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French agency for food, environmental and occupational health & safety

Investigate, evaluate, protect

Co-exposure of bees to stress factors

ANSES Opinion
Expert Report

July 2015 Scientific publication

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The Director General

Maisons-Alfort, 30 June 2015

OPINION
of the French Agency for Food, Environmental and Occupational Health & Safety

on co-exposure of bees to stress factors

ANSES undertakes independent and pluralistic scientific expert assessments.
ANSES's public health mission involves ensuring environmental, occupational and food safety as well as assessing the potential health risks they may entail.
It also contributes to the protection of the health and welfare of animals, the protection of plant health and the evaluation of the nutritional characteristics of food.

It provides the competent authorities with the necessary information concerning these risks as well as the requisite expertise and technical support for drafting legislative and statutory provisions and implementing risk management strategies (Article L.1313-1 of the French Public Health Code).

Its opinions are made public.
This opinion is a translation of the original French version. In the event of any discrepancy or ambiguity the French language text dated 30 June 2015 shall prevail.

ANSES issued a formal internal request on 13 July 2012 concerning the issue of co-exposure of bees to stress factors.

1. BACKGROUND AND PURPOSE OF THE REQUEST

Over the last 50 years or so, the number of pollinators has been on the decline in industrialised countries. This decrease seems to have accelerated over the past 20 years, particularly among honeybees in France, with deleterious consequences for plant species and bee products. According to France Agrimer, between 2004 and 2010, the number of beekeepers decreased by 40%, the number of hives fell from 1,350,000 to 1,074,200, and honey production dropped by 28%.

As a result, many studies have been carried out over the last few years to understand the mechanisms underlying phenomena such as colony weakening, collapse and mortality observed in most of the countries practicing intensive agriculture, in particular Europe and the Americas. AFSSA published a report in 2009 that highlighted the multifactorial aetiology of the phenomenon: infectious, chemical, physical, climate and nutritional factors, among others. The report concluded that there was a need to evaluate the individual and combined effects of bee and colony exposure to infectious agents and plant protection products, and to undertake research on chronic exposure to pesticides in the presence of latent and recurrent infections caused by various infectious agents that are likely to have potentiating effects. In 2012, ANSES issued three opinions relating to two scientific publications (Henry et al. 2012, Vidau et al. 2011) that reported the effects on bees and/or colonies of sub-lethal pesticide doses in the presence of infectious agents. In May 2012, EFSA published a "Statement on the findings in recent studies investigating sub-lethal effects in bees of some neonicotinoids".

In this context and given the ongoing studies being performed by EFSA on the chronic effects of neonicotinoids, ANSES's internal request concerns co-exposure, i.e. concomitant or successive exposure of individual bees and bee colonies to various stress factors, the mechanisms of action and interaction of these factors, and their respective roles in bee colony weakening and mortality phenomena. The focus is on interactions between infectious and parasitic agents on the one hand, and toxic factors at sub-lethal doses, on the other. The other factors, whether intrinsic (genetic makeup and diversity) or extrinsic (beekeeping practices, environmental factors) have been taken into account in terms of their ability to modulate these interactions and their effects. Because of the number of individuals making up a colony and its highly structured nature (for example distribution of work based on the age of worker bees), the effects on the individual bee at the molecular, cellular, tissue, and systemic levels have been considered separately from consequences at the colony level, or superorganism.

2. ORGANISATION OF THE EXPERT APPRAISAL

The expert appraisal was carried out in accordance with French Standard NF X 50-110 "Quality in Expert Appraisals – General Requirements of Competence for Expert Appraisals (May 2003)".

ANSES analyses the links of interest declared by the experts prior to their appointment and throughout the work, in order to avoid potential conflicts of interest with regard to the matters dealt with as part of the expert appraisal.
The experts' declarations of interests are made public *via* the ANSES website (www.anses.fr).

ANSES entrusted this expert appraisal to the working group on "Co-exposure of bees to stress factors", reporting to the Expert Committee (CES) on Animal Health. This group, set up on 8 January 2013 following a call for candidates, was made up of 17 experts with complementary skills in beekeeping, bee physiology and pathology, and toxicology/ecotoxicology, specifically interactions between xenobiotics and infectious and parasitic agents, epidemiology and modelling.

The objectives of the working group set up by ANSES to address this internal request were as follows:

(1) to better understand the role of stress factors in colony weakening, mortality and collapse phenomena, in particular:

- co-exposure of bees to pathogens and to chemical substances at sub-lethal doses,
- mechanisms of action including additive and synergistic effects, and potentiation,
- the modulator role of other stress factors (genetic factors, nutritional factors, climate, and electromagnetic fields, etc.) on these individual and joint effects,

and determine, as far as possible, the respective roles of each of these factors and their interactions, while also taking into account the impact of beekeeping practices and environmental factors.
Results of analysis of data collected on the health status of the honeybee population and on exposure of bees to various stress factors in mainland France were examined and evaluated in view of the literature data;

(2) to determine whether it would be appropriate and feasible to develop methods that take into account possible interactions between infectious agents and toxic factors when assessing plant protection products, in particular if this were done in a standardised manner. Where appropriate, these kinds of methods could be proposed by the working group;

(3) to issue recommendations in terms of beekeeping practices and research.

The activities of the working group required 23 plenary meetings between 8 February 2013 and 20 March 2015, and seven hearings with stakeholders from the beekeeping sector. The methodological and scientific aspects of this work were regularly submitted to the CES. The report written by the working group takes account of the observations and additional information provided by the CES members.

The final collective expert appraisal report was validated by the working group on 20 March 2015 and adopted by the CES on Animal Health on 7 April 2015.

Concerning the bibliographic parts of the report, the expert appraisal approach involved critical analysis by the experts of original scientific articles published mainly in peer-reviewed scientific journals. Older articles were included when relevant. Three subgroups of experts were set up to collect these articles and discuss their relevance. For articles regarding pesticides, specific attention was paid to the quality of the analytical and sampling methods. The literature data were updated throughout the working group's activities, up to the date of validation of the report.

Data recorded in France on the health status of apiaries and on co-exposure to infectious agents and xenobiotics were collected and underwent statistical analysis by ANSES, with regular input from the working group. The results of these analyses were communicated to the working group which discussed them and took them into account when drafting this report.

3. Analysis and conclusions of the Working Group adopted by the CES on Animal Health

3.1. Summary

In its collective expert appraisal report, the working group tasked with responding to this internal request first studied bee and bee colony health by defining, as far as possible, the "normal" state of health of a bee colony, by describing assessment tools for bee and bee colony health and by proposing health indicators that can be used by beekeepers, bee health technicians, veterinarians, and researchers.

On the basis of bibliographic data, the working group then presented, in no specific order, the main stress factors to which bees can be exposed and which are likely to induce interactions: biological, chemical and nutritional factors, as well as beekeeping practices, weather conditions and physical factors. Co-exposure and interactions between these stress factors, as reported in the literature, were then studied, after providing the background to mechanisms of immunity and detoxification of bees, some of which are involved in the observed interactions.

In addition to this bibliographic review, the working group discussed the results of statistical analyses on nine datasets concerning the health status of apiaries in mainland France, obtained by various national bodies.

The experts also examined the relevance of taking into account certain interactions between stress factors when authorising applications for plant protection products (PPPs).

3.2. Conclusions

3.2.1. On the state of colonies and tools for assessing the health of bee colonies

Findings based on the available data showed a large number of infectious and parasitic agents that affect bee colonies and many xenobiotics present in bee matrices. These elements define the current context in which bee colonies live, and their annual biological cycle must also adapt to other environmental factors such as climate and food. In this changing context it appeared necessary to define the state of health of bee colonies and to better determine what constitutes a normal or abnormal situation. Some of the tools currently used to evaluate bee health need to be renewed or adjusted to this new setting. This is already underway for some of these tools. They need to achieve distinct objectives for single time-points and follow-up analysis at various levels, i.e. individual bees, colonies, regions, and so on, and at different levels of study, whether molecular, cellular, or behavioural, etc.

The experts pointed out how difficult it is to compare data on the health and strength of colonies because of the variability of geographic, climate, floristic, or agricultural factors that strongly

influence the annual biological cycle of colonies. These data should be compared to reference standards and include the notion of change over time.

3.2.2. On the stress factors

The range of stress factors that bees can be exposed to concomitantly or successively appears to be very wide. For each factor, significant variability may be found from one apiary to another, or even from one colony to another. It is therefore often difficult to determine the exact role played by a specific factor, or their joint effects, when colonies develop disorders, and to make comparisons between apiaries. These various stress factors jointly contribute to weakening of colonies and colony disorders, although a single factor can be found in some cases.

For many biological agents, more knowledge of their pathogenicity needs to be developed both in the laboratory and within bee colonies. Asymptomatic carriage of infectious and parasitic agents is very widespread in bee colonies and this should be distinguished from clinical disease. Maintaining the balance of microbial populations is related to factors that are intrinsic to the beehive and to the environment, and changes in these factors can lead to colony disorders. It is important to look into the predictive nature of carrier states for the development of subsequent disorders, specifically using an approach based on colony demographic data as well as geographic and temporal data during beekeeping seasons.

There is a very high number of diverse chemical factors. A wide range of substances are found in beehive matrices to which bees are exposed outside and inside the colony. As part of this study, the substances of interest retained were insecticides, fungicides, and varroacide acaricides. A certain number of substances involved in bee disorders, occasionally at sub-lethal doses, are well documented (for example pyrethroids, neonicotinoids, and fipronil). Some studies have described disorders and identified the underlying mechanisms. Laboratory studies are more common than tunnel studies or field studies because of the difficulties involved in carrying out and interpreting non-laboratory studies. Exposure of bees in the field is not comparable to controlled exposure in the laboratory and the results for the same substance can differ, mainly depending on the method and monitoring of exposure (type, number of substances and their quantity).

Abundance and diversity of food sources and environmental resources play an important role in reproduction, development and maintenance of bee colonies. These factors influence health and tolerance of bees to other stress factors whether chemical or biological. Studies mainly carried out in the laboratory have demonstrated the adverse effects of nutritional deficiencies on metabolism and immunity. It is important to determine whether the observed effects can be transposed to natural conditions.

Certain beekeeping practices may generate stress likely to be added to other factors and lead to the development of disorders. The possible negative impact may be inherent to the practice itself or be related to unsuitable practices or others that are not implemented.

The working group highlighted the importance of compliance with good beekeeping practices based on in-depth training in beekeeping and regular monitoring of colonies to maintain the health of apiaries.

The intensity and duration of weather phenomena can change the physiological balance and dynamics of bee populations in a colony and cause natural weakening.

In this context, the working group highlighted the benefit of using and maintaining bee populations suited to local conditions.

3.2.3. On co-exposure and interactions between stress factors

Apiaries are co-exposed to multiple combinations of stress factors: the *Varroa* mite, bacteria, viruses, microsporidia and xenobiotics such as insecticides, fungicides and acaricides have all been identified as stressors.

The overview of the suspected/confirmed role of interactions between stress factors showed that several infectious and/or chemical agents may interact on the same functional targets in the larva and the adult bee, and lead to additive or synergistic effects. Chemical substances may also disrupt detoxification mechanisms and thus alter the sensitivity of bees to other substances. Moreover, certain biological agents, such as *Varroa,* and certain substances have immunosuppressant effects and contribute to amplification of infections/infestations in general. *Varroa* also acts as a vector (ABPV, KBV), or even a multiplier (DWV[1]) of infection by certain viruses it transmits. Lastly, some substances like neonicotinoids and acaricides may have an effect on the cohesion of the colony and the hygienic behaviour of worker bees and thus on the infectious and parasitic risks. As such, specifically the interactions between *Varroa* and viruses (DWV, AKI[2] complex virus), neonicotinoids and *Nosema*, fipronil and *Nosema*, neonicotinoids and viruses (DWV and BQCV[3]), fungicides and insecticides, show synergistic effects that threaten the health of colonies.

These mechanisms may act simultaneously and their effects depend on the season. The level of infection of the colony at the start of winter depends on the interaction between these factors during the foraging period. They may only become visible after a period of latency. Beekeeping practices may compensate for or amplify them.

3.2.4. On the results of data analysis (single-factor aspects and interactions)

Results of analysis of datasets confirm the high number and diversity of biological and chemical hazards detected in bee colonies in France. These results have not enabled conclusions to be drawn on the prevalence of biological or chemical hazards in apiaries in the country since the conditions for representativeness of samples were not met and only certain studies were designed for systematic and standardised assessment of biological and chemical hazards.
These observations point to certain hazards that should be detected, provide indications, and highlight the methods to use and the needs concerning matrices to sample.

Given this context of co-exposure of bees to many stress factors, associated with high qualitative and quantitative variability in exposure and the possible resulting interactions, the working group emphasised the difficulty in determining the health status and the “normality” of a bee colony as well as the role to be allocated to each co-factor identified in a bee colony with disorders. The observed disorders can result from concomitant co-exposure but also successive exposure to stress factors. One factor may induce effects, for instance on immunity, which will only have visible consequences later on, even though the factor may no longer be present in the hive.

3.2.5. On the issue of taking interactions into account when assessing plant protection products

Although it is not realistic to take into account all the possible interactions, the working group deemed it useful to consider some of them when assessing PPPs, while distinguishing between the marketing authorisation (MA) phase for the product and the post-MA phase. Evaluation of PPPs pre-MA in interaction with one or more stress factors among the most common and most important should be carried out using validated methods already available. Post-MA monitoring of products containing new active substances would make it possible to detect and assess possible interactions when disorders are observed in the field once these substances have been used.

The various conclusions from analysis of the literature and from results of analysis of datasets led the working group to propose several recommendations.

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[1] DWV: Deformed wing virus

[2] Complex of three closely related viruses belonging to the *Dicistroviridae* family, that are often difficult to differentiate: Acute Bee Paralysis Virus (ABPV), Kashmir Bee Virus (KBV) and Israeli Acute Paralysis Virus (IAPV)

[3] BQCV: Black Queen Cell Virus

3.3. Recommendations

This part summarises the recommendations made in the various chapters of the report. The working group, which was made up of experts from multiple fields, wanted to highlight priority recommendations in bold type, without overlooking the importance of the other recommendations.

3.3.1. On the tools for assessing the health of bee colonies

As a preamble, it is important to note the need to define characterisation tools, in terms of physical, chemical and biological parameters, for the average "normal" health status of a bee colony in its environment.
The working group recommends:

- distinguishing between tools for beekeepers and those intended for research and/or diagnosis;
- support the development of innovative and validated methods and tools to better understand the health and strength of bee colonies. In the clinical and pathology areas, the development of an illustrated guide to bee pathology would be a useful diagnostic support tool;
- **developing validated and harmonised schemes to assess colony disorders** (loss of forager bees, queen egg-laying, etc.).

The experts also recommend the **creation of reference apiaries**[4]**, organised in networks,** to achieve coverage of the French territory that is as extensive as possible. These apiaries would help to define regional reference standards for the various players on the basis of standardised collection of data on populations and production. An identified national stakeholder should collate and compile the data and make them easily available to all interested parties in the sector.

3.3.2. On the stress factors

- ✓ For infectious and parasitic agents, the working group recommends further studies:
  - o aimed at **defining the prevalence of infectious agents in colonies with and without symptoms, and their regional differences;**
  - o aimed at **identifying virulence factors for infectious and parasitic agents** (specifically *Nosema ceranae* and certain viruses), **in the laboratory and within colonies;**
  - o to **determine the pathophysiological mechanisms involved in host sensitivity, at the colony and individual level;**
  - o on the predictive nature of quantities of infectious agents present in the development of subsequent disorders, in association or not with the presence of chemical stress factors.
- ✓ For chemical agents, further studies should be conducted:
  - o aimed at **developing suitable analytical tools to measure actual (co)-exposure during field studies;**
  - o aimed at better describing and clarifying exposure and the toxic effects of chemical substances to which colonies are exposed;
  - o **on the direct effects or interactions of fungicides and insecticides,** given the frequency and plurality of exposure to these substances;
  - o to **determine the toxicity mechanisms involved, at the individual bee level, at the various stages of development (larva, nymph and adult), and at the colony level;**

[4] Bee colonies located in specific environments and monitored with physico-chemical and biological tools; through self-correlation of these parameters (comparison of a colony with itself), it would be possible to deduce the average normal state over time, the environment, and time-environment interaction in a given region, like reference farms in other livestock production sectors.

- on the **multiple and repeated nature of these exposures over time and its effects in co-exposure with other factors.** It is important to carry out studies on the **fate of chemical substances** (degradation kinetics, accumulation, etc.) **in the various bee matrices,** including bees and wax.

✓ In addition, for other stress factors, the working group:

- recommends implementing studies to evaluate the effects of nutritional deficiencies in natural conditions;
- highlights the benefits of **compliance with good beekeeping practices to maintain apiary health,** specifically biosafety measures and control of infectious agents and **use and maintenance of bee populations adapted to local conditions**;
- emphasises the importance of training veterinarians and bee health technicians concerning the complexity of the disorders occurring in bees;
- takes note of the benefits of studies on the physiological response processes of colonies to climate change.

3.3.3. *On epidemiological studies and data collection aimed at elucidating the issue of* in situ *interactions*

It is difficult to determine the health status of colonies and identify the cause(s) of disorders. As a result, the experts recommend continued and reinforced surveillance of apiaries, especially concerning biological and chemical factors. The working group stresses that infectious, parasitic and chemical agents, including acaricides in wax, should be screened for concomitantly during active surveillance, like during outbreak or routine surveillance (for example colony disorders).

For epidemiological studies in the beekeeping sector aimed at identifying risk factors, it is essential to use methods enabling comparisons of exposure profiles to these factors (in terms of diversity and quantity), between case and control epidemiological units and over time. Measured parameters in colonies should include:

✓ availability of reserves,
✓ the demographic structure within the colony,
✓ population size,
✓ and foraging activity.

Sampling must take into account the structure of apiaries. It is very important to keep information on the relationship between the scale of the colony and that of the apiary, and to carry out statistical analyses taking this structure into account. It is also important to take into account seasonal and geographic factors which strongly affect colony biology.

Epidemiological surveillance requires standardisation of data collection. This standardisation in particular requires centralised coordination ensuring compliance with protocols, training of surveyors, information reporting, information feedback, and relevant statistical analyses based on sufficient sample sizes. There are sampling rules that make it possible to achieve the required accuracy based on the question asked. With these criteria in mind, most current surveillance schemes are insufficient; the debate underway for the mortality and alerts observatory should support these recommendations. Regional observatories should be developed with the aim of having beehives that can serve as references, both for normal production and for regular exposure to the risk factors specific to the region.

Carrying out epidemiological studies seeking to explain the phenomena described through surveillance requires a protocol enabling cases to be compared with a reference population. Given the complexity of the phenomena involved in bee disorders, an extremely strict methodology is essential when developing and implementing protocols for epidemiological surveys.

The working group highlights the importance of a reinforced cross-disciplinary approach before implementation of surveys in order to ensure the suitability of analytical tools, sampling tools, data collected by questionnaire, and statistical analyses with the questions posed, while keeping feasibility in mind.

Active programmed surveillance of infectious and parasitic agents should be done using methods that are specific, sensitive and quantitative, as well as validated and standardised. The main potential pathogens in France should be screened concomitantly, whether there are symptoms or not. This screening should be associated with:

- quantification of the degree of infestation with *Varroa.* This parameter strongly influences the dynamics of infections transmitted by this mite and the immune state of bees;
- detection of the main toxic factors (at least those for which the sub-lethal effects can influence immunity, whether individual or collective).

This surveillance should help to **provide qualitative and quantitative data on asymptomatic carriage in colonies,** data that are currently insufficient. It will also make it possible to compare the levels of infectious agents present in asymptomatic hives with those observed in the context of outbreak surveillance, and thus **help determine the role of a specific infectious agent in the development of disorders.**

Strategies for detection of pesticides should have the following characteristics:

- target a range of substances known to be used in the region;
- depending on the question asked, take account of multiple treatments applied to the foraging zone over time and target the matrix/matrices to analyse;
- use validated quantitative methods (existing or to be developed) with detection/quantification thresholds that are compatible with studies on the potentiation of substances and their adverse effects on bee colonies. Multiple-residue methods should be given preference provided they have satisfactory sensitivity for the specific objective. For highly toxic pesticides, single-residue analyses on the active substance and its toxic metabolites are essential on the matrices of interest, i.e. pollen, nectar, wax, bees, bee bread. For surveillance of emerging issues and for toxicovigilance of veterinary and PPPs, it is necessary to standardise and centralise data collection when disorders occur and to standardise the multiple-residue methods used.

Moreover, the fate of chemical substances should be studied in the various bee matrices, including bees. Better knowledge of this aspect will help to determine the matrices to sample when disorders occur and to identify possible co-exposures to chemical agents and interactions, whether concomitant or successive.

It is very important to have **validated and harmonised quantification methods for infectious and parasitic agents, as well as for chemical agents.** Validation of diagnostic methods will enable surveillance using suitable tools whose sensitivity, specificity, reproducibility, repeatability, and detection and quantification limits have been determined, and that are used in a harmonised manner between the reference and accredited laboratories in order to carry out studies with comparable results.

3.3.4. On taking into account interactions when assessing the risks associated with plant protection products

- ✓ Concerning pesticide-pesticide interactions, the working group recommends that **the pre-MA procedures to assess the toxicity of a PPP include tests to measure the effect of chronic chemical co-exposure, by oral or topical route, to another substance (chosen for its potential to interact).** Co-exposure of the PPP under investigation should specifically be tested with:
  - an anti-*Varroa* acaricide;
  - a fungicide also known to inhibit detoxification mechanisms in bees;

- an insecticide with the same mechanism of action as the product under investigation and known to be present in bee matrices, if the PPP tested is an insecticide.

Given the plurality of potential stress factors, although difficult, it would be beneficial to **establish a hierarchy of substances to test in interaction**, on the basis of criteria such as their prevalence and effects, including mode of action, by characterising the effects of the most common co-exposures.

These proposals should be **discussed at the European level** since their implementation requires integration into the European regulatory framework, after development of the necessary tests and procedures.

In terms of research, studies on the ecotoxicological risks related to multiple exposures to pesticides should contribute to:

- development of operational tools to collect and process data on exposure, of various origins;
- **understanding the role of exposure of bee colonies to several pesticides in phenomena of excess mortality, weakening and decrease in production;**
- evaluation of the effect of pesticide mixtures, especially over the long term;
- development of risk assessment methods considering co-exposure to pesticides, particularly at low doses, and the cascade effects at the population level;
- development of research into the effects of fungicides in combination with other pesticides, specifically insecticides;
- **development of mathematical models enabling assessment of additive and synergistic effects, mainly of pesticides.**

✓ Concerning pesticide-biological agent interactions, it is necessary to:
  - determine in the laboratory the effects of these co-exposures that induce synergies, potentiation or antagonism on bee mortality or disruption of reproduction processes;
  - describe interaction mechanisms;
  - subsequently test the effects in the field at the colony level.

Epidemiological studies will provide evidence on the specific pesticides that tend to change the prevalence of certain infectious and parasitic agents or the response of host individuals.
Accumulation of laboratory data and field data on co-exposure to infectious agents/pesticides will help to fuel the development of mathematical individual-centred models. These models, that will take account of biological and ecological features of bees, aim to predict the development and survival of colonies in the presence of stress factors in different contexts (landscapes, populations, and climates).
In the context of PPP approvals, it will be useful to carry out tests in the laboratory by co-exposing bees to the PPP and to infectious or parasitic agents that have a high prevalence and “relatively low” pathogenicity to determine the possible occurrence of additive effects, synergistic effects, potentiation, or antagonism.

For the study of these interactions, certain existing methods can already be used in the laboratory, in semi-natural conditions, or in the field to take into account interactions in the methods for assessing PPPs. Other methods would need to be developed to better test exposure and the state of infection of experimental colonies, at the start and end of testing.

3.4. Outlook

Co-exposure of honeybees to multiple stress factors is now a proven reality. Management of health risks, whether chemical and/or biological, must now be adapted to this reality and this report demonstrates how complex and interdependent disorder development mechanisms can be.
In view of the plurality and the extent of exposure to chemical substances used in plant and livestock health, it is essential to work towards an overall reduction in these inputs by all means possible.
The aim is to minimise treatments, or at least their adverse effects, specifically the development of resistance and the presence of residues. This requires an integrated approach using, as a priority, available agro-ecological and zootechnical levers and if necessary rational use of chemical treatments. Concerning bee health more specifically, the experts wish to encourage dialogue between researchers in other animal sectors and those in the beekeeping sector, taking into account its specific characteristics, particularly its very strong link to the land.

4. AGENCY CONCLUSIONS AND RECOMMENDATIONS

The French Agency for Food, Environmental and Occupational Health & Safety endorses the conclusions and recommendations of the working group on “Co-exposure of bees to stress factors”, adopted by the CES on Animal Health.

The quantity of scientific studies published since the last report by the Agency on the subject of bee health (2009) today enables us to reach more robust conclusions on the contribution of the various factors involved in bee and bee colony disorders. Although cases of bee mortality are sometimes the result of a single factor, the experts emphasise that multiple factors are often the cause of bee colony mortality, but insist specifically on the importance of co-exposure to pesticides and biological agents in the occurrence of colony collapse. The presence of multiple infectious agents (parasites including primarily *Varroa*, bacteria, fungi, and viruses) within colonies, which are often asymptomatic at first, and the colonies’ exposure to pesticides of various origins and mechanisms of action (insecticides, fungicides and acaricides in particular), most likely result in the change from a normal state of health to the development of disease, leading to colony collapse. The mechanisms leading to this change primarily involve decreased immunity of individuals or of the colony, or decreased mechanisms of detoxification in bees. These phenomena are particularly marked since bees are exposed to multiple substances that sometimes have synergistic effects. Disorders related to co-exposure to factors have been demonstrated in publications for certain pesticides and infectious agents. Research is however still needed to study others.

ANSES notes in general that despite the seriousness of weakening phenomena in bee colonies and the long-term nature of these phenomena, multiple scientific studies carried out by a wide range of stakeholders over the past few years have not been able to develop a consolidated diagnosis of the state of health of colonies in mainland France, nor of their co-exposure to biological and chemical hazards.

In this context, although it is not possible to act in the short term on stress factors such as the climate, ANSES emphasises the need to intervene on all the other factors identified as contributors to colony weakening and specifically points to the importance of:

- maintaining biodiversity;
- adopting and complying with good beekeeping practices;

  Although insufficient on their own, these two measures nonetheless appear necessary to maintain bees and colonies in good health.

- reducing overall exposure of bees to plant protection products through greater control of use of inputs in agricultural practices;
- using chemical treatments rationally with substances that have been tested in terms of their additive, synergistic, or antagonistic effects;
- using quantitative methods to qualify the status of beehives with regard to infectious agents;
- creating reference apiaries, within a network, to achieve coverage of the entire country that is as extensive as possible, enabling establishment of regional reference standards for the various players;

  The last two measures should help in time to develop harmonised references and a structured observation network with national coordination that can produce reports of the health status of colonies, their co-exposure to biological and chemical agents, and changes over time.
- as part of the pre-MA assessment procedures, integrating tests on the toxicity of a PPP (within the context of discussions to initiate at the European level) to measure the effect of chronic chemical co-exposure by the oral or topical route to another substance:
  - ✓ an anti-*Varroa* acaricide;
  - ✓ a fungicide also known to inhibit detoxification mechanisms in bees;
  - ✓ an insecticide that has the same mode of action as the product under investigation that is known to be present in bee matrices, if the PPP to test is an insecticide.
- using data from the recommended observation network as a phytopharmacovigilance tool enabling the observed effects of PPPs on the health status of colonies to be adequately taken into account as part of procedures to re-assess the conditions for authorisation or use of substances and products.

Marc Mortureux

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Co-exposure of bees to stress factors

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Working Group on "Co-exposure of bees to stress factors"

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Presentation of participants

FOREWORD: Outside experts, Expert Committee and WG members, or designated rapporteurs are all appointed in their personal capacity, *intuitu personae,* and do not represent their parent organisation.

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Glossary

Emerging bee: bee passing from the nymphal stage to the adult stage

Infectious agent: microscopic element, alien to an organism, able to multiply or reproduce in it to the detriment of this organism

Pathogen (biological): agent whose presence or excess is responsible for the emergence of a disease (definition in the context of the report)

Anamnesis: history of a disease in a sick organism

Antagonism: phenomenon occurring when the combined effect of at least two substances is less toxic than the individual effects of these substances

Drone colony: colony in which all the haploid eggs yield males

Case history: patient's prior history

Sub-lethal dose: dose of a toxic substance slightly below the lethal dose

Additive effect: phenomenon that occurs when the combined effect of at least two chemical products is equal to the sum of the effects of each individual chemical product

Synergistic effect: when the combined effect of two chemical products is greater than the sum of the effects of each individual product

Enzootic: disease, whether or not clinically expressed, usually affecting animals in a given region (enzootic disease)

Swarming: formation of a new bee colony by emigration of part of the population of workers and the queen (swarm)

Strength of a colony: number of individuals, adults and immature, constituting the colony at any given time, in a region and for a given genotype

In silico**:** set of numerical methods using mathematics approaches for simulating or modelling a biological phenomenon using a computer tool

Introgression: transfer (natural or not) of genes from one species into the genome of another species

Bee matrix: live or dead bee, pollen, nectar, honey, wax, propolis, bee bread, royal jelly

Honeydew: sugary excretion produced by certain sap-feeding insects, particularly aphids and scale insects

Bee bread: pollen harvested by bees, mixed with honey and salivary secretions, and stored in the alveoli. It constitutes the protein resource of the colony

Parasite: (1) broad sense: Foreign entity that lives at the expense of a host; (2) strict sense: Uni- or multicellular eukaryote whose life cycle is only possible in close association with a host, if only for a limited time

Pesticide: according to the WHO, a pesticide is defined as "*any substance or mixture of substances, or micro-organisms including viruses, intended for repelling, destroying or controlling any pest, including vectors of human or animal disease, nuisance pests, unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage, transport or marketing of food, agricultural commodities, wood and wood products, or animal feeding stuffs, or which may be administered to animals for the control of insects, arachnids and other pests in or on their bodies. The term includes substances intended for use as insect or plant growth regulators; defoliants; desiccants; agents for setting, thinning or preventing the premature fall of fruit, and substances applied to crops, either before or after harvest, to protect the commodity from deterioration during storage and transport. This term also includes pesticide synergists and safeners; where they are integral to the satisfactory performance of the pesticide*. In the context of the WG's work, the chemical products considered were those used for the treatment or protection of

plants. Pesticides include fungicides, insecticides and acaricides, rodenticides, corvicides and herbicides

Population: number of bees in a colony

Potentiation: phenomenon occurring when a substance that does not usually have a toxic effect is combined with a chemical product, which has the effect of making the latter far more toxic

Preparations as defined in Regulation (EC) No 1107/2009: mixtures or solutions composed of two or more substances intended for use as a plant protection product or as an adjuvant

Requeening: replacement of the colony's queen by a new one

Residues as defined in Regulation (EC) No 1107/2009: one or more substances present in or on plants or plant products, edible animal products, drinking water or elsewhere in the environment, and resulting from the use of a plant protection product, including their metabolites, breakdown or reaction products

Stress: all the responses to factors threatening the integrity and health of an organism

Substances as defined in Regulation (EC) No1107/2009: chemical elements and their compounds, as they occur naturally or by manufacture, including any impurity inevitably resulting from the manufacturing process

Superorganism: body composed of many individuals, organised in a society (colony), where the isolated individuals are not able to live by themselves. Each individual works for the society, and the cohesion between all the components of the social group is ensured by a highly sophisticated system of communication, in particular chemical communication based on numerous pheromones

Supersedure: Phenomenon of natural requeening

Synergy: situation that occurs when the simultaneous exposure to at least two chemical products causes health effects that are greater than the sum of the individual effects of these products

Trophallaxis: regurgitation of liquid food to feed other bees. This transfer also helps to circulate information in the colony *via* chemical messages

Xenobiotic: chemical substance that is alien to a living organism

Acronyms and abbreviations

ai Active ingredient
IPA Infectious and Parasitic Agent
ABPV Acute Bee Paralysis Virus
ADARA Association for the development of beekeeping in Rhône-Alpes
AKI Complex including ABPV, KBV and IAPV
ALPV Aphid lethal paralysis virus
MA Marketing Authorisation
BQCV Black Queen Cell Virus
CBPV Chronic Bee Paralysis Virus
CCD Colony Collapse Disorder
CETIOM French technical centre for research and development of production procedures for oilseeds and industrial hemp
LC_{50} Lethal Concentration 50
LD_{50} Lethal Dose 50
EBI Ergosterol Biosynthesis Inhibitor
ILPT Inter-Laboratory Proficiency Test
ILVT Inter-Laboratory Validation Test
ELISA Enzyme Linked ImmunoSorbent Assay
EPA US Environmental Protection Agency
EPPO European and Mediterranean Plant Protection Organization
EAGF European Agricultural Guarantee Fund
HPG Hypopharyngeal Gland
HMF Hydroxymethylfurfural
IAPV Israeli Acute Paralysis Virus (Israeli variant of the acute paralysis virus)
IGR Insect Growth Regulator
INPN French National Inventory of Natural Heritage
ITSAP Technical and scientific institute for beekeeping and pollination - French Bee Institute
KBV Kashmir Bee Virus
LOEC Lowest Observed Effect Concentration (smallest concentration in an experiment inducing an observed effect)
LSV Lake Sinai Virus
MNHN French Natural History Museum
OECD Organization for Economic Cooperation and Development
PCR Polymerase Chain Reaction (gene amplification technique)
PER Proboscis Extension Reflex
PPP Plant Protection Product
qPCR quantitative PCR
RFID Radio-Frequency Identification
RT-PCR Reverse Transcription - Polymerase Chain Reaction
SBV Sacbrood Virus
LT_{50} Lethal Time 50
VdMLV *Varroa destructor* Macula-like Virus
VdV1 *Varroa destructor* Virus 1

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1 Background, purpose and procedure for responding to the request

1.1 Background

Numerous species of insect pollinators around the world contribute to the survival and evolution of over 80% of plant species. These pollinators include some 20,000 species of bees in the world, of which 850 are found in France, including the honeybee *Apis mellifera*. Over its geographical range (Europe, Africa and the Middle East), this species has diversified into almost 30 sub-species with specific characteristics adapted to their environment. In France, the native sub-species is *A. mellifera mellifera* (called the European dark bee).

Over the past fifty years or so, the number of pollinators has been on the decline in industrialised countries. This decrease seems to have accelerated in the past twenty years, particularly among honeybees in France (AFSSA 2009) with deleterious consequences for plant species and bee products. Other countries in Western Europe have also reported abnormal mortalities in apiaries, reaching as much as 80% in some countries (Neumann and Carreck 2010; Potts *et al.* 2010). The AFSSA (2009) report recalled however that *"honey bee colony losses have long been reported in beekeeping journals, since the time that beekeeping moved on from traditional hives to frame hives."*

In mainland France in 2010, the beekeeping sector had 41,836 beekeepers declaring 1,074,200 hives (compared with 1,350,000 in 2004), and producing 18,330 tonnes of honey (FranceAgriMer 2012). Among them, 91% were amateur beekeepers owning 1 to 30 hives, 4% were professionals with more than 150 hives, and 5% were beekeepers with multiple activities having from 31 to 150 hives (FranceAgriMer 2012). According to these estimates, between 2004 and 2010, the number of beekeepers fell by 40%, the number of hives decreased from 1,350,000 to 1,074,200, and honey production dropped by 28%. It should be noted that the overall number of hives depends on both losses and renewals of populations by beekeepers. A decrease in yield at the hive has been observed, in parallel with phenomena of excess bee mortality[1].

In this context, phenomena of weakening, collapse and mortality of bee colonies, observed in most countries practising intensive agriculture (Europe, the Americas), have over the past few years been the subject of several studies aimed at understanding the mechanism(s) involved in these disorders. The AFSSA (2009) report stressed their multifactorial aetiology (including infectious, chemical, physical, climate and nutritional factors). This report concluded, in particular, with the need to assess the individual and combined effects of exposure of bees and bee colonies to infectious agents and plant protection products, and to carry out research on chronic exposure to pesticides in the presence of latent, recurrent infections by various infectious agents likely to have potentiating effects. In 2012, ANSES issued three opinions relating to two scientific publications (Henry *et al.* 2012; Vidau *et al.* 2011), which reported the effects on bees and/or bee colonies of sub-lethal doses of pesticides (ANSES 2012a; ANSES 2012b; ANSES 2012c). In May 2012, EFSA published a "Statement on the findings in recent studies investigating sub-lethal effects in bees of some neonicotinoids" (EFSA 2012b).

1.2 Purpose of the internal request

In the above-mentioned context, and in view of EFSA's ongoing work on the chronic effects of neonicotinoids, the internal request relates to co-exposure (concurrent or successive exposure) of

[1] See the introduction of the AFSSA 2009 report on Weakening, collapse and mortality of bee colonies, and chapter 1 of the ANSES report on the prioritisation of health hazards in bees (2013-SA-0049) for information on the beekeeping sector

individual bees and bee colonies to various stress factors, these factors' mechanisms of action and interaction, and their respective roles in phenomena of mortality or weakening of bee colonies. The emphasis is on the interactions between infectious and parasitic agents on the one hand, and toxic factors at sub-lethal doses, on the other. The other factors, whether intrinsic (genetic makeup and diversity) or extrinsic (beekeeping practices, environmental factors), have been taken into account in terms of their ability to modulate these interactions and their effects. Because of the number of individuals making up the colonies and its highly structured nature (for example the allocation of work according to the age of the workers), the effects on the individual bee (at the molecular, cellular or tissue scale, or relating to the whole organism) have been distinguished from the consequences at the colony scale (superorganism).

The objectives of the Working Group (WG) set up by ANSES to address this internal request are as follows:

(1) to better understand the role of stress factors in phenomena of weakening, mortality and collapse of colonies, in particular:

- co-exposure of bees to pathogens and chemical substances at sub-lethal doses,
- the mechanisms of action (additive, synergistic, potentiation effects),
- the modulator role of other stress factors (genetic factors, nutritional factors, climatic factors, electromagnetic fields, etc.) on these individual or joint effects,

and to determine, as far as possible, the respective share of these factors and their interactions, while also taking into account the influence of beekeeping practices and environmental factors. Results of analyses of data collected on the health status of the honeybee population and on exposure of bees to various stress factors in mainland France were examined in the light of the literature data;

(2) to determine whether it would be appropriate and feasible to develop methods taking into account, in the assessment of plant protection products, the possible interactions between infectious agents and toxic factors, in particular if this were done in a standardised manner. Where appropriate, such methods may be proposed by the WG;

(3) to issue recommendations in terms of beekeeping practices and research.

1.3 Procedure: methods used

ANSES entrusted this expert appraisal to the Working Group on "Co-exposure of bees to stress factors", reporting to the Expert Committee on Animal Health.

The methodological and scientific aspects of this group's work were regularly submitted to the CES. The report produced by the working group takes account of the observations and additional information provided by the CES members.

This work was therefore conducted by a group of experts with complementary skills.

The expert appraisal was carried out in accordance with French Standard NF X 50-110 "Quality in Expert Appraisals – General Requirements of Competence for Expert Appraisals (May 2003)".

Concerning the bibliographical parts of the report, the expert appraisal approach involved critical analysis by the experts of original scientific articles published mainly in peer-reviewed scientific journals. Older articles were included when relevant. Three sub-groups of experts were set up to collect these articles and discuss their relevance. For articles relating to pesticides, particular attention was paid to the quality of the analytical and sampling methods. The literature data were updated throughout the work of the WG; up to the date of validation of the report.

Data recorded in France on the health status of apiaries and on co-exposure to infectious agents and xenobiotics were collected and underwent statistical analysis by ANSES, with regular input from the working group. The results of these analyses were communicated to the WG, which discussed them and took them into account when drafting this report.

2 Status of the colony: definitions, measurement tools, health indicators

Different types of disorders have been reported in bee colonies: weakening, collapse and mortality.
In its report on weakening, collapse and mortality of bee colonies, AFSSA (2009) had defined several bee colony disorders, as follows:
"Bee die-off indicates the ultimate destruction of bees with no precise expression of the nature or speed of this destruction." (Petit Robert 2007 Dictionary). A number of terms are commonly used in beekeeping journals and conference reports to designate and characterise this. In particular, scientists and beekeepers use the terms weakening, collapse, mortality, excess mortality and depopulation (Haubruge et al. 2006).
Weakening describes a lack of strength of a bee colony and is linked to a decrease in the density of the colony population over time, generally accompanied by a reduction in hive activity (for a period of the year when such reductions are not expected). Disorders can be observed among the bees, such as developmental or behavioural abnormalities, for example. The term "weakening" covers a multitude of clinical signs, left to the observer's subjective assessment. Weakening of a colony is accompanied by a reduction in its honey production.
Colony depopulation is a specific nosological[2] entity, characterised by a gradual reduction in the number of bees in a colony over time, with no apparent cause, until it disappears completely, due to the inability of the surviving bees to perform the elementary tasks essential to the survival of the colony. This syndrome[3] can be linked to a series of signs, such as a reduction in honey production and pollen collection resulting from the gradual loss of bees. (Higes et al. 2005).
Collapse is characterised by a rapid loss of bees within a colony, leading to its total destruction. This syndrome is known as Colony Collapse Disorder or CCD." Often, cases of depopulation fall within the field of description for CCD.
Table 1 (AFSSA 2009) summarises these various disorders.

Table 1: Die-off, weakening, depopulation and collapse of bee colonies (schematisation) (source: AFSSA (2009).

Description	Decrease in the number of bees		Decrease in colony activity		Decrease in honey production	
	Fast	Gradual	Yes	No	Yes	No
Die-off	X	X	X		X	X*
Weakening	(X)	X	X		X	
Depopulation		X	X		X	
Collapse	X		X		X	X*

**: Bees do not produce large quantities of honey all year round. There are periods known as "honeyflow"[4] during which large quantities of nectar are accumulated. If the collapse occurs after the last honeyflow, there will be no noticeable reduction in honey production.*

[2] Nosology: medical discipline studying the distinctive characteristics of diseases with a view to their methodical classification.
[3] Syndrome: set of clinical signs, symptoms and morphological, biological or functional changes of an organism, forming a morbid entity that may be triggered by causes that are varied or of unclear origin (Toma B, Bénet J-J, Dufour B, Eloit M, Moutou F, Sanaa M (1991) 'Glossaire d'épidémiologie animale.' (Maisons-Alfort, 365 pages)
[4] Honeyflow: transport by bees of nectar secreted by the nectaries of flowers, and making of honey.

2.1 Health status of bee colonies

2.1.1 Introduction

In the course of its evolution, the species *Apis mellifera* has spread to the south of the Southern hemisphere (South Africa), to the north of the Northern hemisphere (close to the Arctic Circle), also taking in the equatorial regions (Ruttner 1988). In the east, it has extended as far as Iran (to the south) and the Ural Mountains (to the north) (Rinderer 1986). Then, since humans began keeping bees[5], the species has spread virtually everywhere, with bee colonies developing in very different climate and environmental conditions and evolving into almost 30 subspecies with specific characteristics adapted to their environment. The range of the bee has therefore considerably extended, to virtually the whole planet, including North and South America, as well as Oceania. In France, *A. mellifera* was represented by the subspecies *A. mellifera mellifera*, called the European dark bee, and its different ecotypes, some of which have been described (this is the case with the *Abeille Landaise* for example). Since the 1970s, beekeepers have frequently used inter-breed hybrids, obtained by crossing bees from various subspecies that they have imported. Other beekeepers use bees selected from other subspecies for their productivity, their docility or their tendency not to swarm. Buckfast bees, very popular with some beekeepers, are an example of hybridisation to obtain bee colonies with the characteristics of interest of several geographical subspecies. These different subspecies, which have been imported for many years, are today responsible for the very high levels of mitochondrial introgression in French bee populations.

The fact that bees can be bred by humans does not imply that all bee colonies live in hives belonging to beekeepers. There are bees that have reverted to the wild as the result of swarming - at some point in the past - from their hive of origin. The number of wild colonies in France is not known, but it should be emphasised that they take part in the pollination of cultivated and wild plants and the maintenance of bee biodiversity, just like the colonies owned by beekeepers. When considering the honeybee, it is not possible to consider only those colonies raised by beekeepers.

The ecological success of the species *Apis mellifera* is due to several factors that rely greatly on the highly-developed social organisation of this species - in particular the division of labour between workers - as well as the permanent optimisation of the gathered food (pollen, nectar, honeydew) or water (Seeley 1995; Winston 1987). The key factors also include its exceptional cognitive abilities, its social immunity and its thermoregulation ability, which explain how this species can thrive in hot or cold climates, if food and water are present in sufficient quantities.

2.1.2 Annual population growth in a colony

The vast majority of the population of bees in a colony consists of workers, whose number varies from 40,000 to 60,000 individuals during the warm season, falling to 15,000 or even 5,000 in winter. There is only one queen, and the drones account for only a few thousand individuals, present for only a few months.

[5] Human beekeeping does not imply that the bee is completely domesticated ("honeybee"), since its reproduction is generally not controlled. Queens are fertilised freely, by males from different genetic origins.

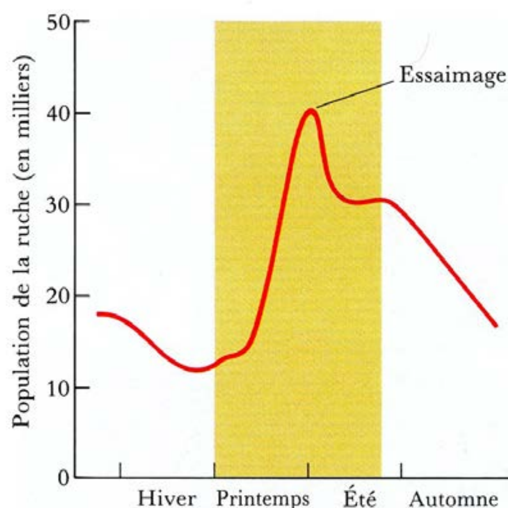

Figure 1: Annual population growth of a bee colony in a temperate climate, from Gould and Gould (1993)

Figure 1 shows an example of an "average" annual cycle for a bee colony.
The population growth of a bee colony during the year depends on many parameters related to the colony, such as the age of the queen, or genetic factors such as the subspecies. Within a subspecies, it may depend on the ecotype[6], which is adapted to a given environment.
The population growth of a colony also depends on its location, the rhythm of the seasons and, especially, on the available vegetation in the colony's foraging area.

There are generally four main phases in the development cycle of a bee colony:

- *A development phase* (population explosion), which begins at the end of the winter. During this period, the queen lays intensively (from 1,500 to 2,000 eggs per day), and the workers gather abundant amounts of pollen, nectar and honeydew. The lifespan of workers at this time is a few weeks (approximately 5 to 7 weeks).
Concerning the beekeeping activity, this phase of development can be stimulated by inputs of sugar and/or pollen. A great deal of vigilance is needed regarding the quality of these inputs;

- *A phase related to the reproduction of the colony* which includes, in particular, the annual production of drones and, possibly, the breeding of a new queen followed by swarming. The drones are present from the end of the winter until the beginning of the autumn. Swarming occurs when the population reaches its peak, towards the end of the spring (June). The queen, along with some of the workers, then leaves her hive and will form a new colony some distance away. A new queen will hatch in the original colony to replace the old queen having left with the swarm. Several successive swarms may leave the hive (secondary swarms), each containing a young virgin queen and a group of workers. This period is crucial for the level of the population, as the number of bees will fall considerably, and the harvesting activity falls correspondingly. An important point should be emphasised here: a decrease in population during this period, even if not immediately (or ever) detected by the beekeeper, is not equivalent to the weakening of a colony as a result of a disorder.
After swarming, the young queen begins to lay eggs, which will lead to a temporary increase in the population. Under certain circumstances, swarming does not take place for various reasons. In this case, the curve shown in Figure 1 does not show the sharp fall in population due to swarming.
Concerning the beekeeping activity, the beekeeper may decide to prevent the swarming, in order to maintain a high population in the colony and ensure a larger crop of honey;

[6] For example, the ecotype of the *Abeille Landaise* of the subspecies *Apis mellifera mellifera* (European dark bee) presents a different cycle at the end of summer since the queen starts laying eggs again just before the honeyflow of the *Calluna* heather (Louveaux *et al.*, 1966).

- *A wintering preparation phase*, which begins at the end of the summer. This is a period of natural decrease in the population of the colony. This phase will enable the best possible development of the colonies the following spring. The colony produces the workers that will survive the winter ("winter bees") and that will live longer (several months) than the summer bees (a few weeks).

Concerning the beekeeping activity, the role of the beekeeper will be to ensure that the level of reserves is sufficient and to provide, if necessary, a nutritional supplement (feeding);

- *a winter season, called* "*wintering*", during which time the population, reduced to a few thousand worker bees around the queen, lives on reserves accumulated during the warm season. Wintering worker bees will have the task of starting up work again in the colony in the spring. The health of these wintering individuals is of key importance for the survival of colonies over the cold season.

Concerning the beekeeping activity, the role of the beekeeper will be to ensure that the colony has sufficient reserves during the winter and to provide additional feed, if necessary.

2.1.3 Health status of bee colonies

There is generally a consensus that the size of the colony population represents its strength, its vigour, and that the "stronger" the colony, the more it can harvest food and resist certain stressors. Colonies in good health and abundant food gathering are important both for the development and survival of the colony itself, and for the beekeeper. As the colony size varies greatly throughout the beekeeping season, depending on the resources available, the assessment of a colony's strength refers to the "usual" situation in a given geographical context and at a given time of year. It can therefore be highly subjective, and strictly speaking empirical, when it relies on the multi-year experience of the beekeeper or health visitor, who evaluates the quantity of bees and brood, and has a good understanding of the apiary context. More objective parameters, as shown below, will need to be compared with a reference that takes into account the evolutionary aspect throughout the season and the local context (see section 2.2.4.2). This variability is one of the main difficulties in studying health factors in the honeybee.

2.1.3.1 Population level of adult bees

In temperate climates, a healthy bee colony is one whose annual development generally follows the development cycle shown in Figure 1, in the context in which it is found. The criterion of population level is particularly important. Apart from the case of swarming, which leads to a large fall in population, the population of the colony must be at the level generally found in the climatic conditions and the environment in which the colony is located. This assessment of the size of the population can be made by an experienced beekeeper.

2.1.3.2 Level of egg-laying by the queen

One important factor contributing to the good health of bee colonies is the rate of egg-laying by the queen. Egg-laying must be sufficient to allow an increase in the number of workers at the end of the wintering period, in order to reach the optimal size for the colony, and to replace bees that died in the course of the season. It must also enable the development of the drone population, and the production of new queens, when this proves necessary. It is generally accepted that the fertility of the young queen is regarded as good when less than 10% of cells are unoccupied (Jean-Prost and Le Conte 2005).

The fact that the queen can increase her egg-laying to compensate for abnormal mortalities of workers may, in some cases, make it possible to return to a normal number of workers, but this "catching-up" has a biological cost, not only because of the number of eggs needed, but especially because of the cost of raising these workers in terms of food and care by the nurses, and then the cost in food (honey and pollen) that they will need in the course of their adult lives.

While in normal conditions, the average lifespan of queens is estimated at 3 years (maximum 4-5 years) (Jean-Prost and Le Conte 2005), many testimonies by beekeepers and bee scientists show that it is now often closer to just one year, and that the fertility of the queens may have also

decreased, hence the frequent, sometimes annual, renewal of queens by beekeepers (Le Conte, personal communication).

2.1.3.3 Level of activity of the colony

In temperature, rainfall and brightness conditions that are conducive to foraging, and if blossoms attractive to bees (nectar and/or pollen) are found in its foraging range, a colony in good health must show sustained activity at the entrance of the hive. In particular, there should be many forager bees leaving and returning to the hive loaded with food (pollen, nectar, honeydew, water).

The foraging radius of the bees around their colony has been shown to vary from a few hundred metres up to 10 km or more (von Frisch 1987). Thus, bees have been seen collecting nectar up to 13.5 km from their colonies (Eckert 1933). The foraging distance varies depending on the environment, food and water needs, colony genetics, *etc*. The publications by Visscher and Seeley (1982), Beekman and Ratnieks (2000) and Steffan-Dewenter and Kuhn (2003) have reported results on the average (1.5 to 5.5 km), the median (1.2 to 6.1 km) and the maximum foraging distance (10 to 12 km).

These foraging radii around the colony correspond to foraging areas potentially visited by the bees. Thus, for a radius of 1 km, the exploitable area is 3.14 km² (314 ha), for 2 km it is 12.56 km² (1,256 ha), for 5 km it is 78.5 km² (7,850 ha) and for 10 km it is 314 km² (31,400 ha).

2.1.3.4 Normal level of bee mortality in a colony

The normal level of bee mortality in a healthy colony is not easy to calculate, because it depends on many factors. In a recent scientific opinion, EFSA (2012a) estimated the normal daily mortality of a colony at approximately 1% of the total number of individuals, based on the following publications: (i) Sakagami and Fukuda (1968), whose results were used by DeGrandi-Hoffman *et al.* (1989) and Schmickl and Crailsheim (2007), and (ii) Gary (1960), whose results were used by Moritz and Southwick (1992). A mortality rate of 1% corresponds to 400 to 500 bees per day in a colony of 40,000 during the beekeeping season. It must be stressed that this figure is a very rough estimate, ultimately based on just two early scientific studies carried out in specific contexts. New experiments on this subject therefore need to be carried out, based on the use of specific tools, for example bee counters that determine daily the number of bees not returning to their hive (see section 2.2). These experiments should be carried out in areas representative of characteristic regions and landscapes, on healthy colonies located in areas not exposed (*a priori* and *a posteriori*) to pesticides.

2.1.3.5 Level of infectious agents

A number of infectious agents are found in healthy bee colonies. Moreover, a high level of these agents in a colony is not necessarily a sign of poor health or poor honey production. It just reflects asymptomatic carriage, which is covered in a paragraph in the section devoted to biological agents.

2.2 Tools for assessing the health of bees / bee colonies

The bee colony must always be considered as a whole when assessing its state of health: the interrelationships between individuals are essential to the physiological balance of this superorganism[7]. Any alteration to one part of its population (forager bees or nurses for example) leads to compensation by the other part (versatility in the distribution of tasks between the workers), as far as possible. Any assessment of the health of bees and bee colonies must be able to meet two requirements: firstly, verify their good health (or conversely their poor state of health) at a time T, and secondly, have specific information and measurement tools for monitoring their change over a period P: indeed, two normal observations at two different times does not necessarily mean a normal change over the corresponding period. These two objectives, although closely related, remain separate and rely on different methods and tools. Generally speaking, an "abnormal" colony

[7] Superorganism: body composed of many individuals, organised in a society (colony), where the isolated individuals are not able to live by themselves. Each individual works for the society, and the cohesion between all the components of the social group is ensured by a highly sophisticated system of communication, in particular chemical communication based on numerous pheromones;

state should be defined by identifying and quantifying the parameters defining a "normal" state for this same colony in its specific environmental conditions. This is a field of research that should be developed further. In addition, the expert eye of trained, experienced beekeepers can detect particular states (weak, inactive, *etc.*) which should also be taken into account since these observations auto-correlate the behaviour of a colony with its previous state.

2.2.1 Assessing the health of a bee colony

2.2.1.1 The clinical examination

The bee colony is comparable to an animal which, when in good health, fulfils the functions essential to its survival and development, such as reproduction and nutrition (see section 2.1). As such, the health of this animal can be assessed during a classical clinical examination by comparing the examined animal with known physiological constants. In the case of the bee colony, the veterinary clinician should therefore compare the examined colony's state of development with the theoretical stage of development of a colony placed in the same conditions, taking into account the subspecies and the environment (season, climate, food, *etc.*). As with other domestic species, the anamnesis (history of the disease) and the case history (background, age of the queen, *etc.*) are also vitally important in bee health: the history of the technical background, management of the parasitism, certain beekeeping practices (changing queens) or the state of production yields (honey and pollen in particular) can in particular influence the clinical assessment.

The model followed by the examination may vary depending on the epidemiological context and the aim of the observation: either there is a high probability of encountering a particular disease (high prevalence of the disease in the area under study) and the examination will be a key factor in verifying the absence or presence of suggestive symptoms; or the examination is performed with a surveillance objective (low or even zero prevalence of the disease). In both cases, objective health parameters will then be useful. For example, a clinical examination in the context of bee imports must take account of the epidemiological context of the exporting country.

Semiology[8] is a science that remains little developed in beekeeping, relying mainly on observation (absence of tools normally used for other species such as the stethoscope or thermometer). The clinician must therefore be experienced not only in veterinary and medical diagnosis in particular, but also in bee observation: for the non-initiated, the physiological aspect can easily be confused with a lesion. The clinical examination is therefore more than a tool to assess bee colony health, it is also the method that sums up the assessment of the health of a bee colony, even if, in the absence of diagnostic tools, it has its limitations. The clinical examination may nevertheless rely on other tools for assessing bee health.

2.2.1.2 Additional examinations in suspected cases of infectious disease or poisoning

Some disorders observed in the colony or in individual bees are suggestive of a known infectious or toxic cause. When faced with these disorders, an attempt should be made to confirm the suspicion by detection, in the colony, of the suspected biological or chemical agent. To do this, samples must be taken under conditions that enable their analysis and interpretation by laboratories, specifically:

- as soon as possible after the onset of the disorder, due to the often rapid degradation of infectious and/or chemical agents;
- if possible before the administration of any treatment in the case of infectious diseases;
- on targeted matrices, adapted to the agent being screened for, which varies depending on the causative agent sought (for example, some infectious agents need to be screened for in the brood rather than among adult bees). The analytical laboratories may specify the types of matrices to target;
- in sufficient quantity, these quantities may also be specified by the laboratory receiving the samples;

[8] In medicine, study of the signs of disease in order to be able to make a diagnosis

- dispatched rapidly to the laboratory and in conditions suited to the type of sample and analysis requested (e.g. in cold conditions).

In addition, it is important that these samples are accompanied by a complete, precise description of the apiary and its context (size of the apiary, environment, monitoring, description of the disorders, photos, *etc.*), as these data are necessary for interpreting the results of the analysis. In suspected cases of poisoning, the difficulty is to first identify the type of xenobiotic (pesticide or other pollutants), then the class of pesticide (insecticide, fungicide, herbicide, *etc.*) or more specifically the compound(s) to screen for. In this regard, detailed field surveys on the environment are a valuable tool without which *a priori* identification cannot really be substantiated before analysis. During the analysis, the technical limitations of multi-residue analytical methods often broaden the search to encompass compounds of little interest (pesticides not found in the environment) while also limiting it to compounds that are "analysable" in terms of cost, relevant sampling and relevant detection/quantification (Bonmatin *et al.* 2015). When a suspicion of poisoning is established and supported by consistency with field surveys, it is then possible to undertake more detailed screening for a more limited number of compounds. In most cases, due to a lack of resources, the analyses do not result in identification/quantification of residue levels concerning the toxic metabolites.

Several laboratories, in particular private or departmental, can conduct screening for infectious and/or chemical agents in bee matrices. In France there is a network of accredited laboratories for the diagnosis of bee diseases. The national and international reference laboratories (ANSES Sophia Antipolis NRL-EURL) are responsible for developing, optimising and validating (according to the applicable standards) reference methods (for detection, identification and/or quantification) for conducting microbiological or chemical examinations, including those to be used for regulatory controls. This covers the number and type of samples to be taken and the laboratory analytical methods to be used to ensure the best chance of detection, and the reliability of the method. The NRL-EURL is also tasked with ensuring the harmonisation of methods within the networks of laboratories, in particular the networks of accredited French laboratories.

In principle, the presence of the causative agent in the sample in connection with its load, together with the presence of specific clinical signs in some cases, makes it possible to confirm the suspicion of an infectious or toxic cause in order to explain the observed disorders. The aetiological diagnosis is thus established and may lead to the establishment of suitable treatment. In addition, for certain regulated infectious diseases, it may lead to the establishment of specific measures, mainly to avoid the spread of the causative agent to neighbouring apiaries. In the case of chemical agents, determining the substance in question sometimes enables the source of the exposure to be identified and remedied.

However, it is unusual for the observed disorders, which are rarely specific, to be suggestive of one particular cause. They can also relate to multifactorial determinism. In this framework, the cause of the weakening can enable the clinical expression of healthy carriage: the expression of a disease is then only the result of a primary cause. Sometimes there can be a substantial period of time between exposure to this primary cause and the clinical manifestations. In this case, regular monitoring of hives will enable early detection of weakening associated with a primary cause, and may prevent the later collapse of the colony. More precisely, in this multifactorial context, a battery of laboratory tests will be prescribed, on the basis of a careful examination of the disorders, the history and context (for which the results will be attached to the samples).

It should be noted that it is often difficult to interpret the results of tests of multiple residues and multiple infectious agents: indeed, chemical substances and infectious agents can be detected jointly, often at low levels of contamination, without it being possible to attribute the origin of the disorders with certainty to one or other of the detected agents.

2.2.2 Assessment of the health of bees, at the scale of the individual

2.2.2.1 Clinical examination of the bee

Although the health of a bee colony must be assessed as a whole, the individuals making up this colony can each express symptoms. They can provide information to the veterinary clinician as to the good or bad health of the superorganism.

Accordingly, morphological abnormalities are sometimes found in certain individuals: abnormal positioning of wings, shape and size of wings, shape and size of abdomen, colour of abdomen, absence of hair, tongue continuously protruding, presence of parasites, *etc*. Some digestive symptoms such as diarrhoea, noted in the near environment and in the hive, can sometimes affect the bees. Some behavioural disorders may also be noted by the observer (sometimes in relation with the morphological disorders): inability to fly, increased aggressiveness, tremors, pruritus, *etc*. When they affect a group of individuals, these disorders can translate into social behaviour disorders: marked aggressiveness, blockage of the flight hole, abnormal arrangement of a group of bees, increased refusal by guardians to allow entry, *etc*. and can affect foraging activity.

This non-exhaustive list of symptoms must be interpreted by the clinician, who will have to weigh the importance of each observed anomaly on the scale of the colony. Some signs, observed in a single individual, may be more important in the diagnostic process than others that concern a greater number of individuals. For example, a few trembling bees is suggestive of poisoning and may constitute the only observable indicator.
The presence of bee corpses must also be regarded as an important individual sign. When the number of bees dying daily is higher than it should be (several hundred a day sometimes (EFSA 2012a)) carpets of dead bees can be observed. Again, the observer will have to get sufficient perspective to determine whether or not they are faced with an anomaly, in particular in the context of suspected poisoning. Similarly, the absence of corpses does not rule out certain diagnostic assumptions, for example in the case of mortalities occurring far from the hive, or when the bee corpses have been eaten by predators.

A few rapid tests can provide additional information on this individual examination, such as the appearance of the intestine or a microscopic examination of the respiratory system. Concerning the corpses, the precise dating of their death would be a great help. Nevertheless, the "washed out" appearance, the smell and the weight can only give approximate indications about when death actually occurred.

Concerning the health of the queen, any condition that could affect her is a real danger for the colony as a whole. As soon as she emerges, her state of health and anatomical development are key determinants for her later egg-laying abilities (flight ability, integrity of the reproductive system). Thus, some queens are born dwarf or suffering from hyperplasia of their reproductive system. A sometimes non-negligible proportion of births are regarded by queen breeders as incapable of ensuring the reproductive function for which they are intended. Later, in the course of their lives, many pathogens can affect them in the same way as workers. A queen's theoretical life expectancy, of several years, makes her the only individual to survive from one bee season to the next, and therefore the only individual permanently exposed to a potentially hazardous environment. For example, the adverse effects of some xenobiotics (coumaphos, tau-fluvalinate, *etc*.) on the health of queen bees and their breeding are already known (Haarmann *et al.* 2002; Pettis *et al.* 2004). The physiological changes related to her ageing (degeneration, calcification) or pathological disorders contracted with age (infections, melanosis, ovarian atrophy) may also have an adverse effect on her egg-laying qualities. The assessment of these anomalies can generally only be performed by microscopic laboratory examination (anatomical pathology) or using physiological markers (Provost 2013). Clinically, such impairments in queens correspond to supersedures (when they are possible) or to drone colonies.

2.2.2.2 Clinical examination of the brood

A careful examination of the brood is of paramount importance because it is revealing about the colony's state of health and its future. While its "quantity" can be an interesting indicator for

estimating the strength of the colony, its "quality" confirms a satisfactory state of health and good care provided by the young house-cleaning bees. As for adult bees, some symptoms may be identified in the open or closed brood: desiccated larvae, larvae of abnormal colour or shape, presence of flakes, pierced wax capping, bulging wax capping, abnormal odour, presence of parasites or insect larvae, *etc.* Foulbroods, some parasites and some viruses can all affect the brood. Some toxins can also affect the development of larvae by preventing their moulting, for instance.

All brood anomalies, whatever they may be, should be regarded by the observer as of major severity. In some cases, when the colony is able, the work of undertaker bees (hygienic behaviour) is sufficiently effective to quickly clean affected cells. The brood then resembles a "mosaic". Only careful observation and repeated examinations at regular intervals can sometimes enable a sign of a brood disorder to be detected. Given the development cycle of the brood (21 days for the worker bee between egg-laying and emergence), it can therefore be considered that a monthly visit should be indicated, during the brood development period, to verify its state of health.

2.2.2.3 Tools available in scientific research for assessing bee health

2.2.2.3.1 Behaviour tests

The behaviour of internal and forager bees is an important parameter of their state of health. The cognitive capacities of bees are highly developed. If impaired, this may not only affect the simple individual, but also disrupt the operation of the whole colony. A few assessment tests are regularly used by researchers, in particular to measure the effects of some xenobiotics.

For example, observing orientation behaviour towards the hive (in particular the time taken to return to the colony of origin) is useful for improving understanding of the effects of certain toxins. New technologies are now helping to achieve a better understanding of this type of measurement. Social communication through dances has been known for several decades, and is an essential means of sharing information between worker bees: proper transmission of a message can be fairly easily observed with the naked eye, thereby revealing whether or not behaviour is impaired. Lastly, other tests such as the "proboscis extension reflex" or the "T-maze" are used in fundamental research to assess the behaviour of bees exposed to disruptors. All the behavioural and neural tests are described more precisely in section 3.1.2. devoted to chemical stresses.

2.2.2.3.2 Individual biomarkers

The life expectancy of a bee depends on many extrinsic (such as the season) and intrinsic (social) factors. When a colony is in good health, the life expectancy of the bees should theoretically not be reduced. Certain biochemical parameters, which are measurable, may constitute genuine biomarkers of the bee's age. For instance, cellular senescence can be measured by assaying lipofuscin (Münch *et al.* 2013), while the vitellogenin titre in haemolymph contributes to modulate the tasks performed by each bee and therefore to shorten or lengthen the life expectancy of each individual (Amdam 2011). However, the modularity of bee life expectancy (winter bees *vs* summer bees - possible reversion of tasks) complicates the use of these tools, which are mainly reserved for fundamental research.

2.2.2.3.3 Weight of emerging bees

The weight of emerging bees could be an interesting parameter, according to some authors (Scheiner 2012), due to the demonstrated link between the morphology of the adult bee at emergence and its subsequent cognitive abilities.

2.2.2.3.4 Radioentomology

Other techniques have been tested that are based on the principles of medical imaging applied to the scale of the colony, such as magnetic resonance imaging (MRI) and ultrasound (Greco 2010). MRI can achieve better differentiation of tissues than a scanner, but gives lower resolution digital images. Ultrasounds (high frequencies), which are not transmitted through the air, only give an low-resolution image for the internal structures of the bees. In contrast, the scanner seems applicable to

individual examination of bees with digital images of very high precision (Butzloff 2011). The authors point out that X-ray exposure of bees by this technique is far lower than the minimum dose leading to an adverse biological effect (the dose of 0.14 mGy per bee, maximum, is nearly 3,800 times lower than the toxic dose measured for Drosophila, even though it is probably not appropriate to compare these two insects). According to the same authors, this tool could be interesting for studying the morphology and internal structures of bees (Greco *et al.* 2008). Compared with dissection, it has the advantage of keeping the individuals alive (examination required under anaesthesia), therefore making it possible to repeat the same examination several times over the bee's life. Lastly, it also allows a greater range of possibilities (infinite number of angles of observation) than conventional microscopic examination. The main disadvantage of this tool is its cost and accessibility: it cannot be transposed to field use and is reserved for scientific research.

2.2.2.3.5 Pathological examination

The microscopic examination of tissues is a technique that can be applied to insects (bees especially) to observe the internal and external structures. However, there are still relatively few documented microscopic images for the bee. Fixation techniques have been developed recently that should make it possible to consider this method for diagnostic and scientific purposes (Scudamore *et al.* 2012).

2.2.3 Tools available to assess the strength of a colony at a time T

2.2.3.1 Assessment of the total number of bees

The total population of bees found within a healthy colony fluctuates greatly, especially according to its development cycle: daily egg-laying by the queen and raising by the workers vary the number of emerging young and balance the hive population with regard to "normal" mortalities of bees at the end of their life. The size of the population is often viewed by beekeepers and researchers as an indicator of health (excluding the issue of swarming). Assessing the number of bees in a colony has long been of interest to the beekeeping world as an indicator of the hive's future productivity. Thus, from the 1950s, the bee population was estimated by shaking all the frames and by weighing the swarm alone (Moeller 1958). This method remains the most accurate and is still used for scientific purposes (Costa *et al.* 2012; CST 2003; Odoux *et al.* 2014). However, because it is relatively invasive, it has been abandoned by beekeepers in their practices in favour of an estimate of the surface area of the frame occupied by the bees (Burgett and Burikam 1985). To be as accurate as possible, this examination must take place early in the morning or late in the evening in order to include the population of forager bees (outside foraging periods therefore). Each side of a frame covered with a uniform layer of bees is considered to represent approximately 1400 bees for one Dadant hive and 1100 for one Langstroth hive (Imdorf *et al.* 2010). More generally, it is considered that 130 bees cover each dm^2 of frame. Therefore, measuring the number of dm^2 covered with bees in the hive makes it possible to estimate the approximate size of the population. Even though this method remains the most widespread, it is still relatively imprecise (surfaces unevenly occupied, bees inside the cells or not clinging to the frame, foragers outside the hive, bees on the walls of the hive, *etc.*), and the number of bees per surface element can easily vary (from 130 to 400 bees per dm^2 (Imdorf *et al.* 2010)). Imdorf recommends calibrating this measurement on a few hives, supplementing the result of the assessment with the weighing of the number of bees swept up and collected in a container (Imdorf *et al.* 2010). This calibration is used in the field within the framework of research programmes (for example currently in the framework of the ColEval method developed by INRA and the UMT-Prade in monitoring of colonies on lavender).

2.2.3.2 Assessment of the brood surface area

Outside the winter period when the brood may be absent, the number of cells containing eggs, larvae and pupae is an indicator of the colony's development and the queen's fertility. These cells are usually placed at the heart of the hive and constitute the brood nest. It is possible to estimate the surface area occupied by these cells (capped and uncapped brood) by a careful observation of frames containing brood. A "standard frame", whose surface is subdivided with wires into dm^2, can

be laid over each frame to be assessed, in order to better determine the surface areas observed (Imdorf *et al.* 2010).

Modern tools, such as digital photographs of each side of the frame, can facilitate measurement and improve its quality. These images can then be interpreted by computer software (Emsen 2006; Imdorf *et al.* 2010; Yoshiyama *et al.* 2011).

One of the methods used to measure these two parameters (number of bees and surface area of brood) is the "Liebefeld" method developed by the Swiss Bee Research Centre (Imdorf and Gerig 1999). It is probably the most widespread method currently used for assessing the strength of a colony. It has a proven track record and, provided it is performed carefully by the operator, obtains good correlation between the assessments and the measurements, in particular for the number of bees and the surface area of the closed brood. Concerning the open brood, the assessment is often overestimated (Imdorf *et al.* 2010). In order to speed up assessment of the frames, another technique (derived from the Liebefeld method) involves virtually dividing the frames into four quarters: recording the observations made for each quarter then enables a semi-quantitative estimate to be made. However, this traditional method is more invasive and can generate errors that have to be corrected by additional methods (example of ColEval).

2.2.3.3 Estimation of foraging activity

Estimating foraging activity in the strict sense has the benefit of measuring the effort expended by a colony in collecting environmental resources: intense activity is a positive sign, generally evocative of good colony health. The intensity of foraging by bees can be assessed through careful, continuous observation of the flight board. Automatic bee counters were developed very early on, with the aim of also estimating the number of foragers failing to return to the hive (Pham-Delègue *et al.* 2002). The earlier counters recorded electrical pulses or photoelectric signals when the bees passed by. The various devices were finally adapted to the hives, and linked to powerful computer systems and algorithms that made it possible firstly, to monitor foraging activity over time and secondly, to measure the balance between bees arriving and leaving in order to assess mortalities outside the hives. Today, many automated systems can measure normal and abnormal behaviour in bee colonies (Devilliers and Devilliers 2014).

Among the most effective are the apiSCAN counters, with technology based on infrared detectors (Struye *et al.* 1994), and which are used in certain experimental protocols because of their accuracy (Danka and Beaman 2007). These tools sometimes rely on metal detection or even colour detection counters when they are combined with cameras (Le Conte and Crauser 2006; Poirot *et al.* 2012). The bees are then identified as soon as they emerge, either from metal pellets glued to their thorax, or paint markings (Dussaubat *et al.* 2013; Le Conte and Crauser 2006), which has finally made it possible to monitor a cohort of bees in a colony and their lifestyle (*i.e.* the sequence of tasks carried out by the bees). The use of cameras or radars has finally made it possible to measure activity on the flight board in a non-invasive way (Campbell *et al.* 2008; Devilliers and Devilliers 2014).

2.2.3.4 Estimation of mortality in forager bees

Certain modern technologies applied to bees, such as RFID (Radio Frequency Identification), now enable this measurement to be supplemented by an assessment of the time spent by monitored foragers away from the hive (Decourtye *et al.* 2011b; Devilliers and Devilliers 2014; Streit *et al.* 2003). This technique requires foragers to be individually marked. This automated measurement technique has high accuracy and is a valuable tool in the establishment of certain experimental protocols (Henry *et al.* 2012). Bee counters can also calculate the difference (in the evening) between the number of bees returning compared to the number that left the hive; it may be assumed that many of them were foragers.

2.2.3.5 Estimation of mortality in the colony

The number of bees dying daily within a colony is sometimes high. These losses may be the consequence of bees dying at the end of their life, or of a disorder that may affect adult bees by reducing their life expectancy. Egg-laying by the queen, which can reach an average of 1,500 eggs

per day during the season (Jean-Prost and Le Conte 2005; Winston 1987), must at least compensate for the losses during the population development period. Disorders may be manifested by the presence of many corpses at the bottom of the hive or at the foot of the flight board. Unfortunately, the tools available for counting dead bees (dead bee traps on the bottom or at the entrance of the hive, collectors in front of the flight board) are often imprecise: firstly, they only measure the number of bees that died inside and do not count foragers dying on the outside (which are however the most exposed) and secondly, they do not take into account the activity of undertaker bees, for example, that carry the corpses out of the hive. The values obtained are therefore always underestimated and must be corrected in order to be interpreted. The most reliable method is probably the one that relies on bee counters (*see* above, any missing bees are, by definition, dead, lost or have strayed off course). This number needs to be added to the bees found in the dead bee collectors in order to obtain the total mortality.

2.2.3.6 Estimation of egg-laying by the queen

Successful mating of the emerging young queen is verified by the beekeeper-breeder by noting regular egg-laying on the part of the frame where she has chosen to lay her eggs: the fertility of the queen is generally regarded as good when less than 10% of cells are unoccupied. Regular observation of all the frames of the brood nest may make it possible to measure the daily egg-laying of the queen, and therefore to determine the population of workers and drones to be born (this is estimated at 1,500 eggs per day on average during the beekeeping season according to Winston (1987) and can reach 3,000 eggs laid daily (Jean-Prost and Le Conte 2005). It is more difficult to determine the fertility rate, which measures the number of viable adults obtained, but this is important as it yields information about disruptions to the correct development of eggs.

2.2.3.7 Other assessment methods and techniques

Diagnostic Radioentolomogy (DR) is presented by some authors as promising for assessing the health of insects, including bees (Greco 2010). The technique is based on the use of sophisticated and modern medical imaging tools such as scanners (see previous section on this technology). This technique therefore uses X-rays and computed tomography to obtain scanned images of the internal structures of the hive (when the populated hive is concerned by the examination). It has the advantage of being non-invasive (because it is not necessary to open the hive to examine it) and precise (because it can observe details such as eggs or larvae and pupae). Nevertheless, the examined hive still has to be moved and therefore closed, which can disrupt the observations and, more importantly, as mentioned previously, the use of such methods is still limited to research.

2.2.4 Tools available to monitor changes in a colony over a period P

Monitoring the dynamics of a colony is a parameter that should be taken into account when measuring its state of health. Repeating examinations at different times and then comparing them can enable these dynamics to be defined, but certain parameters are more specifically suited to assessing this trajectory. The frequency of hive visits by the beekeeper is an essential factor for ascertaining the satisfactory health status of colonies. Good beekeeping practices require visits in spring and autumn, as well as visits during the season "as often as necessary" (ITSAP 2014). During periods of brood development or at-risk honeyflows and pollenflows, visits need to be more frequent in order to trigger an alert, where necessary, as early as possible.

In addition to these visits by the beekeeper, monitoring tools are currently in development, such as the Ecobee scheme (Odoux *et al.* 2014) or swarm monitoring (Bencsik *et al.* 2011), with the aim of improving knowledge about variables in bee population dynamics under natural conditions.

2.2.4.1 Production data

A healthy bee colony accumulates reserves by collecting various nectar and pollen from its environment in order to store it as honey and bee bread. This results in a significant increase in the weight of the hive. The difference in the weight of the hive between two weighings, carried out under the same conditions, can therefore be a relevant criterion for assessing health, especially since the total weight of the bees generally remains constant over a short period. Specific production data

(measurement of weight increases, amount of pollen collected in pollen traps) can thus be useful parameters. The surface area of bee bread stored in the cells around the brood nest, and which is vital to raising bees, is also an interesting parameter. Similarly, a healthy colony is capable of producing large quantities of wax to extend the structures in which it can store its reserves. This production - and its change over time - are sometimes used in experimental monitoring of colonies (Mattila and Seeley 2007).

In order to determine and monitor the colony's harvest, the weight of the hive is commonly used. It can be measured in a simple, continuous and automated manner using weighing scales placed under the hive that are directly connected to computer systems. Buchmann and Thoenes (1990) were the first to propose the use of high-precision electronic weighing scales for monitoring bee colonies. This tool is now marketed for beekeepers wishing to monitor the weight of their hives remotely. This measurement can also indicate a sharp variation in the bee population such as swarming or mass mortality (bearing in mind for example that a kilogram of bees corresponds to approximately 10,000 individuals).

Lastly, the weight of the hive, measured precisely and continuously, can be sufficiently informative to enable the colony's activity in the broad sense to be assessed for scientific purposes, and as a decision-support tool in beekeeping (Meikle *et al.* 2008).

2.2.4.2 Monitoring of the population

Like the mortality rate, the rate at which the queen lays eggs is crucial for the survival of the colony. Coupled with mathematical models of population dynamics, this rate can be used to predict the colony's population growth (Schmickl and Crailsheim 2007). Monitoring of the total bee population can be achieved by multiplying the counts according to the methods described above or by information provided by automated weighing scales. Similarly, the daily ratio of bees born/dying may be a useful parameter for determining whether the colony's population is declining or growing: its calculation requires knowledge of the number of bees emerging and the number of bees dying per unit of time.

Swarming, a natural phenomenon, leads to a sudden, major decline in the colony's population that has an adverse effect on hive productivity. This collapse in the number of bees should not be confused with an abnormal weakening of the colony. Early detection of swarming is possible through the continuous recording of sounds emitted by the colony. Indeed, as well as an increase in temperature within the hive, some authors (Ferrari *et al.* 2008) have shown a continuous increase in the amplitude and frequency of sounds emitted by the bees when swarming is imminent. The European "Swarmonitor" programme (FP7-SME-2012-2 project) should help develop these non-invasive population monitoring techniques, in cases of swarming or impaired health.

However, the population curve is not a sufficient indicator for measuring the good health of a colony because it does not show, for example, how tasks are divided between workers or the rotation of bee activities: the decline of a colony is not necessarily a consequence of mortality in the oldest bees (i.e. the foragers). Even though foragers are the most exposed to external stress factors, the impact of these factors on house-cleaning bees may cause an imbalance in the population according to the tasks to be performed. The reversion of tasks (foragers becoming house-cleaning bees again) or their acceleration (house-cleaning bees quickly becoming foragers) therefore constitutes an imbalance, and a particular effort (organisation and energy) is required to maintain a proper balance in the colony. This redistribution is not measured by a simple population monitoring curve.

Two apiary observatories were set up by scientists more than 7 years ago, in order to understand the evolution of colonies according to the landscape, climate, toxicological or parasitic context. The first one, Ecobee, is being coordinated by the CNRS in Chizé (CEBC) and INRA (Le Magneraud Entomology Unit and UR 406 Avignon). In particular, it enables the ecological study of the relationship between the available resources and colony development (Odoux *et al.* 2014; Requier *et al.* In press). The second observatory is investigating factors favouring or penalising lavender

honeyflow and is being coordinated by the PrADE joint technology unit (INRA Biostatistics and Spatial Processes Unit and ADAPI[9]; Decourtye, personal communication).

2.2.4.3 Monitoring of the temperature of the brood

For normal development, the temperature of the brood nest should ideally be maintained between 32°C and 36°C with an average of 34.5°C (Kronenberg and Heller 1982). Managing heat or cold around the brood is vital for the survival of the colony: healthy bees in sufficient quantity must be able to maintain this average temperature. This thermoregulation is essentially made possible by the action of workers (ventilation and provision of water against heat, and thermogenesis against cold). The temperature around the brood and the monitoring of this data could therefore be used as parameters of colony health. A few experimental techniques can be used to assess and monitor this temperature. Thermocouples were previously used. More recently, Becher and Moritz (2009) used sensors ("thermistors") placed in the centre of a colony and linked to a computer to monitor changes in the temperature of the nest over three days. Infrared thermometers are sometimes used to measure the temperature within colonies, especially in studies on the physiology of thermogenesis by bees, in particular within winter clusters (Stabentheiner *et al.* 2003; Stabentheiner and Schmaranzer 1987). Nevertheless, these methods are still experimental and are not used routinely because of their cost.

2.2.4.4 Mathematical models

The physiological development of a bee colony and the dynamics of its population are fairly well known and depend on many intrinsic (age of the queen, subspecies, *etc.*) and extrinsic (climate, region, season, food resources, *etc.*) factors. This development can be modelled and mathematical equations now enable the evolution of a colony to be projected based on certain initial parameters. The issue of mathematical models is discussed in section 5.4.2.

2.2.5 Lack of tools

Most of the tools and methods proposed in the previous paragraphs cannot be used routinely by beekeepers: either the technology is too expensive, they are adapted rather to scientific use, or they are too invasive. Today, only assessments based on observations or on measured weights are possible on a routine basis. Additional tools are therefore expected, in order to improve the assessment of colony health.

2.2.5.1 Diagnostic tools

The recording of clinical and lesional signs in colonies currently relies solely on observations that are highly dependent on the experience and knowledge of bee health technicians and veterinary clinicians. Like with other species, veterinary clinicians could refine their presumptive diagnosis if they had simple measuring tools that were innovative in terms of semiology during the clinical examination. These tools would need to be practical and usable in the field. Similarly, there is still too little access to additional examinations, rapid tests in particular, in beekeeping. Rapid tests are available that can be conducted directly at the apiary to assist in the diagnosis of foulbrood[10], but there is no guide to bee diseases, in particular concerning microscopic examinations of tissues (histology). Lastly, the epidemiological data (valuable as an aid to diagnosis and in risk management) available to the beekeeping sector are still ad hoc, partial and recent: the efforts made over the past two years (European Epilobee programme) have helped gain a better understanding of the prevalence and incidence of bee diseases and disorders in France, but only continual monitoring will provide information over the long term. The other animal production sectors have monitoring systems that have proven their worth over several decades and these models could be applied to beekeeping.

[9] Association for the development of beekeeping in Provence

[10] Currently, in bee pathology, only two tests based on immuno-chromatography (ELISA) are available for American and European foulbrood

2.2.5.2 Estimation of mortalities

It is not currently possible to count the disappearance of forager bees on a routine basis. Firstly, dead bee traps alone are not enough to measure bee mortalities within colonies. New tools for measuring mortality, that can be used on a routine basis, are therefore needed: in addition to precise measurement, they must be able to detect mortalities early in order to help the beekeeper trigger an alert as soon as possible, especially in cases of poisoning where the interval between exposure and screening for the toxin must be short due to the rapid metabolism of the toxin. Early detection necessarily implies remote monitoring with triggering of alerts. Tools for early detection of sharp population falls are not available on a routine basis (weighing scales, forager bee arrival-departure counters) and the means available are often too expensive to be accessible to all and/or to cover a large number of colonies.

2.2.5.3 Estimation of the distribution of age groups

A more detailed knowledge of the distribution of tasks within the population would help to better understand the phenomena of mortalities and weakening. A count of bees flying away from the hive cannot be used to determine precisely the share of the population assigned to foraging. Indeed, a significant proportion of the bees entering and leaving the hive are not foragers (Van der Steen *et al.* 2012). Similarly, the different age groups are evenly distributed among the frames of a hive (Van der Steen *et al.* 2012), which makes it difficult to assess the age pyramid within a colony.

2.2.5.4 Predictive data for estimating the fate of a colony

Mathematical models are able to project and simulate population dynamics depending on possible situations. However, some biological parameters cannot yet be incorporated into these models. Nevertheless, predicting the evolution of a colony would be valuable to the beekeeper for colony management (management of unproductive assets, management of population renewal, improvement of productivity) and it is now known that some biomarkers are predictive factors of colony health (Dainat *et al.* 2012b). A better knowledge of these predictive factors, grouped together for example in the form of colony health assessments, would therefore improve the prevention of certain risks of collapse.

2.3 Proposals for indicators of the health of bees / bee colonies

2.3.1 Indicators that can be used by the beekeeper

Among the various health indicators mentioned in the previous paragraphs, the beekeeper can monitor healthy growth of his/her colonies by relying, for example, on the following tools:

- observation of activity on the flight board;
- observation of brood frames and bees in order to estimate the strength of the colony (first impression on opening the hive, number of inter-frame gaps occupied, surface area of open and capped brood, *etc.*);
- observation of the queen and of the quality of her egg-laying;
- production of wax (recently produced wax has a whitish appearance), monitoring of arrivals of pollen, nectar and honeydew;
- scales for weighing the hives (to obtain production data and population data);
- remote monitoring of this weight, combined with climate data on the apiary site.

A few additional tools are currently under development:

- remote video monitoring of activity on the flight board;
- monitoring of vibrations and sounds emitted by the colony to predict swarming;
- "thermobuttons" to monitor the temperature inside the hive.

Certain information, which is currently incomplete or unavailable, would help improve the beekeeper's assessment of the development of his/her colonies: this includes reference values, available locally, which are lacking. The definition of a colony with "normal" behaviour is also lacking,

especially since, in contrast, a colony's behaviour is described as "abnormal". Averages should be defined by region and/or by subspecies used, starting with older data such as those generated by the work of Louveaux *et al.* (1966) in order to update and develop them. Bee colonies could also be positioned in several specific environments and monitored with the available physico-chemical and biological measurement tools, in order to self-correlate these parameters (comparison of a colony with itself) and deduce a normal average state as a function of the time, the environment, and the time-environment interaction, in a given region. These normal average states, once defined, should be readily accessible, like those derived from reference farms in other animal sectors, for example. The use of free online sites (such as http://hivetool.net/ in the United States), would help and support the beekeeper with good zootechnical management of his/her apiary.

2.3.2 Indicators that can be used by the veterinary clinician

In addition to the previous information collected by the beekeeper and constituting the case histories that will be taken into account by the veterinary clinician, the following items can be used by the veterinary clinician:

- the clinical examination;
- some quick tests that can be carried out "at the bedside of the sick colony" (such as the ELISA for foulbrood);
- some additional laboratory tests, including screening for residues;
- some epidemiological data (number of colonies affected, animal epidemic, animal movements, regional context, etc.).

Compared to other species, these tools are very limited (this is the case with the laboratory tests and rapid tests) or fragmentary (the case with epidemiological data). Other tools that can be used "at the bedside of the sick colony" should be developed in order to advance the semiological examination and improve diagnostic guidance in the field. Defining physiological constants would also assist veterinary clinicians in their diagnostic approach or in the development of health protection tools such as health assessments.

2.3.3 Indicators that can be used by the researcher

There are many tools available to the scientist for assessing the health of bees/bee colonies. Standardised methods for bee research, based on the currently available consolidated tools, were recently identified by the Coloss group in the *Journal of Apicultural Research* (Beebook, 2013[11]). Researchers thus have access to the following tools:

- at the individual level (bee only): behavioural tests, weight of emerging bees, radioentomology, pathological examination, individual biomarkers, *etc.*;
- at the scale of the colony: high-precision weighing scales, various bee counters, monitoring of brood temperature using temperature sensors, monitoring of foragers identified by radiofrequency (RFID) or by camera, *etc.*;
- at the scale of the apiary: the previous measures added to landscape data (example of Ecobee);
- mathematical models.

[11] http://www.coloss.org/beebook

2.4 Conclusions and recommendations

Defining the state of health of bee colonies, in order to better determine their "normal" or "abnormal" status and better characterise a colony's disorders, seems to be a necessity at the present time. Assessing the health of a bee colony is based on the estimation of several criteria: size of the population, egg-laying levels, hive activity, normal daily mortality of bees and infectious agents and parasites. It is based on a clinical examination, associated with further examinations where appropriate. Several tools are available for these estimates, at a given time and/or in the framework of monitoring of the dynamics of a colony.

The working group identified a lack of several tools, and stressed the value of distinguishing tools that can be used by the beekeeper from those intended for diagnosis, or even research. Research should thus be encouraged to:

- establish physiological constants in bees and bee colonies;
- improve and develop mechanisms for assessing rates of mortality and disappearance of worker bees, especially foragers;
- develop tools to measure the balance of different castes and age groups in the colonies;
- develop validated and harmonised diagnostic reference tools that can be used at several levels (in the field for assessing colonies and in the laboratory for analysis). These reference tools will mainly help to ensure the quality, representativeness and comparability of the results.
- produce a guide to bee diseases;
- develop mathematical models for understanding the potential effects of disruptors on colony health, and as an alert for colony health;
- obtain reference data (average status of a colony) in a given environment and region.

3 Stress factors

As a preamble, it is important to note that the internal request only concerns the hazards present in mainland France. As a result, exotic biological hazards such as *Aethina tumida*, that recently appeared in Italy, and *Tropilaelaps spp.*, as well as substances not currently used in France, are not addressed.

3.1 Literature data

In biology, the term stress refers to all the responses of an organism to factors that threaten its integrity. In the context of co-exposure of bees to stress factors, a certain number of factors were identified in the literature and are described below. These factors are biological, chemical, nutritional, or physical, or are related to beekeeping practices or climatic conditions.

We would like to point out that the stress factors in this chapter are not presented in order of priority, whether between the different types of factors or within a group of factors.

3.1.1 Biological factors

3.1.1.1 Introduction

Like in the case of infectious and parasitic diseases in vertebrates, the colony will be considered as a host individual, or superorganism. The various compartments (age groups and combs) can be parasitised or contaminated by infectious agents or other organisms. Reserves taken by humans (honey, pollen, etc.), as well as young queen bees being bred, are considered the “production” of the colony.

Several biological hazards, whether bacterial, viral, fungal, protozoal, parasitic, or predatory, have been found to cause specific clinical entities through pathogenic mechanisms that have an impact on one of the compartments of the colony or on production (Evans and Schwarz 2011; Genersch 2010). In certain well-known disease entities, called "infectious or parasitic" diseases, the deleterious consequences for the host organism are directly attributable to the spoiling effect of the “pathogenic” biological organism. This pathogen is, in biological terms, always an infectious or parasitic agent that lives to the detriment of its host. The intensity of the disorders is often correlated to the abundance of this infectious agent. Damage can involve diversion of certain metabolic pathways or even tissue damage sometimes leading to destruction of cells. The death or weakening of individuals leads to lower production and can result in decline of the entire colony.

If we consider that the epidemiological unit used to measure the health status is the colony, a biological agent is incriminated as a causal factor by detecting and quantifying it in the affected colony and by taking into account, in some cases, the induced clinical signs that may be characteristic. The degree of infection by an infectious agent can vary from one individual to another in the colony, making it important to carry out representative sampling of several individuals within the colony (from the compartment of interest) in order to evaluate the infection quantitatively (Ribière *et al.* 2010). Within the same class of infectious agents, there are often more or less virulent genetic variants, that can be characterised by genetic markers.

In the descriptions that follow, the agents recognised as being potentially pathogenic are presented with their pathogenic mechanisms. Experimental studies have often focused on the effect of each agent on individual bees. Effects on the colony can be more complex and gradual. Certain agents may cause an imbalance between age groups, leading to weakening of the colony. Moreover, the effects on the colony may differ depending on the bee caste affected, i.e. the queen, drones, or worker bees.

However, a large number of asymptomatic colonies are found that carry infectious or parasitic agents known to be able to cause bee diseases (Chauzat *et al.* 2010). Infection and infestation are dynamic phenomena that rely on exposure to contamination, e.g. an infectious dose, host resistance, and the stage of development of infection, but also on the effects of other exacerbating factors. In well-balanced colonies, several types of infectious agents may be found simultaneously, at the same time as commensal flora, which includes bacteria, protozoa, fungi, and viruses that are non-pathogenic or even beneficial for the colony (Cornman *et al.* 2012b). In broad terms, the microflora of bee bread for example can also be considered part of the colony's microflora. New high-throughput sequencing techniques have recently made it possible to determine the composition of this microflora (Runckel *et al.* 2011).

The final paragraph, concerning asymptomatic carriage, will address the studies showing that colonies may carry various potentially pathogens without the development of clinical signs.

3.1.1.2 Presentation of biological hazards of interest in the context of co-exposure and interactions in mainland France

3.1.1.2.1 Bacteria

Among the bacteria that can be pathogenic in bees, two main species can lead to larval mortality: *Paenibacillus larvae* and *Melissococcus plutonius*, the agents responsible for American foulbrood and European foulbrood, respectively (Forsgren 2010; Genersch 2010).

History of discovery

Foulbrood disease is a contagious disorder that affects the brood stage and has been known since the 18th century. It was however not until the early 20th century that European foulbrood was differentiated from American foulbrood, caused by distinct infectious agents (review in Forsgren 2010).

3.1.1.2.1.1 American foulbrood

- ***Infectious agent***

Paenibacillus larvae

In 2006, Genersch *et al*. showed that the two sub-species *P. larvae* subsp. *larvae* and *P. larvae* subsp. *pulvifaciens* in fact belong to a single species known as *Paenibacillus larvae,* on the basis of biochemical, genetic and virulence criteria (Genersch 2010; Genersch *et al.* 2006a).

- ***Disease***

American foulbrood / AFB

- ***Change in geographical distribution, current situation***

P. larvae is distributed worldwide. Four genetic clusters have been identified through Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR) molecular typing, including two genotypes with high virulence, ERIC I and ERIC II, which co-circulate in Europe (Morrissey *et al.* 2014; Peters *et al.* 2006; Rusenova *et al.* 2013). The virulence of ERIC types III and IV is less well known.

- ***Morphological and molecular description***

P. larvae is a Gram-positive bacterium producing spores. These spores are extremely resistant and constitute the method of dissemination and contamination with this bacterium. *P. larvae* was assigned to the *Paenibacillus* genus, distinct from the *Bacillus* genus, through 16S rRNA genotyping. The genome of *P. larvae* has been sequenced in full (Chan *et al.* 2011; Qin *et al.* 2006). In a comparative genomics study, Djukic *et al.* (2014) showed that the two genotypes have the ability to secrete many toxins and collagenases and are characterised by significant genomic plasticity (presence of transposases, integrases and recombinases) that support acquisition of virulence factors by horizontal gene transfer. The two genotypes are rather different at the genomic level, a characteristic that is reflected in their virulence. The two genotypes may be observed simultaneously in the same colony (Rusenova *et al.* 2013).

There are no known pathogenicity islands in *P. larvae*, but adaptation to the host, and thus virulence, are related to the presence of a large genetic cluster coding for polyketides/non-ribosomal peptide synthetases (PK/NRPS gene clusters). These enzymes enable the synthesis of antimicrobial molecules (antibiotics, antiparasitics, and antifungals) through which the bacterium destroys its competitors and the commensal flora of the larva (Genersch 2010; Yue *et al.* 2008). Specifically, an antibacterial non-ribosomal tripeptide was recently identified (Garcia-Gonzalez *et al.* 2014a; Genersch 2010; Yue *et al.* 2008). Garcia-Gonzalez *et al.* (2014b) also characterised a protein involved in degradation of the chitin of the peritrophic membrane during infection of larvae. Other virulence factors, including in particular toxins and other secreted metabolites, have also been demonstrated (Djukic *et al.* 2014; Fünfhaus *et al.* 2013; Krska *et al.* 2015; Schild *et al.* 2014).

- ***Clinical manifestations, infectivity/pathogenicity***

P. larvae infects larvae during the first days following hatching. The bacteria proliferate in the digestive tract before invading the haemocoel and killing the larva by releasing chitin-degrading proteins. Clinically, the disease manifests by brown threadlike cell content which then dries out and flakes (Garcia-Gonzalez *et al.* 2014b; Yue *et al.* 2008). Only some of the cells on a brood frame are affected. Adults show no clinical signs. The spores are extremely resistant in the hive environment, both in cells and wax, and can be carried by nurse bees and during evacuation of dead larvae or cleaning of cells. ERIC-I and ERIC-II genotypes, which co-circulate in Europe, differ by the time needed for systemic infection of larvae, which leads to larval death before or after capping of the brood and thus alters accessibility for cleaning. This affects clinical manifestations. As a result, virulence within the colony is inversely proportional to the rate of invasion of larvae by the bacterium (Rauch *et al.* 2009). The slow infection genotype (ERIC I) causes higher mortality within the colony and higher production of spores because of delayed and less effective elimination of diseased larvae by worker bees.

The hygienic behaviour of worker bees, genetically determined, is an essential component of a colony's response to this disease.

Swarming has a curative effect on this disease, by moving adults from the contaminated environment, which emphasises the importance of shook swarm methods (Fries *et al.* 2006; Pernal *et al.* 2008).

- ***Situations of co-infection/co-exposure to other stress factors***

In this disease, the hygienic behaviour of worker bees plays a decisive role concerning the consequences of infection at the colony level. This behaviour depends on genetic factors and can be altered by chemical hazards, for instance that disrupt the sense of smell (Kadala *et al.* 2014). In addition, interactions are possible at the larval microbiota level, promoting proliferation of the bacterium or invasion of the haemocoel through intestinal weakening. de Smet *et al.* (2014) showed that the sugar content of intestinal fluid and the haemocoel in the larva had regulating effects on the expression of these different genes, and on the growth of *P. larvae* in general. It is therefore also possible that the composition of the larval diet may be involved in the dynamics of infection.

- ***Detection***

The infection manifests through a mottled appearance of the brood, with collapsed and pierced caps and brown threadlike larval contents. Diagnosis can be performed using the "matchstick test" which involves inserting a matchstick into a cell that is suspected of being infected. When pulling the matchstick out, a brown viscous filament is found if the larva is infected. This highly viscous appearance is characteristic of American foulbrood. The diagnosis must however be confirmed by laboratory testing. The cells with diseased larvae contain *P. larvae* in large amounts. The Terrestrial Manual of the OIE (OIE 2014) lists the reference bacteriological and molecular methods for detection and identification, particularly a set of primers to detect the gene coding for the 16S fragment of ribosomal RNA. In the OIE Scientific and Technical Review, Rivière *et al.* (2013) recommend generalisation of quantitative PCR methods in order to have information on the infectious load that is more sensitive and easier to implement than counting spores. The infectious load is highly variable within an infected apiary and within a colony. As part of diagnosis, samples

must be taken from several colonies (Forsgren and Laugen 2013; Lindström 2008; Rauch *et al.* 2009).

There is currently no standardised sub-specific genotyping method, nor any international database. However, sub-typing methods that rely on PCR profiles on repeated sequences such as ERIC, REP and BOX, appear to be the best candidates to characterise geographic sub-types correlated with virulence (Genersch 2010; Rusenova *et al.* 2013).

- ***Treatment, control and prevention methods***

The preferred method to eliminate clinically diseased colonies is to destroy the hive through fire. Only spores are able to cause disease and they are extremely resistant to environmental conditions, heat, and chemical agents.

Shook swarm methods are effective but only on infected colonies that are not clinically diseased, where the spore load in adults is low (20 CFU *vs* 6000 CFU in adults from diseased colonies) (Pernal *et al.* 2008; Vidau *et al.* 2009). In the case of diseased colonies, these methods are not sufficient.

Although antibiotic treatment is prohibited in France, *P. larvae* is susceptible to oxytetracycline and to sulfathiazole, but resistant strains develop due to a mobilisable plasmid pMA67 (*tetL* resistance gene (Ammor *et al.* 2008)), that is likely to be harboured by other bacteria of broods such as *Paenibacillus alvei*. Antibiotics are not active on spores and their use in beekeeping is prohibited in the European Union (see section 3.1.2.4.1).

- ***Regulations covering the disease***

American foulbrood is classified as a category 1 health hazard (Ministerial Order of 29 July 2013). It is included on the OIE list of notifiable diseases (OIE 2015). In European regulations, it is included in list 1 of notifiable disease in Council Directive 92/65/EEC of 13 July 1992 and within the European Union, exchanges of live animals are subject to certification requirements (Commission Regulation (EU) No 206/2010).

3.1.1.2.1.2 European foulbrood

- ***Infectious agent***

Melissococcus plutonius is a Gram-positive bacterium that does not produce spores.

- ***Disease***

European foulbrood / EFB

- ***Change in geographical distribution, current situation***

European foulbrood is distributed worldwide, except in New Zealand. A resurgence of severe clinical cases has been observed in Europe since the 1990s, particularly in Switzerland (Roetschi *et al.* 2008) and in Great Britain (Haynes *et al.* 2013).

- ***Morphological and molecular description***

The bacterium *M. plutonius* is the only species in the *Melissococcus* genus, related to the *Enterococci*. It is fairly pleomorphic[12] on direct examination (Gram-positive, no formation of spores). Genetically, the species is remarkably homogenous worldwide. Recently however, local genotypes have been identified using an MLST (multi-locus sequence typing) approach (Haynes *et al.* 2013). More recently, this approach made it possible to distinguish geographic variants related to cases of varying severity (Budge *et al.* 2014). Nonetheless, in this recent study, the variables concerning other stress factors were not assessed.

- ***Clinical manifestations, infectivity/pathogenicity***

M. plutonius infects the gut in larvae, with infection often being lethal within 4 to 5 days, before capping of the brood.

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[12] Likely to take different forms

The disease manifests as a non-capped mottled brood with some of the larvae having been killed. Larvae die displaced in the cell, and are yellow in colour, then brown and finally greyish-black. Since larvae are susceptible to infection at any age, a capped brood can also be affected, with an appearance similar to that of American foulbrood. However, larvae may survive if they were infected late. Emerging adults are then smaller and carry the infection, spreading the bacterium via their excrement in the hive (Forsgren 2010). The complete genome of the type-strain has been sequenced (Okumura *et al.* 2011), opening avenues on mechanisms of virulence and genes that could be used for more precise diagnosis, and support genes for typing variants. The sequenced strain harbours in particular CRISPR (clustered regularly interspaced short palindromic repeat) sequences, conferring resistance to infection by phages.

As a result, the genome of *M. plutonius* appears far less plastic than that of *P. larvae* but the vast global distribution of this bacterium demonstrates its general parasitic nature in bees.

- ***Situations of co-infection/co-exposure to other stress factors***

The direct pathogenicity of *M. plutonius* has been correlated experimentally with the infectious dose. However, this bacterium is often found in co-infection with other opportunistic infectious agents such as *Achromobacter euridice*, *Enterococcus faecalis, Brevibacillus laterosporus*, or *Paenibacillus alvei*, whose roles in pathogenicity mechanisms remain poorly understood. These saprophytes are often more abundant in diseased cells at the time of diagnosis than actual *M. plutonius*.

- ***Detection***

Diseased larvae are yellow-brown in colour and not threadlike, unlike those affected by American foulbrood. *M. plutonius* is most abundant in the cells containing diseased larvae but may be undetectable in neighbouring cells. It may also be difficult to detect at the end of the course of infection once other microorganisms have proliferated (see above). In a diseased colony, worker bees for the hive body generally carry the bacterium, which is detectable and quantifiable by quantitative PCR (Rivière *et al.* 2013; Roetschi *et al.* 2008). The reference bacteriological and molecular detection methods are listed in the Terrestrial Manual of the OIE (OIE 2014).

- ***Treatment, control and prevention methods***

The shook swarm method can be used but in the United Kingdom, where the disease requires notification, highly affected colonies are destroyed (Budge *et al.* 2014). However, a study has shown that systematic destruction as practiced in Switzerland did not stop clinical cases from occurring. This is probably the result of persistent infection in neighbouring colonies, particularly when apiaries are located close to one another (Roetschi *et al.* 2008). Transmission through contaminated imported honey is possible and could explain the occurrence of new emerging genetic variants locally. Although *M. plutonius* is susceptible to oxytetracycline, antibiotic treatment (prohibited in the European Union in beekeeping, see section 3.1.2.4.1) is insufficient in cases of severe colony infection. Unlike *P. larvae*, there is no known resistance to tetracyclines in this species.

- ***Regulations covering the disease***

European foulbrood is not a category 1 or 2 health hazard in the Order of 29 July 2013. It is included in list B of diseases that could be subject to national programmes in European regulations (Council Directive 92/65/EEC of 13 July 1992) and is among the notifiable diseases to the OIE (OIE 2015).

3.1.1.2.2 Viruses

By 2011, 19 viruses had been described in the bee (Chen and Siede 2007; Evans and Schwarz 2011). Most are small positive sense single-strand RNA viruses that were classified as viruses belonging to the *Picornaviridae* family. More recently, Runckel *et al.* (2011), in a study based on temporal analysis of the honeybee microbiota, identified four novel RNA viruses that can infect *Apis mellifera*. Lastly, Li *et al.* (2014a) suggested that a pathogenic plant virus (tobacco ringspot virus - TRSV) could replicate in honeybees.

The main viruses described below include Deformed Wing Virus - DWV (Lanzi *et al.* 2006), Black Queen Cell Virus - BQCV (Leat *et al.* 2000), Chronic Bee Paralysis Virus - CBPV (Olivier *et al.* 2008a), Sacbrood Virus - SBV (Ghosh *et al.* 1999), and the AKI complex virus consisting of three

different viruses: Acute Bee Paralysis Virus - ABPV (Govan *et al.* 2000), Kashmir Bee Virus - KBV (de Miranda *et al.* 2004) and Israeli Acute Paralysis Virus - IAPV (Maori *et al.* 2007a).

Many infections remain asymptomatic; some may cause brood diseases or disease in adult individuals associated with malformations and paralysis sometimes leading to weakening, and/or mortality of colonies (Chen and Siede 2007; Olivier and Ribière 2006). Importantly, the development of the parasitic mite *Varroa destructor* appears to have altered the balance between viruses and bees through its ability to transmit and/or cause activation of replication in some viruses (de Miranda and Genersch 2010; Mondet *et al.* 2014; Nazzi *et al.* 2012; Ryabov *et al.* 2014; Tentcheva *et al.* 2004).

3.1.1.2.2.1 Deformed Wing Virus

- ***Infectious agent***

Deformed Wing Virus, DWV

- ***History of discovery:***

DWV was isolated in the early 1980s from symptomatic bees in Japan and was initially described as the Japanese strain of Egypt Bee Virus (EBV) (Bailey and Ball 1991; Ball 1989; Bowen-Walker *et al.* 1999). The transmission of DWV is strongly promoted by the parasitic mite *Varroa destructor*, but not necessarily (Ball and Allen 1988).

- ***Change in geographical distribution, current situation***

The virus is now distributed worldwide (Allen and Ball 1996; Ellis and Munn 2005). DWV infects not only *Apis mellifera* but also the Asian honeybee *Apis cerana* F. (Allen and Ball 1996), the dwarf honeybee *Apis florea* F. (Allen and Ball 1996; Ellis and Munn 2005), the giant honeybee *Apis dorsata* F. in southern India (Desai *et al.* 2012), and the buff-tailed bumblebee *Bombus terrestris* (Furst *et al.* 2014; Genersch *et al.* 2006b). DWV is now present in most apiaries in France. Recent analyses have shown that strains of DWV may have different virulence levels (Ryabov *et al.* 2014).

- ***Morphological and molecular description***

DWV belongs to the *Iflavirus* genus. The virus is a small 30 nm icosahedral particle composed of a positive sense single-strand RNA genome of 10,140 nucleotides, coding for three major structural proteins: VP1 (44 kDa), VP2 (32 kDa) and VP3 (28 kDa) (Lanzi *et al.* 2006).

- ***Clinical manifestations, infectivity/pathogenicity***

DWV is transmitted horizontally by excretion in faeces, cannibalism, and oral transmission (de Miranda and Genersch 2010), but also vertically since it has been found in sperm of drones and in queens (de Miranda and Fries 2008; Fievet *et al.* 2006; Yañez *et al.* 2012; Yue *et al.* 2006; Yue *et al.* 2007). It persists at all stages of honeybee development (adults, nymphs, larvae to a lesser extent, and eggs) (Bailey and Ball 1991; Chen *et al.* 2006; Lanzi *et al.* 2006; Yue *et al.* 2006), and pupae are overall less infected than adult bees (Tentcheva *et al.* 2004).

Reported DWV infections with clinical manifestations, i.e. wing deformations, malformed shortened abdomen, etc., are closely related to vectorial transmission by *Varroa destructor* (transmission by injection of the virus to pupae). Clinical signs of DWV have also been observed in the absence of *Varroa* (Forsgren *et al.* 2012; Shutler *et al.* 2014). Although there is a consensus in the literature that transmission of DWV to pupae by parasitic mites is a prerequisite for the development of deformed wings (Ball and Allen 1988; Bowen-Walker *et al.* 1999; Shen *et al.* 2005b; Yue and Genersch 2005), the precise mechanisms underlying these malformations remain to be elucidated (de Miranda and Genersch 2010).

A higher prevalence of DWV has been recorded in bees collected in autumn versus bees collected in spring or summer. This increase over the course of the year may be related to increasing infestation rates of apiaries by *Varroa* until the administration of anti-*Varroa* treatment in late summer/early autumn. Nymphs parasitised by *Varroa* have much higher viral loads than those that are not parasitised (Shen *et al.* 2005b). DWV is therefore implicated as one of the causes of colony loss during the winter (Dainat and Neumann 2013; Highfield *et al.* 2009).

In symptomatic adult bees from colonies that are highly infested with *Varroa destructor*, the presence of DWV is described as often being associated with wing deformation: vestigial or crumpled wings, bloated abdomen, paralysis, and in asymptomatic bees, premature death at the nymphal stage (Dainat *et al.* 2012a), along with decreased colony performance (Bowen-Walker *et al.* 1999). However, despite a viral load 10 to 100 times higher in bees with deformed wings than in bees without symptoms (Tentcheva *et al.* 2004), the process leading to wing deformation is still not known, and apparently symptom-free bees can also carry high concentrations of DWV. These results confirm those obtained by Bowen-Walker *et al.* (1999) who concluded that the number of viral particles present in the bee was a decisive factor for wing deformation at emergence. Queen bees can also carry DWV and may be symptomatic but they are probably eliminated early by their half-sisters given their deformity and thus their inability to ensure continuation of the colony (Williams *et al.* 2009). DWV multiplies slowly during the immature stages of bee development and although it rarely kills the nymph, it shortens lifespan at the adult stage. The virus is concentrated in the head and abdomen of adult bees and is also found in lower concentrations in the thorax and wings of infected individuals, but never in the legs (Lanzi *et al.* 2006). In reproductive individuals, DWV has been localised by *in situ* hybridisation in the cytoplasm of cells in the adipose tissue of queen bees, and to a lesser extent by quantitative PCR in the ovaries, the head, and digestive tract. In drones, DWV is present in the digestive tract and throughout the reproductive/genital tract (Fievet *et al.* 2006). The virus replicates in the bee, but exceptionally high doses of DWV in *Varroa* appear to indicate that the virus may also replicate very effectively in the mite vector to ensure dissemination (Bowen-Walker *et al.* 1999; Shen *et al.* 2005b). However, immunolocalisation tests for DWV in *Varroa destructor* that showed the presence of the virus in the digestive tract lumen did not confirm the hypothesis of the parasitic mite as the site of viral replication (Santillán-Galicia *et al.* 2008). The hypothesis of activation or induction of DWV replication in the bee by *Varroa* has also been put forward, specifically following parasite-induced immunosuppression (Shen *et al.* 2005b). This immunosuppression is thought to increase the sensitivity of bees to “opportunistic” infectious agents such as DWV (Nazzi *et al.* 2012; Yang and Cox-Foster 2005).

- ***Co-infection/co-exposure to other stress factors***

Among nymphs naturally parasitised by *Varroa* (n = 46), Shen *et al.* (2005b) demonstrated that 70% of them were co-infected with DWV and KBV. This co-infection is nonetheless not essential since nearly 22% of nymphs were carriers of only one of the viruses. This study also showed a synergistic effect of DWV with parasitism by *Varroa destructor*. No synergistic action between *Nosema ceranae* and DWV was however observed (Martin *et al.* 2013). Instead, it appears there may be competition between the two during their development in the gut (Doublet *et al.* 2015). Recently, negative effects of exposure to a neonicotinoid (clothianidin) were found on antiviral immunity, leading to DWV replication in bees carrying the virus (Di Prisco *et al.* 2013).

- ***Detection***

DWV virus is one of the main bee viruses likely to cause visible damage in the infected host. Its presence may be suspected in a colony when there are adult bees with deformed wings, or abnormally short, crumpled wings. However, since DWV is also found in asymptomatic colonies, simple observation is not sufficient to formally establish absence of the virus, which requires validation using more specific tests.

The most sophisticated current methods to determine the presence of DWV make use of antibody detection, for instance ELISA, or molecular techniques using RT-PCR (Tentcheva *et al.* 2004; Yue and Genersch 2005) or quantitative RT-PCR (Chen *et al.* 2005; Dainat *et al.* 2011).

- ***Treatment, control and prevention methods***

Like for all bee viruses, there is currently no treatment available for DWV. Strategies involve the use of treatments based on RNA interference (RNAi). This technique has been used against IAPV (Maori *et al.* 2009).

In terms of beekeeping practices, since *Varroa* is now a recognised vector of DWV, careful treatment of colonies against this parasite is strongly recommended in order specifically to limit viral dissemination and parasite-related increases in viral loads (Locke *et al.* 2012).

Lastly, vertical transmission of DWV, via drones but more importantly via queen bees (since they alone return to the hive after the mating flight and represent a potential risk of contamination for the colony), points to the possibility of testing for the presence of virus in samples of sperm used for artificial insemination (de Miranda and Fries 2008).

- ***Regulations covering the disease***

None.

3.1.1.2.2.2 Black Queen Cell Virus

- ***Infectious agent***

Black Queen Cell Virus, BQCV

- ***History of discovery***

BQCV was first described in 1974 by Bailey and Woods in larvae and pupae of *Apis mellifera* queen bees. Its name comes from the dark colour found on some parts of the surfaces of queen cells containing infected pupae (Bailey and Woods 1977; Benjeddou *et al.* 2002). This RNA virus, initially classified in the Picorna-like viral group, was reclassified by the International Committee on Taxonomy of Viruses (ICTV) in 2002 and now belongs to the *Cripavirus* genus in the *Dicistroviridae* family (Mayo 2002).

- ***Change in geographical distribution, current situation***

The virus is found worldwide (Allen and Ball 1996). It has been found in bee samples from Europe, Africa, Asia, the Americas and Australia. The complete genome sequence of a strain from South Africa has been obtained (Leat *et al.* 2000). The phylogeny generated from BQCV viral sequences collected from bees worldwide shows a high degree of genome conservation for isolates from different geographical locations, particularly between sequences coding for structural proteins. The most variable region corresponds to the coding sequence for a non-structural protein whose function is currently unknown (Reddy *et al.* 2013a).

BQCV has also been found in the *Bombus huntii* bumble bee (Peng *et al.* 2011), which indicates a potentially broad spectrum of BQCV hosts in terms of pollinating species.

- ***Morphological and molecular description***

The isometric viral particles of BQCV measure 30 nm in diameter. They contain single-strand RNA with an estimated length of 8550 bp. Sequencing of a strain from South Africa (Leat *et al.* 2000) showed two open reading frames (ORFs). The first, in 5', codes for a replicase type protein, and the second, in 3', codes for a capsid polyprotein. The molecular masses of mature proteins are 34, 32, 29 and 6 kDa, respectively.

- ***Clinical manifestations, infectivity/pathogenicity***

Worker bees, larvae and pupae of *Apis mellifera* may carry BQCV but remain asymptomatic. Workers appear to transmit the virus to larvae and more specifically to queen bee larvae when bringing royal jelly and then larva pollen to the brood. Of note, BQCV has never been detected in the parasite *Varroa destructor* (Gauthier *et al.* 2007; Tentcheva *et al.* 2004), which appears to rule out the hypothesis of transmission via the mite.

The pathogenic mechanism has not to date been described and is still unknown. The virus injected into the pupae multiplies but does not spread between captive adult bees. However, it may multiply in adult bees when it is ingested with spores of the microsporidian *Nosema apis* (Bailey *et al.* 1983).

The virus has been found in many asymptomatic apiaries and colonies investigated (Mouret *et al.* 2013; Tentcheva *et al.* 2004) (Provost, personal communication). The virus was detected in most samples of adult bees and in almost a quarter of pupae. The viral load in adult bees shows peak infection in the spring and early summer (like *Nosema apis*) and then decreases slightly in the autumn (Tentcheva *et al.* 2004) unlike parasite pressure related to *Varroa destructor,* which would corroborate the above-mentioned hypothesis that BQCV is not transmitted by the parasite.

The role played by BQCV in bee mortality is still poorly understood but it is thought that the effects on the health of workers and drones are limited, independently of the level of infection (Retschnig *et al.* 2014b). BQCV has been described as the most common cause of queen bee death in Australia (Anderson 1993), with these bees being found dead at the prepupal or pupal stage in the royal cell. In addition, symptoms described as being related to BQCV infection include abdominal hypertrophy and jerking movements (Higes *et al.* 2007a).

- ***Co-infection/co-exposure to other stress factors***

Several authors have described frequent co-infections with BQCV and the microsporidian *Nosema apis* (Allen and Ball 1996; Bailey *et al.* 1983) since BQCV could be involved in the death of bees co-infected with this parasite. Higes *et al.* (2007a) suggested that co-infection with the two infectious agents may influence the clinical course by increasing the pathogenicity of *Nosema*. These co-infections were confirmed by Dainat *et al.* (2012b) and Mouret *et al.* (2013). Synergistic interactions have also recently been documented between BQCV and the species *Nosema ceranae* (Doublet *et al.* 2014).

- ***Detection***

The most reliable and relevant technique to date is reverse transcription (RT) followed by quantitative PCR (RT-qPCR) (Gauthier *et al.* 2007). It has replaced conventional PCR which provides only a presence/absence type diagnosis. For a number of years, conventional PCR has also enabled detection of several infectious agents in a single reaction, using the Multiplex technique (Grabensteiner *et al.* 2007; Sguazza *et al.* 2013; Topley *et al.* 2005).

- ***Treatment, control and prevention methods***

No treatment method is currently available. Only strict disinfection of materials is recommended to avoid contamination.

- ***Regulations covering the disease***

None.

3.1.1.2.2.3 Chronic Bee Paralysis Virus

- ***Infectious agent***

Chronic Bee Paralysis Virus, CBPV

- ***Disease***

Chronic bee paralysis disease

- ***History of discovery***

CBPV is the aetiological agent of an infectious and contagious disease in adult bees and was isolated and characterised by Bailey *et al*. in 1963 (Bailey *et al.* 1963). Along with ABPV, it was one of the first bee viruses to be identified. Since it was not possible to assign the virus to an existing family, it appears to represent a new virus family (Morimoto *et al.* 2012).

- ***Change in geographical distribution, current situation***

CBPV is found worldwide (Morimoto *et al.* 2012) and can be detected throughout the year, mostly in asymptomatic bees (Bailey 1967; Bailey *et al.* 1963). The symptoms, associated with mortality near the hive entrance, are most commonly observed during the spring and summer (Bailey 1967; Ribière *et al.* 2002). Queen bees can also be infected through contact with symptomatic worker bees (Amiri *et al.* 2014). CBPV also infects the Asian honeybee *Apis cerana* (Ai *et al.* 2012; Choe *et al.* 2012).

- ***Morphological and molecular description***

The viral particle in CBPV is small (30 to 60 nm) and anisometric (Bailey *et al.* 1968). It is a positive sense, fragmented, single-strand RNA virus (Overton *et al.* 1982). Its genome contains two primary RNAs (RNA1 of 3674 nucleotides and RNA2 of 2305 nucleotides) and has been sequenced (Olivier *et al.* 2008a). Analysis of these sequences shows the presence of seven open reading frames (ORFs), three for RNA1 and four for RNA2.

- ***Clinical manifestations, infectivity/pathogenicity***

The disease is sometimes called black bee paralysis by beekeepers (Faucon 1992) and is characterised by chronic paralysis manifesting as the presence of trembling bees, colony weakness, and decreased production (Ball and Bailey 1997), and it can sometimes lead to colony losses (Kulinčević and Rothenbuhler 1975). This viral infection may lead to two types of syndromes called Type 1 and Type 2 (Bailey and Ball 1991) which can present within the same colony. In the case of Type 1 syndrome described in England, wing and body trembling is observed. Bees are not able to fly and crawl on the ground or on the stems of plants and die a few days after developing symptoms (Ribière *et al.* 2010). Type 2 syndrome, first described primarily in continental Europe, is characterised by loss of hair, giving the bees' bodies a black shiny appearance. These bees are sometimes rejected by the colony and many bodies may be found at the entrance to the hive (Ribière *et al.* 2010).

In symptomatic bees, large quantities of virus are found in different regions of the brain (Blanchard *et al.* 2007; Olivier *et al.* 2008b).

CBPV is transmitted mainly by contact (Bailey *et al.* 1983; Ribière *et al.* 2007) and transmission appears to be favoured during periods of confinement during the beekeeping season through increased contacts between healthy and infected bees. All bee castes can be affected: worker bees, drones and queen bees (Blanchard *et al.* 2007; Chen *et al.* 2005; Chen *et al.* 2006; Tentcheva *et al.* 2004).

Bees can be infected experimentally via the oral or topical route and by direct injection (Bailey 1965; Rinderer and Rothenbuhler 1975) but efficacy is much higher with injection. Symptoms develop 5 to 6 days after experimental infection (Chevin *et al.* 2012). However, Toplak *et al.* (2013) indicate that replication of CBPV appears more effective when bees are infected orally.

One study suggests that ants may also be reservoirs for the virus but their possible role in transmission has not been demonstrated. Transmission could also take place through *Varroa* (Celle *et al.* 2008).

- ***Co-infection/co-exposure to other stress factors***

In experimental conditions, a higher replication of CBPV was found along with more rapid mortality in bees co-infected with the microsporidian *Nosema ceranae* (Toplak *et al.* 2013).

- ***Detection***

The symptoms, which include trembling and presence of crawling bees in front of the hive, may be confused with those of other diseases or result from chemical intoxication. This is why it is necessary to have reliable and validated diagnostic tools enabling interpretation of results, in particular quantification tools for this virus which is widespread in bee colonies, with or without associated clinical signs.

An initial RT-PCR test was developed specifically to reveal hidden infections because many colonies are carriers of the virus but show no symptoms (Ribière *et al.* 2002). The availability of the complete genome sequence for CBPV (Olivier *et al.* 2008a) enabled development of a new RT-PCR method used to detect the various isolates of the virus (Blanchard *et al.* 2007; Blanchard *et al.* 2009). A real-time RT-PCR (RT-qPCR) method based on TaqMan technology was also developed to measure the viral load of CBPV (Blanchard *et al.* 2007; Celle *et al.* 2008).

In order to propose this test as a reference method, it was characterised in an intra-laboratory study during which the reliability and repeatability of test results and performance were confirmed. The qPCR test alone and the entire quantification method, from sample RNA extraction to analysis, were validated in accordance with the ISO/CEI 17025 Standard and the recent U47-600 XP Standard provided by the French Standards Institute (AFNOR). The performance of the quantification analysis method for CBPV by RT-qPCR was validated and the limit of detection established. This quantification protocol for CBPV by RT-qPCR has been approved by the French Accreditation Committee (COFRAC) (Blanchard et al. 2012).

Above 10^{10} viral genome copies per bee, chronic paralysis is considered overt (Blanchard *et al.* 2012; Ribière *et al.* 2010). The virus can be detected in all stages of development, from the egg to adult bees (Blanchard *et al.* 2007) but the disease only manifests in adults (Ribière *et al.* 2010).

The diagnosis of chronic paralysis is thus based on the measurement of the viral load coupled with clinical symptoms observed in the field.

- ***Treatment, control and prevention methods***

There is no treatment currently available.

- ***Regulations covering the disease***

None.

3.1.1.2.2.4 Sacbrood disease

- ***Infectious agent***

Sacbrood Virus, SBV

- ***Disease***

Sacbrood disease

- ***History of discovery***

The viral aetiology of Sacbrood disease was established in 1917 (White 1917) and the causative agent, SBV, described in 1964 (Bailey *et al.* 1964). It was the first bee virus to be fully sequenced genomically (Ghosh *et al.* 1999). The disease name comes from the appearance of dead larvae which form small liquid-filled sacs.

- ***Change in geographical distribution, current situation***

In *Apis mellifera,* SBV is found on all the continents (Allen and Ball 1996). In general, it does not have a major impact on colony survival, although it can sometimes affect brood development and cause colony losses. In *Apis cerana*, the virus is a significant cause of colony mortality in Asia (Liu *et al.* 2010).

- ***Morphological and molecular description***

SBV is a positive sense single-strand RNA virus and viral particles are about 28 nm in size (Bailey 1968). Like DWV, it belongs to the *Iflavirus* group, a group of viruses related to the Picornaviruses (King *et al.* 2011; Lanzi *et al.* 2006). The full genome has been sequenced and contains 8832 nucleotides (Ghosh *et al.* 1999) and a single ORF encoding a polyprotein with 2858 amino acids. Other SBV strains isolated in Vietnam and Korea were recently sequenced (Choe *et al.* 2012; Nguyen and Le 2013).

- ***Clinical manifestations, infectivity/pathogenicity***

Sacbrood is a contagious disease of bee broods. At the start of infection, the larva becomes pale yellow in colour and then appears as a sac filled with liquid. At the advanced stage, the disease manifests as an irregular, mosaic brood with collapsed capping. Dead larvae become dark coloured and unlike American foulbrood disease, they can easily be removed from the cell. SBV is also found in adult bees, particularly in the presence of *Varroa* (Tentcheva *et al.* 2004) and leads to decreased life expectancy. The virus accumulates in hypopharyngeal glands in worker bees, and in the adipocytes, muscle cells, and tracheal cells in larvae (Lee and Furgala 1967).

The frequency of infection is higher in spring and summer (Chen and Siede 2007).

Analyses carried out in France on apparently healthy bee colonies have shown that SBV is present in 86% of adult bee samples, 80% of nymph samples, and 45% of samples of *Varroa destructor* (Tentcheva *et al.* 2004). This suggests that *Varroa* plays a role in SBV transmission.

- ***Co-infection/co-exposure to other stress factors***

As mentioned above, the presence of the virus is often associated with *Varroa* infestation of colonies.

- ***Detection***

Methods using quantitative RT-PCR (RT-qPCR) have been developed to detect and quantify SBV (Chantawannakul *et al.* 2006; Evison *et al.* 2012; Gauthier *et al.* 2007; Kukielka and Sánchez-Vizcaíno 2009; Locke *et al.* 2012; Yoo *et al.* 2012). More recently, Blanchard *et al.* (2014b)

developed an RT-qPCR approach using TaqMan technology that can quantify the virus in larvae, pupae and adults. They defined a threshold of 10^{10} viral genome copies per bee from which clinical signs are observed. In order to propose this test as a reference method, the technique was validated in accordance with the AFNOR U47-600 Standard, in which the reliability and repeatability of results, and test performance were tested and validated.

- ***Treatment, control and prevention methods***

Like for other bee viruses, there is currently no antiviral treatment available in beekeeping. Good hygiene conditions and high honey flow can enable colonies to resist this viral infection. Research has shown that the use of RNA interference (RNAi) could help to control Chinese Sacbrood Bee Virus (CSBV) (Liu *et al.* 2010).

- ***Regulations covering the disease***

None.

3.1.1.2.2.5 AKI complex (ABPV, KBV, IAPV)

The AKI complex brings together three closely related viruses belonging to the *Dicistroviridae* family that are often difficult to differentiate: Acute Bee Paralysis Virus (ABPV), Kashmir Bee Virus (KBV) and Israeli Acute Paralysis Virus (IAPV) which was described more recently (de Miranda *et al.* 2010). These viruses are distributed worldwide and are often responsible for asymptomatic infections but can also be involved in colony losses, particularly when they are associated with the *Varroa destructor* parasite. Their prevalence is higher in adult bees but they can also be found in larvae and nymphs. Their virulence is high when they are inoculated experimentally. 100 viral particles injected into the haemolymph are sufficient to result in death, while about 10^{11} particles are needed to cause death via the oral route (Bailey and Ball 1991; Bailey *et al.* 1963; Bailey and Woods 1977; Maori *et al.* 2007a; Nordström 2000; Ribière *et al.* 2008).

3.1.1.2.2.5.1 Acute Bee Paralysis Virus

- ***Infectious agent***

Acute Bee Paralysis Virus, ABPV

- ***History of discovery***

ABPV was identified in 1963 in England (Bailey *et al.* 1963) as part of studies on Chronic Bee Paralysis Virus (CBPV) by inoculating nymphs with extracts of diseased bees suffering from chronic paralysis.

- ***Change in geographical distribution, current situation***

The virus has worldwide distribution and is the most common of the AKI complex in Europe (Baker and Schroeder 2008; Blanchard *et al.* 2008; Gauthier *et al.* 2007; Siede and Buchler 2006; Tentcheva *et al.* 2004) and in South America (Antúnez *et al.* 2006; Weinstein-Teixeira *et al.* 2008). Transmission of this virus is strongly promoted by the parasitic mite *Varroa destructor*.

The original host is probably *Apis mellifera*. Infection testing has also demonstrated that the virus may replicate in various bumble bee species (Allen and Ball 1996; Bailey and Gibbs 1964; Ribière *et al.* 2008).

- ***Morphological and molecular description***

ABPV is a positive sense, single-strand RNA virus belonging to the *Dicistroviridae* family (*Cripavirus* genus). Viral particles have a diameter of about 30 nm. The full genome of an isolate from the United Kingdom (about 9.5 kb) has been sequenced (Govan *et al.* 2000). Like all the viruses in the *Dicistroviridae* family, the genome has two open reading frames (ORFs) separated by an intergenic spacer. The ORF in 5' codes for non-structural proteins (helicase, protease and RNA-dependent polymerase) involved in particular in replication. The 3' ORF is shorter and codes for capsid proteins (de Miranda *et al.* 2004; Govan *et al.* 2000; Maori *et al.* 2007a). Other isolates from various geographic regions have also been sequenced (Bakonyi *et al.* 2002b).

- ***Clinical manifestations, infectivity/pathogenicity***

ABPV is able to multiply in adult bees and in brood, and its prevalence increases during the beekeeping season with a peak in late summer (Bailey and Ball 1991; Bailey *et al.* 1981; Ball and Allen 1988; Gauthier *et al.* 2007; Siede and Buchler 2006; Tentcheva *et al.* 2004). Like many other infectious agents, ABPV can be detected in colonies with no clinical signs. During experimental infections, bees are sluggish, trembly and flightless (Bailey *et al.* 1963; Ribière *et al.* 2008). In some cases, symptoms of early paralysis were reported. The wings of young bees may be asymmetrical or outspread. A mosaic brood and high mortality in the larval or nymphal stages have occasionally been observed and can lead to population decline.

The virus has been found in large quantities in the brain and hypopharyngeal glands in adults (Bailey and Milne 1969) and can also be found in excrement (Ribière *et al.* 2008). The transmission routes are the same as those for the other two viruses in the AKI complex: horizontal transmission, orofecally or vectorially by the *Varroa* parasite, and vertical transmission, via the ovaries (Beebook, 2013[13]). The viruses of the AKI complex have been found in the ovaries, on eggs and in the sperm in males (Francis *et al.* 2013a; Yue *et al.* 2006).

- ***Co-infection/co-exposure to other stress factors***

The role of ABPV in colony collapse has been suspected since the appearance of *Varroa*. Correlations between a high level of this virus (and the other two viruses in the AKI complex), infestation by *Varroa*, and winter mortality have been demonstrated (Francis *et al.* 2013b; Genersch *et al.* 2010). *Varroa* mainly plays a mechanical vector role (Di Prisco *et al.* 2011). Moreover, its immunosuppressor role often causes increased viral replication.

- ***Detection***

ABPV is often found at low levels in healthy, asymptomatic colonies. In addition, clinical diagnosis is difficult since symptoms, when present, are not always specific. Diagnosis therefore relies on laboratory testing and the most effective methods involve molecular biology with RT-PCR or RT-qPCR that enable differentiation of the three viruses in the AKI complex. As a result, several conventional RT-PCR approaches (Bakonyi *et al.* 2002a; Benjeddou *et al.* 2001; Gauthier *et al.* 2007; Grabensteiner *et al.* 2007; Tentcheva *et al.* 2004) and RT-qPCR methods using SYBR-Green (Kukielka and Sánchez-Vizcaíno 2009; Siede *et al.* 2008) or a TaqMan probe (Chantawannakul *et al.* 2006; Jamnikar Ciglenečki and Toplak 2012), have been developed to detect ABPV. The RT-qPCR method developed by Jamnikar Ciglenečki and Toplak in 2012 is 230 times more sensitive than conventional RT-PCR methods and makes it possible to detect different variants of ABPV. Validation of these methods based on applicable standards appears essential.

- ***Treatment, control and prevention methods***

There is no treatment available for ABPV. Research studies have shown that approaches using small interfering RNAs (siRNAs) could be considered. Since *Varroa* is a vector of the viruses in the AKI complex, it is important to continue to combat this mite to limit dispersion of these viruses.

- ***Regulations covering the disease***

None.

3.1.1.2.2.5.2 Kashmir Virus

- ***Infectious agent***

Kashmir Bee Virus, KBV

- ***History of discovery***

The Kashmir Bee Virus (KBV) was identified during experimental infections in *Apis mellifera* in 1974 on the basis of extracts of Asian honeybees *Apis cerana* from the Kashmir valley (Bailey *et al.* 1976; Bailey *et al.* 1979). In the laboratory, it appears to be the most virulent bee virus but it is also found in apparently healthy colonies.

- ***Change in geographical distribution, current situation***

[13] http://www.coloss.org/beebook/II/virus/table-2

KBV is found in *Apis cerana* and *Apis mellifera* in various regions of the world (Allen and Ball 1995; Allen and Ball 1996; Ball and Bailey 1997; Choe *et al.* 2012) but its prevalence is higher in North America (Cox-Foster *et al.* 2007; Hung *et al.* 2000; Hung *et al.* 1996) and New Zealand (de Miranda *et al*. 2010; Todd *et al*. 2007). It has also been found in bumble bees in New Zealand and in wasps in Australia (Anderson 1991). KBV is particularly virulent in bees in the presence of the mite *Varroa destructor* and can very rapidly result in mortality among brood and adults, without specific symptoms.

- ***Morphological and molecular description***

KBV is a positive sense, single-strand RNA virus of the *Cripavirus* genus and belongs to the *Dicistroviridae* family (Liljas *et al.* 2002). Study of capsid protein profiles and serological analyses have shown that the virus is more variable than ABPV (Allen and Ball 1995; Bailey *et al.* 1979). Its genome has been fully sequenced and is about 70% identical to that of ABPV (de Miranda *et al.* 2004). Genotypes of different geographic origins have also been sequenced (Reddy *et al.* 2014).

- ***Disease, clinical manifestations, infectivity/pathogenicity***

Infection with KBV is among the most frequent viral infections in honeybees. It affects all stages of development of the brood and is found in adult bees. No symptom clearly defines the infection which may even be hidden. When inoculated experimentally, KBV is the most virulent of the bee viruses (Chen and Siede 2007). It is lethal for adults and larvae after injection or via the oral route at high doses (Bailey *et al.* 1963; Nordström 2000). The virus can infect several tissues in the bee body.

KBV can be detected in *Varroa* (Shen *et al.* 2005b). Mites from colonies infected with KBV are able to transmit the virus to nymphs from healthy colonies with 70% effectiveness of transmission (Chen *et al.* 2004). Like with other viruses, infection by *Varroa* causes activation of KBV replication. It can therefore result in colony losses in combination with *Varroa* (Hung *et al.* 1996; Ribière *et al.* 2008; Todd *et al.* 2007). KBV can thus be transmitted in several ways: vectorial transmission via *Varroa*, oral transmission, and vertical transmission with virus detected on the surface of eggs (Chen *et al.* 2006).

The prevalence of KBV increases over the season with a higher peak observed in the autumn (Gauthier *et al.* 2007; Tentcheva *et al.* 2004).

- ***Co-infection/co-exposure to other stress factors***

In a latent state in the hive, the infection develops when KBV is associated with *Varroa destructor*, the fungal parasite *Nosema apis,* or certain environmental factors.

- ***Detection***

The first molecular diagnostic technique for KBV by RT-PCR was developed by Stoltz *et al.* (1995) and has been used to study the prevalence of the virus in various countries (Blanchard *et al.* 2014a; Blanchard *et al.* 2008; Evans 2001; Siede *et al.* 2005; Tentcheva *et al.* 2004). However, the primers developed by Stoltz *et al.* (1995) were not specific to KBV and also amplified IAPV (Blanchard *et al.* 2008; de Miranda *et al.* 2010). More recently, a more specific test by RT-PCR was developed by Blanchard *et al.* (2012). There are also approaches using RT-qPCR (Antunez *et al.* 2012; Ward *et al.* 2007).

- ***Treatment, control and prevention methods***

No treatment is used to combat KBV. Like for other viruses, strategies using small interfering RNAs could be considered.

- ***Regulations covering the disease***

None.

3.1.1.2.2.5.3 Israeli Acute Bee Paralysis Virus

- ***Infectious agent***

Israeli Acute Paralysis Virus, IAPV

- ***History of discovery***

The Israeli Acute Paralysis Virus (IAPV) was detected more recently in Israel in 2002 (Maori *et al.* 2007a; Maori *et al.* 2007b). Like in the cases of ABPV and KBV, it was discovered during experimental infections.

- ***Change in geographical distribution, current situation***

IAPV was first detected in Israel but has since been identified in several countries worldwide, including France in 2008 (Blanchard *et al.* 2008; Cox-Foster *et al.* 2007). It is dominant in the Middle East and in Australia (Maori *et al.* 2007a; Palacios *et al.* 2008). *Varroa* is a viral vector for IAPV (Di Prisco *et al.* 2011).

A metagenomic study carried out in the United States suggests that the presence of the virus could be correlated with colony collapse disorder (CCD) (Cox-Foster *et al.* 2007). The only known host for IAPV is *Apis mellifera* (Chen and Evans 2007; Maori *et al.* 2007a; Maori *et al.* 2007b). However, an experimental study has shown that IAPV, like KBV, can infect *Bombus terrestris* (Meeus *et al.* 2014).

- ***Morphological and molecular description***

IAPV is a new virus belonging to the *Dicistroviridae* family and is closely related to ABPV and KBV (Maori *et al.* 2007a). It is a positive sense, single-strand RNA virus with 9487 nucleotides. Its genome has the same type of organisation as the two other viruses in the AKI complex. Several strains isolated in South Korea have also been sequenced (Reddy *et al.* 2013b). There is high genetic variability between IAPV strains (Chen *et al.* 2014).

- ***Clinical manifestations, infectivity/pathogenicity***

IAPV is able to infect all the stages of development of bees (eggs, larvae, nymphs, and adults) and the various castes of *Apis mellifera*. It causes systemic infection in bees and is detected in the haemolymph, brain, fat body, salivary gland, hypopharyngeal gland, gut, muscle cells, etc. with highest concentrations found in the gut (Chen *et al.* 2014). *In situ* hybridisation approaches show that IAPV can be found in the eggs, gut, ovaries, and spermatheca in infected queen bees (Chen *et al.* 2014).

Like for the other two viruses in the AKI complex, transmission of this virus can occur horizontally and/or vertically, and *Varroa* plays a vectorial role.

Recent studies on the response of bees to this viral infection show that IAPV alters the transcription activity of genes involved in various fundamental cell functions such as the ribosome synthesis machinery (Boncristiani *et al.* 2013) or mitochondrial activity (Chen *et al.* 2014). These authors also demonstrated that the viral infection triggers immune response pathways in adult bees. IAPV could therefore play an important role in bee colony weakening, associated with colony collapse disorder (Hou *et al.* 2014).

- ***Co-infection/co-exposure to other stress factors***

The mite *Varroa destructor* plays a vectorial role in transmission of IAPV (Di Prisco *et al.* 2011). Moreover, this parasite inhibits immune response in bees and activates IAPV viral replication.

- ***Detection***

IAPV can be detected by RT-PCR (Blanchard *et al.* 2008; Cox-Foster *et al.* 2007) or by PCR Multiplex methods (Carletto *et al.* 2010).

- ***Treatment, control and prevention methods***

Studies carried out by Maori and Hunter, carried out in colonies in the field, have shown that use of a double-stranded RNA helped to control the infection with IAPV in bee colonies (Hunter *et al.* 2010; Maori *et al.* 2009). In regulations regarding maximum residue limits (MRLs) in food of animal origin, this RNA was added to the list of substances that are pharmacologically active, with the remark “no MRL required” for honey[14].

[14] Commission Implementing Regulation (EU) No 489/2013 of 27 May 2013 amending the Annex to Regulation (EU) No 37/2010 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin, as regards the substance double stranded ribonucleic acid homologous to viral ribonucleic acid coding for part of the coat protein and part of the intergenic region of the Israel Acute Paralysis Virus.

The *Varroa destructor* parasite plays a vectorial role and combating the mite is a prophylactic measure to be maintained to control this viral infection.

- ***Regulations covering the disease***

None.

3.1.1.2.2.5.4 Conclusions on the AKI complex viruses

In France, two studies carried out in 2002 (Tentcheva *et al.* 2004) and in 2009 (Mouret *et al.* 2013) provided data on the prevalence of the AKI complex. The study by Tentcheva *et al.* (2004) included 360 asymptomatic colonies from 36 apiaries from across the country. 23% of the apiaries were carriers of ABPV and the prevalence of IAPV was 6%. Studies by Mouret *et al.* (2013) were carried out on 90 asymptomatic colonies from 18 apiaries located in western France. Bees from five hives in each apiary were analysed at four time points in the year. KBV was the most prevalent (75% of apiaries), followed by IAPV (65%) and ABPV (14%).

Some studies have suggested that the viruses in the AKI complex may play a role in colony collapse. It is however important to remain cautious about the causes of observed mortality since the number of studies is low and it cannot be assumed that the presence of these viruses in collapsed colonies indicates that they were the responsible agents. *Varroa* plays a major role as a mechanical vector and through its immunosuppressive role which promotes or activates proliferation of these viruses. This is also true for other bee viruses and many other infectious agents.

3.1.1.2.3 Fungi

Several biological hazards classified as fungi lead to diseases in bees. Among the most frequent are the parasitic microsporidia of the gut *Nosema apis* and *Nosema ceranae* that cause nosemosis, and *Ascosphaera apis,* the agent inducing chalkbrood (ascospherosis).

3.1.1.2.3.1 Nosema apis/Nosema ceranae

- ***Infectious agent***

Nosema apis/Nosema ceranae

- ***Disease***

Nosemosis is a disease in adult bees that affects the digestive tract and can cause acute diarrhoea and in some cases can cause mortality in the affected colonies. Two species of *Nosema* are found in honeybees: *Nosema apis* and *Nosema ceranae. Nosema* fungi are members of the microsporidia, a group of eukaryotic, obligate intracellular, single-cell parasites.

- ***History of discovery***

Nosema apis has been known to infect the European honeybee *A. mellifera* for more than a century (Zander 1909). In 2005, sampling from Spain and Taiwan showed that *A. mellifera* may be infected by a second species, *Nosema ceranae* (Higes *et al.* 2006; Huang *et al.* 2007). Subsequent studies have suggested that *N. ceranae* has been present in *A. mellifera* since at least 1998 in Europe (Paxton *et al.* 2007), 1995 in the USA (Chen *et al.* 2008), and even 1990 in Uruguay (Invernizzi *et al.* 2009). *N. ceranae* was first described in China in the Asian honeybee *Apis cerana* (Fries *et al.* 1996). *N. ceranae* appears to have recently crossed the species barrier from the Asian honeybee to the European honeybee (Botias *et al.* 2012a).

Adult bees are contaminated by ingesting spores in wax, pollen, nectar or water soiled with excrement from contaminated bees. Spreading of the infection may occur within a colony through exchanges between bees, cleaning activities, trophallaxis, etc., and between colonies via drift, robbing, migratory beekeeping, and so on.

- ***Change in geographical distribution, current situation***

Trade has rapidly led to the geographical spread of *N. ceranae* within colonies of *A. mellifera*. This species is now widely distributed worldwide (Klee *et al.* 2007). In addition to its wide geographic presence, *N. ceranae* today seems to have a much higher prevalence than *N. apis* in bee colonies (Botias *et al.* 2012c; Chaimanee *et al.* 2010; Chen *et al.* 2009; Stevanovic *et al.* 2010). Replacement

of *N. apis* by *N. ceranae* in *A. mellifera* has in fact been suggested (Botias *et al.* 2012a; Chen *et al.* 2012; Martinez *et al.* 2012). Nonetheless, in some countries, for instance Germany (Gisder *et al.* 2010), or Sweden (Forsgren and Fries 2013), both species are still commonly found, with *N. apis* showing a higher prevalence than *N. ceranae*. The fact that *N. ceranae* spores are more sensitive to low temperatures (Fenoy *et al.* 2009; Fries 2010) and that their germination, which is necessary for infection of host cells, is significantly reduced after treatment at 4°C (Gisder *et al.* 2010) may inhibit the infectious potential and spread of this species in climates characterised by colder winters. Aside from these exceptions, *N. ceranae* appears to dominate in terms of prevalence in many regions with warmer climates (Fries 2010; Higes *et al.* 2010; Higes *et al.* 2013). *N. ceranae* is also able to infect other bee species such as *A. cerana*, *A. florea*, *A. dorsata* and *A. koschevnikovi* (Botias *et al.* 2012a; Chaimanee *et al.* 2013; Suwannapong *et al.* 2010) and some species of bumble bees such as *Bombus atratus*, *Bombus morio*, *Bombus bellicosus* and *Bombus terrestris* (Graystock *et al.* 2013; Plischuk *et al.* 2009).

- ***Morphological and molecular description***

Nosema are eukaryotic, obligate intracellular, single-cell parasites classified as fungi that produce small resistant spores of a few µm and that can persist in the environment for many months. These pathogens belong to the microsporidia, a group that includes about 1500 species parasitising all the organisms in the animal kingdom, including humans (Vavra and Lukes 2013). Microsporidian spores that infect honeybees are oval, with those of *N. apis* (6 x 3 µm) being slightly larger than those of *N. ceranae* (4.4 x 2.2 µm) (Chen *et al.* 2009; Fries *et al.* 1996).

The spore is surrounded by a membrane and a very thick rigid extracellular wall made up of two parts: the exospore, a dense fibrous glycoprotein matrix, and the endospore, a matrix composed primarily of chitin and proteins. The inside of the spore contains the sporoplasm which is the infectious material (Keeling and Fast 2002). In *N. apis* and *N. ceranae,* the sporoplasm has two nuclei in a close diplokaryotic arrangement (Chen *et al.* 2009; Fries *et al.* 1996). The sporoplasm also contains a polar tube or coiled polar filament, a structure involved in the process of invading cells in the intestinal epithelium (Vavra and Lukes 2013). The invasion process starts with a germination phase during which the microsporidian spore evaginates its polar tube, enabling transfer of the sporoplasm to the cytoplasm of the host cell. The full genomes of both *N. ceranae* (Cornman *et al.* 2009) and *N. apis* (Chen *et al.* 2013) are now known. Currently, no correlations have been established between genetic variants and virulence. However, differences in susceptibility to *Nosema* among colonies have been reported (Fontbonne *et al.* 2013).

- ***Clinical manifestations, infectivity/pathogenicity***

Nosemosis is a disease of variable severity depending on whether conditions are more or less favourable for multiplication and dissemination of the parasite. Nosemosis mainly develops when weather conditions are unfavourable, such as long rainy winters, when colonies are weak, or when queen bees are older. Although nosemosis has no characteristic clinical signs, it can cause digestive manifestations (diarrhoeal marks found on frames or the floorboard). In some cases, it is possible to observe crawling bees with bloated abdomens, dead bees in front of hives, or depopulation.

The parasite fulfils its developmental cycle within the epithelial cells of the midgut (Higes *et al.* 2010). In these cells, different parasitic stages can be observed: meronts, sporonts, sporoblasts and spores. Observing spores of *N. ceranae* in intestinal cells of bees three days after infection suggests rapid development of the parasite, completed in only 72 h (Higes *et al.* 2007b). Natural infection by *N. ceranae* often leads to production of millions or even several dozen million spores within a single individual (Higes *et al.* 2008; Mulholland *et al.* 2012; Paxton *et al.* 2007; Smart and Sheppard 2012). These spores are released with the bees' excrement and can thus contaminate the hive and its environment.

The presence of *N. ceranae* has also been detected by PCR in other tissues in worker bees: the hypopharyngeal, mandibular and salivary glands, Malpighian tubules, the fat body, and the venom sac (Chen *et al.* 2009; Copley and Jabaji 2012). However, despite molecular detection of the parasite in various tissues, microscopic analyses have not been able to demonstrate spores or intracellular development stages in tissues outside the digestive tract (Huang and Solter 2013).

Natural infections of *A. mellifera* with *N. ceranae* were first detected in the worker bee caste (Higes *et al.* 2006; Huang *et al.* 2007), and more recently in drones (Traver and Fell 2011), and in queen bees (Traver and Fell 2012). Detection of spores in their ovaries suggests possible vertical transmission of the parasite to individuals of successive generations (Traver and Fell 2012).

Many studies on *N. ceranae* have been performed at the individual level in laboratory conditions and at the colony level in semi-field studies to identify the effects of infection on behaviour, physiology, and even survival of bees. Infection has many consequences, specifically effects on the nutritional and energy status of bees (Alaux *et al.* 2010a; Aliferis *et al.* 2012; Dussaubat *et al.* 2012; Martin-Hernandez *et al.* 2011; Mayack and Naug 2009; Mayack and Naug 2010; Naug and Gibbs 2009), on foraging (Dussaubat *et al.* 2013; Kralj and Fuchs 2010; Mayack and Naug 2010), on hormone and pheromone production in bees (Alaux *et al.* 2011b; Antúnez *et al.* 2009; Ares *et al.* 2012; Dussaubat *et al.* 2010; Goblirsch *et al.* 2013), on the intestinal epithelium (Dussaubat *et al.* 2012), and on survival in bees (Chaimanee *et al.* 2013; Forsgren and Fries 2013; Higes *et al.* 2007b; Martin-Hernandez *et al.* 2011).

- ***Co-infection/co-exposure to other stress factors***

Interactions, sometimes synergistic, with other infectious agents (Bromenshenk *et al.* 2010; Doublet *et al.* 2015; Hedtke *et al.* 2011; Ravoet *et al.* 2013; Schwarz and Evans 2013; Toplak *et al.* 2013) and classes of insecticides (Alaux *et al.* 2010a; Aufauvre *et al.* 2012; Pettis *et al.* 2012; Retschnig *et al.* 2014a; Vidau *et al.* 2011; Wu *et al.* 2012) have been demonstrated in recent years (see chapter on interactions). Inhibited expression of immunity genes by certain insecticides could also enhance the effects of infection by *N. ceranae* (Aufauvre *et al.* 2014).

- ***Detection***

There is no clinical sign that is characteristic of this disease. Nosemosis induced by *N. apis* manifests as digestive symptoms, with diarrhoeal marks being observed on the walls of the hive or frames. Intestinal examination of bees can be carried out. The gut in affected bees is generally white in colour, while in healthy bees it is brown-red. Confirmation of diagnosis is carried out in the laboratory and involves identifying and counting spores under the microscope. However, the presence of spores is not absolute proof that the parasite is the cause of the disease seen in colonies or of the observed losses. Also, it does not enable differentiation between *N. apis* and *N. ceranae*. Only molecular diagnostic testing using PCR on adult bees enables differentiation of the two species of *Nosema*. Various markers can be used (Gisder and Genersch 2013; Roudel *et al.* 2013). Multiplex PCR (Carletto *et al.* 2013; Hamiduzzaman *et al.* 2010) and quantitative PCR approaches (Bourgeois *et al.* 2012) have also been developed.

- ***Treatment, control and prevention methods***

Control methods for *N. ceranae* and *N. apis* are relatively limited. Colony infection can be controlled through the use of an antiparasitic agent called fumagillin. Fumagillin is an antibiotic originally produced by *Aspergillus fumigatus*. This substance appears to target the methionine enzyme aminopeptidase 2 which is a protease that cleaves N-terminal methionine during protein maturation (Didier *et al.* 2006). Treating colonies infected with *N. ceranae* with fumagillin in the autumn significantly reduces the intensity of infection (parasitic load) the following spring (Williams *et al.* 2008). In addition, treating colonies with fumagillin appears to significantly reduce the risk of depopulation although it does not prevent subsequent reinfections (Higes *et al.* 2008). This risk of subsequent reinfection is a serious problem since low concentrations of fumagillin, that remain for several months after treatment, may have a negative effect and lead to hyperproliferation of *N. ceranae* in treated bees (Huang *et al.* 2013). Many countries in the world still use fumagillin to control infection of colonies with *N. ceranae*. However, treating bee colonies with this antibiotic is prohibited in the European Union because of the absence of marketing authorisation and the absence of a maximum residue limit established for honey (Fries 2010; Higes *et al.* 2010).

Alternatively, good management in beekeeping activities could in some cases prevent the development of nosemosis, for instance by replacing frames and queen bees in infected colonies (Higes *et al.* 2010). Replacing the queen bee in an infected colony with a younger queen bee leads to a significant reduction in the number of infected bees in the colony, enabling colony survival (Botias *et al.* 2012b). To reduce the risk of nosemosis, it is also advisable to avoid overly shady

positioning and damp areas, to start overwintering quite early, to avoid delayed honeyflow and feeding, and to regularly disinfect materials.

- ***Regulations covering the disease***

In France, *N. apis*-related nosemosis is a category 1 health hazard (Ministerial Order of 29 July 2013). This is not the case for *N. ceranae.*

3.1.1.2.3.2 Ascosphaera apis

- ***Infectious agent***

Ascosphaera apis

- ***Scientific name/Common name/Nomenclature/Abbreviation***

Ascospherosis/Chalkbrood disease

- ***History of discovery***

Chalkbrood disease was described in the early 20th century (Maassen 1913) in Europe. The causative agent, initially called *Pericystis apis,* was renamed *Ascosphaera apis* by Spiltoir in 1955. Transmission occurs via ingestion of *A. apis* spores by larvae.

- ***Change in geographical distribution, current situation***

This disease is now found in most countries worldwide and its incidence has been on the rise in the past few years (Kluser and Peduzzi 2007).

A recent publication indicated that DWV, BQCV and IAPV can infect and multiply in *Ascosphaera apis* (Li *et al.* 2014b). Further studies are needed to assess the potential effects of virus-fungus combinations on bee health.

There is are also other species of the *Ascosphaera* genus that can parasitise solitary bees (Wynns *al.* 2013).

- ***Morphological and molecular description***

Ascosphaera apis is a fungus belonging to the Ascomycota phylum (Lumbsch and Huhndorf 2007). Sexual reproduction of this fungus results from fusion of two mycelia (vegetative forms that are difficult to distinguish morphologically) of different sexual types. This fusion gives rise to asci in which spores are formed called ascospores with a size of 2 to 3 µm. Ascospores are the infectious stage that will then germinate in the gut of larvae to again form mycelia. Ascospores can persist for several years in mummified larvae (a mummy can contain between 10^8 and 10^9 ascospores). They also persist in pollen, honey and wax, which are therefore major sources of contamination (Flores *et al.* 2005a; Flores *et al.* 2005b). Spores are also resistant in the external environment but this is not the case for mycelia.

Enzymes involved in penetration of the fungus through the peritrophic membrane have been identified (Theantana and Chantawannakul 2008). Two strains of different sexual types (ARSEF 7405 and 7406) were isolated by Murray *et al.* (2005). These two isolates were named MAT1-1 and MAT1-2 by Aronstein *et al.* (2007).

Sequencing the *A. apis* genome (Qin *et al.* 2006) along with transcriptome studies (Cornman *et al.* 2012a) enabled many virulence factors to be demonstrated, particularly genes coding for chitinases, proteases and toxins.

- ***Disease, clinical manifestations, infectivity/pathogenicity***

The disease is mainly observed in the spring. It is spread by ascospores which are ingested by larvae. When conditions are favourable, the spores germinate in the larval gut and form a filament or mycelium. The mycelium then invades all the tissues in the larva, resulting in death. Infested larvae are first soft and whitish-yellow in colour, and then become firm and yellow. The mycelium forms a white or green-black coating. The larva then dries out and enters into a process of mummification. This is why the disease is called chalkbrood.

Although infection is fatal in larvae, it is rarely the cause of total destruction of a colony. It can however cause weakening and lower colony productivity.

Consumption of contaminated food (honey, pollen), trophallaxis, and contaminated materials, lead to spread of the disease. Robbing, drift and beekeeping handling operations may facilitate spread of the disease between colonies.

All the castes (worker bees, drones, and queens) can be affected but only larvae are susceptible. Adult bees are resistant but can nonetheless harbour spores in the gut and serve as vectors (Aronstein and Murray 2010).

Importantly, several strains of *A. apis* have been found with different virulence levels (Glinski 1982; Lee *et al.* 2013; Vojvodic *et al.* 2011).

Biotic and abiotic factors can promote development of the fungus and occurrence of the disease. As such, a drop in temperature and high humidity promote germination of spores and thereby fungal growth.

- ***Co-infection/co-exposure to other stress factors***

Hedtke *et al.* (2011) have shown that infestation of bee colonies with *Varroa* in summer promotes occurrence of ascospherosis. A possible role of *Nosema ceranae* in susceptibility of colonies to *Ascosphaera apis* has also been suggested. The use of antibiotics and the presence of plant protection product residues also appear to be supporting factors (see chapter on interactions).

- ***Detection***

The presence of white or black mummified larvae on the flight board or in front of the hive is a specific characteristic of this disease. Diagnosis is based on microscopic detection of spores in dead larvae.

Given that *A. apis* spores are often found in asymptomatic colonies, diagnosis by PCR is required. This is carried out using the rDNA region and ITS1 and 2 (Borum and Ulgen 2008; Chorbinski 2004). A PCR strategy based on repeat sequences (Rep-PCR) was also used by Reynaldi *et al.* (2003) to characterise various isolates of *A. apis*. More recently, an approach using multiplex PCR was developed to evaluate the prevalence of *A. apis* and of bacteria that cause American and European foulbrood (Garrido-Bailon *et al.* 2013).

- ***Treatment, control and prevention methods***

No treatment is currently available. Should *A. apis* be detected in a hive, it is necessary to clean and disinfect the hive to limit spread. Alternative control strategies have also been suggested: selection of resistant lines based on hygienic behaviour (Evans and Spivak 2010; Invernizzi *et al.* 2011), replacement of queen bees, improved sanitary management of apiaries, use of natural products with antifungal activity such as essential oils (Kloucek *et al.* 2012), or microorganisms (e.g. *Bacillus subtilis*) capable of inhibiting *A. apis* growth (Sabaté *et al.* 2009).

Sterilisation methods can be used to reduce the spore load of *A. apis* in hives since spores can persist for several years in pollen, wax, or honey (Aronstein and Murray 2010).

It is also important to select a sunny location for the apiary, check ventilation of the hives and the quality of food that is brought to the colonies, and to replace old frames that may be contaminated with spores. In the event of significant infection, transfer of the colony may be considered.

- ***Regulations covering the disease***

None.

3.1.1.2.4 Parasites

3.1.1.2.4.1 Varroa destructor

- ***Parasite***

Varroa destructor

- ***Disease***

Varroasis, varroatosis

- ***History of discovery***

The first species of *Varroa, Varroa jacobsoni Oudemans,* was described in the early 20th century in Indonesia in bees on Java island belonging to the *Apis cerana* species. Another species, *Varroa destructor,* from South-East Asia (Anderson and Trueman 2000), rapidly developed in its new host *Apis mellifera* and is today considered to be the greatest threat to honeybees and one of the major causes of their decline (Le Conte *et al.* 2010).

- ***Change in geographical distribution, current situation***

The adaptation of the mite to *A. mellifera* probably occurred during the 1960s following the gradual increase in *A. mellifera* populations in Asia in order to improve production of Asian bee colonies and from a very limited number of *Varroa* (Solignac *et al.* 2005). Transport of infested swarms, on the one hand, and exchanges between beekeepers on the other, resulted in the spread of this pathogen worldwide. Varroasis now affects all countries, except Australia. In France, the first colonies infested with *Varroa* were identified in 1982 (Colin *et al.* 1983).

- ***Morphological and molecular description***

Varroa destructor is a parasitic mite of the adult bee, larvae and nymphs. It is found in worker bees, drones, and rarely in queen bees. The presence of a brood is necessary for its development. It displays sexual dimorphism that is easily observed at the adult stage. Females are brown in colour and measure 1 to 1.8 mm in length and 1.5 to 2 mm in width. Males are yellowish-white and measure 0.8 mm in diameter. Only the female, by perforating the integument, feeds on haemolymph. *Varroa* mites are flat shaped and have eight very short but very strong legs that enable them to attach to bees.

The genome of this parasite has been partially sequenced (Cornman *et al.* 2010).

- ***Disease, clinical manifestations, infectivity/pathogenicity***

The development cycle of this ectoparasite mostly occurs in the brood and lasts for approximately eight days. Adult females invade the cells of the brood a few hours before capping by worker bees (Beetsma *et al.* 1999). Approximately 60 hours after capping, the female mite lays its first egg which produces a male, the following eggs producing females. The duration of *A. mellifera* worker bee brood capping enables production of about two mature female *Varroa,* and capping of drone broods enables production of three to five mature females (Martin 1998; Rosenkranz *et al.* 2010). The male fertilises the females inside the capped cell. The parasite feeds on the haemolymph of immature stages and adults. *V. destructor* infestation is extremely damaging to honeybee colonies (Bailey and Ball 1991). The major harmful effects are caused by the reproducing females which, by feeding on the haemolymph, weaken the larvae, nymphs and worker bees, with repercussions on the entire colony (Kanbar and Engels 2003). The mite is also a vector of other infectious agents, particularly viruses. It can itself be infected with a specific virus (VDV1), which is however not known to be pathogenic in bees (Ongus *et al.* 2004; Zhang *et al.* 2007), although recombinant viruses of VDV1 and DWV do exist (Moore *et al.* 2011). Many of the clinical signs seen within the colonies appear to be due to transmitted infections rather than infestation itself (Ball 1985; Gliński and Jarosz 1995). Moreover, Benoit *et al.* (2004) have shown that *V. destructor* is able to transmit microorganisms such as *Aspergillus* sp. and *Penicillium* sp. in honeybee colonies. The mite is also considered to be a potential vector for stonebrood disease and/or chalkbrood disease (Benoit *et al.* 2004; Liu 1996).

In particular, the consequences of infestation are:

- a reduction in weight and in volume of haemolymph (Romaniuk and Wawrzyniak 1991; Yang and Cox-Foster 2007);
- underdevelopment of the hypopharyngeal glands (De Jong *et al.* 1982);
- reduced lifespan (Amdam *et al.* 2004a; Ellis and Delaplane 2009; Kovac and Crailsheim 1988);
- early foraging activity by worker bees in their lifecycle (Janmaat and Winston 2000b);
- impaired ontogenesis and expression of spermatozoal glycoproteins (Martí *et al.* 1996).

Furthermore, an immunosuppressive effect has been demonstrated during infestation by this parasite in emerging bees (Yang and Cox-Foster 2007; Yang and Cox-Foster 2005). Effects related

to synergies or combinations with other pathogens, such as other mites, bacteria, viruses and fungi, may also appear to be related to this immunosuppression (Gregory *et al.* 2005; Yang and Cox-Foster 2005).

As a general rule, a high level of winter mortality is observed in severely infested apiaries (Amdam *et al.* 2004a), along with the loss of many colonies (Caron *et al.* 2005; Faucon *et al.* 2002; Morse and Goncalves 1979; Oldroyd 2007; Wenning 2001). In the United States, a significant proportion of colony losses appears to be the result of *V. destructor* in combination with viral attacks (Johnson 2007).

Varroa females spread through the hive and to neighbouring hives by attaching to worker bees and drones.

- ***Co-infections/co-exposure***

Colonies infested with *Varroa* are often co-infected with other infectious agents (other mites, bacteria, viruses, and fungi), particularly specific viruses including DWV, KBV, ABPV or SBV, known to be or suspected of being transmitted to bees by this mite species. In addition, the immunosuppressive action of *Varroa* amplifies development of these viruses and their effects (Gregory *et al.* 2005; Yang and Cox-Foster 2005). As a result, higher levels of virus are found in colonies severely infested with *Varroa*.

- ***Treatment, control and prevention methods***

Several control methods are used but it is currently impossible to completely eradicate this ectoparasite. The aim is to decrease *V. destructor* infestation to a "tolerable" level for the colony. There are three types of acaricides used to control *Varroa* (see section 3.1.2.5): synthetic organic substances (*tau*-fluvalinate, amitraz or coumaphos), natural products containing thymol, and organic acids (formic acid, oxalic acid). Use of coumaphos is not authorised for beekeeping in France. Another approach to control *Varroa* aims at selecting colonies that are resistant to this parasite (Rinderer *et al.* 2014).

- ***Regulations***

Varroasis is a category 2 health hazard in France (Ministerial Order of 29 July 2013) and is included on the OIE list and list B of diseases that could be subject to national programmes in European regulations (Council Directive 92/65/EEC of 13 July 1992, Annex B).

3.1.1.2.4.2 Acarapis woodi

- ***Parasite***

Acarapis woodi

- ***Disease***

Acarapisosis, acariosis, acarine disease

- ***History of discovery***

Acariosis of the trachea was associated with "Isle of Wight disease" (Rennie 1921), a bee disease causing extremely high losses that appeared in 1904 on the Isle of Wight (United Kingdom). In 1906, approximately 90% of the Island's bee colonies were believed to have been affected and in 1918, colony losses throughout the British Isles were estimated to be 90% (Borchert 1970; Sammataro *et al.* 2000). Bailey (1961) reported that adverse harvesting and weather conditions together with the disastrous beekeeping practices associated with the unstable, unsafe situation during World War I promoted the development of this acariosis. According to Bailey, however, the disease was not only due to the tracheal mite. From an analysis of bee health data obtained on the Isle of Wight, many other diseases were believed to have contributed to this situation with occasionally similar clinical signs. The clinical signs described for this disease are in fact also very similar to those described for chronic paralysis, a disease of viral origin (Ball and Bailey 1997; Ribière *et al.* 2008). Isle of Wight disease therefore appears to be a fatal, infectious disease related to several causes, including *A. woodi* (Borchert 1970; Wilson *et al.* 1997).

- ***Change in geographical distribution, current situation***

A. woodi has a worldwide geographic distribution, except for Oceania (Wilson *et al.* 1997). Like varroasis, acariosis is harmful to beekeeping.

Since it was first identified in the United States in 1984, *A. woodi* has been responsible for the loss of millions of colonies at an estimated cost of several million dollars (Delfinado-Baker 1984). In 1989, bee sampling from 55 beekeepers revealed firstly *A. woodi* to be present in 50% of samples and secondly, a significant relationship between the impact of the mite and winter mortality (Frazier *et al.* 1994). It currently has a hypothetical presence in apiaries in France and acaricide treatments used to control *Varroa* have decreased its prevalence in bee colonies.

- ***Morphological and molecular description***

A. woodi is a parasitic mite that is specific to honeybees and lives and reproduces in the respiratory tract, mainly in the first pair of thoracic tracheae. It can parasitise the three castes of adult bees (queen bees, workers and drones). The mite is brown in colour and measures about 150 µm and is therefore not visible to the naked eye. It has mouthparts with thin pointed mandibles that it uses to perforate the tracheal wall in order to feed on haemolymph.

- ***Disease, clinical manifestations, infectivity/pathogenicity***

Acariosis is an adult bee disease. Clinically, the disease manifests as tracheal necrosis that takes on a black appearance, characteristic of infestation. The mite invades part of the respiratory system (particularly the first pair of tracheae). It perforates the *A. mellifera* tracheal wall feeding on its haemolymph and sometimes severely compromising the host's respiration. Whereas all of the development stages (development cycle lasting approximately fourteen days) of *A. woodi* take place within the respiratory tract, the reproducing females leave the trachea to infest another adult bee (Morgenthaler 1933). As *A. woodi* only survives a few hours outside of the trachea, direct contact transmission between adult bees is therefore necessary (Pettis *et al.* 2007), and any prolonged confinement of colony individuals, particularly under adverse weather conditions, is conducive to transmission of the pathogen.

The clinical signs in adult bees depend on the number of parasites present in the tracheae and are usually attributed to mechanical injury and physiological disturbances from obstruction of the first pair of tracheae.

The symptoms of colony infestation only appear once the number of parasites exceeds a critical threshold, and once the parasites are able to obstruct the trachea, generally at the beginning of spring: These symptoms result in:

- ✓ paralysed and/or flightless bees (Faucon 1992; McMullan and Brown 2006);
- ✓ shortened lifespan (Bailey and Ball 1991; De Guzman *et al.* 2005; Gary and Page 1989);
- ✓ adult mortality in the spring higher than natural mortality (Bailey and Ball 1991; Otis and Scott-Dupree 1992; Root 1990);
- ✓ high winter mortality, particularly in temperate regions (Bailey 1958; De Guzman *et al.* 2005; Phibbs 1996);
- ✓ reduction of brood and honey production (Eischen *et al.* 1989; Eischen 1987; McMullan and Brown 2006).

Apart from its plundering and damaging activities, *A. woodi* is believed to be able to transmit viruses to the honeybee (particularly acute bee paralysis virus: ABPV) (Shimanuki *et al.* 1994).

Some symptoms seen in colony collapse disorder (CCD) in the United States appear very similar to those of "Isle of Wight Disease" (van Engelsdorp *et al.* 2007), but CCD has not been found to be associated with this parasitosis.

- ***Co-infection/co-exposure to other stress factors***

During biting, the mite may inoculate other infectious agents, particularly viruses.

- ***Detection***

Acariosis can only be detected in the laboratory. Detection of *A. woodi* is performed by microscopic examination of the trachea after dissection. An immunological ELISA test has been developed (Grant *et al.* 1993). PCR approaches were designed more recently (Cepero *et al.* 2015; Kojima *et al.* 2011).

- ***Treatment, control and prevention methods***

Natural products containing formic acid, menthol, or thymol, or synthetic products (amitraz in fumigation) can be used to combat acariosis (Underwood and Currie 2004). Acaricide treatments to control *Varroa destructor* have also led to lower prevalence of *A. woodi* and thus this acariosis.

- ***Regulations***

Acariosis is included on the OIE list (OIE 2015), and on list B of diseases that could be subject to national programmes in European regulations (Council Directive 92/65/EEC of 13 July 1992).

3.1.1.2.4.3 Crithidia mellificae

Crithidia mellificae is a flagellate protozoon of the *Trypanosoma* genus parasitising the honeybee that was described for the first time in 1967 in Australia (Langridge and McGhee 1967). It shows high distribution as it has since been detected in many countries worldwide including in the United States, Europe and Asia. Forty years after its discovery and with the availability of new molecular detection techniques, studies have reported a strong correlation between the presence of *Crithidia mellificae* and colony loss in the United States and Belgium (Cornman *et al.* 2012b; Ravoet *et al.* 2013). For example, parasitic loads of *C. mellificae* were 6.15 times higher in CCD colonies than in unaffected ones in the study carried out in the USA. However, no relationship between the occurrence of CCD and the prevalence of the parasite has been observed (Cornman *et al.* 2012b). In Belgium, a slightly higher prevalence of the parasite was observed in collapsed colonies during the winter (81.3%) versus surviving colonies (71.3%) (Ravoet *et al.* 2013). In addition, the bees were often co-infected with *C. mellificae* and *Nosema ceranae* (Ravoet *et al.* 2013; Runckel *et al.* 2011). The pathogenicity of *C. mellificae* is not yet known but a laboratory study has identified the immune response in bees to this parasite (Schwarz and Evans 2013). In France, the parasite is not currently monitored and the disease is not regulated.

3.1.1.2.5 Predators: Asian hornet

- ***Biological agent***

Vespa velutina Lepeletier 1836/Asian hornet, Yellow-legged hornet/no abbreviation

- ***History of discovery***

Introduced species in France, reported for the first time in 2004 in the Lot-et-Garonne *département* (Haxaire *et al.* 2006).

- ***Change in geographical distribution, current situation***

Its original distribution range extends from Afghanistan to eastern China, Indochina and Indonesia (Villemant *et al.* 2011). Since it was first observed in 2004 in southern France, it has gradually colonised most of mainland France and northern Spain. Its current distribution is monitored through nationwide notification campaigns, coordinated by the French National Museum of Natural History (MNHN) (see website of the INPN[15] for the current situation). About twenty other species of exotic hornets may be imported into Europe accidentally and become established (review in Beggs *et al.* (2011)). Like all potentially invasive species, effective local establishment of a given taxon is unpredictable. Establishment depends on its characteristics and on the carrying capacity of the host ecosystem.

- ***Morphological and molecular description***

V. velutina is smaller than the European hornet (*Vespa crabro*) and has a different appearance (mainly black, orange and yellow borders on the abdomen, orange face, legs yellow at the tips) (Rome and Villemant 2011).

[15] INPN - French National Inventory of Natural Heritage

- ***Observed effects***

The Asian hornet is a colony predator and attacks foragers near the flight board, then the brood if hive configuration permits.

The effect on bee colonies is direct through predation, and indirect as a result of the threat to foragers of hornets flying stationary near the hive or near resources (Arca *et al.* 2014; Monceau *et al.* 2013). The threat can thus have a disproportionate effect on foraging. Experimental tests in *Apis cerana* have shown that the bees reduce foraging by 55% to 79% on resources where the predators are present (Tan *et al.* 2013).

Vespa velutina colonies do not survive the winter. Founding females leave the colony in late summer, are fertilised, and survive alone through the winter in crevices. They raise a small number of workers that then build a nest of several thousand individuals. The nest is often difficult to detect during the season, since it is masked by tree foliage.

- ***Detection***

The diagnostic criteria for workers and nests are published by the National Museum of Natural History[16].

There is currently no mention of *Vespa velutina* in OIE documentation but since this agent is not likely to be transmitted by living animals or products of animal origin, there is no reason to implement a health certification scheme, like for *Tropilaelaps* for example.

Progression of the species in France is monitored by a network of voluntary observers.

- ***Control and prevention measures***

Because of the difficulty in detecting founding females, control methods can only target predators near the entrance to the hive but selective trapping is still ineffective. Destruction of a nest during the season, when it is accessible, temporarily resolves the attacks until subsequent establishment (Beggs *et al.* 2011).

- ***Regulations covering the disease***

The Asian hornet is classified as a category 2 health hazard in the Ministerial Order of 29 July 2013. Its introduction is prohibited across the country (it is absent from Overseas *Départements* and Territories) (Order of 22 January 2013 prohibiting introduction of *Vespa velutina* yellow-legged hornet specimens into the country).

3.1.1.3 Asymptomatic carriage

When a colony shows specific clinical signs, aetiological diagnosis is based on identifying and quantifying the causal agent in the colony and in its environment (apiary, other sources of contamination). Diagnosis will also aim to distinguish between saprophyte contaminants and highly virulent agents (for example between *Paenibacillus alvei*, saprophyte, and *Paenibacillus larvae*, the causative agent of American foulbrood). Today, this is often done through molecular detection and quantification, using molecular biology techniques. Interpreting a laboratory analysis result therefore requires a good understanding of the “parasitic and microbiological setting” in which the colony lives.

In bees, most infectious agents have little or no virulence. They persist at low levels in some apiaries without causing disorders but they may affect beekeeping performance (Evans and Schwarz 2011). If necessary, it may be useful to determine the health status of a colony in order to select the best beekeeping techniques so as to reduce carriage. In this case, it is beneficial to compare the combinations of infectious agents, which sometimes have potentiating effects, with indicators of colony strength that reveal subclinical impact.

Although a low-grade infectious state may be well tolerated by the colony, a co-factor, whether toxic or meteorological, etc., can break the balance and result in occurrence of symptoms and mortality. Consequently, it is important to be aware of the normal situation in order to assess the risk related to this type of disruption.

16 http://inpn.mnhn.fr/docs/Vespa_velutina/Fiches_Identification_Vespa_velutina_MNHN.pdf

Currently available studies on the prevalence of infectious agents in bees reveal the diversity of settings in which efforts are made to determine asymptomatic carriage. The objectives of each team are also highly diversified. This section gives an overview of these prevalence studies to provide information on the following:

- the infectious agents detected in asymptomatic colonies, their possible combinations, and the status of the apiary in which they were identified;
- infectious load found at given time points;
- frequency of detection of infectious agents. Because of the colony life cycle, the season must be taken into account;
- geographic variations;
- the possible predictive nature for a subsequent risk in the event of presence of infectious agents at time point T, and if so, the probable mechanism.

Table 2 summarises the information from studies (shown in *Table 3*) on the infectious agents in asymptomatic colonies in Europe. Some of the studies address several of the aspects mentioned above.

van Engelsdorp *et al.* reviewed the criteria for the methodological quality for epidemiological studies in bee health (van Engelsdorp *et al.* 2013a). Caution is required when considering the term prevalence since it is used differently by the authors for asymptomatic infection (prevalence of infection) and occurrence of disorders (prevalence of cases of disease, with a specific definition of the case). In *Table 3*, the prevalence level of an infectious agent (IA) observed in the colony population or apiaries without symptoms (P_{asy}_IA) sometimes, depending on the study, makes it possible to extrapolate to the whole study area. The methodological limitations of each study are presented below.

The data provided by these studies help to make recommendations concerning sampling, with four objectives:

- aetiological diagnosis of disorders,
- screening of epizootic agents and qualification of disease-free zones,
- management of the infectious state,
- standardisation of evaluation tests for plant protection products before and after approval.

3.1.1.3.1 Infectious and parasitic agents found in asymptomatic colonies. Status of the apiary. Associated agents

Table 2 (2a and 2b) presents most of the infectious and parasitic agents known to be in circulation in Europe and detected in the studies listed in *Table 3*. Only recent European studies with sampling after 2002 were reviewed.

Table 2: Infectious and parasitic agents circulating in Europe

Table 2a: Bacterial and parasitic agents circulating in Europe

	Varroa	*Paenibacillus larvae*	*Melissococcus plutonius*	*Nosema apis*	*Nosema ceranae*	*Crithidia mellificae*
Frequency in apiaries (/n) (reference)	35-50% (/24) (B)	99% (/18) (A) 23-40% (/24) (B) 70% (/27) (J)	76% (/18) (A) 18-29% (/24) (B) 14% (/7) (H)	29% (/18) (A) 13 - 80% (/24) (B) 33% (/9) (K) 8-15% (/456) (M)	99% (/18) (A) *Nosema* spp. 13-80% (/24) (B) *N. ceranae* 88% (/9) (K) 40% (/456) (M)	
Frequency in colonies (/n) (reference)	15-24% (/120) (B) 51.16 - 62.12% (/1931) (P)	66% (/90) (A) 7-11% (/120) (B) 82% (/73) (J) not detected (/1073) (P)	26% (/90) (A) 3-8% (/120) (B) 4% (/7) (H) not detected (/1073) (P)	5% (/90) (A) 3-60% (/120) (B) 5-15% (/220) higher in spring than in autumn (G) 8% *N. apis* (/61) (K) 5.4-18.9% (/2278) (P) 8-15% (/456) (M) 10.2% PCR (/363) (Q)	71% (/90) (A) *Nosema* spp. 3-60% (/120) (B) 65% *N. ceranae* (/61) (K) 3.02 — 14.46% (/2278) (P) 50% (/456) (M) 92.6% PCR (/363) (Q)	70.5% (/363) (Q)
Abundance (measurement unit/sample) (reference)	2-20 (individuals/day*colony) (C) 1 to 112 (individuals/100 bees in a colony) (P) 0-500 individuals/week (Q)	10^3— 10^8 (gene copies/bee) (A) 6.10^4 spores/100 bees (J)	1 — 10^6 (gene copies/bee) (A)	10^{-1}—1 (gene copies/bee) (A) *Nosema* spp. see opposite (B) 24 10^3 - 16 10^6 spores/bee (K) 10^5-10^9 spores/bee (Q)	10^{-2} — 10^5 (gene copies/bee) (A) 2. 10^4 — 2. 10^7 (spores/bee) (B) significant differences depending on season (RT-PCR relative abundance) (C) 24 10^3 - 16 10^6 spores/bee (K) 10^5-10^9 spores/bee (Q)	
Co-occurrence	DWV, ABPV, SBV (P)	*M. plutonius* (B)	*P. larvae* (B)	*N. ceranae* (K) (P) 8.8% co-infection with *N. ceranae* (/363) (Q) BQCV (L)	*N. apis* (K) (P) 8.8% co-infection with *N. apis* (/363) (Q) ALPV, VdMLV (Q)	

A: (Mouret *et al.* 2013); B: (Chauzat *et al.* 2010); C: (Dainat *et al.* 2012b); D: (Antunez *et al.* 2012); E: (Baker and Schroeder 2008); F: (Tentcheva *et al.* 2004); G: (Gisder *et al.* 2010); H: (Forsgren *et al.* 2005); I: (Gauthier *et al.* 2007) (same samples as F, but viral loads); J: (Lindström and Fries 2005); K: (Chauzat *et al.* 2007), detail *Nosema apis* and *N. ceranae*, Chauzat *et al.* (2010); L: (Berényi *et al.* 2006); M: (Martín-Hernández *et al.* 2012); O: Beenet 2011-2013; P: (Hedtke *et al.* 2011); Q: (Ravoet *et al.* 2013); R: (Genersch *et al.* 2010); S: (Belloy *et al.* 2007)

Table 2b: Viral agents circulating in Europe

	DWV	ABPV	KBV	IAPV	CBPV	BQCV	SBV	VdV1
Frequency in apiaries (/n) (reference)	96% (/A) 100% (/23) (E) 97% (/36) in adults, 94% in pupae (F)	14% (/18) (A) 4.3% (/23) (E) 58% (/36) in adults, 23% in pupae (F)	75% (/18) (A) 17% (/36) in adults, 6% pupae (F)	65% (/18) (A)	90% (/18) (A) 28%(/36) in adults, 0% in pupae (F)	83% (/18) (A) 86% (/36) in adults, 23% in pupae (F)	85% (/18) (A) 86% (/36) in adults, 80% in pupae (F)	94% (/18) (A)
Frequency in colonies (/n) (reference)	84% (/90) (A) 56 — 100% (/456) (C) 6-19% (D) 97% (/69) (E) 97% (/360) except Ouessant (F) 26.29% (/445) (P)	4% (/90) (A) 1% (/456) (D) 29% (/69) (E) 5.39% (/445) (P)	42% (/90) (A) 0% (/69) (E)	24% (/90) (A)	54% (/90) (A) 0% (/69) €	52% (/90) (A) 10% (C) 1.4% (/69) (E) 2 - 75% depending on season (F)	56% (/90) (A) 1% (C) 1.4% (/69) (E) 67% (/360) (F) 0.9% (/445) (P)	56% (/90) (A)
Abundance (measurement unit/sample) (reference)	1-10^8 (gene copies/bee (A) 10^3-10^6 (gene copies/bee) (C) 10^5 — 10^{10} copies/bee)	10^2 — 10^6 (gene copies/bee) (A) 10^5-10^8 (gene copies/bee) (I)	10^4— 10^8 (gene copies/bee) (A) 10^5-10^{10} (gene copies/bee) (I)	10^2 — 10^8 (gene copies/bee) (A) 13-25% (C)	10^{-3} — 10^8 (gene copies/bee) (A) relative abundance depending on season (B); 10^5-10^{10} gene copies/bee (I)	1 — 10^8 (gene copies/bee) (A) 10^5-10^8 gene copies/bee (I)	10^{-1}—10^7 (gene copies/bee) (A) 10^5-10^{10} gene copies/bee (I)	10^2 — 10^8 (gene copies/bee) (A)
Co-infection with	BQCV, IAPV (D) other viruses (L) BQCV KBV SBV (M) ABPV, SBV (P)	other viruses (L) DWV, SBV (P)	other viruses (L)		other viruses (L)	DWV, IAPV (C) LSV (Q)	DWV (P)	

Table 3: Prevalence studies on infectious and parasitic agents in Europe

* same sample set

** same sample set or subset of the same scheme

*** same sample set

WM = winter mortality

SM = season mortality

Asy = presence of infection without symptoms

Inf = presence of infection with or without symptoms

Asyq = quantification of infectious load without symptoms

AF = American foulbrood; EF = European foulbrood; Na = *Nosema apis*; Nc = *Nosema ceranae*

Biogeographic regions: Atl = Atlantic; C = Continental; Med = Mediterranean; Alp = Alpine; Bor = Boreal

Se Sp PPV NPV: sensitivity, specificity, Positive Predictive Value, Negative Predictive Value

Study	Region	Case	Agents	Protocol design	Type	Duration	Associated variables	Statistics	Uncertainty	Number of apiaries	Number of colonies	Samples	Colony strength variables
A	France West (Atl)	WM, Asyq	AF, EF, Na, Nc, 8 Viruses	Observational	Cohort	04/2009-03/2010	WM = f (Asy; Asyq)	Prevalence Asy; parametric univariate	No	18	90	frame bees	Liebefeld rating
B *	France (Atl, C, Alp, Med)	WM, SM, AF, EF, Acariosis, Chalkbrood	AF, EF, *Nosema* sp, *Varroa*, *Acarapis*	Observational	Cohort	03/2002-10/2005	WM = f (inf prev. year) No of bees = f (inf, practices)	Odds Ratio; mixed linear model	No	24	120	frame bees + brood	size adult and brood populations
C	Switzerland, Bern (C)	WM, Asyq, Quantification immunity	*Varroa*, 8 Viruses, Na, Nc	Experimental	Cohort	08/2007-02/2008	WM= f (*Varroa*, virus, *Nosema*, season, Immuq)	bilateral t-tests, Kruskal-Wallis, survival analysis, mixed linear models	Not applicable	1	29	frame bees	Liebefeld
D ***	Spain (Med, Alp, Atl)	Asy	6 viruses	Observational	Cross-sectional	03/2006-11/2007	Prevalence virus = f (season, other virus)	Chi^2	No	456	456	frame bees	No
E	Southwest England (Atl)	Asy	6 viruses	Observational	Cross-sectional	09-10/2006	History of *Varroa*	No	No	23	69	frame bees	No

F *	France (Atl, C, Alp, Med)	Asy, Infection by *Varroa*	6 Viruses, *Varroa*	Observational	Cohort	03/2002-10/2002	Prevalence virus/*Varroa* = f (Prevalence virus adults prev. season)	Chi^2	No	36	360	adults, pupae, (3 seasons) Varroa (autumn)	No
G **	Northeast Germany (C)	WM, Asy	Na, Nc	Observational	Cohort	03/2005-04/2009	Prevalence *Nosema* = f (season); WM =f (*Nosema*)	-	No	22	220	adults bottom of hive (spring), frame adults	No
H	Switzerland (C, Alp)	clinical EF, AsyEF	EF	Observational	Comparison between colonies with or without symptoms, same location	NA, in season	infEF	Chi^2	No	12	92	diseased and healthy larvae from same brood	No
I *	France (Atl, C, Alp, Med)	Asyq	Viruses	Observational	Seasonal variability in viral loads	03/2002-10/2002	season, stage	Mann-Whitney, ANOVA on ranks	? range	36	360	adults, pupae (3 seasons)	No
J	Central Sweden (Bor)	clinical AF, AsyAF	AF	Observational	Comparison between colonies with or without symptoms, same location	NA, in season	InfAF; Se Sp PPV NPV; brood vs. honey super	Wilcoxon test	No	59	459	brood adults, honey super	No
K *	France (Atl, C, Alp, Med)	Asy	*Na, Nc*	Observational	Subtyping and co-infections	03/2002-10/2005	(proportion Na/Nc)/pos gold standard N..sp	Aggregation/exclusion co-infection	No	9	61	frame adults	No
L	Austria (C, Alp)	Weakening, mortality, chronic bee paralysis, Asy Asyq	N. spp., *Malpighamoeba, Acarapis, Varroa*, Virus, Virusq	Observational	Comparison between colonies with or without symptoms, same location	01/2003-01/2004	Geography, IA combinations	No	No	NA	90 diseased, 15 healthy from same apiaries, 26 diseased from neighbouring countries	dead worker bees	No
M ***	Spain (Med, Alp, Atl)	InfNosema	*N. apis, N. ceranae*	Observational	Cohort	03/2006-11/2007	bioclimatic region, season, year	Estimated prevalence level Chi^2	Yes for prevalence rate	456 cross-sectional study including 30 longitudinal study	456 cross-sectional study including 30 longitudinal study	frame bees + foragers	No

O	Italy (C, Alp, Med)	InfNc, InfDWVq, InfABPVq, Inf CBPVq, Infvarq	Nc, *Varroa*, DWV, ABPV, BQCV	Observational	Cohort	01/2011-12/2013	region (province)	No	No	23			
P **	Northeast Germany (C)	WM, SM, clinical Chalkbrood	*Ascosphaera apis*, *Varroa*, Na, Nc, Viruses	Observational	Cohort	10/2004-05/2010	season, year, IVarroa, Inc, INa	Prevalence at the colony level (IA), logistic model CB=f (Inf_var_season, inf_nosema_season_year_owner)	Yes	22	120	frame adults, larvae	Not used
Q	Belgium (Atl)	WM, Asy	*Varroa*, Nc, Na, *Crithidia*, Viruses	Observational	Cross-sectional	07/2011	No	WM= mixed linear model prevalence Asy; Chi^2 association between IAs	Yes	170	363	bees on flight board	No
R **	Germany (Atl, C, Alp)	WM, Asy, ratio winter weakening	*Varroa*, N. spp., 5 viruses, AF	Observational	Cohort	10/2004-05/2008	size population, exposure intensive crops, pesticide residues	Kruskall-Wallis		112-123	596-1924 (depending on IAs)	frame adults, bee bread	Yes No. of frames of bees covered
S	Switzerland (C, Alp)	AsyEF	EF	Observational	Case-control	NA	presence of clinical EF in region, distance to a colony with EF	Chi^2	Not applicable	13	80	frame adults + adults from flight board	No

The range of infectious agents targeted by the studies is highly variable depending on the objective. Few of them are strictly speaking observational studies of current prevalence or year-on-year follow-up (cross-sectional studies or cohort monitoring) (Antunez *et al.* 2012; Gisder *et al.* 2010; Hedtke *et al.* 2011; Martín-Hernández *et al.* 2012; Mouret *et al.* 2013; Ravoet *et al.* 2013)[17]. An experimental study (Dainat *et al.*, 2012) and individual observations in a zone (Berényi *et al.* 2006; Forsgren *et al.* 2005; Lindström and Fries 2005) have been included because they provide data on carriage in asymptomatic colonies in the vicinity of diseased colonies.

The following were described in asymptomatic colonies:

- *Varroa destructor*: easily identifiable mite with no room for confusion, but with detection methods of variable sensitivity;
- the agents causing European foulbrood and American foulbrood: often detected when molecular methods are used but difficult to detect with the OIE reference microbiological methods. These methods aim to identify spores or bacteria in pure cultures. They have poor sensitivity in the absence of clinical signs such as threadlike or scaly larvae (Forsgren *et al.* 2005; Lindström and Fries 2005). For example, in the German cohort studies (Genersch *et al.* 2010; Hedtke *et al.* 2011), neither of the two agents were detected using methods from the OIE Standards Manual for five years;
- *Nosema ceranae* and *Nosema apis*: the spore counting method provides a quantitative indication but cannot distinguish between the two species. There is a specific PCR method for each species;
- DWV, ABPV, IAPV, KBV, SBV, CBPV, BQCV, VdV1 and others discovered recently: some of these viruses are genetically similar, for example those within the AKI complex (ABPV + IAPV + KBV) (de Miranda *et al.* 2010). The PCR methods used must be able to distinguish between species for prevalence data to be reliable (Gauthier *et al.* 2007). Certain PCR methods yield quantitative results for viral loads, while others only enable measurement of virus presence or absence (see section 3.1.1.2.2). Importantly, most of these viruses have high evolutionary potential and emerging genotypes (Maori *et al.* 2007a) may no longer be detected if they have evolved. One often finds several viruses simultaneously in a single apiary or colony, or even in a single individual.

Ravoet *et al.* (2013) found numerous co-infections among all classes of infectious agents but found significant combinations only between *Nosema ceranae* and *Varroa destructor*, and *Crithidia mellificae*. *C. mellificae* is a Trypanosoma known for some time but that has been rarely incriminated in disease outbreaks until now.

3.1.1.3.2 Frequency in apiaries and colonies. Seasons and geographic considerations

The epidemiological unit for prevalence studies may be the apiary (group) or the colony.

In several studies, the dependency between colonies and apiaries is not indicated, in other words the results published for colonies are not subject to the infectious status of the apiary, or conversely, there is no indication of the rate of infected colonies per apiary (for example Genersch *et al.* (2010)). We therefore do not have aggregated data on positive colonies, resulting in a high risk of statistical bias for prevalence at the colony level.

Of the 17 publications analysed, only the Spanish sampling system, used for prevalence studies on viruses and *Nosema spp.,* presents a randomisation of epidemiological units, needed to infer the rate of actual asymptomatic prevalence (with a confidence interval) (Antunez *et al.* 2012; Martín-Hernández *et al.* 2012). In this second study, a correlation was demonstrated between prevalences

[17] Italian National Rural Network http://www.reterurale.it/flex/cm/pages/ServeBLOB.php/L/IT/IDPagina/1092

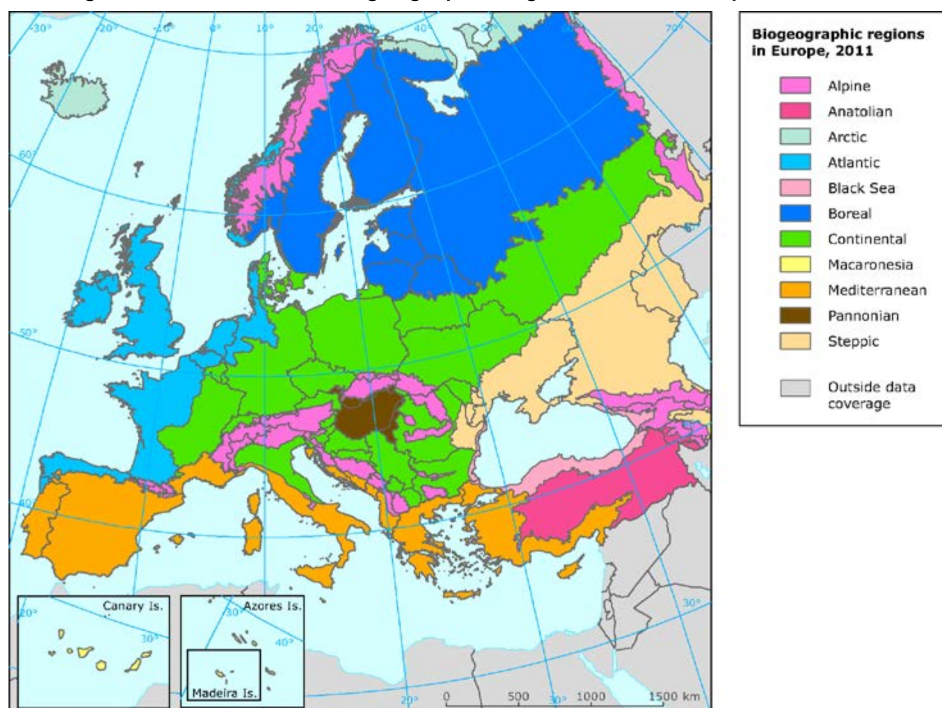
of *Nosema ceranae*, *Nosema apis* and the “bioclimatic unit”, “season” and “year” variables. The results appear to indicate that *Nosema apis* is more frequently found in colder climates with a contrasted winter. Its prevalence is seasonal with a peak in the spring, unlike *Nosema ceranae* which is present year-round. This is consistent with data on the biology of this infectious agent and with year-on-year follow-up in North-eastern Germany on a cohort of non-randomised colonies (Gisder *et al.* 2010). However, in Spain, a single colony per apiary was analysed, which leads to a risk of error by default, i.e. an underestimation of the rate of detection in affected apiaries. This could also partly explain the low prevalence of DWV in Spain compared to other European countries, where this virus is practically ubiquitous. Information on the concomitant presence of *Varroa* is not available, although it is known that *Varroa* not only facilitates the transmission of DWV but also serves as a host and amplifies its population dynamics. The study in North-eastern Germany shows that where the prevalence of DWV is lower than 30%, there is good compliance with acaricide treatment in the autumn and a relatively low *Varroa* infestation level (Hedtke *et al.* 2011).

The "geographic region" variable reflects both the bioclimatic characteristics of the zone and the socio-economic structure, particularly the density of apiaries and agricultural practices. As such, it is expected that prevalences would vary depending on the bioclimatic context and resources.

The year-on-year follow-up system in Germany and Italy involves stratified cohorts that are representative of the geographic diversity and beekeeping demographics of each country (Genersch *et al.* 2010)[18].

In *Table 3*, the administrative region of each study is matched with the defined biogeographic region for the European continent (*Figure 2*) (source: http://www.eea.europa.eu/data-and-maps/data/biogeographical-regions-europe-1 consulted on 20/02/2015), enabling matching of the corresponding bioclimatic context for each study. The Spanish study shows that more precise biogeographic differentiation may be needed, with subclasses or variables for altitude and seasonal contrasts (Martín-Hernández *et al.* 2012).

Figure 2: Cross-border biogeographic regions for the European continent



[18] Italian National Rural Network http://www.reterurale.it/flex/cm/pages/ServeBLOB.php/L/IT/IDPagina/1092

In conclusion, these studies only partially elucidate the "normal infectious and parasitic setting" of bee colonies in the various European countries. The data for the same infectious agent are not comparable because of the widely differing protocols and missing associated variables. The Epilobee project, carried out in 17 countries in the European Union, has nonetheless prompted standardisation of methods and associated variables. Its main objective is to determine the prevalence of mortality taking into account the seasonal factor and the history of infection the previous year. It does not take account of exposure to pesticides but its randomised approach ensures minimal representativeness of exposure to non-biological factors. For this appraisal, it is important to remain cautious when interpreting the results of geographic variables in terms of possible confounding effects between the bioclimatic context and agricultural practices.

It is important to mention the need to validate identification, detection and quantification tools for the biological agents that may affect colony health in accordance with current standards (ISO, AFNOR), as well as the need to harmonise methods based on references in force (example: OIE). Validation and harmonisation of diagnostic methods enable surveillance using suitable tools whose sensitivity, specificity, reproducibility and detection and quantification limits are known, and that are used in a harmonised manner between the reference laboratories in order to carry out studies with comparable results.

3.1.1.3.3 Quantitative aspects of infectious or parasitic load in the absence of clinical signs

More recent studies have aimed to go beyond detection of infectious agents in order to quantify the infectious load and changes over time.

Various analytical techniques can be used to obtain quantitative data on the infectious and parasitic loads. It is nonetheless difficult to compare quantitative values from one study to another because of the many differences in detection thresholds or the types of samples used:

- direct counting: for *Varroa*, Chauzat *et al.* (2010), Hedtke *et al.* (2011) and Genersch *et al.* (2010) counted phoretic mites, i.e. those attached to the bodies of adult bees, possibly bringing the number to 100. Dainat *et al.* (2012b) and Ravoet *et al.* (2013) counted *Varroa* falling to the hive floorboard with or without acaricide treatment on a daily or weekly basis;
- spore counting: for spore-forming organisms, i.e. *P. larvae*, *N. ceranae* and *N. apis*, spore counting is carried out based on established OIE methods. For *Nosema*, the method cannot distinguish between *N. ceranae* and *N. apis* and results are therefore indicated as *Nosema* spp. (Rivière *et al.* 2013). Note that the study by Berényi *et al.* (2006) mentions *N. apis* in the results obtained with this method, although no subsequent subtyping took place;
- quantitative PCR: for all organisms, quantitative PCR, qRT-PCR or qPCR is possible if a specific genetic marker is available. It was used in studies by Mouret *et al.* (2013), Dainat *et al.* (2012b), Gauthier *et al.* (2007), Berényi *et al.* (2006) for viruses (no subtyping of *Nosema* by molecular methods) and by Beenet (2011-2013) for viruses, *Nosema, P. larvae* and *M. plutonius.* The detection threshold is usually lower than for direct PCR and for counting of spores for *Nosema*. However, qPCR is sometimes only carried out on samples that were positive on spore counting (Gisder *et al.* 2010). In this case, method sensitivity (number of carrier samples detected as positive) is limited by that of the spore counting, since samples with low levels of infection are not analysed by the most sensitive method.

  In the absence of harmonisation of techniques and of detection and quantification methods, qPCR data are not comparable from one study to another, even when an internal calibration of the method has been performed. This enables comparisons only between results from the same protocol, since many internal laboratory parameters affect the sensitivity of detection. This aspect can be corrected by implementing harmonised methods through inter-laboratory testing (ILT), both for validation and for proficiency. This

harmonisation process is currently in its infancy, with the nomination in 2011 of the European Union Reference Laboratory for bee health, whose main task is to develop, validate and harmonise diagnostic tests used in national reference laboratories (NRLs), in particular. However, within a single study, these tools help to observe seasonal variations in infectious loads in particular, or the predominance of one agent over another in the event of co-infection. In the case of DWV, Dainat *et al.* (2012b) showed growing seasonal dynamics from spring to autumn, following a peak in the *Varroa* population in summer. This is because the transmission of this virus is facilitated by *Varroa*, vector and competent host for DWV (de Miranda and Genersch 2010; Sumpter and Martin 2004). Through modelling, Sumpter and Martin studied comparative seasonal dynamics between DWV, which multiplies in the *Varroa* vector, and ABPV, which is transmitted by *Varroa* but does not multiply in this host. The quantitative difference found by Gauthier *et al.* (2007) for these two viruses and their seasonal variations are consistent with this dynamics model.

For viruses, the reported viral loads, which range from 10 to 10^{12} copies per bee, are to be taken into account as a size measurement and a relative measurement, within the same study. DWV is reported to be the most abundant in all the studies for the reasons mentioned above. Of note, Francis *et al.* (2013a) report similar viral concentration levels for DWV and the viruses in the AKI complex in queen bees.

In Italy, monitoring has been implemented in a representative manner for the whole country. This bee colony monitoring system was initiated in 2009 with the Apenet project and was carried forward in 2011, by Beenet[19]. It aims to study interactions between bees and the environment and to follow-up mortality and colony losses in Italy. In 2012, it assessed 303 apiaries (97 in 2011) located across the country and covering about 3000 colonies. Each monitoring unit includes five apiaries of 10 hives each, followed-up by a referring beekeeping expert. Through the year, four visits are organised to the apiary: (1) in late winter, (2) in spring-summer, (3) in late summer-early autumn, and (4) before overwintering. Each visit includes examination of the following parameters: colony health, food, number of bees and brood, age of the queen bee, climate, and local context. During the first and third visits, samples of bee bread and live bees are taken and are used to detect pesticides, infectious agents (*Nosema*, viruses, and at the third visit, *Varroa*), and nutritional analyses (crude proteins in the bee bread). These data are then collected and analysed. This follow-up provides consistent results with data on *Varroa* and DWV, in the spring and autumn.

A good example demonstrating the difficulties in comparing results of counting between studies is the causative agent of European foulbrood, *M. plutonius* (see section 3.1.1.2.1.2). This agent is very difficult to detect through conventional bacteriological methods, even in diseased colonies, since it is abundant essentially in sick larvae and only transiently (Forsgren *et al.* 2010). Asymptomatic carriage at the colony level has been demonstrated by Forsgren *et al.* (2005) using a non-quantitative semi-nested PCR method[20] on larvae samples. Using this method, Forsgren found three positive asymptomatic colonies out of 72 (4%), with a detection threshold estimated to be 100 bacteria/mL in larvae and honey. Importantly, the colonies of interest all belonged to an apiary in which there had been clinical cases of American foulbrood. In asymptomatic colonies, without clinical cases, Mouret *et al.* (2013) detected a concentration range of 0 to 10^6 copies per bee by qPCR, in 26% of 90 colonies, distributed among 76% of the 18 asymptomatic apiaries studied. Although this involved two different biogeographic regions (Sweden versus France), it is probable that the higher prevalence found here is due to greater sensitivity of the qPCR method, used on frame bees. These individuals, particularly those that clean the brood, are likely to be contaminated by contact with larvae in which the bacterium is present. A sample of worker bees is thus a type of control of the infectious status of a colony concerning *M. plutonius*. In Switzerland, Belloy *et al.* (2007) compared the proportion of positive asymptomatic colonies between apiaries in

[19] http://www.reterurale.it/flex/cm/pages/ServeBLOB.php/L/IT/IDPagina/1092

[20] Semi-nested PCR is a variant of PCR in which the product from the first PCR is amplified using a primer pair in which one of the primers hybridises with an internal part of the DNA, the other primer is one of the two primers used during the first amplification

an enzootic zone and in zones where no symptoms of European foulbrood had been observed. In the enzootic zone, they found 91% (10/12) of asymptomatic colonies to be carriers of *M. plutonius* less than 10 metres from colonies with clinical signs, and 31% (10/32) at a distance of more than 500 metres. In the zone where no clinical signs had been observed, no positive colony was detected among the 16 belonging to 2 different apiaries. As a result, the distribution pattern shown by qPCR is probably more accurate, although it confirms that asymptomatic carriage for this agent is rather low.

Given this data and considering the situation in Switzerland and Great Britain (Belloy *et al.* 2007; Haynes *et al.* 2013; Roetschi *et al.* 2008), we can hypothesise that the absence of detection of *M. plutonius* for five years in the German monitoring study (Genersch *et al.* 2010) is attributable to low sensitivity of the method used: bacteriological detection on frame bees or bee bread.

Overall, these studies agree that:

- carriage of high viral concentrations for DWV is frequent in autumn;
- lower loads are detected for ABPV in spring and summer;
- carriage varies little in quantity during the year for *Nosema ceranae* in a large proportion of colonies.

3.1.1.3.4 Predictive nature of carriage for subsequent disorders, specifically winter mortality

Most of the studies reviewed above aimed to identify a relationship between the status of infection with one or more infectious agents and the occurrence of subsequent disorders, especially winter mortality (Berényi *et al.* 2006; Vidau *et al.* 2010; Dainat *et al.* 2012b; Genersch *et al.* 2010; Gisder *et al.* 2010; Hedtke *et al.* 2011; Mouret *et al.* 2013; Ravoet *et al.* 2013). The study tools and statistical methods used to demonstrate correlations between carriage and subsequent mortality are quite varied but very often rely on linear models. Only Mouret *et al.* (2013), Chauzat *et al.* (2010), Dainat *et al.* (2012b) and Genersch *et al.* (2010) measured variables of colony strength to find possible subclinical effects related to the infections.

Ten of these studies had consistent findings regarding co-infections (Antunez *et al.* 2012; Berényi *et al.* 2006; Chauzat *et al.* 2010; Chauzat *et al.* 2007; Dainat *et al.* 2012b; Gisder *et al.* 2010; Hedtke *et al.* 2011; Martín-Hernández *et al.* 2012; Mouret *et al.* 2013; Ravoet *et al.* 2013).

The most recent aim to demonstrate "interactions" between infectious agents in the occurrence of mortality, i.e. stronger statistical correlations between certain infectious agents (for example Ravoet *et al.* (2013)). These findings do not exclude interactions with chemical substances that may be present in the tested bees' environment. This suggests a synergistic effect between infectious agents. However, it is important to remember that simultaneous abundance of two infectious agents can be related to a common confounding factor, specifically decreased immunity or disorders of hygienic behaviour. As such, Dainat *et al.* (2012b) remain very cautious about the causal nature of infection by DWV or *Nosema* in the development of a disorder, and only report high infectious loads.

Experimental studies today aim to demonstrate the mechanism or mechanisms that lead to these carriage states with lower winter survival. van Dooremalen *et al.* (2013) showed that high levels of parasitism with *Varroa* in summer had an impact on the lifespan of winter bees.

In the United States, a more integrated approach was used in recent studies based on the concept of microbial balance in the colony (Anderson *et al.* 2011). Studies focussed on the interactions between microorganisms as a group within the colony (Cornman *et al.* 2012b; Evans and Schwarz 2011; Runckel *et al.* 2011). Novel high-throughput sequencing techniques are being used in these studies to describe multiple infections and relative abundances of organisms in the microbiota. Using these methods, the researchers also reveal co-infections, which appear to be general, seasonal variations, and shifts in the populations of commensal flora associated with clinical disease. Although these techniques are costly, they are promising since they combine quantitative and population data that could be correlated to environmental factors that influence the state of the colony. These approaches also pave the way to mechanistic studies on these interactions, or even

for the molecular dialogue that can occur between the various compartments of the microbiota, and the bees of the colony. These are cutting-edge techniques that cannot be used routinely at this time. The gut microbiota is increasingly well described but the effects of stress factors, e.g. pesticides, malnutrition, and disease, on the microbiota are not known.

3.1.2 Chemical factors

There is a very wide range of chemical substances that bee colonies may be exposed to and they cannot be listed in full. Some of these substances may be toxic in bees at high doses, generally in an acute manner, but also at sub-lethal doses. In this case, the effects are less overt and more difficult to demonstrate. Methods have been developed to identify these effects, particularly at low doses.

After a description of the main methods of detecting toxic effects at the individual level in bees, chemical factors will be listed. These include factors of interest for interactions, given current knowledge. It should be noted that the order of presentation of these chemical factors does not result from any ranking of importance or potential impact.

3.1.2.1 Methods to detect toxic effects at the individual level in bees

3.1.2.1.1 Neuronal and behavioural tests

Since no evaluation method for the neuronal and behavioural effects of chemical agents has been validated at the international level, scientists have attempted to develop such methods in order to put forward routine tests that could be validated internationally.

A specific test has been the subject of many publications: ***the proboscis extension reflex (PER)***. The proboscis is a mouthpart of about 6 mm in length that can be retracted under the mouth into a cavity, or extended, specifically to collect nectar from flowers. The proboscis is extended as a reflex when a gustatory stimulus (water or sucrose) touches the antennae (sensory organs for taste and smell). Experimental use of this reflex helps to evaluate possible changes in a particular ability of the central nervous system in bees, specifically the abilities of:

- gustatory perception, for example perception of water or a sucrose solution;
- discrimination between smells;
- associative learning and memorisation when associated with conditioned, olfactory, or tactile stimuli:
  i. in olfactory learning, an odour that represents the conditioned stimulus (CS) is directed towards the bee's antennae for x seconds, then y seconds after the start of olfactory stimulus, the antennae are touched with a sucrose solution (unconditioned stimulus, US), which induces extension of the proboscis. After several associations of olfactory and gustatory stimuli, the bee responds to the odour alone by extension of the proboscis, which constitutes a conditioned response;
  ii. in tactile learning, an object is brought near the antennae (CS) and the bee explores it for a few seconds; gustatory stimulation with sucrose water (US) is applied to the proboscis. This protocol is used to dissociate the unconditioned stimulus pathway from the conditioned stimulus pathway.

  In both learning processes, the extension of the proboscis is rewarded by the fact that the bee can collect several microlitres of sucrose water with its proboscis.

Use of the PER in learning tests makes it possible to test several parameters, such as:

- memorisation abilities in bees, short and long term;
- parameters related to learning, such as habituation, recovery, or generalisation.

Use of this test also helps in the study of the effects of several types of exposure of bees to pesticides: acute (before, during or after conditioning) or chronic.

Depending on the results obtained for learning or perception abilities, use of the test enables evaluation of the effects of pesticides on the functioning of the central nervous system in bees in a more general manner.

The ***T-maze test*** was proposed by Han *et al.* (2010) to study the visual learning abilities of bees. It is a simplified version of the maze test described earlier (Zhang *et al.* 1996). It includes an entrance tube of 20 cm in length leading to two arms of 12 cm in length, with a tube diameter of 1.6 cm. The two arms are of different colours, blue and yellow, to carry out visual learning. A more complex version of the maze, developed by Zhang *et al.* (1996), can also be used for ecotoxicological purposes. It consists of 20 identical cubic boxes with a central hole of 4 cm in diameter on each surface of 30 cm, into which the bees can crawl. After being conditioned for a visual cue, foragers must fly through the maze following this cue to reach a syrup feeder. The spatial performance of foragers exposed to a pesticide is compared to that of non-exposed foragers (Decourtye *et al.* 2009).

3.1.2.1.2 Motricity tests

The locomotor activity of bees can be assessed in a vertical glass enclosure. One can then measure parameters such as the distance covered, time spent immobile, and the vertical level reached.

3.1.2.1.3 Physiological tests

Several physiological tests are used to examine in particular the lifespan, development of hypopharyngeal glands, breathing rhythm, production of pheromones, production of heat, and expression of detoxification, immune, and development pathways, at the molecular or enzyme levels. The larval test in *in vitro* conditions enables evaluation of the short- and medium-term lethal and sub-lethal effects of any stress factor by ensuring complete control of exposure to this factor and environmental development conditions.

3.1.2.1.4 Molecular tests

Transcriptome and proteomic analyses (Di Prisco *et al.* 2013) can be carried out to identify in particular the physiological functions of bees that are affected or not by given substances and characterise molecular markers (Aufauvre *et al.* 2014; Derecka *et al.* 2013).

3.1.2.2 Insecticides

Insecticides are a type of pesticide. Some co-formulants are not considered to be active pesticide substances but can have a major effect on the action of active substances that they are combined with, specifically by increasing their bioavailability (see section 4.1.3.2.1). They may also be toxic to bees on their own. Zhu *et al.* (2014) demonstrated that N-methyl-2-pyrrolidone (NMP), which is a common co-formulant in insecticides (e.g. neonicotinoids) or fungicides and is present in wax (parts per million), has high toxicity in bee larvae with 50% mortality in 4 days at 0.01% NMP. This calls into question the classification of some co-formulants as so-called “inert” substances.

Depending on their target, pesticides are classified as insecticides, acaricides, fungicides, or herbicides. There are several chemical families including many that are of particular interest to this report: neonicotinoid insecticides, pyrethroids, organophosphorus compounds, carbamates, triazole fungicides, and carboxins.

3.1.2.2.1 Neonicotinoid insecticides and fipronil and their sub-lethal effects

Neonicotinoids are systemic neurotoxic insecticides that interact with nicotinic receptors found in the nervous system of insects.

Several neonicotinoid insecticides are currently on the market worldwide and account for about one third of the insecticides used (Casida and Durkin 2013; Simon-Delso *et al.* 2015). They include imidacloprid, thiamethoxam, clothianidin, thiacloprid, dinotefuran, acetamiprid, nitenpyram and

sulfoxaflor. Imidacloprid alone accounts for 41% of the neonicotinoid market and is the second most common plant protection product used worldwide (Jeschke *et al.* 2011; Pollack 2011).

In the European Union, Commission Implementing Regulation (EU) No 485/2013 of 24 May 2013 limited professional use of clothianidin, thiamethoxam and imidacloprid and prohibited sale of seeds treated with these substances, as well as non-professional use. The restrictions on use relate to treatment of seeds and treatment of ground and foliage and concern more than 75 different crops including rapeseed and maize, but also fruit crops considered attractive to bees. These restrictions followed re-evaluation of these substances by the European Food Safety Authority (EFSA) (EFSA 2013a; EFSA 2013b; EFSA 2013c), carried out because of shortcomings in the evaluation methods used until now, as recently identified in an opinion from EFSA (EFSA 2012a). Their risks related to use as treatment of seeds or in granule form was evaluated, particularly acute and chronic effects on survival and development of bee colonies, on larvae, and on bee behaviour, as well as the risks associated with sub-lethal doses. The three main exposure routes were considered to be nectar and pollen, dust emitted by coated seeds at the time of seeding, and water droplets produced by treated plants. Some re-evaluations could not be finalised because of a lack of available data. Finalised re-evaluations led to the following conclusions for the three substances: (1) for exposure to pollen and nectar, only use of these substances on crops that do not attract bees presents a low risk, (2) a risk for bees exposed to dust was reported or could not be excluded (with the exception of sugar beet, greenhouse crops, and use of certain granules), and (3) a risk of exposure of bees to guttation droplets could only be evaluated for thiamethoxam, with an acute effect on bees.

Many studies have been carried out since the 1990s to analyse the effects of neonicotinoids on bees. Several literature reviews have been published recently (Blacquière *et al.* 2012; Casida and Durkin 2013; Cresswell 2011; Decourtye and Devillers 2010; Godfray *et al.* 2014; Goulson 2013; Hopwood *et al.* 2012; van der Sluijs *et al.* 2013).

Very recently, all of the available data in the scientific literature on neonicotinoids and fipronil was assessed as part of a worldwide integrated assessment concerning impacts on biodiversity and in particular, on invertebrates (Pisa *et al.* 2015). This evaluation, which took the form of a meta-analysis, also looked into the metabolites of neonicotinoids (Simon-Delso *et al.* 2015). It also reviewed all the published data on exposure for various environmental compartments and those in Draft Assessment Reports (DARs). More specifically, all the data concerning pollen and nectar were analysed in detail (Bonmatin *et al.* 2015). Honeybees and bumble bees were given particular attention because of the large number and high quality of available studies. The conclusions in this meta-analysis clearly tend towards direct and major effects of neonicotinoids and fipronil on pollinators (van der Sluijs *et al.* 2015). According to the authors, the meta-analysis shows the sum of the four main characteristics of these neurotoxic insecticides: their very high toxicity (acute, sub-lethal and through chronic exposure), their significant bioavailability in real conditions through pollen and nectar, their long active periods in ecosystems (soil, water, plants), and their highly intensive prophylactic use, particularly on nectariferous and polliniferous plants. Another major exposure route was also confirmed for pollinators via the emission of dust during planting of coated seeds (www.tfsp.info, see video).

Experimental results are presented below chronologically and in the following order: the insecticide analysed (imidacloprid, thiamethoxam, clothianidin and acetamiprid), and the category of test performed (laboratory, tunnel, or open field).

3.1.2.2.1.1 Imidacloprid

Imidacloprid is an insecticide with high toxicity in bees with an oral LD_{50} of 0.0037 µg/bee and a contact LD_{50} of 0.081 µg/bee. Importantly, imidacloprid is the archetype of substances in the neonicotinoid class. This neurotoxic agent is the most studied of the substances in the class since it was the first to be marketed, and many research findings on imidacloprid can potentially be extrapolated to the other neonicotinoids. Mean imidacloprid contamination via pollen (or via bee bread) from treated plants ranges from 1 to 39 µg/kg depending on the study, the crops, and the

methods of application. This mean contamination ranges from 1 to 73 µg/kg when it comes to nectar or honey (Bonmatin *et al.* 2015).

The effects of this insecticide have been analysed in detail by various French and European expert committees. In France, the Ministry of Agriculture and the Ministry of Ecology jointly set up the Scientific and technical committee for the multifactorial study of bee disorders (CST) in 2001. The CST analysed 245 study reports or related documents provided by the Directorate General for Food (DGAL) within the Ministry of Agriculture. It also assessed 93 documents from the scientific and technical literature based on an exhaustive bibliographic analysis that brought together all the data on bee toxicology and all the data on behavioural problems related to the use of imidacloprid at the various stages of the bee lifecycle. Field data, as reported by beekeepers belonging to the group of experts and interviewed for the study, were also taken into account, along with data provided by the manufacturer of imidacloprid. The CST published its final report in 2003 (http://agriculture.gouv.fr/IMG/pdf/rapportfin.pdf).

The work carried out by the experts on the CST was innovative since, in comparison with the conventional assessment methods that were standard at the time, the experts defined validation criteria for studies, which led them to disregard studies that had scientific or technical shortcomings. In addition, this work helped to highlight the need to improve assay techniques, data on toxicity of substances and metabolites in different categories of bees, as well as a lack of standardisation of measurement protocols in the field.

The conclusion of the report was as follows: "*In the current state of knowledge and based on the scenarios developed to evaluate exposure and the uncertainty factors chosen to assess hazards, the PEC/PNEC*[21] *ratios obtained are of concern. They are consistent with the field observations reported by many beekeepers in large crop-growing areas (maize, sunflower), on mortality of foraging bees (scenario 4), their disappearance, behavioural problems and some winter mortality (scenario 5)*". *As a result, Gaucho® coating of sunflower seeds leads to a significant risk for bees of different ages, with the exception of foragers when they ingest pollen during the production of pollen loads (scenario 3). Concerning Gaucho® coating of maize seeds, the PEC/PNEC ratio is also of concern, like for sunflower seeds, in the context of consumption of pollen by nurse bees, which could lead to increased mortality in this population and represent one of the explanations for weakening of bee populations that is still observed despite the ban on Gaucho® in sunflowers".* This study also recommended expanding the analysis to other factors involved in bee losses, such as diseases, beekeeping and agricultural practices, genetic varieties for treated plant crops, and the influence of terpenes, etc.

3.1.2.2.1.1.1 Laboratory experiments

Laboratory experiments have focused on evaluating the lifespan of bees and analysing behavioural and physiological effects.

- **Effects on lifespan**

Imidacloprid can affect the lifespan of bees exposed to the substance either in an acute manner, following single exposure that kills the insect within a few hours or days, or following repeated (chronic) oral exposure. In this type of exposure, the bee is exposed to the insecticide for a period of several days, and dies prematurely in terms of the usual lifespan.

In the framework of this report which deals with interactions, it appears logical to address only chronic exposure to the insecticide, which for a certain amount of time can be combined with the presence of other factors, such as infectious agents, to alter bee functions. However, it is important to remember that bees can also be exposed on a one-off basis (single exposure) to this insecticide, while they are already under significant pressure from infectious agents. If the exposure dose is close to the LD_{50} (acute toxicity), it is possible that the combined action of the infectious agent and the insecticide could result in death, at a lower exposure than that of the mean LD_{50}.

[21] PEC = Predicted Environmental Concentration; PNEC = Predicted No Effect Concentration

A study on chronic toxicity was performed by feeding bees aged about 3 weeks with a sucrose solution containing 0.1, 1 and 10 µg/L imidacloprid and its metabolites for 10 days (Suchail *et al.* 2001). The 50% level of mortality was reached between the 7th and 10th day, depending on concentrations, and is equivalent to exposure of 0.01; 0.1 and 1 ng/bee (i.e. content of 0.1, 1 and 10 µg/kg, where one bee = 0.1 g). These doses are 30 to 3000 times (for the olefinic derivative), 60 to 6000 times (for imidacloprid), and 200 to 20,000 times (for 5-OH-imidacloprid) lower than those required to produce the same effect in acute intoxication. Importantly, bee death only began 72 h after intoxication. One of the systemic metabolites, 6-chloronicotinic acid, also showed very high toxicity. Similar effects were recently confirmed concerning mortality induced by chronic exposure of *Drosophila* to imidacloprid (Charpentier *et al.* 2014a). The researchers also found that reproductive functions such as mating and fertility were significantly affected at concentrations seven fold lower than the acute LC_{50}.

Dechaume Montcharmont *et al.* (2003) measured the lifespan of captive bees from emergence that were exposed to imidacloprid. Lifespan was significantly shorter than that of control bees, i.e. 28 days and 31 days, respectively for treatments with imidacloprid at 4 µg/L and 8 µg/L sucrose solution (respectively equivalent to consumption of 0.08 and 0.16 ng/day).

Decourtye *et al.* (2003) found higher lethal doses than those established by other researchers earlier, in winter bees (lowest observed effect concentrations - LOEC = 24 µg/kg) and in summer bees (LOEC = 8 µg/kg).

In response to the article by Suchail *et al.* (2001) which showed very high chronic toxicity for imidacloprid and its metabolites via the oral route, the company Bayer sponsored four studies (Schmuck 2004). These studies however did not examine imidacloprid, but only its urea and 6-chloronicotinic acid metabolites (CST 2003)[22]. They showed no abnormal increase in mortality after ingestion of these metabolites in food. During its analysis of the reports for these four studies, the CST examined all the studies and concluded that "*the studies requested by the company Bayer only enable us to establish a validated NOEC higher than 10 µg/kg*".

In a study examining the interactions between the microsporidian *Nosema ceranae* and imidacloprid, Alaux *et al.* (2010a) analysed the chronic toxicity of imidacloprid via food (0.7 µg/kg, 7 µg/kg and 70 µg/kg) for 10 days and found that exposure to imidacloprid led to a mortality level higher than that of controls after 10 days at all the tested concentrations. The results concerning interactions between *Nosema* and imidacloprid are presented in section 4.1.2.3.

On the basis of recent analysis data on residues in pollen and nectar or honey, and those on the toxicity of pesticides, Sanchez-Bayo and Goka (2014) adopted a new approach for the risks of pesticides on bees by taking into account the effects of accumulation over time. They determined the time needed to reach LD_{50} (acute toxicity). Concerning contact exposure, the authors showed that three neonicotinoids, i.e. imidacloprid, thiamethoxam and clothianidin, present a high risk with contaminated pollen. Regarding exposure by ingestion of contaminated pollen and nectar, imidacloprid and thiamethoxam also showed a high risk.

Considering the toxicity data from the literature on imidacloprid in bees, Rondeau *et al.* (2014) used a toxicological model derived from Haber's rule that takes into account the change in toxicity of the pesticide over time. They demonstrated that current acute toxicity tests, which only last for two days, or four days in some cases, and even so-called chronic tests with a duration of only 10 days, are too short to characterise possible effects on bee survival beyond these periods. Indeed, the lifespan of bees is about 30 days in summer and 150 days in winter. By extrapolating the results of their model, the authors suggest that winter bees that consume honey with an imidacloprid content of 0.25 µg/kg could die before the end of overwintering and the renewal of activities in the colony. They propose that regulatory toxicity tests should last at least 30 days, and that time/effect curves be used to precisely establish changes in insecticide toxicity over time.

- **Neuronal and behavioural effects**

[22] CST 2003 p. 48

Imidacloprid, like the main neonicotinoids, interacts with nicotinic acetylcholine receptors. It acts as an agonist on this neuromediator by mimicking its action on the post-synaptic membrane (van der Sluijs *et al.* 2013). The bee nervous system is particularly rich in cholinergic synapses, specifically in the brain (Bicker 1999). Many studies have focussed on the possible effects of sub-lethal doses of imidacloprid on the functioning of the nervous system in bees and on certain bee behaviours (see also summary by Belzunces *et al.* (2012)).

- ✓ *Neuronal effects*

Using electro-physiological recordings, Palmer *et al.* (2013) showed that disrupted behaviour and learning caused by imidacloprid could result from its action on the functioning of mushroom body Kenyon cells. These cells comprise 40% of neurons in the bee brain and are the centre of multi-sensorial information integration in the brain, and learning and memory processes. Also, the authors found that cumulative exposure to several cholinergic pesticides, such as clothianidin and coumaphos, led to increased neurotoxicity.

- ✓ *Effects on learning and conditioning*

  - *Proboscis extension reflex tests*

Contact exposure of bees to sub-lethal doses of imidacloprid (0.1, 1 and 10 ng/bee) altered the number of trials needed to habituate honeybee response to multiple sucrose stimulation in 7 and 8 day-old bees (Guez *et al.* 2001).

Contact exposure at doses of 5, 10, and 20 ng/bee led to an increase in the gustatory threshold, defined as the lowest concentration of a sucrose solution able to elicit the proboscis extension reflex (PER). The dose of 1.25 ng/bee has no effect on gustatory function but has a facilitating effect on habituation (Lambin *et al.* 2001). Oral exposure (0.21 or 2.16 ng/bee) to imidacloprid temporarily increased the response threshold to sucrose one hour after treatment (Eiri and Nieh 2012).

Oral exposure to sub-lethal doses of imidacloprid results in decreased olfactory learning abilities. The lowest dose inducing a sub-lethal effect (LOEC) was 12 µg/kg in summer bees and 48 µg/kg in winter bees (Decourtye *et al.* 2003). Chronic oral exposure of bees for 7 days to imidacloprid-contaminated pollen at a sub-lethal dose of 48 µg/kg caused decreased olfactory learning abilities (Han *et al.* 2010).

Sub-lethal exposure of bee larvae to imidacloprid (0.04 ng/larva) affected their learning abilities once they became adults (Yang *et al.* 2012).

Decourtye *et al.* (2004a) administered imidacloprid orally (12 ng/bee) 15 min or 1 h after conditioning. They found that imidacloprid changed medium-term memory formation through the cells of mushroom bodies in the brain, centres of multi-sensory integration, learning, and memory formation and retrieval. These findings confirm that imidacloprid causes variable behavioural effects in bees depending on the dose and type of learning, whether associative such as the olfactory conditioning performed, or non-associative such as habituation.

Decourtye *et al.* (2004b) studied the sub-lethal effects of oral imidacloprid (24 µg/kg sucrose solution), both in laboratory conditions and in an outdoor flight cage. Imidacloprid induced a decrease in both the foraging activity on the food source and activity at the hive entrance. In addition, negative effects of imidacloprid were observed in olfactory discrimination tests (PER).

Williamson and Wright (2013) exposed bees to sub-lethal solutions of imidacloprid (100 nM and 10 nM). During this experiment, they also exposed bees to coumaphos (organophosphate) and to a mixture of the two compounds. Using the PER method, they confirmed that these substances, used separately or in combination, affected learning performance in bees and memory formation, with imidacloprid primarily affecting long-term memory and coumaphos short-term memory.

  - *T maze test*

Chronic oral exposure of bees to pollen contaminated with imidacloprid for 7 days at the sub-lethal dose of 48 µg/kg leads to a decrease in visual learning abilities (Han *et al.* 2010).

- *Conclusion of the two tests*

The main results obtained show changes in olfactory and visual learning abilities, habituation and the sucrose response level. More generally, these findings show that the nervous system in worker bees is affected by sub-lethal exposure to imidacloprid, which is consistent with the fact that the imidacloprid target, acetylcholine receptors, are highly abundant in the worker bee brain.

✓ *Locomotor activity*

The motor activity of bees exposed by contact (thorax) to doses of 1.25, 2.5, 5, 10 and 20 ng/bee imidacloprid was evaluated in a vertical glass structure (30 x 30 x 4 cm). Measured parameters included the distance covered, time spent immobile, and the vertical level reached (Lambin *et al.* 2001). Locomotor activity was diminished at all tested doses, with the exception of the 1.25 ng/bee dose, at which it was increased (Lambin *et al.* 2001). These results were confirmed by Teeters *et al.* (2012), who showed that oral administration of imidacloprid at 0.05, 0.5, 5.0, 50, and 500 µg/kg led to an inhibitor effect on locomotion at the highest doses, and a stimulatory effect at the lowest doses.

- **Physiological effects**

✓ *Hypopharyngeal glands*

Worker bees function as nurse bees for the first 10-12 days of their lives. Over this time, their hypopharyngeal glands (HPGs) are well developed and produce secretions for feeding larvae. Given the importance of this function for the development of the colony, several studies have focused on the analysis of possible effects of sub-lethal orally administered doses of imidacloprid on the development of these glands. Findings have been consistent. Smodiš Škerl and Gregorc (2010) exposed bees of different ages for periods ranging from one to three days, and showed a decrease in the size of acini (HPG lobes), from an exposure duration of a single day. Heylen *et al.* (2011) exposed bees aged 7 days for a single day and also found a decrease in the size of acini. Lastly, Hatjina *et al.* (2013) exposed bees to contaminated pollen and a sucrose solution continuously for 14 days. They confirmed a reduced sized of acini that was maintained until the end of the experiment.

✓ *Respiratory rhythm*

In the same experimental study, Hatjina *et al.* (2013) analysed respiratory rhythm in bees exposed orally to sub-lethal imidacloprid doses and demonstrated that it was significantly affected.

✓ *Pheromones*

Dussaubat *et al.* (2010) analysed the effects of imidacloprid on the production of ethyl oleate, a pheromone compound in worker bees. Tested insects were exposed for 10 days at 10 h per day to a sucrose solution containing 7 µg/kg imidacloprid. No effect was demonstrated.

✓ *Bee metabolism*

Nicodemo *et al.* (2014) showed that imidacloprid (25 to 100 µM) is an inhibitor of ATP (adenosine 5'-triphosphate) production by mitochondria, organelles involved in cell metabolism. ATP is the compound that provides energy to all cells, and reduced production could, in particular, have negative effects on motor activities such as flying.

3.1.2.2.1.1.2 Tunnel experiments: foraging behaviour

The effectiveness of foraging behaviour was assessed on small bee colonies (2300 bees) placed in a tunnel (Colin *et al.* 2004). Oral exposure to imidacloprid was performed by means of feeders containing a sucrose solution contaminated with imidacloprid at 6 µg/kg (concentration 70 times lower than the LD_{50}, based on data available in 2003). Imidacloprid caused a decrease in the number of active bees and thus affected the effectiveness of foraging behaviour.

In an experiment performed in a flight cage where bees are free to fly, colonies with a total of 10,000 bees were exposed to sucrose solutions containing 48 µg/kg imidacloprid. At this concentration, imidacloprid led to decreased foraging activity and the quantity of sucrose solution harvested (Ramirez-Romero *et al.* 2005). The authors state that the tested concentration was 16 times higher than that found in sunflower pollen, for example, and that it would be useful to test concentrations lower than 48 µg/kg.

Previously, using the same system, decreased foraging activity on a feeder was recorded with the help of electronic counters after contamination of syrup with imidacloprid at a concentration of 24 µg/kg (Decourtye *et al.* 2004b).

3.1.2.2.1.1.3 Field experiments

- **Effects on the brood**

Imidacloprid solutions at concentrations of 0.1, 6, 50, 500, 1000, 1500 and 2000 mg/L were placed once daily in cells containing larvae (Yang *et al.* 2012). The larvae were thus exposed both orally and by contact. The brood combs were replaced in the colonies where larvae had been raised by nurse bees. Exposure was repeated for 4 consecutive days and the total exposure doses were 0.4, 24, 200, 2000, 4000, 6000 and 8000 ng/larva. The rate of capping of cells containing larvae was significantly reduced from the dose of 24 ng/larva, indicating that the nurse bees extracted dead or unhealthy larvae from the cells. Derecka *et al.* (2013) analysed the effects of low doses of imidacloprid (2 mg/L) on development and larval physiology in colonies placed in open fields. They showed that the level of expression of 300 genes was altered in the larvae, either a reduction for 195 genes, or an increase for 105 genes.

- **Behavioural effects**
  - ✓ *Foraging behaviour*

Studies on the effects of imidacloprid on bee foraging have primarily focused on the time interval between two visits to a feeding site and the rate of return to the hive.

Yang *et al.* (2008) trained bees marked with coloured points to visit a feeding site placed at a distance of 35 m from their hive and tested the effects of various sub-lethal concentrations of imidacloprid. The time interval between two visits increased from a concentration of 50 µg/L.

Bortolotti *et al.* (2003) tested the effects of three imidacloprid concentrations in a sucrose solution (100 µg/L, 500 µg/L and 1000 µg/L) on the ability of bees marked with coloured numbers to return to the hive from a distance of 500 m. Bees fed with the solution at 100 µg/L returned to the hive, but only returned to feed on the sucrose solution after 24 h. Bees fed with solutions containing 500 and 1000 µg/L were not found again. Of note, the protocol used did not provide data on the quantity of imidacloprid that was actually consumed by the forager (to provide it with energy) and thus to which it was exposed, since the bees brought some of the solution harvested back to the hive.

An automated method of following up bee foraging activity using radiofrequencies (RFID) was developed for the bumble bee by Streit *et al.* (2003) and for the honeybee by Decourtye *et al.* (2011b). Using this technique, Schneider *et al.* (2012) showed that bees subjected to acute exposure through a contaminated sucrose solution at sub-lethal doses of imidacloprid (0.15 to 6 ng/bee) showed a significant reduction in foraging activity from the dose of 1.5 ng/bee.

Another follow-up method for bees when returning to the hive, the harmonic radar, was used by Fischer *et al.* (2014). The research team tested the effects of three neonicotinoids, imidacloprid (7.5 ng/bee or 11.25 ng/bee), clothianidin (2.5 ng/bee) and thiacloprid (1.25 µg/bee) on honeybee orientation and navigation abilities. After ingesting a sucrose solution containing one of the neonicotinoids, the bee, with a transponder, was released and tracked by radar enabling precise monitoring of its itinerary and return to the hive. The main findings showed that the rate of return to the hive was reduced for the three neonicotinoids versus controls. The authors concluded that these doses blocked navigation memory retrieval or altered this form of memory. They however did recognise that these doses are high and represent the worst case in terms of doses consumed by

bees during a single foraging flight. The doses used could be equivalent to the quantity taken by a forager cumulatively over about 15 foraging flights.

✓ *Communication by dances*

Eiri and Nieh (2012) tested the effects of sub-lethal doses of imidacloprid on bees visiting a feeding site placed 1.5 m from the entrance to the colony and containing a sucrose solution. Foragers ingesting imidacloprid at a dose of 0.21 ng/bee produced significantly fewer recruitment dances 24 h after treatment. In the long term, this dance reduction can affect colony strength by decreasing the quantity of honey collected.

3.1.2.2.1.2 Thiamethoxam

Thiamethoxam is a highly toxic insecticide in bees with an oral LD_{50} of 0.005 µg/bee and a contact LD_{50} of 0.024 µg/bee. Mean concentrations measured in pollen or bee bread from plants treated with thiamethoxam range from 1.7 to 122 µg/kg depending on the study, the crop, the method of application, and whether the metabolite clothianidin is included. This mean contamination ranges from 0.6 to 9.9 µg/kg concerning nectar and honey (Bonmatin *et al.* 2015).

3.1.2.2.1.2.1 Laboratory experiments

- **Behavioural effects**

Following acute exposure of bees to oral thiamethoxam doses of 0.1, 0.5 and 1 ng/bee, no effect was found on locomotor activity, sensitivity to sucrose, and olfactory learning (El Hassani *et al.* 2008).

After chronic exposure by contact to sub-lethal thiamethoxam doses, i.e. 1 and 0.1 ng/bee, for 11 days, bees were assessed using the PER test (Aliouane *et al.* 2009). They showed a significant decrease in olfactory memory at 0.1 ng/bee, and a significant decrease in learning performance with no effect on memory at 1 ng/bee. Moreover, the response to antenna stimulation with sucrose was significantly reduced for high concentrations (1 ng/bee).

- **Physiological effects: enzyme activity**

The activity of certain enzymes including carboxylesterases (CaE1, CaE2, CaE3), glutathione-S-transferase (GST), catalase (CAT), and alkaline phosphatase (ALP) is altered after contact exposure on the thorax to sub-lethal doses of thiamethoxam, 5.12 ng/bee (LD_{50}/10) and 2.56 ng/bee (LD_{50}/20) (Badiou-Bénéteau *et al.* 2012).

3.1.2.2.1.2.2 Field experiments

- **Foraging behaviour**

Bees were subjected to acute thiamethoxam exposure at sub-lethal doses and were followed-up using the RFID technique. Significant bee mortality was found because a proportion of bees were not able to return to their colony (Henry *et al.* 2012). Further analyses showed that weather conditions had a marked impact along with the complexity of the landscape and bee sensitivity to the insecticide (Henry *et al.* 2014). Thiamethoxam induces a moderate risk of non-return to the hive, increasing from 3% to 26% when weather conditions become unfavourable. This level of disappearance related to the insecticide is moreover modulated by the landscape environment, reaching 35% in hedged farmlands versus 18% in open lands with a less complex structure.

- **Long-term effects on the colonies**

Pilling *et al.* (2013) carried out an open field study lasting 4 years to assess thiamethoxam. Colonies were exposed to maize and rapeseed crop fields with an area of 2 ha. The colonies had abundant food reserves (15 to 20 food combs). The control and treated fields were 2 km away. The authors found no difference between the control and treated field colonies for the following parameters: bee mortality, foraging behaviour, colony strength, colony weight, brood development, quantity of stored food, overwintering, and the general health status of colonies.

However, the selected experimental conditions, particularly the exposure of bees to fields of only 2 ha, are different from conditions found by colonies in large field crop areas, where simultaneous or successive flowering of a large number of rapeseed or maize fields for several weeks, or more than a month, exposes them to higher quantities of pesticide residues. It was therefore not demonstrated that bees were significantly exposed in terms of the usual field conditions. Also, no statistical analysis was performed in this study.

3.1.2.2.1.3 Clothianidin

Clothianidin has high toxicity in bees, with an oral LD_{50} of 0.00379 µg/bee and a contact LD_{50} of 0.0275 µg/bee. Mean contamination in pollen and bee bread from plants treated with clothianidin ranges from 1.8 to 9.4 µg/kg depending on the study, crop, and method of application. This mean contamination ranges from 1.9 to 89 µg/kg concerning nectar or honey (Bonmatin *et al.* 2015).

3.1.2.2.1.3.1 Laboratory experiments

- **Neuronal effects**

Palmer *et al.* (2013) showed that clothianidin has the same effects as imidacloprid on cells in the higher brain centres in bees (see “Neuronal effects of imidacloprid”).

- **Effects on immunity**

Di Prisco *et al.* (2013) demonstrated that clothianidin (and imidacloprid) negatively modulate the NF-κB transcription factor involved in immunity and thus affects antiviral defences in bees. These neonicotinoids promote the replication of DWV in this way. These findings bring up the question of possible nerve circuits that control immunity in insects, like those known in mammals. This result shows the close link between various co-exposure factors, in terms of a well-defined cause-effect (neonicotinoid-virus) relationship.

3.1.2.2.1.3.2 Field experiments: foraging behaviour

Bees were exposed acutely to sub-lethal clothianidin doses of 0.05^{-2} ng/bee and were tracked by RFID. Clothianidin resulted in a significant decrease in foraging activity and an increase in the duration of foraging flights from a dose of 0.5 ng/bee for the first three hours following treatment (Schneider *et al.* 2012).

Another study was performed to assess the effects of sub-lethal doses of clothianidin on return flights to the hive (Fischer *et al.* 2014). The results are described in the section concerning imidacloprid (see "Foraging behaviour").

3.1.2.2.1.4 Acetamiprid

The oral LD_{50} is 14.53 µg/bee with a contact LD50 of 8.09 µg/bee. Mean contamination in pollen or bee bread from plants treated with acetamiprid ranges from 3 to 59.3 µg/kg depending on the studies, crops, and methods of application. This mean contamination is 2.4 µg/kg while a maximum is observed at 112.8 µg/kg concerning nectar and honey (Bonmatin *et al.* 2015), although few studies are available.

Laboratory experiments

Following acute acetamiprid exposure of bees via the oral route at doses of 0.1, 0.5 and 1 µg/bee, the sensitivity of bees following antennal stimulation to sucrose (PER test) was increased at a dose of 1 µg/bee. Long-term olfactory learning memory was affected by a dose of 0.1 µg/bee (El Hassani *et al.* 2008). After thoracic application, acetamiprid did not produce an effect with these two tests but increased locomotor activity at the 0.1 and 0.5 µg/bee doses, and the proboscis extension effect induced by antennal exposure to water, at the 0.1, 0.5 and 1 µg/bee doses.

The effects of chronic sub-lethal acetamiprid doses (0.1 and 1 µg/bee) administered for 11 days were evaluated for three different functions: locomotor activity, responsiveness to water and sucrose, and learning performance (Aliouane *et al.* 2009). The only significant effect observed on

oral administration of 0.1 µg/bee acetamiprid was increased responsiveness to water. At the highest dose (1 µg/bee), acetamiprid causes limited effects on sensory, motor and cognitive functions in the bee.

3.1.2.2.1.5 Thiacloprid

The oral LD_{50} is 17.32 µg/bee and contact LD_{50} 38.82 µg/bee. Mean contamination in pollen or bee bread from plants treated with thiacloprid ranges from 10 to 187.6 µg/kg depending on the studies, crops, and methods of application. This mean contamination ranges from 1.8 to 6.5 µg/kg concerning nectar or honey, although few studies are available (Bonmatin *et al.* 2015).

Field experiments

A study was carried out to assess the effect of sub-lethal doses of thiacloprid on return flights to the hive (Fischer *et al.* 2014). The findings are mentioned in the section concerning imidacloprid (see foraging behaviour for imidacloprid).

3.1.2.2.1.6 Fipronil

Fipronil is a systemic insecticide belonging to the pyrazole group. It acts as a reversible inhibitor of the GABA receptor and of chloride channels activated by glutamate.

The substance has high toxicity in bees with an oral LD_{50} of 0.00417 µg/bee and a contact LD_{50} of 0.00593 µg/bee.

In France, the Ministry of Agriculture and the Ministry of Ecology jointly set up the Scientific and technical committee for the multifactorial study of bee disorders (CST). The committee released a report in 2006[23]**.** The conclusion was as follows: "*In the current state of knowledge and based on the scenarios developed to evaluate exposure and the uncertainty factors chosen to assess hazards, the PEC/PNEC ratios obtained may appear to be of concern (table XXXI) and unacceptable risks cannot be ruled out."*

Fipronil was also the subject of the very recent worldwide meta-analysis cited above (Bijleveld van Lexmond *et al.* 2015). In particular, mean contamination of pollen and bee bread ranges from 0.8 to 28.5 ng/g (µg/kg) depending on the studies, crops, and methods of application. This mean ranges from 1.2 to 70 µg/kg for nectar or honey (Bonmatin *et al.* 2015). Like for neonicotinoids, the conclusions of the meta-analysis point to significant risks and impact for pollinators (Bonmatin *et al.* 2007; van der Sluijs *et al.* 2015).

3.1.2.2.1.6.1 Laboratory experiments

- **Chronic mortality**

Bees were fed for 14 days (chronic exposure) with a sucrose solution containing fipronil at concentrations of 2.2 to 9 µg/L (Decourtye *et al.* 2005). These doses led to bee mortality. The lowest tested concentration that resulted in death (2.2 µg/L) is equivalent to a dose of 0.1 ng/bee/day, i.e. about 60 times lower than the LD_{50}. Bees were exposed for 11 days, orally and by contact, to two doses of fipronil, 0.1 and 0.01 ng/bee (Aliouane *et al.* 2009). The dose of 0.1 ng/bee (orally and by contact) led to death of all bees one week after the start of treatment. Mortality increased significantly from D3 for the oral exposure and from D5 for contact exposure. At the 0.01 ng/bee dose, mortality was not significantly different from that of control bees.

- **Learning and conditioning**

Bees were exposed orally or by contact to sub-lethal doses of fipronil (0.1, 0.5 and 1 ng/bee) (El Hassani *et al.* 2005). The 1 ng/bee dose given topically led to significantly decreased sucrose sensitivity, while oral exposure had no effect. The dose of 0.5 ng/bee topically disrupted olfactory learning. Locomotor activity was not affected on administration of fipronil.

[23] http://agriculture.gouv.fr/IMG/pdf/080218_rapport_fiproniljuillet2006.pdf

Bees were fed for 14 days (chronic exposure) with a sucrose solution containing fipronil at concentrations of 2.2 to 9 µg/L (Decourtye *et al.* 2005). Decreased learning performance was observed in bees exposed to this substance.

Sub-lethal doses of fipronil were injected in bees (0.1 and 0.5 ng/bee) that were then assessed on the basis of the PER test (El Hassani *et al.* 2009). The dose of 0.1 ng/bee did not affect the learning process but lowered memory performance. The 0.5 ng/bee dose had the opposite effects, since it affected learning but not memory performance. These findings show that sub-lethal fipronil doses affect learning and memory processes through multiple targets, including glutamate and GABA receptors.

Bees were exposed to two doses of fipronil (0.1 and 0.01 ng/bee) orally and by contact for 11 days in another study (Aliouane *et al.* 2009). At the 0.01 ng/bee dose, at least one behavioural parameter was affected. One of the main findings was that fipronil affects discrimination between odours (generalisation). At this dose, locomotor activity was also reduced.

Contact exposure to an acute sub-lethal dose of fipronil (0.5 ng/bee) affected tactile learning processes and memorisation (PER test) (Bernadou *et al.* 2009).

- **Effects on immunity**

Aufauvre *et al.* (2014) analysed the molecular response in bees exposed to *Nosema ceranae* and to fipronil, separately and in combination. The main results of the study are presented in the section on interactions. During the experiment, the authors showed that fipronil alone had an effect on the expression of certain genes and enzyme activity in bees, specifically related to immunity.

- **Effects on bee metabolism**

Nicodemo *et al.* (2014) demonstrated that fipronil (25 to 100 µM) inhibits ATP (adenosine-5'-triphosphate) production by mitochondria, organelles involved in cell metabolism.

3.1.2.2.1.6.2 Tunnel experiments: foraging behaviour

A study on foraging behaviour in a colony of 2300 bees showed that fipronil at a concentration of 2 µg/kg led to a reduced number of active bees (Colin *et al.* 2004). After 4 days of exposure to fipronil, bees no longer foraged (no food intake). In subsequent research using the RFID technique, Decourtye *et al.* (2011b) showed a reduced number of return flights to the hive following acute oral exposure at 0.3 ng/bee (and not at 0.06 ng), that lasted 24 h after application. The return time from the food source to the hive was increased at this dose for 3 days.

3.1.2.2.2 Insect Growth Regulators

Insect Growth Regulators (IGRs) can be classified into four categories:

- Chitin synthesis inhibitors,
- Juvenile hormone analogues,
- Moulting hormone agonists,
- Ecdysone antagonists.

Their mechanism of action varies depending on the given category.

3.1.2.2.2.1 Chitin synthesis inhibitors

Chitin synthesis inhibitors include about a dozen compounds used for their larvicide activity, and one of them, diflubenzuron, has been studied in particular. This compound, belonging to the benzoylurea family, is essentially a larvicide with contact ovicide activity. It disrupts chitin deposition in the cuticle causing serious damage to endocuticular tissue. Because of its mechanism of action, diflubenzuron is thought to have little or no effect on adult insects. It is recommended for the control of Mediterranean corn borer in maize crops, codling moth in orchards, and certain forest pests such as processionary caterpillars or *Lymantria dispar* (gypsy moth). Depending on the type of crop, its usual concentration ranges from 48 g/ha in forests to 125 g/ha in maize crops. Diflubenzuron is soluble in water at 0.02 mg/L, i.e. about 25,000 times less than imidacloprid.

In the honeybee, *Apis mellifera*, Chandel and Gupta (1992) observed higher sensitivity of nymphs to diflubenzuron compared to larvae at the third and fourth instar. These experiments, carried out in colonies in open fields, showed that application of 6 and 4 µg of diflubenzuron per nymph leads to various malformations in more than half the adults, including growths at the abdominal extremity and crumpled wings. In this study, the contact LD_{50} in 4th instar larvae was evaluated at 6.01 ng per individual, and only at 2.42 ng in larvae at the third instar. No delayed lethal or morphological effects were observed after larval treatment, since all the tested insects died. More recently, in laboratory larva rearing conditions, Aupinel *et al.* (2007b) found an LD_{50} at 48 h of 175 ng/larva, after acute oral exposure at the age of 4 days. In the same experimental conditions, a dose of 23 ng/larva was sufficient to induce significant nymphal mortality, higher than 50% for a control value of 20%. The no adverse effect level could not be determined in this experiment since the 23 ng/larva dose was the lowest of the tested doses.

Gupta and Chandel (1995) examined the effects of diflubenzuron in emerging bees, following topical and oral application. They also evaluated the effects of oral exposure in foragers captured at the entrance to the hive. Application of 100 µg diflubenzuron on the thorax of young emerging worker bees affected weight gain from the second day following treatment. This decrease in weight was also observed in foragers exposed orally to 12.5 µg diflubenzuron per individual. In emerging bees, absorption of 50 µg diflubenzuron disrupted development of the hypopharyngeal glands.

In colonies fed with 1 L sucrose solution containing 50 mg diflubenzuron, Chandel and Gupta (1992) found within 10 days of treatment, a reduction in brood and an increase in the laying rate of the queen bee, without honey and pollen stores being affected. With a similar set-up but at a higher concentration (300 mg/L) of active substance, equivalent to the maximum application level for crops, Thompson *et al.* (2005) observed comparable effects, i.e. a decrease in brood and a higher egg and larva replacement rate. The researchers also found decreases in adult bee populations but did not mention long-term effects on renewal of activity after overwintering. Studies in orange trees (Emmett and Archer 1980) treated at concentrations of 0.11, 0.20 and 0.40 kg AI/L showed no effect on colonies in terms of changes in adult and larva populations or mortality. A direct spray on 230 foragers with a solution of 0.40 g/L diflubenzuron led to the same conclusions. Importantly, these studies did not enable evaluation of bee exposure to the insecticide, and spraying onto foragers simulates very brief exposure on a few individuals.

Among the other chitin synthesis inhibitors studied in *Apis mellifera*, penfluron has the same effects as those resulting from diflubenzuron at similar doses, i.e. an LD_{50} of about 2 µg/larva at the third instar, 6 µg/larva at the fourth instar, and 3 µg/nymph (Chandel and Gupta 1992). Production of deformed adults was also found for this compound. In laboratory conditions, Rabea *et al.* (2010) showed that chlorfluazuron had low toxicity in adults with an LD_{50} of 2526 mg/L, corresponding to 10 times the usual concentration. In colonies fed with 1 L of syrup containing 0.25 and 2.5 g triflumuron, Amir and Peveling (2004) showed a significant reduction in flight activity, as well as decreased capped brood. Colonies exposed to the highest concentration present long-term effects characterised by high winter mortality.

In *Bombus terrestris*, diflubenzuron produced effects similar to those found in *Apis mellifera*. Tests on microcolonies raised in the laboratory showed that through contact exposure, contamination of pollen or of syrup led to the same effects at maximum field concentrations (288 mg AI/L), i.e. elimination of brood (Mommaerts *et al.* 2006). These effects, observed two days after exposure, continued for two weeks. In larvae aged 1 to 4 days, exposure to flubenzuron induced total mortality. Gretenkord and Drescher (1995) observed similar effects in larvae aged 1 to 4 days, and higher tolerance in larvae aged 6 days.

In their study, Mommaerts *et al.* (2006) also tested the effects of seven other chitin inhibitors: buprofezin, cyromazine, flucycloxuron, flufenoxuron, lufenuron, novaluron and teflubenzuron. The observed effects were generally similar to those generated by flubenzuron with variable effects on brood.

3.1.2.2.2.2 Juvenile hormone analogues

- ***Methoprene***

Methoprene has a chemical structure related to that of juvenile hormone III found in bees. Its oral or contact LD_{50} in the adult bee is higher than 1000 µg/bee (Redfern and Knox 1974).

Sasagawa *et al.* (1989) assessed whether injected or topical methoprene affected the development of the *corpora allata*[24] (glands producing juvenile hormone) and the hypopharyngeal glands. They also looked at its potential effects on α-glucosidase activity and the behaviour of worker bees. The development of the *corpora allata*, which usually takes place during the first two weeks of life, was inhibited on injection of methoprene (0.1, 0.5, 1, 5 and 10 µg) in oil (0.5 µL) in each bee. The lowest dose (0.1 µg) seemed to stimulate development of the hypopharyngeal glands (the size of which was slightly higher than that of controls), while the highest doses inhibited normal development. Peak α-glucosidase activity in the gland, normally observed in older foragers, was induced in one to two weeks by injection of 0.1 to 10 µg methoprene.

The effects of methoprene on age-related behaviour were studied by Robinson (1987). One-day worker bees were marked individually with coloured tags and treated with a solution of methoprene applied to the abdomen (groups of 50 bees treated with 25, 50, 100, 150, 200 and 250 µg methoprene dissolved in 5 µL acetone). Methoprene resulted in a significant dose-dependent decrease in the frequency of brood and queen bee care in all the tests and at most ages. Methoprene led to large dose-dependent decreases in the frequency of nest maintenance behaviour. Bees treated at the highest doses showed higher general activity peaks. Robinson (1987) did not find significant effects of methoprene on ventilation. However, treated bees began orientation and foraging flights earlier than control bees in all the tests. In another study, Robinson (1985) showed that although workers treated with 250 µg methoprene showed early foraging behaviour, treatments at 2.5 and 25 µg only led to low, non-significant effects. Methoprene also led to premature production of two alarm pheromones, 2-heptanone and isopentyl acetate. Deng and Waddington (1997) confirmed most of these findings. Marked foragers were treated by topical application of 200 µg methoprene dissolved in 5 µL acetone. The authors found that methoprene did not affect preferences (i.e., pollen vs nectar) or forager performance. Adult bees showed circadian rhythm of locomotor behaviour which was associated with division of labour. Since juvenile hormone coordinates various physiological and behavioural processes involved in the division of labour, Bloch *et al.* (2002) tested whether methoprene influenced ontogeny of circadian rhythms and the parameters of the internal clock in young worker bees. Treatment with methoprene (200 µg dissolved in 5 µL acetone), or allatectomy, did not affect the onset of rhythmicity and overall locomotor activity.

- ***Kinoprene***

The effects of kinoprene (Enstar 65% WG) were studied in *Bombus terrestris* by Mommaerts *et al.* (2006). Applications of 650 mg AI[25]/L by contact and orally did not induce death. After 11 weeks, there was no difference with controls. Production of drones after 11 weeks was not affected by applications (650 mg AI/L) by contact or orally: sucrose water and via contaminated pollen. However, with contaminated pollen, significant mortality was observed in larvae. One contact application of 65 µg AI/L positively affected the size of ovaries and the production of eggs.

- ***Pyriproxyfen***

Bitondi *et al.* (1998) showed that bees treated with topical applications of pyriproxyfen (1 µg in 1 µl acetone) during the larval stage displayed pigmentation changes and sclerotisation of the cuticle. The effects varied depending on the stage at which treatment was applied. Using groups of 120 newly hatched bees treated with acetone (1 µl) containing different concentrations of pyriproxyfen (10, 5, 2.5, 1.25, 0.1, 0.01, 0.001 or 0 µg), Pinto *et al.* (2000) showed that this juvenoid affected the synthesis, secretion and accumulation of vitellogenin in young worker bees in a dose-dependent manner. Machado Baptista *et al.* (2009) found that direct spraying of pyriproxyfen (Cordial 100 EC - 0.075) led to an LT_{50} of 466 h. Yang *et al.* (2010) demonstrated an effect on the brood on one-day

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[24] Paired endocrine organs of the head belonging to the retrocerebral system. The *corpora allata* produce juvenile hormone and maintain larval characters during post-embryonal development and stimulate vitellogenesis during fledgling life.

[25] Active ingredient

larvae fed with 0.1 and 1 ppm pyriproxyfen. At 10 and 100 ppm, all the larvae died before hatching. On the basis of a larval test, it was shown (Devillers *et al.* 2013) that a dose of 305 µg/kg pyriproxyfen (cumulative dose of 54 ng/larva) affected the development of the hypopharyngeal glands. At this dose, up to 1/3 of emerging bees could present wing malformations. Emerging bees were often rejected by their fellow bees on introduction into the hive. As a result, larvae treated at 101 and 305 µg/kg led to rejection of 38% and 80%, respectively while this was less than 10% in controls.

The effects of pyriproxyfen (Admiral 10% EC) were evaluated in *Bombus terrestris* by Mommaerts *et al.* (2006). Applications of 25 mg AI/L by contact and orally led to no deaths. After 11 weeks, there was no difference with controls. The production of drones was not affected by the applications (25 mg AI/L) by contact, orally or via contaminated pollen. However, with contaminated pollen, significant mortality was observed in the larvae.

- ***Fenoxycarb***

Aupinel *et al.* (2007a) evaluated the effects of fenoxycarb (98.5% purity) on bee larvae. The doses tested on D4 were 3, 6, 12, 25 and 50 ng/larva. No lethal effect on larvae was observed but hatching was affected at doses higher than 6 ng/larva. In this study, effects on adults stemming from these larvae were not evaluated. Heylen *et al.* (2011) showed that fenoxycarb had an effect on the size and structure of the hypopharyngeal glands at 14 days after oral exposure of 7-day bees at doses of 100 ppm.

Beliën *et al.* (2009) used a feeder containing a sucrose solution of fenoxycarb (Insegar 25 WG, 1 g AI/L) to experimentally contaminate colonies of 18,000 bees (*A. mellifera carnica*). The total number of active bees in the hive was estimated on the basis of photographs of each side of the frames and counting of all bees present in fixed areas. After six weeks, the exposed colonies had fewer active bees than in controls. Beliën *et al.* (2009) also showed that at three weeks, the development of brood and the weight of intoxicated colonies were lower than in controls but that these effects do not last. However, from one week after contamination, the number of foragers versus the number of active bees increased and remained higher than controls for the 10 weeks of the study.

Thompson *et al.* (2005) also used a feeder containing a sucrose solution of fenoxycarb (Insegar 25%, 0.6 kg/200 L) to experimentally contaminate colonies. They considered that this was equivalent to 50 µg fenoxycarb/cell of brood. At this dose, they observed an increase in the rate of egg and brood replacements versus the controls (i.e., 46% vs 24% and 21% vs 5%). No effect on sperm production was observed. However, the rate of mating of queen bee sisters used to test the effects of the compound and the number of eggs laid were strongly affected. One month after contamination, Thompson *et al.* (2005) found a smaller brood and a lower number of bees. The treated colonies declined more rapidly than controls, affecting renewal of activity the following year. One of the treated colonies did not survive overwintering.

The effects of fenoxycarb (Insegar 25% WG) were evaluated in *Bombus terrestris* by Mommaerts *et al.* (2006). Applications of 100 mg AI/L by contact and orally caused no deaths. After 11 weeks, there was no difference with controls. Production of drones was not affected by the applications (100 mg AI/L) by contact, orally or via contaminated pollen. The oral LD_{50} in larvae of 1, 4 and 6 days was estimated to be > 650, > 1740 and > 3710 ng/larva, respectively (Tasei 2002). Honeybees are thus more susceptible than bumble bees.

- ***Azadirachtin***

Azadirachtin is a secondary metabolite[26] present in oil extracted from *Azadirachta indica* seeds (also called chinaberry or neem). It is an ecdysone agonist.

[26] "Secondary metabolite" is a term used for a compound that is not directly involved in the development of plants (in the broad sense), but rather that intervenes in the relationships with biotic and abiotic stresses or improves the effectiveness of reproduction or defence, etc.

A concentration of 100 mg AI/L azadirachtin did not induce deaths in 24 h in adult bees (Akca *et al.* 2009). Larvae of bees treated by contact with 0.5 µL methanol containing 0.25 and 0.50 µg azadirachtin showed reduced survival rates versus controls. Only the last concentration induced decreased weight gain compared to controls (Rembold *et al.* 1982).

Thompson *et al.* (2005) used a feeder containing a sucrose solution of azadirachtin (1 mg AI/L) to experimentally contaminate colonies. They estimated that this was equivalent to 0.067 µg azadirachtin/cell in the brood. No apparent adverse effect was observed on the development of colonies, but 4 of 5 treated colonies did not survive overwintering.

Two formulations of azadirachtin were tested in tunnel conditions on bee microcolonies. Granules of NeemAzal were added to rapeseed seeds at the time of planting (77 g/15 m^2, i.e. twice the recommended dose) or azadirachtin was sprayed (1.5 mL/15 m^2) at the flowering stage. The (systemic) granule formulation did not have effects on mortality, foraging activity, and brood development, while the spray treatment had adverse effects on brood development and reduced foraging activity (Shawki *et al.* 2005).

3.1.2.3 Fungicides and herbicides

3.1.2.3.1 Effects of fungicides

One study described abnormal bee bread, called "entombed" bee bread, found in collapsed colonies and in which the fungicide chlorothalonil was quantified at a mean concentration of 1.3 mg/kg. The authors assumed that fermentation did not occur correctly. Fungicides are known to have an impact on the colony by altering the microflora present in food reserves or in the digestive tract of bees (Batra *et al.* 1973). A study showed a positive correlation between the number of pesticide residues and symptomatic colonies (Simon-Delso *et al.* 2014). Pettis *et al.* (2013) showed a positive correlation between the presence of *Nosema ceranae* and that of fungicides (chlorothalonil and pyraclostrobin).

The fungicides chlorothalonil and myclobutanil, like imidacloprid, increase cellular mortality in the gut (Gregorc and Ellis 2011).

The synergistic effect of certain fungicides (imidazoles or ergosterol biosynthesis inhibitors) in combination with insecticides-acaricides is described in section 4.1.2.2 of this report.

3.1.2.3.2 Exposure of bee colonies to fungicides and herbicides

- ***Current situation***

Bees are exposed to the fungicides and herbicides found in the food that they consume, such as nectar, pollen and bee bread, as well as those that they collect in the atmosphere during their foraging activity. As a result, all the members of the colony are likely to be exposed to these substances (EFSA 2012a) and studies carried out in Europe and the United States show regular exposure of bees to fungicides and herbicides.

In France, surveys performed for three years in five *départements* in which 41 pesticides, including 11 fungicides, were assessed, showed that 16% of 181 pollen samples, 9% of 305 bee samples, 1.1% of 93 wax samples, and 0.7% of 140 honey samples analysed contained at least one fungicide (Chauzat *et al.* 2009). The main fungicides quantified in pollen during these surveys were penconazole, flusilazole, tebuconazole, cyproconazole, myclobutanyl and hexaconazole and for which mean concentrations between 10 and 20 µg/kg were calculated. The fungicides quantified in bees were penconazole, tebuconazole, and tetraconazole with mean concentrations ranging from 5 to 20 µg/kg (Chauzat *et al.* 2009). The survey included an analysis of the frequency of co-detection of fungicides and revealed that bees and pollen are frequently contaminated by several fungicides or are contaminated by a fungicide associated with another pesticide (e.g. imidacloprid). In this study, no herbicides were investigated.

More recently, between 2008 and 2009, a survey on bee, pollen and honey contamination was carried out in 5 apiaries located between Brittany and Pays de la Loire. Of the 22 fungicides screened for, 5 were found in the 141 bee samples (benalaxyl, carbendazim, flusilazole,

propiconazole and thiophanate-methyl), 9 in the 120 pollen samples (bupirimate, carbendazim, cyproconazole, diethofencarb, flusilazole, iprodione, thiophanate-methyl, triadimenol and vinclozolin), and 9 in the 141 honey samples (bupirimate, carbendazim, cyproconazole, diethofencarb, flusilazole, imazalil, prochloraz, tebuconazole and thiophanate-methyl) (Lambert *et al.* 2013). Carbendazim was the most commonly found fungicide with a detection frequency of 41% in bees, 64% in honey, and 34% in pollen. For all the fungicides detected, mean calculated concentrations in these matrices were lower than the quantities found by Chauzat *et al.* (2009) (example: < 10 µg/kg), except for thiophanate-methyl with a mean concentration of 23 µg/kg of honey. In this study, no herbicides were investigated.

In France, during the 2014 beekeeping season, the presence of pesticide residues in pollen collected by colonies was studied in five sedentary apiaries (Vidau 2015). The 165 samples of trapped pollen collected were analysed using a multiple residue method that enabled detection of more than 400 substances (LOQ = 10 µg/kg). The analytical results revealed that 72% of the samples contained less than one pesticide residue and that about 25% contained five or more. Sixty-six substances were detected, including 32 fungicides, 23 insecticides, 8 herbicides, and 3 growth regulators. The most commonly found residues included a pesticide, chlorpyrifos-ethyl (27.9%), two fungicides, fludioxonil (17.6%) and cyprodinil (16.4%), a juvenile hormone analogue, fenoxycarb (14.5%), and a herbicide, pendimethalin (10.9%). The mean concentrations of these substances were usually between 10 and 250 µg/kg, but concentrations that can exceed 500 µg/kg were sometimes found. The results of this study show a continuous but irregular exposure that is generally higher from late winter to early summer. This is primarily related to contamination of pollen by fungicides and insecticides.

In Spain, between 2006 and 2007, an overview of bee bread contamination was carried out in more than 1000 apiaries (Bernal *et al.* 2010). The analysis covered two years with 845 samples collected in the spring, and 176 in the autumn. For all the bee bread collected in the spring, 12 fungicides were found: procymidone, hexachlorobenzene, metalaxyl, difenoconazole, captan, myclobutanil, vinclozolin, chlorothalonil, propiconazole, azoxystrobin, iprodione and flusilazole. The frequency of detection of each fungicide was lower than 2% in the samples. Mean fungicide concentrations were between 67 and 2 µg/kg. Alongside fungicides, four herbicide residues were found: trifluralin, atrazine, simazine and imazamethabenz-methyl, detected in 9.7%, 2.9%, 1.9% and 0.4% of analysed bee bread, respectively. Over the two years, the mean calculated concentrations for the four herbicides were 3.2, 25.15, 43.0 and 9.5 µg/kg. The bee bread collected in autumn was on average less contaminated than that collected in spring, with no herbicide and only two fungicides detected: hexachlorobenzene and vinclozolin in 1.13% of samples. The authors of this study also noted higher contamination in migratory colonies than in colonies belonging to sedentary apiaries.

In Belgium, Nguyen *et al.* (2009) compared the survival of bee colonies placed in environments where maize crops treated with imidacloprid were present or absent. Between 20 August and 20 October 2004, in each of 16 apiaries across the country, three colonies were sampled. In the 48 samples of wax, bees and honey collected, two fungicides and one herbicide were found: flusilazole, trifloxystrobin, and bitertanol respectively in 14.6%, 12.5% and 2.1% of honey, and in 31.3%, 8.4% and 4.2% of wax. However, no fungicide or herbicide was detected in bees.

In the United States, a survey was carried out between 2007 and 2008 by Mullin *et al.* (2010). In this study, 350 samples of bee bread, 140 samples of bees, and more than 200 samples of wax were collected over the beekeeping season analysed. Findings showed higher contamination than that described in France in the studies by Chauzat *et al.* (2009) and Lambert *et al.* (2013), since 63% of wax, 61% of pollen and 13% of bees contained at least one fungicide (Mullin *et al.* 2010). Among the investigated fungicides, 25 residues were detected at least once in pollen, 23 in wax, and 6 in bees, highlighting the wide range of fungicides bees are exposed to. In wax and pollen, the mean concentrations of several fungicides (examples: chlorothalonil, boscalid, captan and iprodione) sometimes exceeded 100 µg/kg. This study also revealed the presence of herbicides in beekeeping matrices. The detection frequency for herbicides in wax, pollen and bees was

respectively 41.8%, 50.3% and 6.4%. A lower number of herbicides are found in wax and pollen compared to fungicides, since 11 and 13 herbicides were detected in wax and pollen, respectively. Like for fungicides, six herbicides were found in bees. Mean herbicide concentrations measured in wax were overall less than 10 µg/kg, except for ethofumesate with a mean concentration of 392 µg/kg. In pollen, mean herbicide concentrations were higher than those calculated for wax since they regularly exceeded 10 µg/kg. Mean herbicide concentrations found in bees were between 2.2 and 15.9 µg/kg.

- ***Case study***

More targeted studies have also been performed with the aim of evaluating bee exposure after application of fungicides to rapeseed crops (Wallner 2009), apple trees (Kubik *et al.* 2000), and cherry trees (Kubik *et al.* 1999).

Wallner (2009) examined the contamination of pollen and crop nectar sampled from foragers in 14 colonies placed near flowering rapeseed fields (from seeds coated with clothianidin) and treated with boscalid 250 g/ha for 7 days. Boscalid was detected in 22 samples of pollen analysed (pooled pollen loads harvested from 150 - 200 foragers). The mean measured concentrations in pollen were respectively 13.9 mg/kg on the day of treatment, 26.2 and 4.7 mg/kg the day after, and the third day after treatment, and reached 3 mg/kg one week later. The nectar collected by foragers was also contaminated by boscalid over the entire study period. Measured concentrations were respectively 1.43 mg/kg on the day of treatment, 0.13 mg/kg and 0.017 mg/kg the day after and the third day, then 0.025 mg/kg one week later. In this study, clothianidin from the coating of rapeseeds planted was also co-detected in crop nectar from foragers at concentrations ranging from 0.001 to 0.003 mg/kg.

Similar studies were performed on bee colonies located near flowering orchard fruit trees (Kubik *et al.* 1999; Kubik *et al.* 2000; Smodiš Škerl *et al.* 2009). In the study carried out by Kubik *et al.* (2000), the formulations sprayed over 10 ha of flowering apple trees contained the fungicides captan (1000 g/ha) and difenoconazole (50 g/ha) and the matrices analysed were honey, pollen loads from apple trees, and bee bread. Analysis of pollen contamination showed persistence of fungicides 13 days after treatment. Captan concentrations measured in pollen for the whole period studied were systematically higher than those for difenoconazole. For both fungicides, peak concentrations in trapped pollen were observed on the third day following treatment. The maximum concentrations of captan and difenoconazole residues measured in these pollens were 18.9 mg/kg and 0.166 mg/kg, respectively. Bee bread and honey were also contaminated with the two fungicides 14 days after spraying. On average, the honey stored in the 10 colonies included in the study contained difenoconazole and captan at respective concentrations of 0.6 µg/kg and 9 µg/kg. Bee bread obtained from pollen was more contaminated and contained mean difenoconazole concentrations of 270 µg/kg and captan concentrations of 6.5 mg/kg.

In the study by Kubik *et al.* (1999), the colonies were placed at the centre of a cherry tree orchard with an area of 4.5 ha at the start of flowering. The treatments carried out in the orchard contained methyl thiophanate (0.7 kg/ha) and iprodione (0.7 kg/ha) for the first treatment, and methyl thiophanate (0.7 kg/ha), iprodione (0.185 kg/ha) and vinclozolin (0.375 kg/ha) for the second treatment 6 days later. The analysed matrices were pollen loads from cherry trees, honey and bee bread. Pollen contamination was assessed daily for 14 days. Over this period, the pollen analysed regularly contained the three fungicides with mean concentrations of methyl thiophanate, vinclozolin and iprodione of 0.25, 0.12 and 0.009 mg/kg, respectively. Peak contamination was observed 11 days after the first treatment: methyl thiophanate at 4 mg/kg, vinclozolin at 3 mg/kg and iprodione at 0.5 mg/kg. Honey and bee bread were collected in five colonies, 14 days after the first treatment and contained mean concentrations of 58.9 +/- 17.1 µg/kg of methyl thiophanate, 107.0 +/- 43.6 µg/kg of vinclozolin and 23.1 +/- 5.4 µg/kg of iprodione for honey, and 1.9 +/- 1.0 mg/kg of methyl thiophanate, 23.6 +/- 7 mg/kg of vinclozolin, and 3.0 +/- 1.4 mg/kg of iprodione for bee bread.

Smodiš Škerl *et al.* (2009) compared contamination of trapped pollen and bee bread collected in colonies placed in an apple tree orchard with those of the same matrices from an area in which orchard plantations are absent. The orchards were treated with formulations containing diazinon

(15 L/ha), difenoconazole (0.2 L/ha) and thiacloprid (0.2 L/ha). Contamination of pollen by diazinon was highest the day after treatment (1.98 mg/kg) then decreased quickly to reach 0.03 mg/kg 10 days after treatment. In bee bread collected 16 days after treatment, a diazinon concentration of 0.09 mg/kg was found. Difenoconazole and thiacloprid, sprayed as a mixture on the orchard, were found the next day in pollen loads at concentrations of 0.01 and 0.09 mg/kg. Contamination of bee bread by these two compounds was not assessed. In pollen loads and bee bread collected in the control colonies, these three compounds were not examined.

3.1.2.4 Antibiotics

3.1.2.4.1 Regulatory aspects

Within the European Union, in accordance with Regulation (EC) No 470/2009 and Commission Regulation (EU) No 37/2010, no maximum residue limits (MRLs) have been defined for antibiotics in beehive products (honey, royal jelly). As a result, *no antibiotics are authorised for use in bees,* and detection of any antibiotic residue in honey prohibits sale in EU countries. However, as per the cascade principle, any veterinarian could theoretically prescribe antibiotics to treat bees using an antibiotic approved for use in another animal species. The prescribing veterinarian then has the responsibility to indicate the dose, the duration, the method of application, and the withdrawal period. In practice, no dose, and no withdrawal time have been officially established. We should note that use of oxytetracycline is authorised in the United Kingdom in the cascade context for the treatment of European foulbrood with a withdrawal time of at least 6 months (EMA 2010). Moreover, a temporary authorisation for use of fumagillin to control *Nosema* spp. was granted in Spain from 2005 to 2007 and in the United Kingdom until recently. These exceptions have been abandoned.

Conversely, in the United States, oxytetracycline, tylosin and more recently lincomycin were registered for the treatment of American foulbrood with an MRL of 200 µg/kg and a withdrawal time of 4 weeks (tylosin, lincomycin) and 6 weeks (oxytetracycline) (USFDA 2014). Tylosin was also approved in Canada for the same indication with an MRL of 200 µg/kg. However, the benefit of using antibiotics to control American foulbrood has been called into question since antibiotics are inactive on the highly resistant spore forms of its aetiological agent, *Paenibacillus larvae*. Fumagillin is authorised in the United States and Canada. To reduce residues, treatment is not allowed during the foraging season (USFDA 2012). Hives are usually treated preventively once in late autumn and once in early spring (Webster 1994). Fumagillin persists within the hive (Higes *et al.* 2011), and degrades over time (Nozal *et al.* 2008).

Bicyclohexylammonium fumagillin is an antibiotic isolated from the *Aspergillus fumigatus* fungus and was the only treatment used broadly against nosemosis in European honeybees *Apis mellifera* (Bailey 1953; Higes *et al.* 2011) for about 60 days (Higes *et al.* 2011). Fumagillin, in a 3% concentration for veterinary use, is considered to be the only effective treatment for infection with *Nosema apis*. It also eliminates the more recently discovered pathogenic microsporidian *N. ceranae* (Williams *et al.* 2008), but its efficacy has been challenged (Botías *et al.* 2013; Huang *et al.* 2013; Williams *et al.* 2011). Fumagillin is no longer authorised in the European Union since its MA was suspended in the absence of an MRL established at the European level.

3.1.2.4.2 Use of antibiotics in bees outside the EU

Control of foulbrood relies on destruction of infectious sites. Most countries recommend destruction of diseased brood and decontamination of the infected frames and hives by fire, after transferring the colony of adult bees to new hives. In some countries outside the European Union (United States, Canada, Argentina), treatment with antibiotics is authorised to control these diseases. In these countries, the main antibiotics used in beekeeping for brood diseases are tetracyclines, streptomycin, sulfonamides and chloramphenicol (Al-Waili *et al.* 2012).

It is important to point out the limitations of antibiotic treatment in foulbrood disease. Antibiotics act by blocking the metabolism of bacteria. However, they must only be used to combat bacteria in the active phase of multiplication, which on the clinical level corresponds in practice to the acute phase of infectious disease. No anti-infectious effect can be expected on resistance forms (spores) or on

bacteria in the latency phase. Another consequence is that the infectious site is not destroyed by antibiotic treatment and remission may be observed with treatment, but the infection may recur once the antibiotic's inhibitory effect is no longer active (*Table 10*).

In the case of American foulbrood (AF), when infection with *Paenibacillus larvae* is moderate, transferal[27] is recommended as the method of treatment (Reybroeck *et al.* 2012; von der Ohe 2003). AF requires strict sanitary measures: all the frames in the colony are incinerated and the body of the hive disinfected and flame-treated. Sulphonamides have been used against severe forms, particularly sulfathiazole and tetracyclines. Some authors (Kochansky *et al.* 2001; Okayama *et al.* 1996) reported anti-bacterial activity against *P. larvae* with lincomycin. Its efficacy against AF has been demonstrated by Feldlaufer *et al.* (2001). Various studies have shown the effectiveness of tylosin in the control of AF (Peng *et al.* 1996). Tylosin was used against AF once it was found that *P. larvae* had acquired resistance to tetracyclines (Reybroeck *et al.* 2012). Erythromycin was first tested in 1955 (Katznelson 1956; Katznelson *et al.* 1955). According to certain authors, erythromycin was found to be effective against AF (Machova 1970; Okayama *et al.* 1996), while others found it ineffective (Alippi *et al.* 1999; Katznelson *et al.* 1955; Moffett *et al.* 1958). Other studies found greater efficacy of penicillin and macrolides than tetracyclines against *P. larvae* (Leighton 1983).

For European foulbrood (EF), sulphonamides had no effect. Some antibiotics, for example oxytetracycline, showed their efficacy against EF. Streptomycin and tetracyclines have been used to combat this disease. Destruction and elimination of diseased combs is mandatory, irrespective of beekeeping practices. The efficacy of erythromycin against EF has been described by some authors (Wilson 1962; Wilson and Moffett 1957). Gunes *et al.* (2008) report use of this substance in southern Turkey by professional beekeepers.

Nosemosis, a fungal disease (see 3.1.1.2.3.1), is also treated with antibiotics outside the European Union. Katznelson and Jamieson (1952) found fumagillin to be effective against nosemosis. Bicyclohexylammonium fumagillin, an antibiotic isolated from the *Aspergillus fumigatus* fungus, was the only treatment used for the control of nosemosis in European honeybees, *Apis mellifera* (Bailey 1953; Higes *et al.* 2011) for about 60 days (Higes *et al.* 2011). According to Williams *et al.* (2008), fumagillin is considered to be an effective treatment for *Nosema apis* and *N. ceranae* infection. However, its efficacy has been questioned (Botías *et al.* 2013; Huang *et al.* 2013; Williams *et al.* 2011). In a recent publication, Huang *et al.* (2013) showed that fumagillin altered the protein structure of intestinal tissue in bees at concentrations that did not inhibit reproduction of microsporidians. In Chile, some beekeepers have also used sulphonamides against nosemosis (Lourdes 2002).

3.1.2.4.3 Consequences of antibiotic use in hives

- **Specific problem of residues in the hive**

The specific methods of administering medicinal products in beekeeping lead to a particular problem of residue in this production sector. Antibiotics applied in the hive rarely undergo metabolism (Table 4). As a result, the residues are not eliminated after a certain amount of time, like the usual withdrawal times established for veterinary medicinal products administered directly to animals. Furthermore, no maximum residue limit (MRL) has been established for honey and royal jelly, products consumed by humans. Therefore, no veterinary product containing antibiotics is authorised for the bee species in Europe, unlike in the United States where some of these antibiotics are approved. In the USA, oxytetracycline, tylosin, fumagillin, and lincomycin are used under certain conditions.

Following antibiotic treatment, residues may be found in the hive products, particularly in honey. The presence of these residues can lead to selection of resistant strains and increase the frequency of resistance of pathogens, both in humans and animals (Al-Waili *et al.* 2012). In fact, in the United States and Argentina, intensive and repeated use of tetracyclines has led to the

[27] Transferal: beekeeping practice involving collection of adult bees from a hive and transfer to a new hive fitted with empty formed frames or embossed wax frames.

selection of tetracycline-resistant strains of *P. larvae* (Reybroeck *et al.* 2012). Following treatment failures related to selection of resistant strains, tetracyclines were replaced by tylosin which has become more widely used.

Several studies have shown persistence of various antibiotics in honey (Adams *et al.* 2009; Granja *et al.* 2009; Martel *et al.* 2006). One study demonstrated increased concentrations of sulfamethazine in wax along with a simultaneous increase in residues of this compound in honey (Reybroeck 2003). In addition, persistence of sulfathiazole in wax 12 months after the last application in powder form was recently reported (Martinello *et al.* 2013). In a survey of 3855 honeys of various origins, 1.7% of samples presented antibiotic residues: streptomycin, sulphonamides, tetracyclines, chloramphenicol, nitrofurans, tylosin and quinolones (Diserens 2007).

Also, in some cases, antibiotics have been used in agriculture, for example streptomycin against fire blight on fruit trees. The antibiotics used as plant protection products can be collected by foragers when they visit flowers and thus contaminate honey. In 2001, 21% of German honeys contained streptomycin (Bogdanov 2006; Brasse 2001). Agricultural use of antibiotics including streptomycin and oxytetracycline is currently prohibited in Europe.

Lastly, trade networks for beehive products are highly globalised. Europe imports honey from various continents where the regulations on MRLs are very different to those of the European Union. In a literature review, Bogdanov (2006) reported that 20 to 50% of imported honey in France, Belgium and Switzerland contained antibiotic residues, mainly streptomycin (in honey mainly from Mexico), sulphonamides (mainly in honey from Turkey), but also chloramphenicol (mainly in honey from China), as well as tetracyclines.

Table 4: Antibiotic residues in honey (Bogdanov, 2006)

Antibiotics	References
Sulfonamides	
sulfathiazole, sulfamerazine, sulfamethazine, sulfamethaxazole, sulfadiazine, sulfamethoxypiridazine, sulfadoxine, sulfadimidine, sulfanilamide	(Martel and Zeggane, 2003; Reybroeck, 2003; Wallner, 2003; Kaufmann and Känzig, 2004)
Aminoglycosides	
streptomycine, dihydrostreptomycine	(Morlot and Beaune, 2003; Reybroeck, 2003; van Bruijnsvoort et al., 2004)
Tetacyclines	
tetracycline, oxytetracycline, chlortetracycline, doxycycline	(Argauer and Moats, 1991; Tantillo et al., 2000; Morlot and Beaune, 2003; Reybroeck, 2003; Sabatini et al., 2003)
Amphenicols	
chloramphenicol	(Dharmananda, 2003; Reybroeck, 2003; Verzegnassi et al., 2003; Ortelli et al., 2004)
Macrolides	
tylosine	(Baggio et al., 2004; Feldlaufer et al., 2004)
myrosamine	(Nakajima et al., 1998)
Beta-lactams	
penicillins	(Nakajima et al., 1997; Reybroeck, 2003)
Nitrofuran metabolites	(Stiftung Warentest, 2004; Jenkins and Young, 2005)
AOZ, SC	

AOZ: 3-amino-2-oxazolidinone; SC: semicarbazide.

Contamination of royal jelly is also possible through antibiotic residues (Matsuka and Nakamura 1990). Chloramphenicol residues were detected in royal jelly produced in China (Dharmananda 2003; Reybroeck 2003).

Use of fluoroquinolones is increasing in Asia (Savoy Perroud *et al.* 2009) and the main residues found are enrofloxacin and norfloxacin.

Analyses of nitrofurans in honey show that furazolidone is the main nitrofuran administered to combat bee diseases (Khong *et al.* 2004).

In 2007, Zhou *et al.* reported from China that five nitroimidazoles were used in the previous years to combat *Nosema apis*, as an alternative to fumagillin. Since then, use of these compounds has been prohibited in China. The main residue found in Chinese honey was metronidazole (Zhou *et al.* 2007).

Most antibiotics are stable in honey, others degrade (Table 5 and

Table 6).

Table 5: Marker residues for antibiotics used in beekeeping (Reybroeck *et al.*, 2012)

Pharmacologically active substance	Metabolite	Marker residue[a]
Streptomycin	–	Streptomycin
Tetracyclines	Epimers	Sum of parent drug and its 4-epimers
Sulfonamides	–	Parent drug
Erythromycin	–	Erythromycin A
Tylosin	Desmycosin (tylosin B)	Tylosin A
Lincomycin	–	Lincomycin
Enrofloxacin	–	Sum of enro- and ciprofloxacin
Ciprofloxacin	–	Sum of enro- and ciprofloxacin
Trimethoprim	–	Trimethoprim
Metronidazole	–	Metronidazole
Chloramphenicol	–	Chloramphenicol
Furazolidone	AOZ (3-amino-2-oxazolidone)	AOZ (3-amino-2-oxazolidone)
Nitroimidazoles	Hydroxymetabolites	Hydroxymetabolites

[a] Commission Regulation (EU) No. 37/2010.

Table 6: Half-life ($t_{1/2}$) of selected antibiotics in honey (Reybroeck *et al.*, 2012)

Pharmacologically active substance	Temperature – storage	Half-life	Reference
Tetracycline hydrochloride	20 °C – lab	242 days	Martel et al. (2006)
	35 °C – lab	121 days	Martel et al. (2006)
	in bee colony	65 days	Martel et al. (2006)
Oxytetracycline	34 °C – lab	12 days	Argauer and Moats (1991)
	in bee colony	2–4 days	Gilliam and Argauer (1981a)
	in bee colony	9–44 days	Thompson et al. (2006)
	in bee colony	11–14 days	Anon. (2002)

- **Effects of antibiotics in bees as reported in the literature**

Before discussing the possible toxic effects of antibiotics on bee health and in consumers of beehive products, it is important to mention that use of antibiotics may create selection pressure promoting the emergence of bacterial strains that are resistant to the compounds found in the environment. This can led to therapeutic failure, both in animals and humans. An example is the broad use of oxytetracycline for the treatment of foulbrood: several studies have shown an increase in the frequency of resistance in *Paenibacillus larvae, Melissococcus plutonius* and *Streptococcus pluton* in the United States (van Engelsdorp and Meixner 2010).

Regarding the toxic effect itself, infection of bees by *Nosema apis* leads to atrophy of the hypopharyngeal glands. In infected bees treated with fumagillin, ultra-structural changes are found in secretion granules that are probably related to changes in the secretory activity of these glands (Liu 1990): the antibiotic appears to have inhibitor effects on the hypopharyngeal glands in infected bees.

Peng *et al.* (1992) have shown that with larval food containing 0.0025% chlortetracycline (CTC), larval mortality was similar to that observed for the control group. At this concentration, chlortetracycline decreased the mortality of larvae inoculated with 1×10^4 to 1.5×10^8 spores/mL of *Paenibacillus larvae*. However, concentrations higher than 0.0025% CTC delayed larval growth and development and led to early pigmentation in young larvae. At 0.05% CTC, the authors found 100% larval mortality. They considered that American foulbrood is controlled with 0.0025% CTC, even if high levels of pathogens are inoculated in larvae.

According to Peng *et al.* (1996), bee larvae can tolerate doses of 0.005 to 0.05% of tylosin in their food without negative effects being observed. A 200 mg terramycine and 100 mg tylosin mix protected colonies for 3 weeks. A dose of 200 mg tylosin protected the colony for an additional week. Doses of 100 mg tylosin eliminated the clinical signs of AF infection. Among the antibiotics, penicillins, erythromycin and tylosin appear to be the most effective, unlike tetracyclines. Tylosin is

more effective than sulfathiazole in the treatment of American foulbrood. However, at tylosin doses of 0.5% or more, larval mortality increases. The few larvae that survived following applications of 0.5 and 1% did not continue their development to the adult stage. In the group fed with 0.03% tylosin, fewer deaths were observed compared to the other groups (controls and groups treated at different doses), and arrival at the adult stage was more common in the group fed with 0.03% tylosin. These authors indicate that colonies fed with 200 mg tylosin are protected for 4 weeks but they point out that residues can be found in honey following this treatment.

Chloramphenicol acts on insect proteins (Ashour *et al.* 1980; Fragouli-Fournogeraki *et al.* 1978). Including 0.5 g/L chloramphenicol (1.6 mM) in the diet led to a significant decrease in protein concentrations of bee haemolymph, from the second to fifth day after the start of treatment (Bounias *et al.* 1982). After 16 days, adding chloramphenicol to food decreased the mortality rate from 21 to 2% in the case of bees receiving sucrose and from 50 to 45% in the case of trehalose.

In an older study, Gilliam *et al.* (1974) reported that the presence of yeasts may be an indicator of stress conditions. Antibiotics decrease intestinal bacterial flora in bees and increase the frequency of yeasts. The combination of oxytetracycline and fumagillin decreased not only bacterial flora but also fungal flora. More recently, Flores *et al.* (2004) studied the possible role of excessive use of oxytetracycline as a condition promoting the development of chalkbrood at three temperatures (25, 30 and 35°C). No significant difference was observed between the treated and non-treated colonies in broods maintained at 25, 30 and 35°C. However, significant differences were observed at the start of the study in broods maintained at 25°C: the percentage of chalkbrood was higher in the presence of oxytetracycline. The researchers believe that in these conditions, oxytetracycline may disrupt the balance in gut microflora in bees, promoting growth of *Ascosphaera apis* (Menapace and Wilson 1979), naturally leading to occurrence of chalkbrood. They conclude that in the conditions of their short and medium-term study, the presence of oxytetracycline does not lead to a major risk of brood mycosis. Nonetheless, they consider that it is important to assess the effects of using oxytetracycline long-term in the colonies.

3.1.2.5 Antiparasitic treatments against *Varroa*: toxic effects in bees

Beekeepers need to use anti-*Varroa* treatments (Rosenkranz *et al.* 2010) that must be toxic for the parasites but with the fewest possible adverse effects in bees. This is a major difficulty given the susceptibility of bees to many pesticides (Atkins 1992).

Varroacides used worldwide can be divided into three categories: organic synthetic compounds, natural products, and organic acids (see review in Johnson *et al.* (2010)).

3.1.2.5.1 Organic synthetic pesticides

- ***Tau*-fluvalinate (Apistan®)**

Tau-fluvalinate is a pyrethroid containing two of the four isomers of the racemic mixture fluvalinate (EMEA 1995). It was the first synthetic varroacide to be authorised in beekeeping in the United States (Ellis *et al.* 1998). It is available as plastic strips of 8 g containing 10% *tau*-fluvalinate. A single strip enables diffusion of the product for 8 weeks (Bogdanov *et al.* 1998b; Vita Europe Ltd 2009).

Like the other pyrethroids, *tau*-fluvalinate kills the mites by blocking voltage-gated calcium and sodium channels (Davies *et al.* 2007), prolonging sodium channel opening in nerve cells of the central nervous system and peripheral nervous system of these mites. Initially, it stimulates nerve cells and induces hyperexcitability, then paralysis and death of the mite. While most pyrethroids are very toxic in bees, they tolerate high concentrations of *tau*-fluvalinate, primarily as a result of rapid detoxification via cytochrome P450 monooxygenases (P450s) (Johnson *et al.* 2006). For *tau*-fluvalinate, the US Environmental Protection Agency (EPA) expects an acute toxicity risk in non-target insects because of the high toxicity of the product in bees, whose acute contact LD_{50} is 0.2 µg/bee (EPA 2005). In adult bees, an increase in mortality related to *tau*-fluvalinate was estimated at 2.7 bees/day for 60 days (Frilli *et al.* 1991).

In agriculture, a presentation of *tau*-fluvalinate is available as an aqueous emulsion that is used widely by beekeepers to soak wood panels that are then suspended between the frames of the brood. This use in hives, which is not authorised but inexpensive, may contribute to the presence of *tau*-fluvalinate residues detected in bee waxes (Berry 2009; Bogdanov 2006; Mullin *et al.* 2010; Wallner 1999).

Tau-fluvalinate is not harmless in bees and affects the health of reproduction castes. In one study, queen bees exposed to high doses of *tau*-fluvalinate were smaller than non-treated queen bees (Haarmann *et al.* 2002). In queen bee cages, contact exposure for 3 days at 1% *tau*-fluvalinate led to significant mortality in accompanying worker bees and increased supersedure among queen bees. Exposure for 7 days led to significant mortality among queen bees (Currie 1999).

In colonies treated with Apistan®, the percentage of emerging males (86%) was significantly lower than in non-treated colonies (97%). However, in both cases, the survival rate was higher than that of colonies infested with *Varroa* (59%). A decrease in the weight of drones and of several glands was found in colonies infested with *Varroa* and in colonies treated with Apistan®. Drones exposed to *tau*-fluvalinate during their development less commonly survived the period of sexual maturity than non-exposed drones. Their weight was lower as was their sperm production (Rinderer *et al.* 1999).

The practical consequences of exposure of drones to *tau*-fluvalinate seem to be limited since exposed insects had the same reproductive abilities as non-exposed individuals (Sylvester *et al.* 1999).

Three experiments were conducted on queen bees and worker bees to investigate the effects of Apistan®. Workers were placed in bee packages (each group weighing 1.4 kg) and treated for 5 days with a strip (2.5 x 13 cm) containing *tau*-fluvalinate (at 2.5%), without any increase in mortality. Egg-laying queen bees after overwintering (n = 30) and queen bees that recently mated (n = 60) were treated for 5 days with Apistan® (Apistan® 1% queen tablets) and kept in Benton cages: all deaths of queen bees were observed on the fourth and fifth days of treatment, i.e. after the recommended treatment duration of 3 days. None of the treated queen bee groups showed a significant increase in mortality. However, in the second test, workers showed a significant increase in mortality during treatment. No difference was observed concerning acceptance of queens, brood viability, or supersedure rates, two and six months after exposure (Pettis *et al.* 1991).

Initially, *tau*-fluvalinate was highly effective in controlling *Varroa*, but resistance developed in several populations of this parasite (Lodesani *et al.* 1995). This resistance was due, at least partly, to mutation of the voltage-gated sodium channels leading to lower binding affinity for *tau*-fluvalinate (Wang *et al.* 2002). Despite decreased efficacy, *tau*-fluvalinate is still used to control *Varroa* in Europe and in the United States (Elzen and Westervelt 2002; Macedo *et al.* 2002; Rosenkranz *et al.* 2010).

Lastly, we should remember that *tau*-fluvalinate is widely used in agriculture as an insecticide. As a result, its presence in beekeeping matrices is due to deliberate acaricide treatment by beekeepers and/or treatments outside the hive that contaminate pollen and/or nectar. This phenomenon was described by Paradis *et al.* (2013) on analysis of nectar collected by foragers in spring in the Vendée *département*. Although hives had not been treated with *tau*-fluvalinate, levels of up to 69.2 µg/kg were found in fresh honey.

- **Amitraz (Apivar®)**

Amitraz is a pesticide belonging to the formamidine family registered for the first time in 1992 in the United States under the brand name Miticur®. The active substance was added to plastic strips suspended between the frames of brood (PAN 2009). However, the product was withdrawn from the market in 1994 since beekeepers reported colony losses after treatment (PAN 2009). This decision was made in the absence of proof confirming that the product led to these losses (PAN 2009). In Europe, amitraz strips (Apivar®) were authorised in 1998 for the control of *Varroa*.

Amitraz is an octopaminergic agonist in arthropods (Evans and Gee 1980) and is therefore able to act on bee behaviour. Elevated levels of octopamine in the bee brain have been associated with increased exploring/foraging behaviour. In addition, young bees fed on octopamine were more

likely to initiate foraging than non-treated bees (Schulz and Robinson 2001). Foraging bees treated with octopamine increased the value of resources collected when they communicated through dancing (Barron *et al.* 2007).

Acute toxicity of amitraz was also observed in larvae, which presented higher apoptosis of cells in the midgut after being exposed to an amitraz solution (Gregorc and Bowen 2000).

In the United States, populations of *Varroa* display resistance to amitraz, possibly due to high esterase-mediate detoxification (Sammataro *et al.* 2005). The mechanism of resistance in *Varroa* may be similar to the resistance to detoxification of amitraz observed in some populations of cattle ticks (Li *et al.* 2005).

Amitraz has relatively low toxicity in bees (Briggs 1992; Thomson 1983) with an LD_{50} of 12 µµg/bee by ingestion and 3.6 mg/L by direct spraying (The Agrochemicals Handbook Third Edition 1994).

- **Coumaphos**

The low toxicity of coumaphos in the form of Périzin® (product withdrawn from the market) in bees was established by the manufacturer, with an LD_{50} of 14.39 µg/bee (Klochko *et al.* 1994). With Périzin®, increased adult bee mortality was estimated to be 15.7 bees/day after 7 days.

Coumaphos is an organophosphate pesticide used to control *Varroa* and to treat the small hive beetle *Aethina tumida*. In the European Union, but not in France, only Checkmite+® strips are approved for the control of *Varroa*. These strips that contain about 600 mg coumaphos are suspended between the frames of brood for 6 weeks. Coumaphos, or its bioactive metabolite coumaphos oxon, acts by inactivating acetylcholinesterase, thus interfering with nerve impulses.

Initially, coumaphos was found to be effective in treating *tau*-fluvalinate-resistant *Varroa* populations (Elzen *et al.* 2000). However, from 2001, coumaphos-resistant *Varroa* populations were detected (Elzen and Westervelt 2002; Pettis *et al.* 2004; Spreafico *et al.* 2001). The mechanism of resistance in *Varroa* to coumaphos is unknown, although a detoxification mechanism mediated by esterase has been suggested (Sammataro *et al.* 2005). This resistance could be related to mechanisms involved in resistance in cattle ticks, *Rhipicephalus microplus*, that include insensitivity to acetylcholinesterase and increased detoxification metabolism (Li *et al.* 2005).

Bees tolerate therapeutic doses of coumaphos partly via a detoxification mechanism involving enzymes produced by cytochromes P450 (Johnson *et al.* 2009). Nonetheless, exposure to coumaphos may lead to adverse effects. Young bee larvae were transferred to cups containing known concentrations of coumaphos (0 to 1000 mg/kg). These larvae were placed in queenless colonies and examined 10 days later to determine the rate of rejection or acceptance, as indicated by a mature sealed queen cell. No queen developed at 1000 mg/kg, and more than 50% of queen bees were rejected at 100 mg/kg. Moreover, queens that survived exposure at 100 mg/kg coumaphos had a significantly lower weight than that of control queens (Pettis *et al.* 2004). Queen bees exposed chronically to 100 mg/kg coumaphos incorporated in bee wax did not develop (Collins *et al.* 2004).

Developing queens in colonies treated with a single soaked strip of coumaphos for more than 24 h showed a high mortality rate. Several queens presented sub-lethal effects, particularly physical anomalies and abnormal behaviour. Queen bees exposed to coumaphos had significantly lower weight to that of queen bees in the control group. The weight of their ovaries was lower than that of control insects (Haarmann *et al.* 2002).

The mean coumaphos residue content in bee samples from 120 French hives in open field conditions was 1545.6 µg/kg. There was no direct link between the detected residue levels in bees and other matrices and colony mortality (Chauzat *et al.* 2009).

An apiary with *A. mellifera carnica* colonies developed anomalies 4 h after installation of coumaphos strips (Checkmite+®): the bees started to leave the hives, fly extensively around them, cluster in front of the flight board, and drop down on the grass in front of the hives. Worker bees formed small groups of 10 to 40 bees and died around the treated hives, with extended wings and curved, trembling abdomens. Bees were also clustered at the back of the hives, and at the entrance. Brood frames were not adequately covered by workers and dead workers were found on

the hive bottom board. The quantities of coumaphos found in worker bee samples from the brood chambers, honey compartments and in front of the hives were 1771, 606 and 514 µg/kg, respectively. Tests for coumaphos were negative in workers from non-treated colonies. Adult bee populations were reduced by about one third in treated colonies (Gregorc 2012).

The viability of sperm was lower in drones treated with coumaphos used at the manufacturer's recommended doses (Burley *et al.* 2008). Exposure of drones to coumaphos during their development and sexual maturity significantly decreased the viability of sperm during the six weeks of observation. Viability decreased significantly from the first sample. It also dropped significantly from the fifth to the sixth week for all the treatments used (*tau*-fluvalinate, thymol) and in controls.

3.1.2.5.2 Products of natural origin

- **Thymol (Apilife Var®, Apiguard®, Thymovar®) and essential oils**

Varroacides containing natural products (Colin 1990; Imdorf *et al.* 1999a) gained in popularity when the efficacy of synthetic pesticides started to decrease (Rosenkranz *et al.* 2010).

Thymol and menthol, monoterpenoid components of essential oils, are used to control *Varroa* and *Acarapis woodi*, respectively. Thymol is the main component of Apilife Var® (strips), Apiguard® (gel) and Thymovar® (sponge or strip).

Varroacides containing essential oils are food additives that are "*generally recognised as safe*" (GRAS) for human consumption (Quarles 1996). However, monoterpenoids such as thymol and menthol are not necessarily safe for bees given that in plants, they are used as broad-spectrum pesticides (Isman 2006). In fact, of all the terpenoids tested by fumigation in bees, thymol and menthol were the most toxic (Ellis and Baxendale 1997). These monoterpenoids probably kill *Varroa* by binding to octopamine (Enan 2001) or to GABA receptors (Priestley *et al.* 2003).

Residue levels in the hive may result from the type and number of treatments and from the interval between the end of treatment and sampling. Given its lipophilic properties, thymol preferentially accumulates in wax: 662-4753 mg/kg (Bogdanov *et al.* 1998a) and 21.6-147.7 mg/kg (Floris *et al.* 2004). Several studies have shown that thymol may also accumulate in pollen, at 0.037-39.7 mg/kg (Rennich *et al.* 2012), and honey, at 2.07-7.54 mg/kg (Bogdanov *et al.* 1998a), 0.4-8.8 mg/kg (Floris *et al.* 2004), 0.75-8.2 mg/kg (Adamczyk *et al.* 2005) and 0.62-2.65 mg/kg (Nozal *et al.* 2002). Pollen and honey are the main components of the larval diet but the risk of exposure of larvae to thymol remains hypothetical. Nonetheless, an open field study has shown that Apiguard® affected the expression of genes involved in detoxification, immunity and development of adult bees at a higher level than *tau*-fluvalinate (Boncristiani *et al.* 2012).

Thymol is a valuable alternative to synthetic products for the control of *Varroa*. However, it accumulates in hive products and is thought to cause adverse effects in colonies, particularly in larvae. The effects of acute and chronic exposure to thymol on larvae raised *in vitro* and fed on contaminated food were studied and compared to theoretical larval exposure based on the quantity of pollen and honey consumed by larvae during their development.

Laboratory tests have shown that the LD_{50} - 48 h of thymol added to the diet of larvae was 0.044 mg/larva. The LC_{50} - 6 D was 700 mg/kg of food. A significant decrease in survival and larval weight was observed from 500 mg thymol/kg of food ($p < 0.0001$). Lastly, expression of vitellogenin, which reaches a maximum at the fifth instar, is delayed in individuals exposed to 50 mg thymol/kg of food ($p < 0.0006$). These results are 10 times higher than the theoretical exposure level. On the basis of thymol residue levels found in honey and pollen, these results suggest that contamination of food by thymol does not involve a major risk for the first larval instars (Charpentier *et al.* 2014b).

Mattila *et al.* (2000) applied Apiguard® in colonies to determine the effect of treatment on the capped brood before application (larval death) and on adult bees. When Apiguard® was applied after capping or when the larvae were 4-5 days old, emergence of adults subsequently was very high in treated colonies (95.5-100%) and non-treated colonies (92.3-100%). Higher mortality was observed in young larvae (less than 3 days) in treated colonies (74.4-87.0%) than in non-treated colonies (89.7-95.2%). Surviving adults were not affected by treatment with Apiguard®.

Although these are products of natural origin, they can have adverse effects in bees: treatment with thymol can result in elimination of brood (Floris *et al.* 2004; Marchetti *et al.* 1984) and increased mortality in queen bees (Whittington *et al.* 2000).

During an experiment testing Apilife Var®, high bee mortality was not observed (Imdorf *et al.* 1994). However, when used incorrectly, overdose may lead to significant bee losses. Small amounts of brood located near the strips may be eliminated by the bees (Imdorf *et al.* 1995a).

Adverse effects in bees after application of Thymovar® were more severe than those observed with Apilife Var® and Apiguard®. In all the tested hives, removal of brood and honey next to the Thymovar® application site was observed. Also, in apiaries located in northern Italy, a marked decrease in colony population and severe disruption of bees was reported. In particular, in one apiary, the test was suspended because of severe bee reactions to treatment including massive elimination of brood, suspended egg laying, and decreased population of adult bees (Baggio *et al.* 2004).

Imdorf *et al.* (1995b) studied dose-response relationships between several volatile acaricide substances and mortalities in bees and mites. For each test, two cages (Liebefeld) each with 100 bees and 20 to 40 *Varroa* were exposed to air contaminated with acaricides at different concentrations. After 72 h, a count of the dead bees and *Varroa* was performed. Concentrations of 5 to 15 µg/L for thymol, 50 to 150 µg/L for camphor, and 20 to 60 µg/L air for menthol led to mortality in *Varroa* of about 100% without particular loss of bees. A concentration of 240 µg/L eucalyptol led to 100% mortality in *Varroa*, but also 25% mortality in bees. Thymol was found to be the main varroacidal component of Apilife Var in different types of hives. Camphor and menthol also had effective varroacide properties. However, eucalyptol is not well suited to the treatment of *Varroa* since its evaporation rate is difficult to control. In addition, a small difference in its toxicity for *Varroa* and for bees was found.

Regarding essential oils, Hoppe (1990) evaluated the toxicity of these substances in bees by placing small cages of 20 bees in a 3-4 L closed glass recipient containing 10 µL of pure essential oil. Mortality in bees and *Varroa* were evaluated at 24, 48 and 72 h. After 72 h, 24 essential oils had led to a *Varroa* mortality rate greater than 90%. Among them, only nine induced a mortality rate in bees of less than 10%. After topical application, only three oils resulted in the same level of mortality, with a maximum observed effect at 48 h. This suggests that passive evaporation is the most appropriate application form for the essential oils and their components. In another toxicity test, 1 mL of an aqueous acetone solution containing 0.5-20% of essential oils was sprayed onto bees in a cage. Only high concentrations of wintergreen oil led to high mortality in *Varroa*, while remaining well tolerated in bees. Among 55 essential oils, only wintergreen oil was chosen for open field studies.

Kraus (1990) studied mortalities in bees and mites after exposure to marjoram, cinnamon, clove, lemongrass and lavender oils. Ten bees, each with a mite, were placed in a cup with a piece of wax containing 0.1, 1 or 10% essential oil. Mortality in bees and mites was assessed after 3 days. 1% clove oil in wax led to mite mortality of more than 80%, with a bee mortality rate identical to that of non-treated bees. At a concentration of 10%, bee and mite mortality was close to 100%. Application of 10% marjoram oil led to mortality of 100% in mites and 20% in bees, a rate that was not statistically different from the controls.

Bunsen (1991) tested the tolerance of bees to lavender, lemon balm, wintergreen, pine needle, mountain pine, Neem and citral oils. In cages of 20 bees, 300 µL of acetone containing 0.1, 1 or 10% oil was evaporated from filter paper. Bee behaviour was observed for 7 hours. All the citral concentrations and only the high concentrations of lavender and lemon balm oil disrupted bee behaviour. The other oils did not induce an effect. High brood mortality was observed after application of bergamot, cinnamon, fennel, pine needle, nerolidol, savory, thyme, anethol, linalool, linalyl acetate, octenol and terpineol.

Other monoterpenes were tested (ALP 2006; Imdorf *et al.* 1999a) based on the same type of test. At concentrations of 400-1000 µg p-cymene, 120-260 µg α-thujone and 30-100 µg isopinocamphone, mortality of nearly 100% was observed in mites, along with good tolerance in bees. Isopinocamphone is the main component of hyssop oil. Exposure to α-terpinene led to high

mortality in mites and bees. Limonene and α-pinene led to low mortality, both in mites and bees, even at high concentrations.

- **Organic acids (formic acid, oxalic acid)**

Two organic acids, formic acid and oxalic acid, have valuable properties to combat *Varroa* since they are naturally present in honey and above all have varroacide activity (Bogdanov 2006; Rademacher and Harz 2006).

✓ *Formic acid*

Formic acid has been used for some time (Stoya *et al.* 1986). It is currently authorised in several European countries, including France since 2014, as a varroacide in a liquid form or a slow evaporation block and acting by fumigation (CMDv 2013). This acid probably acts on *Varroa* by inhibiting electron transport in mitochondria and thus energy metabolism (Keyhani and Keyhani 1980). It can induce neuronal excitation in arthropods (Song and Scharf 2008). Formic acid can have adverse effects in bees by reducing the lifespan of worker bees (Underwood and Currie 2003) and by altering brood survival (Fries 1991).

Loss of queen bees was a serious problem when formic acid was first used, especially with "home-made" fresh preparations. Currently, through modern application methods, these losses have become exceptional. However, problems on uncapping of brood or hatching of young bees cannot be completely ruled out. These problems depend on the ambient temperature and the distance between the brood and the evaporation block. In the conditions observed in Europe, a moderate brood loss did not have a negative effect on overwintering of colonies (Imdorf *et al.* 1999b).

✓ *Oxalic acid*

Oxalic acid is authorised as a veterinary medicinal product in several Member States of the European Union and in Switzerland, but not in France to date. It can be administered by dripping of a sucrose solution between the frames (Mutinelli *et al.* 1997) or by evaporation (Varrox 2007). The mode of action of oxalic acid in *Varroa* is not known but it requires direct contact (Aliano and Ellis 2008), hence the greater efficacy in the absence of brood.

Repeated treatment of colonies with this acid may lead to higher mortality of queen bees and a decrease in capped brood (Higes *et al.* 1999). In the midgut of bees fed with sucrose solution containing oxalic acid, a high level of cell death was observed (Gregorc and Smodiš Škerl 2007). However, in open field conditions, bees generally avoid consuming syrup containing this acid (Aliano and Ellis 2008).

Oxalic acid is readily available and at a low cost worldwide. It has no MA in France but there is an authorisation for organic beekeeping for the control of *Varroa* (DGAL/SDSPA/N2004-8136, 12 May 2004: "*the veterinarian may prescribe a veterinary compounded preparation of oxalic acid in organic beekeeping, without the need to assess beforehand the inefficacy of synthetic chemical allopathic medicinal products that have MAs")*. The ease of obtaining this acid from a number of sources did not lead manufacturers to initiate a long and costly registration procedure for the product (Johnson *et al.* 2010).

The concentration of oxalic acid in the rectum, Malpighian tubules, digestive tract and haemolymph of bees is strongly influenced by the method of administration, whether topical or oral. It has been shown that oxalic acid crosses keratin by the topical route (Nozal *et al.* 2003).

The toxicity of various concentrations of oxalic acid dihydrate in an aqueous and sucrose solution was investigated in *Varroa destructor* and in bees (*Apis mellifera*) using submersion tests of caged bees and by spraying bees in colonies with and without brood (Toomemaa *et al.* 2010). An aqueous solution of 0.5% oxalic acid was able to control *Varroa* effectively without toxicity for bees, while higher concentrations of 1 and 2% oxalic acid were very toxic in bees. The submersion tests in solutions of 0.1% oxalic acid showed an acaricide action in an aqueous solution (59.9 ± 3.7%) and in a sucrose solution at 50% (71.1 ± 4.2%). Concentrations of 0.2-0.5% were found to be highly effective. Oxalic acid in a sucrose solution was more toxic for bees than in an aqueous solution. Spraying the 0.5% oxalic acid solution (25 mL per frame) in May 2003 and April 2004 showed an efficacy of 99.01-99.42% in the control of *Varroa*. Most mites fell after the first spray. In

autumn, one or two sprays of 0.5% oxalic acid solution in colonies with little capped brood enabled effective control of *Varroa* (92.94 ± 0.01% and 91.84 ± 0.02%, respectively) without specific toxicity in bees. In this study, five sprays of 0.5% oxalic acid were applied in April 2004 with similar efficacy (99.42 ± 0.10%). Most of the mites (647.1 ± 154.3, i.e. 78.3%) died in the 2 days following the first application, fewer died in the 2 days following the second spray (139.6 ± 23.7, i.e. 16.9%) and very few after the following sprays. However, 4 of 11 colonies tested saw considerable weakening, indicative of toxicity of the treatment for bees. Major weakening was observed after the fourth and fifth spray, especially after 12 days. The morning following the third application, a large number of dead bees (20-50) was recorded in front of the entrance to certain tested colonies. After the fourth and fifth sprays, most of the tested colonies had many dead bees at the entrance to the hives. In some colonies, bees were observed with signs of intoxication, i.e. falling and crawling in front of the hive.

3.1.2.6 Industrial pollutants

During their various flights, foragers necessarily come into contact with industrial xenobiotics that contaminate the different environments visited. These substances, whether organic or inorganic, are retained at the surface of the body (i.e. cuticle, setae, legs) and/or absorbed and may, depending on their nature and toxicity, lead to the death of the insect in the short or medium term, or accumulate in the body (Hladun *et al.* 2013; Raes *et al.* 1992). These pollutants are of various types and are often brought back to the hive where they can contaminate the other members of the colony by direct contact, trophallaxis, etc. Smith *et al.* (2002) identified nearly 200 volatile or semi-volatile industrial compounds in the hive atmosphere. In the same way, depending on their physico-chemical properties, in particular their lipophilia, xenobiotics are also able to accumulate in the other individuals populating the hive, as well as in wax, honey, and pollen. This is the case for polycyclic aromatic hydrocarbons (PAHs) (Amorena *et al.* 2009; Ciemniak *et al.* 2013; Devillers and Budzinski 2008; Lambert *et al.* 2012; Lourdes *et al.* 2014; Perugini *et al.* 2009), polychlorobiphenyls (PCBs) (Anderson and Wojtas 1986; Devillers and Budzinski 2008), and brominated flame retardants (BFRs) (Mohr *et al.* 2014; Wang *et al.* 2010). However, no relationship has formally been established between their presence and proven toxic effects.

3.1.2.7 Other: GMOs

3.1.2.7.1 Introduction

The main acquired properties of transgenic plants are (1) resistance to the action of some herbicides, (2) resistance to insect pests, and (3) acquisition of new agronomic properties. As such, genetic changes in rapeseed do not target resistance to insect but may enable tolerance of herbicides, changes in their fatty acid composition, or production of sterile males. Malone and Pham-Delègue (2001) reported the effects on bees and bumble bees of the following transgenic products:

- toxins produced by *Bacillus thuringiensis* (Bt) which have various phenotypes depending on the origin strain (Cry1 Ac, Cry1 Ab, Cry 9c);
- serine protease inhibitors (Bowman-Birk soybean trypsin inhibitor (BBI), aprotinin, Kunitz soybean trypsin inhibitor (SBTI), Potato proteinase inhibitor (POT-1 and -2), cowpea trypsin inhibitor (CpTI), cysteine protease inhibitors (oryzacystatin (OC-1), chicken egg white cystatin);
- other transgenic products (chitinase, β-1,3 glucanase, avidin, glyphosate resistance, lectins).

The main crops of interest for these transgenes are soy, maize, cotton plants and potatoes. Other crops such as tomatoes, tobacco, lucerne, rice, apples, kiwi fruit, grapes and melons may also be subject to these procedures. Some of these crops require bees for their pollination (apples, kiwi, tomatoes) or for the production of seeds (rapeseed). Others that do not require insects for their pollination, play an important role in the bee diet (cotton plant, maize and potato). Malone and Pham-Delègue (2001) distinguished direct effects of transgenic products from indirect effects. The direct effects are the consequence for the body of transgenic products after their ingestion by the

insect. In the case of bees, pollen constitutes the main exposure vector given its high protein content compared to nectar. In adult bees, the consumption of pollen is at its highest for the first 10 days since protein intake is necessary at this stage for the maturation of the hypopharyngeal glands. In larvae, Babendreier *et al.* (2004) showed that consumption of maize pollen is about 1.5 to 2.0 mg per individual, i.e. 5% of total proteins. Expression of products of transgenes in pollen is variable depending on the plant species, the product, and the type of promoter. As an example, in the case of maize, depending on the type of promoter, Bt toxins can be measured in the pollen at concentrations ranging from 260 to 418 ng of toxin per mg of pollen. The same gene placed under the control of another promoter will not produce measurable quantities of toxins (Malone and Pham-Delègue (2001), according to Kozeil *et al.*, 1993).

Indirect effects are related to changes in the plant related to the transgene that induce a loss of attractiveness or palpability. Since these cannot be considered a real stress factor, particularly since decreased palpability related to the presence of a toxic substance may turn out to be a protective factor, we will only address direct effects in this section. We will present the results obtained from different types of transgenes: Bt, protease inhibitors (of serine and others), as well as other transgenes (such as chitinases, glyphosate, etc.) in non-target species of the genera *Apis* and *Bombus*.

3.1.2.7.2 Effects related to exposure to Bt toxins

- ***Apis mellifera***

Bt toxins, used as biopesticides against certain pests such as Lepidoptera or Coleoptera, are known for their harmless nature to Hymenoptera. The main plants concerned by these products of transgenes are maize, cotton and potatoes. In their review, Malone and Pham-Delègue refer to five publications that focussed on *Apis mellifera* in the laboratory on larvae and adults, and in the open field in colonies (Anon 2000; Arpaia 1996; Malone *et al.* 1999; Malone *et al.* 2001; Sims 1995). These studies looked into the lethal effects and some sub-lethal effects such as growth, consumption and flight activity. None of this research revealed any effects following exposure that can reach for example 1700 or 10,000 times the concentration levels measured in pollen or nectar of transgenic cotton. More recently, Duan *et al.* (2008) published a meta-analysis of 25 publications selected on the basis of six criteria: studies carried out with active proteins on Lepidoptera or Coleoptera, ingested by *Apis mellifera*, in the laboratory, with measures of mortality, in comparison with a non-treated control, and measure of variability of response. This study supports the conclusions of the previous review and notes the complete absence of lethal effects of these toxins in the honeybee, whether in larvae, nymphs or adults. Many studies carried out more recently in the laboratory or in the field, on larvae and adults, not cited in these two reviews, led to similar conclusions, that there is no effect of Bt proteins on mortality, development of colonies, larvae, diet behaviour, intestinal flora, memorisation abilities, and development of the hypopharyngeal glands (Babendreier *et al.* 2005; Dai *et al.* 2012; Geng *et al.* 2013; Han *et al.* 2010; Hendriksma *et al.* 2012; Hendriksma *et al.* 2011; Hendriksma *et al.* 2013; Lipinski *et al.* 2008; Liu *et al.* 2009; Malone *et al.* 2004; Ramirez-Romero *et al.* 2008; Tian *et al.* 2006).

- ***Bombus***

In *Bombus occidentalis* and *Bombus impatiens*, exposure to realistic doses of Bt toxin (Morandin and Winston 2003) revealed no effect on consumption of pollen, weight of workers, development of colonies, or production of queens and drones. A study on microcolonies of *Bombus terrestris* fed with pollen from transgenic maize in the laboratory (Malone *et al.* 2007) showed no effect of Bt toxin on the survival of workers, consumption of pollen and syrup, ability to produce drones, and their weight. Microcolonies fed with contaminated syrups containing 0.001% and 0.01% of Bt toxin showed normal development and production of drones, no different from controls (Babendreier *et al.* 2008).

3.1.2.7.3 Protease inhibitors

The impact of protease inhibitors on an insect depends on the proteolytic profile of the insect and on the specific activity or activities of the given inhibitor. In the honeybee and bumble bee, serine

proteases are predominant versus cysteine proteases. A predominant effect of serine protease inhibitors is therefore to be expected in these species (Malone and Pham-Delègue 2001).

- ***Apis***

In their review, Malone and Pham-Delègue (2001) referred to several studies that pointed to serine protease inhibitors that can inhibit proteases in the midgut in honeybees and bumble bees. At high concentrations, these substances may cause a reduction in the lifespan of adult insects. More recently, Brodsgaard *et al.* (2003) carried out a study on larvae of honeybees in the laboratory by exposing the insects orally to concentrations of SBTI varying from 0.1 to 1% of larval food, bearing in mind that a concentration of 0.2% is equivalent to a rate of presence of 1% of protease inhibitors in total pollen proteins (Malone *et al.* 2002). The 1% concentration significantly extended the duration of development of larvae, reduced the weights of adults produced, and increased larval mortality. In young adults, Babendreier *et al.* (2005) observed a negative effect of the substance on the development of hypopharyngeal glands (HPGs) following feeding for 10 days with pollen contaminated with 0.1% SBTI. Sagili *et al.* (2005) observed a similar phenomenon from a concentration of 1%, but no effect at 0.1%. Although SBTI was not detected in HPGs, it led to a decrease in consumption of syrup and a drop in raising of brood at a concentration of 1% (Babendreier *et al.* 2005), and significantly reduced the enzyme action of the midgut, along with survival of individuals at the same concentration (Sagili *et al.* 2005). Similar results were observed with POT-1, POT-2 and BBI proteins, which also belong to the serine protease inhibitor group (Malone and Pham-Delègue (2001), according to Belzunces *et al.* 1994, Girard *et al.* 1998, Malone *et al.* 1998, 2000, Pham-Delègue *et al.* 2000, Sandoz 1996). Among the other protease inhibitors tested on adult insects in the laboratory, Malone *et al.* (2004) showed no effect of oral exposure to aprotinin, a trypsin inhibitor, at 1.175 mg/g of pollen on the survival of young adults and the development of HPGs.

Liu *et al.* (2009) observed no reduction in survival in adult bees fed with pollen from transgenic cotton plants expressing proteins of Bt and CpTI. Using the same pollen, Han *et al.* (2010) found no effect after exposure of 7 days to this mixture of proteins on learning abilities as shown by the proboscis extension reflex test.

Of the cysteine protease inhibitors, OC-1 and chicken egg white cystatin have been tested in the short term (exposure for 24 h) and long term (continuous feeding) in adult bees without any observed effect on lifespan (Malone and Pham-Delègue (2001), in Girard *et al.* 1998, Sandoz 1996).

- ***Bombus***

Serine protease inhibitors produce similar effects in *Bombus* to those found in the honeybee (Malone and Pham-Delègue 2001), i.e. reduced lifespan of adults and reduced enzyme activity in the midgut. These phenomena are specifically observed after exposure to SBTI, POT-1 and POT-2. Like observations in the honeybee, aprotinin did not have an effect on lifespan. More recently, Babendreier *et al.* (2008) tested low (0.01%) and high (0.1%) concentrations of SBTI in syrup offered to bumblebee colonies via a feeder in a greenhouse chamber, or in microcolonies in the laboratory setting. Although no effect on foraging behaviour was observed on the colonies, the laboratory tests revealed an effect of 0.1% SBTI on survival and production of drones, and at 0.01% on the weight of worker bees.

3.1.2.7.4 Other transgenic products

- ***Apis***

Oral exposure of adult bees to chitinase doses of 11 µg/individual had no effect on survival at 24 and 48 h (Malone and Pham-Delègue 2001). Injections of 1.69 µg per bee led to the same conclusion. At concentrations of 1, 5 and 10 µg/mL of syrup, no effect was observed on learning performance. Similar results were obtained after adult bees ingested β-1.3 glucanase at a dose of 11 µg/bee, or after injection of 0.3 µg of this product (Malone and Pham-Delègue (2001), in Picard-Nizou *et al.* 1997).

Preliminary studies in young adults exposed orally to 6.7 and 20 µM doses of avidin showed no effects on pollen consumption and lifespan (Malone and Pham-Delègue 2001). Emerging bees fed for 10 days on avidin-contaminated pollen at a concentration of 0.174 mg/g were not affected in terms of survival and development of their HPGs. Moreover, no trace of the compound was found in this organ (Malone *et al.* 2004).

Lehrman (2007) tested the effects of pea lectins (PSL) on larvae in the laboratory using pollen collected from transgenic rapeseed mixed with larval food for the whole duration of development at 1.5%. The author initially verified that this concentration of pollen did not affect larval development, and established that it induced a concentration of 0.0012% PSL in larval food. No effects were observed for the two PSLs tested on mortality, weight and duration of development. Hendriksma *et al.* (2012) carried out a similar study exposing larvae in their fifth day to doses of GNA (*Galanthus nivalis* agglutinin) ranging from 0 to 80 µg. This lectin induced complete mortality of larvae at the maximum dose and no effect was observed for the lower doses.

Huang *et al.* (2004) carried out a study on the direct effects of exposure to pollen from transgenic glyphosate-resistant rapeseed. In a first step, the researchers exposed colonies to crop plots of transgenic or non-transgenic rapeseed and in a second experiment, they artificially fed larvae with transgenic or non-transgenic pollen, and then reintroduced the specimens into the hive. In both scenarios, no effect was observed on larval or nymphal mortality, nymph weight, or concentration of proteins in haemolymph. The first study also showed no evidence of an effect on the adult population, even though the experimental conditions were questionable on some aspects which led to the study not being validated as part of the collective expert appraisal undertaken by the CNRS-INRA[28] on plants tolerant to herbicides.

- ***Bombus***

Colonies of *Bombus occidentalis* fed with contaminated pollen at a concentration of 6 µg/g chitinase were not affected by this treatment in terms of development, i.e. brood quantity and number of workers (Morandin and Winston 2003).

Babendreier *et al.* (2008) observed negative effects of GNA on microcolonies of *Bombus terrestris*. This substance added to feeding syrup significantly reduced the weight and lifespan of workers at a concentration of 0.1%. At this concentration, no male descendants were observed and the consumption of syrup was also lower. The production of male descendants was significantly affected at a concentration of 0.01%.

In conclusion, all the studies involving Bt toxins provide consistent findings on the harmlessness of these proteins in bees. Knowledge of this harmlessness in fact preceded GMOs since insecticides containing Bt are known for their specific action on Lepidoptera and Coleoptera in particular. In this regard, it is important to note that a strain of Bt is marketed to treat formed frames against wax moth.

Concerning other transgenic products, serine protease inhibitors are toxic at relatively high doses. Expression of these products in pollen, which constitutes the main vector in food, must be analysed on a case-by-case basis to assess the risk related to exposure to plants that express these products.

There are few studies on the sub-lethal effects, except proboscis extension reflex tests which, for these products, generally show no effect on learning abilities. It however seems justified to take into account these possible effects and other potential effects related to co-exposure to other stress factors.

3.1.3 Food and environmental resources

Growth and survival of bee colonies are strongly associated with the quantitative and qualitative availability of floral resources from which nectar and pollen can be collected (Brodschneider and Crailsheim 2010; Haydak 1970). Floral nectar stored in the form of honey is the main source of carbohydrates, the energy food of bees, while pollen provides most of the proteins, amino acids

[28] http://inra.dam.front.pad.brainsonic.com/ressources/afile/223293-076bc-resource-expertise-vth-synthese.htmL

and lipids needed to develop specific tissues such as the fat body and hypopharyngeal glands, and as food for larvae (Brodschneider and Crailsheim 2010). As such, bee populations and beekeeping activities depend on environmental resources and any deficit may have an immediate effect by way of weakening of colonies, particularly through significant reductions in brood production (Brodschneider and Crailsheim 2010). More long term, nutritional stress can lead to physiological deficiencies (Alaux *et al.* 2010b; Brodschneider and Crailsheim 2010) and may thus affect the bees' resistance threshold to other stress factors. Moreover, depopulation of colonies related to nutritional stress may limit the response abilities to an additional stress. Colonies may then reach a point of no return in terms of demographic flexibility, for instance replacement of foragers or nurse bees.

In this section, the nutritional needs of a colony will be described before focussing on the current changes in the availability of food resources that may affect these nutritional needs. Finally, the potential effects of nutritional stress on bee health will be addressed. Beekeeping feeding practices will be considered in section 3.1.4.

3.1.3.1 Nutritional needs of a colony

Carbohydrates from nectar are stored in the form of honey and mainly contain fructose, glucose and sucrose with varying content levels. These carbohydrates cover the energy needs of bees required to carry out the various maintenance and development tasks within the colony. Pollen itself is mixed with nectar, and salivary secretions containing enzymes and microorganisms from the bee stomach. This mixture is stored in the form of pollen bread produced from lactic fermentation (Vasquez and Olofsson 2009). It provides the required proteins and amino acids that play a decisive role in brood production and in bee lifespan (see Brodschneider and Crailsheim (2010) for a review). It contains additional nutrients such as vitamins, minerals and lipids, but the importance of these substances for the colony is far less well understood.

A colony of 50,000 bees has an annual requirement of 120 kg of nectar and 20 kg of pollen (Seeley 1995). An adult bee requires a minimum of 4 mg of nectar per day (Barker and Lehner 1974) and consumes 3 to 5 mg of pollen per day during the first few weeks of its life (Crailsheim *et al.* 1992; Pernal and Currie 2000). Lastly, larvae consume about 60 mg of carbohydrates (Rortais *et al.* 2005) and 25 to 37.5 mg of proteins during their development, equivalent to 125-187.5 mg of pollen (Hrassnigg and Crailsheim 2005). To prevent certain dietary deficiencies, beekeepers provide the colony with sugars or protein supplements but these additions do not necessarily have the same nutritional quality as pollen (Cremonez *et al.* 1998; DeGrandi-Hoffman *et al.* 2008) and nectar (Mao *et al.* 2013; Wheeler and Robinson 2014).

Bees also have significant but highly variable water needs for the osmotic balance in adults, preparation of larval milk and to cool the colony during the warmest months. According to the recent analysis by EFSA (2012a), the quantity of water is difficult to calculate since it varies over time and depends on the reference, but is about 20 to 42 litres per colony per year and up to 20 litres per week per colony in the summer.

3.1.3.2 Availability of food resources

The availability of floral food resources, including their quantity and quality, has an impact on the development and survival of colonies. Intensive agriculture leads to reduced or lost foraging areas for bees, floral diversity and natural habitats.

In most large production areas, crop rotation has become highly simplified, leading to a decline in floral biodiversity, particularly melliferous plants in grain-producing zones. The development of single crops along with application of herbicides (reducing the diversity and abundance of flowering plants) results in periods of shortages before and after the flowering period of these single crops. In this case, nectar and pollen are relatively abundant but only for a very short space of time, provided that the plants are melliferous, which poses a problem for honeybees that have an extended period of activity. A possible link between lower environmental resources and colony losses was suggested in the United States (Naug 2009). However, at this time, there are no studies demonstrating a causal relationship between the availability of floral resources and colony losses. van Engelsdorp *et al.* (2009) reported that worker bees from colonies affected by colony

collapse disorder showed no changes in protein levels in the head, thorax, or abdomen. This may be explained by the fact that even if beekeepers face reduced environmental resources, shortages can be avoided by moving hives to areas that are more favourable at certain times and where colonies manage to find adequate resources in their environment to survive. Finally, although there is no direct effect of reduced resources on colony survival, nutritional stress can have more subtle effects and become a co-factor of colony weakening by decreasing tolerance levels to other stress factors (Brodschneider and Crailsheim 2010; Le Conte *et al.* 2011). The presence of crop-free, non-treated areas is a way of restoring floral diversity and providing continuous availability of resources between periods of flowering of major crops (Decourtye *et al.* 2011a). This can help to decrease potential co-exposures such as chemical treatments coupled with decreases in the nutritional quality and quantity of food resources for bees. However, these areas remain poorly developed in practice. They may nonetheless be essential during key periods of the colony cycle: preparation of overwintering requiring storage of enough nutritional reserves to survive the winter, and renewed activity after the winter to support colony development.

3.1.3.3 Effect of availability of food resources on bee health

In natural conditions, bees are rarely confronted with a complete absence of pollen in their environment. Rather, they face variability in abundance, and quality and diversity of resources over time and spatially, like for instance in the agricultural environment (Odoux *et al.* 2012). The influence of these three levels on bee health will be discussed, but there is a strong bias in available information in favour of polliniferous resources.

3.1.3.3.1 Abundance

Abundance of food resources has a direct impact on the population status of colonies. If pollen supply is interrupted, bees naturally maintain brood rearing for a short period of time using bee bread reserves, followed by their own body protein reserves. The resulting bees however present protein deficiencies (Haydak 1970). Young larvae that had lower nutritional intake compared to older larvae may also be cannibalised so that the nurse bees have proteins to feed other larvae (Schmickl and Crailsheim 2001). Rearing larvae can also be compromised by a reduction in the size of the hypopharyngeal glands of nurse bees with pollen deficiencies. This malnutrition of larvae leads to morphological and physiological changes in adult bees (review in Brodschneider and Crailsheim 2010).

Reduced pollen abundance and quality leads the colony to change its foraging efforts by increasing the number of pollen foragers (Pernal and Currie 2001) and young workers become foragers at an earlier age (Janmaat and Winston 2000a). Moreover, in agricultural production zones with a simplified resources layout, pollen foragers cover more distance than in more complex environments (Steffan-Dewenter and Kuhn 2003). Increased foraging distances also require higher energy outlay and thus greater consumption of carbohydrates (nectar, honey). Reduced brood, along with increased foraging efforts, lead irreversibly to a reduction in the colony population.

Over and above the direct consequences on colony size, decreased resources may also affect bee health and tolerance of other stress factors. In fact, experiments in the laboratory setting have highlighted the importance of pollen supply on physiological metabolism in young bees (Alaux *et al.* 2011a; Ament *et al.* 2011). For example, the production of vitellogenin, a glycolipoprotein involved in the production of royal jelly (Amdam *et al.* 2003), lifespan (Seehuus *et al.* 2006), and cell-mediated immunity (Amdam *et al.* 2004b), is reduced to a significant extent (Alaux *et al.* 2011a; Ament *et al.* 2011).

Most of the studies designed to test the effects of nutritional resource abundance on bee health were carried out based on the all or nothing method. However, bees are rarely confronted with these extreme cases of total absence of resources. It is therefore necessary to test intermediate amounts that are more representative of natural situations.

The abundance of resources may also affect the preparation of overwintering and thus compromise the survival of bees over the winter. In this way, availability of environmental resources during preparation of overwintering appears to be a key factor for winter survival

according to beekeepers belonging to the French Bee Institute (ITSAP). Although this notion appears implicit, it remains to be tested.

3.1.3.3.2 Quality

The quality of nutritional resources may differ between flowering species, suggesting that some have higher quality for bees than others. Concerning pollen, its protein, amino acid and lipid contents as well as levels of other nutrients vary from one species to another (Herbert and Shimanuki 1978; Odoux *et al.* 2012; Roulston and Cane 2000). The same is true for nectar which has varying concentrations of carbohydrates (5 to 80%) depending on the species (Baker and Baker 1982; Crane 1980; Cruden *et al.* 1983).
As such, not all pollen has the same nutritive value and certain studies have shown, in bees reared in the laboratory, that their quality can significantly affect major life characteristics such as lifespan (Di Pasquale *et al.* 2013; Maurizio 1950; Schmidt *et al.* 1987; Schmidt *et al.* 1995; Standifer 1967), development of the hypopharyngeal glands (Pernal and Currie 2000; Standifer 1967), and the production of vitellogenin (Di Pasquale *et al.* 2013).
Ten amino acids are essential in bees (de Groot 1953). If poor nutritional quality pollen is available that does not contain one or more of these amino acids, bees should theoretically be affected. However, it is highly likely that some diversity in pollen supply, if the quantity is sufficient, will compensate for this deficiency phenomenon.
Certain fatty acids have antifungal properties (*Ascosphaera apis*) and antibacterial properties (American foulbrood, European foulbrood) *in vitro* (Feldlaufer *et al.* 1993a; Feldlaufer *et al.* 1993b; Hornitzky 2003; Shimanuki *et al.* 1992) but this has not been confirmed in bee larvae (Giersch *et al.* 2010).

Aside from beneficial effects, some resources contain nutrients that are toxic in bees. This is the case for some carbohydrates (e.g. galactose, lactose, stachyose and raffinose) found in pollen, nectar and certain plant exudates (Barker 1977; Barker 1990; Barker and Lehner 1976). For instance, about 40% of carbohydrates present in soybean pollen are toxic to bees (Barker 1977). Use of supplements containing soybean pollen must therefore be monitored.

3.1.3.3.3 Diversity

Concerning diversity of resources, bees tend to prefer multispecific rather than monospecific pollen nutrition, as shown by greater consumption (Schmidt 1984), and thus have a longer lifespan (Schmidt *et al.* 1987). This pollen biodiversity is beneficial for certain immunocompetence traits, such as the activity of glucose oxidase catalysing the production of antiseptics (hydrogen peroxide) in royal jelly (Alaux *et al.* 2010b). It also plays a buffer role at times of poor quality pollen supply (low in nutrients and/or containing toxic components) by improving bee lifespan (Schmidt *et al.* 1987) and tolerance of larvae and adults to pathogens, such as the fungus *Aspergillus fumigatus* (Foley *et al.* 2012) and the microsporidian *Nosema ceranae* (Di Pasquale *et al.* 2013).

Pollen and nectar from flowers are the main source of food but also contain phytochemical components and are rich in carotenoids, flavonoids, alkaloids, and phenolic compounds that have antioxidant properties and antimicrobial activity (Adler 2000; Balch and Balch 1990; Basim *et al.* 2006; Campos *et al.* 2003; LeBlanc *et al.* 2009a; Morais *et al.* 2011). The diversity of nutritional resources in the environment increases the possibilities for bees to find valuable nutrients but also to avoid and find alternatives to toxic plant compounds. A simplified agricultural environment decreases these possibilities.

3.1.3.4 Missing data/outlook

Several studies have demonstrated the effects of nutritional stress on bee health but most of them were conducted in the laboratory setting, far from actual conditions in nature. It is therefore important to determine whether the effects observed in the laboratory can be transposed to natural conditions. As an example, in the laboratory, bees are often fed with pollen loads, while in the colony, they ingest pollen mainly in the form of bee bread. This product has a slightly different chemical composition compared to pollen loads with similar levels of proteins and lipids, but an absence of starch, a higher sucrose level, and a lower pH (Herbert and Shimanuki 1978).

With the aim of better understanding the importance of availability of food resources in colonies, it is necessary to determine (1) the link between quality and diversity of food resources and the development and survival of colonies, (2) interactions/mechanisms between nutrition and other co-factors (infectious agents, parasites, chemicals), (3) the role of intestinal flora, and (4) whether the availability of food resources affects winter survival.

Lastly, although it is possible to find out more about the type of resources bees collect in their environment, it would be useful to know whether they face deficiencies concerning development and maintenance of their colony. If this is the case, the periods and types of deficiencies should be determined.

3.1.4 Beekeeping practices

All beekeepers can use their knowledge and experience to apply methods aimed at helping their colonies to develop and remain healthy, and thus to maintain their production potential. These methods are known as beekeeping practices. There are many books on these practices and some of them were published a long time ago. Today, management has become more technical and practices have become essential to maintain a bee population.

As such, beekeeping practices include the choice of position, prevention measures against disease and zootechnical choices, among others. "*Good beekeeping practices*" are defined in a guide as being the practices of managing a bee population by a beekeeper "*aimed at preserving the health of colonies*" (ITSAP 2014). However, although these measures aim to contribute to bee health, some of them may on the contrary lead to potentially stressful effects on colonies in certain circumstances. In addition, some practices that are sometimes needed (or occasionally incorrectly performed) become risk factors for the development of disease. Here, we will only consider practices that may generate stress or compounding risk factors for bee colonies and that have a potentially negative effect on their health.

Beekeeping practices follow the annual biological cycle of colonies which is divided into four periods: (1) end of overwintering (development period), (2) colony reproduction period (or swarming), (3) period of preparation of overwintering, and (4) overwintering period (see chapter 2, section on annual population growth in a colony). Beekeepers thus apply suitable measures at each of the life phases of the colony: promotion of population development, multiplication of colonies, exploitation of honeyflow and prevention of disease, for example.

3.1.4.1 Potential impact of certain beekeeping practices

Multiplying a bee population, or maintaining it, requires systematic rearing. When beekeepers wish to increase the size of their apiaries, they must spend part of their time and/or financial resources on rearing, but given colony losses which can be normal or exceptional, they must also carry out minimal rearing to maintain the population. Beekeepers thus either rely on self-renewal of their colonies, or purchase of queen bees and/or swarms from France or abroad. In both cases, the necessary operations and the effects they produce can have an impact on the health of colonies.

In the case of rearing by the beekeeper, production of bee packages or division of colonies (the most common conventional method after natural swarming (FranceAgriMer 2012)) breaks down the superorganism and changes, at least temporarily, the distribution of age classes and/or the bee:brood ratio. For queen bees, artificial requeening is, all things being equal, beneficial in a colony with an aging queen. But in the case of virgin queens, interrupted egg laying, i.e. the interval between the last egg laid by the former queen and the first by the new queen, estimated at 2 to 3 weeks, could disrupt the population balance. We can however consider that these disruptions are negligible in natural swarming periods: all "active" rearing methods, such as division or rearing of queen bees, are far better tolerated by colonies and are successful when one reaches the natural swarming period that constitutes the natural bee reproduction period. Most of the imbalances mentioned related to the distribution of age classes will normally not have a lasting effect on the colony.

In some cases however, specifically when other co-factors are involved, the effects on colonies may be significant. An example would be the case of a chilled brood caused by colony division with too few adult bees to maintain the brood at the right temperature, if a cold spell follows division. Disappearance of foragers exposed to infectious and/or toxic agents would have the same effect. Moreover, the period of queen rearing and worker population growth will lead to increased needs in food resources. If these resources are insufficient or absent, major effects can be expected on the colony's development cycle: cannibalism and adult morphology (Brodschneider and Crailsheim 2010; Di Pasquale *et al.* 2013; Naug 2009; Requier *et al.* In press). The beekeeper must therefore provide food supplements to the colony if necessary.

In addition, quality of the queens produced may vary depending on the rearing season: queen bees produced in the spring have a higher number of living spermatozoids in their spermatheca than those produced in the autumn. They also less often have lesions affecting their ovaries and their sting apparatus (Provost 2013). Likewise, studies in semi-controlled conditions have shown that biotic and abiotic environmental stressors, alone or in combination, can affect the quality of sperm in drones (Brunet 2013). Obtaining high quality reproducers guarantees good colony health, and on the contrary, poor reproducers lead to lower egg-laying levels with lower bee numbers and a weaker colony that is therefore potentially more susceptible to co-exposures, for example.

In the case of purchases of queens or swarming, the risk of disease is related either to the introduction of infectious agents to the colony that may disrupt the balance of asymptomatic carriage, or introduction of another bee subspecies that may lead to disruptions in colony functioning from aggressiveness, robbing, and drift[29] for example. Beekeepers must be aware of the risks associated with these practices.

Although each **handling operation by a beekeeper in a colony** involves risks including death of workers by crushing or stinging, death of the queen by crushing, robbing by neighbouring bees, transmission of infectious agents from one hive to another, lowering of the temperature, and disruption of the winter cluster, a minimum number of operations remain essential today to maintain the colony. These include treatments against *Varroa* and checking food reserves, for instance. Visits must therefore be limited to what is needed, both in terms of time and frequency. Good quality prior training will reduce or even practically eliminate the risk of damage to colonies related to beekeeper visits.

Harvesting the honey super, and especially possible sampling of body honey frames, which consists of taking excess honey produced by the colony over the previous weeks, can constitute a form of stress, aside from the possible effects of handling described above. In effect, depending on the region, beekeeping seasons sometimes alternate "honeyflow periods" and "non-honeyflow periods" with low or absent nectar production in a given region at a specific time point. When removal of honey supers coincides with a non-honeyflow period, particularly in spring (when the hive bodies may be almost completely filled with brood and pollen, especially for certain subspecies or strains such as Buckfast), energy resources may not be sufficient. This nutritional stress may be worsened and prolonged over time by other co-factors such as disappearance of nectar foragers, or a period of confinement in bad weather. These phases are therefore critical. They are generally well understood by beekeepers who can feed colonies or move hives once the honeyflow is completed.

Migratory beekeeping constitutes practices that are often pinpointed as stress factors for bee colonies. They correspond to a period of confinement of bees during their removal that may cause overheating or suffocation, particularly for transferals over long distances or for long periods. These effects are usually managed preventively by beekeepers who move colonies at night, in the early morning or evening, avoiding hot periods, and in hives that enable satisfactory airing. In the United States, studies have shown that transferals may also have negative effects on bee colony health (van Engelsdorp *et al.* 2013b; Welch *et al.* 2009). However, distances covered in France are at the most a few hundred kilometres, which are much less than those for American hives. By

[29] In beekeeping, the term "drift" refers to the risk for a forager of choosing the wrong hive on its return flight from foraging. This phenomenon, identified a long time ago, involves a risk of transmission of infectious agents from one colony to another.

contrast, a rather positive effect of transferal on bee health can be observed: prolonged access to nutritional resources that is beneficial to the colony. In France, only imported bees may undergo this type of confinement and its negative effects, in the framework of population renewal for example.

Production of single-flower honeys is generally more valuable than production of multi-flower honeys (FranceAgriMer 2012). As a result, some honeyflows are particularly sought after by beekeepers (acacia, lime and lavender, for example). This transient flowering is accessible to all beekeepers able to move their colonies and with a host site available in areas with a high concentration of the same floral species. These areas thus attract a large number of colonies from different geographical origins for a short period of time, corresponding to the honeyflow of interest. Although they are difficult to calculate, inter-colony and inter-apiary exchanges may be increased. The probability of drift, robbing and contacts in the broad sense, and thus possible exchanges of infectious agents, will increase considerably (Welch *et al.* 2009). Lastly, when the density of colonies is particularly high in an area, a form of competition for access to the same resources may develop and have an effect on colonies (increased distances and flight times, and access to water in dry periods). These are therefore risk areas, both for colonies located there, but also for the beekeeping sector in general on their return and for the joint efforts against bee diseases.

The technical location of colonies (successive locations occupied by a colony during a beekeeping season) may be an aspect to take into account as a co-factor in colony health. The professional beekeepers may be required to follow honeyflows after honeyflows used by the same colonies. Thus, the potential of a colony to exploit a resource is used, but by modifying the biological cycle which must be dynamically adjusted to a sedentary colony. Although the impact of transport over long distances has been highlighted as playing a potential role in excess mortality, particularly in the United States (Oldroyd 2007; Pettis and Delaplane 2010), very few studies have been conducted on this subject. The link between migratory beekeeping and high mortality has however been proven in South Africa (Pirk *et al.* 2014). The development of the hypopharyngeal glands can be affected when long transferals involve workers that have just emerged (Ahn *et al.* 2012). However, despite a higher prevalence of viruses in migratory colonies, they do not display increased mortality versus sedentary colonies (van Engelsdorp *et al.* 2008).

Some of these risks, or rather these risk factors for colony weakening, can easily be compensated for through suitable beekeeping practices. Complying with good practices can suffice in most cases. On the contrary, aside from the practices mentioned thus far, others are inadvisable and can sometimes endanger colonies.

3.1.4.2 Potential impact of certain unsuitable or absent practices

Annual renewal of wax is recommended at a rate of one quarter or one third of formed frames (ITSAP 2014). Wax may accumulate lipophilic xenobiotic substances over very long periods but also infectious agents such as spores of *Paenibacillus larvae* (American foulbrood). It is therefore common to find substances in wax used in certain beekeeping practices against parasites (coumaphos, *tau*-fluvalinate and paradichlorobenzene, for example), hive wood maintenance products (Bogdanov 2004) and pesticides (Chauzat *et al.* 2011). As a result, if these old waxes are not removed from hives, the pressure from contaminants of any kind will increase. This permanent contact of adult bees but also brood with these chemical substances may be harmful to bee colony health (Medici *et al.* 2012; Orantes-Bermejo *et al.* 2010). Likewise, newly installed waxes should not contain xenobiotic substances. It is therefore inadvisable to reuse old wax, even remoulded, since heating alone does not ensure its decontamination (ITSAP 2014), particularly for pesticides that have a high degradation temperature of several hundred degrees Celsius. Moreover, it is important to check the quality of purchased waxes. The self-renewal process of waxes, using capping wax only, appears to be a minimum precaution.

The trade in waxes is not well understood. There are very few means of traceability indicating the origin of waxes and little information on their quality. It appears necessary to encourage implementation of traceability in this sector that has highly globalised exchanges.

Requeening may respond to various objectives generally corresponding to a strategy set up by the beekeeper or a rearing plan. The choice of the new queen and selection of specific genetics will be based on production criteria, behaviour (limited tendency to swarm, early development in spring, docility, etc.) or health considerations (hygienic behaviour, resistance to certain diseases, and so on). This practice of requeening has become almost standard in professional rearing, to reach sometimes annual rates (FranceAgriMer 2012). The theoretical lifespan of queen bees is several years (see chapter 2). However, the lifespan of queen bees reported by beekeepers has been declining over the past 15-20 years. Natural requeening compensates partly for queen mortality but today some colonies remain drone colonies (i.e. the queen dies but is not replaced). These phenomena of reduced life expectancy in queen bees and orphan colonies are still poorly understood. Good beekeeping practices therefore require systematic requeening before this obsolescence takes place. A German study carried out over several years also showed that the risk of winter mortality was much higher once the queen bee reached the third overwintering (Genersch *et al.* 2010). Beekeepers must however be careful to adapt chosen genetics to the installation area of the apiary (see section 4.1.3.1.4. on genetic factors). Inconsistencies between the biological cycle or food needs of the chosen bee with climatic factors and flowering parameters in the landscape of the foraging area can constitute stress factors that disrupt a given colony. As an example, a colony that resumes activity very early after overwintering will not be suitable for a mountain climate.

Combating the parasite *Varroa destructor* must be given special attention by all beekeepers. Its prevalence across France is very high (86% of apiaries visited as part of epidemiological monitoring by *Résabeilles* had some parasitic pressure in autumn 2013, bearing in mind that this estimate was obtained during or at the end of acaricide treatment - Résabeilles Bulletin No 2), and its effects on bee colony health are very severe (see corresponding section). Systematic annual control is therefore indispensable. Strategies can include various methods and must be carefully assessed: evaluation of infestation, use of medicinal products, biotechnical methods, alternating methods, checking efficacy of implemented strategies, compliance with specific requirements, and late honeyflow periods, etc.

If hives are not treated or treatment is ineffective, on top of the harmful effects of the *Varroa* parasite, many risk factors are added to other potential co-factors and may lead to colony death. Treatment must therefore be systematic along with checking of treatment efficacy. Additional treatment may sometimes be needed.

The control of *Varroa* cannot however be carried out using any means available. According to recent epidemiological surveys (ANSES 2013; FranceAgriMer 2012), the antiparasitic management practices of some beekeepers are not in compliance with regulations since they involve the use of medicinal products that do not have a marketing authorisation for this indication. This misuse is dangerous in many ways: (1) "home-made" preparations expose the beekeeper to the risk of ingestion or inhalation of highly toxic substances (organic acids, thymol salts or amitraz in solution for example); (2) their presentations (cardboard holders, soaked cloth, pieces of wood, vermiculite, etc.) do not ensure controlled release of the active substances, leading to variable antiparasitic action; (3) these practices may, in the medium term, promote the development of resistance of the parasite to substances that are active today by exposing *Varroa* to sub-lethal concentrations; (4) these practices contribute significantly to contamination of waxes with active substances with negative effects on brood development (Medici *et al.* 2012); (5) harmlessness in treated colonies is not guaranteed; and (6) these substance may be a danger to the health of the consumer because of the possible persistence of residues in marketed hive products.

These practices ultimately do not contribute to improving colony health but may in fact prove harmful. Authorised veterinary medicinal products for the treatment of *Varroa* must be the only possible option when chemical agents are considered. This guarantees safety for the user and for bees, effectiveness against the parasite (though not complete), absence of residues in hives, and thereby protection of the consumer.

By **choosing a location,** the beekeeper offers the colony a specific foraging zone. This area must supply the colony with the resources it needs, including proteins, carbohydrates and water (see

food factors). A poor location for an apiary may thus have serious consequences. Likewise, along with these food resources, foragers will collect and bring back to the hive a range of other substances, thus creating a faithful image of their foraging area ("Bee sentinel for the environment"). Neighbouring geographical sites to the apiary may therefore be visited by the bees if they are attractive: industrial sites, croplands, urban areas, transportation routes, and farms, etc. The mean/usual foraging range of bees should be known to the beekeeper (see chapter 2) so as to take into account, as far as possible, all the landscape aspects that the colony will be exposed to. Food and water may be more or less easily accessible to the colony and the efforts made to access them can vary if the distances to cover and weather conditions (wind, rain, heat, etc.) are taken into account. Lastly, exposure and location of hives strictly speaking (full sunlight, wetland, presence of pests, etc.) may be risk factors for the development of disease, and/or force the colony to use excessive amounts of energy. Nonetheless, the rarity of locations in some regions, the desire to produce specific honeyflows, or pollination contracts, may lead beekeepers to place their hives in a location that is not ideal, with full knowledge of this fact. The possible effects on the given colonies should therefore be taken into account by providing specific care and increased monitoring.

Feeding schemes (artificial feeding) for honeybee colonies have been practiced for many years. Bee colonies store reserves in order to survive periods of shortage. However, beekeepers have been aware for a long time that their honey harvesting strongly impacts these reserves and that the efforts bees make to maintain a sufficient reserve are significantly increased by this harvesting. It is thus possible to help bees to compensate for these efforts by bringing them food, even if this food supplement is not always necessary. Feeding is primarily intended to assist in winter survival (autumn feeding), but also to help develop the population and renew the hives through new swarms that do not have foragers ("stimulant feeding" or "speculative feeding" – spring feeding, late summer), or to compensate for deficiencies during "non-honeyflow periods" or poor weather conditions. Moreover, the rarity of melliferous and polliniferous crops in agricultural systems, and wild plant life in some environments, make this feeding essential. When feeding, generally necessary, is not carried out, the lack of food may lead the colony to collapse through starvation[30].

To feed colonies, beekeepers provide sugars and sometimes pollen to their colonies. These supplies must be of good quality: sugars must be easily digestible and suitable for the period (candy in winter, syrup in spring). The best feeding product, as a general rule, would be honey, but there may be risks of transmission of infectious agents. Nonetheless, this type of feeding is rare because it is counter-productive. Feeding sugars are therefore generally supplied commercially and there are a number of presentations and compositions: syrups, candy, sugar beet or cane sugar, hydrolysed grain starches, etc. They may in some cases contain substances such as pesticides used in the treatment of sugar plants or GMOs (Lu *et al.* 2012), heavy metals (Dufault *et al.* 2009) or excess levels of hydroxymethylfurfural (HMF), particularly when they are not correctly stored (LeBlanc *et al.* 2009b). Moreover, since high HMF levels are promoted by excessive heating, home-made preparations may be also contain these substances (syrups obtained for instance by heating a mixture of granulated sugar and water). In April 2010 in Belgium, a syrup containing large amounts of HMF (108 to 356 mg/kg) was incriminated in colony mortality (AFSCA 2010; Wilmart *et al.* 2011). Unlike HMF that may be present in honey, which does not seem to pose a significant public health risk (Zirbes *et al.* 2013), HMF present in syrup for feeding bees appears to be toxic to them. According to Jachimowicz and El Sherbiny (1975), a syrup containing 30 mg/kg HMF administered to bees showed no significant difference with a control syrup in terms of lifespan (in France, the legal acceptable content in honey, with some exceptions, is 40 mg/kg - Decree No 2003-587 of 30 June 2003, Annex II on the characteristics of the composition of honeys). By contrast, still according to these authors, a syrup containing 150 mg/kg HMF leads on average to 58.7% mortality after 20 days of administration. LeBlanc *et al.* (2009b) arrived at the

[30] It has also been known for a long time that "bee nutrition during the pre-winter period and during overwintering though sugar syrup can reduce to a minimum the percentage of bees affected by *Nosema* in the hives" (Toumanoff C., *Les maladies des Abeilles*, Ed. 1930, p162)

same conclusions: administration of a maize syrup with a high concentration of fructose (55%) to bees and containing 150 mg/kg HMF leads to 50% mortality after 19 days. At 26 days, testing HMF concentrations of 57 to 250 mg/kg, only the 250 mg/kg concentration led to significantly higher mortality. These authors considered in the end that the 250 mg/kg concentration was to be considered toxic in bees, a threshold that was exceeded during the episode in Belgium in 2010.

Protein feeding is mostly used in the context of rearing but may be used increasingly given the changes in landscape and the effects of lower diversity that are now being recognised (see food factors). However, few data are available on the physiological needs of amino acids in bees and these data are often old (de Groot 1953). The amounts administered by beekeepers are generally from their own pollen harvest or from bee bread, but some commercial preparations also have this indication. Like for sugar feeding, the presence of chemical or biological contaminants in these preparations is possible. Most of them do not have adequate labelling to determine the precise composition. It is not possible for example to tell whether the intakes meet the needs or to check the digestibility of these proteins by bees.

Artificial feeding is also commonly used by beekeepers to compensate for a temporary lack of resources or to ensure sufficient energy intake when the hive has high needs, particularly in periods of rearing drones. Field experiments have been carried out to test the impact of feeding nurse bees responsible for tending to drones in the larval phase, on the quality of sperm in drones. The bees were fed with sugar syrup, or a mixture of honey and pollen, while no supplement was provided to bees in control hives. It was found that at sexual maturity, the control drones, whose nurse bees were not fed artificially, produced more spermatozoids than drones whose nurse bees did receive feeding, irrespective of the type of feed. In addition, the sperm of the control drones always had the highest percentage of living spermatozoids (Provost, personal communication; Report of the Technical Assistance Project EAGF 2011-2014 coordinated by the ADAPRO association). The questions raised by this experiment concern not only the type of artificial food, its quality, its method of administration and time of administration, but also its relevance.

Feeding bee colonies is a widespread practice since it is often essential but it may present a danger for the health of bees and a factor of weakening (bee mortality, toxicity of certain substances). Currently there are no standards in France, and generally little information, on the composition of bee feeding products. Over and above the question of the health and nutritional quality of these products for bees, the question of their digestibility can also be raised. Because of possible crystallisation (which would incidentally concentrate certain toxic substances in the still liquid phase of the syrup (AFSCA 2010)) or the physiological specificities of bees that for instance have no lactase (Chauvin 1968), the question of the value of these products for feeding bees must be considered with a broad perspective. Quality standards based on available scientific data should therefore be established for these products.

Lastly, **the hives chosen** by beekeepers to house their colonies may have flaws that are compounding factors or have indirect effects on colony health. Concerning their function, firstly, they must be suitable for the location of the apiary (protection against the cold, heat, predators) by minimising as far as possible any robbing and effort from the colony. They must also correspond to the space needed by the colony, depending on its size (applying partitions for example to limit spaces unoccupied by the colony). Certain unsuitable beekeeping practices may, despite good intentions, prove harmful to bees. Examples include partitions made from non-inert materials, and toxic paint and coatings. Substances that are harmful to bees and to consumers of hive products may thus be released by the bees and enter into their food chain.

3.1.5 Climatic factors

Among the various stresses that bee colonies can be exposed to, climatic conditions are one of the most commonly cited factors to explain mortality or decreased production. The species *Apis mellifera* has however, over the course of its evolution, proven its ability to colonise various habitats and to adapt physiologically and anatomically, particularly its remarkable abilities in thermogenesis and thermoregulation. Natural selection and geographic isolation have thus made it possible for various strains to develop that are identified by specific morphological, behavioural and

genetic characteristics, with 26 subspecies of *Apis mellifera* now present in very different climates, from the coldest to the hottest. Our aim here is therefore not to question the ability of the species to adapt to highly varied climatic conditions, but to identify and assess how climatic conditions may disrupt bee colonies in the life cycle. Weather, a factor on which we cannot act but whose impact can be mitigated through suitable zootechnical choices (location of apiaries, colony genetics, feeding, etc.), may have direct and indirect consequences on the development of bee colonies.

3.1.5.1 Direct impact on the development of bee colonies

In our temperate climate, the development cycle of colonies is seasonal with, broadly speaking, periods of development of brood (spring and late summer) and interruptions in egg laying (colder periods). Colonies, in particular queen bees, adapt as far as possible to climatic conditions. As an example, the egg-laying period in late winter is naturally delayed when winter lasts longer. Regulation mechanisms such as the availability of pollen help to modulate egg laying by the queen. Other mechanisms intervene to adjust colony development to its climatic environment. The changing seasons alter the social composition of the colony. Variations in outside temperature, more than day length, change the relative distribution of tasks between workers, related to juvenile hormone (Huang and Robinson 1995). Any "abnormal" climatic event may thus be a stress for the colony, with a cost in proteins and carbohydrates.

The winter period is probably the time of year that is most difficult for colonies to get through given that available resources in the environment almost disappear (pollen and nectar) and the need to fight the cold. Reserves accumulated earlier become crucial, both in terms of their quality and quantity. Available epidemiological data mostly confirm the increased risk of loss during the winter. However, the mortality level during the beekeeping season can sometimes be the same as the winter mortality level in France. For example, during the winter of 2012-2013, it was estimated at 14.1% (CI 95 = 10.8 – 17.5) (Chauzat *et al.* 2014), while it was 13.6% during the beekeeping season, i.e. during the spring and summer of 2013. The observatory of winter mortality of the ADARA - ITSAP has, since its creation, mentioned rates of around 20% (mortality data obtained on the basis of voluntary reporting). The same is found in neighbouring European countries and the United States where overwintering is the major cause of losses in beekeeping (van Engelsdorp *et al.* 2008). These losses are much higher than those observed previously on an annual basis, about 5 to 10%.

In their efforts to combat the cold, bees form a winter cluster, with a diameter and bee density that varies depending on the temperature. The colder the weather, the smaller and denser the cluster (Heinrich 1981; Watmough and Camazine 1995). The temperature within the cluster, which can reach more than 30°C irrespective of the outside temperature, is achieved thanks to production of heat by worker bees (Stabentheiner *et al.* 2003). As such, when the number of bees at the start of winter is insufficient to form a large enough cluster, the small colony will not survive. It is accepted that the minimum strength of a colony to get through the winter and renew activity in spring is at least 8000 bees in a temperate climate for colonies of honeybees (Imdorf *et al.* 2010). Also, any event or stress during the overwintering that leads to decreased adult bee populations and/or food reserves may lead to an increased risk of winter mortality (see section 3.1.3). Similarly, subspecies and ecotypes must be suitable for their environment and in particular for the local climate (see section on genetic factors).

Weather conditions may be stress factors for bee colonies outside the winter period. It has long been known that late spring cold spells hinder the development of brood and affect the quality of nurse bees (Dustmann and von der Ohe 1988). Likewise, long rainy periods can change the behaviour of nurse bees that tend less to brood, with less inspection and cleaning of cells (Riessberger and Crailsheim 1997), thus promoting transmission of disease, specifically those of brood. Certain infectious agents and parasites (e.g. *Varroa destructor* and *Nosema*) develop variably depending on the temperature and humidity (Chen *et al.* 2012; Harris *et al.* 2003). Weather conditions can thus increase the sensitivity of bees to disease.

3.1.5.2 Indirect effects on the development of bee colonies

Indirectly, climatic conditions can also affect the physiology of bee colonies by changing the access to or quality of nutritional resources. Foraging behaviour and expansion of the colony are related to flowering in association with climatic conditions.

Concerning the quality of food resources, other than the floral environment (see section 3.1.3), climatic events can change the melliferous or polliniferous potential of a plant: heavy rain can for instance wash acacia flowers making them less attractive to bees and diluting their nectar (Le Conte and Navajas 2008). By contrast, lavender flowers no longer produce nectar when the weather is too dry (Le Conte and Navajas 2008). Colonies are particularly susceptible at specific times, like during development of brood. When the colony and its brood have reached a considerable size thanks to an early spring, sudden interruptions in the availability of pollen can be very damaging (Mattila and Otis 2006). In terms of access to these resources, optimal conditions for foraging vary over time: according to Puškadija *et al.* 2007, sunflower flowers are visited by bees optimally when the temperature is between 20 and 25°C with humidity of 65 to 75%. A higher level of humidity, heavy rains, wind, or low temperature, have a negative effect on flower visits by bees. Similarly, higher temperatures have a positive effect on foraging flights but the intensity of sunlight can, above an optimum level, reduce their number (Burrill and Dietz 1981).

More generally, large-scale climate changes could potentially, in the near or more distant future, result in desynchronisation between the geographic distribution of melliferous and polliniferous plants and the lifecycle of bees (Abrol 2009; Delgado *et al.* 2012; Thuiller *et al.* 2005).

Although honeybee colonies have sophisticated ways of adapting to their environment, which have enabled them to colonise diverse and sometimes hostile areas, climate has a major impact on their development and health. That said, since the honeybee is a general pollinator insect and it is therefore able to collect food from a large number of plants, it would be less affected than pollinator insects that are specialised in certain plants.

3.1.5.3 Conclusion

The effects of the climate on bee colonies can be considered at the loco-regional level or at the global level. The intensity and duration of weather phenomena such as droughts, rainfall, extreme temperatures and wind must be taken into account as a stress factor for colonies. They may, directly or indirectly lead to weakening of a colony by disrupting its physiological balance. The choice of subspecies or a suitable ecotype for the local environment appear therefore to be important aspects. If this is not the case, beekeeping practices will need to compensate for these stresses. An example is winter feeding for large colonies overwintering in areas with long, harsh winters. The physiological processes of colony response to climatic stress are still unknown, though we are aware of the colony's marked thermoregulation abilities, among others.

3.1.6 Physical factors: electromagnetic fields

Bees have a magnetoreception system (Kirschvink *et al.* 1997). The mechanism relies on iron "granules" distributed randomly in the cytoplasm of certain cells in the bee, particularly under the cuticle of the abdomen (Hsu and Li 1994; Kuterbach *et al.* 1982). This system is highly effective since bees can detect fluctuations in the Earth's magnetic field (evaluated at 50 μT) of very low intensity (i.e. from 0.026 μT). It enables them, according to (Hsu *et al.* 2007), to orient themselves using a magnetic "memory" of their environment.

We also know that the electromagnetic fields created by human activities, high-voltage power lines for instance, leads to electrical disruptions of charged objects. Electromagnetic fields produced by these high-voltage lines can affect bee behaviour (Bindokas *et al.* 1988; Lipinski 2006; Sharma and Kumar 2010) and the development of colonies (Greenberg *et al.* 1981; Lipinski 2006). Electromagnetic fields generated by mobile phones in telecommunications can also lead to biochemical changes, such as lower carbohydrate and lipid levels in the haemolymph, in worker bees (Kumar *et al.* 2011), probably related to increased activity, i.e. increased aggressiveness and

frequency of wing movements. They also alter the sounds emitted by the colony (induction of "piping" (Favre 2011)).

However, the effects observed in these publications were found in conditions of close proximity between the emitting source and the colony (mobile phones placed in the hive (Favre 2011; Kumar *et al.* 2011), conditions that are not found in the natural environment. On the topic of high-voltage power lines, a safety distance of 65 metres would be enough to protect bee colonies from the possible harm induced by the highest voltages (Lipinski 2006). Choosing a suitable location for the apiary should therefore limit these risks. If applicable, the expected effects through disruptions by electromagnetic radiation are an increase in consumption of reserves by the colony (Kumar *et al.* 2011) and changes in bee behaviour (Lipinski 2006).

Lastly, recent publications showed the importance of electric fields in exchanges between individuals (honeybees (Greggers *et al.* 2013)) or in exchanges with their environment (bumble bees (Clarke *et al.* 2013)).

More realistic studies closer to field conditions and with a greater scope, i.e. number of tested colonies and duration of study, would make it possible to better understand the real impact of electromagnetic fields on bee health.

3.1.7 Changes in colony structure

Some natural states in a colony cannot be considered stresses strictly speaking, but could participate, as risk factors, in increased exposure to other stress factors. Among the physiological events that have the most effects, important examples are swarming and aging queen bees.

These phenomena essentially generate quantitative changes in the population through abnormal egg laying by the queen. In both cases, the age pyramid between workers can become considerably altered (distribution of age groups) and require adaptations such as plasticity of roles played by worker bees. This imbalance in the age pyramid may also be the result of other causes, for instance intoxication with mortality of foragers. Importantly, when these phenomena occur, the (new) queen may not be fertilised and may even die, e.g. swarming, drone colonies, and supersedure. Poor quality of queen bees is considered by American beekeepers to probably be the main cause of winter colony losses (van Engelsdorp *et al.* 2008). Likewise, the age of the queen at the start of winter seems to be a predictive factor for winter survival of the colony (Genersch *et al.* 2010). Lastly, an anomaly affecting the queen appears to increase the risk of collapse for a migratory colony (van Engelsdorp *et al.* 2013b). Many causes, whether suspected or proven, may harm the queen bee, including:

- pesticides, such as coumaphos (Pettis *et al.* 2004), which can lead to lower fertility in drones (Burley *et al.* 2008);
- certain infectious agents, such as *Nosema ceranae* which affects the physiology of queen bees (Alaux *et al.* 2011b) and viruses that are associated with ovarian degeneration (Gauthier *et al.* 2011);
- or lastly, poor insemination (Richard *et al.* 2007).

If these problems lead to death of the queen bee, colony collapse may occur. When they only lead to "weak queen bees", they increase the susceptibility of the colony to other stress factors. Well-controlled beekeeping practices may compensate for these changes, in some cases.

3.2 Presentation and analysis of data on exposure to biological and chemical factors in France (single-factor aspects)

3.2.1 Objectives of examining available exposure data

To evaluate exposure conditions in France, the working group had monitoring data from public analysis laboratories, studies co-financed by French or European public funds, and from private initiatives of professional networks.

The data reviewed and examined in this report were generated in different contexts for different objectives in France between 2006 and 2013 (Table 7). The nine datasets and their use will be presented briefly below. They include results for microbiological and chemical analyses on matrices including adult bees, larvae, pollen, honey and bee bread. Some of them also include parameters on colony health status, such as colony strength, disorders and mortality.

Table 7: Presentation of analysed datasets

Name or acronym	Title	Objective of the sponsor	Type	Study design	Number of apiaries	Period/ geographic area	Measurement of IPAs	Measurement of chemical residues	Measurement of co-occurrences	Parameters of hive status
ONIRIS	Sentinel bee	Multicentre study on the presence of chemical and biological hazards in various contexts and landscapes	Analytical	Repeated cross-sectional	18 apiaries	2008 and 2009, 4 samples per year (western France)	Yes in 2009 systematically quantitative (method applied for a single laboratory)	Yes in 2008 and 2009 quantitative (method applied for a single laboratory)	Yes	Yes in 2008 and 2009
Epilobee France	Bee mortality	French part of the European multicentre study on the prevalence of bee disorders and mortality (*Résabeilles*)	Analytical	Repeated cross-sectional	391 apiaries	2012 and 2013 (6 French *départements*)	Yes but detection of IPAs depending on symptoms	No	No	Yes
Cruiser	Post-approval maize surveillance plan Cruiser TM	Follow-up of apiaries exposed to crop areas with maize seeds coated with Cruiser over time	Analytical design initially, finally descriptive	Originally, Cohort (exposed/non-exposed) but high recruitment bias. Usable as a case study	56 apiaries	3 years: 2008-2010 (6 regions)	Yes but not systematic	Yes but not systematic and several labs involved without inter-laboratory harmonisation	Yes but not systematic and non-standardised	Yes but not standardised
BNEVP	Follow-up of residues over time	Observation of possible adverse effects related to pesticide residues	Descriptive	Case study	5 apiaries	Spring 2011 (2 *départements* in the South West)	Yes but not systematic	Yes	Yes for residues	Yes

Itsap/ CETIOM	Follow-up of apiaries in oil seed crops over time	Link between the stress factors (residues, infectious agents) and status of colonies	Descriptive	Case study	4 apiaries	Spring 2012 and 2013 (region Centre)	Yes but not systematic	Yes	Yes for residues	Yes
ADARA	Analysis of apiaries with disorders in season	Detection of biological and chemical agents associated with the disorders	Descriptive	Case study	13 apiaries	Winter 2012 (1 case) Spring and summer 2013 (12 cases) (region Rhône-Alpes)	Yes systematically quantitative (method applied by a single laboratory)	Yes but not systematic and several labs involved without inter-laboratory harmonisation	Yes for IPAs	Non-standardised symptoms
DGAL, Ministry of Agriculture	Annual monitoring network for bee disorders	Cause of disorders for governmental actions (regulated diseases, massive intoxications)	Descriptive	Case study	36 apiaries	Year 2013 (national)	Yes but not systematic	Yes but not systematic	No	Non-standardised symptoms
LNR Sophia	Summary of analytical results for samples submitted to the NRL (ANSES Sophia-Antipolis)	Results obtained by a diagnostic and national reference laboratory	Information on type of hazards detected in various matrices	ND; no history in the database	482 + 253 test reports	2011-2013 (national)	Yes but not systematic	Yes but not systematic	No	No
LDA39	Summary of analytical results for samples submitted to LDA39	Results obtained by a departmental diagnosis laboratory	Information on type of hazards detected in various matrices	ND; no history in the database	658 test reports	2006-2012 (several *départements*)	Yes but detection of IPAs depending on symptoms	No	No	No

Statistical processing for the requirements of the formal request was carried out by scientists at ANSES in collaboration with several members of the working group. Descriptive statistics (means, quartiles, ranges) were used to describe the datasets and were mostly a simple observational value that could not be extrapolated to the whole area where they were obtained. Results of statistical association tests are valid *within each dataset*.

The summary of these observations can be used to establish lists of infectious and parasitic agents (IPAs) and chemical substances (residues) that were detected in the bee matrices in France between 2006 and 2013. Some of the methods used were qualitative (presence/absence), others were quantitative. The lists of hazards, then quantitative information (i.e. infectious load or residue dose) will be examined in order. In chapter 4.2 we will present and discuss joint observations of hazards in the same apiary (co-exposure) and their co-occurrence with symptoms or subclinical variables.

This overview aimed to gain knowledge from these observations with a view to improving:

- the diagnosis of diseases in bee health;
- knowledge of co-occurrences;
- monitoring of emerging problems;
- ex-post evaluation of veterinary and plant protection products;
- observational tools for the detection of bee diseases.

3.2.2 Summary presentation of nine datasets examined and information obtained for the request

3.2.2.1 ONIRIS: multicentre study of residues and infectious and parasitic agents (IPAs) in 18 apiaries in Western France (2008 – 2009)

This multicentre study on 18 apiaries was designed to evaluate the usefulness of the honeybee as an indicator of contamination of the environment by chemical hazards. It compared different landscape contexts in the Pays de la Loire region. These were hedged farmlands, large-scale crops, or urban areas, as well as two island apiaries. Apiaries were monitored two years running, four times during the season. The results for pesticide residues were published (Lambert *et al.* 2013) and are presented in section 3.3 on the discussion of detected substances. In the second year (2009), systematic and quantitative microbiological analyses (qPCR) were carried out for the four sampling periods.

None of these apiaries showed symptoms of disease or mortality in season. This was therefore a sample of asymptomatic apiaries. The rate of winter survival of colonies was studied in relation to infectious loads by Mouret *et al.* (2013). The results will be summarised along with those on interactions in section 4.2.3.

In addition to the prevalence of chemical and microbiological hazards, it was possible, at the request of the working group, to calculate the co-occurrences of hazards in the same apiary for this dataset.

Certain health status variables were collected at each visit. The link between the status variables and detection of a given hazard, whether chemical or microbiological, was also assessed.

We should note that the characteristics of the landscapes in foraging zones were known (landscape analysis by Geographic Information System), as well as the botanical families of the pollen collected for the hives at each period (palynological analyses) (Piroux *et al.* 2014). Field surveys were also carried out on treatments applied by users, including farmers, local authorities, companies and private individuals). These additional data will be used for the general discussion (section 4.3).

3.2.2.2 Epilobee France (Résabeilles 2012 and 2013) epidemiological surveillance of cases of mortality in 391 apiaries

This study was motivated by the fact that the real extent of colony losses in European was not known. Epilobee was an epidemiological survey on the prevalence (case numbers) of colony mortalities. It was coordinated at the European level by ANSES, with standardised protocols. Preliminary results were available in April 2014 (Chauzat *et al.* 2014). The French part of this study included 391 apiaries randomly selected in six *départements*. In the event of mortality or clinical cases (for example symptoms characteristic of foulbrood), microbiological analyses were performed, but the type of hazard screened for depended on the associated symptoms with the aim of confirming clinical suspicion through laboratory analysis. It was therefore not possible to calculate the prevalence of IPAs in this sample of apiaries. The study is representative concerning prevalence of cases of mortality and clinically manifest disease, but no statistical association can be calculated between the presence of a given microbiological hazard and occurrence of a disease, since there was no analysis in non-affected apiaries by comparison.

In this survey, chemical hazards were not screened for.

The information provided by the study for this overview concerned which microbiological hazards were detected in the event of mortality or disease, and at what infectious load, when there were quantitative methods.

3.2.2.3 Cruiser Maize – Post-approval monitoring plan 2008-2010

The marketing authorisation for Cruiser (seeds coated with a neonicotinoid insecticide, active substance thiamethoxam) granted in 2008 was associated with a monitoring plan for possible adverse effects in field conditions, implemented by the French Directorate General for Food (DGAL). The seeds used at the time were those marketed in indications for which coated products were approved. In most cases, the coating also contained the fungicides fludioxonil and Metalaxyl M (mefenoxam), as well as adjuvant substances in co-formulation. Coating was performed by seed companies and not by the product manufacturer. A follow-up study was therefore implemented that resembled in its design an explanatory epidemiological study of the exposed/non-exposed type (cohort). The exposure variable initially considered was the presence or absence of Cruiser maize cultivated areas within a radius of 1 km around an apiary, in a landscape already planted with maize crops. Two to three “exposed” apiaries and two to three “non-exposed” apiaries per year were followed-up in six different regions. The study lasted three years and six of the 49 apiaries were followed-up in consecutive years, but not necessarily the same colonies.

Strong recruitment bias affected the implementation of this study. Many non-exposed study apiaries in fact had Cruiser maize in the immediate foraging area. Information on maize-cultivated areas within 3 km, on other crops and other treatments applied in the immediate foraging area were not available for this study. Over these years, maize coated with Cruiser was the only authorised product containing thiamethoxam. Clothianidin, the main metabolite of thiamethoxam, was also marketed in the past as an active substance but did not have an authorisation for the years covered by the study.

Since the "with" and "without" categories for Cruiser in the 1 km area were not valid, new exposure variables were calculated based on the area cultivated with Cruiser maize and the total maize-cultivated area in the immediate foraging area.

Thiamethoxam and clothianidin, as well as acetamiprid and thiacloprid, were screened for in several beekeeping matrices, mainly during the maize flowering period. However, it was found that contamination of beekeeping matrices, in frequency and content, by thiamethoxam and clothianidin was rather similar in the areas with different Cruiser maize densities. This bias prevented effective differentiation of field situations for a study based on a comparison.

There are no palynological analyses that would indicate which resources were actually visited by the bees of these apiaries.

Some IPAs (viruses, bacteria, microsporidia, *Varroa*) were screened for but large amounts of data are lacking.

Symptoms were recorded as well as certain colony health status parameters.

In view of the protocol's insufficiencies, the data are used as case studies where co-exposure could be observed, in association with certain colony health status parameters.

3.2.2.4 BNEVP – Follow-up of residues in five apiaries over time (spring 2011)

Following cases of colony losses in spring 2008, the National Brigade for Veterinary and Phytosanitary Investigation (BNEVP) carried out sampling in 2009 and 2010 on several apiaries that had been affected in 2008, with a view to identifying a possible common causal factor, i.e. one that would be present in the analysis of samples from April-May. The apiaries did not present disorders in 2009 and 2010. In spring 2011 (March to mid-May) these apiaries were then monitored weekly with evaluation of the status of colonies, with screening for 63 residues and detection of specific IPAs, among which only the presence of SBV and quantification of *Nosema* spp. spores could be used for the statistical study.

Although there are not many data, it was possible to observe co-exposures to several substances in trapped pollen and bee bread. There are too many missing data and the data supplied are too inconsistent to quantify the link between the presence of residues and colony health status parameters (rate of filling of frames).

3.2.2.5 ITSAP/CETIOM[31] Follow-up of residues in four apiaries in a context of oil seed crops over time (April - May 2012 and May - June 2013)

Spring is considered to be a period of particularly high toxic risks in oil seed crop areas (rapeseed, sunflowers). This follow-up consisted of two to three visits in the space of 15 days during flowering. Its aim was to measure joint exposure to 33 substances used in agriculture on this type of crop, including three neonicotinoids, in field conditions. Viruses, microsporidia and bacteria (foulbrood) were screened for in symptomatic bees and in dead bees, but large amounts of data are missing. Infestation with *Varroa* was not studied. Among the acaricides used in the control of *Varroa*, only *tau*-fluvalinate was screened for, because of its use in agriculture.

The weight of hives was recorded and weight gain calculated over 15 days, as well as the rate of colony loss and the possible presence of symptoms.

Here again, these are purely case studies since the apiaries were not chosen to be representative of the apiaries in the region.

These data contributed to the list of IPAs and residues detected in beekeeping matrices.

In these four apiaries, 13 colonies presented disorders and 19 did not. The association between disorders, mortality and weight gain in the colonies was examined, as well as the association between disorders and detection of the 33 residues, taken one by one.

3.2.2.6 ADARA[32]: Study of biological and chemical hazards in 12 apiaries with disorders in the 2013 beekeeping season, Rhône-Alpes region

This professionally sponsored study was implemented to strengthen the national epidemiological surveillance network on bee disorders (see section 3.2.1.7). The twelve apiaries reported in Rhône-Alpes because of occurrence of disorders were examined in depth, with systematic screening for IPAs using a calibrated method (standardised for the purposes of the study) that was quantitative (eight viruses + *Nosema apis* and *N. ceranae*) and screening for residues by three different laboratories. Foulbrood was not screened for.

This dataset can thus contribute to the list of IPAs and residues detected, with good reliability and comparability of infectious loads. The analytical method (qPCR) and the test samples were the same as those in the ONIRIS study, which was performed in another region and on apiaries without disorders. By contrast, for residues, the number and quantities of substances are not comparable from one apiary to another since they were obtained by different laboratories.

Co-occurrences between hazards were described but were only quantified for IPAs as a group.

[31] CETIOM: French technical centre for research and development of production procedures for oilseeds, protein crops and industrial hemp

[32] ADARA: Association for the development of beekeeping in Rhône-Alpes

The health status parameters and type of reported symptoms are rather inconsistent but did allow for an overall discussion on standardisation of questionnaires for epidemiological surveillance.

Lastly, the relationship between the various disorders and the presence of biological hazards was assessed.

3.2.2.7 DGAL: Annual network for surveillance of bee disorders - 36 apiaries in 2013

The DGAL has carried out passive epidemiological surveillance since 2011 through voluntary reporting of cases of bee disorders. This involves clinical cases of regulated infectious diseases, i.e. American foulbrood, nosemosis caused by *Nosema apis* and exotic arthropods, and "significant in-season mortality", such as massive intoxication.

The guidance note DGAL/SDSPA/SDQPV/N2012-8113 provides the investigation procedure for 2013 and the questionnaire to use for reported cases. The objective is to enable the authorities to take appropriate regulatory measures to prevent risks: sanitising outbreaks of foulbrood, identifying adverse effects of products authorised for sale, issuing penalties for inappropriate use of approved products or illicit use of non-approved products.

As a general rule, disorders are probably under-reported with 98 cases for the whole country, including 36 that were not sufficiently described to be used in this assessment, and completion of questionnaires was incomplete. Laboratory analyses for IPAs or residues were carried out based on the assessment of the prescriber in view of suspicion and are not comparable among themselves.

This dataset can therefore only contribute to the list of IPAs and residues detected.

The questionnaires filled in can contribute to the overall discussion on the definition of cases of "significant in-season mortality" and standardisation of questionnaires in epidemiological surveillance.

3.2.2.8 LNR Sophia-Antipolis – Summary of results for samples submitted for analysis to the National Reference Laboratory 2011-2013

The NRL performs testing to detect IPAs or residues for public or private contractors. The results for 2011 to 2013 were extracted from the laboratory's internal database. Since the database of results for chemical analyses is separate from that for microbiological analyses, the structure of the data does not indicate which hazards were identified simultaneously in the same apiaries. The reasons for analysis and case history are not included in the database.

The dataset contributed to the list of hazards, whether biological or chemical. There are no co-exposure data nor colony health status parameters.

3.2.2.9 Departmental analysis laboratory for the Jura (LDA39) – Summary of results for samples submitted for analysis to an accredited departmental laboratory between 2006-2012

The LDA39 carried out a certain number of detection analyses for IPAs in bee pathology for public or private contractors. The results for the years 2006 to 2012 were extracted from the laboratory's internal database. There were no results for chemical analyses. The reasons for analysis and case history are not included in the database.

Like the information mentioned above, this dataset was used to build the list of detected hazards. There is no information on co-infections nor colony health status parameters. In the future, it would be beneficial to include this missing information in the databases of the NRL and departmental analysis laboratories. General discussion on hazards detected in this dataset.

3.2.2.10 Wide range of hazards detected in various matrices

3.2.2.10.1 Biological hazards

Table 8 provides an overview of the infectious agents detected in adult bees and/or in brood. Depending on the dataset, "adult bees" are frame bees or foragers sampled from the flight board. Findings are consistent with current knowledge on the sensitivity of methods since certain hazards are known to be more easily detectable in a given matrix. The detection threshold also depends on the method used; molecular methods are generally more sensitive than detection by spore counting (possible for *Paenibacillus larvae*, and *Nosema* spp.). The frequencies of detection in the studies are also given for information purposes in Table 8.

Most of the agents known to circulate in Europe are found in France. Viruses are omnipresent. Foulbrood agents are easy to detect when molecular methods are used. *Nosema* were detected in all studies that screened for them.

These findings are consistent with current knowledge on circulation of infectious agents in France (see sections on asymptomatic carriage and biological hazards).

Table 8: Screening and detection of biological hazards in datasets
(N = number of studies with detected hazard / Number of studies with screening for hazard)

	Bees: prevalence (detection and symptoms – Epilobee)	Bees: detection	Brood: detection	Comments
CBPV	1.2 – 2.6%	Yes (N = 8/8)	No (N = 2)	Virus often detected only in bees
SBV	-	Yes (N = 7/8)	Yes (N = 5/5)	Virus often detected in bees and brood
ABPV	-	Yes (N = 7/8)	No (N = 4)	Virus often detected only in bees
BQCV	-	Yes (N = 7/8)	Yes (N = 3/3)	Virus often detected in bees and brood
DWV	-	Yes (N = 7/8)	Yes (N = 2/3)	Virus often detected in bees and brood
IAPV	-	Yes (N = 7/8)	Yes (N = 3/3)	Virus often detected in bees and brood
KBV	-	Yes (N = 4/8)	No (N = 3)	Virus sometimes detected only in bees
VdV1	-	Yes (N = 3/3)	-	Virus not often screened, but detected
Paenibacillus (American foulbrood)	1.5 – 11.6%	Yes (N = 1/1)	Yes (N = 3/4)	Bacterium present mainly in brood but rarely screened
Melissococcus (European foulbrood)	3.6 – 7.6%	Yes (N = 1/1)	Yes (N = 4/4)	Bacterium present mainly in brood but rarely screened
Nosema (counting)		Yes (N = 5/5)	Yes (N = 1/1)	Microsporidian often detected in bees
N. ceranae (typing)	-	Yes (N = 7/7)	Yes (N = 1/1)	*N. ceranae* more often detected than *N. apis*
N. apis (typing)	0 – 0.3%	Yes (N = 4/6)	No (N = 1)	
Acarapis woodi	-	Yes (N = 3/3)	-	Not often screened, but detected
Varroa destructor	0.9-7.3%	Yes (N = 4/4)	Yes (N = 3/4)	Not often screened

3.2.2.10.2 Chemical hazards

In seven of the nine studies assessed, a total of 115 chemical residues were screened for in adult bees, pollen brought back to the hive, honey, as well as other matrices.

Fifty-five substances were detected at least once. A summary by category is presented in Table 9.

Table 9: Summary of substances screened for and detected at least once in hives, in nine studies across France.

Substance category	Number of substances screened	Number of substances detected
Acaricides	6	3
Herbicides	3	3
Fungicides	38	22
Insecticides	61	27
Other	7	0
Total	115	55

The methods used have varying degrees of sensitivity. In general, multiple residue methods are less sensitive. Results are particularly difficult to compare since no method is standardised from one study to another. Some studies called on several different laboratories and panels of different substances were screened.

Analysis of the data shows that among the acaricides, mainly amitraz I and II were detected. These are the metabolites of amitraz, the main product used in hives against *Varroa*.

Among the 61 insecticides analysed, 34 were not detected, either because they were effectively absent, or because their concentration was below the chosen detection limit. Seven insecticides were noteworthy particularly because of their frequency of detection:

- *tau*-fluvalinate and coumaphos in bees,
- carbaryl, *tau*-fluvalinate, phosmet, coumaphos and pyriproxyfen in pollen,
- carbaryl, imidacloprid, *tau*-fluvalinate, phosmet, coumaphos, pyriproxyfen and piperonyl butoxide in honey,
- desmethyl-pirimicarb, thiacloprid, thiamethoxam, acetamiprid, *tau*-fluvalinate and coumaphos in bee bread.

Of the 38 fungicides screened, 16 were not found or only rarely. 14 fungicides were detected more often:

- thiophanate-methyl and carbendazim in bees,
- prothioconazole-desthio, pyrimethanil, tebuconazole, boscalid and carbendazim in pollen,
- imazalil, cyproconazole and carbendazim in honey.

Carbendazim was found often in the three matrices. These findings are mainly from the ONIRIS study (samples from 2009, authorisation withdrawn since 2008 with limit of use until 31/12/2009). This substance is also a metabolite of other benzimidazoles (thiophanate-methyl and benomyl).

The substances screened for are a subgroup of the substances actually used in France. As an indication of the extent of use, a summary of the amounts sold from 2009 to 2012 for the substances detected in the matrices, and their place in the ranking of sales, will be examined in section 4.2.

The case of the Cruiser study shows the low reliability of quantifying exposure by measuring the surfaces cultivated with the product in an area. Thiamethoxam and its metabolite clothianidin were detected in beekeeping matrices more frequently than expected in view of the treated surfaces in the immediate foraging area, 1.5 km in this study. This was the case even though at the time of the study, no other product was authorised in France for these two substances.

3.2.2.11 Highly variable infectious loads and quantities of residues detected

3.2.2.11.1 Infectious loads

The only method that provides standardised comparable results from one laboratory to another for the quantification of microorganisms is spore counting (for the causative agent of American foulbrood *Paenibacillus larvae* and for *Nosema* spp.) (OIE 2014).

Molecular methods, specifically qPCR quantification for microorganisms, are not reproducible from one laboratory to another, even when they are expressed in the same unit (number of copies per bee). Results can only be compared within a single study, for instance for seasonal variations.

Figure 3 is an example of the range (minimum-maximum) of infectious loads detected by qPCR in the ONIRIS study for colonies without disorders, during the beekeeping season (March to November 2009) in Western France (method see Gauthier *et al.* (2007)).

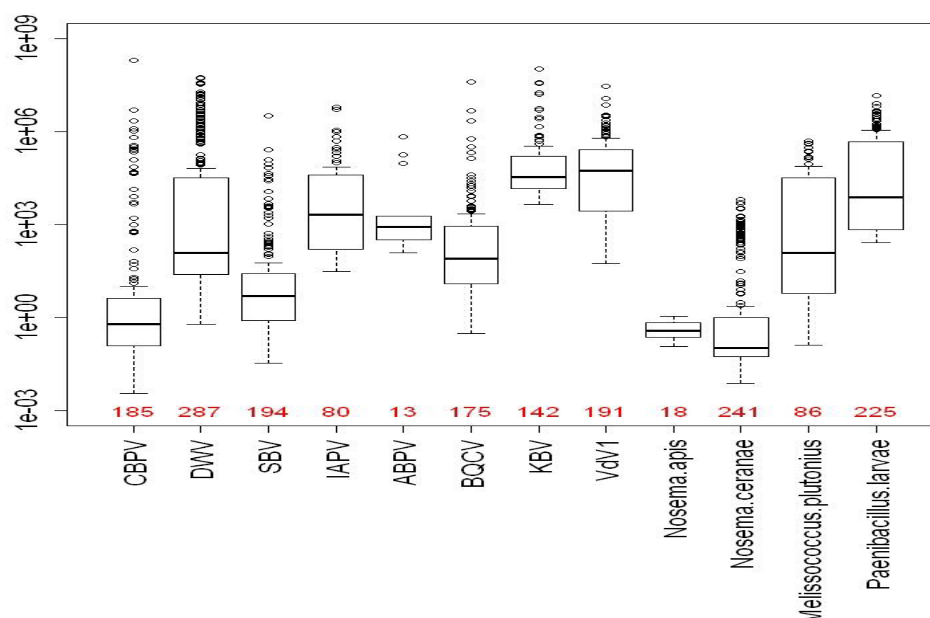

Figure 3: Minimum, maximum, medians as "number of gene copies" per bee detected by qPCR in adult bees from asymptomatic hives over the year 2009, for 12 infectious agents.

The box plots correspond to the first and the third quartile (25 and 75%). In red, the number of samples for which results were available.

The quantities of agents detected vary by a factor of 10 logarithms; this range clearly covers marked seasonal differences. However, it can be seen that they are systematically elevated for DWV and for other viruses transmitted by *Varroa*. The ONIRIS study gives no data on the parasitic load of *Varroa*, but these colonies without disorders clearly harbour many viruses, the bacteria causing European and American foulbrood, as well as occasionally large quantities of *Nosema ceranae*.

In the ADARA results (13 cases of apiaries affected by depopulation or imbalances in the structure of the hive population), infectious loads detected were also highly variable, especially between the spring and summer (Figure 4).

In the summer cases, the amounts of *Nosema ceranae*, CBPV, IAPV, and BQCV measured were higher than in those that occurred in the spring

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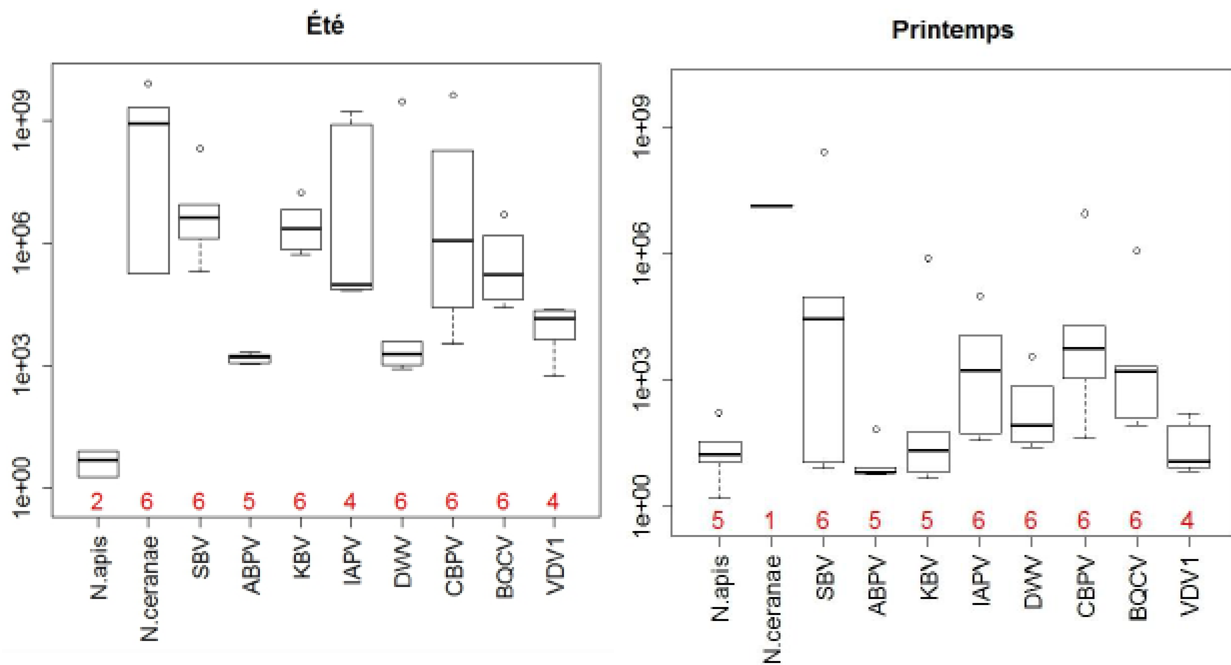


Figure 4: Minimum, maximum, medians and quartiles of the number of gene copies per bee detected by qPCR in bees from 12 symptomatic apiaries in 2013 (ADARA clinical case study), for 10 infectious agents. In red, the number of samples for which results were above the limit of quantification.

3.2.2.11.2 Analysis of residues

For residues, the detected doses, when they were quantifiable, depended on the method, the laboratory, the matrix, the test sample, and the storage conditions before analysis. It is therefore completely impossible to compare quantitative data from one study to the next. In terms of magnitude, most of the positive residues were around 10 times the limit of quantification. We can however see, as a general rule, that all the beekeeping matrices can be contaminated by multiple pesticides, particularly insecticides (including acaricides) and fungicides.

3.2.3 Conclusion

These findings cannot be extrapolated to determine the prevalence of biological or chemical hazards in French apiaries because the representativeness of the samples is not sufficient (statistical biases). Moreover, only some of the studies were designed for the systematic and standardised detection of an array of biological and chemical hazards.

Nonetheless, the data help to target the hazards to detect in the future, as well as the methods to use.

For biological hazards, the results show that methods are needed that:

- simultaneously detect the main agents known to be potentially pathogenic in France;
- are specific, distinguishing between *Nosema ceranae* and *Nosema apis* for example, or between the viruses of the AKI complex;
- are quantitative, with relative quantification between agents, samples and dates of sampling;
- have a rather low detection threshold.

Quantitative PCR methods fulfil these requirements. Their current cost is still high but other technologies (miniaturised) could be used in the future, provided that they also fulfil these criteria.

This type of method is particularly useful for European and American foulbrood, for which the prevalence situation is still poorly characterised. It is remarkable that these two agents are generally screened for only after clinical suspicion. It is quite possible, like for other agents, that they have subclinical additive or synergistic effects with other hazards.

For chemical hazards, it is the wide diversity and multiplicity of detected substances in the various matrices that is striking. The diversity of substances that are actually present is very likely underestimated in these datasets, firstly because the number of substances screened for per study is relatively low. The data show, nonetheless, that insecticides and fungicides are the main chemical agents found in all beekeeping matrices. In all, 115 different substances were screened for, while more than 400 are commercially available in France (BNV-D data, 2012).

An increasingly large number of multiple residue tests are being developed by analytical laboratories, but these often have the drawback of being low in sensitivity, with an excessively high detection threshold. They also have a high implementation cost related to calibration and validation of equipment for each substance and each matrix. Finally, for each matrix, the type of extraction determines the sensitivity of the analysis.

The type of matrix to analyse as a priority depends on the physico-chemical properties of the screened residues (lipophilic, hydrosoluble, etc.), but also the type of risk that is being assessed.

When a case of intoxication with a chemical agent is suspected, multiple residue analytical methods should be given preference, especially if the chemical agent has not been identified by a field survey, for example. Depending on the analytical result, there may be zero, one or many chemical agents present. A negative detection result does not mean that no chemical agent is responsible. It is then necessary to check whether the sensitivity of the analytical method, in terms of limits of detection and quantification, is suitable for the investigation. If the sensitivity is suitable for the context, the spectrum of substances to screen for should be widened. If a single chemical agent is detected, it is necessary to evaluate the coherence of the causal link between (1) the toxicity and/or effects of the agent, (2) the level of the chemical agent found in the analysed matrix (in view of the limits of quantification and detection of the method), (3) its physico-chemical characteristics (stability, etc.), and (4) knowledge of the situation in the field (treatments based on places and dates, for example). When several chemical agents are identified, this approach, applied for each chemical agent, will help to rank the possible causes of intoxication, from the least likely to the most likely. If a chemical agent is strongly suspected or in cases where confirmation or greater sensitivity is sought, single-residue methods that include screening for relevant metabolites should be given preference. There are also multiple residue methods with low numbers of screened agents that have the advantage of combining the benefits of the previous methods in terms of identification and sensitivity for the analysed matrices. Lastly, it is important to remember that some substances have major indirect effects at low concentrations (synergies with other stress factors, inhibition of biological functions, etc.), meaning that corresponding analyses must always be performed with suitable sensitivity, i.e. as high as possible. Interpretation of toxicological results can be complex. Identification of a pesticide or a metabolite in a biological sample is proof of exposure. But it is often difficult to evaluate with precision the dose or duration of exposure because of imprecise intervals between the incident and collection of samples.

The National database of sales of plant protection products by approved distributors (BNV-D) was consulted to determine the position of the substances detected in these nine studies among all plant protection products. The database covers more than 400 substances marketed each year in mainland France, in the form of more than 2500 different products. For example, boscalid, which is found in a number of studies, is in 21st position among the fungicides, in kg of active substance sold (source: Onema and ANSES - National database of sales of plant protection products by approved distributors - BNV-D). As an example, *Figure 5* shows the cumulative quantities sold of 15 substances that were detected in beekeeping matrices for use as fungicides and insecticides, by region and by year[33]. These data, which represent only a fraction of the substances used in

[33] Seven fungicides = thiophanate-methyl; carbendazim; prothioconazole-desthio; pyrimethanil; tebuconazole; boscalid; and cyproconazole. Eight insecticides = *tau*-fluvalinate; carbaryl; phosmet; imidacloprid; pirimicarb-desmethyl; thiacloprid; thiamethoxam; and pirimiphos-methyl

France, show that co-exposure to insecticides and fungicides can take place constantly and may be massive in some regions.

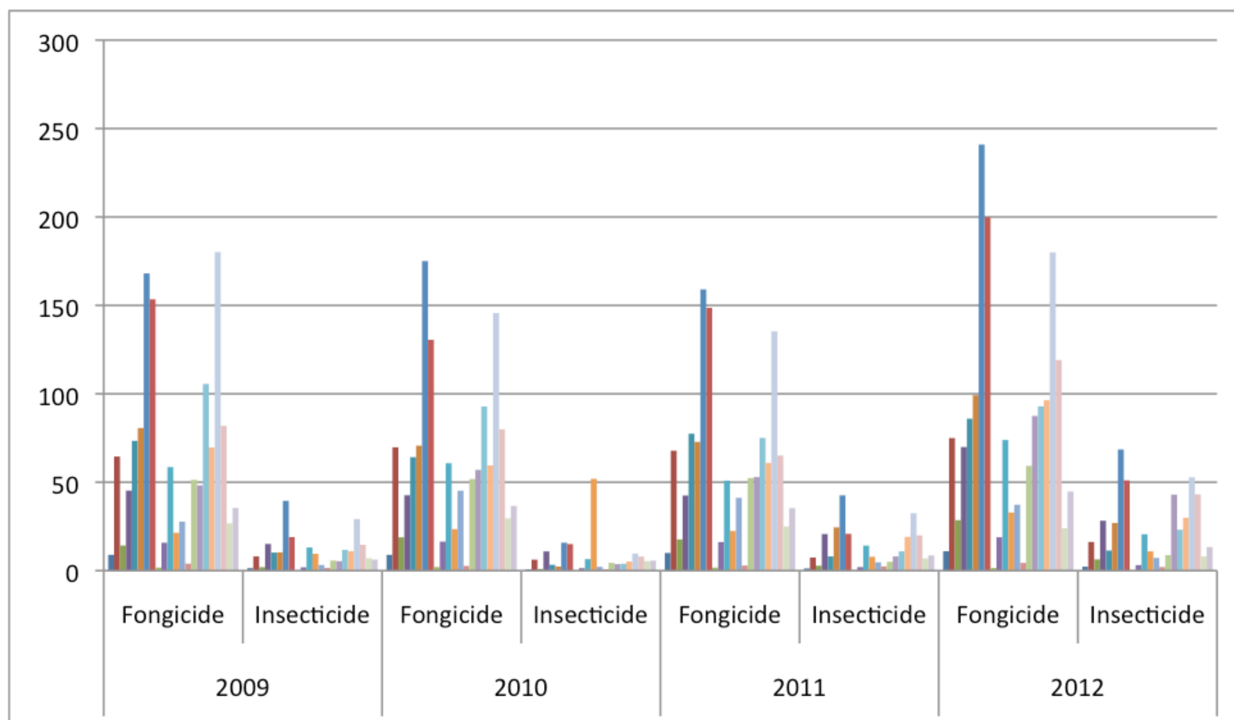


Figure 5: Inter-annual and inter-regional variations in cumulative quantities sold by usage (in tonnes) of 15 active substances detected in beekeeping matrices in France (7 fungicides and 8 insecticides). Each colour in the histogram represents cumulative quantities for one of the 22 regions in mainland France (source: Onema and ANSES – National database of sales of plant protection products by approved distributors – BNV-D)

The wide range of substances that can be found is also reflected in the survey of uses of plant protection products in foraging areas in the ONIRIS study (18 apiaries in Western France). The surface studied was equivalent to about half of the total surface of examined foraging areas (Lambert 2012).

Table 10 and Table 12 extracted from this thesis show the high number of treatments applied and the high diversity of substances used in these 18 foraging areas.

Table 10: Overview, by type of landscape, of the number of plant protection and veterinary treatments applied in foraging areas for 18 apiaries in Western France and relative to one hectare of studied land

(Source: Lambert, 2012, doctoral thesis)

Apiary	Number of plant protection treatments		Number of veterinary treatments	Total	Mean number of treatments per ha of studied land
	Professional agricultural uses	Other uses			
Hedged farmland	5023	420	975	5443	0.52
Crops	5754	133	397	5887	0.74
Urban	872	791	246	1663	0.72
Island	48	86	37	134	2.02

Table 11: Comparison, by type of landscape, of the number of different compounds used in 2008 in study apiaries (results of surveys) and of the number of different compounds screened for and found in beekeeping matrices (results of toxicological analyses)

(Source: Lambert, 2012, doctoral thesis)

Apiary	Number of compounds used	Number of compounds used and screened for	Number of compounds used, screened for, and found in beekeeping matrices	Number of compounds found in beekeeping matrices and not used
Hedged farmland	201	24	9	10
Crops	223	35	16	8
Urban	161	21	6	9
Island	20	6	1	10

There is some inconsistency between screened compounds and those used in an area, which can be explained by the fact that the array of substances to screen for was decided before knowing the results of the surveys. Also, analyses found some substances that had not been recorded through surveys, either because users were not asked or because treated areas were outside the 3 km radius of the foraging area.

In addition, sales and use of plant protection products are highly variable from one region to another for the same year (data from the BNV-D).

As a result, unless a specific substance is suspected from the outset, detection strategies for pesticides should have the following characteristics:

- target a panel of substances known to be used locally, for example by consulting the BNV-D. A minimum quantity sold in the region can be established to avoid screening for substances only used anecdotally;
- use quantitative methods with compatible detection thresholds and potentiation hypotheses and subclinical effects;
- depending on the objective, take into account multiple treatments applied to the foraging area, and thus the possible accumulation of substances in some matrices such as waxes or bee bread. A different matrix may need to be analysed depending on the question posed.

3.3 Conclusion and recommendations

In the area of co-exposure of bees to stress factors, a certain number of factors were identified in the literature and were examined in this report, without ranking them in order of importance in the presentation. The most numerous and important stress factors are infectious and chemical agents.

Concerning biological hazards, a number of bacterial agents, viruses and parasites were identified as stress factors in mainland France. The pathogenic potential of some of these infectious agents, particularly viruses and *Nosema ceranae*, remain to be clarified, both in the laboratory and in bee colonies. We also would like to point out the importance of asymptomatic carriage of infectious and parasitic agents reported in the literature and observed in French datasets examined by the working group. Maintaining the balance of this microbial population is related to factors that are intrinsic both to the beehive and to the environment, and changes in these factors can lead to colony disorders. It is important to distinguish between asymptomatic carriage and clinical disease. Recent studies have examined the predictive nature of carriage for the development of subsequent disorders, specifically using an approach based on colony demographic data and spatial-temporal data during beekeeping seasons.

The working group recommends pursuing studies:

- aimed at determining virulence factors of infectious agents, in laboratories and colonies, as well as the role of infectious loads in the occurrence of disorders;
- to determine the pathophysiological mechanisms involved, at the colony and individual level;
- on the predictive nature of quantities of infectious agents present in the development of subsequent disorders, in association or not with the presence of chemical factors.

Concerning chemical hazards, their number and diversity are extremely high. The substances of interest in this appraisal were pesticides and substances for veterinary use: insecticides, fungicides and acaricides, especially those used in beekeeping against *Varroa destructor*. Some substances were identified as factors involved in bee disorders, sometimes at sublethal doses. Description of the disorders was, in some studies, associated with identification of explanatory mechanisms. Note that laboratory studies are more common than tunnel studies or field studies, which can be explained by the difficulties involved in carrying out and interpreting non-laboratory studies. Exposure of bees in the field is not comparable to controlled exposure in the laboratory and the results for the same substance are different, due to the method of exposure (qualitative and quantitative). The range of substances found in beekeeping matrices was revealed through the literature and also through the results of analysis of the datasets examined by the experts.

The working group recommends pursuing studies:

- aimed at clarifying exposure and the toxic effects of chemical substances to which colonies are exposed;
- to determine the mechanisms involved, at the colony and individual bee level;
- on the multiple nature of such exposure over time and its effects in co-exposure with other factors (chemical and biological).

Food and environmental resources, by their abundance and diversity, play a major role in reproduction, development and maintenance of bee colonies. They also have an impact on bee health and tolerance of bees to other stress factors. Several studies have thereby demonstrated

the adverse effects of nutritional deficiencies on metabolism and immunity. These studies were mainly conducted in the laboratory. It is therefore important to determine whether the effects observed can be transposed to natural conditions.

The working group therefore recommends pursuing studies under natural conditions.

Certain **beekeeping practices**, although their aim is to preserve bee health, may generate stress that is added to other factors and can lead to the development of disorders. The possible negative impact may be inherent to the practice itself or be related to unsuitable practices or others that are not implemented. Compliance with good beekeeping practices, based on solid training in beekeeping, is an important requirement for healthy apiaries.

Concerning the climate, the intensity and duration of weather phenomena must be taken into account as factors that are likely to alter the physiological balance within a colony and lead to its weakening. The physiological response processes of colonies to climate change are still poorly understood and difficult to quantify. Studies should be carried out in this area.

In this context, the working group highlighted the benefit of using and maintaining bee populations suited to local conditions.

The range of stress factors that bees can be exposed to concomitantly or successively therefore appears to be wide. Moreover, for each factor, significant variability may be found from one apiary to another, or even from one colony to another. It is therefore difficult to determine the exact role played by a specific factor, or their joint effects, when colonies develop disorders, and to make comparisons between apiaries. In any event, these diverse stress factors contribute to colony weakening and to colony disorders, even though in some instances, a single type of factor can be identified (e.g. significant infestation by *Varroa*, intoxication with a pesticide, etc.).

Results of statistical analysis of datasets confirm the high number and diversity of biological and chemical hazards detected in bee colonies in France.

These results have not enabled conclusions to be drawn on the prevalence of biological or chemical hazards in apiaries in the country since the conditions for representativeness of samples were not met and only certain studies were designed for systematic and standardised assessment of biological and chemical hazards.

Nonetheless, these findings help to determine the hazards to be screened for, the matrices to sample, and the methods to use.

For biological hazards, methods need to be specific, sensitive and quantitative, and need to simultaneously detect the main potentially pathogenic agents in France.

Strategies for detection of xenobiotics should have the following characteristics:

- target a range of substances known to be used in the region;
- develop and use quantitative methods with detection and quantification thresholds that are compatible with potentiation studies for compounds, as well as their adverse effects on bee colonies;
- depending on the question asked, take account of multiple treatments applied to the foraging zone over time and target the matrix/matrices to analyse;

Furthermore, the fate of chemical substances, i.e. degradation kinetics and accumulation, etc., in the various beekeeping matrices, including bees and wax, should be studied to help in determining the matrices to sample when disorders occur, and to identify possible concomitant and successive co-exposures and interactions for chemical agents.

4 Co-exposure of bees to stress factors and interactions between these factors: mechanisms involved; demonstration methods

4.1 Literature data

Data on contamination of bee matrices were collected by FERA (Thompson 2012), EFSA (EFSA 2012b; EFSA 2013d) and within the framework of the working group's activities:

- Thompson (2012) found 148 publications to describe the routes of exposure of bees and their relative importance, 103 references for mixtures and 112 publications on pesticide/disease interactions;
- EFSA built a database by selecting the highest concentrations found in the publications and in the monographs of plant protection substances and preparations, in order to develop a model of "worst-case" exposure for regulatory calculations;
- The data available in France have been aggregated and analysed in the framework of this internal request to describe co-exposure of bees to substances (see below).

The presence of infectious agents and residues in bee matrices and therefore the fact that individual bees and bee colonies are therefore subject to (co)exposure, is established. Significant advances in demonstrating this reality have been made with the improvement in analytical methods and their limits of detection/quantification. These co-exposures may lead to interactions between infectious agents, chemical agents and infectious agents/chemical agents by means of different mechanisms, especially those concerning immunity and detoxification. This chapter presents the immunity and detoxification mechanisms possibly involved in the interactions, and then the types of interactions reported in the literature.

4.1.1 Immunity and detoxification mechanisms at the individual level and at the colony scale

4.1.1.1 Immunity of bees and bee colonies

4.1.1.1.1 Individual immunity

4.1.1.1.1.1 Immune pathways and responses

Like all insects, the bee has different lines of defence. The first is the cuticle, whose physical and chemical properties prevent infectious agents from entering the body. However, infectious agents can get past the cuticle or simply access the internal organs through food, air or the respiratory route. The defence mechanisms of the innate immune system, including the cellular and humoural defences, then come into play.

The humoural response includes melanisation (a healing process) and the production of antimicrobial peptides in fat bodies that then circulate in the haemolymph (apidaecins, abaecins, defensins, hymenoptaecins and lysozymes), while **the cellular response** is mediated by haemocytes regulating phagocytosis and encapsulation of foreign bodies. These responses help to combat various types of infectious agents such as bacteria, fungi and viruses. They are regulated by different signalling pathways that consist of recognition of the infectious agent, modulation or amplification of the recognition signal, and production of proteins or metabolites directly involved in the inhibition or destruction of this infectious agent.

Four major interconnected signalling pathways have been described in bees: Toll, Imd, Jak/STAT and Jnk (Evans *et al.* 2006). The Toll and Imd pathways are mainly involved in the humoural response, while Jak/STAT regulates both types of responses (humoural and cellular). The Jnk pathway is still poorly understood, but plays a role in the humoural response. Activation of these immune responses is initiated by the detection of pathogen-associated molecular patterns (PAMPs). Accordingly, bacteria are generally recognised by peptidoglycan recognition proteins (PGRPs) and eukaryotes such as fungi by Gram-negative binding proteins (GNBPs).
The analysis of the honeybee genome has enabled identification of the different genes potentially involved in these pathways but it would seem that the bee has only one third of the immunity genes identified previously in the fruitfly. For example, the PGRPs of bees are less diverse than those of flies. There are only four in the bee genome compared to 13 in that of the fruitfly (Evans *et al.* 2006). This immunity gene deficit seems to be a tendency among social insects and thus a consequence of the evolution of sociality and social immunity (see below). It may also be that other as yet unidentified pathways or genes also play a role in bee immunity. For example, in insects, the production of reactive oxygen species represents one of the immediate responses when faced with the intrusion of an infectious agent in the intestine (Ha *et al.* 2005).
Various studies have helped identify the immune responses of bees confronted with bacteria (Evans 2004; Evans *et al.* 2006; Siede *et al.* 2012), fungi (Antúnez *et al.* 2009; Aronstein *et al.* 2010; Aronstein and Murray 2010; Chaimanee *et al.* 2012; Dussaubat *et al.* 2012; Huang *et al.* 2012; Schwarz and Evans 2013), trypanosomes (Schwarz and Evans 2013) and *Varroa* (Navajas *et al.* 2008; Nazzi *et al.* 2012; Yang and Cox-Foster 2005; Zhang *et al.* 2010), although the responses to viral infection are less well known. They seem to be initiated by the recognition of double-stranded RNA, acting as a viral PAMP in the host (Flenniken and Andino 2013), but do not seem to involve a humoural or cellular response, at least with regard to ABPV (Azzami *et al.* 2012).
Stimulation of the immune system induces significant changes in the expression of a large number of genes and not only those involved in the cascades of the immune response (Richard *et al.* 2012). In the solitary bee, *Megachile rotundata,* exposed to varying temperatures, transcription of some genes involved in the regulation of immunity was modulated (Xu and James 2012).

Given the absence of any specific immunity and clonal response in bees, vaccine approaches do not seem feasible at the present time.

4.1.1.1.1.2 Ontogeny of immunocompetence

The ability of bees to produce an immune response to an antigen, known as immunocompetence, is not constant and varies according to age or sex. Thus, in workers, production of phenoloxidase (PO), which is involved in melanin synthesis, healing, encapsulation and phagocytosis stimulation, is lowest in larvae and pupae and increases with age in adults (nurses *vs* foragers) (Schmid *et al.* 2008; Wilson-Rich *et al.* 2008). In contrast, the fat bodies, the main site of humoural immunocompetence, and the number of haemocytes, diminish with age in adults (Schmid *et al.* 2008; Wilson-Rich *et al.* 2008). This decrease in the number of haemocytes was not found in a different study (Amdam *et al.* 2005), which indicated that variations in abundance of haemocytes must depend on other factors than age or behavioural status, such as nutritional or genetic factors. However, the level of haemocytes is higher in larvae and pupae compared to adults (Wilson-Rich *et al.* 2008).
Males have similar immunocompetence to workers with low production of PO in larvae and pupae which then increases on emergence (Laughton *et al.* 2011). Similarly, the investment in the production of antimicrobial peptides produced in fat bodies decreases with age in adults (Laughton *et al.* 2011). However, adult males are able to develop a wider range of immune responses than larvae (Gatschenberger *et al.* 2012), although these responses remain weaker than those in workers (Laughton *et al.* 2011).
Lastly, caution is needed with regard to the link between the immune system's capacity to respond to an infectious agent and bee survival, because the two are not necessarily related (Bull *et al.* 2012).

4.1.1.1.1.3 Modulation of immunocompetence

Apart from developmental processes, the immunocompetence of bees can be regulated by their diet of pollen (Alaux *et al.* 2010b) or honey (Mao *et al.* 2013). For example, stimulation of production of certain antimicrobial peptides by honey constituents suggests that providing food to colonies in the form of honey substitutes (for example high-fructose corn syrup) is not necessarily beneficial to the health of bees (Mao *et al.* 2013). Genetic factors also play a role in shaping immunocompetence (Decanini *et al.* 2007).
Lastly, factors external to bee biology can disrupt the immunocompetence of individuals. This is the case with pesticides, including acaricides used against *Varroa*, which induce stimulation or inhibition of the expression of certain immunity genes (Boncristiani *et al.* 2012; Garrido *et al.* 2013; Gregorc *et al.* 2012). A recent study showed that exposure to neonicotinoids causes inhibition of the Toll signalling pathway, which may weaken the immune system (Di Prisco *et al.* 2013). This study is particulary striking since Toll and Imd pathways are the major regulators of immune response against bacteria in insects, notably regarding the production of defensins. (Bonmatin *et al.* 1992; Il'iasov *et al.* 2012; Randolt *et al.* 2008).

4.1.1.1.1.4 **Cost of the immune response**

While the immune defences are necessary to the host as they reduce the impact of the pathogens, an immune response often has a direct energy cost. For example, a "relatively simple" immune response, such as encapsulation, may increase the metabolic rate by as much as 28% in different species of insects (Ardia *et al.* 2012; Freitak *et al.* 2003). This suggests a high energy cost for this immune response, and perhaps changes in individual behaviour in order to adapt to this increase in energy expenditure. For example, learning and memory underlie behaviour with an energy cost for bees (Jaumann *et al.* 2013), which suggests that cognitive impairment could result from an immune "stress" (Alghamdi *et al.* 2008; Mallon *et al.* 2003).

4.1.1.1.1.5 Microbiota and immunity

The microbiota[34], forming the host's population of symbiontes[35], was recently identified in bees (Engel *et al.* 2012; Olofsson and Vásquez 2008) and also appears to play a role in bee immunity. Indeed, it has been shown that some bacteria in the microbiota can stimulate the immune system of bees (Evans and Lopez 2004) and improve the resistance of larvae against the agents of American (*Paenibacillus larvae*) and European (*Melissococcus plutonius*) foulbrood (Evans and Armstrong 2005; Forsgren *et al.* 2010; Sabaté *et al.* 2009; Vasquez *et al.* 2012). This suggests that a dysbiosis[36] of the bee's symbiotic flora could lead to a weakening of the general state of the colony (Hamdi *et al.* 2011). Indeed, a metagenomic study showed that the presence of certain bacteria was dramatically reduced in bees from colonies suffering from CCD (Colony Collapse Disorder) compared to bees from healthy colonies (without CCD) (Cox-Foster *et al.* 2007).

4.1.1.1.2 Social immunity

Besides their individual immunity, bees have developed collective defence mechanisms resulting from behavioural cooperation between individuals known as social immunity (Cremer *et al.* 2007; Wilson-Rich *et al.* 2009). After all, the hive provides a favourable environment for the development of infectious agents or diseases between the colony members: high concentration of potential hosts in constant interaction and stable microclimate (temperature, humidity). Behavioural adaptations help avoid or resist the spread of parasites or infectious agents. There is a wide diversity of such behaviour that helps prevent or minimise the spread of infection.

4.1.1.1.2.1 Reduction in sensitivity

[34] Community of micro-organisms existing in a given environment
[35] Micro-organisms establishing sustained interactions with their host and forming an enduring and mutually beneficial association
[36] Imbalance in the microbiota

Foragers, which are usually the older workers, are the most likely to introduce disease into the colony. The bees can limit the intrusion of infectious agents by screening individuals at the entrance to the colony and rejecting any sick bees (Waddington and Rothenbuhler 1976). In addition, foragers generally die outside the colony, and any bees dying inside the colony are expelled, which helps to reduce the risk of infection.

Workers also collect materials from some plants in order to seal the hive. This plant-based resin, known as propolis, has antiseptic properties that may limit the development of infection in the colony (Simone-Finstrom and Spivak 2010). Indeed, laboratory tests show that propolis has a certain efficacy against American foulbrood (Antunez *et al.* 2008; Bastos *et al.* 2008) and *Varroa* (Garedew *et al.* 2002). However, in the field, colonies collecting more propolis do not necessarily have lower rates of *Varroa* than colonies collecting less (Nicodemo *et al.* 2013). Note that exposure to propolis may reduce the expression of certain immune functions (Simone *et al.* 2009), suggesting a decrease in investment in individual immunity.

Lastly, since immunocompetence varies according to the genetic profile of the bees (Decanini *et al.* 2007), polyandry[37] may ensure better resistance to diseases, in particular those affecting the brood (Tarpy and Seeley 2006).

4.1.1.1.2.2 Reduction of infection

Self-cleaning or cleaning of congeners is practised by bees to eliminate external parasites such as mites (Boecking and Spivak 1999). Internal infections, bacterial for example, can also be detected by congeners because they induce a change in the profile of cuticular hydrocarbons, an indicator of social and physiological status (Richard *et al.* 2012). A simple immune response may also modify this chemical profile (Richard *et al.* 2008) and therefore the interactions with congeners. Bees that are sick or whose immunity has been stimulated may then be targeted for more cleaning behaviour than healthy bees. However, aggression towards these individuals may also appear (Richard *et al.* 2008; Richard *et al.* 2012).

Hygienic behaviour is another collective response, but here it is directed by the adults to the brood when infected, in particular with American foulbrood (Spivak and Reuter 2001a) or *Varroa* (Boecking and Spivak 1999; Harris 2007; Ibrahim and Spivak 2006). The capacity to develop this behaviour varies greatly between colonies and seems to be an important factor in disease resistance. This behaviour, generally developed by older nurse worker bees, involves detecting any infected larvae or pupae and removing them from the colony, in order to limit multiplication of the infectious agent. They intervene on the open cells of the brood and remove the parasitised or diseased pupae. Hygienic behaviour toward *Varroa* is highly specific and includes a suite of behaviour that ultimately tends to prevent the mites reproducing and shortens the duration of their reproductive cycle. To do this, the workers remove pupae infested with mites from the closed cells.

"Social fever" consists in increasing the temperature in the brood areas, thereby inducing the death of infectious agents (Starks *et al.* 2000).

In the case of infection of the colony, "self-medication" is another form of defence that acts through an increase in propolis collection. This has been observed in the case of colonies experimentally exposed to spores of *Ascosphaera apis,* the agent of chalk brood disease (Simone-Finstrom and Spivak 2012).

Lastly, the early development of foraging behaviour appears as a general response of young workers to parasitism due to *Varroa destructor* (Downey *et al.* 2000; Janmaat and Winston 2000a), *Nosema apis* (Wang and Moeller 1970), *Nosema ceranae* (Dussaubat *et al.* 2013; Goblirsch *et al.* 2013) and to an immune stress (Alaux *et al.* 2012). This behaviour seems adaptive since it may have the effect within the colony of limiting contact with the queen, the brood and the young workers, thereby limiting the spread of parasites in the colony (Cremer *et al.* 2007).

4.1.1.2 The detoxification system of the bee

- *<u>Composition-location</u>*

[37] Fertilisation of the queen by several males

The effects of a toxic chemical (xenobiotic) on an organism depend on several factors, primarily resistance and detoxification. Resistance is characterised by three joint mechanisms: (1) the efficacy with which a compound can reach its target (e.g. the transport of a neurotoxin to the central nervous system), (2) the way in which compounds interact with varying efficacy on their target (e.g. the strength of the bond between ligand and receptor) and (3) the way in which the toxin can be extracted from its target for it then to be metabolised.

Detoxification is a physiological process that enables bees, like other species, to reduce the level of a toxic compound, whether before it has reached its target or after it has been extracted from it. Therefore it often helps decrease the effects of these substances when the metabolites are less active than the initial compound to which the bees have been exposed. This is not always the case (as for example with thiamethoxam metabolised into clothianidin). As such, it would be over-simplistic to suggest that the risk posed by a chemical disappears when the active substance or some of the major metabolites have been metabolised, since the metabolic cascade is often overlooked, the toxicity of all the metabolites has been little studied, metabolism processes in bees have been little described and the reference compounds for analytics are difficult (if not impossible) to obtain unless they can be synthesised. The example of neonicotinoids and fipronil (Simon-Delso *et al.* (2015), see above) is one of the most widely documented, but such cases are comparatively rare.

The process of detoxification calls on metabolism mechanisms, and involves enzymes that degrade the xenobiotics (Claudianos *et al.* 2006; Gilbert and Wilkinson 1974; Gilbert and Wilkinson 1975) and membrane transporters that facilitate their elimination (Hawthorne and Dively 2011). Generally, there are two different types of enzymes. Firstly, metabolism enzymes are located on the membranes of the endoplasmic reticulum. They catalyse oxidation, reduction and hydrolysis reactions. They include carboxyl/cholinesterases (CEs) and cytochrome-P450 (CYP450) monooxygenases. Secondly, transfer enzymes are located in the cytosol. They catalyse conjugation reactions. They include glutathione-S-transferases (GSTs). The action of these enzymes changes the molecular structure of the xenobiotic (for example by dechlorination), reducing its intrinsic toxicity and/or promoting its excretion by making it more soluble.

In bees, the activity of the detoxification system is generally greater in the gut, the head and the fat body than in the other tissues (Gilbert and Wilkinson 1974). In addition to detoxification, CEs and CYPs are also involved in the biosynthesis of hormones and pheromones (Claudianos *et al.* 2006).

Conventional enzymology approaches used to study the detoxification enzymes of insects have rapidly been confronted with the problem of the existence of an endogenous inhibitor in bees. This compound, released during the preparation of microsomal fractions, has an inhibitory effect on CYP activity (Gilbert and Wilkinson 1975). These authors did, however, manage to isolate this compound from a preparation of bee gut. It is a nucleoprotein weighing 19 KDa whose inhibitory effect can be moderated by the action of ribonucleases. The difficulties encountered in studying CYPs from microsomal fractions explain why there are only a limited number of studies relying on the use of biochemical approaches to describe the activity of these enzymes in bees.

The study conducted by Claudianos *et al.* (2006), following the sequencing of the bee genome, has shown that this contains relatively few genes involved in detoxification. Indeed, while the genome of most insects contains around a hundred genes coding for CYPs (Feyereisen 1999), the bee genome has only around half as many. More precisely, the bee has 46 genes coding for CYPs, whereas the genomes of the fruitfly (*D. melanogaster*) and a mosquito (*An. gambiae*) have respectively 85 and 106. An even greater difference can be observed for the genes coding for GSTs. Bees have approximately only one third to one quarter as many as these two species of fly and mosquito (Claudianos *et al.* 2006). Thus, despite its intense foraging activity, which exposes it to a large number of xenobiotics, the bee seems to be less well armed than the other insects for protecting itself from chemical stress. More recently, an analysis of the number of genes coding for CYPs, GSTs and esterases showed similarities among various species such as *Bombus huntii*, *B. terrestris*, *B. impatiens*, *Apis mellifera* and *Megachile Rotundata* (Xu *et al.* 2013).

The direct link between a smaller number of genes coding for the detoxification enzymes and a possible greater sensitivity to xenobiotics is however debatable. Indeed, the fact that there are

fewer of these enzymes does not mean that they are less effective, especially if their spectrum of action is wider. The analysis by Hardstone and Scott (2010) supports this assumption. The authors compared the acute toxicity (LD_{50}) of 62 insecticides in several insects and showed that the bee is not generally more sensitive to these substances. In addition, the assumption of a lower individual immunity to xenobiotics in bees, as found in the work of Claudianos *et al.* (2006), must also be considered in the light of social immunity, whose effects may be significant and compensating for the colony. These two types of immunity provide effective protection for the hive under real field conditions. Thereby, the effects of xenobiotics can have major consequences i) by their effects on the various individuals, depending on the various castes: larvae, workers, queen, etc., and (ii) by their effects on the superorganism, depending on the various functions essential to the colony: hygiene, fertility, recognition, communication, *etc.*

- *Metabolism of pesticides*

The wide variety of enzymes enables the detoxification system to recognise and metabolise compounds of very different natures and this protects the bees, as far as possible, against the many different xenobiotics to which they are exposed in the environment. There are more than a hundred enzymes likely to metabolise xenobiotics, but only a few of them actively take part in the degradation of a particular xenobiotic. The metabolic profile of a xenobiotic, i.e. the nature and quantity of metabolites formed during its degradation, is therefore determined by the activity of a limited number of enzymes. It is thus likely that during co-exposure to more than one xenobiotic (several pesticides for example), the detoxification of each toxin will be lessened because certain mechanisms may be called on at the same time (or within a short timeframe) to act on several active substances or their metabolites (competition for detoxification). This has been illustrated in the case of coumaphos and *tau*-fluvalinate (Johnson *et al.* 2009). A more precise study has shown that three CYP9Q enzymes were all involved in the detoxification of coumaphos and *tau*-fluvalinate (Mao *et al.* 2011). This explains the additional and/or synergistic effects of other pesticides administered together. Johnson *et al.* (2013) in particular illustrated these effects in cases of insecticide + insecticide or insecticide + fungicide (see Table 12). When the compounds are from the same class (for example during co-exposure to two neonicotinoids), detoxification may be reduced even further. As such, the patent filed by Bayer (Andersch *et al.*, US patent 7745375 B2, 29/06/2010)[38] states that "*it has now been found that mixtures comprising in each case at least two ... compounds from the series of chloronicotinyl insecticides ..., act synergistically*". The particular example of imidacloprid and nitenpyram is emphasised here.

[38] http://patentimages.storage.googleapis.com/pdfs/US7745375.pdf

topical pre-treatment (dose)	tau-fluvalinate	coumaphos	fenpyroximate	amitraz	thymol	mode of action
control 1 µl acetone	19.8 16.3-22.4	31.2 22.2-49.6	6.65 4.00-12.0	3.66 2.26-5.56	55.1 42.1-70.0	
acaricides						
tau-fluvalinate 1 µg		6.50[ab] 4.98-8.57	5.54 3.13-12.8	4.87 2.38-8.31	16.1[a] 11.2-21.4	sodium channel modulator [33]
coumaphos 3 µg	0.78[a] 0.13-3.05		2.03[a] 1.31-4.46	2.73 1.82-3.73	20.4[a] 10.4-38.0	acetylcholinesterase inhibitor [33]
fenpyroximate 1 µg	2.40[a] 1.45-3.65	4.12[a] 3.35-5.06		4.57 2.78-6.48	34.9 23.9-47.9	mitochondrial complex I electron transport inhibitor [33]
amitraz 1 µg	3.74[a] 2.14-7.08	9.20[a] 1.12-25.1	1.80[a] 1.61-2.04		43.2 25.3-61.0	octopamine receptor agonist [33]
thymol 10 µg	10.2[a] 7.85-14.0	23.1 14.7-34.5	3.69 2.83-4.98	3.91 2.98-5.52		modulator of GABA receptor [27]
oxalic acid 100 µg	7.05[a] 5.67-8.98	14.7 10.8-22.1	1.50[a] 0.77-3.06	14.6[a] 8.66-38.7	30.7[a] 23.4-39.9	unknown [28]
fungicides						
pyraclostrobin + boscalid 30 µg	5.95[ab] 4.48-8.09	25.9 19.9-34.6	3.16 2.62-3.92	4.04 2.25-10.4	31.9 16.9-44.7	
pyraclostrobin 10 µg	4.43[a] 0.67-61.4	-	2.09[a] 0.48-4.24	1.64 0.899-2.51	28.2 4.96-57.9	mitochondrial complex III ubiquinol oxidase inhibitor [32]
boscalid 20 µg	11.6 7.43-19.9	22.6 15.3-32.4	5.64 2.89-17.2	4.82 2.83-6.74	47.1 35.4-62.1	mitochondrial complex II succinate dehydrogenase inhibitor [32]
chlorothalonil 10 µg	7.24[a] 3.96-12.9	16.6 6.77-85.6	6.41 5.62-7.36	3.34 1.48-8.89	29.8[a] 21.1-39.9	multi-site contact activity [32]
prochloraz 10 µg	0.01[a] 0.006-0.017	0.44[a] 0.38-0.50	0.25[a] 0.17-0.34	2.48 1.45-3.74	39.0[b] 33.2-45.1	sterol biosynthesis (P450) inhibitor [32]
model inhibitors of detoxification						
DEM 100 µg	8.26† 7.57-9.03	19.9‡ 10.5-53.5	4.38 1.90-8.80	2.30 0.306-4.24	64.0 42.6-91.6	glutathione-S-transferase inhibitor [29]
DEF 10 µg	1.96† 0.83-4.17	7.29‡ 4.88-9.22	1.26[a] 0.10-15.2	2.17 1.63-2.87	35.1 21.9-52.0	carboxylesterase inhibitor [29]
PBO 10 µg	0.01† 0.006-0.015	5.04‡ 3.34-7.01	0.27[a] 0.12-0.75	2.41 0.917-6.35	32.4 19.8-49.6	cytochrome P450 inhibitor [29]

LD_{50} fold-change relative to control	<1		1	2	5	20	50	100

Table 12: "*Median lethal dose (LD_{50}) of acaricides (listed horizontally) to honeybees in 2009 following sub-lethal treatment with acaricides, fungicides or detoxicative enzyme inhibitors (listed vertically). Confidence intervals (95%) are indicated below the LD_{50} values. Significant differences compared to the control treatment are indicated with a superscript letter: a = significant pretreatment effect, b = significant pretreatment*acaricide dose effect (see Table S1 in Johnson et al., 2013). LD_{50} values taken from previous work: † = Johnson et al. (2006); ‡ = Johnson et al. (2009). A dash indicates an LD_{50} that could not be calculated because of insufficient data.*" Johnson *et al.* (2013)

It should be noted that the brighter the red in the box, the more the interaction caused the LD_{50} to decrease (see colour key). For example the pretreatment with 10 µg of prochloraz decreased the LD_{50} of *tau*-fluvalinate by a factor of 19.8 / 0.01 =1980, which is equivalent to the effect of PBO (10 µg), a CYP450 inhibitor.

Several detoxification pathways have been identified through the use of enzyme activity inhibitors (specific metabolic inhibitors). Work conducted with piperonyl butoxide (PBO, a CYP inhibitor), S,S,S-tributyl phosphorotrithioate (DEF, a CE inhibitor) and dimethyl maleate or diethyl maleate (DMM or DEM, GST inhibitors) has shown that the addition of these inhibitors may increase the sensitivity of bees to certain xenobiotics. The experience shows that the enzyme (corresponding to the inhibitor with an effect) plays a role during detoxification. The use of these inhibitors by Johnson *et al.* (2006) helped show that the first phase of detoxification of three pyrethroids (cyfluthrin, lambda-cyhalothrin and *tau*-fluvalinate) is mainly related to the action of CYPs. By comparing the acute toxicity (LD_{50}) of these three compounds, these authors showed that these pyrethroids are, respectively, 30, 80 and 980 times more toxic to bees in the presence of PBO.

Inhibition of CEs by DEF also increased the toxicity of cyfluthrin, lambda-cyhalothrin and *tau*-fluvalinate, but by lower degrees than with the PBO inhibitor. In contrast, inhibition of GSTs by DMM had no influence on the toxicity of the three pyrethroids studied, in the way that this toxicity was evaluated (Johnson *et al.* 2006). These results indicate that CYPs are the main enzymes metabolising pyrethroids (in particular *tau*-fluvalinate) and explain the variable sensitivity of bees to some of them. More recently, these authors tested the effect of the same inhibitors on the acute toxicity (LD_{50}) of various acaricides (Johnson *et al.* 2013). This time, inhibition of CYPs and CEs increased the toxicity of *tau*-fluvalinate, coumaphos and fenpyroximate very significantly, but had very little influence on the toxicity of amitraz and thymol. Lastly, inhibition of GSTs had no influence on the toxicity of these acaricides, as evaluated (LD_{50}).

The work of Iwasa *et al.* (2004) considered the effect of metabolic inhibitors on the acute toxicity (LD_{50}) of neonicotinoid insecticides: acetamiprid, thiacloprid and imidacloprid. The authors showed that acetamiprid, imidacloprid and thiacloprid were respectively 6, 1.7 and 154 times more toxic in bees previously exposed to 10 µg of PBO. These results strongly suggest that CYPs are involved far more actively in the detoxification of thiacloprid than in that of acetamiprid or imidacloprid.

PBO was also used by Niu *et al.* (2011) to study the detoxification of two mycotoxins (aflatoxin B1 and ochratoxin A) produced by fungi (e.g. *Aspergillus* spp) frequently found in bee colonies. By comparing the longevity of bees placed in containment, the authors showed that inhibition of CYPs increases the toxicity of aflatoxin B1, but does not influence that of ochratoxin A.

Unlike studies seeking to inhibit detoxification, the researchers attempted to promote it. For example, the addition of quercetin may induce greater production of CYP, which would explain an increase in resistance of bees to *tau*-fluvalinate (Johnson *et al.* 2012). Another example is shown with the addition of coenzyme Q10 (CoQ10, 2,3-dimethoxy, 5-methyl, 6-decaprenyl benzoquinone) which increases the production of antioxidant enzymes including GSTs (Strachecka *et al.* 2014).

- *Molecular biology and biochemistry*

Broad-spectrum metabolic inhibitors cannot be used to determine with precision which enzymes are involved in the metabolism pathways of xenobiotics. This can be achieved, however, with molecular biology and biochemistry techniques, as these enable *in vitro* study of the degradation of xenobiotics by recombinant enzymes.

The first studies using these techniques were those of Mao *et al.* (2011), who studied the metabolism of flavonoids (quercetin, kaempferol, eriodictyol and taxifolin), compounds that are frequently found in pollen and propolis. Using recombinant CYPs, they showed that CYP6AS1, CYP6AS3, CYP6A4 and CYP6A10 are capable of degrading quercetin but not the other three flavonoids. They also confirm, through a docking approach coupled with molecular modelling, that the active sites of CYP6AS enzymes can indeed bind to quercetin. Mao *et al.* also studied the metabolism of *tau*-fluvalinate and coumaphos, with the help of recombinant proteins (CYP6AS3, CYP6AS10, CYP6AQ1, CYP6BD1, CYP338A1 and CYP9). They showed that only the CYP9Q1, CYP9Q2 and CYP9Q3 enzymes are capable of degrading these two acaricides. Mao *et al.* also predicted, using an *in silico* approach, the binding of *tau*-fluvalinate and coumaphos at the active site of these three enzymes. They suggest that the resulting competitive inhibition leads to a slowdown in their metabolism and may explain the synergistic effects observed between these two acaricides during previous studies (Johnson *et al.* 2009; Johnson *et al.* 2006).

- *Radio-labelling*

Other studies have demonstrated the efficacy of the bee's detoxification system in degrading pesticides. These studies have mainly focused on the degradation kinetics of pesticides and on their metabolic profile using radiolabelled compounds.

Pilling *et al.* (1995) studied the metabolism of lambda-cyhalothrin in bees exposed topically. Sixteen hours after exposure, they observed that three main metabolites emerged. They are 4'-hydroxy-3-phenoxybenzyl alcohol, 4'-hydroxy-3-phenoxybenzoic acid and 2'-hydroxy-3-phenoxybenzyl alcohol. They also found that if bees are exposed simultaneously to prochloraz (fungicide), the metabolism of lambda-cyhalothrin is inhibited for the first 16 hours. Confirmation of

this inhibition is provided by the *in vitro* study they conducted in parallel on bee guts, which testified to a change in the metabolic profile of lambda-cyhalothrin in the presence of prochloraz. Pilling and Jepson (1993) suggested that inhibition of lambda-cyhalothrin metabolism by prochloraz explains the synergy observed between these two substances in an earlier study. However the metabolic hypothesis advanced by Pilling and Jepson explaining the synergy between the pyrethroids and prochloraz was challenged a few years later by Chalvet-Monfray *et al.* (1996). After modelling the degradation kinetics of deltamethrin in the presence of prochloraz, Chalvet-Monfray *et al.* argued that inhibition of CYPs cannot alone explain the synergy observed in bee mortality. They formulate new hypotheses according to which the synergy could be due to an increase in the cuticular permeability of pyrethroids in the presence of prochloraz, or to the action of these pesticides on secondary targets such as the Ca^{2+}-ATPases expressed on the surface of cell membranes (Chalvet-Monfray *et al.* 1996).

The work by Suchail *et al.* (2004a) focused on the toxicokinetic parameters of imidacloprid in bees exposed orally to a dose of 100 µg/kg of bee (close to the LD_{50} for imidacloprid). They observed that the half-life of imidacloprid in bees is 4 hours and that it is mainly degraded into five metabolites. These are 4,5-dihydroxy-imidacloprid, 4/5-hydroxy-imidacloprid and olefin (which are distributed preferentially in the head, thorax and abdomen of the bee), and urea derivative and 6-chloronicotinic acid (found in the midgut and the rectum). The nature of these metabolites suggests that they are mainly produced by CYPs and that the transfer enzymes (e.g. glutathione-S-transferase) do not participate in the metabolism of imidacloprid because no conjugated metabolite was detected. In another study, Suchail *et al.* (2004b) suggested that, although minor, the formation of olefin and 5-hydroxyimidacloprid four hours after ingestion of imidacloprid explains the mortality peak observed in bees exposed to imidacloprid.

Similar work conducted by Brunet *et al.* (2005) has explored the toxicokinetic parameters of acetamiprid in bees exposed orally to a dose of 100 µg/kg of bee (a dose 1,500 times lower than the LD_{50} for acetamiprid). After absorption, acetamiprid is preferentially found in the head, abdomen and thorax of bees but it is rapidly metabolised, since 30 minutes after ingestion, less than 50% of the ingested dose remains in the bee's body. It is metabolised into seven metabolites, of which the main two are 6-chloronicotinic acid and an unidentified metabolite (U1).

- *Modulation of the detoxification system*

The detoxification system of insects functions continuously but its activity can be regulated by transcriptional means. Generally, expression of detoxification enzymes is induced by the substances they metabolise, which enables the insects to adapt their response to the chemical stress (Li *et al.* 2007). However, the work by Johnson *et al.* (2012) suggested that regulation of the bee's detoxification system differs from that of other insects. Indeed, they noted that phenobarbital, a compound known to induce CYP expression in a large number of organisms, does not affect gene expression in bees. Contrary to their expectations, they also observed that exposure of bees to phenobarbital increases the bees' sensitivity to *tau*-fluvalinate, lambda-cyhalothrin and aldrin. The inefficiency of phenobarbital in inducing CYP expression does not mean that the bee's detoxification system cannot be induced. The studies cited previously in this chapter have demonstrated that xenobiotics are indeed likely to modulate the expression of detoxification genes in bees.

4.1.2 Interactions between stress factors identified in the bibliography

The foraging activity of bees exposes them simultaneously to many stress factors of abiotic (chemical contaminants etc.) or biotic (infectious agents etc.) origin that can result in adverse effects on their health, their longevity and the effective organisation of the colony. Cases of mortality in bees sometimes result from the action of just one of these factors. However, at present, none of these factors can by itself explain all the colony losses reported in France and at a worldwide level. It would appear therefore that the causes of this decline could also result from interactions between several stress factors (Nazzi and Pennacchio 2014; Neumann and Carreck 2010; Potts *et al.* 2010; vanEngelsdorp *et al.* 2009; vanEngelsdorp *et al.* 2010). Indeed, the stress factors may be interdependent and have more harmful consequences when they act together

(Johnson *et al.* 2009; Locke *et al.* 2012), sometimes causing significantly higher mortality (Alaux *et al.* 2010a; Colin and Belzunces 1992; Doublet *et al.* 2014; Nazzi *et al.* 2012; Vidau *et al.* 2011). Among the stress factors affecting bees, a strong emphasis is placed on parasites and pesticides (Neumann and Carreck 2010; Oldroyd 2007; Potts *et al.* 2010; Simon-Delso *et al.* 2015; vanEngelsdorp and Meixner 2010; vanEngelsdorp *et al.* 2010). Several studies have indeed highlighted the multiplicity and high prevalence of infectious agents (Cox-Foster *et al.* 2007; Dainat *et al.* 2012b; Hedtke *et al.* 2011) and pesticides (Bonmatin *et al.* 2015; Chauzat *et al.* 2009; Lambert *et al.* 2013; Mullin *et al.* 2010; Paradis *et al.* 2013; Pisa *et al.* 2015) found within colonies, suggesting that interactions could occur between these factors. However, the multitude of possible combinations complicates any assessment of the impact of these interactions by scientists, beekeepers and the regulatory authorities. There are also mixtures combining a pesticide with a biological agent (for example Bayer patent EP0627165 A1, 1994, which combines insecticides, including pyrethroids, neonicotinoids, phenylpyrazoles, *etc.*, with entomopathogenic microfungi). In addition, increasing numbers of studies are indicating that unintentional interactions between pesticides or between infectious agents can either have additive effects or a synergistic effect on bee mortality and can therefore potentially affect the health of bee colonies. Synergy is defined as occurring when the combined effect of two factors is greater than the sum of the effects of each individual factor (Holmstrup *et al.* 2010). Thus, associations between certain pesticides such as the insecticide deltamethrin and the fungicide prochloraz (Colin and Belzunces 1992), the fungicide chlorothalonil and the acaricide *tau*-fluvalinate (Zhu *et al.* 2014) or the acaricides coumaphos and *tau*-fluvalinate (Johnson *et al.* 2009) lead to a synergistic effect on bee mortality. A synergy can also occur when the bee is exposed to two biological agents, such as the parasitic mite *Varroa destructor* and the deformed wing virus DWV (Dainat *et al.* 2012a; Nazzi *et al.* 2012); a synergy could result from exposure to *Nosema ceranae* and to the CBPV (Toplak *et al.* 2013). Lastly, the interactions between pathogens and insecticides can also have adverse effects on the health of certain pollinators (González-Varo *et al.* 2013) and several studies assessing their impact on *A. mellifera* were recently published (Alaux *et al.* 2010a; Aufauvre *et al.* 2012; Aufauvre *et al.* 2014; Di Prisco *et al.* 2013; Doublet *et al.* 2014; Pettis *et al.* 2013; Pettis *et al.* 2012; Retschnig *et al.* 2014a; Vidau *et al.* 2011; Wu *et al.* 2012). Many of these studies concern the association between the intestinal parasite *Nosema ceranae* and different neurotoxic insecticides, including neonicotinoids. The honey bee is frequently exposed to these stress factors. Studies have also revealed virus-insecticide/acaricide interactions (Boncristiani *et al.* 2012; Locke *et al.* 2012) as well as *Varroa*-pesticide interactions (Gregorc *et al.* 2012).

4.1.2.1 Between biological agents

4.1.2.1.1 Varroa-virus associations

The parasitic mite *Varroa destructor* is known to act in synergy with several viruses that it can transmit to bees (Ball 1983; Gisder *et al.* 2009; Nordström *et al.* 1999; Shen *et al.* 2005a; Shen *et al.* 2005b; Yue and Genersch 2005). In addition, this mite can cause a weakening of the immune system of the bees, thus favouring the proliferation of viruses and making the bees more vulnerable to infection by other infectious agents (Amdam *et al.* 2004a; Bailey *et al.* 1983; Gregory *et al.* 2005; Yang and Cox-Foster 2007; Yang and Cox-Foster 2005). Yang and Cox-Foster (2005) showed that *Varroa* led to a significant decrease in the rate of expression of three antimicrobial peptides (abaecin, defensin and hymenoptaecin) and four enzymes related to immunity (phenoloxydase, glucose dehydrogenase, glucose oxidase and lysozyme), thus making the bees more susceptible to infection by the deformed wing virus (DWV). These studies indeed showed that bees infected with *Varroa* and presenting a deformed wing phenotype have viral loads 10^6 times higher than bees with normal wings.

A study conducted in the south-west of England (Highfield *et al.* 2009) helped demonstrate that the *Varroa*-DWV combination reduced the longevity of winter bees and that the DWV may play a major role in winter mortalities. More recent studies confirm these *Varroa*-DWV associations (Dainat *et al.* 2012a; Francis *et al.* 2013b; Hedtke *et al.* 2011; Martin *et al.* 2012; Nazzi *et al.* 2012; Ryabov *et al.* 2014). Hedtke *et al.* (2011) monitored 220 colonies from 22 apiaries located in north-east Germany (random selection of 10 colonies from each apiary) for a period of six years (autumn 2004 - spring

2010). During this period, winter losses varied from 22.4% (winter 2005-2006) to 4.8% (winter 2008-2009), with each dead colony being replaced by a colony from the same apiary. The different parameters measured were: the rate of infestation with *Varroa* (two measurements a year, in July and October), the viral load in October (KBV, ABPV, SBV, DWV and IAPV viruses), the presence of the bacteria responsible for foulbrood (*P. larvae* and *M. plutonius*) in October, infection by *Nosema apis* and *N. ceranae* (in October and March) and clinical cases of chalkbrood due to the *Ascosphaera apis* fungus (monitored from May to September). This study yielded data on the prevalence of each of these biological agents over the six years of monitoring, showing in particular that: (1) the prevalence of *Nosema* is greater in the spring (with *N. apis* being more frequent than *N. ceranae*), (2) DWV is the virus with the highest prevalence (> 26%), (3) more than 50% of colonies have *Varroa* despite the acaricide treatments regularly used, (4) the agents of European and American foulbrood were never detected. This work also made it possible to analyse the interactions between the different biological agents detected. The main conclusions of the study are:

(i) The existence of a strong correlation between the presence of *Varroa* in summer and the DWV in autumn;
(ii) An infestation with *Varroa* in autumn is followed by an infection with *Nosema apis* in the spring of the following year, which is not the case with *N. ceranae;*
(iii) Cases of chalkbrood are observed in summer in colonies infested with *Varroa* that were infected with *N. ceranae* in spring;
(iv) Very significant co-occurrences are observed between certain viruses (DWV-ABPV and DWV-SBV) in autumn. A lower correlation is also found between the DWV and *Nosema* in autumn.

This study clearly illustrates the diversity and complexity of interactions that may exist under natural conditions between different biological agents. Most of the observed correlations are positive, i.e. the bees infected by a parasite/infectious agent become more susceptible to infection by another biological agent; the effects may be additive or synergistic.

Dainat *et al.* (2012a) conducted a study in Switzerland on 29 colonies that they monitored for several months (August 2007 - April 2008). These colonies were separated into two groups, one comprising 18 colonies that received a treatment against *Varroa* based on organic acid, and the other made up of 11 colonies that received no anti-*Varroa* treatment. All the colonies had similar populations at the beginning of the experiment (approximately 14,000 workers in August 2007). These colonies were monitored during the winter, any dead bees were collected each day and the loads in DWV and ABPV and *Nosema ceranae* were evaluated. Levels of vitellogenin gene expression were measured as a marker of bee longevity. In April 2008, the 11 colonies not treated against *Varroa* and two of the treated colonies had died. The study showed that workers from the colonies that did not survive the winter had a shorter lifespan. These colonies had higher *Varroa* and DWV loads than those treated against *Varroa* which had survived. This study therefore demonstrated that the DWV-*Varroa* combination reduces the lifespan of winter bees, causing colony die-off, which could contribute to the phenomenon of bee decline in cases where the colonies were not treated with acaricides. However, this study shows no correlation between *Varroa* and *N. ceranae*, nor between *Varroa* and ABPV.

Nazzi *et al.* (2012) studied the relationships between infestation with *Varroa*, host defences and DWV through a metagenomic study on colonies with low (treated by an acaricide) or high (not treated) levels of *Varroa* infestation. The study focused on two apiaries, each with six colonies; one undergoing treatment based on thymol against *Varroa*, the other receiving no acaricide treatment. Monitoring of population dynamics showed a sharp decrease in the number of bees in the untreated colonies that were heavily infested with *Varroa*. All of these colonies died at the end of the season or at the beginning of the following spring. Screening for various infectious agents (*Nosema ceranae*, BQCV, DWV, SBV) revealed more significant DWV loads in these colonies compared to those receiving the anti-*Varroa* treatment. This increase in DWV load linked to heavy *Varroa* infestation and associated with high bee mortality was confirmed by laboratory experiments using larvae experimentally infested with *Varroa*. This study showed a synergistic interaction when the bee is exposed to these two infectious agents, with *Varroa* activating the replication of DWV

which thus becomes more virulent. This association is linked to an immunosuppression syndrome in the colonies with high levels of *Varroa* infestation. Indeed, the authors demonstrate a down-regulation of 19 immune genes, in particular a gene from the NF-kb family that plays a central role in insect immunity but also in the response to stress. DWV may therefore behave as an opportunistic agent taking advantage of the weakening of colonies heavily infested with *Varroa*.

The study by Martin *et al.* (2012) focused on 293 colonies of bees from 35 apiaries located on four major islands of Hawaii. It reveals that the recent exposure of bee colonies to *Varroa* is correlated to a very sharp increase in prevalence (from 10 to 100%) and viral load (10^6 times higher) of the DWV and to a decrease in the diversity of strains of this virus, which is not the case for other viruses such as IAPV, ABPV or KBV. The spread of *Varroa* mites in Hawaii has thus led to the emergence of the DWV, and in particular the selection of a predominant variant, whose prevalence was previously very low.

In the study by Francis *et al.* (2013b), the load in *Varroa* and viruses (AKI complex [ABPV, KBV and IAPV] and DWV) was monitored for one year in Denmark in 23 colonies (from 15 apiaries) treated or not against *Varroa* (11 colonies treated with organic acids, 9 colonies treated with flumethrin and 3 untreated colonies). This study, conducted from April 2011 to April 2012, shows that the viral loads increased dramatically in the colonies not treated for *Varroa*. The number of viral copies is correlated to the presence and number of *Varroa* (from 10^4 copies in the colonies without *Varroa* to 10^{10} copies in the most heavily infested colonies). Most of the colonies that did not survive the winter had significantly higher viral loads (AKI and DWV) than the surviving colonies. In total, seven colonies died during the winter, of which four were treated and three untreated.

It is also important to point out that in the various studies mentioned above, no assaying of pesticide residues was carried out. Because of this, the *Varroa*-virus correlations cannot be examined from the angle of the presence or absence of xenobiotic residues.

4.1.2.1.2 Nosema-virus associations

Several studies have also highlighted associations between certain viruses and the microsporidia *Nosema* spp, microfungal parasites of the adult bee gut (Bailey *et al.* 1983; Bromenshenk *et al.* 2010; Doublet *et al.* 2014; Toplak *et al.* 2013). Historically, the BQCV has been associated with the species *Nosema apis* (Bailey *et al.* 1983). These two pathogens have indeed been found in colonies that collapsed during the winter. In addition, an increase in BQCV load has been observed in the presence of *N. apis*, suggesting that this intracellular fungus facilitated replication of the virus in bees (Bailey *et al.* 1983).

The work of Bromenshenk *et al.* (2010) suggested that an iridovirus (IIV) could be associated with the syndrome of colony collapse disorder (CCD), as the prevalence of this virus as well as that of *Nosema ceranae* is greater in colonies affected by this syndrome. In addition, experimental co-infections with these two pathogens, under laboratory conditions, seem to result in higher rates of mortality.

This *Nosema*-virus synergy was described more recently, under laboratory conditions, by means of experimental co-infections of winter bees with the chronic bee paralysis virus (CBPV) and *N. ceranae* (Toplak *et al.* 2013). Indeed, monitoring of viral load by quantitative PCR indicates a synergistic increase in CBPV replication in bees co-infected with this virus and *N. ceranae*. Moreover, these co-infected bees had very high mortality rates.

In the laboratory, Doublet *et al.* (2014) recently evaluated the interactions between *N. ceranae* and BQCV as well as between *N. ceranae* and the insecticide thiacloprid. With both types of association, a synergistic effect on the mortality of adult bees was observed. Indeed, 11 days after experimental infection, the authors found a mortality rate of 50% in co-infected bees while the mortality rates were only 20% for bees infected solely with *N. ceranae* and less than 5% for bees infected solely with BQCV. In addition, this synergistic effect is greater for bees co-infected by the two pathogens than those exposed to *N. ceranae* and thiacloprid.

While additive or synergistic effects have been observed between *Nosema* spp and some viruses, studies indicate that this is not the case for all viruses. The study by Cox-Foster *et al.* (2007)

showed, in particular, that there seems to be no association between *Nosema ceranae* and the IAPV. Similarly, Martin *et al.* (2013) did not observe any positive interactions between *N. ceranae* and the DWV. This last study, concerning 322 colonies in Hawaii, indeed demonstrated that there is no link between the DWV load and the number of spores of *N. ceranae*. Antagonistic interactions have even been observed between *N. ceranae* and the DWV (Costa *et al.* 2011; Doublet *et al.* 2015). Thus, the lesions caused by *N. ceranae* in the gut may reduce the replication ability of the DWV.

It should be noted that antagonistic virus-microsporidia interactions have also been observed in other insects (Bauer *et al.* 1998).

4.1.2.1.3 Other associations between biological agents

- *Varroa destructor-Acarapis woodi*

Downey and Winston (2001) studied the impact of co-infestation of bees by the parasitic mites *Varroa destructor* and *Acarapis woodi*. They assessed the impact of each mite alone or in association by monitoring different parameters over a period of 16 months: colony mortality, number of bees and mites, and available reserves. Although the tracheal mite did not seem to have any effect on its own, a synergistic interaction was observed when associated with *V. destructor,* with five out of six co-infested colonies being dead after the winter (10 months after the start of the study).

- *Varroa-Ascosphaera apis*

Hedtke *et al.* (2011) showed that the presence of *Varroa* in bee colonies in summer promotes the emergence of cases of chalkbrood. This study also suggested a role of *Nosema ceranae* in the susceptibility of colonies to *Ascosphaera apis*.

- *Crithidia mellificae-Nosema ceranae*

Schwarz and Evans (2013) studied, under laboratory conditions, the immune responses of the bee in the case of infection with the trypanosome *Crithidia mellificae*, the microsporidia *Nosema ceranae* and when the two agents are inoculated simultaneously. Using quantitative PCR, they monitored the expression of genes involved in immunity at different times after the experimental infection. Their work shows that concurrent infection by the two infectious agents significantly affects the transcription of immunity genes.

The study by Ravoet *et al.* (2013) also shows the important role that *C. mellificae* may play. In this study conducted in Belgium, the authors screened for the presence of 18 biological agents in 363 colonies in summer, in order to assess their potential link with winter mortalities. This study indicates, in particular, that *N. ceranae* and *C. mellificae* are predictive markers of winter mortality. A synergistic effect on the rate of winter mortality was thus observed when both agents were present within the same colony.

- *Nosema apis-Nosema ceranae*

Experimental infections under laboratory conditions were carried out with the species *N. apis* and/or *N. ceranae* (Milbrath *et al.* 2015). This study showed that a mixed infection caused more rapid mortality and a higher mortality rate in bees than when they were infected with each species separately. The number of spores produced was also higher with mixed infections. However, the number of *N. apis* spores was higher than or equivalent to that of *N. ceranae*, challenging the assumption that *N. ceranae*, which is now the predominant species, may have replaced *N. apis* by competition. Another very recent study indicates, on the contrary, that the order of infection plays an important role in competition between infectious agents. Thus an infection by *N. ceranae* followed by an infection by *N. apis* shows that the first species strongly inhibits development of the second (Natsopoulou *et al.* 2015).

- *Varroa destructor-Nosema ceranae*

Following the significant bee colony losses observed in different regions of Spain, research has been conducted to identify the factors that may be involved. Bernal *et al.* (2011) showed a very

high prevalence of *Varroa destructor* and *N. ceranae* and suggested that the combination of these two parasites may increase the risk of colony mortality.

- *Associations between viruses*

Several studies indicate significant, often asymptomatic, carriage of infectious agents, and viruses in particular (see the section on biological agents). Hedtke *et al.* (2011) thus demonstrated co-occurrences of DWV-ABPV and DWV-SBV. In the study by Ravoet *et al.* (2013), an association between the LSV (Lake Sinai Virus) and BQCV was also observed (monitoring of 363 colonies in Belgium and screening for 18 biological agents). This study also showed a correlation between the number of infectious agents detected and the winter mortalities. The greater the number of infectious agents, the higher the rate of winter mortalities.

In conclusion, the following points should be emphasised:

- ✓ the importance of *Varroa destructor* as a vector of infectious agents, especially the DWV, and for its role in weakening bee immunity;
- ✓ the multiplicity of biological agents in colonies, and therefore the multiple interactions to be considered;
- ✓ the use of certain co-occurrences as risk factors for winter mortality;
- ✓ the lack of data on the mechanisms involved in the additive, synergistic and antagonistic interactions between various stress factors, hence the working group's recommendation that research projects be targeted on this theme.

4.1.2.2 Between chemical factors

The synergy between pyrethroid insecticides and imidazole fungicides or ergosterol biosynthesis inhibitors (EBIs) in honeybees has been the subject of numerous studies, most of which were conducted in the 1990s. A mixture of deltamethrin, a pyrethroid, and prochloraz, an imidazole fungicide, applied to worker bees in the laboratory by spraying in a Potter tower (at the doses recommended in agriculture of 0.125 g/ha and 25 g/ha respectively) caused corrected mortality of 67.5% 24 h after treatment and 74.1% after 50 h (Colin and Belzunces 1992). This last figure was reduced to 27.5% when an interval of 19 h was respected between application of the deltamethrin and the prochloraz (23.8% when the prochloraz preceded the deltamethrin). This synergistic effect has not been reproduced in winter workers (Meled *et al.* 1998). Sub-lethal effects were also measured on the thermoregulation of workers when deltamethrin (0.5 and 1.5 ng/bee) was combined with prochloraz or difenoconazole (850 ng/bee), causing hypothermia in individuals (Vandame and Belzunces 1998). Still concerning the sub-lethal effects, when deltamethrin and prochloraz were combined, the cardiotoxicity of the insecticide was increased by more than 100 times, whereas that of the fungicide was multiplied by a factor of 10 (Papaefthimiou and Theophilidis 2001). The authors assume that these compounds act on the same biological target. However, the main mechanism reported as possibly underlying the synergy between imidazole fungicides and pyrethroid insecticides is the inhibition of enzymes for detoxification of xenobiotics associated with cytochrome P450 (Johnson *et al.* 2009; Johnson *et al.* 2006; Pilling *et al.* 1995). Pilling and Jepson (1993) tested two fungicides (propiconazole and flutriafol) from the class of EBIs, which all increase the toxicity of lambda-cyhalothrin in bees. Propiconazole had the greatest synergistic effect, reducing the LD_{50} of lambda-cyhalothrin by a factor of 16.2 (68.0 ng *vs* 4.2 ng/bee). The authors calculated the risk quotient associated with spraying lambda-cyhalothrin in real conditions at 110. However, this quotient becomes 366 in the case of a mixture with flutriafol, and 1786 with propiconazole.

In the laboratory, a net synergistic effect was measured between thiacloprid and the fungicide tebuconazole, of the class of EBIs (Schmuck *et al.* 2003b). The synergistic effect between this neonicotinoid and other EBI fungicides (triflumizole, propiconazole) has been confirmed (Iwasa *et al.* 2004). These same authors also found a high degree of synergy between acetamiprid and EBI fungicides (triflumizole, propiconazole, triadimefon, epoxiconazole). However, this synergy was not confirmed after agricultural application of commercial products based on thiacloprid and

tebuconazole (respectively Calypso and Folicur; Schmuck *et al.* (2003b)) or based on acetamiprid and triflumizole (Iwasa *et al.* 2004). In any case, the results of the tunnel experiments by Schmuck *et al.* (2003b) should be considered with caution, since they only used three colonies with small populations (3,000 to 8,000 workers).

The toxicity of acaricides, commonly used by beekeepers against *Varroa* for their relative safety for honeybees, may increase when they act together. With experiments in the laboratory, Johnson *et al.* (2009) found that the impact of *tau*-fluvalinate in young workers (3 days old) was greater when they had previously been exposed to coumaphos. Coumaphos applied after the *tau*-fluvalinate caused only a slight increase in mortality. Anti-*Varroa* acaricides can also influence the effect of insecticides.

Workers treated with Apistan® showed greater sensitivity to bifenthrin than untreated workers (Ellis *et al.* 1997). The toxicity of bifenthrin was increased by a factor of 1.9 when the workers had previously been exposed to this acaricide. Such an effect was not found with two other insecticides, carbaryl and parathion-methyl.

The case of neonicotinoids has been much discussed, and was already mentioned briefly in the section on pesticide metabolism (see above). Firstly, market share of this class of neurotoxic insecticides is increasing; it now represents more than a quarter of the world market (Simon-Delso *et al.* 2015). This can be explained by the large number of proposed applications (seed coatings, sprays, soil granules, trunk injections, dips, irrigation, *etc.*) and by the prophylactic use of these compounds (preventive treatments against pests) on more than a hundred different crops (Bijleveld van Lexmond *et al.* 2015). These chemical contaminants are therefore increasingly found in bee matrices (Bonmatin *et al.* 2015) and at levels that can induce considerable lethal and/or sub-lethal effects in pollinators (Pisa *et al.* 2015; van der Sluijs *et al.* 2015). Secondly, the multiplicity of neonicotinoids used is also increasing, as shown, for example, in *Figure 6* concerning Japan.

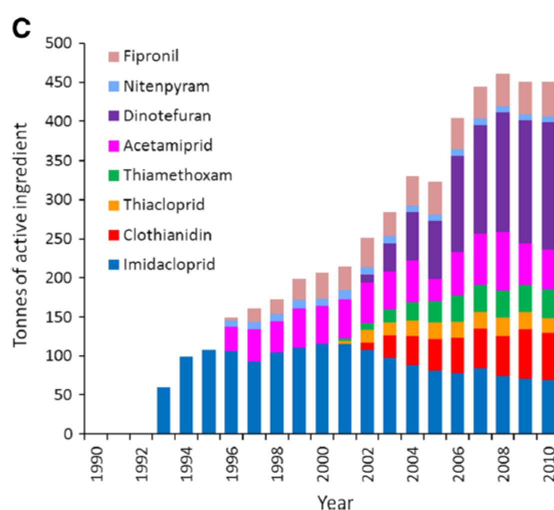


Figure 6: Annual growth in the market for neonicotinoids (and fipronil) in Japan in tonnes of active ingredient. The graph shows the qualitative and quantitative growth for seven neonicotinoids, mainly in terms of the multiplicity of active ingredients used from 1996. These data are from the Japanese National Institute for Environmental Studies. From Simon-Delso *et al.* (2015)

In France, data are available on sales growth between 2009 and 2012 (see *Figure 7*). Tonnages of seed treatment products only began to be integrated in the national database of sales of plant protection products created by authorised distributors from 2012. The tonnages contributed by these products partly explain the sharp increase observed between 2011 and 2012.

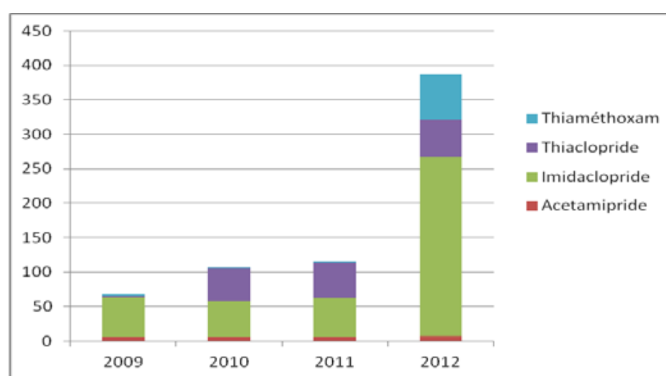


Figure 7: Growth in sales of neonicotinoids between 2009 and 2012 in France, in tonnes of active substance (Source: ONEMA and ANSES - National database of sales of plant protection products created by authorised distributors - BNV-D)

Lastly, neonicotinoids are known to interact with each other in synergy. A patent for this synergistic action was filed by Bayer CropScience in 2010 (Andersch *et al.*, US patent 7745375 B2, 29/06/2010[39]) and it states in particular the mixtures specifically mentioned for the invention, in *Table 13* shown below. Alone, these pairs of associations represent a wide spectrum of synergies.

TABLE

Mixture No.	First active compound	Second active compound	Preferred mixing ratio	Particularly preferred mixing ratio
1	imidacloprid	clothianidin	100:1-1:100	10:1-1:10
2	imidacloprid	dinotefuran	"	"
3	imidacloprid	thiamethoxam	"	"
4	imidacloprid	thiacloprid	"	"
5	imidacloprid	acetamiprid	"	"
6	imidacloprid	nitenpyram	"	"
7	clothianidin	dinotefuran	"	"
8	clothianidin	thiamethoxam	"	"
9	clothianidin	thiacloprid	"	"
10	clothianidin	acetamiprid	"	"
11	clothianidin	nitenpyram	"	"
12	dinotefuran	thiamethoxam	"	"
13	dinotefuran	thiacloprid	"	"
14	dinotefuran	acetamiprid	"	"
15	dinotefuran	nitenpyram	"	"
16	thiamethoxam	thiacloprid	"	"
17	thiamethoxam	acetamiprid	"	"
18	thiamethoxam	nitenpyram	"	"
19	thiacloprid	acetamiprid	"	"
20	thiacloprid	nitenpyram	"	"
21	acetamiprid	nitenpyram	"	"

Table 13: Table of pairs of synergies between neonicotinoids from the Bayer patent (Andersch *et al.*, US patent 7745375 B2, 29/06/2010) for a very large number of insect orders (*Lepidoptera, Coleoptera, Hymenoptera, Diptera, etc.*)

Whether these mixtures of active substances are intentional (formulations) or not (contamination of bee matrices due to varied foraging areas), they are still a source of contamination that can be found in water, pollen and nectar. It should be noted that the ratios of the most effective mixtures range from 100/1 to 1/100, which is a very wide quantitative range of synergies. The recent study

[39] http://patentimages.storage.googleapis.com/pdfs/US7745375.pdf

by Paradis *et al.* (2013) illustrated the simultaneous presence in nectar of four insecticides: three neonicotinoids (acetamiprid, thiacloprid and thiamethoxam) and deltamethrin. Another mixture consisting of *tau*-fluvalinate (from outside the hive) and two neonicotinoids (acetamiprid and thiacloprid) was also observed in the same study conducted in the Vendée *département* of France. A further illustration is found in the CETIOM/ITSAP data (see section 4.2) concerning trapped pollen (Centre region). Here, imidacloprid, thiacloprid and thiamethoxam were found in 2012 and 2013 Thus, co-exposure to several neonicotinoids *via* pollen and nectar has been proven on French territory. While there is no longer any doubt about the synergistic effect of two neonicotinoid compounds, the question is now extended to the synergistic effects of three (or more) neonicotinoids.

Concerning the interactions between antibiotics and other chemical factors, few data are available. Laboratory tests based on mortality rates of foragers have demonstrated interactive effects among acaricides, between acaricides and antibiotics, and between acaricides and fungicides (Johnson *et al.* 2013). These authors showed that *tau*-fluvalinate interacted with two of the three antibiotics tested (oxytetracycline-OTC, more with fumagillin, but not with tylosin). Coumaphos, thymol and amitraz had no interaction with the antibiotics. Other earlier studies had already shown interactions regarding OTC.

Simultaneous exposure to *tau*-fluvalinate and OTC increased the toxicity of the *tau*-fluvalinate (Hawthorne and Dively 2011). OTC blocks the transport enzymes, which increases the sensitivity of the bees to other toxic substances. OTC, in the presence of amitraz, resulted in the programmed death of cells in the bee gut (Gregorc and Bowen 2000). Hawthorne and Dively (2011) showed that bees fed with OTC were significantly more sensitive to coumaphos and *tau*-fluvalinate: the antibiotic must interfere with the excretion or normal metabolism of these pesticides. OTC significantly increases the mortality of bees exposed to coumaphos and *tau*-fluvalinate: (1) the mortality of bees treated with 2 µg/µl of coumaphos increased from 7 to 51% after feeding with OTC and (2) in bees treated with 3 µg/µl of *tau*-fluvalinate, mortality increased from 5.6 to 39% after feeding with OTC. In conclusion, co-application of OTC and acaricides increases the likelihood of poisoning by the acaricides contaminating wax and food reserves. These interactions of OTC with pesticides (coumaphos and *tau*-fluvalinate) contribute to the winter loss of colonies (during the winter or in early spring). Under their experimental conditions, these authors showed that OTC was increasing the sensitivity of the bees to the pesticides.

4.1.2.3 Between biological agents and chemical agents

Many pesticides of agricultural or beekeeping origin are now found systematically in the environment of the bees and in bee matrices (wax, pollen, nectar). In addition, bees are surrounded by a host of parasites, predators and infectious agents. The interactions between these infectious agents and pesticides are therefore potentially numerous and can affect bee health. Among the different combinations of possible interactions, only a limited number have been studied, most often in experimental laboratory conditions (cages) which are, by necessity, imperfect models of the natural conditions of the colony. These interactions were described in part in the DEFRA scientific report by Thompson (2012).

4.1.2.3.1 Controlled conditions

4.1.2.3.1.1 *Nosema*-pesticides

The presence of *Nosema* in artificially infected *A. mellifera* workers increased their sensitivity to an organochlorine insecticide, DDT, banned in France since the 1970s (Ladas 1972), which suggests a link between the two factors in bee survival.

The synergy between *N. ceranae* and imidacloprid has been demonstrated on bees placed in controlled laboratory conditions (cages). In addition, the imidacloprid-*N. ceranae* interaction induced an energy stress and a significant decrease in glucose oxidase, an enzyme involved in social immunity through its larval food sterilisation effect (Alaux *et al.* 2010a). A similar result was demonstrated by Vidau *et al.* (2011) who showed, under the same controlled laboratory conditions, a synergistic effect of sub-lethal doses of fipronil or thiacloprid on the mortality of bees parasitised

by *N. ceranae*. This synergistic effect was not strongly linked to a decrease in the detoxification system. Whereas fipronil seems to decrease the number of *Nosema* spores in the bee gut, thiacloprid, in contrast, seems to increase it significantly. This synergy effect aggravating fipronil-*N. ceranae* co-exposure was confirmed by Aufauvre *et al.* (2012) regarding the survival of worker bees reared in the laboratory; this was regardless of the order in which these two stressors were administered. The authors showed that, regardless of the method of infestation by *Nosema ceranae*, whether sequential or simultaneous with the insecticide, the synergy effect was maintained and more strongly evident when the stressors were applied at the time of the bee's emergence. In a more recent study, the same authors (Aufauvre *et al.* 2014) showed that the combination of *N. ceranae*-chronic insecticide exposure (imidacloprid and fipronil) resulted in significantly higher mortalities than the stressors alone, but did not show any synergy. The global study (RNA-Seq) of gene expression profiles of bees of each form showed no significant effect on detoxification genes but showed a repressor effect on immunity-related genes. Bees treated with *N. ceranae* alone or in combination with one of the insecticides showed a strong alteration of midgut immunity and modifications affecting the cuticle and trehalose metabolism. The impact of these treatments on gene expression increased over time demonstrating an absence of recovery of normal activity, which suggests a link with the high bee mortalities observed in the experiment.

Doublet *et al.* (2014) tested the effects of interactions of sub-lethal doses of a neonicotinoid, thiacloprid, *N. ceranae* and BQCV on larvae and adult bees under laboratory conditions. The authors found an additive interaction between the BQCV and thiacloprid regarding the survival of bee larvae, probably due to the high viral loads. In adult bees, the authors showed two synergistic interactions on bee mortality: *N. ceranae* with BQCV, and *N. ceranae* with thiacloprid. The combination of the two infectious agents had an even greater effect than *N. ceranae*-thiacloprid. The two pathogens seem to be the stressors with the greatest impact on survival of adult bees, and the sub-lethal doses of pesticides cause significant adverse effects on larvae and adults. The authors conclude by posing the question about the effects of these stress factors at colony level. This is an important point because most experiments are carried out under controlled conditions.

Retschnig *et al.* (2014a) also showed a synergistic effect between sub-lethal doses of thiacloprid and *Nosema ceranae* that was dependent on the dose of thiacloprid. In addition, the authors showed a negative effect of the insecticide on *Nosema* reproduction. This result shows that these types of interactions can be dynamic and should be studied in a context that includes a greater number of combinations.

Pettis *et al.* (2013) showed, under controlled conditions in cages, a significant increase in the likelihood of *Nosema* infection in bees that consumed pollen containing fungicides (chlorothalonil and pyraclostrobin).

It is interesting to note that the combination of insecticides and pathogenic fungi, already mentioned previously (Bayer patent EP0627165 A1, 1994), is used in integrated control of crop pests (Maredia *et al.* 2003) through the demonstrated synergistic effects of these interactions on insect mortality, particularly with imidacloprid (Al Mazraáwi 2007; Purwar and Sachan 2006; Ramakrishnan *et al.* 1999; Santos *et al.* 2007). It is therefore not surprising to find the same effects on bees.

4.1.2.3.1.2 DWV-pesticides

Prior to the results of Doublet *et al.* (2014) in the case of the additive toxic effects of thiacloprid and BQCV described above (see section 4.1.2.3.1.1.), the results of Di Prisco *et al.* (2013) showed that exposure of bees to clothianidin was associated with an immunosuppression syndrome (but not with chlorpyrifos), more specifically characterised by an effect on expression of the NF-κb transcription factor (dorsal-1A), and by increased replication of the DWV. Di Prisco *et al.* (2013) studied, under controlled conditions (cages), the effect of clothianidin and imidacloprid on the immune response of the bee and the replication of pathogenic viruses. They showed that the neonicotinoid insecticide clothianidin negatively modulates the immune signalling of the NF-κb transcription factor involved in insect immunity and affects antiviral defences controlled by this

transcription factor. They identified a negative effect on modulation of NF-κb activation. Exposure to clothianidin, by increasing transcription of the gene coding for this inhibitor, reduces the immune defences and induces replication of the deformed wing virus (DWV) in bees. This immunosuppression is also induced by imidacloprid, but not by chlorpyrifos, which does not affect NF-κb signalling. The effect of sub-lethal doses of this insecticide on viral proliferation suggests that neonicotinoids can have a negative effect on bee populations under natural conditions. These experiments under controlled conditions show a strong negative interaction between viruses, *Nosema* and new-generation pesticides (neonicotinoids and fungicides) on bee immunity. Clothianidin, imidacloprid and thiacloprid have the ability to reduce the immune responses of bees and therefore to promote the replication of viruses such as the DWV and BQCV.

4.1.2.3.1.3 Chronic bee paralysis virus-pesticides

By analysing the factors that vary the acute toxicity of insecticides, Bendahou *et al.* (1997) found that the LD_{50} of cypermethrin (a pyrethroid) decreased by a factor of 2.66 (0.06 compared with 0.16 mg/bee) when the emerging honeybee workers were infected with the chronic paralysis virus. The combined administration in the laboratory for 7 days of syrup spiked with cypermethrin (10 mg/L) and with the virus, significantly reduced consumption by workers and their survival compared to bees exposed to a single stressor.

4.1.2.3.1.4 American foulbrood-pesticides

While Morse *et al.* (1965) showed an increase in larvae infected by American foulbrood after treatment with carbaryl, under their experimental conditions, Atkins *et al.* (1981) noted no change in the toxicity of carbaryl, lindane and malathion in bees affected by American foulbrood.

4.1.2.3.2 Natural conditions, colony or apiary level

The aim here is to describe the studies relating to co-exposure under natural conditions and not studies describing single exposure (single-factor studies aimed at observing the influence of an exposure parameter in a specific landscape context). Pettis *et al.* (2012) demonstrated a strong interaction between imidacloprid and *Nosema*. The number of *Nosema* spores in the digestive tract of workers increased very significantly when the colony was exposed under natural conditions to sub-lethal doses of imidacloprid (5 or 20 µg/kg).

Bees exposed to brood combs contaminated by pesticides were more parasitised by *Nosema*, and at a younger age, than bees that had been less exposed to pesticides (Wu *et al.* 2012). These results suggest that pesticides contained in the wax in the frames have an effect on the sensitivity of bees to *Nosema*. In 2011, the same authors showed that exposure of immature bees (larvae and pupae) to pesticides contained in comb wax delayed bee development and thus promoted *Varroa* reproduction. In addition, they showed the ability of pesticide residues to migrate from one frame to another, thereby highlighting their transport and the contamination of bees.

A study of the effects of sub-lethal exposure to an insecticide, chlorpyrifos, and to a product based on two fungicides, boscalid and pyraclostrobin, on the emergence and viral loads of *Apis mellifera* queens under natural breeding conditions and in an enclosed flight area (DeGrandi-Hoffman *et al.* 2013) revealed effects of these pesticides, alone or in association, on the reduction in emergence of the queens and the increase in rates of DWV and BQCV, with a greater impact when both pesticides were applied to the colonies in association.

A study of the effect of chronic exposure to the pyrethroid lambda (k)-cyhalothrin and its interactions with the trypanosome parasite *Crithidia bombi* was conducted on colonies of bumble bees, *Bombus terrestris*, in the laboratory for 14 weeks (Baron *et al.* 2014). Whereas colonies treated with the insecticide produced individuals with lower body mass, no effect of the pesticide-trypanosome interaction was shown. Similarly, no effect of the pesticide on the sensitivity of bees to the trypanosome was shown, nor on the intensity of the parasitic infection.

Concerning anti-*Varroa* treatments, three acaricides used in *Varroa* control (thymol, coumaphos and formic acid) were able to change the bee's metabolic responses under natural conditions

(colonies), such as the expression of four genes involved in detoxification (CYP306, CYP6a514, pkar, pkac) and two immunity genes (DSC37 and BASK). The study showed a tendency to reduce pathogens (other than *Varroa*) with these acaricides (Boncristiani *et al.* 2012).

The *tau*-fluvalinate used to control *Varroa* had an effect on the DWV under natural conditions (colonies). The effect of treatment first led to an increase in viral load, probably by modifying the host's sensitivity. The viral load of DWV then decreased, an effect related to the decrease in *Varroa* parasitism following the acaricide treatment (Locke *et al.* 2012).

A study performed on colonies under natural conditions revealed the adverse effects of *Nosema ceranae* parasitism on the efficacy of anti-*Varroa* acaricide treatments based on amitraz (Botías *et al.* 2012).

It seems that the presence of certain pesticides in the colonies can increase *Nosema* parasitism under natural conditions thereby increasing the negative effects of the parasite, which partly confirms the results obtained under controlled conditions.

In conclusion, regarding the interactions between infectious agents and pesticides, only a few interactions have been studied, compared to the number of infectious agents and especially pesticides to which bees are exposed. The published works focus more particularly on newly identified stressors (neonicotinoids, acaricides, *Nosema ceranae,* viruses, *etc.)*. Although the first studies show considerable effects between these pesticides and infectious agents in bees, there is very little information, given the potentially critical multiple interactions.

4.1.3 Modulation of the effects of chemical or biological factors by other factors

4.1.3.1 Depending on factors intrinsic to bees

4.1.3.1.1 Depending on the age of the workers

The age of the workers is a variability factor for their tolerance to pesticides: newly emerging workers were more sensitive to DDT, dieldrin and carbaryl than older individuals, whereas the latter were more sensitive to malathion and parathion-methyl (Ladas 1972; Mayland and Burkhardt 1970). This high susceptibility of older workers to organosphosphorus was confirmed by Bendahou *et al.* (1997) with fenitrothion. But this fact is not restricted to this class of insecticides, since Wahl and Ulm (1983) also showed that the tolerance of worker bees to a fungicide based on copper oxide and to a herbicide based on 2.4-D (amine salt) decreased as a function of their age. However, the authors showed that the rate of infestation by *Nosema apis*, which increases with age in workers, was a confounding factor.

In 7-day-old workers, the number of tests required to abolish the proboscis extension reflex after administration of imidacloprid increased compared to the control, whereas a similar administration caused an opposite effect in workers just 24 h older (Guez *et al.* 2001).

Bendahou *et al.* (1997) showed that the LD_{50} of cypermethrin in workers less than one day old differed significantly from that calculated for older workers (respectively 0.16 and 0.21 mg/bee); the same was true for fenitrothion (respectively 9.27 and 0.42 mg/bee).

4.1.3.1.2 Depending on the weight of the workers

The lethal and acute toxicity of pesticides decreases with the weight of the exposed bees. This has been shown in honeybees (Gerig 1975; Ladas 1972) and especially in Africanized bees for paraquat (Nogueira Couto *et al.* 1996). It should be noted that the weight of workers measured under laboratory conditions may be different from that of workers in actual conditions due to food intake and the inability of individuals to empty their rectal ampulla.

4.1.3.1.3 Depending on the experience of the bees

Henry *et al.* (2012) showed that the degree of negative effects of thiamethoxam administered by ingestion at a dose of 1.3 ng/bee on the return of foragers to the hive depended on the foragers' experience of the route to be taken. The difference compared to the homing performance of control

foragers was accentuated when the treated foragers were released from points selected randomly around their hive.

4.1.3.1.4 Depending on the genetics of bees

Genetic factors are not strictly speaking stress factors but are potentially involved in modulating the effects of different stresses. This part therefore deals with factors that can affect genetic diversity at the population and colony level, as well as its role in resilience to stress.

- **Genetic diversity of the population**

Domestication processes are often accompanied by profound changes in genetic variability in animals and plants (Brudford *et al.* 2003). Bees have been used by humans since at least 7,000 BC, both for honey, wax production and pollination (Jaffe *et al.* 2010). In the context of bee decline, one of the causes advanced is the reduction in genetic diversity in bee management, with the risk of using bees that are not adapted to local conditions (Oldroyd 2007; Sheppard 2012; vanEngelsdorp and Meixner 2010), since beekeeping promotes the distribution of sub-species with commercial value, producing high yields of honey and pollen, outside their area of origin. However, two relatively recent studies on the evolutionary history of honeybees have raised doubts about the assertion that bee management reduces genetic diversity (Whitfield *et al.* 2006; Zayed and Whitfield 2008).

Although bees are not domesticated in the strict sense of the term (Oxley and Oldroyd 2010), and are subject to numerous environmental constraints, they are actively selected. A direct consequence may be a reduction in genetic diversity. However, a study seeking to compare wild populations with domestic populations in Europe and North America recently showed that human action (beekeeping) has the effect of increasing genetic diversity by crossing individuals of different origins between eastern and western Europe (Harpur *et al.* 2012). Another study showed that, in the Canary Islands, continuous introductions of sub-species of foreign bees have not increased the genetic diversity of local populations (Munoz *et al.* 2012). However, hybridisation between local and imported bees has occurred, and has led to variations in genetic composition described as low, that could lead to a risk of loss of genetic identity, i.e. a loss of local characteristics (Munoz *et al.*, 2012). In addition, it would appear that the bees marketed in Canada are hybrids with greater variability than their ancestors in Europe. These results seem to contradict the suggestion that beekeeping practices, through large-scale genetic homogenisation of mixed/crossed populations, could lead to a substantial loss of local adaptations (De la Rua *et al.* 2013). Indeed, the largest population of wild bees, as well as bee populations with the highest genetic diversity (Harpur *et al.* 2012; Jaffe *et al.* 2010), are found in Africa, where human practices have had little or no impact on populations (Dietemann *et al.* 2009).

The deliberate crossing and use of non-native bees in beekeeping promotes the creation of mixed populations, which will affect native populations. The hybrid bees will have greater genetic diversity, but risk losing some of the traits from natural selection that made them particularly well suited to their local environment (Costa *et al.* 2012; Strange *et al.* 2007).

Very few breeders have the means to implement strict controls using islands for a mating area or even artificial insemination. In France, the EAGF programme funded research on this question for several years, which came to the conclusion that it was possible to maintain a sub-species or an ecotype under defined conditions. Several conservatories were thus set up in a number of regions. Their aim is the conservation, preservation and development of the European dark bee *Apis mellifera mellifera,* and in particular, the selection and production of queens and swarms. Most of these conservatories benefit from scientific support within the framework of an EAGF programme. Some beekeepers mate bees that are native to their region, or take part in conservation programmes aimed at avoiding crosses between domesticated or wild populations (Bouga *et al.* 2011; Chapman *et al.* 2008). These programmes enable different local populations or ecotypes, and therefore a certain diversity, to be conserved on a larger scale. This makes it possible to preserve ecotypes adapted to their environment. Selection targeting phenotypes that are advantageous for beekeeping, for example resistance to *Varroa* (Harbo and Harris 1999; Spivak and Reuter 2001b), may seem to be an ideal solution for improving the health of the population.

However, targeted selection may lead to a reduction in genetic diversity in the population. In addition, it may be to the detriment of other characteristics that are important to the colony or to beekeeping, such as honey production.

- **Inter-colony genetic diversity**

Sperm used for fertilisation are stored in a sperm bank and contain a mixture of semen produced by a dozen males on average (Winston 1987). Queens reproduce during their mating flight; potential selection of males by the queen has not been demonstrated to date. Many studies have convincingly shown the importance of multiple matings or polyandry at different levels. The quality of the mating is crucial, with its effects having an impact on both the queen's physiology and her interactions with workers only a few days after mating (Kocher *et al.* 2009; Kocher *et al.* 2008). Similarly, the quality and quantity of semen used for artificial insemination will affect queen-worker interactions and the queen's physiology (Richard *et al.* 2011; Richard *et al.* 2007).

Intra-colony genetic diversity confers significant adaptive advantages including better thermal stability (Jones *et al.* 2004; Mattila and Seeley 2007; Oldroyd and Fewell 2007) and a reduction in the risk of harm by infectious agents, mainly on the brood (Palmer and Oldroyd 2003; Seeley and Tarpy 2007; Tarpy 2003; Tarpy and Seeley 2006), and is accompanied by better performance at the various tasks due to a better division of labour (Mattila *et al.* 2008; Smith *et al.* 2008), which all lead to higher productivity (more honey) and better chances of survival (Mattila and Seeley 2007; Tarpy *et al.* 2013). Indeed, it was recently shown that queens that mated with more than seven males were 2.86 times more likely to survive during the 10 months of beekeeping activity of the study (Tarpy *et al.* 2013).

However, it has not yet been possible to establish a direct link between the benefits of genetic diversity and the decline in bee populations, even though there is a link between low genetic diversity and the prevalence of brood diseases. Genetically diverse colonies (where the queen has engaged in multiple matings) show a lower probability of contracting severe infections related to brood diseases compared to colonies where the queen was fertilised by a single male (Palmer and Oldroyd 2003; Seeley and Tarpy 2007; Tarpy 2003; Tarpy and Seeley 2006). While these studies show lower virulence in the colonies (mainly chalkbrood caused by *Ascosphaera apis* and American foulbrood caused by *Paenibacillus larvae*), they have not revealed any clear differences in fitness[40] between these two types of colonies and therefore this remains to be demonstrated for diseases affecting adult bees. For example, intra-colony genetic diversity (number of males contributing to the insemination of the queen) does not seem to have any influence on *Varroa* loads within the colony (Neumann and Mortiz 2000). On the other hand, a reduction in genetic diversity at the population level may increase the risks of inbreeding and therefore production of diploid males responsible for a "deficient brood pattern", which would have disastrous consequences on bee colonies (Cook and Crozier 1995; Harpur *et al.* 2013).

In conclusion, there is no evidence of a decline in genetic diversity in honeybees, or of its role in the decline of populations, at least on a worldwide scale.

- **Involvement of genetic factors in the modulation of chemical factors**

It should be noted that the studies on the differential toxicity of pesticides according to breed, mentioned below, do not provide a rigorous genetic characterisation of the biological material used.

After oral administration of imidacloprid, the LD_{50} in *Apis mellifera mellifera* and *Apis mellifera caucasica* was calculated at 5 ng/bee (Suchail *et al.* 2000). In contrast, topical administration of the same insecticide induced an LD_{50} of 24 ng/bee for *A. m. mellifera* and only 14 ng/bee for *A. m. caucasica.* Laurino *et al.* (2010) found comparable toxicity for imidacloprid, clothianidin and thiamethoxam on three strains of *Apis mellifera.* However, the sample sizes used by the authors were very small (10 foragers per dose) and they did not provide a genetic analysis of the strains, examining only morphological criteria.

[40] Or adaptive value: the ability of an individual to reproduce, measured by the number of viable and fertile descendants

4.1.3.1.5 Depending on the type of bees

The sub-lethal effects of imidacloprid on olfactory learning performance in the laboratory were recorded for lower concentrations in summer workers than those causing the same effects in workers reared in winter in a heated apiary (12 µg/kg *vs* 48 µg/kg) (Decourtye *et al.* 2003).

4.1.3.2 Depending on factors extrinsic to bees

4.1.3.2.1 Influence of diet

Wahl and Ulm (1983) showed that the quantity and the quality of pollen consumed by young workers influence their subsequent sensitivity to pesticides. Six commercial preparations (2.4-D Na, Dicopur, Cupravit, Maneb Cela-Merck, Maneb BASF, ZnSO4) out of seven had a lower LD_{50} when the bees received a protein-deficient diet (pollen from dandelion considered to be poor in amino acids; Loper and Cohen (1987)). The pollen substitutes tested increased the toxicity of the pesticides in workers. Colonies maintained in cages and with rich pollen resources at their disposal were less sensitive to pesticides. The authors concluded that the workers most susceptible to toxins were those surviving the winter that had to care for the first larvae of the season. However, the authors based the nutritional quality of pollen only on the level of proteins, which was too restrictive (Brodschneider and Crailsheim 2010; Di Pasquale *et al.* 2013). A recent study demonstrating the positive role of pollen in the diet on the expression of genes coding for detoxification enzymes may explain this phenomenon (Schmehl *et al.* 2014).

In laboratory conditions, a pollen deficiency may alter protein, lipid and energy metabolism in bees (Alaux *et al.* 2011a), induce adverse effects on immunocompetence (glucose oxidase and size of fat bodies, the site of humoural immunity) (Alaux *et al.* 2010b), cause an increase in DWV viral loads (Degrandi-Hoffman *et al.* 2010) and greater sensitivity to American foulbrood (Rinderer *et al.* 1974), microsporidia infections (*Nosema*) (Di Pasquale *et al.* 2013; Rinderer and Elliott 1977) and pesticides (Wahl and Ulm 1983). A decrease in the elimination of brood parasitised by *Varroa* has even been observed in colonies with few pollen reserves (Janmaat and Winston 2000b). In addition, parasites and infectious agents can increase the metabolic needs of individuals, which cannot respond if there is nutritional stress. For example, bees infected by the parasite *Nosema ceranae* increase their consumption of carbohydrates in order to compensate for energy losses induced by the parasite drawing upon the host's resources in order to multiply (Alaux *et al.* 2010a; Mayack and Naug 2009). In contrast, the presence or absence of pollen has no effect on tolerance to *Varroa* at the individual level, since the pathogenicity of *Varroa* is not compensated by a diet rich in pollen (Alaux *et al.* 2011a; van Dooremalen *et al.* 2013).

4.1.3.2.2 Depending on the season

In laboratory experiments carried out in summer, Meled *et al.* (1998) showed a synergistic effect on honeybee mortality between deltamethrin (pyrethroid) and prochloraz (imidazole) from the dose of 31.25 mg/ha, whereas during the winter this synergy was not confirmed, even at doses 4 or 8 times higher. This can probably be explained by the fact that the toxicity of deltamethrin increases when the temperature rises (Bos and Masson 1983).

4.1.3.2.3 Depending on the formulation

To our knowledge, only one study has addressed the differential toxicity of an insecticide according to the formulation that contains it (Bendahou *et al.* 1997). The LD_{50} of cypermethrin and that of fenitrothion in the form of an active substance (respectively 0.16 and 0.27 mg/bee) were significantly lower than those of the formulations Cymbush (0.26 mg/bee) (100 g of cypermethrin per litre of petroleum ether) and Folithion (0.38 mg/bee) (550 g of fenitrothion per litre of petroleum ether). However, a co-formulant can have intrinsic toxicity, such as that of N-methyl-2-pyrrolidone (NMP) on bee larvae (Zhu *et al.* 2014). More generally, co-formulants are especially known for their ability to increase the bioavailability of active substances or their efficacy on their targets. A recent study on nine major pesticides (glyphosate, isoproturon, fluroxypyr, pirimicarb, imidacloprid, acetamiprid, tebuconazole, epoxiconazole and prochloraz) showed that, for eight of them, the toxicity of the formulations for human cells could be several orders of magnitude higher than that of

the active substances alone (Mesnage *et al.* 2014). Only the formulation without an adjuvant and consisting of isoproturon gave the same results in comparisons between active ingredient and formulation in this study. In the particular case of imidacloprid and its formulation Confidor, the increase in toxicity for human cells is due to the co-formulant NMP (Mesnage *et al.* 2014), which supports the observations of Zhu *et al.* (2014) about the direct toxic action of the NMP compound on bee larvae, despite it being considered "inert".

4.2 Analysis of available data on co-exposure of bees to biological and chemical factors in France

4.2.1.1 Reality of co-exposure to biological and/or chemical hazards

In the datasets in which there is simultaneous observation of infectious agents and chemical residues on the same hives or the same apiaries, the following were observed (whether or not there are symptoms):

- ✓ co-infections by several infectious agents (one example in the ADARA study *Figure 8*);
- ✓ co-occurrences of chemical residues (two examples in the ONIRIS and ADARA studies *Figure 10* and *Figure 11*);
- ✓ co-occurrences of several chemical residues and several infectious agents, in the same place on the same date, or in the same place on different dates (one example in the CETIOM/ITSAP monitoring study *Table 15*).

4.2.1.1.1 Co-occurrences of infectious agents

- ***Colonies with disorders***

Figure 8: Example of co-infections by several infectious agents in 13 clinical cases of disorders (ADARA study)
The symbols represent the paired correlation of the presence of infectious agents in the dataset. The narrower the ovals the stronger the correlation, with blue representing simultaneous presences.
Red represents reciprocal exclusions *i.e.* agent A was absent when agent B was present and vice-versa.

In the ADARA dataset, screening for residues was not done systematically, or in an identical way in all the apiaries. For this reason, it is not possible to include the chemical hazards and the correlation calculations for residues in this diagram, nor to confirm whether or not these disorders were caused by these infectious agents or by other factors.

- ***Colonies without visible disorders***

A similar graph was produced for the infectious agents for the ONIRIS study (on apiaries without disorders) (*Figure 9*). The associations are less marked. Co-infections with viruses of the AKI complex can be observed. The molecular tools used have been designed to be specific to each virus of the AKI complex, so this would not be due to any confusion of the method between related genotypes. However, it is possible that this co-infection gives rise to recombinations between these different viruses (de Miranda *et al.* 2010). Several research projects under way are seeking to improve knowledge of the biology of this viral complex and explore its micro-evolutionary dynamics.
Co-infections between the virus CBPV and *Nosema ceranae* or *N. apis* can also be observed on this same dataset. A possible explanation is a neurological effect of the CBPV on the hygienic behaviour of workers (olfaction, memory, motor activity) in eliminating larvae infected by *Nosema*, which may then proliferate in the colony. But a common immunodepressive cause could also be behind the proliferation of the two infectious agents. There were no symptoms in these apiaries during the beekeeping season, nor any quantifiable effect on winter survival (see details in Mouret *et al.* (2013)).

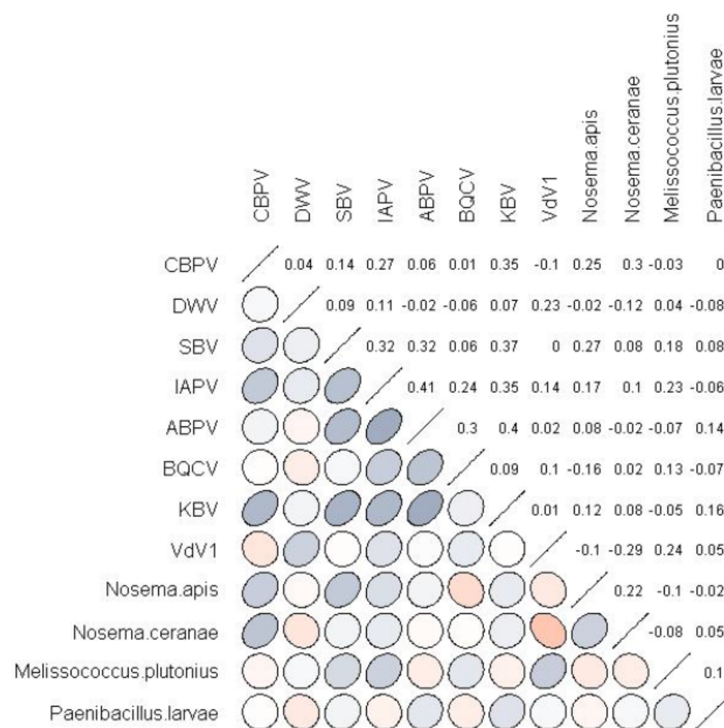


Figure 9: Example of co-infections by several infectious agents in the ONIRIS study

4.2.1.1.2 Co-occurrences of chemical hazards

- ***Colonies without visible disorders***

In the ONIRIS study, whose primary aim was to relate the residues found in bee matrices to the chemical hazards found in the environment, 28 different substances were detected by a multi-residue analysis (Lambert *et al.* 2013).
A comparative analysis of contamination profiles was carried out between the 18 apiaries on the honey matrix (detection data averaged over the two years of the study, for each apiary). *Figure 10*

shows an apiary similarity tree (the greater the similarities in the profiles of detected substances between two apiaries, the closer they are in the graph).

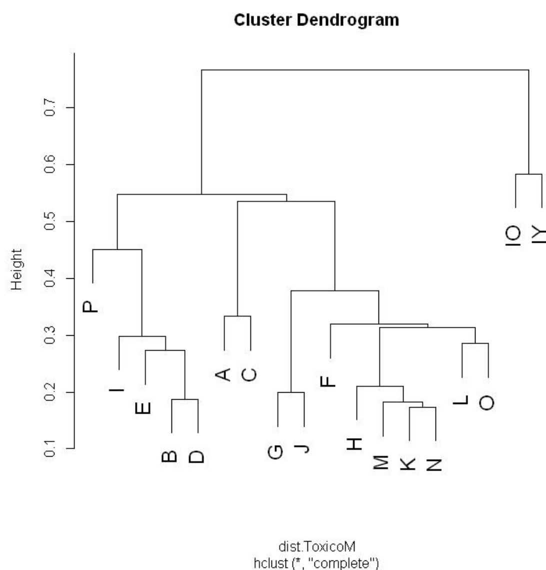


Figure 10: Representation of Bray-Curtis indices for comparing the profiles of 18 apiaries for the honey matrix, in the form of a tree diagram (complete linkage method) (source: report ONIRIS)

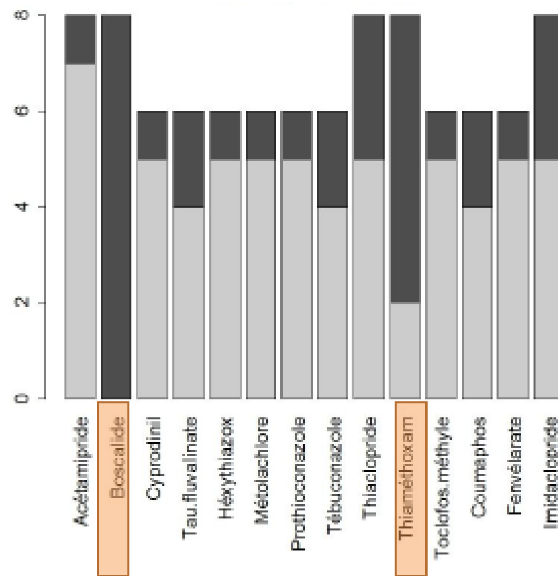
In *Table 14*, the correlation coefficients of paired substances in the ONIRIS study show that there are indeed insecticide/fungicide or acaricide/acaricide co-exposures.

Table 14: The highest correlation coefficients (higher than 0.40) among the 28 residues detected at least once (ONIRIS study)

Residues	Correlation coefficient	p-value
Diethofencarb - Chlorpyrifos-methyl	0.70	2.2e-16
Prochloraz - Fenoxycarb	0.70	2.2e-16
Chlorpyrifos-methyl - Pyriproxyfen	0.63	2.2e-16
Diethofencarb - Pyriproxyfen	0.44	4.498e-08
Amitraz I - *Tau*-fluvalinate	0.44	5.987e-08
Prochloraz - Flusilazole	0.40	1.026e-08
Carbofuran - Flusilazole	0.40	1.026e-08

- ***Colonies with disorders***

This also appears in the observations made on 12 clinical cases of apiaries with disorders, in the Rhône-Alpes region (ADARA study), although the structure of the data means it is impossible to calculate similarity indices or paired correlations so as to detect any possible preferential associations. *Figure 11* shows the number of apiaries tested where substances were detected.



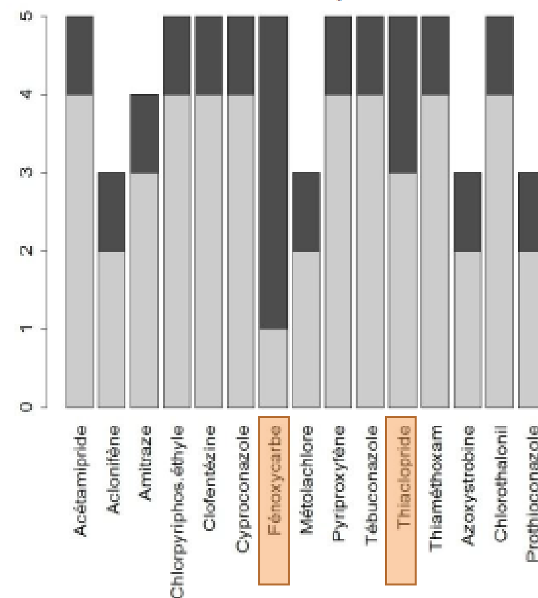


Figure 11: Examples of joint detection of plant protection products in bee bread and pollen from 13 clinical cases of disorders (ADARA study).
On the Y axis, the number of hives in which each residue was screened for
(dark grey = detected; light grey = not detected)

The ADARA study is a compilation of clinical cases reported spontaneously to the network for the monitoring of significant mortalities and investigated in more depth in the Rhône-Alpes region.
As the residues were not detected in a comparable way, nor on all apiaries, it is not possible to quantify the observed associations of pesticides. Note that there was a fungicide-neonicotinoid association here (boscalid + thiamethoxam) in six apiaries out of eight in which the bee bread matrix was analysed. These substances may be present in the food of workers and larvae. This fungicide-neonicotinoid association may generate synergy from the detoxification mechanisms (Johnson *et al.* 2013), especially as thiamethoxam is metabolised into clothianidin.

4.2.1.1.3 Co-occurrences of biological and chemical hazards

Most of the datasets examined by the working group had standardisation failings regarding the joint measurement of biological and chemical hazards, which makes it impossible to obtain a reliable quantitative picture. Nevertheless, specific observations are of interest as a case study. For example,

Table 15 summarises the joint observations of plant protection substances and infectious agents on four apiaries in field crops.

Table 15: Example of co-occurrences, in the same place, on the same date or on different dates, of chemical residues and infectious agents, during monitoring of four apiaries in an area of field crops. Here, the results obtained on bees found dead in front of the hive are given in terms of presence/absence (1/0), or relative quantities, for a given day on an apiary (CETIOM/ITSAP study)

A, B, C and D are the four apiaries monitored in the CETIOM/ITSAP study

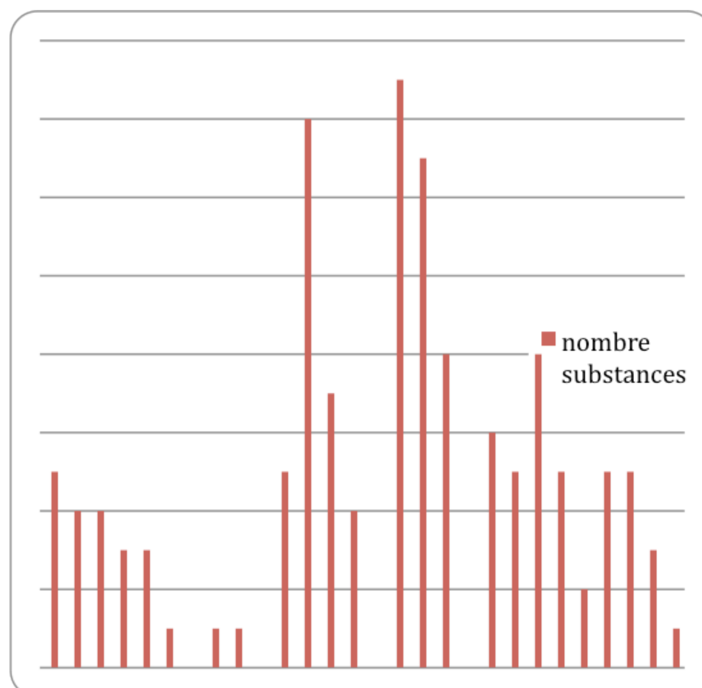
Site	Year	Date	Azoxystrobin	Boscalid	Chlorpyriphos_ethyl	Diphenylamine	Metconazole	SBV	BQCV	CBPV	IAPV	CBPV_quanti	*Nosema* sp.	Disorders
A	2012	19 Apr	0	1	0	0	1	NA	NA	1	NA	3.99e+06	4.32e+06	1
A	2012	24 Apr	0	1	0	0	1	1	1	1	NA	3.05e+11	1.70e+06	1
A	2013	11 May	0	0	0	0	0	1	1	1	NA	55100	<1e+06	1
A	2013	27 May	0	0	0	0	0	1	1	1	NA	2.29e+06	NA	1
B	2012	16 May	1	0	0	1	0	1	1	1	NA	3.49e+12	NA	1
B	2013	31 May	0	0	0	0	0	NA	NA	NA	NA	NA	NA	1
C	2013	23 May	0	0	0	0	0	1	1	0	1	0	1.48e+06	0
D	2012	07 May	0	1	1	0	1	1	1	1	1	5.88e+09	3.34e+06	1

In the same study (monitoring of four apiaries located in an area of field crops at the end of spring in 2012 and 2013), toxicological analyses on the pollen being brought into the hive showed 1 to 15 different substances for the same sample on a given date, and between 0 and 5 substances in new honey (from 30 substances screened for). As an example, in pollen trapped at one site, on 3 May 2013, the following 15 substances were detected: boscalid, chlorothalonil, chlorpyrifos-ethyl, cyproconazole, fluazifop P, flurochloridone, S metolachlor, pencycuron, pendimethalin, phenmedipham, prosulfocarb, prothioconazole-desthio, imidacloprid, thiacloprid, thiamethoxam.

The detail of 30 substances detected in pollen from these four apiaries on different dates is given in Table 16 in Annex 2.

Figure 12 shows the number of substances detected on different dates in the same year of monitoring, for each of the four apiaries. The substances are both diverse and detected repeatedly over time.

Figure 12: Number of substances detected on different dates in the same year of monitoring, for each of four apiaries (A, B, C and D are the four apiaries monitored in the CETIOM/ITSAP study)



The end result is that the co-occurrence of infectious agents and chemical residues seems to be the rule, despite not being described in a satisfactory manner when the agents and substances detected cannot be compared from one apiary to the other, with standardised methods whose sensitivity is always appropriate.

4.2.1.2 Link between the presence of infectious agents and chemical residues and the state of the colonies

4.2.1.2.1 Review of the examination of the datasets

Many criteria on the state of the colony have been identified in the studied datasets, with the degree of detail in the entry of variables differing greatly from one study to the next:

- ONIRIS study: 13 criteria measured (variables on the strength of the colony in the absence of symptoms);
- Epilobee France study: definition of cases of mortality or suspicion of infectious disease;
- ADARA study: 16 disorders of adult bees, 11 brood disorders;
- CETIOM/ITSAP-French Bee Institute study: presence/absence of disorders, mortality, weight gain;
- Cruiser study: various clinical signs and variables on colony strength (filling of frames);
- DGAL review of investigations of the network of bee disorders in 2013: designation of symptoms on adult bees and brood, mortality, "strong/moderate/weak" colonies.

4.2.1.2.2 Test for quantification of the link between presence/absence of hazards and variables on the state of colonies

When there were sufficient data that could be compared within the same dataset, univariate statistical associations were calculated between the detection of hazards and the presence of disorders, or the variables on the state of the colonies. The aim here was to describe co-occurrences of hazards with certain state variables (case studies, prevalence studies) and not to seek a cause and effect relationship. In any case, the conditions concerning representativeness were not met.

In the ONIRIS study for example (variables on the strength of colonies in the absence of proven disorders), a correlation was observed between the presence of CBPV, SBV, KBV and the presence of dead bees in front of the hive. Carbendazim in pollen and cyproconazole in honey were associated with increased activity. Residues of coumaphos and amitraz were associated with larger pollen reserves (trend for coumaphos). These observations should not be interpreted at face value as mechanisms that are favourable or unfavourable to the healthy state of the colonies. Rather, they reflect the complexity of the functioning of the colony, in all its components, including the management of anti-*Varroa* treatments by the beekeeper.

In the Cruiser study, the data were only completed sufficiently during the maize flowering period (whereas the study was also supposed to examine the possible influence of exposure to seed dust during planting, as well as effects later in the season). The characterisation of variables on state varied greatly from one region to another.

During the flowering period for maize (a pollen-rich resource), the apiaries in the "large surface areas with Cruiser in the immediate foraging area" category had a lower percentage of strong colonies. This correlation was not significant, but became more likely (p=0.08) if the interaction with the actual contamination of pollen and bee bread matrices by thiamethoxam and clothianidin was taken into consideration. This should be compared with the observation of contamination of 50% of colonies by thiamethoxam and/or clothianidin, whether or not they are located in a "Cruiser" foraging area.

In addition, a negative link was observed between aggressiveness of workers and detection of *Nosema* > 10^6 spores, between aggressiveness and detection of CBPV, and between symptoms of infectious diseases on the brood and detection of *Nosema* > 10^6 spores.

These observations can be interpreted considering aggressiveness rather as a behavioural trait than as a symptom of an infectious disease or poisoning. Aggressiveness is genetically linked to increased hygienic and defensive behaviour regarding the hive. It may be, therefore, that more aggressive workers reject infected bees more frequently, thereby decreasing the infectious pressure in the hive. Conversely, less aggressive or paralysed bees, or those with olfaction disorders, may enable the proliferation of infections that are not highly pathogenic. Here again, the observed variables may reflect more complex underlying mechanisms.

In this study, the degree of infestation with *Varroa* and the rates of contamination by acaricides for beekeeping use are unknown.

Ultimately, the low number of comparable observations and the selection bias make these results difficult to interpret, even within the same study. A variety of hazards associated with disorders have been revealed, but in any case the reality of the co-occurrence of the hazards described above leads to questions about the value of analysing hazards one by one.

4.2.1.3 Conclusion and recommendations

4.2.1.3.1 Conclusions

The detailed analysis of the observations provided to the working group revealed huge diversity in the presence of biological and chemical hazards in the apiaries, although most of the datasets suffer from a lack of standardisation in the detection measures, small sample sizes and a large quantity of missing data. Part 3.2 showed the high quantitative variation over the course of a beekeeping season.

In most of the datasets, there are three major weaknesses in the approach to comparing disorders with the presence of hazards:

- the failure to take account of seasonal dynamics in the analysis,
- confusion of the apiary and colony scales, whereas there are both common risk factors and great variability in detections within the same apiary,
- an assumption about the single-factor causality of disorders and the expected immediacy of the disorders that can be observed, which leads to a focus on screening for one hazard or

another without querying the interactions between them and the time needed for the disorders to emerge.

4.2.1.3.2 Recommendations

When structuring data, the precise date and place of sampling should be entered in a standardised way, in order to be able to group together the observations by place, date or season and easily calculate results and time series.

The colony scale and apiary scale should be separate, but information on the apiary to which each colony belongs should be known. The number of apiaries and colonies monitored should be sufficient to accurately estimate the frequencies of infection or contamination: the smaller the sampling sizes, the greater the margin of uncertainty.

Confounding factors should also be taken into account: beekeeping practices (especially the history of the colonies), climate, meteorological data regarding temperature, rain and wind in the 15 days preceding the sampling, landscape context and agricultural uses, and local density of apiaries.

Rather than pairs of hazards, profiles of detected agents and substances should be compared from one situation to another, for example using ascending hierarchical classification methods. It would then be possible to identify meaningful associations or accumulations of factors, from the point of view of the underlying biological mechanisms. For example, co-exposure to several substances from the same class (inhibitors of the cytochrome P450 complex, for example) is common, and affects the degree of toxicity of other substances also likely to be present. To be able to draw statistical conclusions in the presence of multiple factors, it is necessary to study a large number of samples. The greater the number of variables studied, the larger the size of the sample needed, which imposes *a priori* methodological choices, depending on the question asked.

Better standardisation is needed in measurement of the state of the colonies, including provision of state variables in asymptomatic colonies and the level of training and information of veterinary clinicians and bee health technicians (little of such information is available in the protocols in general). The development of an illustrated clinical and diagnostic guide to bee diseases would contribute to the training of veterinarians and bee health technicians, and thus to the standardisation of these measurements.

For the diagnosis of acute disorders, quantitative methods sensitive to multiple infectious agents should be used, regardless of the observed symptoms, combined with detection of a battery of chemical residues corresponding to the agricultural uses in the sector and for the season. The presence of veterinary drugs, including antibiotics (OTC, certain macrolides, *etc.*), and approved products likely to be released in the environment should also be screened for.

4.3 Conclusions and recommendations

- **Review of suspected or demonstrated mechanisms of action between stress factors**

The working assumptions resulting from the literature review on the interactions are listed below (non-exhaustive list). These different mechanisms may act simultaneously. Their effects depend on the season. They may only become visible after a latency period. Beekeeping practices may compensate for or amplify them.

- ✓ *Varroa* acts as an immunosuppressant, as it deprives parasitised bees of proteins and also due to specific biochemical factors aimed at keeping it on the bee. It therefore has the potential to amplify infections in general, even those that are not transmitted by this mite (see section 3.1.1.2.4.1.).
- ✓ *Varroa* acts as an amplifier of infection by some viruses that it transmits via haemolymph: the DWV, viruses of the AKI complex and the SBV. The DWV is more strongly amplified by *Varroa*, because the mite is also a multiplier host of the DWV (see sections 3.1.1.2.2.1. and 3.1.1.2.2.5.).
- ✓ Several infectious agents can interact on the same functional targets, for example the

nervous system of the adult, the digestive tube of the larva and the adult, the reproductive function of the queen.

- ✓ Any of the factors acting on olfaction, pheromones and the level of activity may have an effect on the cohesion of the colony and the hygienic behaviour of workers (elimination of sick or dead individuals), and therefore on the risks of infection and parasitism by *Varroa*. This is especially the case with viruses exhibiting neurotropism (e.g. CBPV) (see section 4.1.1.1.2.2.).
- ✓ Some substances, for example neonicotinoids and some acaricides, also act on olfaction, pheromones and the level of activity. They may thus have an indirect effect on amplification of infections or parasitism (see section 3.1.2.2.).
- ✓ Some substances have immunosuppressive effects on bees, and also contribute in absolute terms to the amplification of infections and parasites (see section 3.1.2.2.).
- ✓ The seasonal phenology of plants and weather conditions together influence the trophic level of the colony (available resources, weather conditions favourable to foraging). In particular, sporadic protein deficiencies may be responsible for transient immunosuppression. Adverse weather conditions also influence worker bee cleansing flights, and thus the quantity of infectious agents in the hive. The colony can withstand these fluctuations thanks to its reserves. The foraging history is therefore important, as is the removal of reserves by the beekeeper (see section 3.1.3.).
- ✓ The level of infection of the colony at the start of winter depends on the interaction between all these factors during the foraging period.
- ✓ Certain chemical substances (pyrethroids, neonicotinoids, *etc.*) have an impact on behaviour relating to foraging, memorisation of resources and returning to the hive. They therefore have an influence on the trophic level of the hive. The loss of forager bees can be compensated by other worker bees, but at the expense of the proper functioning of the colony (hygiene, reserves) (see section 4.1.3.).
- ✓ Several substances (insecticides, acaricides, fungicides, *etc.*) can have effects on the same functional target in bees, for example the nervous system or the digestive tract. Their impact can be cumulative and add to that of infectious agents with the same target (see section 3.1.2.).
- ✓ Several substances can interfere with the detoxification mechanisms, which can modify the bees' sensitivity to other substances (see section 4.1.1.2.).
- ✓ Nutritional deficiencies, especially in protein, can also have a suppressing effect on the detoxification function.

- **Recommendations on the surveillance of infectious agents and chemical agents**

- ✓ Infectious and chemical agents, including acaricides in wax, should be screened for concomitantly, both during active surveillance and on the appearance of disorders in the colonies.
- ✓ These hazards should be monitored in a comparable way throughout any given study and between studies. As part of programmed surveillance, it is appropriate to use methods validated according to the standards in force (AFNOR, ISO, OIE), harmonised if possible (to ensure an effective comparison of results), and with the appropriate degree of sensitivity depending on the objectives.
- ✓ Active programmed surveillance of infectious agents should be carried out using quantitative methods targeting several agents, whether or not there are clinical signs. It should always be performed jointly with a quantification of the degree of infestation with *Varroa*, which greatly determines the dynamics of the infections that it transmits, as well as the immune status of the bees.
- ✓ Surveillance of infectious agents should help provide qualitative and quantitative data on asymptomatic carriage phenomena in colonies, as these data are currently insufficient.

- ✓ Surveillance of toxic factors should be focused primarily on the substances applied in the areas concerned, for example in view of the quantities used. However these quantities do not reflect the level of toxic risk, which depends on each substance, association of substances or formulation. Multiple-residue methods should be given preference provided they have satisfactory sensitivity for the specific objective. For highly toxic pesticides (acute or chronic toxicity and sub-lethal effects), single-residue analyses (the active substance and its toxic metabolites in bees) will be essential.
- ✓ For surveillance of emerging issues and for toxicovigilance of veterinary and plant protection products (ex-post assessment), it is also necessary in the first place to standardise and centralise observations of disorders and to standardise the multi-hazard analytical methods used.

- **Recommendations on future studies and data collection aimed at elucidating the issue of interactions in a natural situation**

The conditions of statistical validity for epidemiological studies in beekeeping recently underwent a comprehensive review (vanEngelsdorp *et al.* 2013a), which distinguished descriptive (observation) and explanatory studies (causal link).

Epidemiological surveillance, whose aim is descriptive, requires standardisation of data collection, as this is indispensable for enabling data analysis. This standardisation in particular requires centralised coordination ensuring compliance with protocols, training of surveyors, information reporting, information feedback, and relevant statistical analysis based on sufficient sample sizes. There are sampling rules that make it possible to achieve the required accuracy based on the question asked. With these criteria in mind, the current surveillance schemes are insufficient; the debate under way for the mortality and alerts observatory should support these recommendations. Regional observatories are in place and their aim is to have beehives that can serve as references, both for normal production and for regular exposure to the risk factors specific to the region.

In view of the multiplicity of concomitant exposures, studies aimed at identifying risk factors must use methods enabling comparisons of profiles of exposure to risk factors (in terms of diversity and quantity), between case and control epidemiological units, with the possibility of repeating them over time. Even for studies focused more on infectious diseases or poisonings, it is necessary to measure the other factors, which are likely to have a major influence on the pathological consequences. Beekeeping practices must be taken into account in the factors influencing the health of the colony. The state variables of the colonies measured must include the availability of reserves and the demographic structure inside the colony, the size of the population, etc. Sampling must take into account the structuring in apiaries (for example cluster sampling for cohort studies). It is very important to record information on the relationship between the scale of the colony and that of the apiary, and to carry out statistical analyses taking this structure into account. It is also important to factor in seasonal and geographical parameters, which strongly affect colony biology.

The phenomena described by the epidemiological surveillance protocols could be explored in more detail with epidemiological studies with an explanatory purpose. In this case, the survey protocol should be designed to enable a comparison of cases with a reference population (control or non-exposed population). Given the complexity of the phenomena involved in bee disorders, an extremely strict methodology is essential when developing and implementing protocols for epidemiological surveys, so as to ensure the quality and comparability of the data, as well as their effective collection. The existing protocols suffer from an excessive lack of data, or data that are not comparable.

5 The issue of taking interactions into account when assessing the risks associated with plant protection products

The aim of this section is to determine whether it would be appropriate and feasible to develop methods enabling the possible interactions between infectious agents and toxic factors to be taken into account when assessing plant protection products, in particular if this were done in a standardised manner.

5.1 Review of regulatory assessment of plant protection products

In this request, the issue of changes to regulatory assessment was limited *a priori* to plant protection products. However, it should be noted that pesticide substances found in hive matrices can also originate from the use of biocidal products (e.g. insecticides for anti-vector control) or anti-parasitic products (e.g. treatments against *Varroa destructor* or more generally veterinary pest control products intended for other animal species). In this section, the regulations concerning biocidal and anti-parasitic products, and in particular their requirements relative to the assessment of hazards and risks for bees, will not be presented.

Plant protection products are used by professionals and individuals to destroy and repel pests, or render them harmless. ANSES is responsible for assessing these products, as well as fertilisers and growing media, before they can be put on the market.
This assessment takes place in two stages:

1. The first step, carried out at European level, involves assessing the hazards and risks associated with the active substances used in the composition of plant protection products. This phase is coordinated at European level by the European Food Safety Authority (EFSA), based on the collective assessments carried out by the Member States (ANSES for France);

2. The second step, carried out at the Member State level, involves assessing the benefits and risks associated with the commercial preparations.

The approval of active substances and the placing on the market of plant protection products are associated with a legal period of validity, which must be renewed when it expires. This renewal is subject to a new application that must meet the most recent requirements in force.

Plant protection products are preparations intended to protect plants and crop products. From 1993, the assessment of plant protection products and substances was governed by European Directive 91/414/EEC. This changed in June 2011 with the entry into force of Regulation (EC) No 1107/2009[41]. This regulation is part of a series of legislative texts, called the "Pesticide Package", which was adopted in October 2009.
This regulatory framework defines the data required to assess the hazards and risks of active substances, their degradation products, and plant protection products applied under conditions of use that comply with the principles of good agricultural practices. This assessment includes recommendations for use that seek to keep the identified risks at an acceptable level, the criteria for which are defined in Regulation (EU) No. 546/2011[42]

[41] http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:309:0001:0050:EN:PDF

[42] http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2011:155:0127:0175:EN:PDF

To assess the hazards and risks for bees, the required data are defined in Implementing Regulations (EU) No. 283/2013[43] for active substances and (EU) No. 284/2013[44] for plant protection products, as well as Commission Communications in the framework of the implementation of these regulations[45].

Except when the plant protection products containing the active substance(s) are exclusively intended to be used in situations where bees are not likely to be exposed[46], reports on the following tests must be submitted for active substances[47] and preparations:

- test for acute oral toxicity;
- test for contact toxicity;
- test for chronic toxicity;
- test on the effects on honeybee development and other honeybee life stages.

The last two tests have been required since 1 January 2014 for new active substances and active substances whose approval is being renewed, and will be required from 1 January 2016 for products containing at least one active substance approved under the new requirements.

Tests may be required to analyse the sub-lethal effects, such as effects on behaviour and reproduction, in bees and, where appropriate, in colonies.

When acute or chronic effects on colony survival and development cannot be ruled out, further testing is required (cage or tunnel tests, field tests with honeybees). These tests are conducted by exposing colonies to crops treated with commercial preparations.

The test methods and guidance documents are described in Commission Communications 2013/C 95/01[48] and 2013/C 95/02[49]:

Effects on bees	Guidance Documents
	EU Guidance Document on Terrestrial Ecotoxicology (SANCO/10329/2002 rev 2)
	EPPO Standard PP 3/10 (3) Environmental risk assessment scheme for plant protection products. Chapter 10: honeybees
Sub-lethal effects	OECD Guidance Document 75 on the honeybee (*Apis mellifera* L) brood test under semi-field conditions
	Test methods
Acute oral toxicity	EPPO Standard PP1/170 (4): Test methods for evaluating the side-effects of plant protection products on honeybees. OECD Test Guideline 213: Honeybees, Acute Oral Toxicity Test OECD Test Guideline 237: Honeybee larvae, Acute Oral Toxicity Test
Acute contact toxicity	EPPO Standard PP1/170 (4): Test methods for evaluating the side-effects of plant protection products on honeybees. OECD Test Guideline 214: Honeybees, Acute Contact Toxicity Test
Chronic toxicity to bees	Aupinel *et al.* (2007): A new larval *in vitro* rearing method to test effects of pesticides on honey bee brood. *Redia* **XC**: 87-90 Oomen PA, de Ruijter A and van der Steen J, 1992. Method for honeybee brood feeding tests with insect growth - regulating insecticides. Bulletin OEPP/EPPO Bulletin 22, 613-616.
Effects on honeybee development and other honeybee life stages	Aupinel *et al.* (2007): A new larval *in vitro* rearing method to test effects of pesticides on honey bee brood. *Redia* **XC**: 87-90
Sub-lethal effects	Oomen PA, de Ruijter A and van der Steen J, 1992. Method for honeybee brood feeding tests with insect growth - regulating insecticides. Bulletin

[43] http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2013:093:0001:0084:EN:PDF
[44] http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2013:093:0085:0152:EN:PDF
[45] http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=OJ:C:2013:095:FULL&from=EN
[46] Namely: a) food storage in enclosed spaces; (b) non-systemic preparations for application to soil, except granules; (c) non-systemic dipping treatments for transplanted crops and bulbs; d) wound sealing and healing treatments; e) non-systemic rodenticidal baits; f) use in greenhouses without bees as pollinators.
[47] Tests conducted in the laboratory may be required for the metabolites of the active substance.
[48] http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:52013XC0403(02)&from=EN
[49] http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:C:2013:095:0021:0037:EN:PDF

	OEPP/EPPO Bulletin 22, 613-616.
Cage and tunnel tests	EPPO Standard PP1/170 (4): Test methods for evaluating the side-effects of plant protection products on honeybees.
Field tests with bees	EPPO Standard PP1/170 (4): Test methods for evaluating the side-effects of plant protection products on honeybees.

In 2013, EFSA published a new guidance document on assessing the hazards and risks for honeybees, bumble bees and solitary bees (EFSA 2013d). However, this document was not considered by the SCoFCAH[50] and is therefore not currently applicable (February 2015).

In France, there are two orders in force concerning bees. These orders seek to impose practices with a view to better protecting bees independently of any assessment devoted to hazards, exposure and risks:

- ***The Order of 28 November 2003*** on the conditions of use of insecticides and acaricides for agricultural uses with a view to protecting bees and other pollinating insects[51]. Article 2 of this Order stipulates that "*in order to protect bees and other pollinating insects, treatments carried out using insecticides and acaricides are prohibited during the entire flowering period, and during the period of exudate production, regardless of the products and application device used, on all forest stands and all crops visited by these insects.*"
  Article 3 of the Order of 28 November 2003 stipulates that "*When plants in flower or in a period of exudate production are located under trees or inside a utilised agricultural area intended to be treated by insecticides or acaricides, their aerial parts must be destroyed or rendered unattractive to bees before treatment.*"
  Article 4 stipulates that: "*By derogation from the provisions of Articles 2 and 3, the only insecticides and acaricides that may be used during the period or periods concerned as referred to in Article 2 are those whose marketing authorisation / … / bears one of the following endorsements*:
  - ✓ *'Use authorised during the flowering stage, when bees are not present';*
  - ✓ *'Use authorised during periods of exudate production, when bees are not present';*
  - ✓ *'Use authorised during the flowering stage and during periods of exudate production, when bees are not present'."*

  The derogation is assigned to a product for one or more defined uses and conditions of use provided that the assessed risks to bees and bee colonies are considered acceptable within the meaning of Regulation (EC) No 546/2011.
  The granting of a derogation shall be subject to examination by ANSES of a derogation request dossier submitted by the applicant[52].
  The derogations must be renewed at the time of the ten-year renewal of the marketing authorisation for the product or the re-examination following approval (or re-approval), within the meaning of Regulation (EC) No. 1107/2009, of any active substance that it contains.

  A draft revision of the Order of 28 November 2003 was submitted for public consultation in December 2014[53]. This draft order emanates from the French Plan for Sustainable Development of Beekeeping (PDDA) of February 2013, which proposes to amend the Order of 28 November 2003 (Action 2 of the plan, Point 2.3) in order to clarify the times when treatments using insecticides and acaricides can be administered, to avoid any risk to bees, and to determine, after expert appraisal, the measures that are both relevant to the protection of bees and applicable by farmers.

[50] Standing Committee on the Food Chain and Animal Health
[51] http://www.legifrance.gouv.fr/affichTexte.do;jsessionid=F3CC9A3EF5D13E4268BBD926781D31BA.tpdjo08v_3?cidTexte=JORFTEXT000000799453&dateTexte=20140912
[52] Request indicated by ticking the appropriate box (49A) on the Cerfa administrative document No. 11906*02, which can be done either at the same time as the MA application, or after obtaining MA.
[53] http://agriculture.gouv.fr/Consultation-publique-protection-abeilles

- ***French Order of 7 April 2010*** on the use of tank-mixtures of products mentioned in Article L. 253-1 of the Rural Code[54]. Article 8 of this decree stipulates that *"during the flowering stage or during periods of exudate production, within the meaning of Article 1 of the aforementioned Order of 28 November 2003, a period of twenty-four hours must be respected between application of a product containing an active substance belonging to the chemical class of pyrethroids and application of a product containing an active substance belonging to the chemical classes of triazoles or imidazoles. In this case, the product from the class of pyrethroids must be applied first.*"

5.2 Relevance of taking interactions into account when assessing plant protection products

Scientists have not been able to identify a stress factor that can by itself explain all the bee mortalities occurring in the world, and do not support the assumption that there is a single or universal cause. On the contrary, they believe that a combination of several stressors can explain a certain number of cases, but that in other cases, a single stressor may be responsible for mortalities (*Varroa*, pesticide, etc.). The scientific literature primarily identifies three types of stress factors: parasites (e.g. *Varroa*), infectious agents (e.g. viruses) and pesticides (e.g. insecticides).

There is currently no method that takes interactions into account, with regard to bees, during the assessment of PPPs. Although the regulations issued by the European Union have evolved, interactions are still not taken into account. These regulations aim primarily to improve the assessment of single exposure (e.g. taking into account sub-lethal effects or chronic effects). Product approval is subject to a process of mutual recognition among Member States, with the current trend evolving toward a restriction of the number of PPPs.

However, bees (larvae, nymphs and adults) are exposed continuously to many biological factors whose impact on colony health has been demonstrated. They are also exposed to many PPPs and/or their toxic metabolites found in bee matrices and the colony environment. The concept of mixture effects (action on their health of several stressors to which the bees are exposed simultaneously) is being increasingly mentioned, since it corresponds to the objective reality of the situation in the field. The argument for the impact of multiple exposures to xenobiotics is gaining strength, while the available studies on mixtures have shown the negative effects of combinations of these stress factors on bees. It is therefore appropriate to take the key interactions into account in order to assess PPPs, as described in the ANSES Opinion 2011-SA-0233 (ANSES 2012a). Obviously it is necessary to know which ones to prioritise.

For PPPs, it is impossible to regulate all the possible interactions. But when a field accident associated with co-exposure occurs, the information may be traced back and, where appropriate, can lead to management measures relating to the practice responsible for this accident. It will therefore be necessary to distinguish the product authorisation phase from the post-MA phase, which falls within the scope of phytopharmacovigilance. It may also be relevant to assess PPPs *a priori* in certain well-identified cases (such as known interactions between classes of pesticides) and following their placing on the market, which would highlight the importance of the post-MA monitoring protocol.

Lastly, to test the interactions, it may be relevant to use existing tests and to add one or more stress factors from among the most common (average parasitism, presence of infectious agents). Tests are already available for studying the interactions between chemical factors; tests are less formalised for pathogen-pesticide interactions.

Applied research can provide the methods while basic research can decipher the mechanisms involved.

[54] http://www.legifrance.gouv.fr/affichTexte.do;jsessionid=F3CC9A3EF5D13E4268BBD926781D31BA.tpdjo08v_3?cidTexte=JORFTEXT000022098258&dateTexte=20140912

5.3 Choice of interactions to take into account

The probability of encountering two compounds in a colony is related to their use and their accumulation in the hive. Some mixtures are more frequently encountered. For infectious agents, some of them are found more frequently than others. Pesticide-pesticide interactions are important, but it nevertheless seems relevant to also take infectious agents into account (*Nosema* and DWV for example).

5.3.1 Pesticide-pesticide interactions

The data identified in this report prove firstly, that bees are commonly exposed to several compounds and secondly, that one compound's dangerousness can be increased in the presence of another. They above all raise the question of methods for assessing the potentially toxic compounds that are pesticides. The assessment of the toxicity of a pesticide to bees, conducted prior to its placing on the market, does not currently include an experimental method for testing the impact of co-exposure with another pesticide. However, the working group recommends that the procedure for assessing the toxicity of a pesticide, conducted prior to its placing on the market, should include tests to measure the effect of chemical co-exposure. During laboratory tests, for example, adult worker bees could be exposed simultaneously, by the oral or topical route, to two compounds (one of which is to be tested) on a chronic basis (e.g. 10 days). To detect a possible potentiation, synergistic, or even antagonistic effect, the two compounds should also be tested separately. Three methods of co-exposure should be considered, one using an acaricide compound commonly used by beekeepers to control *Varroa* and another using an approved fungicide compound known to inhibit the pesticide detoxification mechanisms in bees (for example, a fungicide from the class of imidazoles or from the EBIs, *Ergosterol Biosynthesis Inhibitors*). It is recognised that exposure to an acaricide (Ellis *et al.* 1997; Johnson *et al.* 2009) or to a fungicide from the class of the imidazoles or from the EBIs (Colin and Belzunces 1992; Iwasa *et al.* 2004; Pilling *et al.* 1995; Pilling and Jepson 1993; Schmuck *et al.* 2003A; Vandame and M. Belzunces 1998) can aggravate the toxic effect of a pesticide. Lastly, the third method concerns pesticides in interaction with an insecticide, especially if the proposed pesticide is an insecticide from a class already often found in bee matrices. For example, it may be a new neonicotinoid, studied in interaction with a neonicotinoid already regularly detected in bee matrices and found on the national market (imidacloprid, thiacloprid, etc.). It could also be an insecticide from a new class, studied in interaction with one of the most widely used and/or most toxic insecticides (thiamethoxam, lambda-cyhalothrin, deltamethrin, for example). It would be appropriate to conduct testing in semi-natural or natural conditions, combining exposure of bees to the studied compound and to another acaricide (colonies treated or not against *Varroa*), or fungicide (plot treated or not with a fungicide) or even insecticide compound (environment treated or not with a class of insecticide). Standardised guidelines requiring inter-laboratory ring testing will need to be developed for these new methods.

In terms of research, future studies on the ecotoxicological risks associated with multiple exposures to pesticides should contribute to:

- the design of operational tools to assimilate data on exposure, which despite being numerous today, tend to be widely dispersed;
- a better understanding of the role of exposure of bee colonies to several pesticides in phenomena such as excess mortality, weakening and decrease in production;
- an evaluation of the effect of substance mixtures, especially over the long term;
- the development of risk assessment methods considering co-exposure to pesticides, particularly at low doses, and the cascade effects at the population level.

5.3.2 Biological agent-pesticide interactions

There is increasing evidence of co-exposure between infectious agents and pesticides in hives, which now even seems to be the norm. However, there is still little information available on co-

exposure representing a potential hazard for colonies. Some of this co-exposure is now beginning to be analysed in the laboratory and is helping to reveal phenomena ranging from a simple additive effect to synergistic effects. However, confronted with the multitude of co-exposures, coming mainly from the wide diversity of pesticides that can be encountered in the environment, it is important to establish a hierarchy with regard to their prevalence. It then becomes relevant to characterise the effects of the most common co-exposures and accumulate knowledge on the mechanisms of interactions.

Initially, it will be necessary to determine in the laboratory the effects of such co-exposure on bee mortality, if possible according to the caste and the age of individuals. Only co-exposure leading to interactive effects should be selected. The next step will be to describe their mechanisms of interaction and test the effects in the field (at the hive level). This concerns co-exposures inducing synergies, potentiation or antagonisms with regard to bee mortality. Co-exposures resulting in simple additive effects will not be considered as interacting.

As well as interacting with infectious agents, pesticides can increase their prevalence within the colony (e.g. deformed wing virus and *Nosema ceranae*). These amplifications in infectious agent loads can then lead to new pesticide/infectious agent interactions, as described previously. This is therefore a phenomenon that deserves to be studied in more detail, specifically by targeting the different classes of pesticides (e.g. neonicotinoids, Di Prisco *et al.* (2013)) and their impact on the infectious agents. Once again, epidemiological studies will provide clues as to the identity of the pesticides with a tendency to modify the prevalence of certain infectious agents.

Accumulation of laboratory data and field data on co-exposure to infectious agents/pesticides will help fuel the development of mathematical models aiming to predict the development and survival of colonies in the presence of stress factors. In addition, this type of model is of value in determining the outcome of these interactions in different landscape (food resources), population (size of colonies) and climate contexts.

Lastly, in the context of the approval of plant protection products (PPPs), it would be useful to carry out tests in the laboratory by co-exposing bees to the PPP and to infectious agents that have a high prevalence and "relatively low" pathogenicity (e.g. *Nosema*, some viruses) in order to determine the possible occurrence of additive effects, synergistic effects, potentiation or antagonism. Co-exposure with very harmful biological agents such as *Varroa* has little value because even alone it reduces bee longevity very significantly. However, co-exposure with *Varroa* remains relevant in the case of bee colonies with low infestation by this mite, which is the current situation in mainland France. The effects of the PPP on the prevalence or profile of infectious agents in colonies can be directly determined before and after exposure of the colonies.

5.4 Possible methods for taking interactions into account in the methods for assessing plant protection products

5.4.1 Experimental methods in the laboratory, in semi-natural conditions, or in the field

There is no specific method for testing interactions. Therefore all the methods listed in this document could potentially be used, with the first limitation being control of exposure. Maintaining control of exposure is optimal when testing is conducted on individuals in the laboratory, and decreases once the focus turns to effects on colonies and an approach closer to actual field conditions. The nature of the stress factor also modulates this control insofar as it is easier to control the level of exposure to a chemical agent (pesticide) with its relative stability over time compared to a biological agent (infectious and parasitic) for the opposite reasons, even though procedures on exposure of colonies in the field have been described for research purposes for *Varroa*, American foulbrood and chalkbrood (Beebook, 2013[55]). In this regard, depending on the

[55] http://www.coloss.org/beebook

type of infectious or parasitic agent, it is also necessary to take into account the risk of dissemination or contagion when manipulating the agent out of doors.

5.4.2 Use of modelling to study the effects of multiple stressors in bees

A model expresses a more complex and detailed reality in an abbreviated form. Constructed from the essential elements of this reality, it can simulate but never reproduce it exactly. A model's performance is therefore dependent on the right choice of its constituent elements, represented by an algorithm and its data, expressed as variables and constants whose number should be optimised according to the parsimony principle. A model's ability to accept transformations will also depend on the choices made for its constituent elements.
As the functioning of a colony of bees is highly complex, numerous models have been proposed (Devillers *et al.* 2014) to reproduce and study a specific aspect of its biology or behaviour (e.g. swarming, dances, foraging), in order to better understand a process of contamination by a pathogen (e.g. *Varroa*) or a xenobiotic (e.g. *Tau*-fluvalinate), or to simulate the dynamics of the colony in relation to its environment. The process to be modelled is understood in its entirety in the form of a limited number of equations that have to greatly simplify the phenomena, or conversely through sub-models that enable a detailed description of the phenomena under study. The first category includes for instance the models of Martin (2001), Thompson *et al.* (2005), Thompson *et al.* (2007) or Khoury *et al.* (2011), which are represented by a limited number of equations, while the second includes for example the HoPoMo model (Schmickl and Crailsheim 2007), which is made up of 65 equations broken down into interconnected sub-models. While the first category of models can be applied to the study of single chemical or biological stresses (i.e. a manifestation of stress by simulation), the second category is more adapted to simultaneously taking into account different stresses that can act at several levels of colony organisation and/or on different scales. However, the specific structure and functioning of a colony of bees and the need for the models to describe the observed phenomena as precisely as possible have led to the use of individual-centred modelling to simulate the normal or disrupted functioning of bee colonies.
Indeed, in a colony, on a continual basis, through their individual activity, thousands of bees are simultaneously contributing small behavioural elements that interact to constitute the collective behaviour of the colony. The resulting total is greater than the sum of the possibilities of each of the categories of bees taken individually. This definition is conceptually closer to individual-centred modelling in which the model focuses on a specific level of organisation, the individuals, but where the simulation of all or part of these individuals will lead to the emergence of a global, collective action. With this type of stochastic discrete-time modelling, a system is modelled as a collection of autonomous entities, with agents having their own characteristics. They live in an environment with which they interact. They are flexible and can change their behaviour based on experience, facilitating the emergence of new phenomena, which is a characteristic feature of this type of modelling (Devillers *et al.* 2010). We often talk of "bottom-up" modelling in the sense that the models are defined from the bottom and the simulations are observed at the top. They contrast with conventional deterministic models that are referred to as "top-down" (Bonabeau 2002; Topping *et al.* 2009).
Individual-centred modelling is therefore especially well suited to simulating the activities of bees. Because of this, individual-centred models have been proposed to simulate the different tasks of the bees within and/or outside the hive (e.g. de Vries and Biesmeijer (1998), de Vries and Biesmeijer (2002), Thenius *et al.* (2005), Dornhaus *et al.* (2006), Fehler *et al.* (2007), Schmickl and Crailsheim (2008), Johnson (2009), List *et al.* (2009), Johnson and Nieh (2010), Becher *et al.* (2014), Devillers *et al.* (2014)). The categories of bees can each be different types of agents with their own variables and constants. Depending on the phenomenon modelled and the level of organisation considered, this can mean tens of thousands of agents interacting with each other and with their environment, represented by the hive and/or the external environment, as both can be spatialised. The option of coupling the model with a GIS (geographic information system) means that it is possible to work on a real environment with its temporal and spatial components. In these conditions, individual-centred models that simulate the dynamics of a bee colony in its hive and in relation to the external environment are the most realistic from an ecological point of

view and are well suited or easily adaptable to the study of multiple stresses. This is the case with SimBeePop, which has shown its value in the study of the effects of multiple stresses, in larvae and adults, from lethal and sub-lethal concentrations of fenoxycarb (Devillers and Devillers 2013) and pyriproxyfen (Devillers *et al.* 2014). This is also the case with BEEHAVE (Becher *et al.* 2014), which examines the effects of chemical stresses and also those induced by a pathogen (*Varroa destructor*).
Modelling of multiple stresses requires firstly that the studied disruptions can be simulated either directly by the model, or indirectly after it has been modified, and secondly, that the response obtained is as realistic as possible. Even though this second condition is *a priori* difficult to guarantee, it will be far more likely to be met if the model reproduces the population dynamics of bees as faithfully as possible. For this reason, individual-centred models taking the biological and ecological traits of bees into account, because of their flexibility and ability to facilitate the emergence of new phenomena, are more suited to the study of multiple stressors.

5.5 Conclusions and recommendations

The presence of many stress factors both within colonies and bee matrices and outside hives is recognised. The individual impact of many of them on the health of bees has been demonstrated. The impact of multiple exposures of bees to xenobiotics and infectious agents has been argued by studies showing the negative effects of combinations of some of these stress factors. Because of the multiplicity of stress factors and their potential associations, it is unrealistic to take all the possible interactions into account. However, it is appropriate to take some into account for the (re)assessment of plant protection products (PPPs), and strategies can be developed to do this, by distinguishing between the authorisation phase for the product and the post-approval monitoring. The *a priori* assessment of PPPs in interaction with one or more chemical and/or infectious stress factors, among the most common and most important, could be carried out using existing tests. In the context of pharmacovigilance, post-MA monitoring of new compounds should make it possible to detect and assess possible interactions when disorders are observed in the field once these substances have been used.

For taking into account interactions between PPPs, the working group recommends that the procedure to assess the toxicity of a pesticide, prior to its placing on the market, should include tests to measure the effect of chronic chemical co-exposure, by the oral or topical route, to two compounds (one to be tested, the second one likely to interact).
It is also important to identify the mechanism(s) of action of a new compound, in order to be able to consider possible/probable interactions with compounds having similar or antagonistic modes of action.
Co-exposure of the PPP should only be tested by pairing it systematically with a second substance: an anti-*Varroa* acaricide, a fungicide compound known to inhibit the main pesticide detoxification mechanisms in bees, and an insecticide with the same mode of action and known to be present in bee matrices.

Concerning the taking into account of interactions between infectious agents and PPPs, initially, tests should be performed in the laboratory by co-exposing bees to a PPP and to infectious agents that have a high prevalence and a "relatively low" pathogenicity in order to determine the possible occurrence of additive effects, synergistic effects, potentiation or antagonism and to describe the mechanisms of interactions. It will then be necessary to demonstrate the effects in the field at the colony level. The effects of the PPP on the prevalence or profile of infectious agents in colonies should be determined by comparing them before and after exposure of the colonies.
Epidemiological studies should be conducted in order to provide information on the identity of pesticides likely to modify the prevalence of certain infectious agents.
Acquisition of laboratory data and field data on co-exposure to infectious agents/pesticides will also contribute to the development of mathematical models aiming to predict the development and survival of colonies in the presence of stress factors in different landscape, population and climate contexts.

Concerning the possible methods for taking interactions into account when assessing PPPs, there are already methods in the laboratory, in semi-natural conditions and in the field. Other methods should be developed, with special emphasis on maintaining control of exposure and on describing the levels of infectious carriage and contamination of matrices in the tested colonies, at the beginning and the end of the experiment. The development of standardised new methods incorporating these interactions, and requiring inter-laboratory testing, could then contribute to the development of guidelines for assessing PPPs.

Individual-centred mathematical models, taking into account the biological and ecological traits of bees, should be developed to study the effects of multiple stress factors and quantify the additive or even synergistic effects.

In terms of research, it will be necessary to continue:

- in the area of applied research, the development of methods incorporating these interactions;
- in the area of fundamental research, the establishment of studies aimed at better understanding the mechanisms involved in these interactions.

Studies on the ecotoxicological risks associated with multiple exposures to PPPs should in addition contribute to:

- development of operational tools to measure and record data on exposure;
- understanding the role of exposure of bee colonies to several pesticides in phenomena of excess mortality, weakening, reproductive disorders and decrease in production;
- assessment of the effect of pesticide mixtures, especially over the long term;
- development of risk assessment methods considering co-exposure to pesticides, particularly at low doses, and the cascade effects at the population level;
- development of research into the effects of fungicides in combination with other pesticides, specifically insecticides;
- development of mathematical models enabling assessment of additive and synergistic effects, mainly of pesticides.

Because of the multiplicity of possible interactions, a hierarchy should be established of the various chemical and infectious stress factors based on criteria such as their prevalence and effects, by characterising the effects of the most frequent co-exposures.

6 Summary, conclusions and recommendations of the working group

Following several reports and studies highlighting the interactions between stress factors in bees, ANSES issued an internal request in 2012 on the subject of co-exposure of bees to stress factors and interactions between stress factors. In order to understand the phenomena observed by beekeepers of excess mortality and weakening of bee colonies, as well as a decline in production, it was necessary to study these co-exposures and their effects.

The working group tasked with responding to this internal request first studied bee and bee colony health by defining, as far as possible, the "normal" state of health of a bee colony, by describing assessment tools for bee and bee colony health and by proposing health indicators that can be used by beekeepers, veterinarians and researchers.

On the basis of bibliographic data, the working group then presented, in no specific order, the main stress factors to which bees can be exposed and which are likely to induce interactions: biological, chemical and nutritional factors, as well as beekeeping practices, weather conditions and physical factors. Co-exposure and interactions between these stress factors, as reported in the literature, were then studied, after a review of the background to the bees' immunity and detoxification mechanisms, some of which are involved in the observed interactions.

In addition to this bibliographic review, the working group discussed the results of statistical analyses on nine datasets concerning the health status of apiaries in mainland France, obtained by various national bodies.

The experts also examined the relevance of taking into account certain interactions between stress factors when authorising applications for plant protection products.

6.1 Conclusions

6.1.1 On the state of colonies and tools for assessing the health of bee colonies

The available data showed that there are a large number of infectious and parasitic agents that can affect bee colonies and many xenobiotics present in bee matrices. These elements define the current context in which bee colonies live, and their annual biological cycle must also adapt to other environmental factors.

In this context, it appeared necessary to define the state of health of bee colonies and to better determine what constitutes a normal or abnormal situation. Some of the tools currently used to evaluate bee health need to be renewed or adjusted to this new setting. This is already underway for some of these tools. They need to achieve distinct objectives for single time-points and follow-up analysis at various levels, i.e. individual bees, colonies, regions, and so on, and at different levels of study, whether molecular, cellular, or behavioural, etc.

The experts pointed out how difficult it is to compare data on the health and strength of colonies because of the variability of geographic, climate, floristic or agricultural factors, which strongly influence the annual biological cycle of colonies. These data should be compared to reference standards and include the notion of change over time.

6.1.2 On the stress factors

The range of stress factors that bees can be exposed to concomitantly or successively appears to be very wide. For each factor, significant variability may be found from one apiary to another, or even from one colony to another. It is therefore difficult to determine the exact role played by a

specific factor, or their joint effects, when colonies develop disorders, and to make comparisons between apiaries. These various stress factors jointly contribute to weakening of colonies and colony disorders, although a single factor can be found in certain cases.

For many biological agents, more knowledge of their pathogenicity needs to be developed both in the laboratory and within bee colonies. Asymptomatic carriage of infectious and parasitic agents is very widespread in bee colonies and this should be distinguished from clinical disease. Maintaining the balance of microbial populations is related to factors that are intrinsic to the beehive and to the environment, and changes in these factors can lead to colony disorders. It is important to look into the predictive nature of carrier states for the development of subsequent disorders, specifically using an approach based on colony demographic data as well as geographic and temporal data during beekeeping seasons.

There are a very large number of diverse chemical factors. A wide range of substances are found in beehive matrices to which bees are exposed outside and inside the colony. As part of this study, the substances of interest retained were insecticides, fungicides and varroacide acaricides. A certain number of substances involved in bee disorders, occasionally at sub-lethal doses, have already been identified, for example neonicotinoids and fipronil. Studies have described disorders and identified the underlying mechanisms. Laboratory studies are more common than tunnel studies or field studies because of the difficulties involved in carrying out and interpreting non-laboratory studies. Exposure of bees in the field is not comparable to controlled exposure in the laboratory and the results for the same substance can differ, mainly depending on the method and monitoring of exposure (type, number and quantity of substances).

Abundance and diversity of food sources and environmental resources play an important role in the reproduction, development and maintenance of bee colonies. These factors influence the health and tolerance of bees to other stress factors whether chemical or biological. Studies mainly carried out in the laboratory have demonstrated the adverse effects of nutritional deficiencies on metabolism and immunity. It is important to determine whether the observed effects can be transposed to natural conditions.

Certain beekeeping practices may generate stress likely to be infections/infestations

added to other factors and can lead to the development of disorders. The possible negative impact may be inherent to the practice itself, or be related to unsuitable practices or others that are not implemented.

The working group highlighted the importance of compliance with good beekeeping practices based on in-depth training in beekeeping and regular monitoring of colonies to maintain the health of apiaries.

The intensity and duration of weather phenomena can change the physiological balance and dynamics of bee populations in a colony and cause natural weakening.

In this context, the working group highlighted the benefit of using and maintaining bee populations suited to local conditions.

6.1.3 On co-exposure and interactions between stress factors

Apiaries are co-exposed to multiple combinations of stress factors, including the *Varroa* parasite, bacteria, viruses, microsporidia, and xenobiotics such as insecticides, fungicides and acaricides have all been identified as stressors.

The overview of the suspected/confirmed role of interactions between stress factors showed that several infectious and/or chemical agents may interact on the same functional targets in the larva and the adult bee, and lead to additive or synergistic effects. Chemical substances may also disrupt detoxification mechanisms and thus alter the sensitivity of bees to other substances. Moreover, certain biological agents, such as *Varroa*, and certain substances have immunodepressant effects and contribute to amplification of infections/infestations in general. *Varroa* also acts as an amplifier of infection by certain viruses it transmits. Lastly, some substances like neonicotinoids and acaricides may have an effect on the cohesion of the colony and the

hygiene behaviour of worker bees and thus on the infectious and parasitic risks. Thus, certain specific interactions, such as those between *Varroa* and viruses (DWV, AKI complex virus), neonicotinoids and *Nosema*, fipronil and *Nosema*, neonicotinoids and viruses (DWV and BQCV), or fungicides and insecticides, show synergistic effects that threaten the health of colonies.

These different mechanisms may act simultaneously. Their effects depend on the season. The level of infection of the colony at the start of winter depends on the interaction between these factors during the foraging period. They may only become visible after a latency period. Beekeeping practices may compensate for or amplify them.

This analysis is shared by other specialists, as shown in the very recent review by Goulson *et al.* (2015). The authors point out that the three main factors (listed without order of priority) are: parasites and infectious agents, in particular non-native/exotic, cocktails of pesticides (mainly insecticides and fungicides) and the lack of floral diversity. These factors interact with each other and the authors therefore suggest several measures, including increasing the available floral richness, reducing the use of pesticides and better managing the trade in non-native bees between countries/continents (Goulson *et al.* 2015).

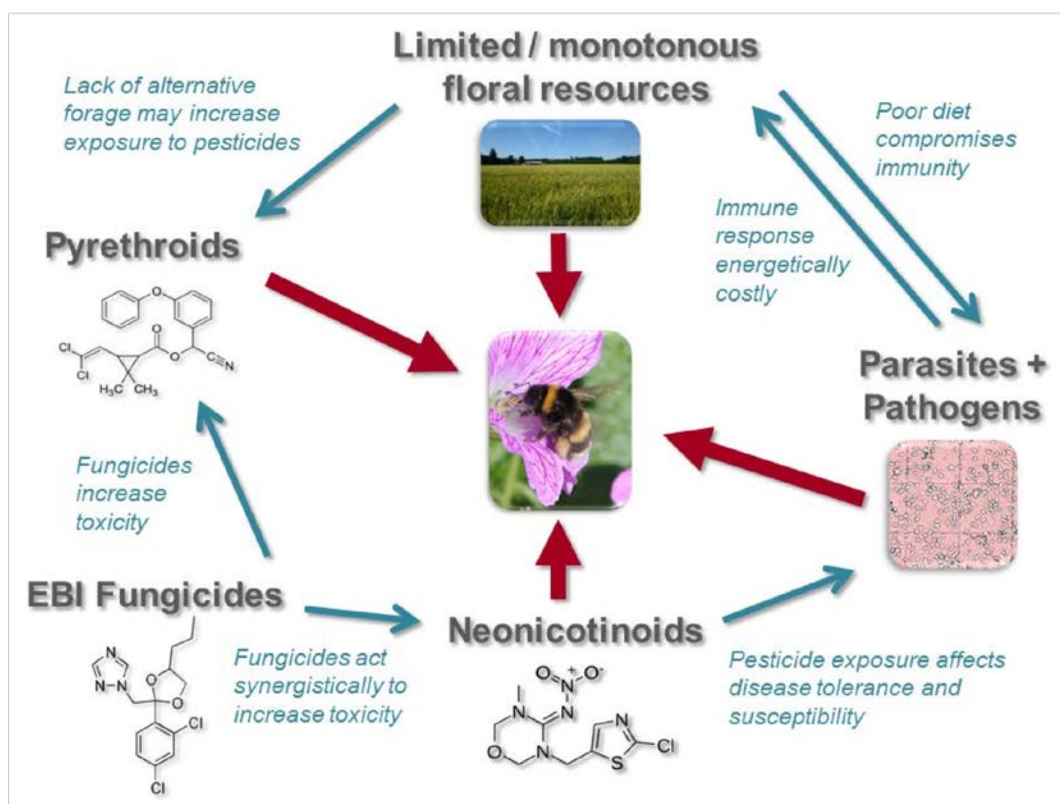


Figure 13: From Goulson *et al.*, Science, February 2015: "Both wild and managed bees are subject to a number of significant and interacting stressors. For example, exposure to some fungicides can greatly increase toxicity of insecticides (references 110 to 112) whereas exposure to insecticides reduces resistance to diseases (references 115-123 and 115-126). Dietary stresses are likely to reduce the ability of bees to cope with both toxins and pathogens (references 127-129). Photo credit: Beth Nicholls; Flickr Commons, AJC1 (https://creativecommons.org/licenses/by-nc-sa/2.0/legalcode_)"

6.1.4 On the results of data analysis (single-factor aspects and interactions)

Results of analysis of datasets confirm the high number and diversity of biological and chemical hazards detected in bee colonies in France. These results have not enabled conclusions to be drawn on the prevalence of biological or chemical hazards in apiaries in the country since the

conditions for representativeness of samples were not met and only certain studies were designed for systematic and calibrated assessment of biological and chemical hazards.
These observations indicate certain hazards that should be detected, provide indications, and stress the needs concerning matrices to sample and the methods to use.

Given the background of co-exposure of bees to many stress factors, associated with high qualitative and quantitative variability in exposure and the possible resulting interactions, the working group emphasised the difficulty in determining the "normal" state of health of a bee colony as well as the role to be allocated to each co-factor identified in a bee colony that has become diseased. The observed disorders can result from concomitant co-exposure but also successive exposure to stress factors. One factor may induce effects, for instance on immunity, which will only have visible consequences later on, even though the factor may no longer be present in the hive.

6.1.5 On the issue of taking interactions into account when assessing the risks associated with plant protection products

Although it is not realistic to take into account all the possible interactions, the working group deemed it useful to consider some of them when assessing plant protection products (PPPs), while distinguishing between the authorisation phase for the product and the post-MA phase. Evaluation of PPPs *a priori* in interaction with one or more stress factors among the most common and most important, should be carried out using the existing laboratory tests. Post-MA monitoring of new compounds would make it possible to detect and assess possible interactions when disorders are observed in the field once these substances have been used.

The various conclusions from analysis of the literature and from results of analysis of datasets led the working group to make several recommendations.

6.2 Recommendations

This section summarises the recommendations made in the previous chapters. The WG, which was made up of experts from multiple fields, wanted to highlight priority recommendations in bold type, without overlooking the importance of the other recommendations.

6.2.1 On the tools for assessing the health of bee colonies

As a preamble, it is important to note the need to define characterisation tools, in terms of physical, chemical and biological parameters, for the average "normal" health status of a bee colony in its environment.

The working group recommends:

- distinguishing between tools for beekeepers and those intended for research and/or diagnosis;
- developing innovative and validated methods and tools to better understand the health and strength of bee colonies. In the clinical and pathology areas, the development of an illustrated guide to bee disorders would be a useful diagnostic support tool;
- **developing validated and harmonised schemes to assess colony disorders (loss of forager bees, queen egg-laying, etc.).**

The experts also recommend the creation of reference apiaries, organised in networks, to achieve coverage of the French territory that is as extensive as possible. These apiaries would help to define regional reference standards for the various players on the basis of standardised collection of data on populations and production. An identified national stakeholder should collate and compile the data and make them easily available to all interested parties in the sector.

6.2.2 On the stress factors

For infectious and parasitic agents, the working group recommends further studies:

- **aimed at defining the prevalence and regional differences of infectious agents in colonies with and without symptoms;**
- **aimed at identifying virulence factors for infectious and parasitic agents (specifically *Nosema ceranae* and certain viruses), in the laboratory and within colonies;**
- **to determine the pathophysiological mechanisms involved in host individual sensitivity, at the colony and individual level;**
- on the predictive nature of quantities of infectious agents present in the development of subsequent disorders, in association or not with the presence of chemical stress factors.

For chemical agents, further studies should be conducted:

- **aimed at developing suitable analytical tools to measure actual (co-)exposure during field studies;**
- aimed at better describing and clarifying exposure and the toxic effects of chemical substances to which colonies are exposed;
- **on the direct effects or interactions of fungicides and insecticides, given the frequency and plurality of exposure to these substances;**
- **to determine the toxicity mechanisms involved, at the individual bee level, at the various stages of development (larva, nymph and adult), and at the colony level;**
- **on the multiple and repeated nature of such exposure over time and its effects in co-exposure with other factors. It is important to carry out studies on changes to the chemical substances in the various bee matrices, including bees and wax.**

In addition, the working group:

- recommends implementing studies to assess the effects of nutritional deficiencies in natural conditions;
- highlights the benefits of compliance with good beekeeping practices to maintain apiary health, specifically biosafety measures and control of infectious agents and use and maintenance of bee populations adapted to local conditions;
- emphasises the importance of training veterinarians and bee health technicians concerning the complexity of the disorders occurring in bees;
- takes note of the benefits of studies on the physiological response processes of colonies to climate change.

6.2.3 On epidemiological studies and data collection aimed at elucidating the issue of *in situ* interactions

The difficulties in determining the state of health of colonies and identifying the cause(s) of disorders led the experts to recommend continued and reinforced surveillance of apiaries, especially concerning infectious, parasitic and chemical agents. The working group stresses that infectious, parasitic and chemical agents, including acaricides in wax, should be screened for concomitantly during active surveillance, and on the appearance of disorders in the colonies (i.e. outbreak or passive surveillance).

For epidemiological observational studies in the beekeeping sector aimed at identifying specific infection risk factors, it is essential to use methods enabling comparisons of exposure profiles to risk factors (in terms of diversity and quantity), between case and control epidemiological units and over time. The measured variables of colony status must include the availability of reserves, the demographic structure within the colony, the size of the population and the foraging activity.

Sampling must take into account the structure of apiaries. It is very important to keep information on the relationship between the scale of the colony and that of the apiary, and to carry out statistical analyses taking this structure into account. It is also important to factor in seasonal and geographic parameters which strongly affect colony biology.

Epidemiological surveillance requires standardisation of data collection. This standardisation in particular requires centralised coordination ensuring compliance with protocols, training of surveyors, information reporting, information feedback and relevant statistical analysis based on sufficient sample sizes. There are sampling rules that make it possible to achieve the required accuracy based on the question asked. With these criteria in mind, most current surveillance tools are insufficient; the debate underway for the mortality and alerts observatory should support these recommendations. Regional observatories should be developed with the aim of having beehives that can serve as references, both for normal production and for regular exposure to the risk factors specific to the region.

Carrying out epidemiological studies seeking to explain the phenomena described through surveillance requires a protocol enabling cases to be compared with a reference population. Given the complexity of the phenomena involved in bee disorders, an extremely strict methodology is essential when developing and implementing protocols for epidemiological surveys.

The working group highlights the importance of a reinforced cross-disciplinary approach before implementation of surveys in order to ensure the adjustment of analytical tools, sampling tools, data collected by questionnaire, and statistical processing with the questions posed, while keeping feasibility in mind.

Active programmed surveillance of infectious and parasitic agents should be done using specific methods that are sensitive and quantitative, as well as validated and standardised. The main potential pathogens in France should be screened for concomitantly, whether there are symptoms or not. This research should be carried out in conjunction with the quantification of the degree of infestation with *Varroa*, which greatly influences the dynamics of infections transmitted by this mite and the immune state of bees and the main toxic factors (at least those whose sub-lethal effects can influence individual or social immunity). This surveillance should help to provide qualitative and quantitative data on asymptomatic carriage phenomena in colonies, data that are currently insufficient. It will also make it possible to compare the levels of infectious agents present in asymptomatic hives with those observed in the context of outbreak surveillance, and thus help determine the role of a specific infectious agent in the development of disorders.

Strategies for detection of pesticides should have the following characteristics:

- target a range of substances known to be used in the region;
- depending on the question asked, take account of multiple treatments applied to the foraging zone over time and target the matrix/matrices to analyse;
- use validated quantitative methods (existing or to be developed) with detection/quantification thresholds that are compatible with studies on the potentiation of substances and their adverse effects on bee colonies. Multiple-residue methods should be given preference provided they have satisfactory sensitivity for the specific objective. For highly toxic pesticides, single-residue analyses on the active substance and its toxic metabolites are essential on the matrices of interest, i.e. pollen, nectar, wax, bees, bee bread. For surveillance of emerging issues and for toxicovigilance of veterinary and plant protection products (post-MA assessment), it is necessary to standardise and centralise data collection when disorders occur and to standardise the multiple-residue methods used.

In addition, the evolution of chemical substances (degradation kinetics, accumulation, etc.) in the various bee matrices, including bees, should be studied, as this will help decide on the matrices to be sampled during disorders, in order to clarify any possible co-exposures and interactions, concomitant and successive, to chemical agents.

It is very important to have validated and harmonised quantification methods for infectious, parasitic and chemical agents. Validation of diagnostic methods will enable surveillance using suitable tools whose sensitivity, specificity, reproducibility, repeatability, and detection and quantification limits have been determined, and that are used in a

harmonised manner by all the reference laboratories in order to carry out studies with comparable results.

6.2.4 On taking into account interactions in the assessment of the risks associated with plant protection products

Concerning pesticide-pesticide interactions, the working group recommends that the procedure to assess the toxicity of a PPP should include tests to measure the effect of chemical co-exposure, by the oral or topical route, to another substance (chosen for its potential to interact) on a chronic basis. Co-exposure of the PPP under investigation should specifically be tested with:

- **an anti-*Varroa* acaricide;**
- **a fungicide also known to inhibit detoxification mechanisms in bees;**
- **an insecticide with the same mode of action and known to be present in bee matrices.**

Standardised guidelines requiring inter-laboratory ring testing will need to be developed for these new methods.

In terms of research, studies on the ecotoxicological risks related to multiple exposures to pesticides should contribute to:

- development of operational tools to assimilate data on exposure;
- **understanding the role of exposure of bee colonies to several pesticides in phenomena such as excess mortality, weakening and decrease in production;**
- assessment of the effect of pesticide mixtures, especially over the long term;
- development of risk assessment methods considering co-exposure to pesticides, particularly at low doses, and the cascade effects at the population level;
- development of research into the effects of fungicides in combination with other pesticides, specifically insecticides;
- **development of mathematical models enabling assessment of additive and synergistic effects, mainly of pesticides.**

Given the plurality of potential stress factors, although difficult, it would be beneficial to **establish a hierarchy of substances to test in interaction**, on the basis of criteria such as their prevalence and effects, including mode of action, by characterising the effects of the most common co-exposures.

Concerning infectious and parasitic agent-pesticide interactions, it will be necessary to determine in the laboratory the effects of these co-exposures that induce synergies, potentiation or antagonisms on bee mortality, colony weakening or disruption of reproduction processes, describe the interaction mechanisms, and then demonstrate the effects in the field at the colony level.

Epidemiological studies will provide evidence on the specific pesticides that tend to change the prevalence of certain infectious and parasitic agents or the response of host individuals, which could lead to the emergence of more virulent strains.

Accumulation of laboratory data and field data on co-exposure to infectious agents/pesticides will help to fuel the development of mathematical models that are centred on individuals, taking into account the biological and ecological traits of bees and able to predict the development and survival of colonies in the presence of stress factors in different contexts (landscapes, populations and climate).

In the context of PPP approvals, it would be useful to carry out tests in the laboratory by co-exposing the PPP with infectious or parasitic agents that have a high prevalence and "relatively low" pathogenicity to determine the possible occurrence of additive effects, synergistic effects, potentiation, or antagonism.

For the study of these interactions, certain existing methods can already be used in the laboratory for this purpose, in semi-natural conditions or in the field, to take into account interactions in the methods for assessing PPPs. Other methods would need to be developed to better test exposure and the state of infection of experimental colonies, at the start and end of testing.

6.3 Outlook

Co-exposure of honeybees to multiple stress factors is now a proven reality. Management of health risks, whether chemical and/or biological, must now be adapted to this reality and this report demonstrates just how complex and interdependent disease development mechanisms can be.

In view of the plurality and the extent of exposure to chemical substances used in plant and livestock health, it is essential to work towards an overall reduction in these inputs by all means possible.

The aim is to minimise treatments, or at least their adverse effects, specifically the development of resistance and the presence of residues. This requires an integrated approach using, as a priority, available agro-ecological and zootechnical levers and if necessary rational use of chemical treatments. Concerning bee health more specifically, the experts wish to encourage dialogue between research in other animal sectors and that in the beekeeping sector, taking into account its specific characteristics, particularly its very strong link to the land.

Dates of validation of the collective expert appraisal report by the working group and the Expert Committee on Animal Health: 20 March 2015 and 7 April 2015

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7.2 Standards

NF X 50-110 (May 2003) Quality in expertise activities – General requirements of competence for an expertise activity. AFNOR (classification index X 50-110)..

7.3 Legislation and Regulations

Order of 7 April 2010 on the use of tank-mixtures of products mentioned in Article L. 253-1 of the French Rural Code

Order of 28 November 2003 on the conditions of use of insecticides and acaricides for agricultural uses with a view to protecting bees and other pollinating insects

Order of 22 January 2013 prohibiting the introduction on the national territory of specimens of the Asian hornet, *Vespa velutina*

Ministerial Order of 29 July 2013 on the definition of Category One and Two health hazards for animal species

Commission Communication in the framework of the implementation of Commission Regulation (EU) No. 283/2013 of 1 March 2013 setting out the data requirements for active substances, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market

Commission Communication in the framework of the implementation of Commission Regulation (EU) No. 284/2013 of 1 March 2013 setting out the data requirements for plant protection products, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market

Decree No. 2003-587 of 30 June 2003 implementing Article L. 214-1 of the French Consumer Code as regards honey

Directive 91/414/EC of the European Parliament and of the Council of 15 July 1991 concerning the placing of biocidal products on the market

Service note DGAL/SDSPA/N2002-8045 of 18 March 2002 relating to veterinary medicinal products intended for the treatment of varroasis in bees

Service note DGAL/SDSPA/N2002-8136 of 12 May 2004 relating to veterinary medicinal products intended for the treatment of varroasis in bees

Service note DGAL/SDSPA/SDQPV/N2012-8113 of 6 June 2012 relating to the annual monitoring of bee disorders

Service note DGAL/SDSPA/2015-134 of 13 February 2015 relating to the conditions for the exercise of certain veterinary medicine acts by bee health technicians

Council Regulation (EEC) 2377/90 of 26 June 1990 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. OJ, L224, 1990, 1-8

Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC

Commission Regulation (EU) No. 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. OJ, 2010, 1-72

Commission Regulation (EU) No. 206/2010 of 12 March 2010 laying down lists of third countries, territories, or parts thereof authorised for the introduction into the European Union of certain animals and fresh meat and the veterinary certification requirements

Commission Regulation (EU) No 546/2011 of 10 June 2011 implementing Regulation (EC) No 1107/2009 of the European Parliament and of the Council as regards uniform principles for evaluation and authorisation of plant protection products.

Commission Regulation (EU) No. 283/2013 of 1 March 2013 setting out the data requirements for active substances, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market

Commission Regulation (EU) No. 284/2013 of 1 March 2013 setting out the data requirements for plant protection products, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market

Commission Implementing Regulation (EU) No. 485/2013 of 24 May 2013 amending Implementing Regulation (EU) no 540/2011 as regards the conditions of approval of the active substances clothianidin, thiamethoxam and imidacloprid, and prohibiting the use and sale of seeds treated with plant protection products containing those active substances

ANNEXES

Annex 1: Internal Request

DECISION N° ANSES-2015-03-097
MODIFIANT LA DECISION N° ANSES-2013-10-275

AUTOSAISINE

Le directeur général de l'Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (Anses),

Vu le code de la santé publique, et notamment son article L. 1313-3 conférant à l'Anses la prérogative de se saisir de toute question en vue de l'accomplissement de ses missions,

Décide :

Article 1er.- L'Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail se saisit afin de réaliser une expertise dont les caractéristiques sont listées ci-dessous.

1.1 Thématiques de l'expertise

- Co-expositions (expositions concomitantes ou successives) des abeilles et des colonies d'abeilles à différents facteurs de stress. L'accent sera mis sur les co-expositions aux agents infectieux et aux facteurs toxiques à des doses sublétales ;
- Mécanismes d'action et rôles (respectifs et conjoints) de ces facteurs dans les phénomènes d'affaiblissement, d'effondrement et de mortalité des colonies d'abeilles.

1.2 Contexte de l'autosaisine

Les phénomènes d'affaiblissement, d'effondrement et de mortalité des colonies d'abeilles ont fait l'objet, au cours des trois dernières années, de plusieurs études visant à comprendre le ou les mécanismes impliqués dans ces troubles. L'Afssa a produit en 2009 un rapport qui en soulignait l'étiologie multifactorielle (facteurs infectieux, chimiques, physiques, climatiques, alimentaires, *etc.*). Ce rapport concluait notamment sur la nécessité d'évaluer les effets, individuels et conjoints, de l'exposition des abeilles et des colonies d'abeilles aux agents infectieux et aux produits phytopharmaceutiques, et de réaliser des recherches sur les expositions chroniques à des pesticides en présence d'infections latentes, récurrentes, par différents agents pathogènes susceptibles de se potentialiser entre eux. L'Anses a émis en 2012, trois avis portant sur des publications scientifiques (Vidau *et al.*, 2011[1] ; Henry *et al.*, 2012[2]) qui ont rapporté les effets sur les abeilles et/ou les colonies d'abeilles, de doses sublétales de pesticides. L'EFSA a publié, en mai 2012, une « déclaration sur les résultats d'études récentes examinant les effets sublétaux de certains néonicotinoïdes sur les abeilles[3] ». Elle poursuit ce travail par une étude des effets aigus et chroniques de cinq néonicotinoïdes sur la survie et le développement des colonies d'abeilles, étude qui prendra en compte les effets sur les larves et sur le comportement des abeilles.

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[1] Vidau C, Diogon M, Aufauvre J, Fontbonne R, Viguès B, Brunet JL, Texier C, Giron DG, Blot N, El Alaoui H, Belzunces LP, Delbac F (2011) Exposure to sublethal doses of fipronil and thiacloprid highly increases mortality of honeybees previously infected by *Nosema cerenae*. Plos One, 6(6), e21550

[2] Henry M, Beguin M, Requier F, Rollin O, Odoux JF, Aupinel P, Aptel J, Tchamitchian S, Decourtye A (2012) A common pesticide decreases foraging success an survival in honeybees. Science 336, 348-350

[3] EFSA (2012) Statement on the findings in recent studies investigating sub-lethal effects in bees of some neonicotinoids in consideration of the uses currently authorized in Europe. EFSA Journal 2012, 10(6):2752.

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Dans ce contexte, et compte tenu du travail en cours de l'EFSA sur les effets chroniques de néonicotinoïdes, l'Anses va constituer un groupe de travail chargé d'étudier les co-expositions (expositions concomitantes ou successives) des abeilles et des colonies d'abeilles à différents facteurs de stress, les mécanismes d'action de ces facteurs et leurs rôles respectifs dans les phénomènes de mortalité des colonies d'abeilles. L'accent sera mis sur les interactions entre agents infectieux et parasitaires, d'une part, et facteurs toxiques à des doses sublétales, d'autre part. Les autres facteurs, intrinsèques (génétiques) ou extrinsèques (pratiques apicoles, facteurs environnementaux) seront pris en considération dans leur capacité à moduler ces interactions et leurs effets. En raison du nombre d'individus constituant les colonies, du caractère très structuré de ces dernières, impliquant une spécialisation des individus par fonction, il conviendra de distinguer les effets sur l'individu abeille (à l'échelle moléculaire, cellulaire, tissulaire, de l'organisme entier) des conséquences à l'échelle des colonies.

1.3 Questions sur lesquelles portent les travaux d'expertise à mener

Les objectifs du Groupe de travail (GT) sont les suivants :

(1) mieux comprendre le rôle des agents pathogènes et autres facteurs de stress dans les phénomènes de mortalité et d'effondrement des colonies, en particulier :

- les co-expositions des abeilles à des agents pathogènes et à des substances chimiques à des doses sublétales,
- la compréhension des mécanismes d'action (effets additifs, synergiques, potentialisation),
- le rôle modulateur d'autres facteurs de stress (facteurs génétiques ; facteurs nutritionnels, climatiques, champs électromagnétiques, *etc.*) sur ces effets individuels ou conjoints,

et déterminer, dans la mesure du possible, la part respective de ces facteurs et de leurs interactions en tenant compte également de l'influence des pratiques apicoles ;

(2) déterminer si l'élaboration de méthodes prenant en compte, dans l'évaluation des produits phytopharmaceutiques, les interactions éventuelles entre agents infectieux et facteurs toxiques serait pertinente et réalisable, notamment de manière standardisée. Le cas échéant, de telles méthodes pourraient être proposées par le GT.

(3) émettre des recommandations en termes de pratiques apicoles et agricoles, ainsi que des recommandations de recherche.

Les travaux d'expertise comprendront :

(1) une mise à jour de l'état des lieux sanitaire en filière apicole en France

(2) une revue des études portant sur les interactions entre différents facteurs de stress :

(a) recensement, objectivation des facteurs de stress ;

(b) méthodes de mise en évidence des interactions :

- au niveau individuel et à l'échelle des colonies ;
- en situation expérimentale, semi-naturelle, ou naturelle ;

(c) interactions entre différents agents pathogènes (infectieux, parasitaires) ;

(d) interactions entre agents pathogènes et pesticides utilisés en pratiques apicole et agricole ;

(e) autres facteurs susceptibles de moduler soit l'exposition à des agents infectieux ou toxiques, soit leurs effets.

Cette revue devra tenir compte de la diversité des conditions d'exposition des abeilles aux facteurs de stress dans les différentes études réalisées (ex : cages de vol, sous tunnel, plein champ), plus ou moins comparables entre elles et plus ou moins proches des conditions naturelles ;

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(3) une revue des mécanismes possiblement mis en jeu ;

(4) des propositions et recommandations :

- sur des mesures visant à adapter les pratiques agricoles et apicoles pour réduire l'occurrence et les effets de co-expositions préjudiciables aux abeilles et aux colonies d'abeilles ;
- de recherche pour améliorer la compréhension des interactions entre facteurs impliqués dans les phénomènes de mortalité des colonies d'abeilles.

1.4 Durée prévisionnelle de l'expertise

Le délai pour la production du rapport est reporté à mai 2015, compte tenu du temps nécessaire pour la finalisation après le traitement des données relatives à l'état des lieux du cheptel apicole français et aux co-expositions des abeilles aux facteurs de stress, achevé en décembre 2014.

Article 2- Un avis sera émis et publié par l'Agence à l'issue des travaux.

Fait à Maisons-Alfort, le 15 AVR. 2015

Marc MORTUREUX
Directeur général

Annex 2: Substances detected in the CETIOM/ITSAP study

Table 16: Detail of 30 substances detected in pollen in four apiaries on different dates (CETIOM/ITSAP study)

Site	A									B				C			D								
Year	2012				2013					2012	2013			2013			2012					2013			
Date	13 Apr	19 Apr	24 Apr	05 May	03 May	11 May	14 May	18 May	27 May	16 May	03 May	16 May	01 June	03 May	14 May	23 May	30 Apr	07 May	12 May	24 May	29 May	27 May	03 June	04 June	10
zaconazole	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	
zoxystrobin	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1	0	0	0	0	0	
boscalid	1	1	1	1	1	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
hlorothalonil	0	0	0	0	0	0	0	0	0	1	0	1	0	1	1	0	0	0	1	1	0	1	1	1	
orpyrifos ethyl	1	1	1	0	1	0	0	0	0	0	1	0	0	1	1	1	1	1	0	1	0	0	0	0	
alothrin-lambda	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	
cymoxanil	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
yproconazole	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	1	1	1	1	1	
cyprodinil	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	
eltamethrin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	
methenamid	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	
phenylamine	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
fenpropidin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	
fluazifop.P	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	
urochloridone	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	
flutolanil	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	
etconazole	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	0	0	1	1	0	
etolachlor- etolachlor.S	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	
icosulfuron	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	

encycuron	0	0	0	0	0	0	0	0	0		0	1	0	0		1	0	0		0	0	0	0	0	0	0	0	
endimethalin	0	0	0	0	1	0	0	0	0		0	1	0	0		1	1	1		0	0	0	0	0	0	0	0	
enmedipham	0	0	0	0	0	0	0	0	0		1	0	0	0		1	0	0		0	0	0	0	0	0	0	0	
rosulfocarb	0	0	0	0	0	0	0	0	0		1	1	1	1		1	1	1		0	0	0	0	0	0	0	0	
thioconazole- thio metabolite	0	0	0	0	0	0	0	0	0		0	1	1	0		1	1	1		0	0	0	0	0	0	0	0	
yrazophos	0	0	0	0	0	0	0	0	0		0	0	0	0		0	0	0		0	0	0	1	0	0	0	0	
u-fluvalinate	0	0	0	0	0	0	0	0	0		0	1	0	0		0	0	0		0	0	0	0	0	0	0	0	
ebuconazole	0	0	0	0	0	0	0	0	0		0	0	0	0		0	1	0		1	0	0	0	0	0	0	0	
midacloprid	1	1	1	1	0	0	0	0	0		NA	1	1	0		1	0	1		0	0	0	0	0	0	1	NA	NA
thiacloprid	1	1	1	1	0	0	0	0	0		NA	1	1	0		1	1	1		1	1	0	0	0	0	0	NA	NA
iamethoxam	0	0	0	0	0	0	0	0	0		NA	1	1	0		1	1	0		0	0	0	0	0	0	0	NA	NA
er of substances	5	4	4	3	3	1	0	1	1		5	14	7	4		15	13	8		6	5	8	5	2	5	5	3	

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