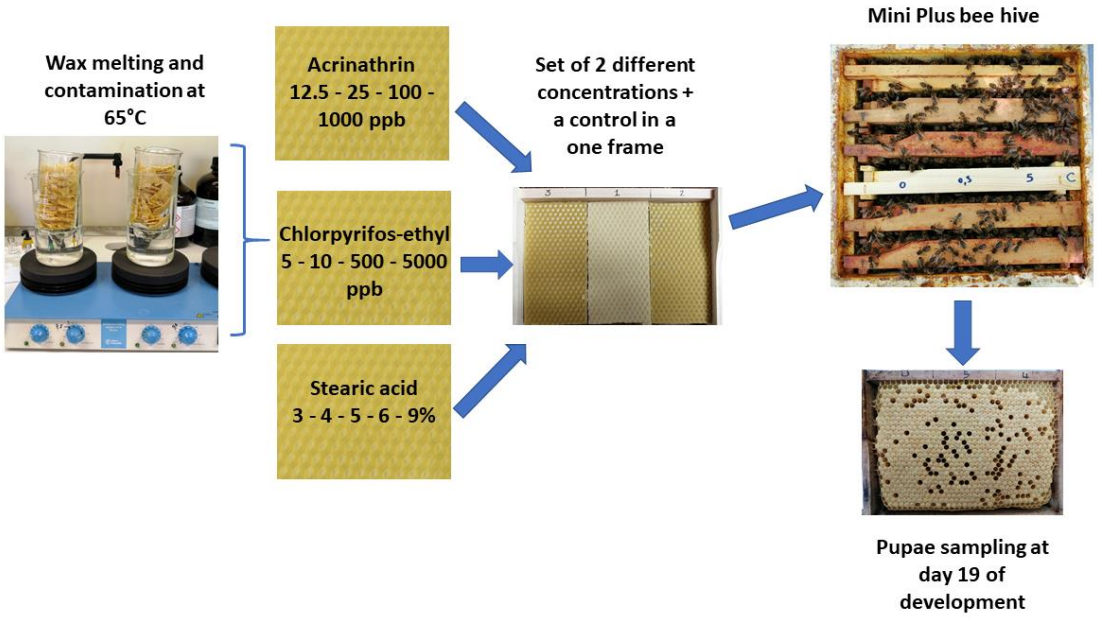


Figure 1: Experimental set to obtain emerging honey bees in contaminated and adulterated beeswax with four concentrations of acrinathrin and chlorpyrifos-ethyl and stearin adulterated bees wax.

Study site and colony establishment

We conducted our study on the site of the Faculty of Veterinary Medicine of the University of Liège, Belgium (50°34'30.913", 5°35'43.832"). The Western honey bee (*Apis mellifera* L.) subspecies that was used was the dark European honey bee (*Apis mellifera mellifera*). A naked swarm was first treated with oxalic acid to eliminate phoretic *Varroa* mites. The swarms were headed by naturally mated

sister queens. The naked swarm was introduced into a Mini Plus hive (Allan and Dean, 2022) on virgin beeswax frames. Once colonies were well established and queens were laying eggs, one experimental frame per colony supporting a control comb section (virgin beeswax) and two contaminated comb sections were placed in the middle of the nest.

Experimental frame

Control and contaminated comb sections were placed side by side within the same frame (**Figure 2**). Per pesticide concentration or stearin percentage, six repetitions were carried out and each concentration was placed in one of the three positions in the frame. Each year, the frames were introduced at the same time in the hives, at the same period of the year and inspected daily to observe the date of the start of oviposition. The frames were then kept in the hive for 19 days after the first observation of oviposition to extract the pupae. Per substance concentration, three honey bee pupae were extracted randomly from each beeswax section, and stored at -80°C until analysis for gene expression.

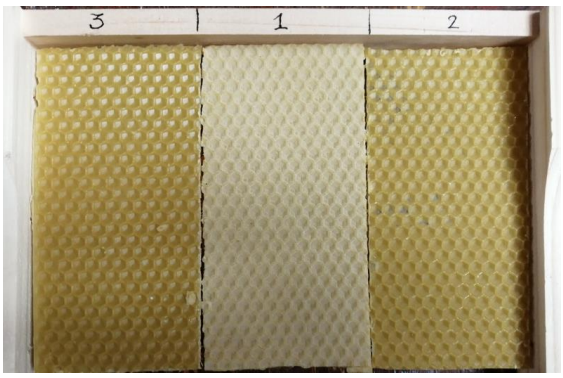


Figure 2: Mini Plus frame with a set of 3 comb sections, 2 contaminated and 1 control placed here in the centre.

Reducing bias

To obtain same-age honey bee pupae in similar conditions and minimize the influence of external stressors such as pathogens and nutritional stress on gene expression, each year, healthy colonies of equal strength were newly formed on virgin beeswax foundations (to avoid cross-contaminations) with sister queens. Sister queens are the progeny of the same queen, which are mated at the same place to minimise genetic variability (OECD Environment, 2007). The colonies were cleared of the phoretic *Varroa* mite before their introduction in the Mini Plus hives. Per year and concentration, six repetitions were performed at the same time of the year. Experiments were conducted at the best time of the year for food sources abundance to avoid nutritional stress. The larvae stayed in the contaminated or adulterated beeswax for their entire development and were randomly sampled from the comb on day 19.

Gene expression

For the gene expression profiling, three frames from each treatment were selected and RNA was extracted from three individual pupae. Total RNA was extracted from individual bees using RNeasy lipid tissue mini kit (Qiagen). The tissues were homogenized by mechanical agitation in a TissueLyser (Precellys) for 90 s at 30 Hz, in the presence of a pair of stainless-steel beads and 1 ml Qiazol lysis reagent. The total RNA was isolated according to the recommendations of the manufacturer's protocol, eluting the RNA in a final volume of 50 µl. The concentration of the total RNA was measured using a Nanodrop (Isogen) equipment. Using random hexamer primers, 1µg total RNA was retro-transcribed with the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific). For the RT-qPCR assays, the Platinum (R) SYBR (R) Green qPCR Supermix-UDG (Live Technologies) was used. Each 15 µl reaction consisted of 7.5 µl master mix, 0.2 µM forward and 0.2 µM reverse primer (Integrated DNA Technologies) and 0.2 µl cDNA template using the CFX96 Real-Time PCR Detection System (Bio-Rad). The PCR program included an activation step of 1 min at 95°C and 40 cycles of a combined denaturation (15 s at 95°C) and annealing step (30 s at 60°C). At the end of this program, a melt curve is generated by measuring fluorescence after each temperature increase of 0.5°C for 5 sec over a range from 65°C to 95°C to verify the presence of the desired amplicon. All reactions were performed in duplicate. No-template controls, containing diethylpyrocarbonate treated water, were included in each run.

Reference gene stability was analysed with the geNormPLUS algorithm within the qBasePLUS environment (Biogazelle NV) with default settings. The geNorm program generates a stability measure (the M value) for every gene, allowing their ranking according to their expression stability (with the lower value indicating increased gene stability across samples). It also generates pairwise stability (v) measures to decide the benefit of adding extra reference genes for the normalization.

Differential gene expression of 6 different target genes, detoxification and immunity genes, was determined using qPCR. The primers used for these different genes are given in **Appendix 1**. The differential expression was analysed per exposure in which the different treatments were compared with each other. The statistical analysis was performed using qBasePLUS, by means of one-way ANOVA. Two-sided significance and correction for multiple testing were performed.

RESULTS

The geNorm algorithm was used to determine the best and most reliable reference genes and to rank the four candidate reference genes according to their stability value for accurate gene expression. Taking into consideration the data obtained from the different treatments, the ranking from the genes from most to least stable is actin > RPL8 > MGST > GADPH (**Figure 3**). It also generates a pairwise

stability measure to decide the benefit of adding extra reference genes for the normalization. The optimal number of reference targets in this experimental setup is two (geNorm V < 0.15 when comparing a normalization factor based on the two or three most stable targets). As such, the optimal normalization factor can be calculated as the geometric mean of reference targets RLP8 and Actin.

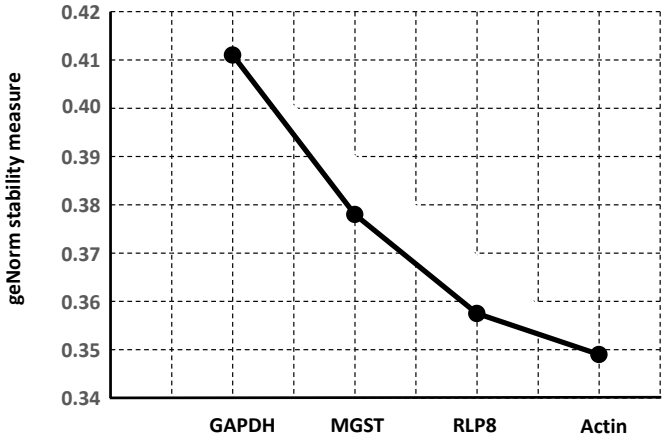


Figure 3. Average expression stability of reference targets with geNormPLUS algorithm.

Exposure to acrinathrin

Pupae raised in beeswax contaminated with 0.0125 mg/kg and 0.025 mg/kg acrinathrin showed a significant upregulation of the *relish* gene when compared to the control group. The detoxification gene, CYP6AS14 was also significantly upregulated when pupae were exposed to beeswax containing 0.0125 mg/kg acrinathrin. Exposure to 0.025 mg/kg showed an upregulation although not significant. Different immunity (*relish*, *defensin* and *dorsal*) and detoxification genes (CYP9Q3 and GST) were upregulated in pupae raised in beeswax contaminated with 0.1 and 1 mg/kg acrinathrin. The upregulation when compared to the control group was not significant. This shows that the immunity system of the bees was triggered by this contamination and that some detoxification processes were stimulated. The results are shown in **Figure 4**.

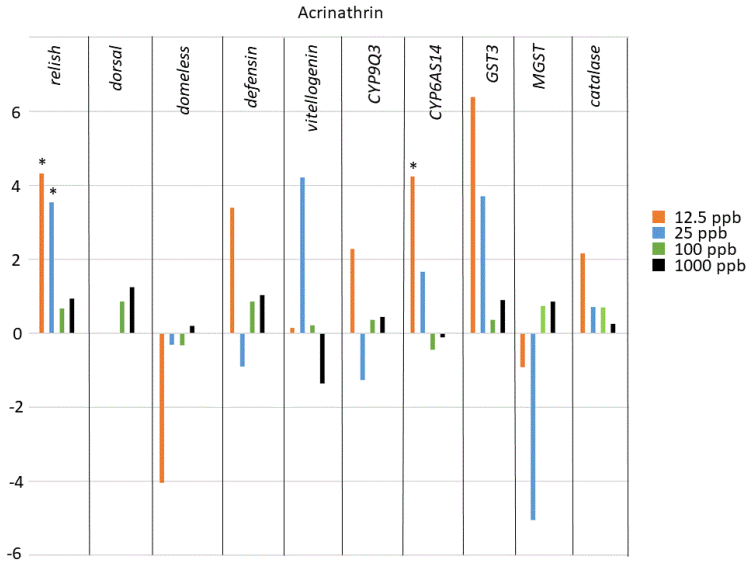


Figure 4. Expression profile of different immunity-related and detoxification genes after pupae exposure to different concentrations of acrinathrin (mg/kg): 0.0125 (orange), 0.025 (blue), 0.1 ppb (green) and 1(black).

Legend: The mean fold changes of mRNA expression for the different condition relative to their appropriate control are given on the y-axis, which represents the log2 transformed fold change. * $p<0.05$, using one-way ANOVA.

Exposure to chlorpyrifos-ethyl

Defensin 1 was downregulated when bee pupae were exposed to 0.005 and 0.0010 mg/kg chlorpyrifos-ethyl in beeswax. However, the down-regulation was only significantly different from the control group when exposed to 0.005 mg/kg in beeswax. It is also worthwhile to notice that both detoxification genes, CYP6AS14 and CYP9Q3, were upregulated when the pupae were exposed to chlorpyrifos-ethyl. These upregulations were not significantly different from the control. When pupae were exposed to higher concentrations, 0.5 and 5 mg/kg chlorpyrifos-ethyl in beeswax, most of the tested immunity genes and detoxification genes were downregulated. In pupae exposed to beeswax containing 0.5 mg/kg chlorpyrifos-ethyl, CYP9, GST3, *catalase* and *domeless* were significantly downregulated when compared with the control group. The results are shown in **Figure 5**. The downregulation of the immunity system is in line with the results when exposed to lower concentrations, although at these lower concentrations the detoxification system was still active. The immunity system was also triggered at lower concentrations while at higher concentrations the bees seem to have suppressed immunity.

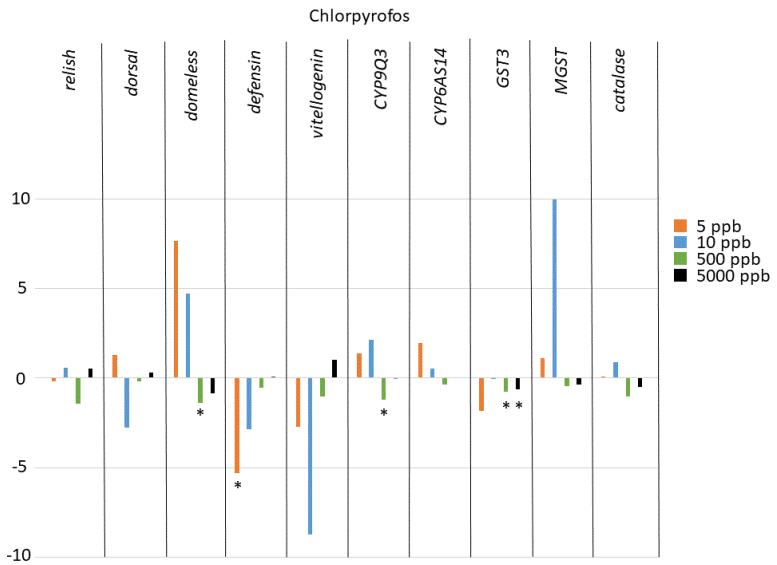


Figure 5. Expression profile of different immunity-related and detoxification genes in bee pupae after exposure to different concentrations of chlorpyrifos in beeswax (mg/kg): 0.005 (orange), 0.01 (blue), 0.5 (green) and 5 (black).

Legend: The mean fold changes of mRNA expression for the different condition relative to their appropriate control are given on the y-axis, which represents the log2 transformed fold change. * $p<0.05$, using one-way ANOVA.

Exposure to stearin

In the adulteration stearin experiment (3, 6 and 9%), no significant differences in gene expression were observed, compared to non-exposed pupae. Vitellogenin showed a very high level of expression when pupae were exposed to beeswax containing 3% stearin, but the variation in gene expression between the samples was also very high. Several genes were expressed at very low levels while the expression levels of the reference genes were normal in this first experiment. In a second experiment, the pupae were raised in beeswax contaminated with 3, 4 and 5 % stearin. In contrast with the previous experiment, the expression levels of all tested genes were similar to the control. Almost all immunity genes and detoxification genes were upregulated which shows that the immunity system and detoxification processes were triggered by the exposure to stearin. CYP9Q3 and *dorsal* were significantly upregulated when exposed to 4% stearin in the beeswax during the pupation (**Figure 6**).

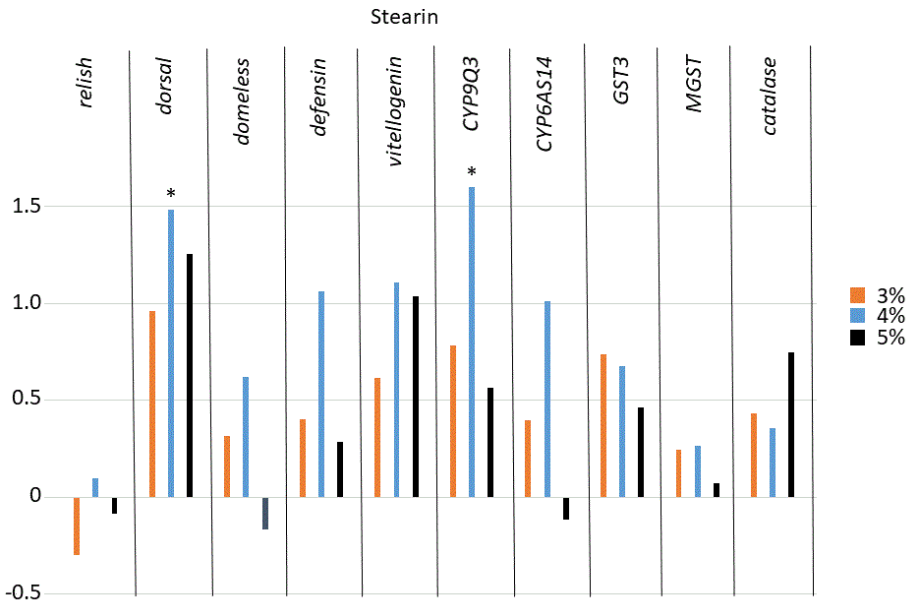


Figure 6. Expression profile of different immunity-related and detoxification genes in bee pupae exposed to different concentrations of stearin in beeswax (%): 3 (orange), 4 (blue) and 5 (black).

Legend: The mean fold changes of mRNA expression for the different condition relative to their appropriate control are given on the y-axis, which represents the log2 transformed fold change. * $p<0.05$, using one-way ANOVA.

DISCUSSION

Pupae rearing in field conditions

Contaminants are ubiquitous in beeswax and highly sensitive honey bee larvae/pupae are exposed to it during their development. As shown by the recent study by Morales et al. 2020, residue transfer to bee brood occurs by direct contact with the beeswax (Murcia Morales et al., 2020). Many studies characterised lethal residues effects (and to lesser extent adulterants) on adult honey bees in *in vitro* conditions, but much is still to be discovered on their unseen/sublethal effects, not only on adult honey bees but also on larvae/pupae, in more realistic *in vivo* conditions.

We used smaller colonies (Mini Plus hives) to conduct the field tests; Mini Plus hives are easier and quicker to handle, measure and observe. The system can sustain stable colonies that function as efficiently as larger colonies. Moreover, the assembly of study colonies is done to a high degree of standardisation in terms of brood quantity and adult bee population. For data verifiability and reproducibility, the basic units of the system, i.e., hive parts and frames, are available from beekeeping equipment suppliers (Allan and Dean, 2022).

The tests in field conditions allowed the brood to develop in its natural environment, in the hive without being disturbed by artificial test conditions, brood could also benefit from the colony effect (stress reduction). It seems that bees in field conditions are able to set up an immune reaction while bees housed in artificial cages suppress this reaction (De Smet et al., 2017). However, we cannot exclude potential environmental contaminations in beebread, though the oral contaminations could have impacted the gene expression results. Nevertheless, the analytical results obtained were comparable, whatever repetition of the experiment, indicating a predominant effect of the tested pesticide on gene expression.

Contamination and adulteration

Although not much is known about the effects of chlorpyrifos on honey bee brood, pupae appeared to be the most sensitive to chlorpyrifos out of five tested substances (Dai et al., 2017). Moreover, the synergistic effects of chlorpyrifos combined with other pesticides led to high larval mortality (Dai et al., 2019). Chlorpyrifos is not used in apiculture, but this highly toxic organophosphate is one of the most ubiquitous chemicals found in hive matrices like beeswax. The accumulative 6-day mortality of larvae exposed repeatedly to chlorpyrifos at 1.5 mg/L of diet preparation was more than 50% (Zhu et al., 2014).

Acrinathrin was used in apiculture to control the mite *Varroa jacobsoni*. It is still found at high concentrations in beeswax samples despite its high toxicity and developed resistance. Its transfer into larvae after direct contact with contaminated beeswax has been shown previously (Murcia Morales et al., 2020).

The emergence of adulterations issue is recent and few studies confirmed an association between the presence of stearin/stearic acid at certain levels and detrimental effects on bee brood. With this pilot research, we tried to assess the possible impact of adulterant stearin on honey bee gene expression.

Gene expression analysis

The effect of the different compounds on the expression of some key enzymes was studied. More and more studies are investigating the use of immune genes as markers for colony health at the field level (Barroso-Arévalo et al., 2019). In this study, gene expression profiling of four genes involved in the major immune response to pathogens and eventually to environmental stress factors was performed: *relish* is involved in the Imd pathway; *domeless*, in the JAK-STAT pathway; and *dorsal*, in the Toll pathway and *defensin* can be used as a marker for antimicrobial peptide production (Brutscher et al., 2015). Next to the immune-related genes, some detoxification genes, CYP6AS14, and CYP9Q3 were also included. The detoxification process of the exposed pesticides and metabolites may be the

initial process in neutralizing the chemicals. Exposure to pesticides may also lead to oxidative stress with can induce pathways involving catalase and GST to neutralize reactive oxygen species (ROS).

Our results suggest that exposure to acrinathrin is activating the immune system. At lower concentrations in beeswax (0.0125 and 0.025 mg/kg) the relish gene was significantly upregulated while at higher concentrations, defensin and *dorsal* were also upregulated although not significant. This likely reflects that acrinathrin is activating the Imd pathway leading to NF-κB activation. Relish regulates the expression of several antimicrobial peptide genes, such as defensin synthesis. The elevated *dorsal* expression suggests that next to the Imd pathway, the Toll pathway is also triggered in pupae raised in acrinathrin-contaminated beeswax at higher concentrations. This may also lead to the production of AMP-like defensin (Schlüns and Crozier, 2007). These results clearly show that the immune system of pupae raised in acrinathrin-contaminated beeswax is triggered, with possible negative impacts on colony health. However, further work is required to confirm this. Next to the triggered immune system, the expression of CYP6AS14 was significantly upregulated when pupae were exposed to beeswax containing 0.0125 and 0.025 mg/kg acrinathrin. For concentrations of 0.1 and 1 mg/kg, the expression of CYP9Q3 and GST3 were elevated although not significant. This shows that detoxification mechanisms are triggered to metabolize acrinathrin and the generated reactive oxygen species (ROS). Considering these results together, it seems that pupae can react and try to cope with acrinathrin exposure.

Exposure to chlorpyrifos results in lowering the expression levels of defensin for the concentration of 0.005 mg/kg in beeswax. At 0.5 mg/kg, the expression of *domeless* was significantly down-regulated. *Domeless* is a key enzyme in the JAK-STAT pathway, while defensin expression may be regulated by the Imd and Toll pathways. This expression profile suggests that the innate immune system is suppressed in pupae raised in chlorpyrifos-contaminated beeswax. The expression levels of CYP9Q3 and GST3 were also significantly lower in pupae exposed to 0.5 mg/kg chlorpyrifos in beeswax. Almost all expression levels of the tested immune and detoxification genes were down-regulated except the expression of the CYPs when exposed to 0.005 and 0.010 mg/kg chlorpyrifos in beeswax, although not significantly different from the control. The expression profile suggests that bees are not or less able to neutralize chlorpyrifos and may be more vulnerable to pathogens and environmental stressors. This reaction to chlorpyrifos could be associated with the lipophilic structure of the compound and the lipid composition of the bee cuticle (Bacci et al., 2006). Lipophilic compounds exhibit greater affinity for the cuticle and are thus more easily absorbed and readily transported to their target site of action (Leite et al., 1998). This hypothesis formulated by Dorneles et al. who assessed organophosphorus pesticides toxicity to stingless bees was based on the low water solubility of chlorpyrifos (1.05 mg/L at 20°C) (Dorneles et al., 2017). Compounds that are more lipophilic (i.e. less soluble in water) are able to penetrate more readily through the cuticle.

Exposure to stearin triggered the immune system and detoxification system of the pupae. CYP9Q3 and *dorsal* were significantly upregulated in the exposure experiment with 4% stearin. This likely reflects that the Toll pathway is activated and that the detoxification mechanisms were initiated. As all immune-related genes were upregulated, although not significantly, this suggests that the pupae reacted to the presence of stearin which may harm their further health status, which should be studied in detail in future experiments.

Conclusions

To the best of our knowledge, this is the first field research that investigates the genomic responses of honey bees reared in beeswax contaminated with chlorpyrifos-ethyl, acrinathrin and adulterant stearin. Exposure to acrinathrin and stearin at the lower concentrations slightly upregulated some immune-related genes. Likewise, exposure to acrinathrin and chlorpyrifos upregulated some of the tested detoxification genes. Immune and detoxification-related genes were downregulated in pupae exposed to higher concentrations of chlorpyrifos which suggest immunosuppression that makes honey bees more susceptible to infections. Our results confirm that pesticide residues at the tested concentrations may lead to decreased or increased honey bee immune response and thus, honey bee health may be challenged. As suggested by Dai et al. (2017), organophosphates (chlorpyrifos) seem to represent a higher risk to honey bee health than pyrethroids. Further research on gene expression is crucial to understand the undelaying mode of actions of pesticides. For an economically important and emblematic species such as honey bees, the identification of substance-specific response factors might ultimately serve to identify molecules that are safer for honey bees and the ecosystem's health.

Abbreviations used

CYP: Cytochrome P450
EFSA: European Food Safety Authority
HQ: Hazard Quotient
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
GST3: Glutathione S-transferase 3
MGST: microsomal glutathione S-transferase 1
RLP8: receptor-like protein
ROS: Reactive Oxygen Species
UGent: University of Gent
ULiège: University of Liège

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work would not have been accomplished without the help of Benoit Dupret, the beekeeper that helped supervise the experimental apiary.

Conflict of interest

The authors declare that they have no conflict of interest.

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Chapter 4 - General discussion and Perspectives

- **Representativity of the data**

To date, there are no precise, comprehensive figures for the entire beekeeping sector in Belgium, as an undefined number of beekeepers, in both regions, are reluctant to register with the competent authority (Federal Agency for the Safety of the Food Chain) or with beekeepers associations. There is a reason for this; most beekeepers have a limited number of hives, do not commercialise their honey, do not want to pay taxes on the sale of honey and fear sanitary controls. We roughly estimate their number in Belgium at 10 000. For our studies, not to base the representativeness of the data on approximations, we always relied on the effective number of beekeepers officially registered on the lists of the Federal Agency for the Safety of the Food Chain (FASFC). Nevertheless, throughout our online survey (**study 6**), we have also been able to reach the beekeepers that are not registered on the FASFC list. Another element that should be taken into consideration is that the beekeeping sector is characterized by its great diversity, which can be materialised by different aspects such as the size and models of the apiary but also by the statutes, the motivations, the forms of engagement in the activity and the associated beekeeping organisations (Fortier et al., 2019). This great heterogeneity is only very partially reflected by statistical data.

- **Cross-sectional studies**

We have used cross-sectional studies in this work (**studies 1, 2 and 5**) to identify risk factors inside BMP and within the pesticide variety. A cross-sectional study is a type of observational study design that involves looking at data from a subset of a population at one specific point in time. In a cross-sectional study, the outcomes and exposures of the study subjects are measured at the same time. It is described as taking a "snapshot" of a group of individuals. The subjects in a cross-sectional study are simply chosen from an available population of potential relevance to the study question. There is no prospective or retrospective follow-up (Wang and Cheng, 2020). Because the outcome and exposure variables are measured at the same time, it is relatively difficult to establish causal relationships from a cross-sectional study. Nevertheless, they are the best way to determine the prevalence and can study the associations of multiple exposures and outcomes. Cross-sectional studies often need to select a sample of subjects from a large and heterogeneous study population. Thus, they are susceptible to sampling bias. In epidemiology, sampling can be defined as the process of selecting certain members or a subset of the whole population to estimate the characteristics of the population. Creating a solid sampling plan in a cross-sectional study is critical because of the considerable heterogeneity usually observed in the target population. To reduce this bias, the sample of beekeepers was stratified and randomised (**studies 1 and 2**). This method of sampling first stratifies the whole study population into subgroups with the same attributes or characteristics, in our case amateur/hobby beekeepers, known as strata, then followed by simple random sampling from the stratified groups, where each element within the same subgroup are selected unbiasedly during any stage of the sampling process, randomly and entirely by chance.

For study 1, we opted for face-to-face interviews by the same investigator to assess BMP and mortality rate. Interviews are more time-consuming than online surveys or self-completion questionnaires, potentially limiting the sample size but leading to a higher response rate, a higher response quality and a better interpretation of the data, we feel that this was a guarantee for the data quality. For **study 5**, an online survey for self-completion was used. Using a survey is the best way to reach a large sample of the population of interest but can result in low response rates, it also presents the risk of 'volunteer bias' as the participants volunteering to take part in a study intrinsically have different characteristics from the general population of interest (Brassey, 2017). To limit the bias, several reminders were sent through different channels (social media, beekeeping networks of ULiège, UGent, local beekeeping unions, etc.) and the representativity was indirectly compared to the target population. The answer rate of the online survey conducted seemed to be acceptable as they are higher by two per cent (6,7%) than the average reported rate of 4,7% for personalised internet surveys (Sinclair et al., 2012).

- **Loss rate and its acceptability**

The colony loss rate was based on beekeepers' declarations. The accuracy of the estimated loss rates depends on the accuracy and representativeness of the data reported by the beekeepers. To assess winter loss rates, beekeepers were asked to state the number of colonies that wintered, and how many of these colonies were alive after winter. The overall proportion of colonies lost was found by subtracting the colonies alive after winter from the stated colonies going into winter, this is divided by the stated colonies going into winter. The colony loss metric is subject to discussion as BMP vary between regions and between professional and amateur/hobby beekeepers. Merging weak colonies into stronger ones decreases the number of colonies in an apiary, but to define those as dead would be inaccurate, so they are considered lost.

We have set the acceptable winter mortality level at 10% according to earlier work (Haubruge et al., 2006; El Agrebi et al., 2020; El Agrebi et al., 2021) and to the EPILOBEE consortium, although no reference values exist for the acceptable level of colony losses during winter, various acceptable rates of winter colony mortality were reported in European countries (Charrière and Neumann, 2010; Genersch et al., 2010b) and outside Europe (Steinhauer et al., 2015). Moreover, according to our data, the level of 10% divides well the population into two subpopulations. We estimate this level as robust for the Belgian context.

- **Multiple factor interaction and the difficulty to establish causal relationships**

Despite honey bees being at risk of several biotic and abiotic factors, no single factor has been shown to be the only cause of colony mortality (Nazzi and Pennacchio, 2014), suggesting that honey bees are not endangered by monocausal stress but rather by a combination of multiple factors (Moritz

et al., 2010). In a study conducted by VanEngelsdorp et al., where 61 variables (stress factors) were weighed in association with colony mortality, the author concluded that "None of these measures on its own could distinguish colony mortality from control colonies" (Van Engelsdorp et al., 2010). The role of interactions between factors is becoming a central paradigm for explaining current colony losses (Moritz et al., 2010), even though instances occur in which a single factor can lead to colony mortality (Le Conte et al., 2010).

In terms of their causal effects on honey bee losses, risk factors may interact additively or synergistically, thereby augmenting, in the case of positive interaction, the individual effects of each other it is then extremely difficult to establish the causal relationship leading to colony mortality. Risk factors can occur in the short term (e.g., environmental pesticides and pathogens) or the long term (e.g., loss of genetic diversity and environmental resources) with regard to the lifetime of the bee population, future studies of interactions will need to integrate the differing timescales of individual factors (Le Conte et al., 2011).

Inside BMP, the results obtained from **study 1** confirmed the hypothesis of an interaction between factors in amplifying the risk, the classification tree analysis (CTA) allowed to determine the relative importance and inter-relation among the different risk indicators of colony losses. The CTA showed that the score of the equipment and the use of divider boards were the two predictor variables with the strongest overall discriminating power.

- **Pesticides**

One of the objectives of this work was **to assess the prevalence and the concentrations of pesticide residues in beeswax** on the national level and **estimate the potential toxic risk of wax to bees.** This work resulted in the creation of a pesticide toxicity estimation tool for beekeepers and wax transformers. We also assessed **the current situation of beeswax adulteration** in beekeepers'and commercial wax in Belgium.

We had no doubts on the serious threat that pesticides represented to honey bees for the simple reason that honey bees are insects and, therefore, susceptible to any poison designed to kill insect pests (insecticide). But, what about other pesticides, such as herbicides and fungicides? Could they also affect honey bee health? If the target of such chemicals is not the insects, many argued, they are probably safe to bees. Research conducted in the past few years in countries with a long history of pesticide usage suggests differently (Atkins and Kellum, 1986; Domingues et al., 2021; Johnson et al., 2013).

- **Representativeness of pesticides found in beeswax and accuracy of the multi-residue GC–MS/MS and LC-MS/MS analysis methods**

We only find what we look for. Our approach to documenting pesticides in beeswax samples has been to search for a wide sweep of pesticides (n=293) that are used frequently in hives, around apiaries and further in the environment. For many published studies that document pesticide residues in beeswax, this has not been the case, and more emphasis has been placed on neonicotinoids (Arce et al., 2017; Balfour et al., 2017; Woodcock et al., 2017) and other systemic insecticides (Bundschuh et al., 2019; Tosi et al., 2021) with high bee toxicity or on the in-hive applied acaricides (Giacobino et al., 2015; Menkissoglu-Spiroudi et al., 2001). We should also not forget that a non-neglectable share of the beeswax used in Europe for beekeeping originates from developing countries where obsolete pesticides are still in use.

A focused study on one pesticide or a single class of chemicals allows for use of a more sensitive method of analysis, while an affordable method that detects many pesticides from widely different chemical classes is compromised by not attaining the lowest limit of detection (LOD) for every pesticide analysed and by some uncertainties. We opted for a more complete assessment of the pesticide burden that bees encounter instead of a biased approach to search for only chemicals renowned for their bee toxicity. The main inconvenient of this approach is that the attainable LOD for a focused method will generally be lower; the more chemically variable and greater number of pesticides on the screen increases the costs of analysis while reducing, at least for some pesticides, the sensitivity of their detection (increasing LOD). Nevertheless, we chose for the multi-residue GC–MS/MS and LC-MS/MS analysis methods that incorporates hive miticides and their metabolites in addition to a large number of potential pesticides from their foraging arena as a better way to measure potential sources of risk for honey bees.

From an analytical point of view, sample preparation should guarantee the representativeness and complete extraction of the residues for a high recovery (Niell et al., 2014). As pesticide residues in beeswax samples are not evenly distributed, beeswax was grounded and homogenised using liquid nitrogen. This method allows limits of quantification (LOQs) of 0.01 mg/kg and limits of detection (LODs) of 0.003 mg/kg for most residues, these limits were considered as the lowest successfully validated levels, that is, the levels at which acceptable recoveries (70–120%) were achieved.

The actual chromatographic process includes uncertainties that are reflected in the concentrations of the found pesticide when these are around the LOQ. Uncertainty is a basic characteristic of any measurement; uncertainty is always present, at every step of a procedure. In a typical chromatographic analysis, the main elements of uncertainty are associated with:

i. the amount of sample used for a determination,
ii. the recovery value of the analytical procedure, including the recovery of an analyte from a sample and the recovery associated with the accuracy of final determinations,
iii. the repeatability of determinations for a true sample (represented by the repeatability of signals)
iv. the concentration associated with the upper detection limit, and
v. calibration of the analytical instruments.

According to the definition of the limit of detection, measurement uncertainty is 100% when the concentration level is equal to LOD. Therefore, the higher the concentration calculated from the LOD, the lower the uncertainty (Konieczka and Namie, 2010).

Our aim was also to assess the risk of the pesticide burden to bees to predict hazards and risks of exposure at levels above the no observable effect level (NOEL) or lowest observed effect level (LOEL). For products intended for bees and known acute toxicity or behavioural effects, and chronic sublethal effects on longevity and reproduction, generally a LOD greater than 0.001mg/kg is used which is sufficient even for the most toxic pesticides such as imidacloprid.

Assessing the risk of a pesticide to bees uses the effects after exposure such as the acute LD50 (lethal dose for 50% of treated bees) and long-term chronic or sub-lethal EC50 (effective concentration that reduces by 50% the growth, learning, and longevity etc. of treated bees). The risk of exposure is predicted by both frequencies and mean residue amounts in pollen, nectar, water and wax, and the persistence (time to remove 50% = half-life) and fate (degradation and metabolism rates) of the pesticide in the hive or exposed bee. Knowing the physicochemical properties of a pesticide active ingredient (octanol (oil)/water partition coefficient, water solubility, vapour pressure) will aid in predicting routes of exposure and the potential for bioconcentration.

- **Approximation and imperfections**

Adult bee oral toxicity data for active ingredients are available in dossiers and databases such as Pesticide Property DataBase (PPDB), adult bee contact toxicity is more scarce. Data on active ingredients are also available rather than formulations. Nevertheless, it is the formulations that are used as veterinary medicine and plant protection products. Often, the toxic effects of commercial pesticide formulations are equated to the effects of their active ingredients, which mostly results in an incorrect assessment of their safety (Kalyabina et al., 2021). Recently, research has invested considerably in the separation of effects and the detection of hidden dangers (Beggel et al., 2010; Gomes et al., 2021; Queirós et al., 2018; Vanlaeys et al., 2018), nevertheless, the effects of the adjuvants and their possible

synergies with other substances and the active molecule constituting the final product are still poorly understood. Somehow, the research with active ingredients is relevant, as it is these, rather than the formulations, that are likely to be present in pollen or nectar (Thompson, 2010). In our last study (**study 7**), it is the active molecule of chlorpyriphos-ethyl and acrinathrin that were used to contaminate pesticide-free beeswax rather than formulations as the synergetic effects of adjuvants are poorly defined and minimise the interactions of risk factors.

Thus, with this lack of data on contact toxicity, formulations and adjuvant effect, it is still difficult to perfectly mimic field-realistic exposure scenarios. In terms of toxicity, there are limited data available on the relative sensitivity of adult honey bees and larvae (brood), and therefore direct extrapolation from adult data is not possible (Bodin et al., 2022).

There are, thus many unanswered questions regarding pollinator exposure to pesticides. We do not currently have an accurate picture of what pesticides are used, where and in what amounts, nor do we have accurate measures of just what the maximum exposure is in agricultural or urban settings on blooming plants. Once contaminated pollen is collected, the potential transformations of pesticides in bee bread and royal jelly are also currently unknown. Clearly, the potential for pesticide involvement in declining honey bee health is far from being understood, and it is too early to discount them as key factors associated with colony mortality.

- **The advantage and the complexity of using beeswax as an indicator for assessing environmental contaminations**

With its high content in lipids, beeswax is the perfect matrix to capture pesticides that are mostly lipophilic and maintain them for many years without their degradation. Nevertheless, these same properties make it difficult to assess the real environmental contaminations due to their accumulation in the matrix over time. Experiments designed to assess the persistence, fate and metabolism of various pesticide compounds have been performed over periods of up to 2 years using experimental hives (Martel et al., 2007). The fate of a compound in the hive is determined by several time-dependent processes: uptake, distribution, biotransformation, volatilisation, diffusion within matrices, phase partitioning, advection from the hive by air ventilation and bee turnover and product collection by beekeepers. The relative importance of these processes is essential in determining the fate of the pesticide in the hive and therefore, the contamination effects on bees and their products (Bonzini et al., 2011). To date, very few studies addressed this issue and very little is known about pesticide fate in beeswax. In study 2, the pesticide burden for honey bees has been assessed, nevertheless, determining the origin of these pesticides is a challenge as most beekeepers purchase commercial beeswax from various origins that already have a contamination history before their use in Belgium.

- **Assessing contact pesticide risk to bees with the Hazard Quotient (HQ)**

Typically, hazards are estimated by calculating a Hazard Quotient (HQ), which is based on acute toxicity data for different pesticides and the quantity of those pesticides applied to a field or detected on bees and matrices associated with their hive (honey, wax, pollen, and/or bee bread). In our study, the HQ was primarily calculated using the concentration of pesticides found in beeswax. These amounts are then related to the LD50 values of the detected pesticides (Thompson, 2021), according to the method suggested by Stoner and collaborators (Stoner et al., 2013). Two threshold values were set according to scientific literature and benchmarking: 250 or the relevant HQ, under which no toxic adverse effects are to be expected, and 5000 or the elevated HQ, above which the toxic potential of beeswax could harm the bees (Traynor et al., 2016a). Between these two values, the closer we get to the upper threshold, the greater the toxic potential will be. Moreover, the HQ calculation is based on the cumulative/additive effects of pesticides. Real-life exposure occurs to complex chemical mixtures, pesticides can affect each other according to the additivity which is the most commonly reported pattern of mixture response, in approximately 80% of the cases (Belden, 2022; Woodcock et al., 2017). Nevertheless, our formula does not take into account other synergetic/antagonistic effects. This research work will be developed in the near future.

Contact LD50 values are not available for some substances of interest and therefore, we chose to use the lower, more conservative LD50 value for a pesticide when available, regardless of the likelihood of oral or contact exposure (Traynor et al., 2016a) or went this was also not available, we attributed a value of 200 mg/kg (cause no harm) to the substance LD50. It is concerning to use acute LD50 values to understand metrics of chronic exposure as there is a mismatch in the toxicity metric of an acute LD50.

Another element of the HQ that can be discussed is the threshold and how it was set. Relevant and elevated thresholds used for beeswax are commonly set five times higher than thresholds in other matrix types, owing to the slower release of pesticides to bees in wax compared to pesticides obtained from eating contaminated honey or pollen (Stoner et al., 2013). Moreover, in a recent study by Kast and Kilchenmann where a model to assess the migration of coumaphos from the beeswax into the diet was tested, the dietary coumaphos concentrations were between a fourth and a fifth of the initial concentrations in beeswax, which corresponded well with a 5-fold lower for dietary exposure as compared to exposure through beeswax (Kast and Kilchenmann, 2022). These results confirm the suitability of the HQ threshold for beeswax.

At best the HQ provides an underestimate of total exposure. Unfortunately, the current state of knowledge does not permit the development of more robust models that include these factors, and thus

we use these more simplistic models as a starting point of departure to help understand the risk posed by the real-world exposome for beekeepers and commercial wax suppliers.

- **Hazard Quotient and honey bee health**

Appart from our study (**study 3**), three other studies correlated colony health outcomes to HQ values: Lee et al. (2019) analysed the relationship between unbroken brood pattern, patchy brood pattern and HQ and found that HQ was not correlated with brood pattern (Lee et al., 2019). However, what was found in correlation with the brood pattern was the number of pesticides detected. The two other studies which examined colony health parameters did not find any significant correlation with HQ detections. Smart et al. (2016) examined the percent loss of colonies in six apiaries over 3 years. A strong relationship was found between percent uncultivated forage land and apiary survival; pollen quantity was also found to influence apiary survival more than pollen diversity and did not appear to be related to HQ values at the same sites (Smart et al., 2016). This suggests that the forage quantity (and to a lesser degree, quality) had a larger impact on colony survival than HQ detections. Similarly, we did not find any link between HQ detections of flumethrin and apiaries where colony losses exceeded 10%. As this study examined only one pesticide, other pesticide detections or management practices likely had stronger impacts on colony health than the HQ of a single pesticide.

- **Using gene expression to investigate the effects of chemical stressors on honey bee health**

The analyses of gene expression was used as a supplementary way to probe the effects of chemical stressors on honey bee health, as the effects of subletal doses are still difficult to characterise. The underlying idea is that changes in gene expression can provide a sensitive indication of effects that will eventually negatively impact a variety of physiological systems. The gene expression analysis has provided insights into the mechanisms underlying tolerance or resistance to these stressors.

The discoveries in gene expression opened new research and breeding perspectives and breeding resistant or resilient honey bee to *Nosema* or to *Varroa destructor*. Although, resistance to Varroa is driven by multiple physiological and behavior traits including grooming (where mites are removed from a nestmate's body), hygienic behavior (removal of parasitised brood) and suppressed reproduction of female mites feeding on developing pupae, some bee breeding programs have shown outstanding results.Modern techniques of selective breeding showed high potential to improve important traits of honey bees such as the enrichment of genes, the reduction of defensive behaviour or swarming tendency (Le Conte et al., 2020).

While it has been possible to identify several genomic regions associated with variation in resilience to different stressors, identifying the specific genes and using this information to breed and maintain improved stocks of bees is a challenge. Variation in many of these traits is influenced by

variation in many genes, thus setting up the possibility of many complex interactions among genes in determining phenotypic differences. In other words, a particular genetic variant that is associated with variation in grooming behavior or pathogen resistance in one population may not be casually relevant in a different population. Furthermore, honey bee queens typically mate with an average of 12 drones, always outside the hive (Tarpy et al., 2015). Thus, beekeepers must use instrumental insemination or tightly controlled breeding yards to limit uncontrolled gene flow into selected stocks. Negative effects of inbreeding or low genetic diversity in a colony can have detrimental consequences (Mattila and Seeley, 2007; Seeley and Tarpy, 2007). To date, in most studies, pesticide exposure generally causes changes in expression of detoxification genes, the identities of these genes can vary greatly across pesticides and studies (Boncristiani et al., 2012; Gregorc et al., 2012). It is also important to note that most studies show correlations between stressors and changes in gene expression levels, nevertheless the specific reaction of a honey bee on a treatment/pesticide exposure seems to be more complex and is dependent on several factors. Detailed functional analyses of these processes must be studied.

Other gene expression studies have also suggested that nutrition and diet can mitigate the effects of pesticides. Moreover, natural pollen/honey based diets result in improved overall health, which in turn improves responses to pesticides and other stressors (Schmehl et al., 2014). The use of gene expression analytical methods holds great promise for improvements in the health of honey bees and other critical pollinator species.

Recommendations

- **Recommendations for beekeepers**

Beekeeping management practices were shown to play a crucial role in maintaining the health status of the colony and beekeepers' knowledge is applied at the interface between landscape and hive-scale factors and has a great potential role in supporting a One-Health approach.

Honeybees pose three specific management challenges to beekeepers in monitoring and managing their health. First, though honeybees are semi-domesticated insects, they are reliant on the open **environment**. Foraging excursions are inherently hazardous: bees interact with individuals from other colonies and are potentially contaminated by diseases and pests visiting plants previously visited by infected bees (Anderson et al., 2013). Through their nearby environment, honey bees are also exposed to numerous contaminants at levels endangering their survival and health, their ability to reproduce and their capability to cope with other stressors such as pathogens, and this represents a threat to biodiversity and ecosystem functioning which is now acknowledged (Acevedo-Whitehouse and Duffus, 2009; Kendall, 2010; Marcogliese and Pietrock, 2011). Thus, **the choice of the apiary location is extremely relevant**. It is very important to place the apiaries in protected areas, avoiding windy and humid areas,

at distance from conventional agriculture and industry. An uncontaminated and rich environment to assure qualitative nutrition is of utmost importance.

The second most important challenge is **managing *Varroa*** infestation: managing bee health, therefore, involves a constant process of negotiation between actions that might benefit a colony and actions that might put it at risk. Rather than treating all pathogen and pest species present within a single hive, it may be more useful to work within a conceptual framework of "tipping points", where attention is focused only on levels of a disease or pest that pose a sufficient threat (Hinchliffe, 2015). This more holistic approach to health management is somewhat practised within the beekeeping community under the term "integrated pest management". Integrated pest management within a beekeeping context pushes for intense monitoring of pests and parasites, allowing action beyond prescribed tipping points of symptoms or density of parasites. We recommend beekeepers reduce the use of veterinary drugs to a strict minimum to avoid the appearance of resistance and residues, to carefully follow the instructions for appropriate use and prefer the use of oxalic acid against *Varroa*.

The third challenge will be **to reduce the in-hive contamination burden on bees** and more specifically on honey bee larvae by improving the ecotoxicological quality of beeswax. Both pesticide residues and adulterations showed an impact on honey bees' gene expression (**study 7**). Comb wax should replace more frequently (1/4 to 1/3 of than old brood frames (ITSAP, 2017)) with potentially non-toxic wax. The tool BeeToxWax has been made available for beekeepers and wax transformers to control its pesticide residue content. We also highly recommend the use of greater amounts of beeswax cappings in the manufacturing process of beeswax foundation, the substrate beekeepers purchase to aid their bees in building comb, as well as using organic wax sources to gradually decrease residues in the colony matrix.

Evolution in management practices is needed as honey bees are exposed to frequent changes in land use, pesticide use, climate, emerging predators, diseases. Adapting BMP to these changes and monitoring the needs of evolving colonies is of crucial importance for their survival. The tool BeeBestCheck has been made available to beekeepers to assess and improve their management practices. Improving BMP will not prevent all losses, but a few behavioural changes including proper comb management, equipment hygiene, and *Varroa* management, can lead to a non-negligible reduction of the risk of colony losses. The study on beekeepers' perception of risk to bees (**study 6**) pointed out that most beekeepers had a great level of perceived risk combined with strong perceptions of the benefits of actions, these elements increase the motivation to act in better ways.

- **Recommendations for researchers**

Honeybees live in a complex social-ecological system, influenced by highly varied forms of human management, from the hive to the landscape scale. In the past decades, focusing on single stress factors without taking other biotic and abiotic factors failed to translate into a successful reversal of bee declines. Thus, the development of a holistic approach such as the 'One-Health concept' in research to address the scope of crises facing pollinators is crucial. The One-Health concept can effectively bridge the different sectors/stakeholders and scales involved with beekeeping, focusing as it does on interdisciplinary approaches to understanding health.

Apart from the approach recommended above, many gaps still need to be addressed in terms of research:

- Data in the area of bee toxicology, i.e. dose-response relationships, the toxicity of metabolites, toxicokinetics and toxicodynamics for different types of chemicals including pesticides and their metabolites, contaminants and veterinary medicines for different honey bee subspecies, bumble bees and solitary bee species and in representative categories of bees such as larvae, foragers, queens and drones (Food and Authority, 2014) still need to be generated.
- Further standardised laboratory tests for acute and chronic toxicity of lethal/sublethal endpoints of multiple chemicals including regulated products (pesticides, veterinary medicines) and contaminants in bees (i.e. in different honey bee subspecies, bumble bees and solitary bee species; and in representative categories of bees such as larvae, foragers, queens and drones) should also be generated.
- Many other factors influence bee health, which we have not explored sufficiently, especially seasonal and climatic changes.
- Social sciences have not been interested in date in the parameters, brakes or levers that are nevertheless conditions for change in the beekeeping sector: carrying out plans that imply a transformation of the sector logically requires looking into the behavioural mechanisms of the actors of change, namely the beekeepers, their professional structures and the adequacy of the support systems. To date, the human factor has been too little studied, although the changes are important and impact the context of beekeeping activities.
- Researchers are called on to publish in journals that are peer-reviewed and read by others in their field. They get little if any credit for publishing in lay magazines, this makes the research results non-accessible for beekeepers. Scientific results/information dissemination must be improved (Fabricius Kristiansen et al., 2022)

- **Recommendations for wax transformers**

The role of wax and wax transformers in honey bee health is underestimated. Wax if not recycled by the beekeepers is purchased from trade. Most foundation wax sold in Belgium has three origins: local wax, sold by the beekeepers, wax from the EU countries and Non-EU countries. The lack of standards for the composition and chemical contamination of beeswax specifically aimed at the protection of bee health is problematic. There are no legal standards set to define the toxicological quality of waxes or to set limits on the import of poor-quality waxes. Nevertheless, the limits set for 9 pesticide and veterinary drug residues by the scientific committee of the FASFC to limit the sale of re-melted beeswax that exceeds these limits is recommended (Scientific Committee of the FASFC, 2018). In a quality approach in favour of honey bee health, we recommend the wax transformer to inform about the origin and traceability of beeswax and to analyse it for its toxicological content before buying it. The use of the BeeToxWax tool made available online to estimate wax potential toxicity is also recommended. Ultimately, setting a regional/national joint quality label under all Belgian beeswax transformers will increase wax quality and help beekeepers distinguish safe beeswax from unsafe ones. The development of a Walloon quality label is ongoing.

- **Recommendations for beekeeping federations and associations**

The beekeeping sector is divided and not well organised, it presents a wide variety of profiles and institutions, and this integral part of a complex system is subject to many issues. Greater coordination and collaboration between the different unions, groups and federations dealing with beekeeping is needed and would give the sector more strength to be heard by the authorities. Scientific support-advices of federations by a well-recognized committee should be stimulated.

- **Recommendations for veterinarians**

In Belgium, the biology and diseases of honey bees and other beneficial insects are minimally included in the study curricula of veterinarians if not at all. There is a clear lack in veterinarians' education on honey bee health. It is important to increase the competencies of veterinarians to ensure the health and welfare of honey bee colonies, gain beekeepers' confidence and achieve better relationships and cooperation with beekeepers. In the long term, the establishment of global networks of specialized "honey bee medicine" veterinarians will assist beekeepers with proper integrated pest management.

The competencies that a veterinarian must acquire to be qualified to practise in apiaries include:

- Clinical inspection of a honey bee colony and identification of signs of disease in brood and adult bees.

- Official sampling, and filling out the formal cover documentation for the delivery of samples to an authorized diagnostic laboratory.
- Basic laboratory examinations, knowledge of serious disease control, and preventive and eradication measures (Chauzat, 2014; Gajger et al., 2021).
- Inspect the beekeepers' records, take proper anamnestic data for a proper diagnosis, advise about disease control and prophylaxis, as well as prescribe the proper veterinary medicine products (Mutinelli, 2016).
- The veterinarian is the only one who can select the right veterinary medicine products for honey bee colony treatment, and issue proper recommendations to beekeepers on their responsible use, and information about withdrawal periods, possible side effects such as residues, and risks related to the development of resistance (Rivera-Gomis et al., 2019b).
- Knowledge in bee epidemiology and risk analysis is a key points to assess properly the One health (both bee, human and environmental compartments).
- Insight in breeding programs; insight in the semi-domesticated nature of bees; ecology and evolution.

➢ **Recommendations for authorities**

The National Pollinator Strategy 2021-2030 has been published a few months ago. Focusing on three axes, it aims to increase the population of pollinating insects by 50% and to reduce the number of declining species by half by 2030. This plan is extremely ambitious and many tangible actions to get there are needed:

- Harmonised approaches for setting protection goals for bees are needed on a global scale but applied locally.
- More efforts and means should be given to monitoring honey bees' and other bee species' health. These studies should be conducted over the longer term and with a wider scope of stressors that may affect bees.
- There is also the need for the development of guidelines and harmonised methods to facilitate data comparison between countries.
- The development of a best BMP guide focused on honey bee health rather than on honey production is also necessary.
- An educational campaign for users of pesticides or veterinary drugs is needed to increase awareness and good practices.
- A top-down policy approach will not prove an effective pathway toward integrating a One-Health concept into bee health, and efforts at inclusion must be carefully assessed to assure they move beyond rhetoric (Rauschmayer et al., 2009).

- Bringing together researchers from different fields, with public institutions, beekeeping stakeholders, conservation organisations and farmers, would help to provide the broad range of perspectives needed to solve challenges in pollinator protection.

Reversing pollinator decline will require the integration of hive-specific solutions, a reappraisal of engagement with the many stakeholders whose actions affect bee health, and recontextualising both of these within landscape scale efforts (Donkersley et al., 2020)

Conclusion and prospects

The overall objective of this thesis was **to better understand some of the risk factors (elements of beekeeping management practices, pesticide contaminations as well as adulteration in beeswax) affecting honey bee health** in the Belgian beekeeping context and **to provide tools, guidance and recommendations** to the beekeeping sector **to alleviate these potential risks and initiate this change** to protect domestic bees.

From a global point of view, pollinating insects including honey bees are crucial for the functioning of ecosystems, our food security, and so much more. Since the end of the 1980s, beekeeping has been going through an unprecedented crisis, the most tangible sign of it is the increased mortality rate of honey bee colonies across Europe and the USA. This crisis has made beekeeping visible to the general public by raising the bee to the rank of sentinel of the environment. Protecting them is highly urgent. The studies presented in this thesis have identified and quantified risk factors to honey bees inside the management practices and in beeswax, gave levers that are the conditions for a change of behaviour as well as tools to estimate the risks dealing with pesticides residues and management practices. Our work has been made accessible to beekeepers and was published in regional and even in German beekeeping journals (Deutsche Bienen journal).

The conclusion of the studies presented in this work all point out at detrimental effect of contaminants (pesticide residues and adulterants) on brood and adult honey bees and the importance of considering the risk of these contaminants both for honey bee health and for human health perspective. The benefit of the use of these products should be considered regarding their toxic effects on bees. From the pharmaceutical industry, more transparency is also asked for the commercial formulation of these products. Management practices were shown to play a crucial role in maintaining the health status of the colony. The use of transcriptional signatures to monitor stressors in honey bees and the expression of specific genes is a supplementary tool to understand the biochemical fingerprint of environmental contamination (Grozinger and Zayed, 2020).

Increasing public awareness of pesticides and veterinary drug effects on pollinators' health, in beekeeping, agriculture and the private sector is needed. Educational campaigns such as the one set up for anti-microbial resistance in a One Health approach would increase awareness and good practices. Introducing maximum residue limits for beeswax trade on the European level taking into account residue toxicity for bees and, ideally, for their larvae would also help to reduce the risk factors for bee health. As contaminations are ubiquitous in the environment, research on beeswax decontamination might help increase beeswax quality and decrease risk factors for honey bee colonies. The implementation of local and national bee-monitoring systems could be initiated with the participation of beekeepers for the longitudinal sampling of their hives within a citizen science program, and/or with the assistance of local technology transfer teams or professional apiculturists. Coordination with academic, industry or government laboratories would be needed for sample analysis and data interpretation. Adoption of this approach in agricultural and urban settings has the potential to provide powerful indicators of ecosystem health to inform policy at the local and global scale.

Chapter 5 - References

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Chapter 6 - Appendixes

Study 1

Appendix 1: Detailed list of the interview questions on bee management practices and results (n=186 beekeepers)

Category	Sub-category	Variable	Number or rate
Beekeeper	Typology	Age (year) (Mean±SD)	58±14.9
		Training	87.2%
		Union membership	91.4%
		Use of a logbook	59.6%
		Quick notes	23.4%
		Willing to improve practice	78.6%
	Beekeeping motivation	Interest for honey bees	58.8%
		Family transmission	21.9%
		Ecology	47.6%
		Honey production	23.5%
	Loss rate	Average winter losses	11.4%
		Average seasonal losses	3.0%
		Average year losses	14.4%
		Losses due to mismanagement	41.7%
Apiary	Size	Total number of hives	2175
		Apiary size (Mean±SD)	11.4±9.9
	Environment	Rural	72.2%
		Crop proximity <3000m	92%
	Plant availability estimation	Abundant	52.9%
Equipment	Hive type	Dadant blatt (10-12 frames)	46.5%
		Simplex	26.7%
	Equipment hygiene	Scraping	53.5%
		Burning	44.4%
Wax	Frame renewal	25-50% of the frame	58.3%
	Origin	Recycled	32.6%
		Commercial	57.2%
Honey bee livestock	Honey bee type	Buckfast	40.4%
		A. m. Carnica	38.3%
		A. m. Mellifera	17.7%
	Queen Rearing	Implemented	58.8%
		Nbr. self-produced queens (Mean±SD)	11.6±37
		Number of purchased queens (Mean±SD)	1.19±3.37
	Queen origin	National	91%

	Queen age	<1 year	51%
	Reproduction type	Divisions	48.1%
		Introduction mated queens	42.8%
		Number of implemted splits (Mean±SD)	4.62±8.46
	Swarms origin	Wild	34.2%
		Received	33.3%
	Breeding criteria	Honey bee stock gentleness	75.9%
	Queen introduction season	Spring	60%
		Fall	30%
Monitoring	Spring monitoring	Use divider board(s)	45.3%
		Brood check	88.3%
		Food check	92.8%
		Pollen entries check	90.7%
	Swarming	Swarming control	80.6%
		Royal cell destruction	54.9%
		Artificial swarming	33.5%
	Summer monitoring	Brood quality and uniformity check	85.2%
		Food quantity and position	78.7%
		Colony strength estimation in summer	84.5%
	Transhumance	Transhumance	19.3%
	Wintering preparation	Use of divider board(s)	38.5%
		Queen presence/laying control	65.2%
		Control wintering on bottom board	68.4%
	Colony strength	Estimation before wintering	82.4%
		Number of Strong colonies	63.1%
		Number of acceptable colonies	14.7%
		Number of weak colonies	20.3%
Varroa control	Veterinary advice	For prescription	88%
	Varroa management	Automatical treatment without diagnose	80%
		Infestation rate determination	19.3%
		Counting natural Varroa fall	42.2%
Diseases	Varroa reported infestation rates	Lack of knowledge	82.9%
	Varroa control biotechnical methods/drone brood removal	Implemented	36.4%
	Treatment efficacy check	Implemented	74.3%
	Nosema	Detected	6.95%
	Deformed wing virus (DWV)	Detected	39.6%
		Not significant	80%

BMP	Losses due to mismanagement	Weakness and pillage	34.3%
		Starvation	23%
		Queen failure	22.4%
		Other	20.1%
Harvest 2015	Spring	Implemented	82.3%
	Summer	Implemented	84.5%
	Yearly	Hives in production in kg (Mean±SD)	30.84±19.2
		All hives in kg (Mean±SD)	27.31±17.3

Appendix 2: BeeBestCheck online tool

A freely available online tool (BeeBestCheck) was developed to enable beekeepers to test their present and future management practices. The tool gives automated real-time advice on how to improve management practices in the Belgian beekeeping context (hobbyist beekeepers with small operations).

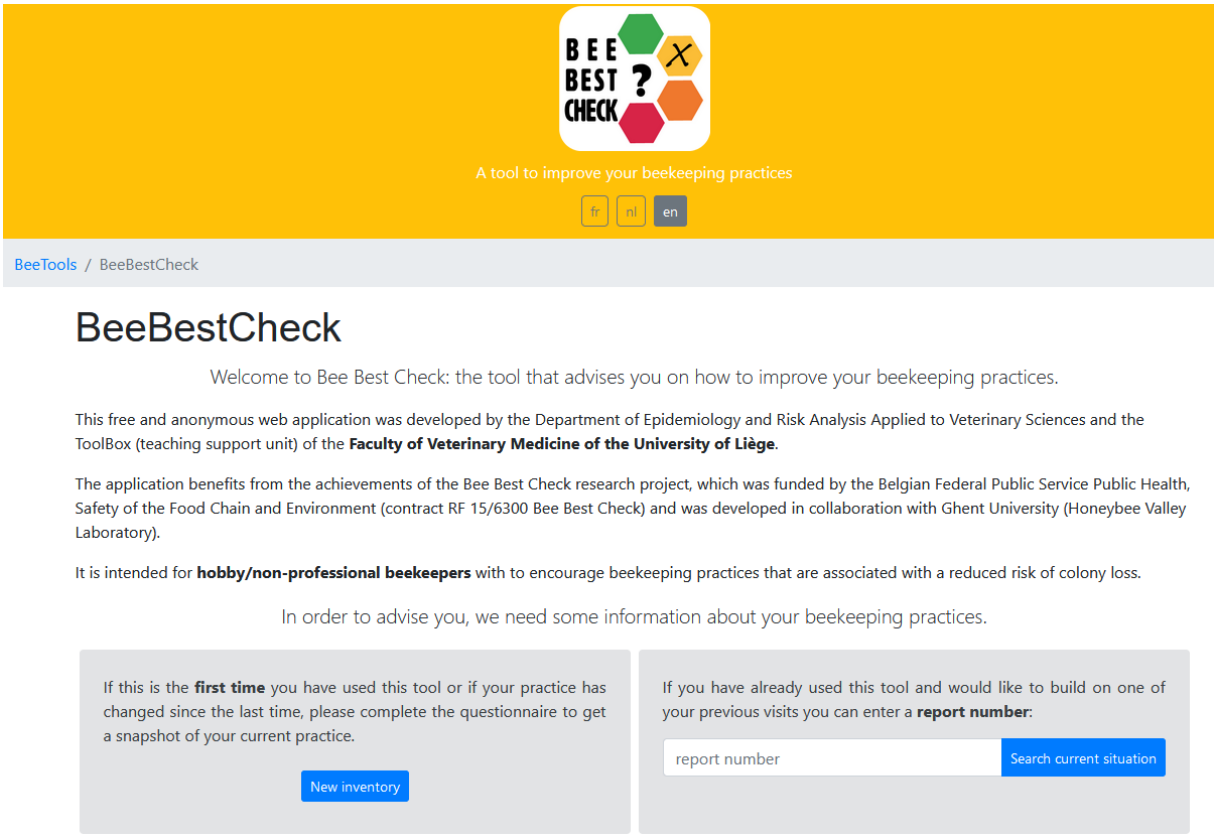
Access

The tool is freely available on smartphone and computer at this address https://www.beetools.uliege.be/beebestcheck/

Users guide

BeeBestCheck is currently available in French, Dutch, and English, click on the sign FR, NL, or EN (https://www.beetools.uliege.be/beebestcheck/) (Figure 1). If this is the **first time** you have used this tool or if your practice has changed since the last time, press the button 'New inventory', and please complete the questionnaire to get a snapshot of your current practice. If you have already used this tool and would like to build on one of your previous visits, you can enter a **report number** and press 'Search current situation'. After completing the questionnaire, positive comments or suggestions will appear to improve the current management practices. To generate a complete report (PDF form) at the end of the procedure, press 'generate a pdf' to have access to the report sheet.

Figure 1. Screenshot application BeeBestCheck start screen with language choice and introduction text



Study 2

Appendix 1: Active ingredients and their limit of quantification, screened in the 4 beeswax types by LC-MS/MS or GC-MS/MS

Active ingredient	LC-MS/MS LOQ [mg/kg]	GC-MS/MS LOQ [mg/kg]	Active ingredient	LC-MS/MS LOQ [mg/kg]	GC-MS/MS LOQ [mg/kg]	Active ingredient	LC-MS/MS LOQ [mg/kg]	GC-MS/MS LOQ [mg/kg]
Abamectin	0.01		Endosulfan, alpha		0.01	Metribuzin	0.01	
Acephate	0.01	0.01	Endosulfan, beta-		0.01	Mevinphos		0.01
Acetamiprid	0.01		Endosulfan-sulfat		0.01	Mirex		0.01
Aclonifen		0.01	Endrin		0.01	Monocrotophos		0.02
Acrinathin		0.01	EPN	0.01	0.01	Monolinuron	0.01	
Alachlor		0.01	Epoxiconazole	0.01		Myclobutanil	0.01	
Aldicarb	0.01		Esfenvalerate		0.01	Nitenpyram	0.01	
Aldicarb sulfone	0.01		Ethiofencarb	0.01		Nitrapyrin		0.01
Aldicarb sulfoxide	0.01		Ethion	0.01	0.01	Nitrofen		0.01
Aldrin		0.01	Ethoprophos	0.01		Nuarimol	0.01	
Amitraz*	0.01		Ethoxyquin	0.01		Omethoate	0.01	
Azinphos-ethyl	0.01	0.01	Etofenprox		0.01	2-Phenylphenol		0.05
Azinphos-methyl	0.01	0.01	Etridiazole		0.01	Oxadixyl	0.01	
Azoxystrobin	0.01		Etrimfos		0.01	Oxamyl	0.01	
Benalaxyl	0.01		Famoxadone	0.01		Oxydemeton-methyl	0.02	
Benfluralin		0.01	Famphur		0.01	Paraoxon-ethyl		0.01

Bifenthrin		0.01	Fenamiphos	0.01		Paraoxon-methyl		0.01
Binapacryl		0.01	Fenarimol	0.01		Parathion-ethyl		0.01
Biphenyl		0.02	Fenazaquin	0.01		Parathion-methyl		0.01
Bitertanol	0.01		Fenbuconazole	0.01		Penconazole	0.01	
Boscalid	0.01		Fenchlorphos		0.01	Pencycuron	0.01	
Bromacil	0.01		Fenhexamid	0.01		Pendimethalin		0.01
Bromophos (-methyl)		0.01	Fenitrothion		0.01	Pentachloroaniline		0.01
Bromophos-ethyl		0.01	Fenoxycarb	0.01		Pentachloroanisole		0.01
Bromopropylate*		0.01	Fenpropathrin		0.01	Permethrin		0.01
Bromuconazole	0.01		Fenpropimorph	0.01		Phenthoate		0.01
Bupirimate	0.01		Fenpyroximate	0.01		Phorate		0.01
Buprofezin	0.01		Fenson		0.01	Phorate-sulfone		0.01
Cadusafos	0.01		Fensulfothion		0.01	Phosalone		0.01
Captan		0.01	Fenthion	0.02		Phosmet		0.01
Carbaryl	0.01		Fenthion-oxon	0.07		Phosphamidon		0.02
Carbendazim	0.01		Fenthion-PO-sulfone	0.04		Piperonyl butoxide		0.01
Carbofuran*	0.01		Fenthion-PS-sulfone	0.02		Pirimicarb	0.02	
Carbofuran, 3-Hydroxy-	0.01		Fenthion-sulfoxide	0.01		Pirimicarb, Desmethyl-	0.01	
Carbophenothion		0.01	Fenvalerate		0.01	Pirimiphos-ethyl		0.01
Chlordane, alpha- (cis-)			Fipronil		0.01	Pirimiphos-methyl		0.01
Chlordane, Oxy-			Fluazifop-P-butyl	0.01		Prochloraz	0.01	
Chlordane, gamma-(trans-)			Fluazinam	0.01		Procymidone		0.01
Chlorfenapyr			Fluchloralin		0.01	Profenofos		0.01
Chlorfenson			Flucythrinate		0.01	Profluralin		0.01
Chlorfenvinphos	0.01	0.01	Fludioxonil	0.01		Propamocarb	0.01	
Chlormephos		0.02	Flufenoxuron	0.01		Propargite	0.01	
Chlorobenzilate		0.01	Fluquinconazole	0.01		Propetamphos		0.01
Chloroneb		0.01	Flusilazole	0.01		Propiconazole	0.01	
Chloropropylate		0.01	Fluvalinate, tau-		0.01	Propoxur	0.01	
Chlorothalonil		0.01	Folpet		0.01	Propyzamide	0.01	
Chloroxuron	0.01		Fonofos	0.01		Prothiophos		0.01
Chlorpropham		0.01	Formothion		0.02	Pymetrozine	0.01	
Chlorpyrifos (-ethyl)		0.01	Halfenprox		0.01	Pyraclostrobin	0.01	
Chlorpyrifos-methyl		0.01	HCH, alpha-		0.01	Pyrazophos		0.01
Chlorthal-dimethyl		0.01	HCH, beta-		0.01	Pyridaben	0.01	
Chlorthion		0.01	HCH, delta-		0.01	Pyridaphenthion	0.01	
Chlorthiophos		0.01	Heptachlor		0.01	Pyrifenox	0.01	
Chlozolinate		0.01	Heptachlor epoxide, cis-		0.01	Pyrimethanil	0.01	
Clofentezine	0.01		Heptachlor epoxide, trans-		0.01	Pyriproxyfen	0.01	
Clomazone	0.01		Heptenophos		0.01	Quinalphos		0.01
Clothianidin	0.01		Hexachlorobenzene (HCB)		0.01	Quinoxyfen	0.01	
Coumaphos		0.01	Hexaconazole	0.01		Quintozene		0.01
Cyanofenphos		0.01	Hexaflumuron		0.01	Rotenone	0.01	
Cyanophos		0.01	Hexythiazox	0.01		Octachlorodipropyl ether		0.01
Cyfluthrin		0.01	Imazalil	0.01				
Cyhalothrin, lambda-		0.01	Imidacloprid	0.01		Spinosad	0.01	
Cymiazole	0.01		Indoxacarb	0.01		Spirodiclofen	0.01	
Cypermethrin		0.01	Iodofenphos		0.01	Spiromesifen		0.01

Cyproconazole	0.01		Iprobenfos		0.01	Spiroxamine	0.01	
Cyprodinil	0.01		Iprodione		0.01	Sulfotep		0.01
DDD, o,p		0.01	Iprovalicarb	0.03		Sulprofos		0.01
DDD, p,p		0.01	Isazofos		0.01	Tebuconazole	0.01	
DDE, o,p		0.01	Isocarbofos		0.01	Tebufenozide	0.01	
DDE, p,p		0.01	Isodrin		0.01	Tebufenpyrad	0.01	
DDT, o,p		0.01	Isofenphos	0.02		Tecnazene		0.01
DDT, p,p		0.01	Isofenphos-methyl	0.01		Teflubenzuron	0.01	
Deltamethrin		0.01	Isoproturon	0.01		Tefluthrin		0.01
Demeton-S-methyl	0.01		Isoxathion		0.01	Terbufos		0.01
Demeton-S-methyl sulfone	0.01		Kresoxim-methyl	0.01		Terbutylazine	0.01	
Diazinon		0.01	Leptophos		0.01	Tetrachlorvinphos		0.01
Dibromo-benzophenone		0.01	Lindane (gamma-HCH)		0.01	Tetraconazole	0.01	
Dichlobenil		0.01				Tetramethrin		0.01
Dichlofenthion		0.01	Linuron	0.01		Tetradifon		0.01
Dichlofluanid		0.01	Lufenuron	0.01		Tetrasul		0.01
Dichlorvos	0.01	0.01	Malaoxon	0.01		Thiabendazole	0.01	
Dicloran		0.01	Malathion	0.01		Thiacloprid	0.01	
Dicofolb*		0.01	Mecarbam	0.01		Thiametoxam	0.01	
Dieldrin		0.01	Mepanipyrim	0.01		Thiodicarb	0.01	
Diethofencarb	0.01		Mepronil	0.01		Thionazin		0.01
Diethyltoluamid (DEET)	0.01		Metalaxyl	0.01		Thiophanate-methyl	0.01	
Difenoconazole	0.01		Metamitron	0.01		Tolclofos-methyl		0.01
Diflubenzuron	0.01		Metazachlor	0.01		Tolylfluanid		0.01
Dimethoate	0.01		Methacrifos		0.01	Triadimefon	0.01	
Dimethomorph	0.01		Methamidophos	0.01	0.01	Triadimenol	0.01	
Dimoxystrobin	0.01		Methidathion		0.01	Triallate		0.01
Diniconazole	0.01		Methiocarb	0.01		Triazophos		0.01
Diphenylamine	0.01		Methiocarb sulfone	0.01		Trichlorfon	0.01	
Disulfoton	0.01		Methiocarb sulfoxide	0.01		Trichloronat	0.01	0.01
Disulfoton-PS-sulfone	0.01		Methomyl	0.01		Trifloxystrobin		
Disulfoton-PS-sulfoxide	0.01		Methoxychlor		0.01	Triflumizole		
Ditalimfos		0.01	Methoxyfenozide	0.01		Trifluralin	0.01	0.01
Diuron	0.01		Metobromuron	0.01		Triforine		
Dodine	0.01		Metolcarb	0.01		Vinclozolin	0.01	0.01

Appendix 2: BeeToxWax online tool

An online tool (BeeToxWax) was developed to enable beekeepers and wax traders to calculate wax toxicity to honey bees based on the residues concentrations reported in a laboratory analysis report and the pesticide residue acute median lethal dose (DL_{50}). The tool gives an automated real-time advice on the reuse or the discard of the tested wax based on threshold defined by the current scientific literature: contact HQ value over 250 are considered to have significant toxicity and elevated toxicity over 5,000 (Traynor et al., 2016b). The tool is a web-based calculator of wax toxicity; its use could be an important strategy to sanitize beeswax sector. A classification based on a colour code expressing the toxicity to bees of contact LD_{50} has been used throughout the tool. Meaning of the LD_{50} has been exposed to avoid confusion.

Access

The tool is freely available on smartphone and computer at this address https://www.beetools.uliege.be/beetoxwax/

Users guide

BeeToxWax is currently available in French, Dutch, English and Spanish, click on the sign fr, nl, eng or sp (https://www.beetools.uliege.be/beetoxwax/) (**Erreur ! Source du renvoi introuvable.**). Scroll down to get to the bottom of the page.

Use the laboratory pesticide analysis report to fill in each box with the concentration (in mg/kg) corresponding to the listed substances (Erreur ! Source du renvoi introuvable.). Substances are classified per alphabetical order; scroll down to see them all. A Hazard Quotient (HQ) will be generated as the boxes are filled (Figure 13), and an advice on re-using the wax or discarding it will appear. When HQ is lower than 250, the wax is not considered as toxic to bees. If the HQ is between 250 and 5,000, the contamination is considered as significant; over 5,000, the contamination is elevated. To calculate a new HQ wax value, use the ‘reset’ button to delete former data (Figure 14). In order to generate a complete report (PDF form) of the analysed wax, fill in the blanks concerning the information about the wax. Press button ‘generate a pdf’ to have access to the report sheet (Figure 15Erreur ! Source du renvoi introuvable.).

Figure 13: BeeToxWax tool screenshot and the generation of the HQ and the wax re-use advice

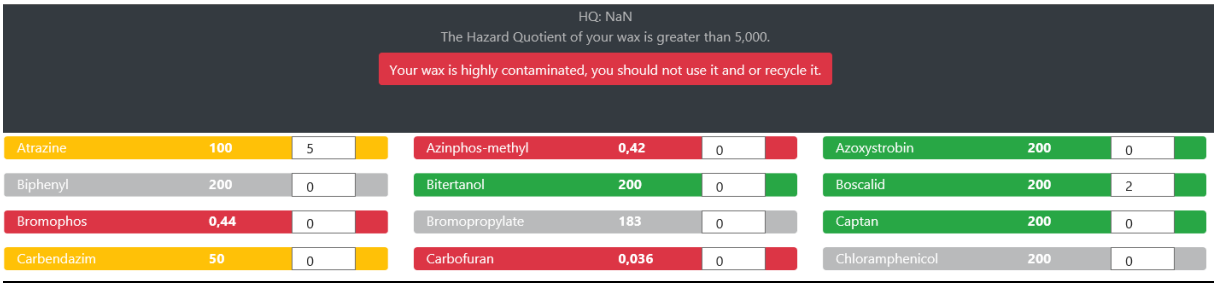


Figure 14: BeeToxWax tool screenshot reset button down the page

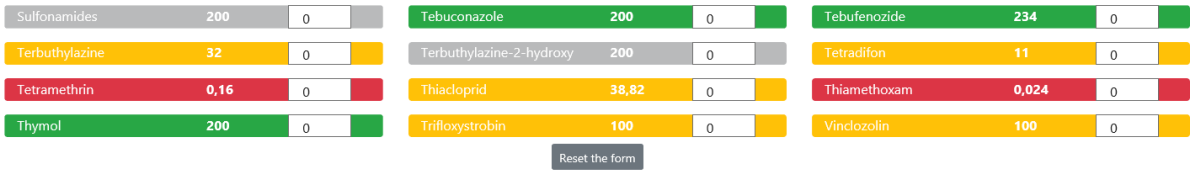
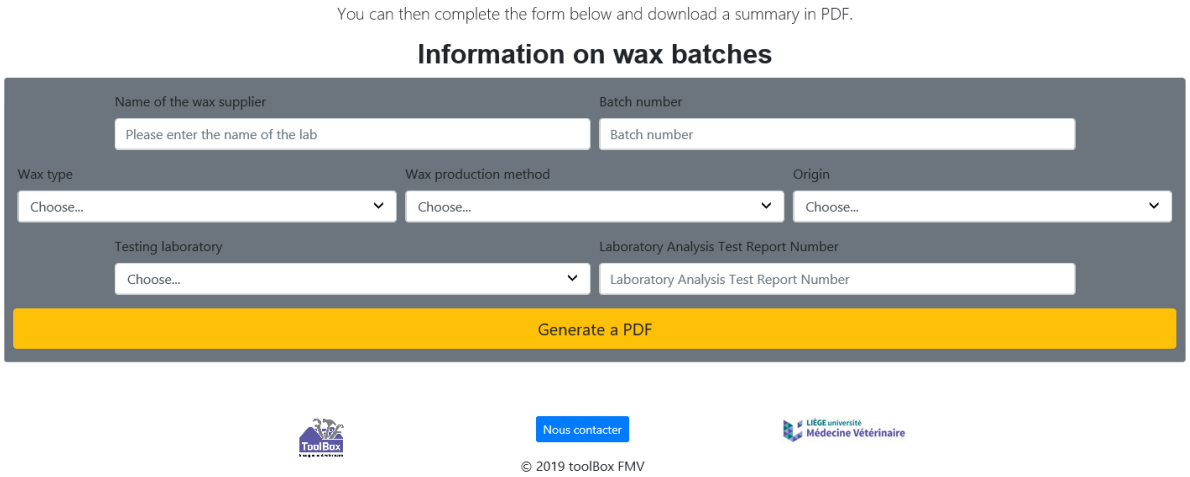


Figure 15: Screenshot BeeToxWax tool, information section on the analysed wax



Study 7

Appendix 1: Characteristics of the RT-qPCR analysis of differentially expressed genes of Apis mellifera

	Sequence (5'-3')	References
Reference genes		
Actin	F: TGCCAACACTGTCCTTTCTG R: AGAATTGACCCACCAATCCA	Cunha et al., 2005
RLP8	F: TGGATGTTCAACAGGGTTCATA R: CTGGTGGTGGACGTATTGATAA	Evans, 2006

MGST	F: TTGCTCTGTAAGGTTGTTTTGC R: TGTCTGGTTAACTACAAATCCTCCTG	Cornman et al., 2012
GAPDH	F: GATGCACCCATGTTTGTTTG R:TTTGCAGAAGGTGCATCAAC	Scharlaken et al., 2008
Target genes		
GST3	F: TGCATATGCTGGCATTGATT R: TCCTCGCCAAGTATCTTGCT	Gregorc et al., 2012
CYP6AS14	F: TGAAACTCATGACCGAGACG R: AAAATTTGGGCCGCTAATAAA	Al Naggar et al., 2015b
CYP9Q3	F: GTAGCCATTCACGCGTTCAC R: GTCTCGTCGATCTCCTGCTG	De Smet et al., 2017
Catalase	F: GGCGGCTGAATTAAGTGCTA R: TTGCGTTGTGTTGGAGTCAT	Collins et al., 2004
Relish	F: GCAGTGTTGAAGGAGCTGAA R: CCAATTCTGAAAAGCGTCCA	Evans, 2006
Domeless	F: TTGTGCTCCTGAAAATGCTG R: AACCTCCAAATCGCTCTGTG	Evans, 2006
Dorsal-2	F: TCACCATCAACGCCTAACAA R: AACTAACACCACGCGCTTCT	Evans, 2006
Defensin-1	F: TGCGCTGCTAACTGTCTCAG R: AATGGCACTTAACCGAAACG	Evans, 2006
Vitellogenin	F: ACGTAATAAATGCCGCCAAG R: TGCATGTTGCTCTCCAACTC	De Smet et al., 2017

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