UNIVERSITÀ DEGLI STUDI DI NAPOLI "FEDERICO II"

PHD IN VETERINARY SCIENCES
XXXII CYCLE

Thesis

"New advances in honeybee pathology: laboratory techniques and pathological findings"

PhD Coordinator
Prof Giuseppe Cringoli

Candidate
Dott.ssa Karen Power

Tutor
Prof.ssa Paola Maiolino

Sommario

LIST OF ABBREVIATIONS 5

LIST OF FIGURES 7

LIST OF TABLES 9

Abstract 11

Introduction 15

I. The important role of honeybees 15

II. Honeybee loss 20

Chapter 1 33

Abstract 34

1.1 Honeybee viruses 35

1.1.1 Viral structure 35

1.1.2 Transmission routes 37

1.1.3 Pathology 40

1.1.4 The role of Varroa destructor 41

1.2 Deformed Wing Virus (Dwv) 44

1.2.1 Genetics 45

1.2.2 Epidemiology 46

1.2.3 Transmission pathways and clinical signs 48

1.2.4 Treatment 51

1.3 Honeybee immune response 53

1.3.1 Impairment of honeybee viral immune defence 56

1.4 Aim of the study 58

1.5 Materials and methods 58

1.5.1 Samples 58

1.5.2 RNA extraction, reverse transcription (RT) and PCR 59

1.5.3 REAL-TIME PCR (qPCR) for detection of relative viral load 60

1.5.4 Anatomopathological and histopathological analysis 61

1.6 Results 62

1.6.1 Biomolecular results 62

1.6.2 Macroscopic and microscopic results 63

1.7 Discussion and conclusions 67

Bibliography 72

Chapter 2 85

Abstract 86

2.1 Drones **87**
2.2 The reproductive system **88**
2.2.1 Sexual maturation of drones 90
2.2.2 Drone semen 90
2.3 Mating **91**
2.4 Sperm competition and cryptic female choice **94**
2.5 Instrumental insemination **95**
2.6 Aim of the study **96**
2.7 Materials and methods **97**
2.7.1 Sampling 97
2.7.2 Anatomopathological analysis 98
2.7.3 Semen analysis 99
2.7.4 Histopathological analysis 100
2.8 Results **100**
2.9 Discussion and conclusions **104**
Bibliography **110**

Chapter 3: **119**
Abstract **120**
3.1 Nosemosis **121**
3.1.1 Life cycle 122
3.1.2 Transmission routes 123
3.1.3 Tissue tropism and pathogenicity 125
3.1.4 Clinical signs 128
3.1.5 Treatment 130
3.2 Diagnostic techniques **132**
3.2.1 Sampling 134
3.2.2 Microscopic examination for spore identification 135
3.2.3 Biomolecular examination 137
3.3 The Mini-FLOTAC technique **138**
3.4 Aim of the study **140**
3.5 Materials and methods **141**
3.5.1 Sampling 141
3.5.2 Anatomo-histopathological analysis 142
3.5.3 Direct smear and hemocytometer examination 142
3.5.4 Examination with the Mini-FLOTAC technique 143
3.6 Results **144**
3.6.1 Macroscopic and microscopic results 144
3.6.2 Parassitological results 149
3.7 Discussion and conclusions **151**
Bibliography **156**

Conclusions **167**

LIST OF ABBREVIATIONS

ABPV: Acute Bee Paralysis Virus
ABC: ATP-binding cassette
Act β: β Actina of *Apis melifera* L
AIV: Apis Iridescent Virus
AmFV: Apis mellifera Filamentous Virus
AMP: Antimicrobial peptides
BQCV: Black Queen Cell Virus
CBPV: Chronic Bee Paralysis Virus
CCD: Colony collapse disorder
cDNA: complementary DNA
DCA: Drone Congregate Areas
DNA: Deoxyribonucleic acid
dsRNA: double strand RNA
DW: honeybees showing clinical signs of DWV
DWV: Deformed Wings Virus
FS: flotation solution
GLD: Glucose Dehydrogenase
H: apparently healthy honeybees
H-E: haematoxylin and eosin
Hsp: Heat shock response
IGR: Intergenic region
II: Instrumental Insemination
Imd: Immune Deficiency
Jak/STAT: Janus kinase/Signal Transducer and Activator of Transcription
JNK: c-Jun N-terminal kinase
KBV: Kashmir Bee Virus
KV: Kakugo virus
LRR: leucine-rich repeat
LSV: Lake Sinai Virus
MAPK: Mitogen-Activated Protein Kinases
NF-κB: Nuclear Factor κB/Dorsal
NTC: No Template Control
OIE: World Organization for Animal Health
ORF: Open Reading Frame
PAMPs: Pathogen Associated Molecular Patterns
PCR: Polymarase Chain Reaction

PO: Phenoloxidase
PP(Asp)2: sucrose-protoporphyrin amide
PPO: Pro-Phenoloxidase
PRRs: Pattern Recognition Receptors
qPCR: Real-Time PCR
RNA: Ribonucleic acid
RQ: Relative Quantization
RT: Reverse Trascriptase
SBV: Sac Brood Virus
siRNA-Dicer: small interfering RNA targeting gene coding Dicer
SPV: Slow Paralysis Virus
ssRNA: single strand RNA
TBE: Tris Borate EDTA
UTR: Untranslated Region
VDV: Varroa Destructor Viruses
Vg: Vitellogenin
VP1-4:Viral proteins 1-4
VPg: genome-linked virus protein

LIST OF FIGURES

Fig. I. Color-coded map showing relative risk (loss rate divided by the loss rate over all regions) of overwinter colony loss from the COLOSS- colony losses monitoring project.
Fig. 1.1. Dicistoviridae. Genome structure of cricket paralysis virus.
Fig. 1.2 Iflaviridae. Genome structure of infectious flacherie virus.
Fig.1.3 Transmission of viruses in honeybees.
Fig.1.4 V. destructor on a A. mellifera worker thorax.
Fig.1.5 V. desctructor life cycle.
Fig. 1.6 Genome structure of DWV.
Fg.1.7 A. mellifera displaying clinical signs of DWV.
Fig.1.8 Honeybee immune pathways, highlighting genes implicated in antiviral immune responses.
Fig.1.9 Anatomopathological analysis of A.mellifera.
Fig. 1.10 Analysis of viral load in honeybee samples showing clinical signs (1-10) compared with apparently healthy honeybee samples (H).
Fig.1.11 A. mellifera showing clinical signs of DWV.
Fig.1.12 A. mellifera infected with DWV. Hypopharyngeal glands.
Fig.1.13 A. mellifera with symptomatic infection of DWV. Flight muscles.
Fig.1.14 A. mellifera with asymptomatic infection of DWV. Midgut.
Fig.1.15 A. mellifera with asymptomatic infection of DWV. Hemocytes.
Fig.1.16 A. mellifera with asymptomatic infection of DWV. Midgut.
Fig.2.1 A. mellifera ligustica drone.
Fig.2.2 Reproductive system of a drone of A. mellifera.
Fig. 2.3 Manual eversion of the endophallus of A ,mellifera ligustica drone.
Fig.2.4 A queen and a drone of A. mellifera mating.
Fig. 2.5 Instrumental insemination of A. mellifera ligustica.
Fig. 2.6 Comb showing the presence of honeybee workers and drones of A.mellifera ligustica.
Fig. 2.7 A. mellifera ligustica. Morphologically normal spermatozoa.
Fig. 2.8. A. mellifera ligustica. Morphologically altered spermatozoa.
Fig. 2.9 A. mellifera ligustica. Normal tesits and normal seminiferous tubules.
Fig. 2.10. A. mellifera ligustica. Altered seminiferous tubules.
Fig. 2.11. A. mellifera ligustica. Altered seminiferous tubules.
Fig. 2.12. A. mellifera ligustica. Altered seminiferous tubules.
Fig. 3.1 Nosema spp. life cycle.
Fig. 3.2 Clinical signs of Nosemosis type A. Diarrohic spots on comb.
Fig. 3.3 Intestine of honeybee infected by Nosema spp. and of healthy honeybee.
Fig. 3.4 Morphological and morphometric differences in N. apis and N. ceranae by scanning electron microscopy.
Fig. 3.5 Mini-FLOTAC components.
Fig. 3.6 Fill-FLOTAC and Mini-FLOTAC.
Fig. 3.7 Sample preparation.
Fig. 3.8 A. mellifera. Midgut.
Fig. 3.9 A. mellifera. Midgut.

Fig. 3.10 A. mellifera. Midgut lumen.
Fig. 3.11 A. mellifera. Malpighian tubules.
Fig. 3.12 A. mellifera. Healthy midgut.
Fig. 3.13 Direct smear.
Fig. 3.14 Hemocitometer.
Fig. 3.15 Mini-FLOTAC.

LIST OF TABLES

Tab. 1 Oligonucleotids used for amplification of DWV and Act β in this study.
Tab. 2.1 Starting and end point of measurement for morphometric analysis of A. mellifera ligustica spermatozoa.
Tab. 2.2 Morphometric parameters of spermatozoa of A. mellifera ligustica.
Tab. 2.3 Morphometric parameters of A. mellifera carnica spermatozoa.

Abstract

During the past decades, many factors have reduced the number of honeybees, with great damage to biodiversity and to agrozootechnical economics.
Honeybee pollination represents a fundamental element in the ecosystem function as it ensures the reproduction of wild plants and agricultural crops as well as genetic variability among species, thus defending global biodiversity.
Moreover, honeybee products are very appreciated and nowadays they are of great interest for the therapeutic properties of their compounds.
Although the synergistic action of different factors (including bacteria, virus, parasites, pesticides) has been identified as the source of loss of honeybees, very little information is available about how these factors actually affect the health of honeybees.
For many years, veterinary pathologists have shown little interest in the study of honeybee pathology, and very few studies are currently available. However, the knowledge possessed by veterinary pathologists could support the studies of researchers pertaining to other fields and unravel the mysteries around colony losses.
The present research collects the experiments and the results of a three year PhD program spent studying honeybee pathology. Each study proposes a new laboratory technique and describes the pathological findings connected to three pathologies, namely Deformed Wing Virus (DWV), male hypofertility and Nosemosis
In Chapter 1 a study on DWV infected honeybees is presented with the aim of enriching knowledge about etiopathogenesis. Honeybee samples with and without clinical signs were collected from a DWV clinically infected hive and analysed to highlight the presence of the virus and determine the relative viral load through biomolecular techniques. Subsequently, using an innovative fixating technique, honeybee samples were subjected to anatomo-histopathological analysis.
The virus was identified in all samples analysed, and the viral load was higher in symptomatic samples compared to the asymptomatic group.

The anatomopathological analysis confirmed the presence of the typical signs of DWV infection: crippled wing, short and discoloured abdomens. In samples showing clinical signs, by histopathology we observed alterations of the hypopharyngeal glands and thoracic muscles, while samples with no apparent clinical signs showed the presence of inflammatory cells, as well as melanisation in the midgut and in the hemocele.
The results suggest a possible pathological action of DWV in both clinical and subclinical infections.

The Chapter 2 describes the methods and the results of a study on the reproductive system and spermatozoa of *Apis mellifera ligustica*, carried out in collaboration with the Unity of General Zootechnics and Genetic Improvement of the Department of Veterinary Medicine and Animal Productions of the University of Naples "Federico II". The reproductive system of drones was analysed by anatomopathology and testes were examined by histopathology. No macroscopic alterations were observed in any sample and through histopathology most of the samples showed unaltered testes, although in some cases they showed degenerated seminiferous tubules, while others appeared immature. Moreover, using an innovative technique, the morphological features and morphometric parameters of spermatozoa were studied. The following morphometric values (mean ± standard deviation) were measured: sperm total length (230,81±17,22 μm), tail length (222,96±17,15μm), head length (7,85±0,65μm), nucleus length (4,44±0,61μm) and perforator length (3,58±1,21μm). Additionally, 7% of the spermatozoa showed the presence of visible defects such as double, split or broken tails. The results obtained provide data about morphology of testes and morphometry and morphology of spermatozoa of drones of *A. mellifera ligustica* and show the presence of alterations.
In Chapter 3 two different techniques for the diagnosis of Nosemosis are presented. Samples of adult honeybees were analysed with anatomo-histopathological analysis and, for the first time, the Mini-FLOTAC technique was applied to the beekeeping field to detect *Nosema spp*.

spores. The anatomopathological analysis did not reveal any alterations of the midgut while the histopathological analysis revealed the presence of spores in the midgut and in the Malpighian tubules, and both organs appeared strongly degenerated. The Mini-FLOTAC technique was compared with two different microscopic techniques, direct smear and microscopic examination with the use of the hemocytometer, in order to assess the possible use in honeybees. The results confirmed the validity of the three techniques to highlight the presence of the spores of *Nosema*. However, the Mini-FLOTAC technique proved to be more user-friendly and a better tool for spore detection, especially when the infection level is low, thanks the high sensitivity, precision and accuracy of the technique. This study was carried out in collaboration with the Unity of Parasitology and Parasitic Diseases of the Department of Veterinary Medicine and Animal Productions of the University of Naples "Federico II".

The results obtained from the different studies show the efficacy and the effectiveness of applying laboratory and diagnostic techniques already validated in other animals and humans in honeybee pathology, although by introducing some modifications due to the particular characteristics of the samples. Moreover, it appears evident that the pathologies here studied, particularly DWV and Nosemosis can impair the immune response of honeybees, corroborating the idea of a key role of immunity in the fight of honeybees against different stressors. Finally, the presence of alterations affecting the male reproductive system, suggests that honeybees have the potential of being bioindicators of the presence of endocrine disruptors in the environment that could also affect fertility in male humans.

Introduction

I. **The important role of honeybees**

Honeybees can be found worldwide, except for the polar regions and the Saharan area.
The relationship between humans and honeybees goes back 40.000 years, as demonstrated by the finding in Europe, North Africa and Middle East of crockery containing wax traces, belonging to first farmers of the Neolithic (Roffet-Salque et al., 2015).
The oldest evidence (VI-V millennium BC) of the anthropogenic use of honey derives from several cave paintings depicting human figures perched on long rope ladders holding a basket, found in the Cuevas de la Arana, Valencia (Hajar, 2008).
Since then, honeybees have followed mankind through its evolution and beekeeping practices have evolved with it.
The species belonging to the *Apis* genus are by far the most affected by anthropic use (Cardinal and Danforth, 2011).
Among these the Western honeybee, *Apis mellifera* is certainly the one that has had the greatest success in the zootechnical field (Preston, 2006) and that mankind, with its migrations and commercial activities, has helped spread in all continents (Michener, 2007).
Honeybees are mainly kept for their products, part of everyday life as well as appreciated nowadays for their therapeutic properties.
The belief that honeybee products (honey, pollen, bee bread, royal jelly) can be used for medical purposes has developed a whole new branch of alternative medicine, obtaining everyday more consensus and scientific validation.
Among the honeybee products, the most common is certainly honey.
Honey is produced from secretions of flowers (nectar) and from secretions of other insects (honeydew) by enzymatic activity and maturation (Crane, 1991).
It is composed mainly by carbohydrates, namely fructose and glucose (Bogdanov et al., 2008), while the content of amino acids and proteins is

relatively small, at the most 0.7%, consisting primarily of enzymes, glucose oxidase and catalase which regulate the production of H_2O_2, although most of the physiologically important amino acids are also present (Cotte et al., 2004).

In most ancient cultures, honey has been used for both nutritional and medical purposes.

The therapeutic properties of honey have been known since 2100-2000 BC, as reported in a Sumerian tablet writing, when honey was used as a drug and an ointment to cure wounds (Mandal and Mandal, 2011).

The ability of honey to improve wound healing, to function as an anti-oxidant and anti-inflammatory, has been repeatedly demonstrated and few products are already available on the market (Bogdanov et al., 2008; Molan and Rhodes, 2015).

Currently many researchers have been focusing their attention on its antibacterial activity as a possible tool to fight antimicrobial resistance (Basualdo et al., 2007), as it was proved that honey can be effective against methicillin-resistant Staphylococcus aureus and vancomycin-resistant Enterococci (Brudzynski and Lannigan, 2012).

The antimicrobial activity is connected to the presence of hydrogen peroxide, to the hygroscopic features, which means it can reduce moistening from the environment and dehydrate bacteria, to its high sugar content and low Ph levels, which prevent microbes from growing (Lusby et al, 2005).

However, it varies with the botanical origin of the honey, and it seems that Manuka honey has inhibitory effects on around 60 species of bacteria and it is the most effective against *Escherichia coli*, *Salmonella typhimurium* and *Staphylococcus aureus* (Molan, 1992).

Bee pollen represents the main source of proteins for honeybees. It is collected from plant anthers, mixed with secretion from salivary glands and nectar and then transported to the hive in the corbiculae. In the hive, pollen is packed in the honeycomb cells and covered with a thin layer of honey and wax, which stimulate anaerobic fermentation which gives origin to bee bread (Couto and Couto, 2006).

Bee pollen can be considered a “perfectly complete food” as it contains about 200 substances including amino acids, carbohydrates, crude fibres, lipids (triglycerides, phospholipids), vitamins, macro and micro-nutrients, and flavonoids (Komosinska-Vassev et al., 2015; Nogueira et al., 2012).
Bee pollen shows a series of therapeutic properties such as antifungal, antimicrobial, antiviral, anti-inflammatory, antimutagenic and immunostimulating activity (Pascoal et al., 2014; Kroyer and Hegedus, 2001). Moreover, it is suggested that flavonoids contained in bee pollen, due to their anti-allergic, antioxidant and anti-inflammatory properties, could be used in treating allergic and immunological disorders (Jannesar et al., 2017).
Beebread is probably the least known among bee products and its commercial use is scarce, probably due to the difficulties of collection. However increased studies have suggested antioxidant and antimicrobial properties of the product, as well as a positive effect on the immune system, due to the presence of lactic and phenolic acids (Kocot et al., 2018). A study by Urcan et al. (2018) analysed the effect of bee bread extract on cultures of some bacteria, causative agents of food poisoning, namely *E.coli*, *Salmonella enterica*, Pseud*omonas aeruginosa*, *Bacillus cereus* and *S.aureus.* The results of the study showed that bee bread decelerated the growth of the bacteria with a greater effect on *S. aureus*.
Propolis is a substance originating from resinous substances of plants which is collected and mixed with beeswax and enzymes found in their saliva (Park et al., 1997). Propolis is mainly composed of balsam resin, wax, essential and aromatic oils, pollen, and other substances, including wood fragments (Sforcin, 2016), and it includes more than 300 different compounds, of which flavonoids are of greater interest in apitherapy (Havsteen, 2002). Propolis has many therapeutic properties such as antibacterial, anti-inflammatory, antifungal, antiprotozoan, antiviral (against *Herpes simplex*) and healing activities (Ramos and Miranda, 2007).
Royal jelly is a white viscous substance secreted by the hypopharyngeal and mandibular glands of worker honeybees and it is essential food for larvae and for the development of the queen (Buttstedt et al., 2013). It is

composed of proteins and carbohydrates in almost the same quantity and, in a lesser amount, of lipids, amino acids, vitamins and minerals (Khazaei et al., 2018). More than 185 organic compounds have been detected in royal jelly, among which flavonoids and hormones (Ramadan and Al-Ghamdi, 2012).

Royal jelly is widely used as a dietary nutritional complex to help combat various chronic health conditions and it shows many therapeutic activities such as antibacterial, antitumor, anti-allergy, anti-inflammatory (Pasupuleti et al., 2017). Moreover, its action on reproductive health (Taavoni et al., 2014) and on neurodegenerative and aging diseases has also been described (Zamani et al., 2012). Although more studies are needed to develop dosages, to analyse potential negative effects and conduct wider experimentations, it seems clear that the use of honeybee products could potentially support traditional medicine especially in the case of infections by antimicrobial resistant pathogens.

Honeybees are not only important for their products; probably, their most meaningful role is pollination.

Insect pollination is responsible for 80-85% of all commercial crops, with fruit, vegetables, oilseed and legumes being mostly pollinated by the Western honeybee *A. mellifera*.

Moreover, given its global distribution and its generalist foraging behaviour, *A. mellifera* can be considered the most important single species pollinator of a wide variety of wild flora, livestock pastures, private gardens (Hung et al., 2018; Garibaldi et al., 2013).

Pollination can be provided by honeybees in a wild way, during everyday flight activity (pollination ecosystem service), or it can be managed, when whole colonies are transported into orchards or fields with the aim of enhancing crop productions.

It has been calculated that commercial pollination is the most important derived value of commercial beekeeping worldwide (Morse and Calderone, 2000), generating annual values in the USA estimated at between USD 1.6 billion and USD 14.6 billion, depending on the methodology used (Allsopp et al., 2008).

The evaluation of wild pollination seems to be more complicated, as it is not always possible to exclude from calculations the contribution to pollination of other insects. However, a rough economic value has been calculated between USD 12.6–30.7 million (Cook et al., 2007), although the greater value of wild pollination is conservation of natural biodiversity. Biological diversity (biodiversity) is “the variability among living organisms from all sources, including, *inter alia*, terrestrial, marine, and other aquatic ecosystems, and the ecological complexes of which they are part: this includes diversity within species, between species and of ecosystems”. This definition was developed by the UN in their Convention on Biological Diversity in 1992.

In other words, biodiversity includes not only the world's different species of animals, plants, microorganism, species with their unique evolutionary histories, but also genetic variability within and among populations of species and the distribution and relationship of species across local habitats, ecosystems, landscapes, and whole continents or oceans (National Research Council US, 1999).

By pollinating different plant species, honeybees guarantee the correct reproduction of plants and seeds ripening, which will give origin to new plants that become food for humans and feed for animals, which in turn can become nourishment for different animals. In this perspective the survival of a wide variety of plants and animals is strictly connected to the survival of honeybees.

As Darwin (1859) observed more than a century ago "the number of humble-bees in any district depends in a great measure upon the number of field mice, which destroy their combs and nests ...the number of mice is largely dependent, as everyone knows, on the number of cats ... it is quite credible that the presence of a feline animal in large numbers in a district might determine, through the intervention first of mice and then of bees, the frequency of certain flowers in that district!" .

With these words, Darwin highlights the importance of variability and interaction among different species, and its relation to the environment, giving great importance to the role of honeybees in the biodiversity balance.

The dependence of many species on honeybee pollination has the potential for a cascade of negative effects on biodiversity resulting from loss of plant-pollinator mutualism, due to the current global loss of honeybee individuals (Kearns and Inouye, 1997).
Given the particular foraging habits and the strict relationship between honeybees and environment, these insects are often used as useful bio-monitoring species. In 1935, Svodoba studied the impact of industrial arsenic on honeybees and he realised that honeybees could provide important data about the substances present in a given area (Svodoba, 1935). Indeed, honeybees, and their products, have the potential to be great bio-indicators and they can help to detect and interpret changes and alterations that happen in the environment (Porrini et al., 2002).
Bio-monitoring with *Apis mellifera* shows great benefits: honeybees through their foraging activities are able to collect many micro-samples of air, soil, pollen, nectar and water from a wide area and subsequently transport them in the hive; the source of any substances can be established by analysing the botanical origin of pollen and locate polluted areas; studies can be performed everywhere and they require easy protocols that can be standardised and reproduced (Ruiz et al., 2013).
To date, many studies have already been performed in different countries and they included bio-monitoring for radioactive isotopes (Haarmann, 1998), industrial pollutants (vad der Steen et al., 2012), pesticides (Henry et al., 2012), Polycyclic Aromatic Hydrocarbons (Perugini et al., 2009).
Bio-monitoring projects should be carried out by the Governments, which should evaluate the environmental risk, and by the private companies to show their environmental responsibilities (Ruiz et al., 2013).
Benefits from bio-monitoring could come both for honeybees and people, as, once the source of contamination is identified, actions could be put in place to reduce the impact on the environment.

II. **Honeybee loss**

Over the past few decades, many concerns about the decline of the

honeybee population have been raised worldwide. The first data about honeybee losses date back to the 1990s and they indicated high honeybee mortality in the United States and Europe, particularly in France and Italy (Williams et al., 2010). In fact, since 1985, according to a report by Greenpeace (2013), Europe has witnessed a commercial loss of 25% of the honeybee population, while in the US the reduction in the number of honeybees reached 45%. Latest data report European losses for 16.4% for the year 2017/2018 (Fig. 1) (Gray et al., 2019).

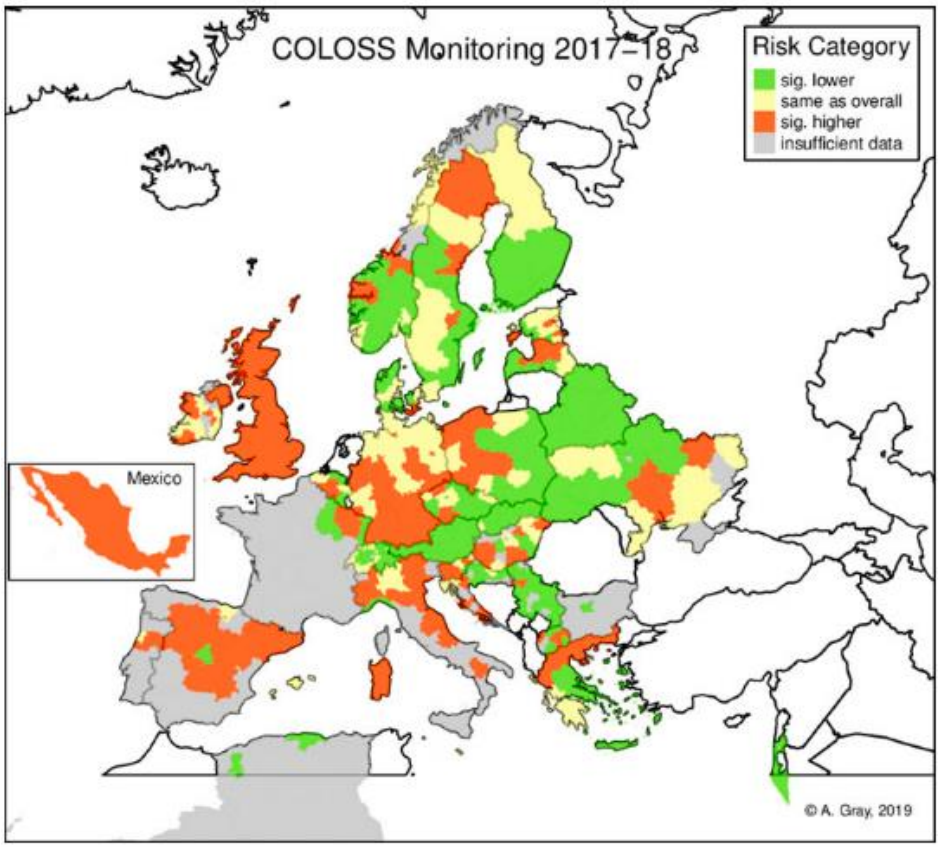

Fig. I. Color-coded map showing relative risk (loss rate divided by the loss rate over all regions) of overwinter colony loss from the COLOSS- colony losses monitoring project. Red/green indicate regions with a relative risk of loss that is significantly higher/lower than 1; yellow indicate regions with a relative risk not significantly different from 1; gray indicate regions were no data were available or data were available from fewer than 6 beekeepers in a region within a participating country, this was treated as insufficient (Gray et al., 2019).

In Italy significant mortalities have been recorded starting from 2003. Since then, data about honeybee mortality in the past years has been collected thanks to two different projects: ApeNet from 2009 to 2010 and BeeNet from 2011 to 2013. Results from the ApeNet project reported in 2009 a colony mortality rate of 19.17%, while in 2010 it was 7.23% (Porrini et al., 2016), showing a significant decrease compared to the two previous years (2007-2008) when mortality rate reached 40% (Mutinelli et al., 2010). In 2011 the annual colony mortality amounted to 13.8%, while average mortality in 2012 amounted to 6.9%. Latest data collected by the International association COLOSS has reported a mortality rate of 10.9 % for the year 2016-2017 (Brodschneider et al., 2018), and of 17.2% for the year 2017-2018 (Gray et al., 2019).

Many are the causes that have been identified and that, by acting synergistically, impair the health of single individuals and of entire colonies (Goulson et al., 2015), above all pesticides, incorrect beekeeping practices, pathogens, reduced availability or quality of food resources and climate change (O' Neal et al., 2018; Steinhauer et al., 2018).

Pesticides are probably the first cause to have been identified as having a detrimental effect on honeybee health, probably due to a lack of genes encoding for detoxification enzymes (Claudianos et al., 2006).

The use of molecules such as neonicotinoids, organophosphates, carbamates and pyrethroids in agricultural and horticultural landscapes, as well as the use of in hive acaricides, has been proved to have acute and chronic effects on honeybees (Ostiguy et al., 2019).

The main signs of acute poisoning are neurological and include paralysis, tremors, and death in front of the hive; while in chronic poisoning the signs are mainly connected to behavioural alterations which can cause inability to perform everyday tasks such as inability to forage and return to the nest, and consequently a slower and progressive reduction in the individuals of the colony (Kiljanek et al., 2016).

Moreover, it has been proved that a complex and harmful interaction exists between pesticide exposure and honeybee immunity, which could make insects more susceptible to the action of pathogens (O'Neil et al., 2018).

At the same time, honeybees displacing alterations of the immune system have been proved to be more susceptible to the action of pesticides.
Bacteria, parasites and viruses can all infect honeybees and cause different degrees of impairment, often silently causing the collapse of the colony, due to asymptomatic infections.
Among the most important bacterial diseases American and European Foulbrood should be mentioned, as they are included in the list of notifiable diseases of the OIE (World Organization for Animal Health) for their severity. They are caused by *Paenibacillus larvae* and *Melissococcus plutonius*, respectively, and affect young larvae, which become infected after ingesting food containing the bacteria (Forsegren, 2010; Genersh, 2010).
The main clinical signs of American foulbrood are that larvae die after capping and transform into a dark semi-liquid mass (Hansen and Brødsgaard, 1999); while European foulbrood is characterised by death of the larvae which become a liquid, brown, ammonia-like smelling mass (Forsegren et al., 2005).
Varroa destructor, Acarapis woodi, Tropilaelaps spp. and *Nosema* spp, are the principal parasites of honeybees and the first three are also included in the list of the OIE.
Varroa destructor is believed to be the leading cause of colony collapse in *A. mellifera* populations (Rosenkranz et al., 2010), due to its feeding habits, immunosuppressive action and viral vector role (Roth et al., 2020) leading to the development of the so called "parasitic mite syndrome" (Tantillo et al., 2015).
Acarapis woodi, also known as the tracheal mite, and *Tropilaelaps* spp, are less frequently encountered, although, due to trade globalization, they should be considered as emerging threats to the Western honeybee (Chantawannakul et al., 2018; Takashima et al., 2020).
Nosema apis and *Nosema ceranae* are globally diffused microsporidia which affect the midgut of honeybees, showing two very different clinical displays. In fact, while *N. apis* shows clinical signs like diarrhoea, *N.ceranae* does not show any clinical signs but it was reported to be associated with colony collapse (Higes et al., 2008).

Viruses are significant threats to the health and wellbeing of honeybees. Although in most cases they cause asymptomatic infections (de Miranda and Genersch, 2010), the association to the presence of the mite *Varroa* often results in the loss of many individuals, probably following a reduction in the immune response (Shen et al., 2005).

Susceptibility to pathogens is not only a direct consequence of immune suppression, but it can also indirectly result from changes in the quantity and quality of feed, which can alter the immune response.

Climate change as well as monoculture farming have altered the floral environment and often reduced colony harvesting capacity (Le Conte and Navajas, 2008).

Climate influences flower development and nectar and pollen production, which are directly linked with colonies' foraging activity and development (Winston, 1987).

During the foraging period, honeybees should try to accumulate enough honey and pollen stores to enable them to survive the winter season while guaranteeing the correct nourishment of young larvae (Le Conte and Navajas, 2008).

Excessively dry climates can result in water and flower shortages, reduced pollen production and impoverishment of its quality, and reduction of nectar.

While carbohydrates from nectar can be replaced with artificial preparations by the beekeeper, proteins from pollen cannot easily be integrated.

Pollen and beebread are necessary for physiological processes such as brood rearing and brood growth, as they are important for the production of royal jelly (Di Pasquale et al., 2013); moreover, it influences the expression of genes affecting longevity, the production of antimicrobial peptides and pesticide detoxification (Schmehl et al., 2014; Alaux et al., 2011).

Reduction of pollen quantity and quality, could result in poorly nourished larvae which, in turn, could develop into weak adults, which are less resistant to the effects of pathogens and pesticides, eventually resulting in colony collapse (Branchiccela et al., 2019).

Bibliography

Alaux C, Dantec C, Parrinello H, Le Conte Y. Nutrigenomics in honey bees: digital gene expression analysis of pollen's nutritive effects on healthy and varroa-parasitized bees. *BMC Genomics*. 2011;12:496. doi:10.1186/1471-2164-12-496

Allsopp MH, de Lange WJ, Veldtman R. Valuing insect pollination services with cost of replacement. *PLoS One*. 2008;3(9):e3128. doi:10.1371/journal.pone.0003128

Basualdo C, Sgroy V, Finola MS, Marioli JM. Comparison of the antibacterial activity of honey from different provenance against bacteria usually isolated from skin wounds. *Vet Microbiol*. 2007;124(3-4):375-381. doi:10.1016/j.vetmic.2007.04.039

Bogdanov S, Jurendic T, Sieber R, Gallmann P. Honey for nutrition and health: a review. *J Am Coll Nutr*. 2008;27(6):677-689. doi:10.1080/07315724.2008.10719745

Branchiccela B, Castelli L, Corona M, et al. Impact of nutritional stress on the honeybee colony health. *Sci Rep*. 2019;9(1):10156. doi:10.1038/s41598-019-46453-9

Brodschneider R, Gray A, Adjlane N, et al. Multi-country loss rates of honey bee colonies during winter 2016/2017 from the COLOSS survey, *Journal Apic Res.* 2018; 57:3, 452-457, doi: 10.1080/00218839.2018.1460911

Brudzynski K, Lannigan R. Mechanism of Honey Bacteriostatic Action Against MRSA and VRE Involves Hydroxyl Radicals Generated from Honey's Hydrogen Peroxide. *Front Microbiol*. 2012;3:36. doi:10.3389/fmicb.2012.00036

Buttstedt A, Moritz RF, Erler S. More than royal food - Major royal jelly protein genes in sexuals and workers of the honeybee Apis mellifera. *Front Zool*. 2013;10(1):72. doi:10.1186/1742-9994-10-72

Cardinal S, Danforth BN. The antiquity and evolutionary history of social behavior in bees. *PLoS One*. 2011;6(6):e21086. doi:10.1371/journal.pone.0021086

Chantawannakul P, Ramsey S, vanEngelsdorp D, et al. Tropilaelaps mite: an emerging threat to European honey bee. *Curr Opin Insect Sci*. 2018;26:69-75. doi:10.1016/j.cois.2018.01.012

Claudianos C, Ranson H, Johnson RM, et al. A deficit of detoxification enzymes: pesticide sensitivity and environmental response in the honeybee. *Insect Mol Biol*. 2006;15(5):615-636. doi:10.1111/j.1365-2583.2006.00672.x

Cook DC, Thomas MB, Cunningham SA, et al. Predicting the economic impact of an invasive species on an ecosystem service. *Ecol Appl*. 2007;17(6):1832-1840. doi:10.1890/06-1632.1

Cotte JF, Casabianca H, Giroud B,et al. Characterization of honey amino acid profiles using high-pressure liquid chromatography to control authenticity. *Anal Bioanal Chem*. 2004;378(5):1342-1350. doi:10.1007/s00216-003-2430-z

Couto RHN, Couto LA, *Apicultura: Manejo e Produtos*, Funep, Jaboticabal, Brazil, 3rd edition, 2006

Crane E. Honey from honeybees and other insects. *Ethol Ecol Evol*. 1991. 3:sup1, 100-105, doi: 10.1080/03949370.1991.10721919

Darwin, C. (1859). *On the origin of species by means of natural selection, or, the preservation of favoured races in the struggle for life*. London: J. Murray

de Miranda JR, Genersch E. Deformed wing virus. *J Invertebr Pathol*. 2010;103 Suppl 1:S48-S61. doi:10.1016/j.jip.2009.06.012

Di Pasquale G, Salignon M, Le Conte Y, et al. Influence of pollen nutrition on honey bee health: do pollen quality and diversity matter?. *PLoS One*. 2013;8(8):e72016. doi:10.1371/journal.pone.0072016

Forsgren E, Lundhagen AC, Imdorf A, Fries I. Distribution of Melissococcus plutonius in honeybee colonies with and without symptoms of European foulbrood. *Microb Ecol*. 2005;50(3):369-374. doi:10.1007/s00248-004-0188-2

Forsgren E. European foulbrood in honey bees. *J Invertebr Pathol*. 2010;103 Suppl 1:S5-S9. doi:10.1016/j.jip.2009.06.016

Garibaldi LA, Steffan-Dewenter I, Winfree R, et al. Wild pollinators enhance fruit set of crops regardless of honey bee abundance. *Science*. 2013;339(6127):1608-1611. doi:10.1126/science.1230200

Genersch E. American Foulbrood in honeybees and its causative agent, Paenibacillus larvae. *J Invertebr Pathol*. 2010;103 Suppl 1:S10-S19. doi:10.1016/j.jip.2009.06.015

Goulson D, Nicholls E, Botías C, Rotheray EL. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science*. 2015;347(6229):1255957. doi:10.1126/science.1255957

Gray A, Brodschneider, R Adjlane N, et al. Loss rates of honey bee colonies during winter 2017/18 in 36 countries participating in the COLOSS survey, including effects of forage sources. *J Apic Res*, 2019; 58:4, 479-485, doi: 10.1080/00218839.2019.1615661

GreenPeace: "Bees in Decline". Greenpeace Research Laboratories Technical Report (Review). 2013.

Haarmann, T K. Honey bees as indicators of radionuclide contamination: comparative studies of contaminant levels in forager and nurse bees and in the flowers of three plant species. *Arch Environ Contam Toxicol.* 1998; 35: 287–294.

Hajar R. Honey and Medicine. In:Selin H(eds) Encyclopaedia of the History of Science, Technology, and Medicine in Non-Western Cultures. Springer, Dordrecht. 2008

Hansen H, Brødsgaard CJ. American foulbrood: a review of its biology, diagnosis and control. *Bee World*. 1999; 80:1, 5-23, doi: 10.1080/0005772X.1999.11099415

Havsteen BH. The biochemistry and medical significance of the flavonoids. *Pharmacol Ther*. 2002; 96(2-3):67-202. doi: 10.1016/s0163-7258(02)00298-x.

Henry M, Béguin M, Requier F, et al. A common pesticide decreases foraging success and survival in honey bees. *Science*. 2012;336(6079):348-350. doi:10.1126/science.1215039

Higes M, Martín-Hernández R, Botías C, et al. How natural infection by Nosema ceranae causes honeybee colony collapse. *Environ Microbiol*. 2008;10(10):2659-2669. doi:10.1111/j.1462-2920.2008.01687.x

Hung KJ, Kingston JM, Albrecht M, et al. The worldwide importance of honey bees as pollinators in natural habitats. *Proc Biol Sci*. 2018;285(1870):20172140. doi:10.1098/rspb.2017.2140

Jannesar M, Shoushtari S, Majd A, Pourpak Z. Bee Pollen Flavonoids as a Therapeutic Agent in Allergic and Immunological Disorders. *Iran J Allergy Asthma Immunol*. 2017;16(3):171-182.

Kearns CA, Inouye DW, Pollinators, Flowering Plants, and Conservation Biology, *BioScience*, Volume 47, Issue 5, May 1997, Pages 297–307, https://doi.org/10.2307/1313191

Khazaei M, Ansarian A, Ghanbari E. New Findings on Biological Actions and Clinical Applications of Royal Jelly: A Review. *J Diet Suppl*. 2018;15(5):757-775. doi:10.1080/19390211.2017.1363843

Kiljanek T, Niewiadowska A, Posyniak A. Pesticide Poisoning of Honeybees: A Review of Symptoms, Incident Classification, and Causes of Poisoning, *J Apic Sci*. 2016;60(2), 5-24. doi: https://doi.org/10.1515/jas-2016-0024

Kocot J, Kiełczykowska M, Luchowska-Kocot D, et al. Antioxidant Potential of Propolis, Bee Pollen, and Royal Jelly: Possible Medical Application. *Oxid Med Cell Longev*. 2018;2018:7074209. doi:10.1155/2018/7074209

Komosinska-Vassev K, Olczyk P, Kaźmierczak J, et al. Bee pollen: chemical composition and therapeutic application. *Evid Based Complement Alternat Med*. 2015;2015:297425. doi:10.1155/2015/297425

Kroyer G, Hegedus N. Evaluation of bioactive properties of pollen extracts as functional dietary food supplement. *Innov Food Sci Emerg Technol*. 2001. 2:171-174.

Le Conte Y, Navajas M. Climate change: impact on honey bee populations and diseases. *Rev Sci Tech*. 2008;27(2):485-510.

Lusby PE, Coombes AL, Wilkinson JM. Bactericidal activity of different honeys against pathogenic bacteria. *Arch Med Res*. 2005;36(5):464-467. doi:10.1016/j.arcmed.2005.03.038

Mandal MD, Mandal S. Honey: its medicinal property and antibacterial activity. *Asian Pac J Trop Biomed*. 2011;1(2):154-160. doi:10.1016/S2221-1691(11)60016-6

Michener CD. The Bees of the World. 2007. 2nd Edition, John Hopkins University Press, Baltimore.

Molan P, Rhodes T. Honey: A Biologic Wound Dressing. *Wounds*. 2015;27(6):141-151.

Molan PC. The antibacterial activity of honey: 1. The nature of the antibacterial activity. *Bee World*. 1992. 73(1), 5-28.

Morais M, Moreira L, Feás X, Estevinho LM. Honeybee-collected pollen from five Portuguese Natural Parks: palynological origin, phenolic content, antioxidant properties and antimicrobial activity. *Food Chem Toxicol*. 2011;49(5):1096-1101. doi:10.1016/j.fct.2011.01.020

Morse RA, Calderone NW. The value of honey bees as pollinators of U.S. crops in 2000. *Bee Cult*. 2000;128:1–15.

Mutinelli F, Sgolastra F, Gallina A, et al. A network for monitoring honeybee mortality and colony losses in Italy as a part of the APENET research project. *Am Bee J*. 2010; 150: 389–390.

National Research Council (US) Committee on Noneconomic and Economic Value of Biodiversity. Perspectives on Biodiversity: Valuing Its Role in an Everchanging World. Washington (DC): National Academies Press (US); 1999. 2, What is Biodiversity? Available from: https://www.ncbi.nlm.nih.gov/books/NBK224405/

Nogueira C, Iglesias A, Feás X, Estevinho LM. Commercial bee pollen with different geographical origins: a comprehensive approach. *Int J Mol Sci*. 2012;13(9):11173-11187. doi:10.3390/ijms130911173

O'Neal ST, Anderson TD, Wu-Smart JY. Interactions between pesticides and pathogen susceptibility in honey bees. *Curr Opin Insect Sci*. 2018;26:57-62. doi:10.1016/j.cois.2018.01.006

Ostiguy N, Drummond FA, Aronstein K, et al. Honey Bee Exposure to Pesticides: A Four-Year Nationwide Study. *Insects*. 2019;10(1):13. Published 2019 Jan 8. doi:10.3390/insects10010013

Park YK, Koo MH, Ikegari M, Contado JL. Comparison of the flavonoid aglycone contents of *Apis mellifera* propolis from various regions of Brazil. *Arq. Biol. Tecnol*., 1997, 40, 97-106.

Pascoal A, Rodrigues S, Teixeira A, et al. Biological activities of commercial bee pollens: antimicrobial, antimutagenic, antioxidant and anti-

inflammatory. *Food Chem Toxicol*. 2014;63:233-239. doi:10.1016/j.fct.2013.11.010

Pasupuleti VR, Sammugam L, Ramesh N, Gan SH. Honey, Propolis, and Royal Jelly: A Comprehensive Review of Their Biological Actions and Health Benefits. *Oxid Med Cell Longev*. 2017;2017:1259510. doi:10.1155/2017/1259510

Perugini M, Di Serafino G, Giacomelli A, et al. Monitoring of polycyclic aromatic hydrocarbons in bees (Apis mellifera) and honey in urban areas and wildlife reserves. *J Agric Food Chem*. 2009;57(16):7440-7444. doi:10.1021/jf9011054

Porrini C, Ghini S, Girotti S et al. Use of honey bees as bioindicators of environmental pollution in Italy. In: Honey bees: Estimating the Environmental Impact of Chemicals (Devillers, J and Pham-Delègue, M H Eds). Taylor & Francis, London, p. 186- 247. 2002

Porrini C, Mutinelli F, Bortolotti L, et al. The Status of Honey Bee Health in Italy: Results from the Nationwide Bee Monitoring Network. *PLoS One*. 2016;11(5):e0155411. doi:10.1371/journal.pone.0155411

Potts SG, Biesmeijer JC, Kremen C, et al. Global pollinator declines: trends, impacts and drivers. *Trends Ecol Evol*. 2010;25(6):345-353. doi:10.1016/j.tree.2010.01.007

Preston C. Bee. 2006. London, England: Reaktion Books

Ramadan MF, Al-Ghamdi A. Bioactive compounds and health-promoting properties of royal jelly: a review. *J Funct Foods*. 2012; 4 (1):39–52.

Ramos AFN, Miranda JL. Propolis: a review of its anti-inflammatory and healing actions. J Venom Anim Toxins Incl Trop Dis. 2007;13:697–710

Roffet-Salque M, Regert M, Evershed RP, et al. Widespread exploitation of the honeybee by early Neolithic farmers. *Nature*. 2015;527(7577):226-230. doi:10.1038/nature15757

Rosenkranz P, Aumeier P, Ziegelmann B. Biology and control of Varroa destructor. *J Invertebr Pathol*. 2010;103 Suppl 1:S96-S119. doi:10.1016/j.jip.2009.07.016

Roth MA, Wilson JM, Tignor KR, Gross AD. Biology and Management of *Varroa destructor* (Mesostigmata: Varroidae) in *Apis*

mellifera (Hymenoptera: Apidae) Colonies, *J Integr Pest Managt*. 2020; 11 (1) https://doi.org/10.1093/jipm/pmz036

Ruiz JA, Miriam Gutiérrez M, Porrini C. Biomonitoring of Bees as Bioindicators. *Bee World.* 2013; 90:3, 61-63. doi: 10.1080/0005772X.2013.11417545

Schmehl DR, Teal PE, Frazier JL, Grozinger CM. Genomic analysis of the interaction between pesticide exposure and nutrition in honey bees (Apis mellifera). *J Insect Physiol*. 2014;71:177-190. doi:10.1016/j.jinsphys.2014.10.002

Sforcin JM. Biological Properties and Therapeutic Applications of Propolis. *Phytother Res*. 2016;30(6):894-905. doi:10.1002/ptr.5605

Shen M, Yang X, Cox-Foster D, Cui L. The role of varroa mites in infections of Kashmir bee virus (KBV) and deformed wing virus (DWV) in honey bees. *Virology*. 2005;342(1):141-149. doi:10.1016/j.virol.2005.07.012

Steinhauer N, Kulhanek K, Antúnez K, et al. Drivers of colony losses. *Curr Opin Insect Sci*. 2018;26:142-148. doi:10.1016/j.cois.2018.02.004

Svoboda J. Prumyslove otravy vcel arsenem [Industrial poisoning of bees by arsenic] *Ved. Pr. vyzk. Ust. vcelaf* CSAZV. 1961; 2 : 55-60

Taavoni S, Barkhordari F, Goushegir A, Haghani H. Effect of Royal Jelly on premenstrual syndrome among Iranian medical sciences students: a randomized, triple-blind, placebo-controlled study. *Complement Ther Med*. 2014;22(4):601-606. doi:10.1016/j.ctim.2014.05.004

Takashima S, Ohari Y, Itagaki T. The prevalence and molecular characterization of Acarapis woodi and Varroa destructor mites in honeybees in the Tohoku region of Japan. *Parasitol Int*. 2020;75:102052. doi:10.1016/j.parint.2020.102052

Tantillo G, Bottaro M, Di Pinto A, et al. Virus Infections of Honeybees *Apis Mellifera*. *Ital J Food Saf*. 2015;4(3):5364. doi:10.4081/ijfs.2015.5364

Urcan A, Criste A, Dezmirean D, et al. Antimicrobial Activity of Bee Bread Extracts Against Different Bacterial Strains. Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. Animal Science and Biotechnologies, [S.l.], v. 75, n. 2, p. 85-91, dec. 2018. ISSN 1843-536X.

van der Steen JJ, de Kraker J, Grotenhuis T. Spatial and temporal variation of metal concentrations in adult honeybees (Apis mellifera L.). *Environ Monit Assess*. 2012;184(7):4119-4126. doi:10.1007/s10661-011-2248-7

Williams GR, Tarpy DR, vanEngelsdorp D, et al. Colony Collapse Disorder in context. *Bioessays*. 2010;32(10):845-846. doi:10.1002/bies.201000075

Winston ML.The biology of the honey bee. 1987.Harvard University Press, Cambridge, Massachusetts.

Zamani Z, Reisi P, Alaei H, Pilehvarian AA. Effect of Royal Jelly on spatial learning and memory in rat model of streptozotocin-induced sporadic Alzheimer's disease. *Adv Biomed Res*. 2012;1:26. doi:10.4103/2277-9175.98150

Chapter 1

Symptomatic and asymptomatic honeybees affected by Deformed Wing Virus (DWV): a macroscopic, microscopic and biomolecular study

Abstract

The Deform Wing Virus (DWV) is a single-strand RNA virus widely spread in beekeeping farms across the world. It is capable of infecting bees at every stage of development, from brood to adults, causing peculiar clinical signs as wings deformation, swollen and discoloured abdomens, high mortality. The presence of this virus is associated with the persistence, in hive, of the Varroa mite which facilitates its transmission, by feeding on honeybees. Given the significant presence of DWV in apiaries, the aim of the work was to investigate the presence/absence of the virus, and determine the relative viral load in honeybee samples with symptomatic and asymptomatic infections, through qPCR. Moreover, honeybee samples were analysed by anatomopathology and, using an innovative fixating technique, subjected to histopathology to highlight any tissue alteration.

The results obtained showed the presence of the virus in all samples analysed through biomolecular techniques, and the viral load was higher in symptomatic honeybees compared to the asymptomatic group.

Histopathology showed degenerative aspects of the hypopharyngeal glands and flight muscles in samples showing clinical signs, while samples with no apparent clinical signs highlighted the presence of plasmatocytes and granulocytes, as well as melanisation phenomena in the midgut and in the hemocele suggesting an inflammatory response.

The results suggest a possible pathological action of DWV in both symptomatic and asymptomatic infections, however further studies are needed to better define the mechanisms underpinning the evidenced microscopic alterations.

1.1 Honeybee viruses

Among the factors that threaten the health and wellbeing of honeybees, a noteworthy variety of pathogens such as bacteria, viruses, fungi and parasites are mentioned.
In recent decades, honeybee viruses have been studied for their potential impact on beekeeping productions, acquiring more and more importance in the research world.
In addition to causing high economic losses, viruses negatively affect the morphology, physiology, and behaviour of honeybees and, although they don't always show clinical signs, they are frequently associated with weakening and colony collapse
Viruses in honeybees, were first described in 1913 (White, 1913) when an American researcher attributed to a virus the "sac" appearance showed by some diseased larvae; although the causative agent (Sacbrood virus) was not characterized until 1964 (Bailey, 1964).

1.1.1 Viral structure

To date, about 22 viruses that can infect honeybees have been described, primarily pertaining to the families of Iflaviridae and Dicistoviridae (order Picornavirales) and with positive single-strain RNA (ssRNA) genomes, with two exceptions such as the Apis mellifera Filamentous Virus (AmFV) and the Apis Iridescent Virus (AIV) that harbouring a DNA genome (Tantillo, 2015).
The Chronic Bee Paralysis Virus (CBPV) and Lake Sinai Virus (LSV), are the exceptions to the rule as they have not been assigned to any family yet.
Morphologically, all viruses show isometric shaped protein capsids of approximately 20-30nm in diameter, appearing quite similar and difficult to differentiate by electron microscopy (Chen and Siede, 2007).
The capsid consists of 60 protomers, each one composed by a single molecule of 3 subunits in the outer portion Viral Protein 1 (VP1), Viral Protein 2 (VP2) Viral Protein 3 (VP3) and an additional smaller Viral Protein named VP4, located internally, in some viruses.

The size of the honeybee viruses genome ranges from 8550 to 10140 bp, and it includes a small genome-linked virus protein (VPg) and a long untranslated region (UTR), containing a clover-leaf secondary structure, at the 5' tail, and at the 3' end a polyA tail, whose length varies among viruses.

The genome organization of the open reading frames (ORF) differs between Dicistoviridae and Iflaviridae viruses. In the first case, two non-overlapping ORF, the larger (ORF1) located in the 5' half of the genome, the shorter (ORF2) located towards the 3' end; the ORF regions are separated by an intergenic region (IGR), flanked by UTRs and they encode respectively for a non-structural polyprotein (helicase, protease, a RNA-dependent RNA polymerase and VPg) and for a structural polyprotein (Valles et al., 2017a) (Fig.1.1).

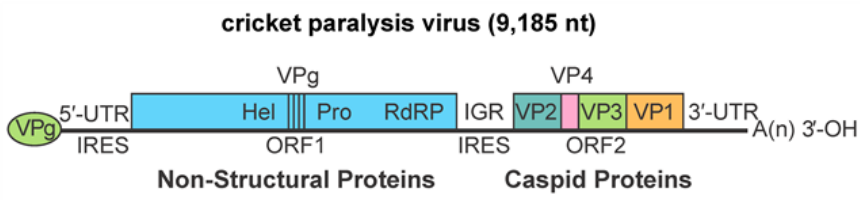

Fig 1.1. Dicistoviridae. Genome structure of cricket paralysis virus (Valles et al., 2017a).

In Iflaviridae viruses, only one large ORF is found and it is flanked by a long UTR at the 5' end and a shorter UTR at the 3' end and it translates into a single replicase polyprotein which encodes both for non-structural proteins (helicase, protease, a RNA-dependent RNA polymerase and VPg) and for structural proteins (Valles et al., 2017b) (Fig. 1.2).

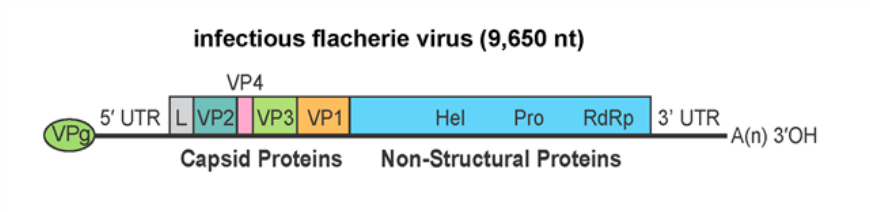

Fig 1.2. Iflaviridae. Genome structure of infectious flacherie virus (Valles et al., 2017b).

1.1.2 Transmission routes

Transmission processes determine the spread and persistence of pathogens in a population and they are strongly influenced by the behaviour and the ecology of the host.

Honeybees live in densely crowded hives, where individual workers, under the influence of queen pheromones, act together through coordinated activities to guarantee the survival of the whole colony.

This greatly organized structure generates the social interactions which establish a high contact rate between colony members, providing great opportunities for viral transmission.

Transmission of viral particles can occur via a horizontal route, when the pathogen is transmitted among individuals of the same generation, or via a vertical route, when the virus is passed form the mother to the offspring (Chen et al., 2006a) (Fig.1.3).

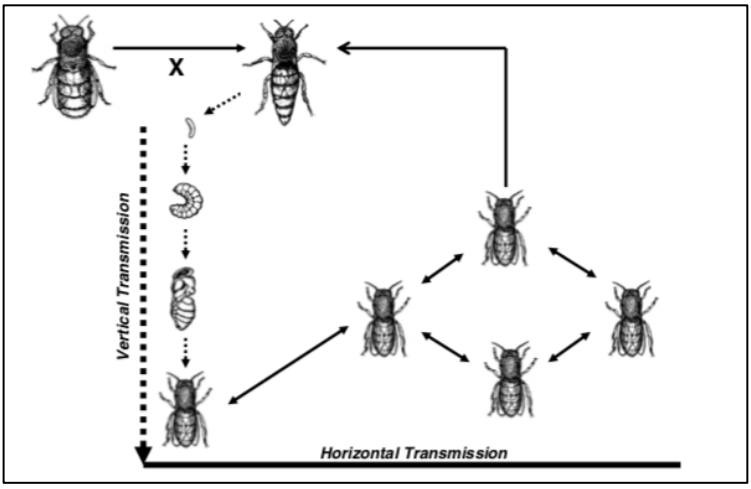

Fig.1.3. Transmission of viruses in honeybees. Solid lines represent horizontal transmission; dotted lines represent vertical transmission (Chen et al., 2006).

Moreover, horizontal transmission can be further classified in horizontal direct transmission, when the pathogen is passed from one honeybee to another, and horizontal indirect transmission, when transmission involves the passage of the virus to a biological or physical vector that subsequently passes it to a honeybee.

Direct food-borne and oro-fecal transmissions are important horizontal direct routes for spreading viral diseases in honeybee colonies, as demonstrated by the presence of viruses in pollen, nectar, bee bread, royal jelly, honey and feces (Mazzei, 2014; de Miranda and Fries 2008; Shen et al., 2005; Chen et al., 2006a). Moreover, the high trophallaxis rates, as well as behaviour like brood feeding and removing feces and dead bees form cells, make it very easy for a virus to spread through the colony.

One of the most underestimated routes of transmission is the venereal, the horizontal direct pathway from drones to queens via infected semen.

The first report of viral sequences in semen of drones, was described by Yue et al., (2006). They found Deformed Wing Virus (DWV) and Acute Bee Paralysis Virus (ABPV) sequences suggesting not only mating as a route of transmission, but also the possibility of transmission of more viruses at the same time through semen.

Further studies, have shown by electron microscopy the presence of viral particles, probably DWV, in mucus glands and testicles of drones (Cruz-Landim et al., 2012). Moreover, the presence of viral genome of 11 different honeybee viruses, was found in 91% of samples of drone ejaculates used for instrumental insemination, indicating collected semen as a risk factor for virus spreading, as well as confirming the possibility of venereal transmission in honeybees (Prodělalová et al., 2019).

Considering that honeybee queens can naturally mate with a great number of drones, and that instrumental insemination is gaining everyday more importance in beekeeping practices, the chance of a queen to become infected remains high even if a small number of drones are carrying a virus (Yue et al., 2006).

Thus, virus-containing semen, is a possible source of virus-positive eggs, offspring and queens (Chen et al., 2005). The venereal transmission was confirmed by the presence of viral RNA in the queen spermatheca and ovaries (Chen et al., 2006b).

Moreover, this specific finding suggested the possibility of vertical transmission, from the queen to eggs and offspring, hypothesis that was confirmed by finding viruses in queen tissues and feces and the detection of the same viruses in queens' eggs and larvae (Chen et al., 2005).

To date, probably, the most studied route is the vector-born and particularly great attention has been given to the role played by the mite *Varroa destructor* in the transmission of viruses to honeybees, and in the collapse of colonies.

Varroa has been associated to numerous outbreaks of viral disease such as Slow Paralysis Virus (SPV), Black Queen Cell Virus (BQCV), Kashmir Bee Virus (KBV), Sac Brood Virus (SBV), ABPV, CBPV, and DWV, and the role of the mite in acquiring and transmitting these viruses from one individual to another has been demonstrated in several studies. The role of *Varroa* as a vector will be further discussed in a specific paragraph.

The different transmission pathways strongly influence host-pathogen interaction as well as the virulence of the virus, which are both important for the survival of the virus. A specific model has been designed for honeybee viral epidemiology: when a colony is in healthy conditions,

viruses spread via vertical transmission and exist in latent and or persistent state without causing any signs of infections, while under stressful conditions viruses leave the latent state, increase the number of virions produced, propagate via horizontal transmission, becoming more virulent and leading to the death of the honeybees and to the possible collapse of the colony (Chen et al., 2006a).

1.1.3 Pathology

Depending on the different pathways of infection and on the health status of the colonies, viruses can cause symptomatic infections, i.e. overt or clinical, and asymptomatic infections, i.e. covert or subclinical (de Miranda and Genersch, 2010). The former is characterized by high levels of virus particle production, to which the insect either succumbs or survives according to the status of the immune system. Symptomatic infections show evident symptoms of the disease, which therefore can be diagnosed on a clinical basis, demonstrating a causal relationship between the pathogen and the clinical picture.

These symptomatic infections can be further divided into acute and chronic: the acute involve the active multiplication of the virus, with a high titre of viral particles in a short time, and cause rapid death of the host with evident clinical signs. A special case of symptomatic acute infections is hyperacute infections, characterized by production of high viral titres in a short time showing no signs before the sudden death (Dimmock and Primrose, 1987).

Chronic infection, on the other hand, implies a slow but constant production of viral particles during the life of the host, or during the duration of the infected life stage, with subsequent appearance of clinical signs.

Both types of infection are transmitted horizontally and negatively act on the vitality and fitness of the host to varying degrees (de Miranda and Genersch, 2010).

On the contrary, asymptomatic infections are characterized by the absence of obvious symptoms, although there could still be a hidden cost for the host, persistence of the virus beyond life stage and vertical transmission.
Asymptomatic infections can be classified as latent and persistent (Dimmock and Primrose, 1987). In the first case there is either the integration of the viral genome into the host genome but without replication occurring or if it occurs it is incomplete, or the viral genome is present as an extrachromosomal episome. In the second case, there is a constant but low production of viral particles in the host cells, without the death of the host; this type of infection represents an important source for the transmission of the virus within a population. For persistence, viruses must: (1) infect the host cells through his whole life stages; (2) escape the host's immunological surveillance system; (3) regulate the expression of its genes and host genes allowing replication and residence within the affected cells without causing their lysis. Either the infected cell survives or the limited number of dead cells is counterbalanced by the production of new cells and no net cell loss occurs. Persistent infections, therefore, represent a balance between host and persistent viral replication, where despite the infection, the host cell is not destroyed.
To date, true persistence and true latency processes have not yet been demonstrated in honeybee virology, but they cannot be excluded as the presence of covert infection was demonstrated for many viruses (de Miranda and Genersch, 2010).
Moreover, asymptomatic infections can become symptomatic when the host homeostasis is unbalanced by stressors such as other pathologies, food deficiencies, and other environmental factors (Chen et al., 2006a).

1.1.4 The role of Varroa destructor

Numerous studies have shown that most viruses establish a close association with other pathogens, particularly with *V. destructor* (Genersch and Aubert, 2010; Anguiano-Baez et al., 2016) (Fig.1.4).

Fig.1.4. V. destructor on a A. mellifera worker thorax (Roth et al., 2020).

Their synergistic action determines a negative effect superior to the one deriving from the single presence of the virus and could be the basis of honeybee mortality and colony collapse (de Jong et al., 1982; Allen et al., 1986).

In fact, honeybee viruses have been known for more than 50 years and where generally considered harmless until *V. destructor* made its appearance in the European honeybee colonies in the 70s (de Jong et al., 1982). Since then, there has been an increase in viral outbreaks, connected to the presence of high loads of mites in hives and to the lack of specific defences that *A. mellifera* still does not posses (Locke, 2016).

V. destructor is an obligated parasite; this means that the entire lifecycle is spent with its host. The lifecycle of *Varroa* takes place in two steps: a phoretic phase, in which adult mites, parasitize adult honeybees, nurse honeybees preferably (Ramsey et al. 2018), and a reproductive phase, during which the mite reproduces and it occurs within capped brood cells, preferably drone cells (Fig. 1.5).

Mites can transfer between colonies naturally through robbing, drifting of drones, and worker honeybees homing errors (Seeley and Smith, 2015), or anthropogenically by moving or combining brood frames and food frames between colonies (Fries and Camazine, 2001).

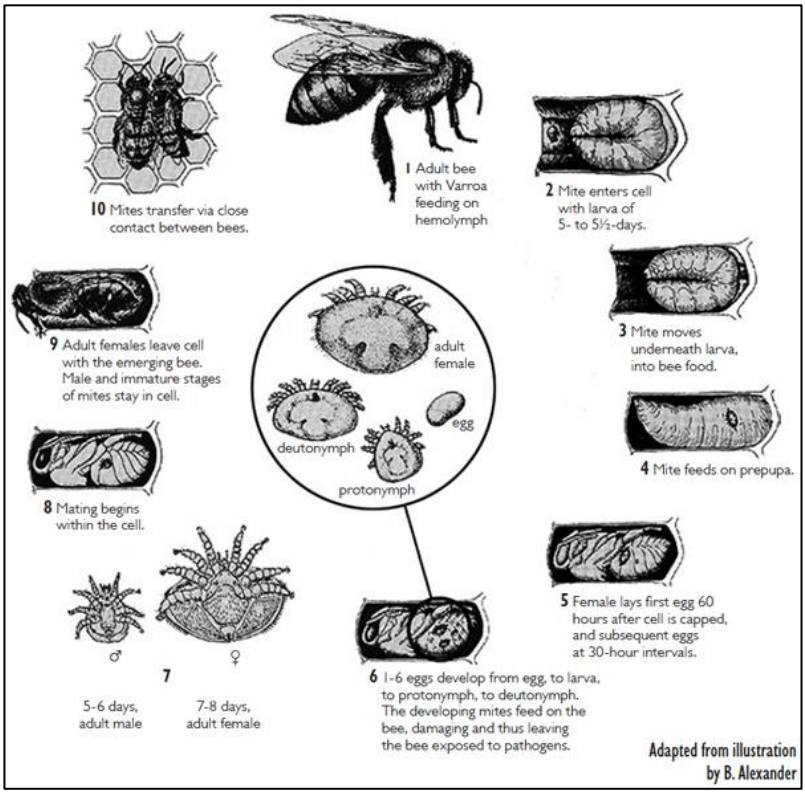

Fig.1.5. V. destructor life cycle (Huang adapted from B.Alexander 1985- https://bee-health.extension.org/varroa-mite-reproductive-biology)

Field observations indicate that in colonies heavily infested with *Varroa* there is a very broad pathological picture, indicated as "parasitic mite syndrome", characterized by the emergence of secondary diseases that are known to be transmitted by *V.destructor* (Shimanuki et al., 1994).
The mechanisms by which *Varroa* acts are:

1) transmission of viruses and activation of latent infections by viral multiplication (Chen and Siede, 2007);
2) traumatic injuries caused by feeding *Varroa* on the larvae, pupae and adults and substances subtraction (Ramsey et al., 2018);
3) inhibition of the release of digestive enzymes, which normally function as defence mechanisms that limit viral replication (Bowen- Walker et al., 1999)

According to studies published so far, *V.destructor* feeds on hemolymph and the saliva released during the meal could contain immunosuppressive proteins that would promote viral transmission and replication. Consequently, *Varroa* could cause weakening of the immune system, increasing the receptivity of honeybees towards secondary pathologies and reducing the life span of the host. Especially pupae parasitized by mites can have a compromised immune system and therefore are more susceptible to virus infections (Yang and Cox-Foster, 2005).
This theory has recently been questioned by a study that has instead highlighted the ability of *Varroa* to feed on the substances contained in the fat body, a tissue similar to the liver of mammals, and not hemolymph. The fat body is considered the "honeybees' liver" due to its role of detoxification. Moreover, it is important for its immune function, and larval and winter sustenance; therefore, by feeding on this tissue, the parasite causes a depletion of the defensive function of the organism making it more susceptible to viral pathologies (Ramsey et al., 2019).
On the contrary, a study by Annoscia et al., (2019), has corroborated the importance of hemolymph feeding (removal) in destabilizing viral immune control, thus enhancing pathogen virulence.
The outcome of the mite infestation, actually depends on numerous factors such as: the absolute number of *Varroa* mites, the presence and titre of viral infection, the prevalence and viral titre in the population of *Varroa*, the pathogenicity of the viral strain, the susceptibility of bees.

1.2 Deformed Wing Virus (Dwv)

DWV is currently the most common virus in apiaries worldwide and is one of the most widely studied.
Three variants have been described DWV-A, DWV-B and DWV-C, which differ in virulence and distribution (Mordecai et al., 2016b).
Master variant A contains the classical DWV strains and Kakugo virus (KV), while DWV-B includes the Varroa destructor viruses (VDV); several recombination between different strains and master variants have

been found and described in the field (Zioni et al., 2011; Ryabov et al., 2014).

DWV is a major pathogen of honeybees and its prevalence, strictly connected with the ectoparasite *V.destructor*, strongly increases honeybee colony mortality (Dainat et al., 2012). DWV is a low pathogenic virus that is capable of infecting all stages of development of honeybees, from eggs to adults, although it shows a higher replication in pupae (Chen et al., 2005; Sanpa and Chantawannakul, 2009). It takes its name from the characteristic symptom that manifests itself in newly-hatched honeybees with deformed or underdeveloped wings; these honeybees, unable to fly, die shortly after emerging from the cell. Initially, the deformity of the wings had been attributed to the action of the *Varroa* mite, as the symptoms were more evident in conjunction with a strong infestation by the parasite. Subsequently, experimental studies confirmed DWV as the etiological agent of wing deformity, emphasizing the association between the virus and the clinical sign. However, although DWV is one of the few honeybee viruses to have its own characteristic clinical manifestation, it is known also to be present in apparently healthy colonies (Brettell et al., 2017).

1.2.1 Genetics

DWV is a virus belonging to the genus Iflavirus, family Iflaviridae of the order Picornavirales (Lanzi et al., 2006) and it produces 30 nm icosahedral particles.

It has a positive single-stranded RNA genome of approximately 10,000 nucleotides and the whole genome encodes one large, uninterrupted open-reading frame that is translated into polyproteins that are co-translationally and post-translationally cleaved by viral proteases to produce structural (capsid-forming) from the N-terminal part and non-structural proteins from the C-terminal part (Bowen-Walker et al., 1999). The three major structural proteins VP1, VP2 and VP3 form a protomer, an elementary building block of the virus capsid (Škubník et al., 2017; Koziy et al., 2019),

while a small VP4, is located internally. The whole capside consists of 60 promoters. The non-structural proteins can be regarded as a replicase unit composed by a RNA helicase, 3C protease and RNA-dependent RNA polymerase (Fig. 1.6).

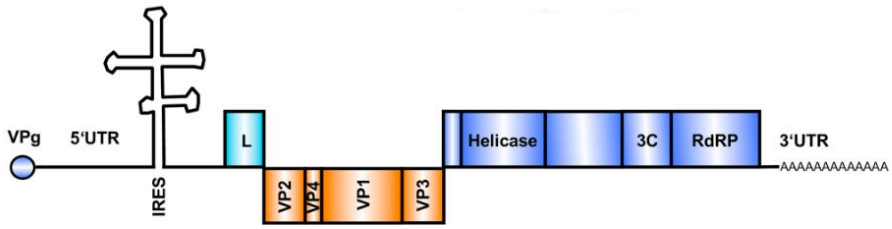

Fig. 1.6. Genome structure of DWV (modified from Lamp et al., 2016).

1.2.2 Epidemiology

DWV is the most prevalent virus in honeybees, with a minimum average of 55% of apiaries infected across 32 countries (Martin and Brettell, 2019). The virus was first isolated in the 1982 in the UK by Bill Baley and Brenda Ball from dead Japanese honeybees showing particular deformity of wings (Baily and Ball, 1991).

Soon after honeybees from colonies from UK, Belize and South Africa died showing DWV symptoms.

Ten years after, in the UK the virus was found in *Varroa*-infested colonies and it was then found in every location where *Varroa* was well established. Due to the link with *Varroa* and following the huge spread of it around the world between 1970 and 1980, DWV altered its epidemiology and has currently a global distribution (Yue and Genersh, 2005).

Except for Australia, Uganda and Canadian island of Newfoundland, where the *Varroa* mite has not been found, the presence of this particular virus has been reported in Africa, Asia, Europe, North America and South America (Allen and Ball, 1996).

Although the pathology and virulence of DWV remain linked to horizontal vectored transmission by *Varroa*, the presence of DWV has been demonstrated also in the absence of *Varroa*.
In 2008, in Leksand many DWV infected colonies were discovered in complete absence of mites and no prior contact with mite-infested bees or mite-infested regions (Forsgren et al., 2012).
As previously stated, DWV appears in three master variants DWV-A, DWV-B and DWV-C, plus numerous recombinations, often more virulent than the masters, probably do to the RNA-dependant RNA polymarase (Gisder et al., 2018).
DWV-A is the most common genotype found in North America (Kevill et al., 2019); while DWV-B has been recorded mainly in Europe, with a high prevalence in the UK, Africa, Asia (Natsopoulou et al., 2017). However, while in 2010 the virus was found only in 3% of colonies around the USA, in 2016 it was found in 65% of colonies at the expense of DWV-A (Ryabov et al., 2017), suggesting the possibility of competition among these two variants. Disparities in virulence, differences in fitness, such as replication rate, host interaction and mortality or a combination of factors could be the causes.
Studies on the relative virulence of DWV-A and DWV-B, have highlighted that both can similarly produce symptoms, such as wing deformities, and mortality after injection into pupae, but DWV-B has been suggested to be considerably more virulent than DWV-A at colony and at individual level (McMahon et al., 2016). Moreover, DWV-B has shown to have a protective role against DWV-A infections by superinfection exclusion, that is high infections with DWV-B are able to prevent infections with DWV-A by competing for the replication site of the virus (Mordecai et al., 2016a).
DWV-C is only occasionally found in *Apis mellifera* colonies, although it is regarded as a dominant variant in the Brazilian *Melipona subnitida* bees.
Recent studies have shown that DWV is present in more than 64 species and highly prevalent not only in honeybees, but also in more than 29 arthropod species associated with honeybee hives (Martin and Brettell, 2019). However, within insects, DWV was found only in bumblebee

species *Bombus terrestris* and *Bombus pascuorum,* wasp species *Vespula vulgaris and Vespa crabro* and *Lasius* spp. ants (Forzan et al., 2017; Martin and Brettel, 2019; Schläppi et al., 2020). However, further studies are needed to better understand the virus prevalence and pathogenicity in different hosts.

1.2.3 Transmission pathways and clinical signs

A recent study on adult honeybee populations has shown that about 90% of apiaries have a latent DWV infection. According to the epidemiological model proposed by Chen et al (2006a), viruses choose the mode of transmission based on the epidemiological, ecological, physiological and developmental conditions of the colony. As stated in previous paragraphs, two distinct moments of viral presence and infection can be recognized: in healthy and viable colonies, viruses carry out a vertical transmission, remaining latent / persistent without determining evident clinical signs. Vice versa, in weak "stressed" colonies, viruses can abandon the state of latency, considerably reproduce, and are transmitted horizontally, increasing their virulence and causing the death of single individuals and depopulation of the colony (Chen et al., 2006a).
The main trigger for DWV remains the uncontrolled *Varroa* infestation.
In the absence of *Varroa*, the DWV can be present in colonies with no damaging effects, and can be found in all developmental stages, from eggs to adults, as well as in drones and queens. It is transmitted vertically, from queens to offspring, which leads to the selection of less virulent viruses that do not affect bee health, leaving the animal in the conditions to reproduce and guarantee its transmission to the offspring (Fries and Camazine, 2001). The vertical transmission was suggested by the detection of viral sequences in the ovaries and spermatheca of the queen, and as well as in the eggs and sperm.
The vertical route of transmission was confirmed by Yue et al., (2007), when virgin queens from mite-infested hives but laying DWV-negative eggs, were inseminated with DWV-positive sperm that resulted in DWV-

positive fertilized and unfertilized eggs, which developed respectively in DWV-positive workers and drones, but showing no signs of infection.
This study also highlighted the existence of a venereal pathway of transmission by instrumental insemination methods, but it has been shown that the virus can be transmitted and infect queens also during natural mating leading to impairment of the reproductive capacity, negatively affecting the performance and productivity of the colony (Amiri et al., 2016).
The extent of the transmission of DWV is determined by the ability of drones to inseminate a virgin queen with infected sperm; subsequently by transovaric route, the queen transmits the virus to her offspring (de Miranda and Fries 2008). This type of transmission is normally characterized by the absence of clinical signs of disease and it can condition the persistence of DWV in the colony.
DWV is transmitted via horizontal direct routes among adult honeybees through common visits to flowers, pollen and nectar collection, trophallaxis, hygienic behaviour, care behaviour and cannibalism. Food sources, such as flowers have been regarded as a hotspot of viral particles. It has been proved that honeybees are able to deposit viruses on flowers and that traits like flower morphology or number of open flowers, can strongly influence deposit modalities (Durrer and Schmid-Hempel, 1997; Alger et al., 2019). Moreover, the presence of viral RNA in pollen and nectar, may suggest the infection of workers during foraging activities and the transportation of the virus in the hive. Here the virus is passed between worker honeybees and nurse honeybees and larvae, through feeding and trophallaxis, as suggested by the presence of DWV in larval food (Yue and Genersch, 2005; Yue et al., 2007).
In a study by Lamp et al., (2016), a molecular clone of DWV was inoculated in pupae, and the presence of DWV particles was detected in hypopharyngeal and mandibular glands by immuhistochemistry, strengthening the importance of horizontal route of transmission.
The detection with RT-PCR of DWV in feces and in the midgut epithelium and in the midgut content by in-situ hybridation, indicates the presence of a faecal-oral transmission (Fievet et al., 2006; Chen et al., 2006b).

As no signs of disease nor evident fitness impairment, have been described in larvae, pupae or adults infected via horizontal direct transmission, it could be that this route generates asymptomatic infections (de Miranda and Genersch, 2010).
Varroa-mediated vector transmission from adult honeybees to developing pupae is responsible for the display of clinical signs (Dainat et al. 2012; Bowen -Walker et al., 1999) such as early pupal death, deformed wings, shortened and swollen abdomen and discoloration of the cuticle in adult bees, and learning deficiencies (Yue and Genersch 2005) (Fig.1.7).

Fig.1.7. A. mellifera displaying clinical signs of DWV (Roth et al., 2020).

Symptomatic DWV infection occur primarily during autumn and in highly mite-infested colonies, where it constitutes predictive marker for winter colony losses (Dainat and Neumann, 2013; Bowen-Walker et al., 1999).
Varroa mites, in which the virus replicates (Gisder et al., 2009), pierce the thin areas of the bee integument, and through the penetration of its mouthpart, it inoculates the virus inside the host's hemolymph, determining high viral titres and clinical signs (Reyes-Quintana et al., 2019; Brettell et al., 2017; Bowen-Walker et al., 1999).
It was initially hypothesized that the parasitic mite had a central role in challenging its host immune system through feeding and by releasing particular secretions contained in its saliva (Richards et al., 2011).

Nowadays, this theory has been challenged by many studies, also highlighting the capacity of the DWV to directly reduce honeybee immunocompetence (Di Prisco et al., 2016).
The mite is with no doubt one of the main factors, jointly with other stressors, to promote uncontrolled viral replication and passage from common asymptomatic infections to dramatically catastrophic symptomatic infections (Nazzi et al., 2012).
Among the other stressors, temperature decline could increase severity of viral infections in newly emerged honeybees (probably explaining the high levels of winter losses), while pesticides and poor nutrition could trigger honeybee immune system making them more susceptible to viral infections, leading to colony collapse (Nazzi and Pennacchio, 2018; Di Prisco et al., 2011).

1.2.4 Treatment

There is no doubt that the impact of viral diseases, especially DWV, in apiaries is a global threat to beekeeping and it is associated to honeybee colony loss (Tehel et al., 2019). Possible treatments against viral infections in honey bees are not known and legally recognized to date. However, a deep knowledge of the crucial aspects of the viral pathogenicity, are very important for realizing an effective control program. Currently, a suitable treatment against *Varroa* is the best approach to fight DWV, since, after treatment, there is a gradual reduction of viral titres in colonies (Locke et al., 2017; Martin et al., 2010). In Italy, for the acaricidal treatment, products based on thymol, oxalic acid dihydrate, formic acid, amitraz, flumethrin, tau-fluvalinate have been authorized; the latter three are not authorized in organic beekeeping.
The therapeutic protocol must take into account the reproductive cycles of *Varroa* and include two treatments to be carried out in the spring-summer period and in the pre-winter period, possibly simultaneously by all beekeepers in the area (Note of the Ministry of Health n.13022 / 2020- Linee guida per il controllo dell'infestazione da *Varroa destructor*, 2020).
Depending on the molecule used, it will be necessary to consider that the

administrations during the autumn must be done in the absence of brood and in the absence of a honey super, while for the summer treatment the absence of the honey super is sufficient. At the same time it is important to carry out suitable interventions of beekeeping techniques such as removal of the drone brood, blockage of the brood or confinement of the queen, production of swarms and nuclei (Note of the Ministry of Health n.8845 / 2019- *Varroa* control plan, 2019).

Different mixes of various essential oils, vitamins, minerals and herb treatments, evaluating heat, water, and ultrasounds, have been evaluated as potential mite control techniques (Rosenkranz et al., 2010; Tlak-Gajger et al., 2013).

Researcher's are always working in trying to identify any possible treatment to fight directly DWV.

An increasing interest has been increasing in RNA interference (RNAi)-based methodologies and their potential application in controlling honeybee viruses ad RNAi is the strongest defence mechanism in honeybees (Niu et al., 2014).

Deasi et al., 2012 have demonstrated that by feeding instar larvae of *A.mellifera* with a double-stranded RNA DWV construct before infecting them under laboratory conditions, the viral titres as well as the wing deformities reduce, increasing honeybee longevity, probably acting on RNAi.

The success of using this treatment method also in other pathologies, suggests that RNAi could be potentially used for reducing viral infections in honeybee colonies around the world (Hunter et al., 2010; Maori et al., 2009).

A new study has evaluated the effects of the oral administration of 1,3-1,6 β-Glucans, in DWV naturally infected newly emerged honeybees; the results showed a reduction of the viral load and a dose-dependent activation of phenoloxidase (PO) (Felicioli et al., 2020), confirming that the use of β-glucans as a dietary supplement improves honeybees' immune defences (Mazzei et al., 2016).

1.3 Honeybee immune response

Honeybees, as many other social insects, have developed both social and individual strategies to fight diseases.

"Social immunity" is a term used to define the behavioural cooperation among individuals in colonies led to create a collective defence against predators and pathogens (Evans and Spivak, 2010).

These behaviours range from acts like grooming, detection and transport of sick bees out from the hive (hygienic behaviour), removal of dead material (undertaking behaviour), social fever, can reduce the spread of pathogens through members of the hive (Wilson-Rich et al., 2009).

Honeybee "social fever" is a defensive function consisting in elevating the temperature inside the nest in order to defend the colony against predators and pathogens infections, like Nosemosis and Chalkbrood (Starks et al., 2000; Martín-Hernández, 2009).

Another example of social behaviour is the use of propolis, a mixture of substances, mainly resins, with antibacterial, antifungal, antiviral, antiparasitic, anti-inflammatory, antioxidant activity (Przybyłek and Karpiński., 2019). It is used by honeybees not only to mummify intruders, disinfect comb cells, sterilise nurseries, creating a safer environment in the colony, but also as an up-regulator of transcription of individual immunity genes, namely three CYP6AS cytochrome P450 genes (Mao et al., 2013; Mura et al., 2020).

Also, antimicrobial venom peptides are often rubbed on the comb wax, as an antiseptic device (Baracchi et al., 2011).

Regarding the individual immunity, honeybee venom is also present on the cuticle of adult bees, and can be considered as a chemical barrier and a first line defence against pathogens in individuals. The exoskeleton cuticle and the peritrophic membranes of the digestive tract, also are considered as a first line defence as they prevent pathogens from entering the body, and have access to the cells (Evans and Spivak, 2010).

If unfortunately, a pathogen manages to surpass these physical barrier, cellular and humoral immune responses will be activated as a second line of defence (Evans et al., 2006). The cellular response consists in activation of hemocytes function including, phagocytosis, nodulation, encapsulation

of the pathogen, what in pathology is defined as “granulomatosus reaction”, and melanisation (DeGrandi-Hoffman and Chen, 2015), catalysed by pro-phenoloxidase (PPO) (Decker and Jaenicke, 2004). The humoral response involves secretion of antimicrobial peptides (AMP), and other effectors, melanisation, and the enzymatic degradation of pathogens by different pathways (Evans et al., 2006).

Richardson et al., (2018), have identified and described the presence of two predominant cell types involved in the cellular response: granulocytes and plasmatocytes. Granulocytes exhibit a strong propensity for phagocytosis while plasmatocytes are involved in the encapsulation activity (Ribeiro and Brehélin, 2006).

Various AMP have been described in the hemolymph of honeybees, and are grouped in four families: apidaecins, abaecin, hymenoptaecin and defensins. They act by generating holes in the prokaryotic membranes and by inhibiting the bacterial protein translation and folding (Danihlík et al., 2015).

The main pathways include: Janus kinase/Signal Transducer and Activator of Transcription (Jak/STAT); RNAi; Toll via Nuclear Factor κB/Dorsal (NF-κB); Immune deficiency (Imd) via NF-κB/Relish; c-Jun N-terminal kinase (JNK); and Mitogen-Activated Protein Kinases (MAPK), as well as orthologues of genes involved in the heat shock response (Hsp), autophagy, eicosanoid biosynthesis, endocytosis, melanisation, and PPO response (Evans et al., 2006; Brutscher et al., 2016) (Fig.1.8).

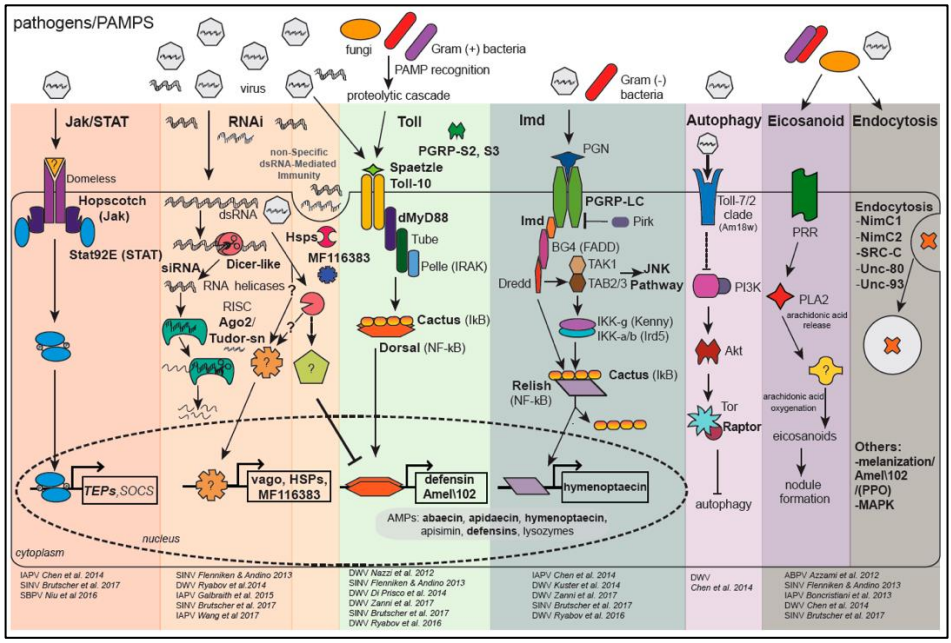

Fig.1.8. Honeybee immune pathways, highlighting genes implicated in antiviral immune responses (McMenamin et al., 2018).

The activation of the immune response is stimulated by the interaction between the pathogen-associated molecular patterns (PAMPs), such as viral double strand RNA (dsRNA), bacterial peptidoglycans, and fungal β-glucans located on the pathogen surface, and the pattern recognition receptors (PRRs); this interaction triggers different signalling pathways according the type of pathogen (McMenamin et al., 2018). Gram-positive bacteria and/or fungi are thought to stimulate the Toll pathway, leading to up-regulation of Dorsal, and while the Gram-negative can stimulate the Imd pathway, leading to up-regulation of Relish (Galiana-Arnoux et al., 2006). The antiviral defence in insects is achieved mainly via RNAi, however recent data suggest that the Toll and IMD pathways also contribute to defence against viral pathogens (Nazzi and Pennacchio, 2018). The initial evidence of the role of RNAi in antiviral defence was a study demonstrating that feeding sucrose containing IAPV-specific dsRNA, diminished the titres of IAPV caused by further infections and increased honeybee survival, generating higher honey yields and bigger colonies (Maori et al., 2009). Since then, great effort has been put in the

study of RNAi due also to its potential role as a commercial treatment or prevention tool (Brutscher el al., 2016; Desai et al., 2012).

1.3.1 Impairment of honeybee viral immune defence

A strong and efficient immune system is the key for honey bee health and colony fitness. The immune system is constantly challenged by numerous stressors leading to a higher susceptibility to pathogens.
One of the most studied relation is the one between nutrition and immunity. A balanced diet, in which not only carbohydrates are present but also high quality proteins, is required for building a strong immunity (DeGrandi-Hoffman and Chen, 2015). However, while the role of carbohydrates is well known and numerous substitutes are present in commerce, the importance of pollen and beebread, which provide amino acids and nutrients required for the synthesis of peptides involved in the immune pathways and AMP components, is often underestimated (Di Pasquale et al., 2013; Yi et al., 2014).
Moreover, it has been proved that the lack of proteins causes an unbalance in the hemocytes population by increasing the percentage of granulocytes, while decreasing the percentage of plasmatocytes (Szymas and Jedruszuk, 2003).
Quantity and quality of the pollen influences the immune competence of honeybees. A study by Alaux et al., (2010), demonstrated that diet diversity improved hemocytes concentration, fat body content and PO and glucose oxidase (GO) activity.
Honeybees, as many other pollinators, are exposed to a great number of pesticides that, when not acting acutely by killing whole colonies, could generate sublethal effects on the immune system. A growing body of literature have provided strong confirmation to the theory of the impact of pesticides on the impairment of honeybee immune system.
Chlorothalonil, a broad spectrum organochlorine fungicide, is not considered as acutely toxic in adult honeybees but has been proved to decrease the survival of honeybees in viral infections, probably as a consequence of immunity impairment (O'Neal et al., 2019).
Neonicotinoids such as thiaclorpid, imidacloprid and thiamethoxam are

considered as immunosuppressive as it was demonstrated their ability to reduce hemocyte density and encapsulation response, and to down-regulate AMPs (Brandt et al., 2016; Tesovnik et al., 2017).
Moreover, Di Prisco et al., (2013), have demonstrated that the neonicotinoid clothianidin can, as well as imidacloprid, negatively modulate the NF-κB immune pathway in insects and adversely affect the antiviral defences, by intensifying the transcription of a gene which encodes for a leucine-rich repeat (LRR) protein, a negative modulator of the NF-κB activation, promoting the replication of DWV.
Also acaricides such as thymol, coumaphos and formic acid, can alter metabolic pathways entailed in the cellular response and JNK pathways, thus interfering with the health of individual honeybees and colony survival (Boncristiani et al., 2016).
However, it should be noticed that the effects of pesticides are emphasized by the synergistic action of *Varroa*, especially in DWV infections.
The honeybee immune competence is strongly influenced by the interaction between DWV and *Varroa*, that can generate the so called "immunosuppressive syndrome"
The parasite *V.destructor* by feeding on honeybee hemolymph is able to destabilise the immune balance that keeps under control the asymptomatic infections of DWV, determining increased replication of the virus and taking advantage of the immune suppression exerted by the virus (Nazzi and Pennacchio, 2018).
DWV adversely affects humoral and cellular immune response by down-regulation of the NF-κB pattern, and interfering with numerous responses such as melanisation, encapsulation and AMP synthesis, thus weakening the antiviral response controlled by the Toll pathway (Di Prisco et al., 2016). In fact, it was demonstrated that melanisation and encapsulation was reduced in DWV infected honeybees, subsequent to a reduction of the transcription of the gene *Amel/102* that was negatively correlated with the levels of the viral titre. Moreover, a strong effect was also registered on Dorsal 1A, a transcription factor in the NF-κB family as demonstrated by RNAi mediated silencing of this gene that was associated to an increase in viral titres (Nazzi et al., 2012).
The reduction of clotting and melanisation, promotes a better food uptake for the mite, resulting in higher feeding capacity and a consequent higher

viral replication (Di Prisco et al., 2016), that only stops with the death of the host.

1.4 Aim of the study

In May 2018, during a regular visit to a beehive at the apiary of the equestrian center “La Vega”, it was possible to observe the presence of numerous small honeybees with deformed wings and shortened and discoloured abdomens, suggesting the presence of a DWV infection.
Symptomatic honeybees as well as apparently healthy honeybees, were captured and taken to the laboratory of Veterinary General Pathology and Anatomical Pathology of the Department of Veterinary Medicine and Animal Productions to investigate the presence/absence of the virus, and determine the relative viral load.
Moreover, honeybees showing clinical signs were subjected to anatomopathological analysis to study any morphological alterations of organs and, by using an innovative fixating technique, to histopathological analysis to highlight any alterations of tissues.
The methods used and the results obtained are reported in the following paragraphs.

1.5 Materials and methods

1.5.1 Samples

After clinical inspection of the suspected hive infected with DWV, 50 adult honeybees were captured from the frames and transported in 50 ml tubes to the laboratory.
General data about the apiary, productions, and colony health were collected using a specific form. Once in the laboratory, 25 honeybees were placed individually in sterile 2 ml tubes and stored at -80°C for

biomolecular analysis, while the remaining 25 were fixed in 10% formalin for anatomopathological and histopathological analysis.

1.5.2 RNA extraction, reverse transcription (RT) and PCR

10/25 honeybees showing clinical signs of DWV infection (DW) and 5/25 apparently healthy bees (H) were subjected to biomolecular investigation to verify and, in case of positive results, quantify the presence of viral RNA.
Samples were individually chopped up with a sterile blade to facilitate subsequent homogenization with the TissueLyser mechanical homogenizer (Qiagen). Each sample was put in 2ml tubes along with a grinding metal bead and subjected to lysis by two steps of five minutes at 50 Hz, interspersed with a cycle of ice cooling of 2 minutes to avoid overheating and preserve the integrity of the biological molecules.
RNA was extracted and purified from genomic DNA using the RNeasy Mini Kit Plus (Qiagen) kit, according to the protocol provided by the manufacturer, and RNA concentration was measured by spectrophotometric reading.
For each sample, 250 ng of RNA were subjected to RT using the commercial iScript cDNA Synthesis Kit (Bio-Rad Laboratories), according to the manufacturer's recommendations.
Subsequently, 12.5 ng of cDNA for each sample were subjected to PCR to amplify a segment of DWV genetic material and verify the presence/absence of the virus in the samples.
At the same time, the housekeeping gene β-actin (Act β) of *A. mellifera* was amplified to ensure the presence of amplifiable cDNA in each sample.
One no template control (NTC) was included in each PCR reaction as negative control.
The set of primers used for the amplification of the genetic material of the virus and β-actin were found in literature and are shown in Tab 1.

Tab.1. Oligonucleotids used for amplification of DWV and Act β in this study.

primers 5'→ 3'	GenBank access	Product (bp)
DWV DWV-F (5- GGATGTTATCTCCTGCGTGGAA -3) * DWV-R (5-CCTCATTAACTGTGTCGTTGATAATTG -3) *	AY224602	69
Act β Actβ -F (5- ATGCCAACACTGTCCTTTCTGG -3) ** Actβ -R (5- GACCCACCAATCCATACGGA -3) **	AB023025/ GB44311	151

*(Tentcheva et al., 2006) **(Purac et al., 2016)

The thermal protocol was carried out as follows:

- 95°C for 10 min,
- 45 amplification cycles at 95°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec,
- 72°C for 5 min.

Amplification products were migrated by electrophoresis on 2.5% agarose gel in TBE buffer (Tris-Borate-EDTA) along with a 50bp molecular marker (Bioline), stained with ethidium bromide and observed under UV with the ChemiDoc gel scanner (Bio-Rad).

1.5.3 REAL-TIME PCR (qPCR) for detection of relative viral load

In order to determine a relative quantization (RQ) of viral load of the samples, a Real-Time PCR (qPCR) was carried out using the previously described primers.

For each sample tested positive for DWV in PCR, 12.5 ng of cDNA were subjected to qPCR using iTaq Universal SYBR Green Supermix kit (Bio-Rad), according to the manufacturer's instructions.

The thermal protocol was carried out as follows:

- ✓ 95°C for 3 min,
- ✓ 45 amplification cycles at 95°C for 15 sec, 60.2°C for 30 sec,

- ✓ 95°C for 10 sec, 65°C for 5 sec with 0.5°C increments until 95°C, to obtain a melting curve.

Amplification of honeybee β-actin as reference gene was also performed in parallel to allow normalization of the results and a NTC was included in the reaction as negative control.
The relative viral load was calculated as described by Mazzei et al., 2018. For each sample, a Cq value for viral amplicon was measured and a ΔCq value was generated by comparison with the corresponding Cq value of the reference gene. Then, a ΔΔCq value was obtained by comparing the ΔCq value of each sample to mean ΔCq value obtained from the healthy bees group, considered as the negative control group. This ΔΔCq value represents a normalised measure of viral load and was calculated using CFX Maestro™ software (Bio-Rad). Relative quantization using the $2^{-\Delta\Delta Cq}$ method was performed to estimate the fold change of DWV viral copies relative to the β-actin reference gene in each DW sample compared to H group.

1.5.4 Anatomopathological and histopathological analysis

After immobilization, 25/25 honeybees where observed under a stereo microscope (Microscope Axioskop HBO50, Zeiss, Milan, Italy) (Fig.1.9) to better identify any macroscopic alterations noticed with the naked eye and then placed in a 50 ml tube containing 10% buffered formalin.

Fig.1.9. Anatomopathological analysis of A. mellifera.

As the honeybee exoskeleton often prevents formalin from penetrating in the hemocele, a new protocol for fixation was developed.
After 1hour fixation in formalin, honeybees were individually injected with 10 µl of 10% buffered formalin using a micropipette and a 10 µl tip. While holding the thorax of the honeybee between the thumb and the index finger of one hand injection was performed laterally between the 3rd and 4th tergite, holding the tip parallel to the tergite in order to avoid puncturing of the gut and contamination of specimens with pollen and feces (Human et al., 2013).
After injection, honeybees were moved to 50ml tube containing 10% formalin.
24 h after, each sample was cut lengthwise in two specular halves, placed in an embedding cassette with the cut edge down and then in an automatic embedding processor.
For each half, paraffin blocks were manually created using an embedding console system and 3 µm thick sections were obtained with a microtome.
In order to facilitate sectioning, a disposable stainless steel blade for fine cuts of hard tissues was used.
Single sections were placed on the surface of hot water and then collected on a slide and dried at room temperature for 12 h.
Slides were mechanically stained with haematoxylin and eosin using an automatic tissue slide stainer and finally mounted.
Tissue preparations were observed by light microscopy (Microscope Nikon Eclipse E-600, Tokyo, Japan).

1.6 Results

1.6.1 Biomolecular results

A fragment of the expected size (69bp) of DWV was successfully amplified from 10/10 (100%) DW and 5/5 (100%) H samples by RT-PCR but not in negative control (NTC). Act β amplification (151bp products) confirmed the integrity of all analysed cDNAs. To gain insights on the

possible difference of viral load between DW bees and H group, samples were further investigated by qPCR. A successful and reproducible Cq of reference and target genes was obtained in 10/10 (100%) DW and 5/5 (100%) H samples. RQ analysis according to $2^{-\Delta\Delta Cq}$ method suggested that the viral load was higher in 9/10 DW samples (90%) compared to the H group.

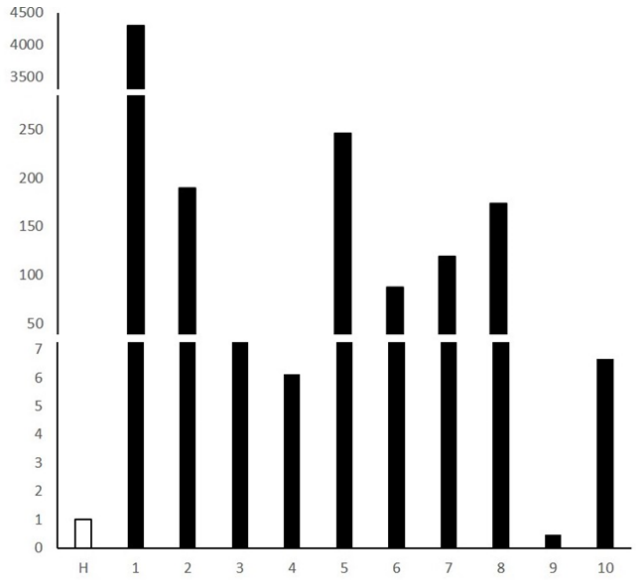

Fig.1.10. Analysis of viral load in honeybee samples showing clinical signs (1-10) compared with apparently healthy honeybee samples (H). Relative quantization data obtained by Real-time qPCR are expressed as fold change with respect to a pool of apparently healthy honeybee samples (n=5), which were set equal to 1, according to the $2^{-\Delta\Delta Cq}$ *method.*

1.6.2 Macroscopic and microscopic results

Observation of honeybees under the stereo microscope confirmed the presence of macroscopic alterations in 19/25 honeybees, namely deformed and crippled wings, discoloured and shortened abdomens (Fig.1.11), while 6/25 honeybees showed no macroscopic alterations.

Fig.1.11 A. mellifera showing clinical signs of DWV.

The histopathologic analysis of honeybees showing clinical signs revealed alterations of the hypopharyngeal glands in 19/19 honeybees and of flight muscles in 6/19 honeybees.
The hypopharyngeal glands were characterized by small irregularly shaped acini, consisting of cells showing hypercromic often fragmented nuclei and more or less abundant cytoplasm filled with numerous eosinophilic granules, probably of proteic origin, and few vacuoles. Moreover, in the gland lumen, it was noticed the presence of small cells with strongly basophilic nuclei and eoshinophilic cytoplasm. (Fig. 1.12).

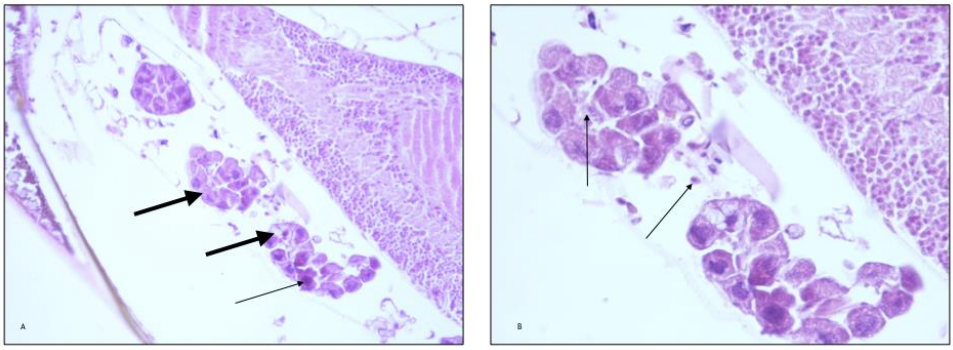

Fig.1.12. A. mellifera with symptomatic infection of DWV. Hypopharyngeal glands: A) small irregular acini showing hypercromic nucle (thin arrow)i, cytoplasm filled with eosinophilic granules and vacuoles (thick arrow). H-E. 20X. B) small cells with basophilic nuclei and eosinophilic cytoplasm in the gland lumen (thin arrow). H-E. 40X.

The flight muscles showed fibres with only few myofibrils and often not completely formed, tonofibrils were absent and the presence of many new

muscle-forming nuclei indicative of an ongoing myogenesis and incomplete maturation. Trophocytes showing nuclear fragmentation or absence of nuclei and the presence of eosinophilic material between the muscle cells, suggested degeneration of the larval fat body (Fig.1.13).

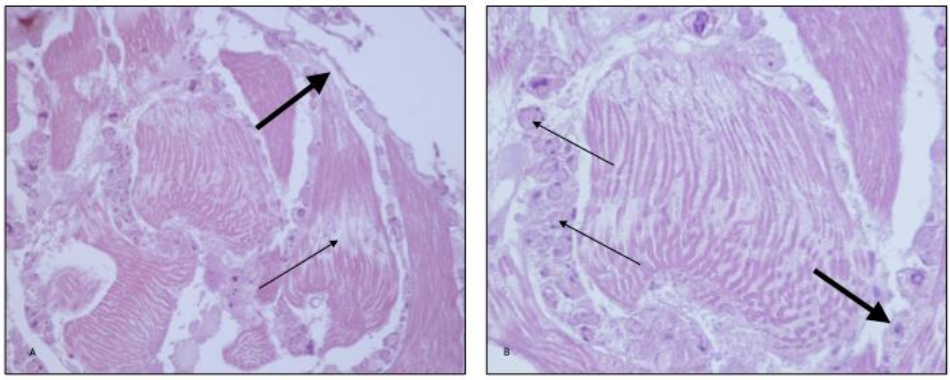

Fig.1.13. A. mellifera with symptomatic infection of DWV. Flight muscles: A) fibres with few not completely formed myofibrils(thin arrow) and absence of tonofibrils (thick arrow). H-E. 20X. B) numerous muscle-forming nuclei (thin arrow), presence of eosinophilic material between the fibres, nuclear fragmentation of trophocytes. H-E. 40X.

The microscopic observation of slides prepared from the 6 asymptomatic honeybees highlighted the presence of a great amount of melanin accumulated between the folds of the villi of the midgut, in the midgut lumen and scattered in the hemocele in 4/6 honeybee samples (Fig.1.14).

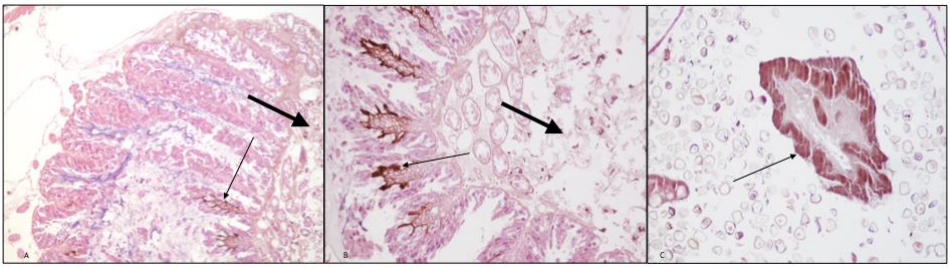

Fig.1.14. A. mellifera with asymptomatic infection of DWV. Midgut: A) melanin accumulation between the fold of the villi (thin arrow) and in the hemocele (thick arrow). H-E. 10X. B) melanin accumulation between the fold of the villi (thin arrow) and in the

hemocele (thick arrow). H-E. 20X. C) melanin accumulation in the midgut lumen (thin arrow). H-E. 40X.

Moreover, the presence of two cell populations, probably of inflammatory origin, was observed: one population was characterized by small cells, showing small and hyperchromic nuclei, often localized at the periphery of the cell, and clear, bright eosinophilic cytoplasm, identified as plasmatocytes; the second population was characterized by bigger cells with dark nuclei and granular light eosinophilic cytoplasm, compatible with granulocytes.
Plasmatocytes were localized in the hemocele and in the epithelium; granulocytes were mainly present near the fat body (Fig 1.15).

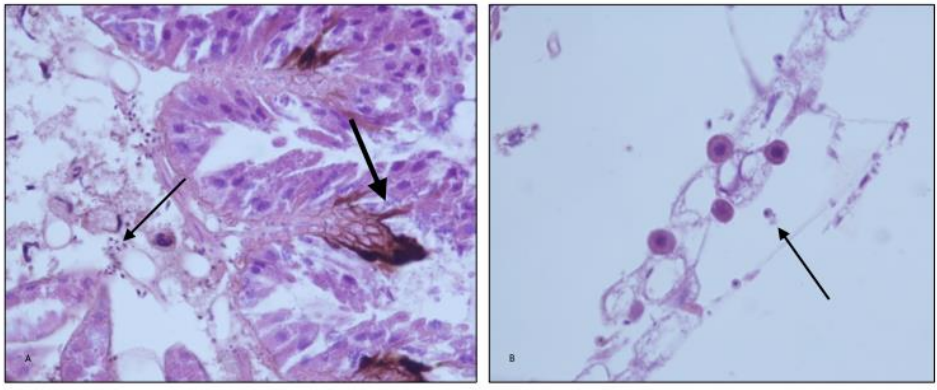

Fig.1.15. A. mellifera with asymptomatic infection of DWV. Hemocytes: A) plasmatocytes in the hemocele (thin arrow) and in the epithelium of the midgut (thick arrow). H-E. 40X. B) granulocytes in the hemocele near the fat body (thin arrow). H-E. 40X.

Where melanin deposition occurred at the basal lamina level and high infiltration of plasmatocytes was seen, high level of epithelial cell exfoliation and only few regenerative cell nests were observed; in the severest cases, epithelial cells showed pyknotic nuclei, necrosis, and disruption of whole villi was present (Fig.1.16).

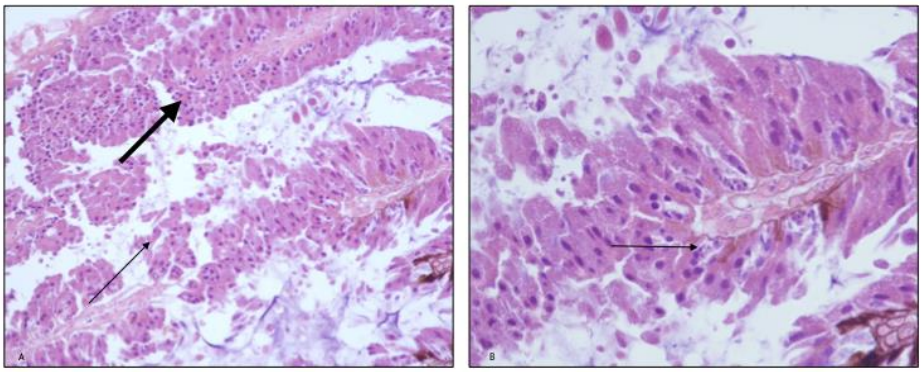

Fig.1.16. A. mellifera with asymptomatic infection of DWV. Midgut: A) epithelium exfoliation (thin arrow) and disruption of the villi (thick arrow). H-E. 40X. B) plasmatocytes infiltration in the epithelium (thin arrow). H-E. 40X.

1.7 Discussion and conclusions

DWV is recognised, in association with *V.destructor*, as one of the main causes of colony collapse.
Unlike many other viruses, it is characterised by typical clinical signs such as high pupal mortality, wing deformities, shorten, bloated and discoloured abdomens; however, many studies have shown that the virus is capable of infecting entire colonies without showing any clinical signs (Brettell et al., 2017).
In this study, honeybee samples, with and without clinical signs, were collected and subjected to biomolecular analysis in order to highlight the presence of viral genome and to anatomopathological and histopathological analysis in order to highlight the presence of any alterations of organs and tissues.
The diagnostic techniques used in our study allowed to highlight the presence of DWV RNA even in clinically healthy honeybee samples and the viral load was found to be very high in DW samples compared to H samples, corroborating the data present in literature (Brettel et al., 2017). Therefore, the absence of clinical signs may not reflect the actual state of health of a colony and, for this reason, it is necessary to identify and

develop new techniques and markers that allow the diagnosis of the asymptomatic infections.
An early diagnosis could allow the application of effective measures to contain the spread of the virus in the colony and through colonies, thus reducing the possibility of collapse of the infected colony.
Although many elements, such as the viral structure, the association with the *Varroa* mite and the transmission pathways are nowadays known (Škubník et al., 2017; Wilfert et al., 2016), many gaps in the pathogenesis still need to be filled. To accomplish this goal, researchers have mostly relied on electron microscopy or molecular techniques while histology has been used in very few studies (Organtini et al., 2017; Koziy et al., 2019). Although for other species histological protocols are well established and jointly accepted, in the case of honeybee pathology researchers are still trying to optimize the technique and fight the challenges that the peculiarity of the samples present (Silva-Zacarin et al., 2012; Hidemi, 2014; Higes et al., 2020). Small size of individuals, highly keratinized cuticle, body divided into different segments and a hollow abdomen are few of the elements that make preparation of honeybee histologic samples difficult (Hidemi, 2014). A sample that is not accurately prepared, cannot be considered reliable and could induce misinterpretation of alterations due to the presence of artefacts, thus leading to incorrect results of a study. Probably, the most challenging step is fixation, as the strongly keratinized and waxed exoskeleton makes it hard for formalin to penetrate in the hemocele and stop autolysis of the tissues, guaranteeing preservation of the morphology. Therefore, in this study, an easy and effective technique for histological fixation of honeybee samples is presented. This technique could help solving problems connected to the fixation step and limit the possibility of artefacts that could lead to inconclusive interpretation. Following this technique, organs appear compact and easy to cut with the microtome, sections appear clear and well coloured, cytological characteristics are well defined. The next step in creating and efficient protocol, would be thinking about using the same sample for both biomolecular studies and histopathology.

Biomolecular results showed an increase of DWV viral titres in DW samples compared to H samples.

Despite the low number of samples analysed, the results obtained agree with previous studies (Brettell et al., 2017; Locke et al., 2017). We can therefore imply that the also the samples used for histopathological analysis follow this rule, that is: high viral titres in honeybees with symptomatic infections and lower viral titres in honeybees with asymptomatic infections.

Honeybees exhibiting macroscopic alterations, and consequently high viral titres, showed microscopic alterations of the hypopharyngeal glands and of the flight muscles.

In *A. mellifera* the hypopharyngeal glands are part of the digestive system and, according to the role played in the colony, they are responsible for the production of royal jelly, storage of glycogen for the flight muscles, synthesis of enzymes important for the transformation of nectar into honey and for social immunity (GO) (Chan, 2009; Costa and Cruz-Landim 1999). Moreover, it has been demonstrated the presence in the hypopharyngeal glands of vitellogenin, a glycoprotein necessary for the production of immune system components and for longevity (Corona et al., 2007).

In this study hypopharyngeal glands appeared small with numerous eosinophilic granules. This seems to suggest an alteration of the secretory activity, particularly a shift towards an increase production of serous secretion, typical of foragers.

A modification in secretion, could lead to an altered production of the components of royal jelly and a consequent altered development of the larvae, which are weaker and more susceptible to the action of the virus and of other pathogens.

Moreover, it can be hypothesized that alterations of the hypopharyngeal glands could also lead to a reduced secretion of vitellogenin, and a consequent, at least partial, impairment of the immune system.

These effects, in the long run could compromise colony fitness and survival. Alterations of hypopharyngeal and mandibular glands of honeybees infected with DWV, have already been described by Koziy et

al., (2019) and our observations match what previously found, corroborating the theory of an action of the virus on these organs

At the thoracic level, DWV honeybees with clinical signs showed incomplete development of the flight muscles. In healthy honeybees, the mature muscles begin to form during pupal development by replacement of the larval muscles with mature muscles, starting from new muscle nuclei with an end-to-end trend. At the same time there is a gradual reduction of the fat body due to the degeneration of the trophocytes. The myogenesis process ends 70 hours after cell capping with the attachment of the muscles to the epidermis of the cuticle using tonofibrils (Snordgrass, 1910). The microscopic study of the flight muscles has highlighted the presence of eosinophilic material (also in the form of granules) and nuclear debris of trophocytic origin between the myofibrils and the presence of numerous immature muscle fibres detached from the cuticle, consequent to the absence of tonofibrils.

These aspects, found in adult honeybees, could be indicative of incomplete myogenesis and could be responsible of an altered development of honeybees and of a reduction of their size and inability to fly. Localization of DWV in the flight muscles was described by Lamp et al., (2016), but no microscopic alterations were described at this level.

Also in this study not all samples showed incomplete myogenesis, and the reason could be found in the different developmental moment in which the virus infects the honeybee or in the titre of the virus.

Interestingly, honeybees showing no macroscopic alterations, and lower viral titres, did not show the same tissue alterations of the ones with a clinical infection but revealed the presence of a high number of hemocytes and melanin accumulation in the hemolymph and between the midgut villi. These findings suggest a strong activation of the immune system, particularly of the cellular response.

It could be questioned whether the immune response was triggered by the presence of other pathogens, that share the same tropism for the midgut. Although it cannot be excluded that “not visible” pathogens are present (viruses), the presence of bigger pathogens such as fungi (*Nosema* spp.)

and bacteria was not evident, as no element was found neither in the cells of the midgut nor in the hemocele.
Moreover, the midgut epithelium appeared necrotic and strongly exfoliating, and as only few regenerative cell nests were present the adequate turnover that could restore the non-functional epithelium was not guaranteed.
It is intuitive that a midgut showing these alterations cannot be functional both in absorption of nutrients and secretion of substances useful for the wellbeing of the peritrophic membrane, and consequently of the honeybees.
It seems evident that, although no clinical signs are evident, something is happening to tissues and cells and that the activation of the immune response comes with a cost for the host.
This small scale study has highlighted the presence of significant morphological alterations in honeybees with or without clinical signs of DWV. The results could suggest a possible pathological action of the virus in both clinical and subclinical infections, and a possible role of the immune cellular response in keeping under control the virus in asymptomatic infections, although with a cost for the host. However, the limited number of samples does not allow to confirm the hypotheses and further studies, comprehending a bigger number of samples and the use of other techniques such as FISH and immunohistochemistry, are necessary to deepen the study and better undestrand the etiopathogenesis.

Bibliography

Alaux C, Ducloz F, Crauser D, Le Conte Y. Diet effects on honeybee immunocompetence. *Biol Lett*. 2010;6(4):562-565. doi:10.1098/rsbl.2009.0986

Alger SA, Burnham PA, Brody AK. Flowers as viral hot spots: Honey bees (Apis mellifera) unevenly deposit viruses across plant species [published correction appears in PLoS One. 2019 Nov 11;14(11):e0225295]. *PLoS One*. 2019;14(9):e0221800. doi:10.1371/journal.pone.0221800

Allen MF, Ball BV, White RF, Antoniw JF. The detection of acute paralysis virus in Varroa jacobsoni by the use of a simple indirect ELISA. *J Apicult. Res* 1986;25:100-5.

Allen MF, Ball BV. The incidence and world distribution of honey bee viruses. *Bee World*. 1996; 77:141–162

Amiri E, Meixner MD, Kryger P. Deformed wing virus can be transmitted during natural mating in honey bees and infect the queens. *Sci Rep*. 2016;6:33065. doi:10.1038/srep33065

Anguiano-Baez R, Guzman-Novoa E, Md Hamiduzzaman M, Espinosa-Montaño LG, Correa-Benítez A. Varroa destructor (Mesostigmata: Varroidae) Parasitism and Climate Differentially Influence the Prevalence, Levels, and Overt Infections of Deformed Wing Virus in Honey Bees (Hymenoptera: Apidae). *J Insect Sci*. 2016;16(1):44. doi:10.1093/jisesa/iew029

Annoscia D, Brown SP, Di Prisco G, et al. Haemolymph removal by Varroa mite destabilizes the dynamical interaction between immune effectors and virus in bees, as predicted by Volterra's model. *Proc Biol Sci*. 2019;286(1901):20190331. doi:10.1098/rspb.2019.0331

Bailey L, Gibbs AJ, Woods RD. Sacbrood virus of the larval honey bee (apis mellifera linnaeus). *Virology*. 1964;23:425-429. doi:10.1016/0042-6822(64)90266-1

Bailey L., Ball B.V., Honey bee pathology, Academic Press, London, 1991

Baracchi D, Francese S, Turillazzi S. Beyond the antipredatory defence: honey bee venom function as a component of social immunity. *Toxicon*. 2011;58(6-7):550-557. doi:10.1016/j.toxicon.2011.08.017

Boncristiani H, Underwood R, Schwarz R, Evans JD, Pettis J, vanEngelsdorp D. Direct effect of acaricides on pathogen loads and gene expression levels in honey bees Apis mellifera. *J Insect Physiol*. 2012;58(5):613-620. doi:10.1016/j.jinsphys.2011.12.011

Bowen-Walker PL, Martin SJ, Gunn A. The transmission of deformed wing virus between honeybees (Apis mellifera L.) by the ectoparasitic mite varroa jacobsoni Oud. *J Invertebr Pathol*. 1999;73(1):101-106. doi:10.1006/jipa.1998.4807

Brandt A, Gorenflo A, Siede R, Meixner M, Büchler R. The neonicotinoids thiacloprid, imidacloprid, and clothianidin affect the immunocompetence of honey bees (Apis mellifera L.). *J Insect Physiol*. 2016;86:40-47. doi:10.1016/j.jinsphys.2016.01.001

Brettell LE, Mordecai GJ, Schroeder DC, et al. A Comparison of Deformed Wing Virus in Deformed and Asymptomatic Honey Bees. *Insects*. 2017;8(1):28. doi:10.3390/insects8010028

Brutscher LM, McMenamin AJ, Flenniken ML. The Buzz about Honey Bee Viruses. *PLoS Pathog*. 2016;12(8):e1005757. Pudoi:10.1371/journal.ppat.1005757

Chan QW, Melathopoulos AP, Pernal SF et al. The innate immune and systemic response in honey bees to a bacterial pathogen, *Paenibacillus larvae*. *BMC Genomics*. 2009;10**,** 387 https://doi.org/10.1186/1471-2164-10-387

Chen YP, Siede R. Honey bee viruses. *Adv Virus Res*. 2007;70:33-80. doi:10.1016/S0065-3527(07)70002-7

Chen Y, Evans J, Feldlaufer M. Horizontal and vertical transmission of viruses in the honey bee, Apis mellifera. *J Invertebr Pathol*. 2006a;92(3):152-159. doi:10.1016/j.jip.2006.03.010

Chen Y, Pettis JS, Collins A, Feldlaufer MF. Prevalence and transmission of honeybee viruses. *Appl Environ Microbiol*. 2006b;72(1):606-611. doi:10.1128/AEM.72.1.606-611.2006

Chen YP, Pettis JS, Feldlaufer MF. Detection of multiple viruses in queens of the honey bee Apis mellifera L. *J Invertebr Pathol*. 2005;90(2):118-121. doi:10.1016/j.jip.2005.08.005

Corona M, Velarde RA, Remolina S, et al. Vitellogenin, juvenile hormone, insulin signaling, and queen honey bee longevity. *Proc Natl Acad Sci U S A*. 2007;104(17):7128-7133. doi:10.1073/pnas.0701909104

Costa RAC, Cruz-Landim C. Occurrence and morphometry of the hypopharyngeal glands in *Scaptotrigona postica* Lat. (Hymenoptera, Apidae, Meliponinae). J Biosci. 1999; 24,97-102. https://doi.org/10.1007/BFo2941113

Da Cruz-Landim C, Roat TC, Fernadez FC. Virus present in the reproductive tract of asymptomatic drones of honey bee (Apis mellifera l.), and possible infection of queen during mating. *Microsc Res Tech*. 2012;75(7):986-990. doi:10.1002/jemt.22024

Dainat B, Neumann P. Clinical signs of deformed wing virus infection are predictive markers for honey bee colony losses. *J Invertebr Pathol*. 2013;112(3):278-280. doi:10.1016/j.jip.2012.12.009

Dainat B, Evans JD, Chen YP, Gauthier L, Neumann P. Dead or alive: deformed wing virus and Varroa destructor reduce the life span of winter honeybees. *Appl Environ Microbiol*. 2012;78(4):981-987. doi:10.1128/AEM.06537-11

Danihlík J, Aronstein K, Petřivalský M. Antimicrobial peptides: a key component of honey bee innate immunity. J Apic Res. 2015; 54(2):123-136

de Jong D, Morse RA, Eickwort GC. Mite pests of honeybees. *Annu. Rev. Entomol*.1982; 27:229–25

de Miranda JR, Fries I. Venereal and vertical transmission of deformed wing virus in honeybees (Apis mellifera L.). *J Invertebr Pathol*. 2008;98(2):184-189. doi:10.1016/j.jip.2008.02.004

de Miranda JR, Genersch E. Deformed wing virus. *J Invertebr Pathol*. 2010;103 Suppl 1:S48-S61. doi:10.1016/j.jip.2009.06.012

Decker H, Jaenicke E. Recent findings on phenoloxidase activity and antimicrobial activity of hemocyanins. *Dev Comp Immunol*. 2004;28(7-8):673-687. doi:10.1016/j.dci.2003.11.007

DeGrandi-Hoffman G, Chen Y. Nutrition, immunity and viral infections in honey bees. *Curr Opin Insect Sci*. 2015;10:170-176. doi:10.1016/j.cois.2015.05.007

Desai SD, Eu YJ, Whyard S, Currie RW. Reduction in deformed wing virus infection in larval and adult honey bees (Apis mellifera L.) by double-stranded RNA ingestion. *Insect Mol Biol*. 2012;21(4):446-455. doi:10.1111/j.1365-2583.2012.01150.x

Di Pasquale G, Salignon M, Le Conte Y, et al. Influence of pollen nutrition on honey bee health: do pollen quality and diversity matter?. *PLoS One*. 2013;8(8):e72016. doi:10.1371/journal.pone.0072016

Di Prisco G, Annoscia D, Margiotta M, et al. A mutualistic symbiosis between a parasitic mite and a pathogenic virus undermines honey bee immunity and health. *Proc Natl Acad Sci U S A*. 2016;113(12):3203-3208. doi:10.1073/pnas.1523515113

Di Prisco G, Cavaliere V, Annoscia D, et al. Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bees. *Proc Natl Acad Sci U S A*. 2013;110(46):18466-18471.

Di Prisco G, Pennacchio F, Caprio E, Boncristiani HF Jr, Evans JD, Chen Y. Varroa destructor is an effective vector of Israeli acute paralysis virus in the honeybee, Apis mellifera. *J Gen Virol*. 2011;92(Pt 1):151-155. doi:10.1099/vir.0.023853-0

Dimmock NJ, Primrose SB, Introduction to modern virology. 3rd Edition. Blackwell Scientific Publications, Oxford. 1987

doi: 10.1080/00218839.2015.1109919

Durrer S, Schmid-Hempel P. Shared use of flowers leads to horizontal pathogen transmission. *Proc R Soc Lond B.* 1997; 258: 299–302. http://doi.org/10.1098/rspb.1994.0176

Evans JD, Aronstein K, Chen YP, et al. Immune pathways and defence mechanisms in honey bees Apis mellifera. *Insect Mol Biol*. 2006;15(5):645-656. doi:10.1111/j.1365-2583.2006.00682.x

Evans JD, Spivak M. Socialized medicine: individual and communal disease barriers in honey bees. *J Invertebr Pathol*. 2010;103 Suppl 1:S62-S72. doi:10.1016/j.jip.2009.06.019

Felicioli A, Forzan M, Sagona S, et al. Effect of Oral Administration of 1,3-1,6 β-Glucans in DWV Naturally Infected Newly Emerged Bees (*Apis mellifera* L.). *Vet Sci*. 2020;7(2):E52. doi:10.3390/vetsci7020052

Fievet J, Tentcheva D, Gauthier L, et al. Localization of deformed wing virus infection in queen and drone Apis mellifera L. *Virol J*. 2006;3:16. doi:10.1186/1743-422X-3-16

Forsgren E, Friesm I, de Miranda JR Adult honey bees *(Apis mellifera)* with deformed wings discovered in confirmed varroa-free colonies. *J Apic Res*. 2012; 51(1): 136-138. doi: 10.3896/IBRA.1.51.1.17

Forzan M, Felicioli A, Sagona S, Bandecchi P, Mazzei M. Complete Genome Sequence of Deformed Wing Virus Isolated from *Vespa crabro* in Italy. *Genome Announc*. 2017;5(40):e00961-17. doi:10.1128/genomeA.00961-17

Fries I, Camazine S. Implications of horizontal and vertical pathogen transmission for honey bee epidemiology. *Apidologie*. 2001;32(3): 199-214

Galiana-Arnoux D, Dostert C, Schneemann A, Hoffmann JA, Imler JL. Essential function in vivo for Dicer-2 in host defense against RNA viruses in drosophila. *Nat Immunol*. 2006;7(6):590-597. doi:10.1038/ni1335

Genersch E, Aubert M. Emerging and re-emerging viruses of the honey bee (Apis mellifera L.). *Vet Res*. 2010;41(6):54. doi:10.1051/vetres/2010027

Gisder S, Möckel N, Eisenhardt D, Genersch E. In vivo evolution of viral virulence: switching of deformed wing virus between hosts results in virulence changes and sequence shifts. *Environ Microbiol*. 2018;20(12):4612-4628. doi:10.1111/1462-2920.14481

Gisder S, Aumeier P, Genersch E. Deformed wing virus: replication and viral load in mites (Varroa destructor). *J Gen Virol*. 2009;90(Pt 2):463-467. doi:10.1099/vir.0.005579-0

Hidemi A. Preparation of honey bee specimens for histopathological studies, including a technique for the preparation of whole sections. *J Apic Res*. 2014; 53:3, 385-391, doi: 10.3896/IBRA.1.53.3.06

Higes M, García-Palencia P, Urbieta A, Nanetti A, Martín-Hernández R. *Nosema apis* and *Nosema ceranae* Tissue Tropism in Worker Honey Bees (*Apis mellifera*). *Vet Pathol*. 2020;57(1):132-138. doi:10.1177/0300985819864302

Human H, Brodschneider R, Dietemann V et al. Miscellaneous standard methods for Apis mellifera research. In V Dietemann; J D Ellis; P

Neumann (Eds) The COLOSS BEEBOOK, Volume I: standard methods for Apis mellifera research. *J Apic Res*.2013; 52(4) http://dx.doi.org/10.3896/IBRA.1.52.4.10

Hunter W, Ellis J, Vanengelsdorp D et al. Large-scale field application of RNAi technology reducing Israeli acute paralysis virus disease in honey bees (Apis mellifera, Hymenoptera: Apidae). *PLoS Pathog*. 2010;6(12):e1001160. doi:10.1371/journal.ppat.1001160

Kevill JL, de Souza FS, Sharples C, Oliver R, Schroeder DC, Martin SJ. DWV-A Lethal to Honey Bees (*Apis mellifera*): A Colony Level Survey of DWV Variants (A, B, and C) in England, Wales, and 32 States across the US. *Viruses*. 2019;11(5):426. doi:10.3390/v11050426

Koziy RV, Wood SC, Kozii IV, et al. Deformed Wing Virus Infection in Honey Bees (*Apis mellifera* L.). *Vet Pathol*. 2019;56(4):636-641. doi:10.1177/0300985819834617

Lamp B, Url A, Seitz K, et al. Construction and Rescue of a Molecular Clone of Deformed Wing Virus (DWV). *PLoS One*. 2016;11(11):e0164639. doi:10.1371/journal.pone.0164639

Lanzi G, de Miranda JR, Boniotti MB, et al. Molecular and biological characterization of deformed wing virus of honeybees (Apis mellifera L.). *J Virol*. 2006;80(10):4998-5009. doi:10.1128/JVI.80.10.4998-5009.2006

Locke B, Semberg E, Forsgren E, de Miranda JR. Persistence of subclinical deformed wing virus infections in honeybees following Varroa mite removal and a bee population turnover. *PLoS One*. 2017;12(7):e0180910. Published 2017 Jul 7. doi:10.1371/journal.pone.0180910

Locke, B. Natural Varroa mite-surviving Apis mellifera honeybee population. *Apidologie*. 2016; 47, 467-482. https://doi.org/10.1007/s13592-015-0412-8

Mao W, Schuler MA, Berenbaum MR. Honey constituents up-regulate detoxification and immunity genes in the western honey bee Apis mellifera [published correction appears in Proc Natl Acad Sci U S A. 2016 Jan 26;113(4):E488]. *Proc Natl Acad Sci U S A*. 2013;110(22):8842-8846.

Maori E, Paldi N, Shafir S, et al. IAPV, a bee-affecting virus associated with Colony Collapse Disorder can be silenced by dsRNA ingestion. *Insect Mol Biol*. 2009;18(1):55-60. doi:10.1111/j.1365-2583.2009.00847.x

Martin SJ, Brettell LE. Deformed Wing Virus in Honeybees and Other Insects. *Annu Rev Virol*. 2019;6(1):49-69. doi:10.1146/annurev-virology-092818-015700

Martin SJ, Ball BV, Carreck NL. Prevalence and persistance of deformed wing virus (DWV) in untreated or acaricide-treated Varroa destructor infested honey bees (Apis mellifera) colonies. *J Apic Res.* 2010; 49, 72–79.

Martín-Hernández R, Meana A, García-Palencia P, et al. Effect of temperature on the biotic potential of honeybee microsporidia. *Appl Environ Microbiol*. 2009;75(8):2554-2557. doi:10.1128/AEM.02908-08

Mazzei M, Forzan M, Carlucci V, et al. A study of multiple Felis catus papillomavirus types (1, 2, 3, 4) in cat skin lesions in Italy by quantitative PCR. *J Feline Med Surg*. 2018;20(8):772-779. doi:10.1177/1098612X17732255

Mazzei M, Fronte B, Sagona S, et al. Effect of 1,3-1,6 β-Glucan on Natural and Experimental Deformed Wing Virus Infection in Newly Emerged Honeybees (Apis mellifera ligustica). *PLoS One*. 2016;11(11):e0166297. Pubdoi:10.1371/journal.pone.0166297

Mazzei M, Carrozza ML, Luisi E, et al. Infectivity of DWV associated to flower pollen: experimental evidence of a horizontal transmission route. *PLoS One*. 2014;9(11):e113448. doi:10.1371/journal.pone.0113448

McMahon DP, Natsopoulou ME, Doublet V, et al. Elevated virulence of an emerging viral genotype as a driver of honeybee loss. *Proc Biol Sci*. 2016;283(1833):20160811. doi:10.1098/rspb.2016.0811

McMenamin AJ, Daughenbaugh KF, Parekh F, Pizzorno MC, Flenniken ML. Honey Bee and Bumble Bee Antiviral Defense. *Viruses*. 2018;10(8):395. doi:10.3390/v10080395

Mordecai GJ, Brettell LE, Martin SJ, Dixon D, Jones IM, Schroeder DC. Superinfection exclusion and the long-term survival of honey bees in

Varroa-infested colonies. *ISME J.* 2016a;10(5):1182-1191. doi:10.1038/ismej.2015.186

Mordecai GJ, Wilfert L, Martin SJ, Jones IM, Schroeder DC. Diversity in a honey bee pathogen: first report of a third master variant of the Deformed Wing Virus quasispecies. *ISME J.* 2016b;10(5):1264-1273. doi:10.1038/ismej.2015.178

Mura A, Pusceddu M, Theodorou P, et al. Propolis Consumption Reduces *Nosema ceranae* Infection of European Honey Bees (*Apis mellifera*). *Insects*. 2020;11(2):124. Published 2020 Feb 15. doi:10.3390/insects11020124

Natsopoulou ME, McMahon DP, Doublet V, Frey E, Rosenkranz P, Paxton RJ. The virulent, emerging genotype B of Deformed wing virus is closely linked to overwinter honeybee worker loss. *Sci Rep*. 2017;7(1):5242. Published 2017 Jul 12. doi:10.1038/s41598-017-05596-3

Nazzi F, Brown SP, Annoscia D, et al. Synergistic parasite-pathogen interactions mediated by host immunity can drive the collapse of honeybee colonies. *PLoS Pathog*. 2012;8(6):e1002735. doi:10.1371/journal.ppat.1002735

Nazzi F, Pennacchio F. Honey Bee Antiviral Immune Barriers as Affected by Multiple Stress Factors: A Novel Paradigm to Interpret Colony Health Decline and Collapse. *Viruses*. 2018;10(4):159. Published 2018 Mar 30. doi:10.3390/v10040159

Niu J, Meeus I, Cappelle K, et al.The immune response of the small interfering RNA pathway in the defense against bee viruses. *Curr. Opin. Insect Sci.*2014; 6, 22–27. doi: 10.1016/j.ibmb.2015.1 2.006

O'Neal ST, Reeves AM, Fell RD, Brewster CC, Anderson TD. Chlorothalonil Exposure Alters Virus Susceptibility and Markers of Immunity, Nutrition, and Development in Honey Bees. *J Insect Sci*. 2019;19(3):14. doi:10.1093/jisesa/iez051

Organtini LJ, Shingler KL, Ashley RE, et al. Honey Bee Deformed Wing Virus Structures Reveal that Conformational Changes Accompany Genome Release. *J Virol*. 2017;91(2):e01795-16. Published 2017 Jan 3. doi:10.1128/JVI.01795-16

Prodělalová J, Moutelíková R, Titěra D. Multiple Virus Infections in Western Honeybee (*Apis mellifera* L.) Ejaculate Used for Instrumental Insemination. *Viruses*. 2019;11(4):306. Published 2019 Mar 29. doi:10.3390/v11040306

Przybyłek I, Karpiński TM. Antibacterial Properties of Propolis. *Molecules*. 2019;24(11):2047. doi:10.3390/molecules24112047

Purać J, Nikolić TV, Kojić D, et al. Identification of a metallothionein gene in honey bee Apis mellifera and its expression profile in response to Cd, Cu and Pb exposure. *Mol Ecol*. 2019;28(4):731-745. doi:10.1111/mec.14984

Ramsey SD, Ochoa R, Bauchan G, et al. *Varroa destructor* feeds primarily on honey bee fat body tissue and not hemolymph. *Proc Natl Acad Sci U S A*. 2019;116(5):1792-1801. doi:10.1073/pnas.1818371116

Ramsey S, Gulbronson CJ, Mowery J, et al. A multi-microscopy approach to discover the feeding site and host tissue consumed by *Varroa destructor* on host honey bees. *Microsc Microanal.* 2018; 24*:*1258–1259

Reyes-Quintana M, Espinosa-Montaño LG, Prieto-Merlos D, et al. Impact of Varroa destructor and deformed wing virus on emergence, cellular immunity, wing integrity and survivorship of Africanized honey bees in Mexico. *J Invertebr Pathol*. 2019;164:43-48. doi:10.1016/j.jip.2019.04.009

Ribeiro C, Brehélin M. Insect haemocytes: what type of cell is that?. *J Insect Physiol*. 2006;52(5):417-429. doi:10.1016/j.jinsphys.2006.01.005

Richards EH, Jones B, Bowman A. Salivary secretions from the honeybee mite, Varroa destructor: effects on insect haemocytes and preliminary biochemical characterization. *Parasitology*. 2011;138(5):602-608. doi:10.1017/S0031182011000072

Richardson RT, Ballinger MN, Qian F et al. Morphological and functional characterization of honey bee Apis mellifera, hemocyte cell communities. *Apidologie*. 2018; 49, 397-410. https://doi.org/10.1007/s13592-018-0566-2

Rosenkranz P, Aumeier P, Ziegelmann B. Biology and control of Varroa destructor. *J Invertebr Pathol*. 2010;103 Suppl 1:S96-S119. doi:10.1016/j.jip.2009.07.016

Ryabov EV, Childers AK, Chen Y, et al. Recent spread of Varroa destructor virus-1, a honey bee pathogen, in the United States. *Sci Rep*. 2017;7(1):17447. doi:10.1038/s41598-017-17802-3

Ryabov EV, Wood GR, Fannon JM, et al. A virulent strain of deformed wing virus (DWV) of honeybees (Apis mellifera) prevails after Varroa destructor-mediated, or in vitro, transmission. *PLoS Pathog*. 2014;10(6):e1004230. Published 2014 Jun 26. doi:10.1371/journal.ppat.1004230

Sanpa S, Chantawannakul P. Survey of six bee viruses using RT-PCR in Northern Thailand. *J Invertebr Pathol*. 2009;100(2):116-119. doi:10.1016/j.jip.2008.11.010

Schläppi D, Chejanovsky N, Yañez O, Neumann P. Foodborne Transmission and Clinical Symptoms of Honey Bee Viruses in Ants *Lasius* spp. *Viruses*. 2020;12(3):321. doi:10.3390/v12030321

Seeley TD, Smith ML. Crowding honeybee colonies in apiaried can increase thei vulnerability to the deadly ectoparasite *Varroa destructor. Apidologie*. 2015; 46, 716-727. https://doi.org/10.1007/s13592-015-0361-2

Shen M, Yang X, Cox-Foster D, Cui L. The role of varroa mites in infections of Kashmir bee virus (KBV) and deformed wing virus (DWV) in honey bees. *Virology*. 2005;342(1):141-149. doi:10.1016/j.virol.2005.07.012

Shimanuki H, Calderone NW, Knox DA. Parasitic mite syndrome: the symptoms. *Am Bee J.* 1994; 134:827-8.

Silva-Zacarin ECM, Chauzat MP, Zeggane S et al. Protocol for optimization of histological, histochemical and immunohistochemincal analyses of larval tissues: application in histopathology of honey bee. *Current microsocpy contribution to advances in science and technology.* 2012; 1:696-703

Škubník K, Nováček J, Füzik T, Přidal A, Paxton RJ, Plevka P. Structure of deformed wing virus, a major honey bee pathogen. *Proc Natl Acad Sci U S A*. 2017;114(12):3210-3215. doi:10.1073/pnas.1615695114

Snodgrass RE. The Anatomy of the Honey Bee. U.S. Government Printing Office. Washington 1910

Starks PT, Blackie CA, Seeley TD. Fever in honeybee colonies. *Naturwissenschaften*. 2000;87(5):229-231.

doi:10.1007/s001140050709

Szymas B, Jedruszuk A. The influence of different diets on haemocytes of adult worker honey bees, Apis mellifera. *Apidologie*. 2003; 34: 97–102.

Tantillo G, Bottaro M, Di Pinto A, Martella V, Di Pinto P, Terio V. Virus Infections of Honeybees *Apis Mellifera*. *Ital J Food Saf*. 2015;4(3):5364. doi:10.4081/ijfs.2015.5364

Tentcheva D, Gauthier L, Bagny L, Fievet J, Dainat B, Cousserans F, Colin ME, Bergoin M. Comparative analysis of deformed wing virus (DWV) RNA in Apis mellifera and Varroa destructor. *Apidologie*. 2006;37(1):41-50.

Tehel A, Vu Q, Bigot D, et al. The Two Prevalent Genotypes of an Emerging Infectious Disease, *Deformed Wing Virus*, Cause Equally Low Pupal Mortality and Equally High Wing Deformities in Host Honey Bees. *Viruses*. 2019;11(2):114. doi:10.3390/v11020114

Tesovnik T, Cizelj I, Zorc M, et al. Immune related gene expression in worker honey bee (Apis mellifera carnica) pupae exposed to neonicotinoid thiamethoxam and Varroa mites (Varroa destructor). *PLoS One*. 2017;12(10):e0187079. doi:10.1371/journal.pone.0187079

Tlak-Gajger I, Tomljanovic Z, Stanisavljevic L. An environmentally friendly approach to the control of Varroa destructor mite and Nosema ceranae disease in Carniolan honeybee (Apis mellifera Carnica) colonies. *Arch Biol Sci,* 2013*;* 65*,* 1585-1592.

Valles, S.M., Chen, Y., Firth, A.E., Guérin, D.M.A., Hashimoto, Y., Herrero, S., de Miranda, J.R., Ryabov, E., and ICTV Report Consortium. 2017a, ICTV Virus Taxonomy Profile: *Dicistroviridae*, Journal of General Virology, 98: 355–356

Valles, S.M., Chen, Y., Firth, A.E., Guérin, D.M.A., Hashimoto, Y., Herrero, S., de Miranda, J.R., Ryabov, E., and ICTV Report Consortium. 2017b, ICTV Virus Taxonomy Profile: *Iflaviridae*, Journal of General Virology, 98, 527–528.

White GF. Sacbrood, a disease of bees. US Department of Agriculture, Bureau of Entomology, Washington, DC, USA. 1913

Wilfert L, Long G, Leggett HC, et al. Deformed wing virus is a recent global epidemic in honeybees driven by Varroa mites. *Science*. 2016;351(6273):594-597. doi:10.1126/science.aac9976

Wilson-Rich N, Spivak M, Fefferman NH, Starks PT. Genetic, individual, and group facilitation of disease resistance in insect societies. *Annu Rev Entomol*. 2009;54:405-423. doi:10.1146/annurev.ento.53.103106.093301

Yang X, Cox-Foster DL. Impact of an ectoparasite on the immunity and pathology of an invertebrate: evidence for host immunosuppression and viral amplification. *Proc Natl Acad Sci U S A*. 2005;102(21):7470-7475. doi:10.1073/pnas.0501860102

Yi H, Chowdhury M, Huang Y et al. Insect animicrobial peptides and their applications. *Appl Microbiol Biotechnol*. 2014; 98, 5807-5822. https://doi.org/10.1007/s00253-014.5792-6

Yue C, Schröder M, Gisder S, Genersch E. Vertical-transmission routes for deformed wing virus of honeybees (Apis mellifera). *J Gen Virol*. 2007;88(Pt 8):2329-2336. doi:10.1099/vir.0.83101-0

Yue C, Schröder M, Bienefeld K, Genersch E. Detection of viral sequences in semen of honeybees (Apis mellifera): evidence for vertical transmission of viruses through drones. *J Invertebr Pathol*. 2006;92(2):105-108. doi:10.1016/j.jip.2006.03.001

Yue C, Genersch E. RT-PCR analysis of Deformed wing virus in honeybees (Apis mellifera) and mites (Varroa destructor). *J Gen Virol*. 2005;86(Pt 12):3419-3424. doi:10.1099/vir.0.81401-0

Zioni N, Soroker V, Chejanovsky N. Replication of Varroa destructor virus 1 (VDV-1) and a Varroa destructor virus 1-deformed wing virus recombinant (VDV-1-DWV) in the head of the honey bee. *Virology*. 2011;417(1):106-112. doi:10.1016/j.virol.2011.05.009

Chapter 2

Morphological and morphometric study of the male reproductive system of *Apis mellifera ligustica*

Abstract

It is well known that factors acting on the decrease of population of honeybees, can act on the male and female reproductive system, compromising the fertility of queens and drones. While there are many studies on female fertility, only few studies have focused on male fertility and the possible alterations of the reproductive system. The reproductive system of drones of *Apis mellifera ligustica* was analysed by anatomopathological techniques. Using an innovative, low cost, easy technique, the morphological features and morphometric parameters of spermatozoa were studied. Moreover, testes and spermatozoa were analysed by histopathology and cytopathology and the alterations that were found are here described. No macroscopic alterations were observed in any sample and by histopathology most of the samples showed unaltered testes although in some cases, testes showed degenerated seminiferous tubules, while others appeared immature. Regarding the morphometry of sperm, the following morphometric values (mean ± standard deviation) were measured: sperm total length (230,81±17,22 μm), tail length (222,96±17,15μm), head length (7,85±0,65μm), nucleus length (4,44±0,61μm) and perforator length (3,58±1,21μm). 7% of the spermatozoa were characterized by the presence of visible defects such as double, split or broken tails. The results obtained provide data about morphology of the reproductive system and morphometry and morphology of spermatozoa of drones of *A. mellifera ligustica* and show the presence of alterations.

This study was carried out in collaboration with the Unity of General Zootechnics and Genetic Improvement of the Department of Veterinary Medicine and Animal Productions of the University of Naples “Federico II”.

2.1 Drones

Drones are born from unfertilized eggs with haploid number of chromosomes (n=16).
The main role of drones in the colony organization is to spread genetic material from the colony of origin to a new colony by mating with queens from other colonies (Boes, 2010). However, drones participate to colony life also by controlling temperature and humidity by ventilation, and by helping circulation of food and pheromones.
In order to fulfil their role, drones have developed specific anatomical features: long antennae with long sensilla, big eyes with a great number of omatids, complex mating organs (Koeniger and Koeniger, 2000) (Fig. 2.1).

Fig. 2.1. A. mellifera ligustica drone showing long antennae, big eyes and everted endophallus.

Furthermore, as they do not participate actively to many colony tasks they show smaller mandibles and mandibular glands, and they lack hypopharyngeal glands as well as pollen collection devices (Hrassnigg and Crailsheim, 2005).
In colonies of *Apis mellifera*, drones typically represent 5-10% of the adult population, however the production and maintenance is regulated by the colony in accordance with several environmental factors, namely food availability, size of colony, number of drones already present in the colony, queen presence/absence and season (Boes, 2010). Drone-producing season in Italy is from mid-April to late August, but in the past

years it was not unusual to see drone brood during the Autumn. Since sexual maturation of drones is longer compared to queens (36 days vs. 22 days, respectively), drones must be produced and raised earlier to be sexually mature when mating season arrives and they are more likely to successfully mate (Koeniger et al., 2014).
Drone development requires 24 days and they can live between 20 and 40 days (Page and Peng, 2001; Rueppell et al., 2005). Drone eggs are laid in brood cells bigger then female ones; embryo development requires 3 days after which a small larva hatches from the egg and is fed by nurse honeybees. After 4-5 days of feeding, drone cells are capped and larvae develop into pupae. Finally, after 13-15 days adult drones emerge from the cells, they start to explore the comb and feed autonomously (Page and Peng, 2001). Before they become mature, young drones, aged from five to eight days, perform orientation flights to locate the Drone Congregation Area (DCA), where they will attempt to mate with a virgin queen (Koeniger et al., 2005).

2.2 The reproductive system

The male reproductive system of *A. mellifera* consists of a pair of bean shaped testes, from which start two vasa deferentia that enlarge distally forming the seminal vesicles, which open in the mucus glands. Mucus glands are elongated accessory glands that produce white mucus, a proteic substance used to produce a mating sign in the queen after a successful copulation (Koeniger et al., 2014). The mucus glands open into paired, lateral ejaculatory ducts, which in turn convey into a long, slender, common ejaculatory duct. The common ejaculatory duct opens in the bulb of the endophallus, the copulatory organ.

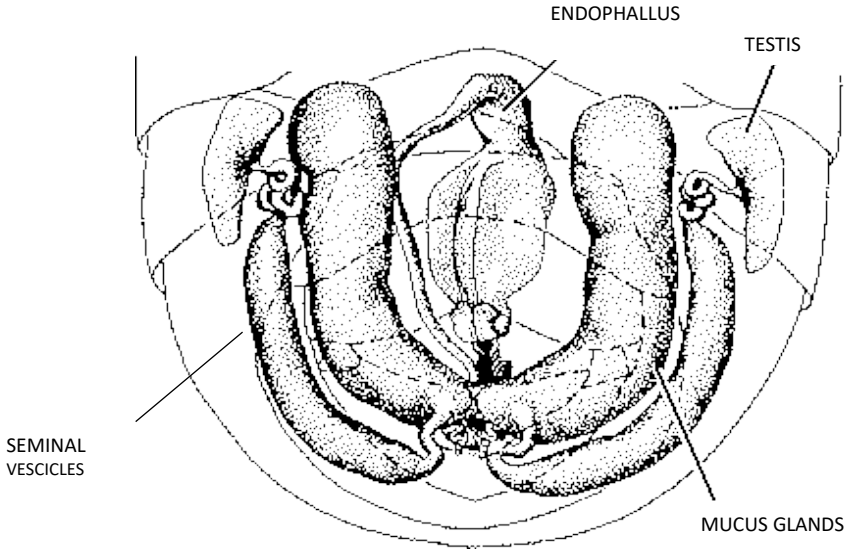

Fig.2.2. Reproductive system of a drone of A. mellifera (http://www1.biologie.uni-hamburg.de/b-online/ibc99/koning/bees.html)

The honeybee copulatory organ is located internally, in the ventral region of the abdomen and it is composed of three main elements: the bulb, provided with the chitinous plates, the cervix and the vestibulum, presenting two yellow cornua (Fig.2.2). Two more accessory sex glands, found near the endophallus bulb, are recognized: the bulbous glandand the cornual glands. The cornual glands secrete an orange-colored secretion that reinforces the attachment of the mating sign in the queen's reproductive tract (Fig. 2.3).

Fig. 2.3. Manual eversion of the endophallus of A. mellifera ligustica drone. The bulbus gland and the cornua are evident.

2.2.1 Sexual maturation of drones

In *A. mellifera* drones, formation of the male reproductive system starts during the first stages of embryonic development. Testes are formed soon before the larva hatches from the egg while spermatogenesis starts on the third day of the larval stage. Spermatogonia, the undifferentiated germ cell, undergo multiple mitoses, developing in primary spermatocytes (Snodgrass, 1910)). The primary spermatocytes are subjected to a reductional meiosis where a secondary haploid spermatocyte and one cell containing only cytoplasm, are formed. The secondary spermatocytes then undergo a non-reductional meiosis, giving origin to two spherical spermatids. Spermatid multiplication stops prior to pupation (Snodgrass, 1910) and honeybee drones seem to be the only insects in which spermatogenesis occurs only during their developmental stages, therefore, drones have a predetermined quantity of sperm in adult life (Bishop, 1920; Page and Peng, 2001). Spermiogenesis, which is the morphological differentiation resulting in spermatozoa formation, starts 2-3 days after pupal moulting, when sperm migrates from the testes to the seminal vesicles, where it remains for approximately 13 days (Koeniger et al., 2014). In the seminal vesicles, it is spermatozoa absorb nutrients to become fully functional (Ruttner, 1976). Drones are sexually mature twelve days after emergence, when they are able to evert their endophallus and creamy coloured semen, containing spermatozoa, can be located at the posterior extremity of the ejaculate, on top of the white mucus, which is void of spermatozoa (Koeniger et al., 2014).

2.2.2 Drone semen

Drone semen originates from the combination of sperm and seminal fluid produced by the seminal vesicles. The seminal fluid is mostly composed of proteins that provide for different functions, such as immunity against microbial attacks or defence against oxidative stress, energy for

spermatozoa, and metabolism of carbohydrates and lipids (Baer et al. 2012; Gorshkov et al., 2015). Moreover, it has been showed that proteins within the seminal fluid are implicated in the so called mechanism of "sperm incapacitation": inside the female reproductive tract proteins can recognize and target spermatozoa of rival males and kill them (den Boer, 2010). The seminal fluid also contains sugars such as fructose, glucose, and trehalose, which serve as energy sources for the spermatozoa (Blum et al., 1962; Verma, 1974). The composition of seminal fluid is important in maintaining viability of the spermatozoa and could influence sperm competition in the female oviducts by enhancing or reducing fertilization success of sperm (Baer et al., 2012).

Drones produce an average of 1.5-1.7 microliter semen with approximately 7.5 million spermatozoa/µl (Rousseau et al., 2015). The semen of mature drones is usually yellow-creamy in colour and it becomes darker and thicker with age (Czekonska et al., 2013; Rousseau et al., 2015). An active debate is still open on the influence of age on sperm quality, quantity and viability: some have shown that sperm viability decreases with age (Locke and Peng, 1993), while others observed an increased viability with age (Czekonska et al., 2013). According to Rousseau et al. (2015), age has no effect on spermatozoa viability and motility, while for Stürup et al. (2013) senescence negatively influences sperm viability only from 20-25 days after emergence, but the length of viability decrease is influenced by colony factors, especially genetics. Sperm viability seems to be also influenced by high temperature and immune stress, while lack of pollen had no significant effect on sperm viability. While it is still unclear whether sperm viability increases or decreases with age, the drone's ability to mate certainly varies with age (Rhodes et al., 2011).

2.3 Mating

Among livestock, honeybees show unique reproductive features. Honeybees are polyandrous, this means that queens mate with multiple

males, and this behaviour is not found in many other social insects (Gençer and Kahya 2011). The honeybee exhibits the most extreme degree of polyandry, as queens can mate with 6-18 drones in one or more nuptial flights (Woyke, 1956). Polyandry is an important reproductive phenomenon for honeybees because it guarantees diversification of honeybee workers' genetic profiles optimising their productivity, task performance, reducing parasitism, while queens mated with only one drone produce weaker colonies (Tarpy, 2003; Page et al., 2006; Koeniger et al., 2014). Moreover, polyandry shows two important consequences: sperm competition and cryptic female choice, that will be discussed in the next paragraphs.

Honeybee mating takes place in the afternoon, when weather is optimal to fly to DCAs, so when it is sunny and there is no wind or rain (Ruttner, 1956; Koeniger et al., 2014). The DCA is already established when the virgin queen, escorted by a group of workers, arrives to mate (Winston et al., 1991). The young queen mates for a short period of time (2-3 days) and can go on one or multiple mating flights until she reaches the optimum volume of semen deposited (Tarpy and Page, 2001). As soon as she starts laying eggs, she never conducts any more mating flights (Winston, 1987). To avoid inbreeding, queens and drones from the same colony fly to different DCAs, with queens flying up to 2-3 km away from the colony of origin and drones remaining nearby the colony, in order not to consume too much valuable energy while flying (Koeniger et al., 2014).

In a DCA more than 10,000 to 30,000 drones of different genetic lineages, originating from approximately 240 colonies, await (Baudry et al., 1998). Therefore, the numerical sex ratio is intensively male-biased as it is estimated at 1000-2000 males per female and it might be at the reason of the "suicidal male mating", a tool to lower the possibility of a second copulation (Boomsma et al., 2005).

During the mating flight, drones fly in the DCA at 15 to 60 m above the ground and form a big circle (Loper et al., 1987). Drones are visually attracted to the queen when she arrives and releases her sex pheromones (Koeniger and Koeniger, 2000). Once in the DCA, over 20-100 drones pursue the queen, forming a "mating comet" behind her, ready to copulate

(Koeniger, 1990). These drones follow her and, eventually, one will grasp the queen and mate (Fig. 2.4). Female mate choice has been considered as successful copulation is consequent to the queens' decision to allow a male to insert his endophallus (Strassman, 2011), but in the end it is the fittest males who can overcome obstacles and survive to copulate with the queen. As only the strongest and fastest drones are able to rapidly find the DCA and the queen, queens potentially mate with the fittest drones (Jaffe and Moritz, 2010).

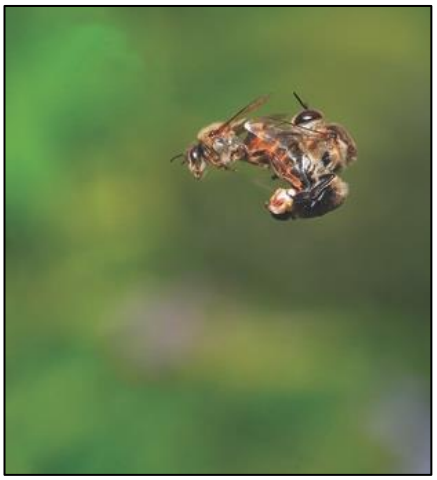

Fig. 2.4. A queen and a drone of A. mellifera mating (https://ppp.purdue.edu/resources/ppp-publications/the-complex-life-of-the-honey-bee/).

Copulation takes less than 5 seconds (Gary, 1963). The flying drone mounts the queen from behind, once the male holds the queen tightly, the drone or inserts the first half of his endophallus, thanks to the contraction of abdominal muscles, in the queen's open vagina (Woyke, 2008). When the queen contracts the vagina, full eversion of the drone's endophallus is completed and ejaculation occurs with sperm being transferred to the queen's lateral oviducts (Koeniger et al., 2014).

Once ejaculation is completed, the drone dies leaving the detached bulb from the endophallus as a "mating sign" in the queen's sting chamber (Woyke, 2010), sign that will be removed by the following drone. The

mating sign increases the queen's visibility to attract additional drones in the DCA, in order to reduce the time of queen's mating flight and save energy (Koeniger, 1991; Koeniger, 1990).
After this mating period, the queen returns to the hive and the honeybee workers remove the mating sign of the last copulation (Koeniger et al., 2014). The queen receives approximately 200 million spermatozoa in her lateral oviducts, but the spermatheca can only contain 5-7 million sperm (Koeniger, 1991). The excess will be expelled from the queen's oviducts, by contraction of the abdominal muscles, while the spermatozoa migrate into the spermatheca where they will be stored for progressive fertilization during her lifetime.

2.4 Sperm competition and cryptic female choice

Sperm competition "occurs when ejaculates of different males compete for the opportunity to fertilize a given set of eggs" (Baer, 2005). It is still unclear whether sperm competition occurs in honeybees and how it works (Moritz, 1986; Harbo 1990; Woyciechowski and Krol, 1996; Shafir et al., 2009). Sperm competition may occur after mating, during sperm storage, so when spermatozoa compete for access to the spermatheca, or when eggs get fertilized (Woyciechowski and Krol, 1996; Shafir et al., 2009; Tofilski et al., 2012). However, different findings indicate that competition during sperm storage is unlikely as sperm completely mixes after being stored and paternities are generally not highly biased towards one or few males (Franck et al., 2002). Therefore, competition most likely occurs during fertilization and it is in the interest of the colony that the healthiest, fittest and most viable spermatozoa fertilizes each egg, so natural selection for superior semen is important (den Boer et al., 2008)
For each egg, the queen releases 4 to 25 sperm (Yu and Omholt, 1999) from the spermatheca (Harbo, 1990) and drones producing more viable motile sperm increase their chance to fertilize eggs.
Cryptic female choice "occurs when a female influences male reproductive success after two or more males have copulated with her"

(Baer, 2005), it is defined as cryptic as the process is hidden to the male. As a matter of fact, the migration of sperm from lateral oviducts of the queen to the spermatheca, only occurs once the queen has returned to the hive, so any manipulation of the ejaculates happens when drones are no longer present. The main reason for this process, seems to be the attempt to equalize the proportion of the sperm from different males in order to increase genetic variability among the offspring, thus the performance and fitness of the colony (Schlüns et al., 2005).
Although in other species, cryptic female choice can occur during egg fertilization, in honeybees it is more likely to occur during the sperm storage process, as spermatozoa from different males are stored mixed in a single spermatheca and it would be difficult for the queen to select sperm from specific males (Baer, 2005).
Therefore, it can only occur during the sperm storage process as the queen is able to reject entirely or partially the ejaculates, by contracting the muscles of the oviduct backwards or by closing the bursa copulatrix (Snodgrass, 1910).

2.5 Instrumental insemination

Instrumental insemination (II) is a crucial technique to control animal breeding, to accelerate genetic selection and to improve desirable characters such as higher productions. Considering the peculiar reproductive behaviour of honeybees, II is a valuable tool to control the source of males and avoid undesired mating with drones that could negatively influence genetics of the colony.
Numerous improvements have been made since the first methods for artificial inseminations in honeybees were developed (Watson, 1928), and although techniques are quite difficult to execute, it has become a successful tool for beekeepers and researchers in breeding programs and genetic studies (Collins, 2004).
The techniques consist in anesthetizing and immobilizing the queen with CO_2, manually opening the sting chamber, collecting drone semen and

injecting it with a syringe in the vagina (Cobey, 2013) (Fig. 2.5).

Fig. 2.5. Instrumental insemination of A. mellifera ligustica. Queen is immobilized, sting chamberis opened and semen is injected in the vagina.

The sophisticated equipment and the wide knowledge required, together with lack of standardization in the techniques, make II in honeybee queens, not an easy task.
Moreover, as in all species, quality of semen highly influences the fertility outcome. The minimum sperm viability should be around 43% (Collins, 2000), and different diluents, cryoprotectants and semen dilutions ratios have been proposed to improve viability of honeybee sperm, especially cryopreserved ones (Taylor, 2009). Less importance has been given in honeybees to parameter such as morphology and motility, that as in other species, should also be considered.

2.6 Aim of the study

The study was developed and carried out by the sector Veterinary General Pathology and Anatomical Pathology in collaboration with the Unity of General Zootechnics and Genetic Improvement, of the Department of Veterinary Medicine and Animal Productions- University of Naples "Federico II".

Drone samples of *A. mellifera ligustica* were collected and processed for semen analysis, in order to study the morphological and morphometric features of spermatozoa and highlight the presence of any abnormalities, and for anatomopathological and histopathological analysis in order to describe any alterations of the reproductive system.
Methods used and results obtained are reported in the following paragraphs.

2.7 Materials and methods

2.7.1 Sampling

125 mature drones (10-27 days old) of *A. mellifera ligustica* (Hymenoptera, Apidae) were collected in 5 different apiaries located in Campania (Italy) (25 drones/apiary) during March-June 2017, a time span that includes the natural reproductive season of local honeybees. Apiaries were located in small beekeeping farms (less than 30 hives) surrounded by orchards and tomato crops. Drones were individually collected from apparently healthy hives, with low levels of *V.destructor* infestation (<2%). Individuals were selected according to size, vitality and absence of clinical signs of pathologies, they were manually caught from the comb (Fig. 2.6) and subsequently transported in 50 ml sterile tubes to the laboratory of Veterinary General Pathology and Anatomical Pathology of the Department of Veterinary Medicine and Animal Productions.

Fig. 2.6. Comb showing the presence of honeybee workers and drones of A.mellifera ligustica

General data about the apiary, productions, and colony health were collected using a specific form. All drones were immobilised with chilling for 3 minutes at -20°C (Human et al., 2013). 100 drones (20/apiary) were used for macroscopic examination and semen analysis, while the remaining 25 (5 /apiary) were used for microscopic examination.

2.7.2 Anatomopathological analysis

After being sacrificed by removing the head with cuticle scissors, drones were pinned trough the thorax to a piece of cork with their back uppermost. The dissection of the abdomen was performed under a stereo microscope (Microscope Axioskop HBO50, Zeiss, Milan, Italy) as described by Carreck et al. (2013). Briefly, the roof of the abdomen and the gut was removed and the reproductive system was exposed. Testes, seminal vesicles and mucus glands were carefully excised and testes were analysed

to study any possible alterations.

2.7.3 Semen analysis

The reproductive organs previously removed were placed in a 1.5 ml tube (Eppendorf, Germany) containing 500 µl of 0.9% sodium chloride solution. Samples were then centrifuged (Centrifuge 5424, Eppendorf, Germany) at 250g for 10 minutes; a drop of pellet was swiped on a slide, air-dried, stained with Diff-Quick and observed with a Nikon ECLIPSE 80i (Nikon, Tokyo) light microscope (100X objective, 10X ocular).
Morphological analysis was performed on 10 spermatozoa per sample (1000 total spermatozoa), while morphometric analysis was performed only on healthy spermatozoa (930/1000). Total length of the sperm, nucleus length, head and perforator length, tail length, were measured using an image analysis software (Nikon NIS Elements 4.00.02, Nikon, Tokyo). Starting and end point of measurement are reported in Tab. 2.1. Mean values and standard deviation were calculated for each parameter.

Tab. 2.1. Starting and end point of measurement for morphometric analysis of A.mellifera ligustica spermatozoa

Parameter	**Starting and end point of measurement**
Total sperm length	Anterior tip of the perforator-caudal tip of the tail
Tail length	Point of insertion of the tail in the nucleus -caudal tip of the tail
Head length	Anterior tip of the perforator- point of insertion of perforator in the nucleus
Nucleus length	Caudal tip of the perforator- point of insertion of the tail in the nucleus
Perforator length	Anterior tip of the perforator-point of insertion of perforator in the nucleus

2.7.4 Histopathological analysis

After immobilization, 25 drones where processed for histopathological analysis as described in Chapter 1. Briefly, samples were 10%formalin fixed, embedded in paraffin wax and 3 μm thick sections were cut, stained with haematoxylin and eosin (H-E) and tissue preparation were observed by light microscopy (Microscope Nikon Eclipse E-600, Tokyo, Japan) to identify any possible alterations.

2.8 Results

The macroscopic examination of the testes, seminal vesicles and mucus glands did not reveal the presence of any visible alterations.
The spermatozoa of the Italian honeybee were characterised by a long tail and an elongated head, formed by a nucleus and a perforator (Fig. 2.7).

Fig. 2.7. A. mellifera ligustica. Morphologically normal spermatozoa showing tail, nucleus and perforator. Diff-Quick. 100X

Morphometric parameters (mean values and standard deviation) are summarized in Tab. 2.2.

Tab. 2.2. Morphometric parameters of spermatozoa of A. mellifera ligustica

Parameter	mean value ± standard deviation (μm)
Total sperm length	230,81±17,22
Tail length	222,96±17,15
Head length	7,85±0,65
Nucleus length	4,44±0,61
Perforator length	3,58±1,21

Moreover, 7% (n=70) of the samples revealed visible defects, such as split and broken tails (Fig. 2.8).

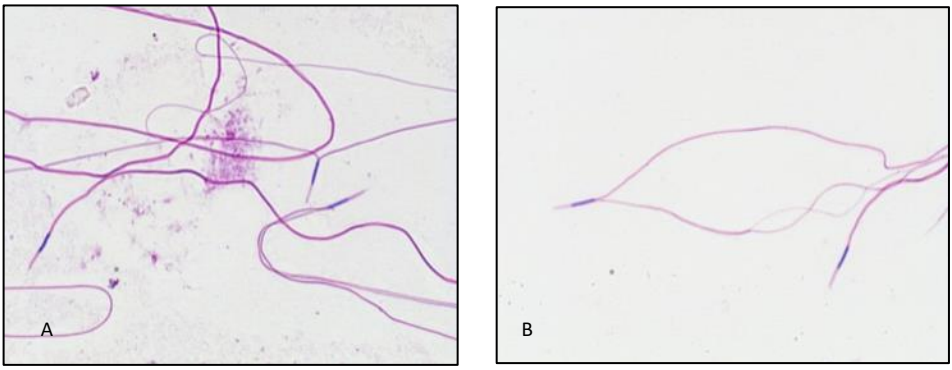

Fig. 2.8. A. mellifera ligustica. A-B) Morphologically altered spermatozoa showing split and broken tails. Diff-Quick. 100X.

Microscopically, 17/25 samples showed healthy testes that appeared as elongated, bean-shaped structures with numerous seminiferous tubules, surrounded by an external epithelial layer (seminiferous epithelium). The seminiferous tubules contained follicular and germ cells which encapsulate to form a thin wall cyst; the tubular lumen was filled with many coiled spermatozoa (Figure 2.9).

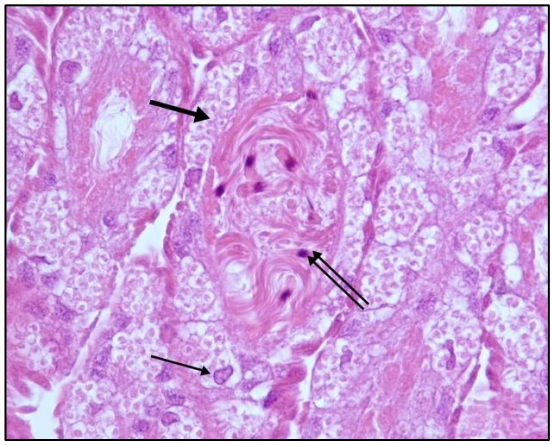

Fig. 2.9. A. mellifera ligustica. Normal testes. Seminiferous tubules. Presence of follicular (thin arrow) and germ cells (thick arrow); tcoiled spermatozoa in the lumen of the tubules (double arrow). H-E. 40X.

In 5/25 samples, the seminiferous tubules presented severe and widespread degenerative phenomena characterized by the appearance of small cytoplasmic vacuoles followed by necrosis of follicular and germ cells, disappearance of the external epithelial layer and of the tubular lumen, until the complete disruption of the normal tissue structure. It was possible to distinguish numerous spermatozoa and the complete absence of spermatogonia and spermatocytes (Fig. 2.10).

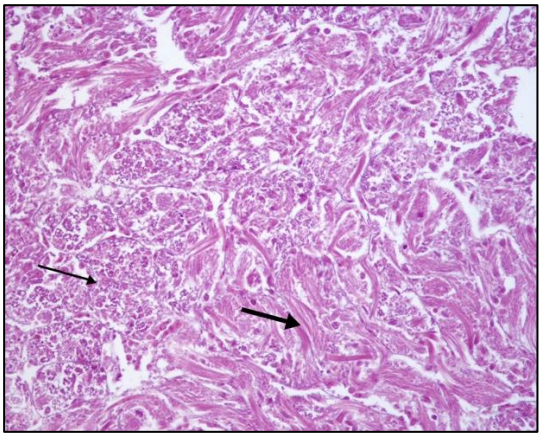

Fig. 2.10. A. mellifera ligustica. Altered seminiferous tubules. Disappearance of the

seminiferous epithelium and of the lumen; necrosis of the follicular and germ cells (thin arrow); numerous spermatozoa (thick arrow;, absence of spermatogonia and spermatocytes. H-E. 20X.

In 2/25 samples, the seminiferous tubules seemed as they hadn't reached complete maturation as they were characterized by a small number of tubules of reduced dimensions, absence of tubular lumen and numerous spermatogonia but no spermatozoa. Between the tubules it was possible to observe the presence of trophocytes and of eosinophilic granules, attributable to residues of trophocytes (Fig. 2.11).

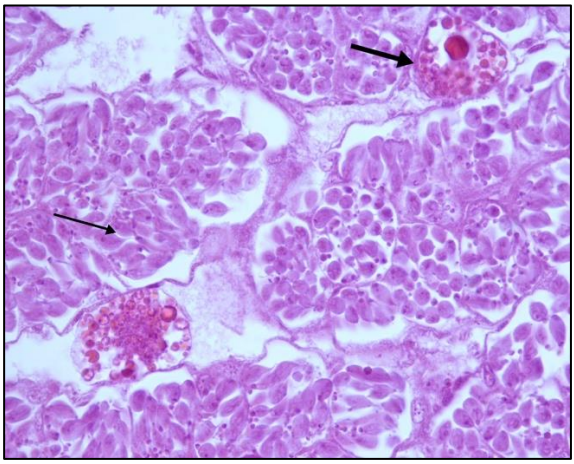

Fig. 2.11. A. mellifera ligustica. Altered seminiferous tubules. Absence of tubular lumen; presence of numerous spermatogonia (thin arrow); absence of spermatozoa. Trophocytes between the tubules (thick arrow). H-E. 40X.

In 1/25 sample it was possible to observe the detachment of the germ cells from the basal lamina of the tubules and the rupture of the membranes of the spermatogonia, probably a consequence of severe and diffuse "intratubular edema" (Fig. 2.12).

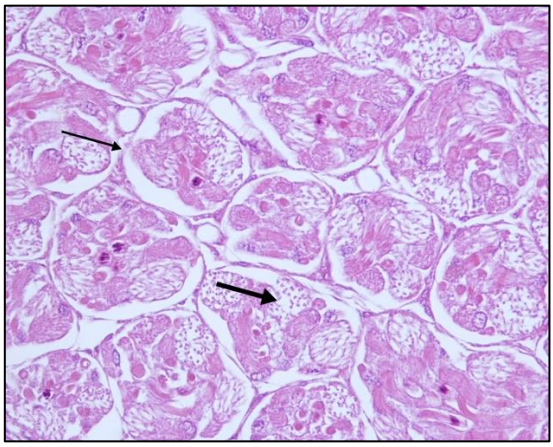

Fig. 2.12. A. mellifera ligustica. Altered seminiferous tubules. Detachment of germ cells from the basal lamina (thin arroa); rupture of the membranes of the spermatogonia (thick arrow). H-E. 40X.

2.9 Discussion and conclusions

When a drastic reduction in the number of individuals of a species occurs, effective reproduction is potentially the key to perpetuation and conservation of the species. Ineffective reproduction, caused by numerous factors such as heavy metals, chemicals, diseases, could lead to a reduction in the offspring, increasing the risk of a further decrease in the population (Kairo, 2016). Although male and female contribution is equally critical to the success of reproduction, more attention has been given to the female side rather than the male side of the issue.

The reason could be that female fertility impairment show evident signs such as: decrease of brood; abnormal pattern of the brood; excessive number of drones; early replacement of the queen and presence of orphan colonies.

In recent years, however, male hypofertility / infertility has been arousing greater interest, since malformations, genetic alterations, infectious diseases, food shortages, managerial errors have been identified as

responsible for a decreased reproductive capacity of many zootechnical species (Kastelic, 2013; Kunavongkrit et al., 2005).

Spermiogram is the keystone to evaluate male hypofertility / infertility: total number of spermatozoa, sperm concentration, sperm motility, viability and morphology are considered essential parameters to be analysed (Freshman, 2002). However, to date, many studies have focused on the viability and motility of spermatozoa and little importance has been given to studies on morphology and possible alterations (Gatimel et al., 2017).

The morphology of spermatozoa has been shown to be a sensitive bio-indicator of exposures to toxic substances, as well as of mutagenesis, and to have predictive value in cases of hypofertility / infertility in mammals and in many other species (Malmgren, 1997), and it should not be different in honeybees.

In order to study any possible alteration in the fertility of drones, it is important to establish what is normal.

The cytologic staining of spermatozoa with Diff-Quick has proved to be easy, quick and very useful to make observations on morphology of the single portions of the spermatozoa structure, however it did not allow a clear highlighting of the acrosome.

The spermatozoa of *A. mellifera*, as described by Peng (1993) are long and filamentous, with a total length of about 250-270 μm, the head measures 8-10 μm and the total length of the acrosomal complex measures 5 μm. A study by Gontarz et al. (2016) has focused the attention on the analysis of *Apis mellifera carnica* spermatozoa.

In our study, Italian honeybee spermatozoa appeared smaller with a mean total length of 230,81 (SD±17,22) μm. It has been shown that the creation of short spermatozoa consumes less energy allowing the production of a greater number of them and the reduction of the resources allocated to the production of the spermatozoa themselves (Pitnick, 1996). Furthermore, shorter spermatozoa allow more sperm to be stored in the queen's spermatheca, resulting in greater long-term fertilization potential (Boomsma et al., 2005).

Our results show the presence of a high variability of the total length of

spermatozoa, as underlined by the SD values (SD±17,22). The reasons for this high degree of variability in spermatozoa morphometry is not known, but has already been highlighted by previous studies and is probably linked to sperm competition (Blengini et al., 2014; Ros-Santaella et al., 2015).
Moreover, our results highlighted the presence of 7% of altered spermatozoa. Morphological alterations could be responsible for a reduced ability of the defective spermatozoa to carry out the normal meandering movements that allow them to go up the female genital tract, reach the spermatheca and after fertilize the egg.
It is known that some elements during the collection and handling of sperm, such as temperature, centrifugation or freezing, can influence sperm viability and induce morphological changes.
In our experiment, sperm manipulation was minimal since the collection of spermatozoa was performed by washing and centrifugation of the testes and seminal vesicles, ensuring a high percentage of vital and morphologically normal spermatozoa. Moreover, centrifugation was performed at 250g x 10 min, this combination falls within the range suggested by Collins (2003), so we can be sure that no damage and changes to sperm morphology have been caused by sperm manipulation. Although it is possible that this procedure could generate a contamination with other tissues, this makes the sample unsuitable for artificial insemination but not for the study of morphometric parameters and morphological characteristics.
The abovementioned alterations of the spermatozoa could be a consequence of the modification of the normal gametogenesis process due to genetic variations and / or environmental factors. It is reasonable to think that they occurred during the larval and pupal phases since, as previously stated, unlike mammals, the drones emerge from their cells with the entire pool of spermatozoa formed. Thus, during adult life, drones will no longer be able to produce sperm and therefore the quality of the sperm cannot be restored (Baer et al., 2012).
All the drones examined in our study did not show any macroscopic alterations or clinical signs but according to the histopathological results it is obvious that they would not have been able to produce an adequate

number of healthy spermatozoa and therefore guarantee the success of fertilization. A queen who would have mated with these drones would have preserved in her spermatheca a reduced number or altered spermatozoa, which would have not been able to fertilize eggs and consequently would have induced a higher deposition of haploid eggs, and the consequent birth of a large number of drones.

The high number of drones in a colony is regarded as unfavourable for the colony and is often cause of intervention from the worker honeybees who generally act by replacing the queen. If this does not occur, queen replacement is realised by the beekeeper.

However, since all the drones came from the same apiary, it is reasonable to think that there may be an environmental or genetic factors.

In this study most of the samples did not show any alterations, however conversely to previous descriptions, the lumen of the seminal tubules appeared filled with coiled spermatozoa. Spermatozoa maturation and migration to the seminal vesicles have been often described as completed during the first week of adult life, however, no histological study has ever been performed, therefore comparable results are currently unavailable. On the contrary, a study by Metz and Tarpy (2019) found that the transfer of spermatozoa from testes to seminal vesicles can actually begin in the first week, but no data is reported for the end of migration.

Five samples showed clear degenerative phenomena affecting the seminiferous tubules and reduced maturation of the testes.

In other species, degenerative alterations have been associated with high levels of heavy metals and pesticides, such as organophsphates (Babazadeh and Najafi, 2017; Ebrahimi and Taherianfard, 2011), in the environment which could induce oxidative stress in tissues. Oxidative stress occurs following the accumulation in organisms of reactive oxygen species (ROS) which can determine high molecular damage, degeneration of tissues, and premature aging (Finkel and Holbrook, 2000). It has been shown that drones are able to survive acute oxidative stress due to individual tolerance and resistance, and not to repair of oxidative damage of lipids and cells, thus leading to a subclinical disease (Li-Byarlay et al., 2016).

For this study, only adult drones ready for mating were collected. Nonetheless, the histopathological analysis highlighted samples with testes that showed reduced maturation, as can be inferred from the presence of many spermatogonia and degenerate trophocytes in the intertubular space. Testis development and spermatogenesis of drones of A. mellifera have been precisely described by Lago et al. (2020) based on histological sections. Changes in whole testicular architecture, as well as of the seminiferous tubules, are described from the first-instar larvae to the pharate-adult stage. According to the histological descriptions, our findings correspond with changes occurring in testes of a fifth-instar stage larvae, however, the presence of trophocytes is not described in the cited research.

It is possible to hypothesize that the organs did not complete their development during the passage from larva to adult insect or that they are drones with homozygous diploid genetic asset. These drones develop from fertilized eggs and normally they do not survive as they are removed by honeybee workers, despite being perfectly viable (Woyke, 1963).

However, the ability of honeybee workers to remove anomalous colony members from the hive can be modified and reduced by many factors, above all the action of neonicotinoids (Brandt et al., 2016).

Moreover, in other species neonicotinoides, and other pesticides, are also considered as endocrine disruptors able to induce both hormonal and morphological alterations of the male reproductive system, by mimicking the effects of estrogens and inducing signs of feminization and demasculinization (Huang et. al., 2016; Hayes et al., 2011; Tyler et al.,1998).

Although endocrine disruptors are found in minimal quantities, in the long run their chronic accumulation can interfere with honeybee health and the correct function and development of the male reproductive system (Sandroc et al., 2014; Baines et al., 2017). Furthermore, endocrine disruptors seem to induce the production of vitellogenin (Vg) in male specimens of many animal species, and probably also in honeybees (Tufail et a., 2014). Vg is a protein present in the fat body and in the hypopharyngeal glands of workers, queens and drones which plays a key

role in phenomena related to egg laying, immunity and longevity (Amdam., 2003; Colonnello-Frattini et al., 2010). It cannot be excluded that in the drones analysed in this study there may be an up-regulation of vitellogenin during the developmental stages which could have influenced the correct maturation of the male reproductive system or induce alterations.

Degeneration or delayed or incomplete maturation could also have been related to the presence of subclinical viral and parasitic diseases.

Deformed Wing Virus (DWV) as well as *Nosema apis* have been localized in the testes of mature drones (Peng et al., 2015; Fievet et al., 2006) suggesting a possible action of these pathogens in drones' fertility impairment although no histopathological findings in the reproductive tissues have been described in previous studies.

In the present study, drones did not show any clinical signs of either disease, but, while we can exclude the presence of *N. apis* and *Nosema ceranae,* as no spores were identified in the gastrointestinal tissue neither in the reproductive tissue, the presence of low levels of DWV cannot be excluded.

Moreover, samples were collected from colonies infested with low infestation levels of *V. destructor*, which is correlated with a low number of spermatozoa (Duay et al., 2002) and oxidative stress (Lipinski and Zółtowska 2005) but also to the spreading of DWV.

In conclusion, the results obtained, although carried out on a limited number of samples, have allowed us to display that the morphological alterations of the spermatozoa as well as the testicular ones also exist in honeybees and we can hypothesise that the alterations can be ascribed to the same causes as those in humans and other animals.

Honeybees have the potential of being bio-indicators of the presence of endocrine disruptors in the environment that could also affect fertility in male humans. The alterations found in honeybee testes and spermatozoa could be a red flag for similar issues affecting humans.

Bibliography

Amdam GV, Norberg K, Hagen A, Omholt SW. Social exploitation of vitellogenin. *Proc Natl Acad Sci U S A*. 2003;100(4):1799-1802. doi:10.1073/pnas.0333979100

Babazadeh M, Najafi G. Effect of chlorpyrifos on sperm characteristics and testicular tissue changes in adult male rats. *Vet Res Forum*. 2017;8(4):319-326.

Baer B, Zareie R, Paynter E, et al. Seminal fluid proteins differ in abundance between genetic lineages of honeybees. *J Proteomics*. 2012;75(18):5646-5653. doi:10.1016/j.jprot.2012.08.002

Baer B. Sexual selection in *Apis* bees. *Apidologie.* 2005; 36:187-200. doi: 10.1051/apido:2005013

Baines D, Wilton E, Pawluk A, et al. Neonicotinoids act like endocrine disrupting chemicals in newly-emerged bees and winter bees. *Sci Rep*. 2017;7(1):10979. doi:10.1038/s41598-017-10489-6

Baudry E, Solignac M, Garnery L, et al. Relatedness among honeybees (Apis mellifera) of a drone congregation. *Proc R Soc Lond B.* 1998; 265:2009-2014 doi: 10.1098/rspb.1998.0533

Bishop, G. H. 1920. Fertilization in the honey-bee I The male sexual organs their histological structure and physiological functioning. *J Exp Zool.* 1920;31: 225-265.

doi:https://doi.org/10.1002/jez.1400310203

Blengini CS, Sergio N, Gabriela C, et al. Variability in sperm form and function in the context of sperm competition risk in two Tupinambis lizards. *Ecol Evol*. 2014;4(21):4080-4092. doi:10.1002/ece3.1262

Blum, M, Glowska Z, Taber S. III. Chemistry of the drone honey bee reproductive system. II. Carbohydrates in the reproductive organs and semen. *Ann Entomol Soc Am.* 1962; 55: 135-139. doi: https://doi.org/10.1093/aesa/55.1.135

Boes KE. Honeybee colony drone production and maintenance in accordance with environmental factors: an interplay of queen and worker decisione. *Insectes Soc.* 2010; 57:1-9. https://doi.org/10.1007/s00040-009-0046-9

Boomsma JJ, Baer B, Heinze J. The evolution of male traits in social insects. *Annu Rev Entomol*. 2005;50:395-420. doi:10.1146/annurev.ento.50.071803.130416

Brandt A, Gorenflo A, Siede R, Meixner M, Büchler R. The neonicotinoids thiacloprid, imidacloprid, and clothianidin affect the immunocompetence of honey bees (Apis mellifera L.). *J Insect Physiol*. 2016;86:40-47. doi:10.1016/j.jinsphys.2016.01.001

Carreck NL, Andree M, Brent CS, et al. Standard methods for *Apis mellifera* anatomy and dissection, *J Apic Res*. 2013; 52:4, 1-40 doi: 10.3896/IBRA.1.52.4.03

Cobey S, Tarpy D, Woyke J. Standard methods for instrumental insemination of *Apis mellifera* queens. *J Apic Res.* 2013;52: 1-18. doi: https://doi.org/10.3896/IBRA.1.52.4.09

Collins AM. Sources of variation in the viability of honey bee, Apis mellifera L., semen collected for artificial insemination. *Inverteb Reprod Dev*. 2004; 45, 231–237 doi: 10.1080/07924259.2004.9652594

Collins AM. A scientific note on the effect of centrifugation on pooled honeybee semen. *Apidologie.* 2003; 34:469-470. doi: 10.1051/apido:2003030

Collins AM. Survival of honey bee (Hymenoptera: Apidae) spermatozoa stored at above-freezing temperatures. *J Econ Entomol*. 2000;93(3):568-571. doi:10.1603/0022-0493-93.3.568

Colonello N, Hartfelder K. Protein content and pattern during mucus gland maturation and its ecdysteroid controlin honey bee drones. *Apidologie.* 2003; 34: 257-267.
doi: https://doi.org/10.1051/apido:2003019

Colonello-Frattini NA, Guidugli-Lazzarini KR, Simões ZL, Hartfelder K. Mars is close to venus--female reproductive proteins are expressed in the fat body and reproductive tract of honey bee (Apis mellifera L.) drones. *J Insect Physiol*. 2010;56(11):1638-1644.
doi:10.1016/j.jinsphys.2010.06.008

Czekonska K, Chorbinski P, Czekońska K, et al. The Influence of Honey Bee (Apis Mellifera) Drone Age on Volume of Semen and Viability of Spermatozoa. *J Apic Sci.* 2013; 57: 61-66. doi: 10.2478/jas-2013-0007

den Boer SP, Baer B, Boomsma JJ. Seminal fluid mediates ejaculate competition in social insects. *Science*. 2010;327(5972):1506-1509. doi:10.1126/science.1184709

den Boer SPA, Boomsma J, Baer B. Seminal fluid enhances sperm viability in the leafcutter ant *Atta colombica*. *Behav Ecol Sociobiol.* 2008;62: 1843-1849. doi:10.1007/s00265-008-0613-5

Duay P, De Jong D, Engels W. Decreased flight performance and sperm production in drones of the honey bee (Apis mellifera) slightly infested by Varroa destructor mites during pupal development. *Genet Mol Res.* 2002; 1: 227–232.

Ebrahimi M, Taherianfard M. The effects of heavy metals exposure on reproductive systems of cyprinid fish from Kor River. *Iran J Fish Sci.* 2011; 10(1):13-24

Fievet J, Tentcheva D, Gauthier L, De Miranda J, Cousserans F, Colin ME. Bergoin M. Localization of deformed wing virus infection in queen and drone Apis mellifera L. *Virol. J*. 2006; 3: 16. doi: 10.1186/1743-422X-3-16

Finkel T, Holbrook N.J. Oxidants, oxidative stress and the biology of ageing. *Nature* .2000; 408: 239–247. doi: 10.1038/35041687

Franck P, Solignac M, Vautrin D, et al. Sperm competition and last-male precedence in the honeybee. *Anim Behav.* 2002;64: 503-509. doi:10.1006/anbe.2002.3078

Freshman JL. Semen collection and evaluation. *Clin Tech Small Anim Pract*. 2002;17(3):104-107. doi:10.1053/svms.2002.34326

Gary NE. Observation of mating behavior in the honeybee. *J Apic Res.* 1963; 2: 3-18. doi: 10.3390/insects5030513

Gatimel N, Moreau J, Parinaud J, Léandri RD. Sperm morphology: assessment, pathophysiology, clinical relevance, and state of the art in 2017. *Andrology*. 2017; 5: 845-862. doi:10.1111/andr.12389

Gençer HV, Kahya Y. The viability of sperm in lateral oviducts and spermathecae of instrumentally inseminated and naturally mated honey bee (*Apis mellifera* L.) queens. *J Apic Res* 2011;50: 190-194. https://doi.org/10.3896/IBRA.1.50.3.02

Gontarz A, Banaszewska D, Gryzinska M and Andraszek K. Differences in drone sperm morphometry and activity at the beginning and end of the season. *Turk J Vet Anim Sci.* 2016; **40**:598-602. doi:10.3906/vet-1511-6

Gorshkov V, Blenau W, Koeniger G, et al Protein and Peptide Composition of Male Accessory Glands of Apis mellifera Drones Investigated by Mass Spectrometry. *PLoS One*. 2015;10(5):e0125068. doi:10.1371/journal.pone.0125068

Harbo JR. Artificial mixing of spermatozoa from honeybees and evidence for sperm competition. *J Apic Res.* 1990; 29: 151-158. doi: 10.1080/00218839.1990.11101212

Hayes TB, Anderson LL, Beasley VR, et al. Demasculinization and feminization of male gonads by atrazine: consistent effects across vertebrate classes. *J Steroid Biochem Mol Biol*. 2011;127(1-2):64-73. doi:10.1016/j.jsbmb.2011.03.015

Hrassnigg N, Crailsheim K. Differences in drone and worker physiology in honeybees (Apis mellifera). *Apidologie.* 2005, 36 (2):255-277. doi: 10.1051/apido:2005015

Huang GY, Liu YS, Chen XW, et al. Feminization and masculinization of western mosquitofish (Gambusia affinis) observed in rivers impacted by municipal wastewaters. *Sci Rep*. 2016;6:20884. doi:10.1038/srep20884

Human H, Brodschneider R, Dietemann V et al. Miscellaneous standard methods for Apis mellifera research. In V Dietemann; J D Ellis; P Neumann (Eds) The COLOSS BEEBOOK, Volume I: standard methods for Apis mellifera research. *J Apic Res*.2013; 52(4) http://dx.doi.org/10.3896/IBRA.1.52.4.10

Jaffe R, Moritz RFA. Mating flights select for symmetry in honeybee drones (Apis mellifera). *Naturwissenschaften*. 2010; 97, 337-343. Doi: https://doi.org/10.1007/s00114-009-0638-2

Kairo G, Provost B, Tchamitchian S, et al. Drone exposure to the systemic insecticide Fipronil indirectly impairs queen reproductive potential. *Sci Rep*. 2016 ;6:31904. doi:10.1038/srep31904

Kastelic JP. Male involvement in fertility and factors affecting semen quality in bulls, *Anim Front*. 2013; 3 (4): 20–25, https://doi.org/10.2527/af.2013-0029

Koeniger G, Koeniger N, Jamie E, Lawrence C. Mating biology of honey bees (*Apis mellifera*) Wicwas Press. United States. 2014

Koeniger N, Koeniger G, Gries M and Tingek S. Drone competition at drone congregation areas in four Apis species. *Apidologie.* 2005b 36:211-221. doi: https://doi.org/10.1051/apido:2005011

Koeniger N, Koeniger G. Reproductive isolation among species of the genus *Apis*. *Apidologie*. 2000;31: 313-339. doi: https://doi.org/10.1051/apido:2000125

Koeniger, G. The role of the mating sign in honey bees, *Apis mellifera*, does it hinder of promote multiple mating. *Anim Behav.* 1990;39: 444-449. doi: https://doi.org/10.1016/S0003-3472(05)80407-5

Koeniger, N. An evolutionary approach to mating behaviour and drone copulatory organs in *Apis*. *Apidologie.* 1991; 22: 581-590. doi: https://doi.org/10.1051/apido:19910602

Kunavongkrit A, Suriyasomboon A, Lundeheim n, et al. Management and sperm production of boars under differing environmental conditions. *Theriogenology.* 2005; 63, 657–667.

Lago DC, Martins JR, Rodrigo PD, et al. Testis development and spermatogenesis in drones of the honey bee Apis mellifera L. *Apidologie* 2020. https://doi.org/10.1007/s13592-020-00773-2

Li-Byarlay H, Huang MH, Simone-Finstrom M, et al. Honey bee (Apis mellifera) drones survive oxidative stress due to increased tolerance instead of avoidance or repair of oxidative damage. *Exp. Gerontol.* 2016; 83, 15–21. doi: 10.1016/j.exger.2016.07.003

Lipinski Z, Zółtowska K.Preliminary evidence associating oxidative stress in honeybee drone brood with Varroa destructor. *J Apic Res*. 2005; 44: 126–128. doi: 10.1080/00218839.2005.11101163

Locke SJ, Peng YS. The effects of drone age, semen storage and contamination on semen quality in the honey bee (*Apis mellifera*). *Phys Entomol*. 1993;18: 144-148. doi: 10.1111/j.1365-3032.1993.tb00461.x

Loper GM, Wolf WW, Taylor OR. Detection and monitoring of honeybee drone congregation areas by radar. *Apidologie*. 1987;18: 163-172. doi: 10.1051/apido:19870206

Malmgren L. Assessing the quality of raw semen: a review. *Theriogenology.* 1997 Sep;48(4):523-30. doi: 10.1016/s0093-691x(97)00268-9.

Metz, B.N.; Tarpy, D.R. Reproductive Senescence in Drones of the Honey Bee (Apis mellifera). *Insects* 2019; 10, 11. https://doi.org/10.3390/insects10010011

Moritz RFA. Intracolonial worker relationship and sperm competition in the honeybee (*Apis mellifera* L). *Experientia.* 1986; 42: 445-448. doi:https://doi.org/10.1007/BF02118652

Page RE Jr, Peng CY. Aging and development in social insects with emphasis on the honey bee, Apis mellifera L. *Exp Gerontol*. 2001;36(4-6):695-711. doi:10.1016/s0531-5565(00)00236-9

Page RE Jr, Scheiner R, Erber J, Amdam GV. 8. The development and evolution of division of labor and foraging specialization in a social insect (Apis mellifera L.). *Curr Top Dev Biol*. 2006;74:253-286. doi:10.1016/S0070-2153(06)74008-X

Peng CYS, Yin CM and Yin LRS. Ultrastructure of honey bee, *Apis mellifera*, sperm with special emphasis on the acrosomal complex following high-pressure freezing fixation. *Physiol Entomol*. 1993; 18:93-101. doi: 10.1111/j.1365-3032.1993.tb00454.x

Peng Y, Baer-Imhoof B, Millar AH, Baer B. Consequences of Nosema apis infection for male honey bees and their fertility. *Sci. Rep.* 2015, 5, 10565. doi: 10.1038/srep10565

Pitnick S. Investment in testes and the cost of making long sperm in Drosophila. *Am Nat.* 1996; 148:57-80.

Rhodes JW, Harden S, Spooner-Hart R, et al. Effect of age, season and genetics on semen and sperm production in *Apis mellifera* drones. *Apidologie.* 2011; 42:29-38. doi:10.1051/apido/2010026

Ros-Santaella JL, Pintus E, Garde JJ. Intramale variation in sperm size: functional significance in a polygynous mammal. *PeerJ.* 2015;3:e1478. doi:10.7717/peerj.1478

Rousseau A, Fournier V, Giovenazzo P. *Apis mellifera* (Hymenoptera: Apidae) drone sperm quality in relation to age, genetic line, and time of breeding. *Canadian Entomologist*. 2015; 147: 702-711. doi: https://doi.org/10.4039/tce.2015.12

Rueppell O, Fondrk MK, Page RE Jr. Biodemographic analysis of male honey bee mortality. *Aging Cell*. 2005;4(1):13-19. doi:10.1111/j.1474-9728.2004.00141.x

Ruttner F. The instrumental insemination of the queen bee, Apimondia International Beekeeping Technology and Economy Institute. 1976

Ruttner F. The Mating of the Honeybee, *Bee World*. 1956; 37:1, 3-15,
doi: 10.1080/0005772X.1956.11094913

Sandroc C, Tanadini LG, Pettis JS, et al. Sublethal neonicotinoid insecticide exposure reduces solitary bee reproductive success. *Agr Forest Entomol.* 2014; 16: 119-128. doi:10.1111/afe.12041

Schlüns H, Moritz RFA, Neumann P, et al. Multiple nuptial flights, sperm transfer and the evolution of extreme polyandry in honeybee queens, *Anim Behav.* 2005; 70, 125–131. https://doi.org/10.1016/j.anbehav.2004.11.005

Shafir S, Kabanoff L, Duncan M, Oldroyd BP. Honey bee (*Apis mellifera*) sperm competition in vitro - two are no less viable than one. *Apidologie*. 2009;40: 556-561. doi:10.1051/apido/2009014

Snodgrass RE. The Anatomy of the Honey Bee. U.S. Government Printing Office. Washington 1910

Strassmann J. The rarity of multiple mating by femals in the social Hymenoptera. Insectes soc. 2001; 48, 1-13. https://doi.org/10.1007/PL00001737

Stürup M, Baer-Imhoof B, Nash DR et al. When every sperm counts: factors affecting male fertility in the honeybee *Apis mellifera*, *Behav Ecol* 2013;24 (5): 1192-1198, https://doi.org/10.1093/beheco/art049

Tarpy DR. Genetic diversity within honeybee colonies prevents severe infections and promotes colony growth. *Proc Biol Sci*. 2003;270(1510):99-103. doi:10.1098/rspb.2002.2199

Tarpy DR, Page RE. The curious promiscuity of queen honey bees (*Apis mellifera*): evolutionary and behavioral mechanisms. *Ann Zooli Fenni*. 2001;38: 255-265.

Taylor MA, Guzmán-Novoa E, Morfin N, Buhr MM. Improving viability of cryopreserved honey bee (Apis mellifera L.) sperm with selected diluents, cryoprotectants, and semen dilution ratios. *Theriogenology*. 2009;72(2):149-159. doi:10.1016/j.theriogenology.2009.02.012

Tofilski A, Chuda-Mickiewicz B, Czekonska K, Chorbinski P. Flow cytometry evidence about sperm competition in honey bee (*Apis mellifera*). *Apidologie*. 2012;43: 63-70. doi: 10.1007/s13592-011-0089-6

Tufail M, Nagaba Y, Elgendy AM and Takeda M. Regulation of vitellogenin genes in insects. *Entomol Sci*. 2014; 17: 269-282. doi:10.1111/ens.12086

Tyler CR, Jobling S, Sumpter JP. Endocrine disruption in wildlife: a critical review of the evidence. *Crit Rev Toxicol*. 1998;28(4):319-361. doi:10.1080/10408449891344236

Verma LR. Honeybee Spermatozoa and their Survival in the Queen's Spermatheca, *Bee World.* 1974; 55:2, 53-61, DOI: 10.1080/0005772X.1974.11097497

Watson L. Controlled mating of queen bees. The quarterly review of biology. 1928;3(3):377-390.

Winston ML, Higo HA, Colley SJ, et al. The role of queen mandibular pheromone and colony congestion in honey bee (*Apis mellifera* L) reproductive swarming (Hymenoptera, Apidae). *J Insect Behav.* 1991;4: 649-660. doi:10.1007/BF01048076

Winston ML. The biology of the honey bee. Harvard university press. USA. 1987

Woyciechowski M, Krol E. On intraoviductal sperm competition in the honeybee (*Apis mellifera*). *Folia Biologica-Krakow.* 1996; 44: 51-53

Woyke J. Three substances ejected by Apis mellifera drones from everted endophallus and during natural matings with queen bees. *Apidologie*. 2010;41(6):613-21. doi: 10.1051/apido/20010007

Woyke J.Why the eversion of the endophallus of honey bee drone stops at the partly everted stage and significance of this. *Apidologie.* 2008; 39 (6): 627-636. doi: https://doi.org/10.1051/apido:2008046

Woyke J. Anatomo-physiological changes in queen bees returning from mating flights and the process of multiple mating. *Bull Acad Pol Sci Cl II*. 1956 4:81–87

Woyke, J. What happens to diploid drone larvae in a honeybee colony. *J Apic Res.* 1963; 2(2), 73-75

Yu R, Omholt SW. Early developmental processes in the fertilised honeybee (Apis mellifera) oocyte. *J Insect Physiol.* 45(8):763-767. doi:

10.1016/s0022-1910(99)00056-6.

Chapter 3:

Histopathology and Mini-FLOTAC

New diagnostic tools for the diagnosis of subclinical Nosemosis

Abstract

Nosemosis is a worldwide parasitic disease that affects adult honeybees, caused by two species of unicellular fungi: *Nosema apis* and *Nosema ceranae*. In a short time, it has become one of the first causes of honeybee death, and it has been linked to the Colony Collapse Disorder. As the infection is not always symptomatic, a sensitive and rapid technique for a correct diagnosis is required. For these reason, samples of adult honeybees were analysed with histopathological techniques and, for the first time, the Mini-FLOTAC technique was applied to the beekeeping field to detect *Nosema spp*. spores.

The histopathological examination revealed the presence of spores in the midgut and in the Malpighian tubules. Both organs appeared strongly degenerated, suggesting an impairment of their physiological function.

The Mini-FLOTAC technique is quick, easy, highly sensitive, precise, accurate, it can be used directly in the field and it has already been extensively validated in parasitology for the uro/copromicroscopic diagnosis of animal and human endoparasites. The Mini-FLOTAC technique was compared with two standard microscopic techniques, direct smear and microscopic examination with the use of the hemocytometer in order to assess the possible use in honeybee samples. The results confirmed the validity of the three techniques to highlight the presence of the spores of *Nosema*. However, the Mini-FLOTAC technique proved to be more user-friendly and a better tool for spore detection, especially when the infection level is low, thanks the high sensitivity, precision and accuracy of the technique.

This study was carried out in collaboration with the Unity of Parasitology and Parasitic Diseases of the Department of Veterinary Medicine and Animal Productions of the University of Naples “Federico II”.

3.1 Nosemosis

Nosemosis is a globally diffused parasitic disease of adult honeybees and one of the most significant pathogens thretening honeybee survival.
It is caused by the two microsporidia *Nosema apis*, responsible for Nosemosis type A, and *Nosema ceranae*, responsible for Nosemosis type C (Higes et al., 2010). Recently the existence of new species also affecting honeybees, *Nosema neumanni*, was described in Uganda, but, to date, not much is known about it (Chemurot et al., 2017).
Infection of *A. mellifera* with *N. apis* was already described by Zander in 1909 (Zander, 1909), whereas *N. ceranae* was initially detected in Apis cerana by Fries in 1996 (Fries et al., 1996), while the first detection of an infection of *A. mellifera* with *N. ceranae* only occurred in 2005 in Taiwan (Huang et al., 2007). Although it is generally thought that the original host on *N.ceranae* is the *A.cerana*, new studies have found this parasite also in historical samples of *Apis dorsata* from 1968 and in *Apis mellifera* samples from 1975 (Traver and Fell, 2015).
Since first detections, the *N.apis* and *N.ceranae* have been reported in every continent, although in with variable prevalence, probably due to the time of sampling (Martín-Hernández et al., 2018). In fact, in temperate areas, type A Nosemosis explodes in April-May to regress in July-August and then recur in the autumn, type C Nosemosis can be found all year round, even if the peak of infection occurs in June-July and depopulation phenomena are usually detectable around the month of September. (Carpana and Lodesani, 2014). Therefor, the level of infection found in a colony varies widely over the season depending on whether it is *N.apis* or *N. ceranae*. *N. apis* infection typically shows low infection levels during the summer, a peak during the autumn, and a gradual increase during the winter, to reach maximum levels in the spring. For *N. ceranae* this seasonality has not been observed in fact, as already mentioned above, it is found all year round.
In Italy, Nosemosis is now considered an endemic pathology as it appears to be present in the latent state in all Italian apiaries. The data of the last Bulletin of the national beekeeping monitoring project "BeeNet" dating back to 2013 (Porrini et al., 2016), highlighted the absence of positive

samples for *N.apis* and the ubiquitous presence of the new species, *N. ceranae*, confirming the higher prevalence of Nosemosis type C, compared to Nosemosis type A (Martín-Hernández et al., 2018).
From a regulatory point of view, Nosemosis type A is one of the "Infectious and diffusive diseases of animals subject to health measures" according to the current national Regolamento di Polizia Veterinaria (Veterinary Police Regulation) (DPR 8 February 1954, n. 320), whereas it is not subject to notification at Community level or on the recommendation of the OIE (World Organization for Animal Health).
In 2011, the Ministry of Health diversified the health measures to be adopted in case of infection from one or the other species of *Nosema*. In the event that clinical Nosemosis caused by *N. apis*, it will be necessary to proceed according to the Regolamento di Polizia Veterinaria, that is reporting of the disease and destruction of the affected hives. In the case Nosemosis is caused by *N. ceranae*, however, the provisions of the Regolamento di Polizia Veterinaria do not apply: the reporting or destruction of the hives is not mandatory, but only it is recommended to follow good beekeeping practices.

3.1.1 Life cycle

Microsporidia are eukariotic obbligate intracellular parasites that can only complete their life cycle inside an infected host cell (Han et al., 2017). The infectious agent of Nosemosis is the spore which enters the organism through ingestion and within 10 minutes reaches the midgut, where stimulated by the intestinal enzymes, germinates. The germination of the spores of microscopridia is a very particular process, characterized by the accumulation inside the spore of a strong pressure that causes the eversion of a long polar filament, which penetrates the host cell and allows the transfer of a binucleate sporoplasm (Goblirsch, 2018). Within hours after penetration, the merogony phase starts and it proceeds via binary fission, which leads to the production of a grat number of meronts, which are often quadrinucleate and show a single thin plasms membrane (Fries et al., 1996). Between 48 and 96 h after infection (Higes et al., 2007), meronts

can develop into primary spores (sporont) through binary fission which gives origin to two sporoblasts, showing a thin wall and a short polar filament. This primary spore is able to infect the adiacent cells through spontaneous germination (Becnel and Andreadis, 1999). Meronts can also transform in mature spores, which are characterised by a think wall and a long polar filament. Multiplication of parasites in the cells leads ultimately to cell lysis and release of mature spores in the midgut lumen. From here mature spores can either pass through the intestine, reach the rectum and be expelled in the environment through the feces, or they can persist in the midgut, germinate again and infect other cells (Goblirsch, 2017) (Fig.3. 1). Once the infection has settles in a cell, if the temperature is stable around 30°C, it takes two weeks for the entire epithelium of the midgut to become infected.

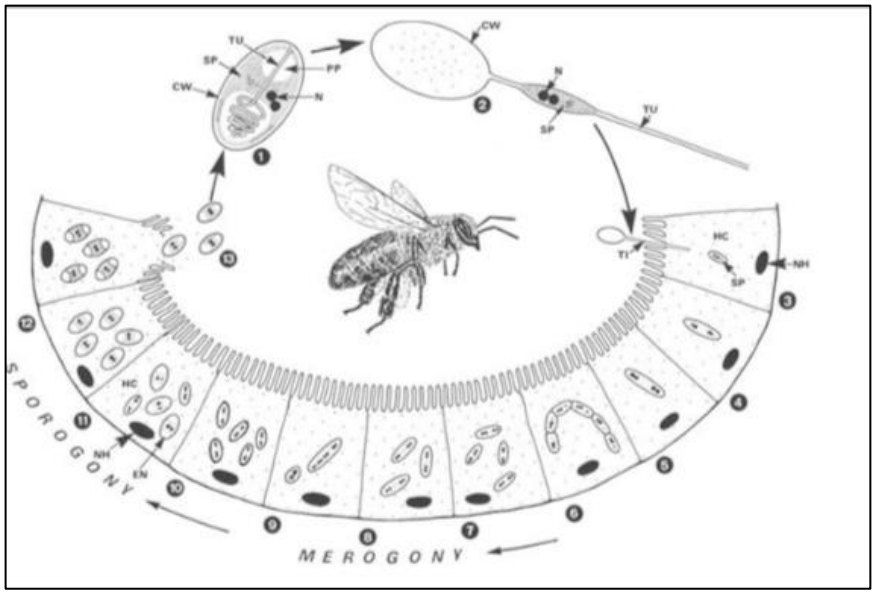

Fig. 3.1. Nosema spp. life cycle (Mehlhorn, 2001).

3.1.2 Transmission routes

Nosemosis is mainly transmitted through horizontal routes.

Spores of *Nosema spp*. are, in fact, transmitted through ingestion of contaminated food or water or feces, and great importance play trofallaxis and the undertaking duties of honeybee workers (Martin-Hernandez et al., 2018).

The median infective dose for Nosemosis type A is 94.3 spores per bee (Fries, 1988), whereas for Nosemosis type C is higher, approximately 149 spores per bee (McGowan et al., 2016).

By food exchange, removal of dead individuals, and feces removal from cells, spores are passed from one worker to another, quickly affecting the whole colony (Chen et al., 2006). Particularly, as spore are eliminated in the environment through feces, honeybees can easily become infected in the cases of *N.apis,* which is known to cause severe diarrhea.

Fecal material can also be spread in the hive by cluncky beekeeping practices, such as not paying too much attention while taking out and putting back the combs, which could lead to honeybee crushing and dispersion of fecal material (Costa, 2014).

A study by Higes et al. (2009), has confirmed the presence of a horizontal route of infection also from workers to queens, but no study has highlighted the existence of a vertical route from the queen to her offspring. A study by Webster et al. (2008) which experimentally infected queen with *N.apis*, showed detection of the parasite in the midgut, but neither in the ovaries nor in the eggs, larvae or pupae born from those queens.

On the contrary, the presence of *N. ceranea* in ovaries and in the spermatheca suggests a possible vertical transmission of this specific species (Traver and Fell, 2012).

Spores of both species of *Nosema* have been detected in semen of both immature and mature infected drones and queens that were artificially inseminated with infected semen became infected themselves (Traver and Fell, 2011; Roberts et al., 2015). This data confirms the veneral transmission between drones and queen, at least through instrumental insemination.

Pollen, honey and royal jelly have been demonstrated as sources of spores contributing to the oral transmission of the parasite, while robbery of

stored food by honeybees from different hives, could easily spread the disease through colonies (Cox-Foster et al., 2007; Higes et al., 2008c; Giersch et al., 2009). Transmission could also occurr to other colonies through food sharing, honeybee drifting, water sources and flowers (Webster et al., 2004; Bordier et al., 2017; Graystock et al., 2015).
A very recent study by Sulborska et al., (2019), has proved that honeybee microsporidia are also transmitted by air paired with typical airborne fungi such as *Ganoderma spp.*, *Leptoshaperia spp*. and *Cladosporium spp*.. Moreover, as *N.ceranae* conversely to *N.apis* is not host specific, and can be found also in other pollinators or other animals that visit the hive, they should also be considered as a source of infection to commercial honeybees and of diffusion through colonies (Li et al., 2012). Another vector of the spores could be the migratory birds *Loxostege sticticalis* (Malysh et al., 2018) and *Merops apiaster*, which is a bee eating bird. During its long migratory flights from Northern Africa to southern Europe, the bird stops to feed on honeybees and then regugitates the undigested parts (Valera et al., 2017). It has been shown that this bird can regurgitate pellets containing numerous spores of *Nosema spp.,* (Higes, 2008b) thus contributing in diffusing the pathogen across contries. Beekeeping activities such as commerce of honeybee queens, colonies and products, as well as well as collecting swarms, and nomadism could also improve diffusion of spores across the world, and could probably be the reasons of the global distribution of the disease (Martin-Hernandez et al., 2018).

3.1.3 Tissue tropism and pathogenicity

Nosemosis is the most common adult honeybee disease, regardless caste and sex, and it affects mainly the digestive system (Higes et al., 2020).
However, both in vitro experiments and on-farm studies, have shown that *Nosema spp*. can also be detected in brood and in other developmental stages (Traver and Fell, 2011; Eiri et al., 2015; Urbieta-Magro et al., 2019), as well as in various organs (Ptaszyńska et al., 2012).

Initially, it was thought that *N.apis* infections were restricted to the midgut epithelium (Fries,1988), while *N.ceranae* could be found also in other tissues, such as Malpighian tubules, hypopharyngeal glands, salivary glands, and fat bodies (Chen, et al., 2009).
Different studies have shown that both species can be found in the venom sac as well as different glands of the body, suggesting that the two parasites are not tissue specific (Copley and Jabaji, 2012; Ptaszyńska et al., 2012)
However, no evidence that the spores are replicating and no significant alterations, that could be undoubthly related with the presence of *Nosema spp*., have been found in organs different from the midgut, proposing a possible role as parasite reservoir rather that infection site (Ptaszyńska et al., 2012).
Nonetheless, more recent studies have restated the specificity of tissue tropism for both species towards the midgut epithelium, so to date the matter remains still controversial (Huang and Solter, 2013; Maiolino et al., 2014; Higes et al., 2020).
Midgut alterations and pathogenesis are similar in the two species, although clinical signs are different; in fact, while *N.apis* causes diarreha, presence of honeybee dead in the proximity of the hive, and seasonality, *N.ceranae* silently impairs honeybee heatlh, yet resulting in death of the honeybees and colony colapse (Higes et al., 2020).
High rates of multiplication of the parasite in the midgut epithelium, leads to degeneration and lysis of the tissue, and possibly to malabsorption of nutrients and water, that ultimately could cause the death of the host as a result of dehydration (Maiolino et al., 2014).
An interesting study by Dussaubat et al. (2012), has analysed the interaction between the host and the parasite, and it showed that the latter can induce a reduction of a great number of genes involved in cell signaling, as an example the expression of the transmembrane receptor protein tyrosine kinase signaling pathways, or in tissue homeostasis, and in biological processes implicated in morphogenesis of epithelium and development of the tracheal system.

These molecular findigs were also confirmed at an histopahological level in the same study and in many others (Higes et al., 2007; Maiolino et al., 2014; Higes et al., 2020).
From his side, the host responds by activating the production of reactive oxygen species (ROS), as demontrated by the upregulation of genes involved in oxidation reduction, and by enahcing the glucidic methabolism needed for the production of ROS (Dussaubat et al., 2012).
The increase in sugar metabolism, as confirmed by the upregulation of the α-glucosidase (an enzyme that hydrolizes sucrose to glucose) has been shown in many studies (Mayack and Naug, 2009; Martin-Hernandez et al., 2011; Dussaubat et al., 2012), but the reasons of this phenomena are still unclear as some suggest that a higher demand of sugar in honeybees infected by *Nosema* is not related to the ROS methabolism, but to the dependancy of the parasite on the host Adenosine triphosphate (ATP).
Microsporidia, thus *Nosema*, are usually amitochondriate and rely, especially for their germination, on energy produced by its host (Keeling and Fast, 2002) which in turn could need more energy and therefore enhances its sugar methabolism.
Microscopical changes such as the disappearence of the Golgi complex, mitochondria, and rough endoplasmic reticulum, as well as the presence of myelin-like coils lysosoms, have also been described in the hypopharengeal glands of worker honeybees infected by *Nosema* (Wang and Moeller, 1971). Thus, a large number of spores could lead to the reduction of the secretion of royal jelly, honey and bee bread, as well as alterations of the vitellogenin output (Copley and Jabaji, 2012). However, the possibility that the alterations are caused as a result of malnutrition, dehyadration or exhaustion of the honeybees, cannot be excluded (Ptaszyńska et al., 2012).
Both species of *Nosema* can also act on the immune system, producing significant changes in the expression of the main antimicrobic peptides (AMP), although in different ways (Antúnez et al., 2009).
N.apis increases the expression of genes encoding AMPs and other immunity-related enzymes such as abaecin, defensin and hymenoptaecin as well as the expression of pro-phenoloxidase (PPO) (Antúnez et al.,

2009), which favours the processes of phagocytosis, encapsulation and nodulation (Glinski and Jarosz, 2001).
On the contray, *N.ceranae* reduces significantly the expression of abaecin, hymenoptaecin, defensin, apidecin, glucose dehydrogenase (GLD) and vitellogenin (Vg), partially suppressing cellular and humoral immune response (Antunez et al., 2009; Chaimanee et al., 2012). Moreover, a decrease in the expression of Vg is consistent and justifies the reduced lifespan and quicker senescence noticed in honeybees affected by *N.ceranae*. However, in a study by Alaux et al. (2010), neither haemocyte population number, nor the phenoloxidase (PO) activity were apparently affected by infection. Nevertheless, Li et al. (2018) described suppression of Toll and Imd pathways and of the expression of pattern recognition receptors regulator (PRRs) genes, that was persistent and intensified in time.

3.1.4 Clinical signs

Although *Nosema* is an intestinal parasite, clinical signs are only seen in Nosemosis type A. The disease is characterised by the presence of diarrohic spots inside and outside the hive, honeybees showing swallen soft abdomens, weak crawling honeybees, increased winter mortality (Fries, 1993) (Fig. 3.2). Conversely, honeybees affected by nosemosis type C do not show any gastroenteric signs but the disease is associated with weakness and increased colony mortality all year round (Higes et al., 2008a; Paxton, 2010; Botías et al., 2013).

Fig. 3.2. Clinical signs of Nosemosis type A. Diarrohic spots on comb (Formato-http://www.fao.org/3/CA3136EN/ca3136en.pdf).

Other signs, such as senescence, reduced fitness, behaviour alterations, quicker transition from nurse to forager, associated to Nosemosis are shared by both pathogens.

Infected honeybees show reduced longevity and impaired function of the hypopharyngeal glands, probably due to downregulation of the Vg expression (Antúnez et al., 2009). As reported by Ptaszyńska et al. (2012) morphological alterations of the hypophryngeal glands on one hand reduce the production of proteolytic enzymes, thus compromising the ability to digest protein sources, on the other reduce the secretion of Vg.

Vg is a powerful antioxidant (Nelson et al., 2007) and as oxidative modifications of intracellular proteins has been linked to senescence (Remolina et al., 2007), honeybees showing high levels of Vg show higher longevity, compared to those with lower levels of Vg. Therefore, as *Nosema* infected honeybees express Vg weakly, they are more susceptible to oxidative stress and have a shorter lifespan (Antúnez et al., 2009).

However, the alterations of the midgut epithelium, impairs the capacity to digeste and absorb carbohidrates, lipids and proteins (Higes et al., 2020) and causes signs of starvation, mainly depletion of the fat body and of the hypopharingeal secretions, namely proteins, Vg and, as a consequence, the production of royal jelly (Vidau et al., 2014).

Starvation has also been linked to less glycogen in the hypopharyngeal glands and consequent weaker capacity to fly and reduction of the foraging activity (Mayack and Naug, 2009).
However, alterations in bahaviour could also be caused by the direct action of the parasite on neurohormonal pathways (Mayack et al., 2015).
The infection with *Nosema spp*. also causes a reduction in orientation ability and homing success is reduced: infected bees have been shown to return to the hive less frequently and have more difficulty finding the hive (Paris et al., 2018).
All the afore mentioned alterations can cause a great loss of honybees and therefore a slow and progressive depopulation of the colony, making *Nosema* one of the factores involved in colony depopulation syndrome also known as Colony Collapse Disorder, or CCD. (Higes et al., 2008; Kralj and Fuchs, 2010).

3.1.5 Treatment

Considered the importance of Nosemosis in apiaries across the world, much effort has been spent in identifying a cure that could contrast the parasite (Martin-Hernandez et al., 2018).
To date, bicyclohexylammonium fumagillin (commercial names Fumidil and Fumagillin-B) is probably one of the few drugs acting directly against the two microsporidia (McCowen et al., 1951). Fumagillin is an antibiotic derived from the fungus Aspergillus fumigatus, able to suppresse both replication and maturation of *Nosema spp*. (Huang et al., 2013). It works by inhibiting the activity of the Methionine aminopeptidase 2 enzyme, but in an unspecific way, thus affecting also honeybees and mammalian (Martin-Hernandez et al., 2018).
Moreover, the antibiotic is mixed with dicyclohexylamine, another component toxic to humans and that can be, together with fumagillin, found in honeybee products, becoming of great risk for consumers as well as honeybees (van den Heever et al., 2016).

Considering its toxicity, fumagillin is not licensed in most European countries, including Italy, where to date no therapeutic treatment for Nosemosis has been legally recognized.
Alternative treatments, such as complementary herbal feeds, have become highly popular, although not always their effectiveness is clearly proved and documentated.
The effect of probiotics and prebiotics on the survival rates of honeybee infected with *Nosema spp*. was studied by different authors and the outcome showed that there were no strong beneficial effects (Endler, 2014; Ptaszyńska et al., 2016). However, a study by Borges (2015) has shown that acacia gum is the most effective prebiotic, but with strong side effects, while the probiotic ProtexinC1 was able to reduce spore number, improving honeybee fitness.
Moreover, the administration of a mixture of four *Lactobacillus kunkeei* in controlled laboratory models was safe for larvae and honeybees and it was able to decrease the counts of *N. ceranae* spores from adult honeybees (Arredondo et al., 2018).
Contrasting results are probably due to the different bacterial strains used.
Researchers are continuing to experiment different possibility to fight Nosemosis. Among the last hypothesis, the use of small interfering RNA which target *N.ceranae* gene coding Dicer (siRNA-Dicer) was used to reduce the number of spores in infected honeybees (Huang et al., 2019).
By feeding infected honeybees with siRNA-Dicer, parasite genes for cell proliferation, ATP-binding cassette (ABC) transporters and hexokinase were downregulated while the honeybee gene mucin-2-like showed significantly upregulation in the siRNA-Dicer group compared with the control group suggesting that the siRNA-Dicer feeding promoted the strength of the mucus barrier (Huang et al., 2019).
An other innovative study has demonstrated that sucrose-protoporphyrin amide PP(Asp)2, exhibited significant inactivation of microsporidia, preventing the development of the parasite and diminishing the mortality of infected honeybees (Ptaszyńska et al., 2018).
Probably, the most interesting finding is that propolis extracts, containing caffeic acid, ferulic acid, ellagic acid and quercetin, is able to reduce spore

loads and it could represent and effective and safe product to control at least *N.ceranae* (Mura et al., 2020). However, the same study shows that honeybees don't seem to use propolis for self-medication, at least when infected by the parasite, so it should be andministrated by the beekeeper.
Nowadays in Italy, no therapeutic treatments have been recognized for Nosemosis, therefore it is necessary to act through prevention and control of the infection by appliying good beekeeping practices.
The "Good beekeeping practices: Practical on how to identify and control the main diseases of honeybees" (FAO, 2020) lists management reccomandations that could help control honeybee diseases and also Nosemosis.
Particularly, it is suggested to position the hives where it is sunny and ventilated, to reduce multiplication of the parasite and facilitate the possibility of flight and therefore evacuation of feces, avoiding its accumulation inside the hive; to ensure enough food is available in the hive during winter and avoid feeding honey or pollen to honeybees, as they can be spore carriers; to apply appropriate treatments against *Varroa destructor*; to remove and burn combs with signs of diarrhoeal feces and not to reuse combs originating from depopulated or collapsed hives, in order to reduce the probability of contamination; to buy queens from queen breeders with stock that are free from *Nosema*.
It is also suggested to periodically send samples of foragers to a laboratory for analysis, in order to identify the presence of the pathogen in the hive before it causes severe damages.
Above all, it is important to respect the general rules of hygiene, especially by cleaning and disinfecting hives, honeycombs and tools before using them for new families and during wintering, before the new season starts (FAO, 2020).

3.2 Diagnostic techniques

From a clinical point of view, the diagnosis of Nosemosis is not always possible. In fact, while Nosemosis type A is more easily recognized, especially when diarrheal feces are found in the hive (Martinez-Hernandez

et al., 2018), Nosemosis type C is more difficult to identify, as the clinical signs detected during the visit at the hive may be rather generic and attributable to many diseases (Maiolino et al., 2013). As a matter of fact, honeybees affected by Nosemosis present abdominal swelling and distension, whitish and swollen intestine, disappearance of the typical folds of the midgut and rectus filled with watery feces (Carpana and Lodesani, 2014) (Fig. 3.3). However, these alterations, are not specific to Nosemosis and to obtain a certain diagnosis, it is necessary to use more specific techniques, such as histopathology.

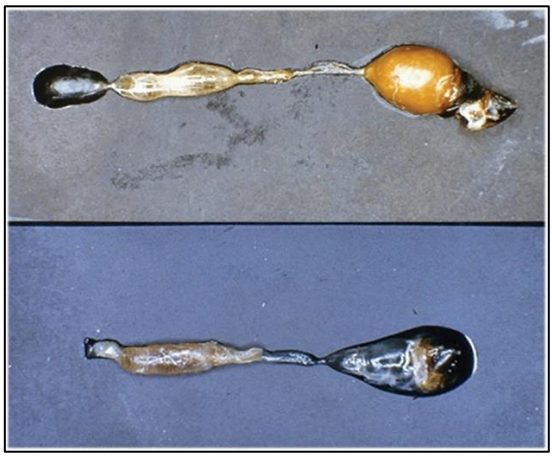

*Fig. 3.3. Intestine of honeybee infected by Nosema spp. (top) and of healthy honeybee (bottom). Altered intestine shows swelling, disappearance of the folds and rectum filled of feces (*https://www.beeculture.com/practical-beekeeping-beekeeping-with-the-new-parasite/*).*

Histopathology consists in highlighting lesions induced by *Nosema spp.* at the level of tissues and cells (Maiolino et al., 2014) and allows detection of the parasite in the cells. The diagnosis of the disease is therefore certain (Higes et al., 2020).

Diagnosis of Nosemosis is, thus, achived by indentification of spores or by amplification of parasite's genetic material in honeybee samples. The spores (Fig. 3.4) of the two species share similar morphometrical measurement and are not easily distinguishible as the largest of *N.ceranae* spores (3.6-5.5 μm lenght and 2.3-3.0 μm width) have the same dimension

of the smallest *N.apis* spores (4-6 μm lenght and 2-4 μm width) (Fries et al., 1996; Higes et al. 2007). Therefore, for species identification, especially in cases of only few spores, biomolecular tests need to be carried out, (Chen et al., 2009; Higes et al., 2010).

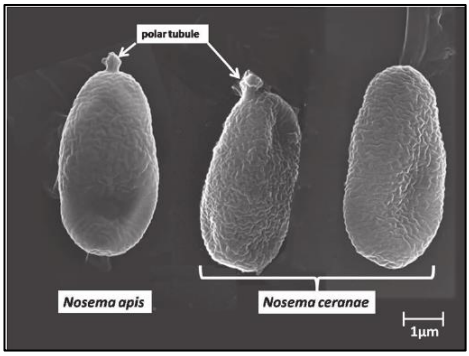

Fig. 3.4. Morphological and morphometric differences in N. apis and N. ceranae by scanning electron microscopy (Ptaszyńska et al, 2014).

Degree of infection is achieved by quantification. Traditional methods for quantifying Nosema infection have high sensitivity and specificity, however they are not always userfriendly as they cannot be used in field conditions by beekeepers. Snow et al., (2019) have presented a field-portable and cost-effective smartphone-based platform for detection and quantification of Nosema spores in honeybees. The innovative technology, consists of a smartphone-based fluorescence microscope, a custom-developed smartphone application, and an easy sample preparation protocol. This device provides results that are comparable with other methods, having a limit of detection of 0.5 × 106 spores per bee, identifying infected colonies and providing accurate quantification of infection levels. This method is potentially adaptable for diagnosis of *Nosema* infection in the field by beekeepers (Snow et al., 2019).

3.2.1 Sampling

According to the OIE manual (2013), to know the real level of infection of a colony, at least 60 foragers must be collected while returning to the hive. It is known that newly hatched honeybees are most likely to be free from infection, so sampling foragers outside the entrance of the hive, diminishes the possibility of collecting younger honeybees (Smart and Sheppard, 2011).
If samples cannot be collected outside the hive entrance, due to unfavourable wheather conditions, samples should be collected from peripheral combe in the brood area whithout hatching bees, or from a super.
However, when sampling occurs just before wintering, honeybees from inside the hive should also be sampled (Fries et al., 2013).
Both pooled and individual samples can be analysed and they should preferably be stored at -20°C until processing (OIE, 2013; Higes et al., 2008a).

3.2.2 Microscopic examination for spore identification

Microscopic examination aims at identifying the spores of *Nosema* spp. in the faeces and /or in the gastrointestinal tract of suspicious or sick bees. The examination can be carried out on the entire bee, on the isolated abdomen, on the entire intestinal tract or only on the midgut (Fries et al., 2013).
Microscopic examination can be qualitative or quantitative, when also the spore number is evaluated
The main techniques used for qualitative diagnosis are the direct smear (Gisder et al., 2010), electron microscopy (Ptaszyńska et al, 2014), and histopathology (Maiolino et al., 2014; Martin-Hernandez et al., 2018). For a quantitative diagnosis, samples are put in a hemocytometer and then observed at the microscope.
For the direct smear technique, samples of whole bees, their abdomens, or intestinal tracts, or ventriculus are macerated with water (1ml / bee) and the obtained macerate is subsequently spread on a slide and observed with a light microscope at 400 X. The use of phase-contrasting microscope is

suggested as thanks to their light refraction properties, *Nosema* spores are easily visible even without staining. However, the standard technique used for diagnosis of microsporidia by light microscopy is contrast staining with Giemsa (Fries et al., 2013). Stained spores appear to have a thick unstained wall and a blue-stained featurelss interior. The nuclei within the spores are not visible. This staining can help to distinguish *Nosema spp.* from pollens, yeasts and other microbes found in bees (OIE, 2013).

With electron microscopy it is possible to differentiate the two species of *Nosema*, based on the number of polar filament coils (Burges et al., 1974). The number of coils in *N.apis* is often more than 30 (Fries, 1988), whereas in *N. ceranae* is lower and it varies between 20 and 23 in mature spores (Fries et al., 1996). On the contrary, immature spores do not show any polar filament and thay can be distinguished from mature ones thanks to a less developed wall. Preparation of the sample requires the use of many steps and it requires highly specific tools and solutions, which are often toxic (Ptaszyńska et al., 2012).

The extent of infection is determined by counting spores on a microscope grid and calculating the average number of spores per area. Thus, for the quantitative microscopic examination, a hemocytometer should be used. This is a glass instrument, generally used for counting of blood cells. With this tool it is possible to evaluate the number of spores of *Nosema spp* / honeybee or the spores/pooled bees (Human et al., 2013). To facilitate counting it is advisable to use 1ml of water / bee for maceration. To avoid the movement of the spores from one square to another, the solution must rest for at least two minutes before starting the count. The size of the chambers can vary with manufacturer but each showes a defined volume ($0.1mm^3$) containing a marked counting grid with an area of 1 mm^2. The whole grid consist of 3 × 3 large squares, separated by triple lines. Each large square is further subdivided into 16 smaller squares subdivided by double lines, in total 144 squares. The spores are counted in the smaller squares with the area of 1/25 mm^2. When the counting is completed, the number of spores per bee in the sample can be calculated according to the formula:

S =St/Sq× Df × 250,000

Where:

S = spore numbers per bee St = total number of spores counted Sq= number of squares counted Df= dilution factor

The number 250,000 is used because the volume in each counted square is 1/250 000 ml and the equation uses the average number of spores per counted square. If no spores are seen, the result should be designated 'not detected', but that does not mean that the bees are not infected (OIE, 2013). The level of infection is considered low if <100,000 spores / bee, medium if between 100,000 and 1 million spores / bee, high if > 1 million spores / bee (Doull and Eckert., 1962; Gross and Ruttner., 1970).

3.2.3 Biomolecular examination

Biomolecular examination consists in the identification of the presence of genetic material in honeybee samples, therefore it allows to obtain a certain diagnosis of the disease but above all to identify "precisely" the species of Nosema, and it often follows the identification of spores in a sample.
The most frequently used technique to amplify nucleic acids and obtain large quantities of genetic material is the Polymerase Chain Reaction (commonly known as PCR).
Multiplex PCR allows detection of both microsporidia (*N.apis* and *N. ceranae*) in one PCR thanks to the use of specific primers. The manual for apidological research (Fries et al., 2013) describes in detail the technique and the protocol in order to unify researches and results.
This technique can be considered both qualitative and quantitative: it is qualitative when applied to individual bees of a sample, it can be considered quantitative, since a result can be expressed in terms of percentage of infected bees (OIE, 2013).

3.3 The Mini-FLOTAC technique

Copromicroscopic techniques are widely used for the diagnosis of helminth and protozoa in humans and animals.
However, not always the performance of the test is highly sensitive, accurate and precise. Moreover, the use of different protocols across diagnostic and research laboratories could implicate discrepancies in results (Bogoch et al., 2006).
In 2004, the FLOTAC technique made his way into the world of parasitological diagnosis and research (Cringoli, 2006), and since then numerous studies have shown that the FLOTAC technique is strongly competitive in terms of precision, accuracy and sensitivity (Cringoli et al., 2010).
The FLOTAC technique is based on a combination of flotation and translation facilitated by a novel apparatus, and allows detection and quantification of a wide range of eggs, larvae, oocysts, and cysts of parasites in fecal samples. However, it requires laboratory equipment that can impede its application on-farm (Barda et al., 2013).
The Mini-FLOTAC technique is the "logical evolution" (Cringoli et al., 2017) of the FLOTAC technique, as it combines highly reproducible, accurate results with an easy and fast protocol, able to analyse many samples in a short time also in spaces were no great equipment (such as centrifuges) is present (Maurelli et al., 2014).
Today, the Mini-FLOTAC technique is used internationally both in veterinary and human parasitology, for the qualitative and quantitave copromicroscopic identification of eggs, larvae, cysts and oocysts of protozoa, nematodes, trematodes and cestodes (Cringoli et al., 2017).
The Mini-FLOTAC is a polycarbonate disk-shaped device, composed of base and reading disk, which, together, delimit two flotation chambers of 1 ml each (total volume = 2 ml). Each chamber is divided into 12 sections, by two ruled grids designed on the surface of the reading disk and that allow quantitative examination of fecal samples. The Mini-FLOTAC is also equipped with two accessories: the key, necessary for the translation

of the reading disk, and the adapter for the microscope (Cringoli et al., 2017) (Fig. 3.5).

Fig. 3.5. Mini-FLOTAC components. i) base; ii) reading disk with two ruled grids; iii) key; iv) microscope adaptor (Cringoli et al., 2017).

In order to protect users against potential biohazards, it is recommended to use the Mini-FLOTAC in combination with the Fill-FLOTAC (Fig. 3.6), a special tool for the collection, storage, measurement, homogenization and filtration of feces, structured to limit the contact between samples and laboratory personnel (Maurelli et al., 2014). The Fill-FLOTAC is a kit consisting of a graduated container for samples, a lid with a filter presenting 250 μm holes and a collecting / homogenizing pole. The lid hermetically seals to the container and it has two screw caps that close two openings, one central for the collector / homogenizer pole and a lateral one that allows sample filtering. The Fill-FLOTAC also presents two accessories: a tip useful for the Mini-FLOTAC and a device for disassembling the Fill-FLOTAC filter (Cringoli et al., 2017).

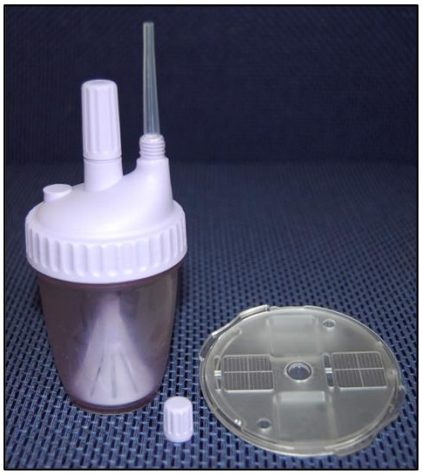

Fig. 3.6. Fill-FLOTAC and Mini-FLOTAC.

The main advantages of the Mini-FLOTAC technique are that it can be used on fresh or fixed samples, it allows to process pooled samples, it is reusable up to 50 times, and, as it is easy to use, it is a valuable tool for laboratories with limited resources or for application directly on-farm (Barda et al., 2013; 2014).
However, the technique also shows some limitations such as the duration of fecal preservation before observation and the selection of choice of the correct flotation solution (FS), that could affect the percentage of parasitic elements detected (Cringoli et al., 2017).
Probably the biggest constraint is the lower sensitivity of the Mini-FLOTAC technique (5 egg/larvae/oocyst/cyst per gram of feces) compared to the one of the FLOTAC (1 egg/larvae/oocyst/cyst per gram of feces) therefore, when highly precise diagnosis is required the use of the FLOTAC should be preferred (Cringoli et al., 2017).

3.4 Aim of the study

The diagnosis of Nosemosis consists in highlighting the spores of *Nosema spp*. in honeybee samples.

Generally, it is made observing the direct smear of the abdomen or fecal material of honeybees from poorly productive colonies or showing clinical signs.
This technique is certainly quick and easy to perform but it does not always allow spore identification and count, and therefore to establish the degree of infection. For a quantitative evaluation it is necessary to use a hemocytometer.
As, the Mini-FLOTAC is quali-quantitative technique that has been used with great success for the parasitological diagnosis both in humans and animals for many years.
In this study the Mini-FLOTAC was tested as a potential tool for the field diagnosis of Nosemosis and a specific method was developed.
Results were compared with the ones obtained by direct smear and microscopic examination with the use of the hemocytometer to evaluate the efficiency of the test.
Anatomo-histopathological analysis was carried out to confirm the presence of the disease in the hives and to study the presence of any organ and tissue alterations.
The methods used and the results obtained are reported in the following paragraphs.

3.5 Materials and methods

3.5.1 Sampling

Pools of 50 forager honeybees of *Apis mellifera ligustica* from 10 different apiaries (500 total) located in the province of Naples and Caserta (Campania region), were collected at the hive entrance, during the months of October- November 2017.
Hives were chosen between the less productive of the precedent season.
The honeybees were held in 50 ml Falcon tubes and transported to the laboratory of Veterinary General Pathology and Anatomical Pathology of the Department of Veterinary Medicine and Animal Productions. For each apiary, the half (25/50) collected sample was frozen at -20 ° C

for parasitological examinations, after removing head and thorax (Fig. 3.7); the other half (25/50) was fixed in 10% formalin for the anatomo-histological analysis after being immobilised with chilling for 3 minutes at -20°C (Human et al., 2013).

Fig. 3.7. Sample preparation. The abdomens are separated from the head and the thorax.

3.5.2 Anatomo-histopathological analysis

All formalin fixed honeybees were observed with a stereo microscope (Microscope Axioskop HBO50, Zeiss, Milan, Italy) to detect any external morphological changes not seen with the naked eye.
After, for each apiary, part of the formalin fixed samples (12/25) were subjected to necropsy. Honeybees were pinned to a piece of cork with their back uppermost and dissection of the abdomen was performed as described by Carreck et al. (2013). The intestine was removed and analysed to identify any macroscopic alterations.
The remaining (13/25) were processed for histopathological analysis using the new protocol described in Chapter 1.

3.5.3 Direct smear and hemocytometer examination

From each frozen sample, pools of 10/25 honeybees were placed in a dish with spring water (in a ratio of 1 ml of sampled water / abdomen), and grounded with a pestle. Both tools were washed in between samples.
A drop of the obtained fluid was placed on the edge of a glass slide and with a second glass slide, the drop was "swiped" with rapid and continuous movement by capillarity. Slides were air-dried for two minutes, stained with Diff-Quick staining by dipping the slide few times in an eosinophilic solution and few times in a basophilic solution, and then rinsed in distilled water and air-dried. Slides were then mounted and observed by light microscopy (Microscope Nikon Eclipse E-600, Tokyo, Japan).
20 µl of the previously prepared macerate, were used to fill each of the two wells of the hemocytometer.
After waiting for 60 seconds to allow the spores to settle, the two chambers of the hemocytometer were observed by light microscopy (Microscope Nikon Eclipse E-600, Tokyo, Japan).

3.5.4 Examination with the Mini-FLOTAC technique

10/25 of the remaining frozen samples were transferred in the Fill-FLOTAC container and were crushed by pumping the fecal collector for ten times while turning it left and right.
10 ml of sodium chloride flotation solution were added (in a ratio of 1 ml of sampled water / abdomen), and samples were carefully homogenized by pumping the fecal collector for twenty times while turning it left and right.
After putting the tip on the top hole, the Fill-FLOTAC was gently shaken to allow nice mixing of the sample with the FS. The solution was then carefully poured using the tip into the two chambers of the Mini-FLOTAC through the filling holes. The Mini-FLOTAC was kept in a 45° position to avoid the formation of bubbles and to create a meniscus. After 10 minutes, the reading disk of the apparatus was turned 90° clockwise using the key that was then removed to allow examination of the apparatus under the microscope (Cringoli et al., 2017).

3.6 Results

Of the 10 apiaries examined, spores were observed only in samples collected in the province of Caserta (4/10).

3.6.1 Macroscopic and microscopic results

The anatomopathological analysis did not reveal any alterations in any of the samples analysed.
In every sample, the midgut appeared wrinkled, firm and light brown, and the individual circular constrictions were perfectly visible; the small intestine was transparent and clear; the rectum appeared full and solid.
On the contrary, the histopathological analysis revealed the presence of alterations of the midgut and Malpighian tubules of honeybees collected in four apiaries (4/10) from the province of Caserta.
The cytoplasm of numerous epithelial cells of the midgut showed the presence of many translucent spores, which filled and enlarged the cells.
Parasites at different developmental stages were present: mature spores appeared smaller and clearer, containing a basophilic elongated mass, and were visible in the apical region of the epithelial cells, while immature spores were larger and more eosinophilic and localized at the bottom of the cells next to few regenerative nests (Fig. 3.8).

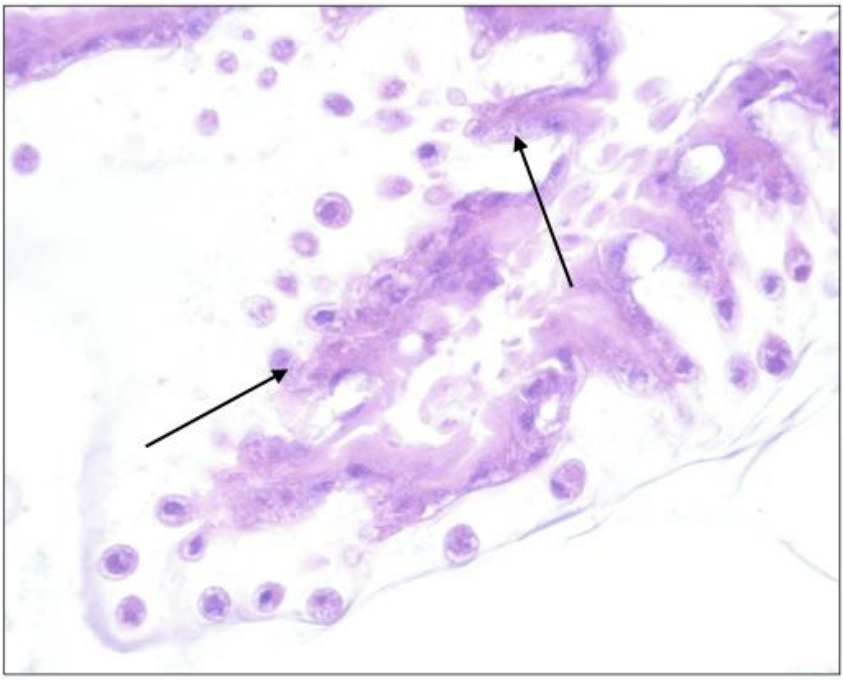

Fig. 3.8. A. mellifera. Midgut. The cytoplasm of epithelial cells shows the presence of numerous translucent spores at different developmental stages, which fill and enlarge the cells. H-E. 20X.

Many cells showed pyknotic nuclei and moderate vacuolization.
The peritrophic membrane was unevenly lining the midgut and many secretive droplets, were present in the lumen (Fig. 3.9).

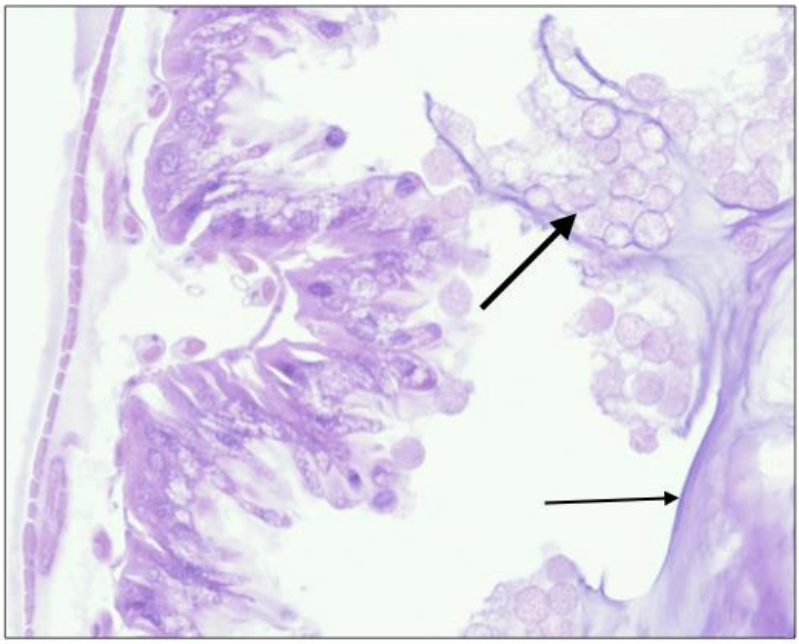

Fig. 3.9. A. mellifera. Midgut. The peritrophic membrane is unevenly lining the midgut (thin arrow); presence of many secretive droplets filled with Nosema spp. spores (thick arrow). H-E. 20X.

Moreover, numerous spores, uniform in shape and size, were also observed in the lumen of the midgut and in secretive droplets (Fig. 3.10).

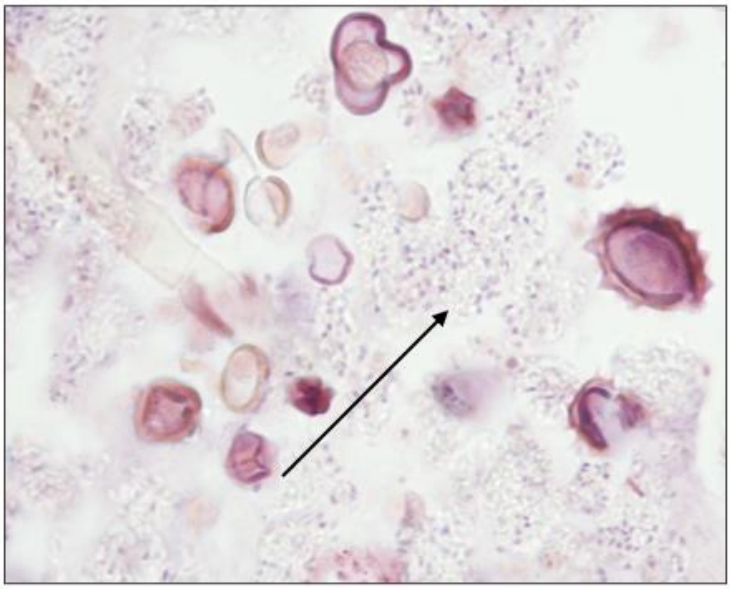

Fig. 3.10. A. mellifera. Midgut lumen.Presence of many spores uniform in shape and size (thin arrow). H-E. 100X.

Translucent spores were also observed in the lumen of the malpighian tublues and in the epithelium, which showed exfoliation, bright eosinophilic material accumulation and higher microvilli (Fig. 3.11).

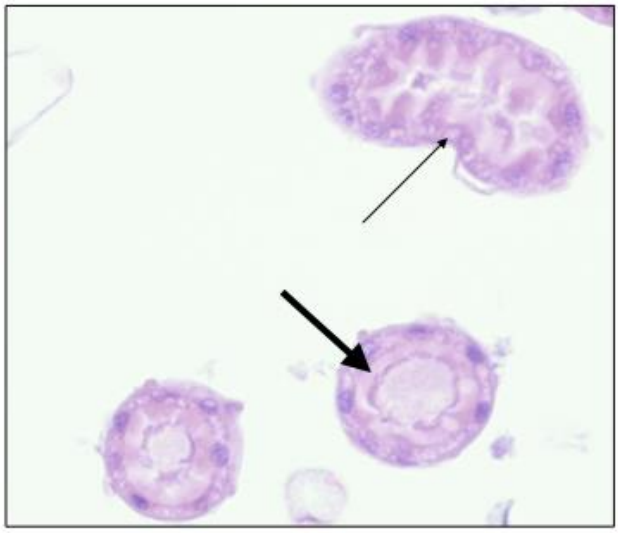

Fig. 3.11. A. mellifera. Malpighian tubules. Presence of translucent spores in the lumen and in the epithelium (thin arrow), which showed exfoliation, bright eosinophilic material accumulation (thick arrow) and higher microvilli. H-E. 40X

The samples collected from the other six apiaries (6/10) did not show any alterations of the midgut (Fig. 3.12).

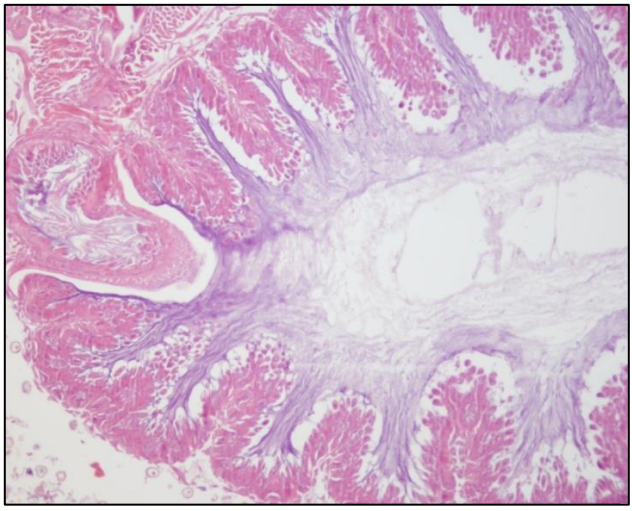

Fig. 3.12. A. mellifera. Healthy midgut.H-E. 10X.

3.6.2 Parassitological results

All of the parasitological techniques used, highlighted the presence of numerous spores in the examined samples from the four (4/10) apiaries in the Caserta province, while the other six (6/10) did not show the presence of spores.
Samples analysed by direct smear (Fig.3.13) and hemocytometer (Fig.3.14), appeared filled with a large amount of fecal debris, pollens, and cells, that often covered the spores making them scarcely detectable or not detectable at all

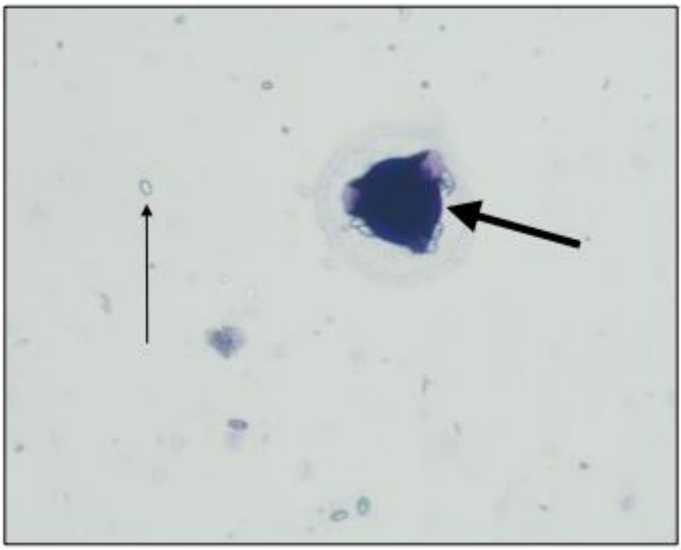

Fig. 3.13. Direct smear. Few spores of Nosema spp. are visible (thin arrow), while others are partially covered by a pollen grain (thick arrow). Diff-Quick. 40X.

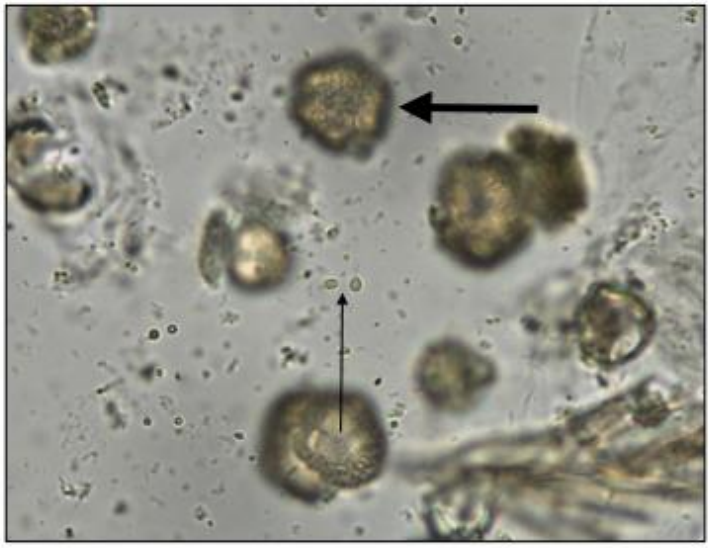

Fig. 3.14. Hemocytometer. Nosema spp. spores (thin arrow) are mixed with pollen and fecal debris (thick arrow). 40X.

Samples obtained using the Mini-FLOTAC technique appeared cleaner and clearer, that is, with scarce or completely absent debris, and therefore spores appeared more easily recognisable (Fig. 3.15).

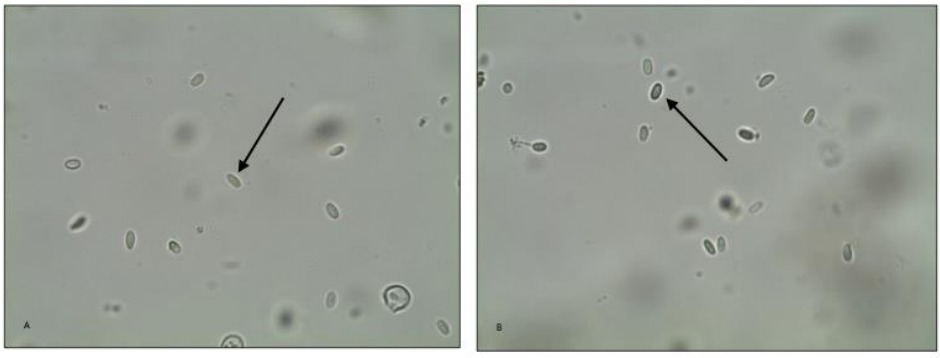

Fig. 3.15. Mini-FLOTAC. Presence of numerous spores of Nosema spp. (thin arrow) and absence of debris. 40X

Morphologically the spores were approximately 3-5 μm long and 2-3 μm wide, highly refractive oval to elliptical in shape, with a thick wall, and surrounded by a dark halo. These characteristics made us assume that are spores of *N. ceranae* rather than *N.apis.*

3.7 Discussion and conclusions

Nosemosis is one of the most serious diseases affecting adult honeybees worldwide, causing colony depopulation and threatening colony survival. More than 400 studies have targeted this topic (Martin-Hernandez et al., 2018), analysing routes of transmission, population structure and genetic diversity, pathogenesis, and possible treatments, but not many have focused on diagnostic techniques.

Nonetheless, an accurate and precise diagnosis is the first step towards correct prevention, control and treatment.

As the parasite represents a major hazard for beekeepers, a sensitive, rapid and affordable technique for a proper on-field diagnosis is necessary, which could allow the visualization of *Nosema* spores even when the disease is not clinically manifesting yet.

The lack of clinical signs does not always reflect the health status of a colony and underlines the importance of introducing periodical analysis of samples in the beekeeping practices of all apiaries, corroborating what recommended by FAO (2020).

Usually, the field diagnosis of Nosemosis is performed by microscopic examination of macerated suspension taken from symptomatic or asymptomatic honeybees, to evaluate the presence of spores (OIE, 2013).

The results of this study show that the direct smear is an easy and quick technique that requires minimal equipment; however, it is often inaccurate as the presence of pollens, fecal debris and honeybee cells on the slide, make visualization of spores more difficult, especially when there are only few. So, it may happen that low-intensity infections may be missed and

when the disease outbreaks, it could be to late to put in place control measures.
Moreover, the direct smear can only give a positive or negative result, by showing presence or absence of spores (Fries et al., 2013).
As a quantitative evaluation is necessary for applying the correct beekeeping procedures, the use of an hemocytometer is required.
However, the equipment required is more expensive, and the operator, needs to be previously trained to carefully mount the aemocytometer and to identify the spores, but also to efficiently count them.
Furthermore, also in the case of the sample examination with the hemocytometer, the observation field appeared often opaque, yellowish, showing the presence of pollens and fecal debris, which did not always allow easy counting of the spores, as they were sometimes covered.
Quantitative evaluation is particularly important when treatments and other measures are applied after a certain threshold.
In this study, for the first time the Mini-FLOTAC technique, a quantitative, very sensitive, accurate and precise copromicroscopic technique (Cringoli et al., 2017) has been used for the diagnosis of Nosemosis.
Compared to the hemocytometer, the use of the Mini-FLOTAC as an equipment appears easier, more userfriendly and safe. In fact, the whole apparatus is made of polycarbonate amorphous thermoplastic (Cringoli et al., 2017) that makes the tool light, manageable and safe when used. The operator is further protected from biohazards if he decides to use the Fill-FLOTAC for stool processing. On the contrary, the hemocytometer, being made of glass, appears heavier and fragile, potentially causing damage to the operator if it breaks. Although both instruments are reusable, the Mini-FLOTAC instrument can by used up to 50 times (Cringoli et al., 2017), while the multiple uses of the hemocytometer are tied to the integrity of the tool. The Mini-FLOTAC technique is based on the flotation, which allows separation of elements based on the weight, so spores flow at the top while fecal debris and pollen accumulate at the bottom, and translation, which allows to “cut” the apical portion of the flotation suspension (Maurelli et al., 2014). Consequently, as proved by the results of this study,

the reading field appeares clear, with no or only few pollen grains and fecal debris per sample, therefore counting of the spores is not affected, and spores are evident also in cases of low infection.
The histopathological analysis highlighted the presence of translucent mature spores both in the midgut and for the first time, to the best of our knowledge, also in the Malpighian tubules.
Localization of *Nosema spp*. in the midgut has been described many times by researchers (Higes et al., 2020; Dussaubat et al., 2012; Maiolino et al., 2014), while tropism for other organs is still debated.
The presence of genetic material pertaining to *Nosema spp.* in the Malpighian tubules, has also been described, but the result was attributed to possible contamination from the near midgut rather then to a real infection (Huang and Solter, 2013).
Here, spores are present both in the lumen and in the cytoplasm of the cells of the epithelium of the tubules, where they accumulate and, probably cause alterations.
In fact, the cytoplasm of those cells appeared filled with eosinophilic material and showing higher microvilli, suggesting alteration of the excretory activity
Malpighian tubules are the main excretory organs of honeybees, as their main role is to eliminate excretory products and toxic compounds from the haemolymph into the hindgut. They are also involved in osmoregulation, through reabsorption of ions and water of the microvilli (Nicolson, 2008).
The presence of higher microvilli could suggest the need of greater water reabsorption, probably to compensate losses of water occurring at the midgut level, whereas the accumulation of bright eosinophilic material, probably of proteic nature, suggests alterations of the excretory function.
As Nosema survives and mutiplicates at expenses of its host, it could be speculated that the energy produced by the host mithocondria is used by the parasite and it is not sufficient to support adequate excretion of urates, thus leading to accumulation of the same in the cell.
Numerous parasites were identified in the midgut cells which showed pyknotic nuclei and vacuolization of the cytoplasm, suggesting the presence of an ongoing degenerative process.

Moreover, the presence of parasitized regenerative nests at the basis of the epithelium, does not guarantee a correct turnover of the destroyed cells and reepithelization of the tissue as demonstrated by Dussaubat et al., 2012.

It seems evident that these alterations significantly impair the absorbing function of the organ, causing starvation and dehydration of the honeybees, which could eventually lead to the death of the insect (Maiolino et al., 2014).

Nosema infected honeybees have been shown to be more susceptible to other pathogens, as the parasite is able to downregulate the immune system of the host (Antunez et al., 2009).

The first line of immune response is the presence of physical barriers namely the cuticle and the perithrophic membrane, which is often fragmented or unlined in honeybees infected with *Nosema* (Higes et a., 2020).

The histopathological analysis of the midgut has confirmed the presence of alterations in the production and in the lining of the perithrophic membrane, probably caused on one hand by changes of the secretion of the cells of the midgut due to *Nosema* infection, on the other hand by alteration of the uptake and metabolism of the nutrients.

Alterations of the peritrophic membrane make the honeybee more susceptible to secondary pathogens, especially those uptaken by ingestion that have tropism for the midgut epithelium (i.e. DWV).

The results reported by this study highlighted the usefulness and validity of the histopathological analysis in the diagnosis of this disease, in particular when the symptoms in the hive are not yet evident, and as a helpful research tool to better understand the seriousness of the disease.

In conclusion, the examination with the Mini-FLOTAC proved to be useful, effective and advantageous compared to the other techniques used. In addition to showing high precision, and sensitivity, the Mini-FLOTAC is also simple and easy to use, inexpensive, and it can be used both in the laboratory, as the material can be fixed in formaline, and in the field, as the equipment required is very basic.

Particularly, the Mini-FLOTAC should be used for an initial screening to identify positive bees that could be subsequently analyzed through biomolecular techniques (such as PCR) for the identification of the species (*N.apis* and / or *N. ceranae*).

Bibliography

Alaux C, Brunet JL, Dussaubat C, et al. Interactions between Nosema microspores and a neonicotinoid weaken honeybees (Apis mellifera). *Environ Microbiol*. 2010;12(3):774-782. doi:10.1111/j.1462-2920.2009.02123.x

Antúnez K, Martín-Hernández R, Prieto L, et al. Immune suppression in the honey bee (Apis mellifera) following infection by Nosema ceranae (Microsporidia). *Environ Microbiol*. 2009;11(9):2284-2290. doi:10.1111/j.1462-2920.2009.01953.x

Arredondo D, Castelli L, Porrini MP, et al. Lactobacillus kunkeei strains decreased the infection by honey bee pathogens Paenibacillus larvae and Nosema ceranae. *Benef Microbes*. 2018;9(2):279-290. doi:10.3920/BM2017.0075

Barda B, Cajal P, Villagran E, et al. Mini-FLOTAC, Kato-Katz and McMaster: three methods, one goal; highlights from north Argentina. *Parasit Vectors*. 2014;7:271. Published 2014 Jun 14. doi:10.1186/1756-3305-7-271

Barda BD, Rinaldi L, Ianniello D, et al. Mini-FLOTAC, an innovative direct diagnostic technique for intestinal parasitic infections: experience from the field. *PLoS Negl Trop Dis*. 2013;7(8):e2344. Published 2013 Aug 1. doi:10.1371/journal.pntd.0002344

Bogoch II, Raso G, N'Goran EK, Marti HP, Utzinger J. Differences in microscopic diagnosis of helminths and intestinal protozoa among diagnostic centres. *Eur J Clin Microbiol Infect Dis*. 2006;25(5):344-347. doi:10.1007/s10096-006-0135-x

Becnel JJ, Andreadis TG. Microsporidia in insects. In M Wittner & LM Weiss (Eds). The Microsporidia and Microsporidiosis (pp.447-501). Washington, DC: ASM Press. 1999

Bordier C, Suchail S, Pioz M, et al. Stress response in honeybees is associated with changes in task-related physiology and energetic metabolism. *J Insect Physiol*. 2017;98:47-54. doi:10.1016/j.jinsphys.2016.11.013

Borges, D. Control of the intestinal parasite Nosema ceranae in apis mellifera using mutraceuticals, prebiotics and probiotics. Thesis School of Environmental Sciences. Ontario, Canada, University of Guelph. 2015

Botías C, Martín-Hernández R, Barrios L, et al. Nosema spp. infection and its negative effects on honey bees (Apis mellifera iberiensis) at the colony level. *Vet Res*. 2013;44(1):25. Published 2013 Apr 10. doi:10.1186/1297-9716-44-25

Burges HD, Canning EU, Hulls IK. Ultrastructure of Nosema oryzaephili and the taxonomic value of the polar filament. Journal of invertebrate pathology. 1974 Mar 1;23(2):135-9.

Carpana E, Lodesani M. Patologie e avversità dell'alveare. Springer Verlag, 2014.

Carreck NL, Andree M, Brent CS, et al. Standard methods for *Apis mellifera* anatomy and dissection, J Apic Res. 2013; 52:4, 1-40 doi: 10.3896/IBRA.1.52.4.03

Chaimanee V, Chantawannakul P, Chen Y, et al. Differential expression of immune genes of adult honey bee (Apis mellifera) after inoculated by Nosema ceranae. *J Insect Physiol*. 2012;58(8):1090-1095. doi:10.1016/j.jinsphys.2012.04.016

Chemurot M, De Smet L, Brunain M, De Rycke R, de Graaf DC. Nosema neumanni n. sp. (Microsporidia, Nosematidae), a new microsporidian parasite of honeybees, Apis mellifera in Uganda. Eur J Protistol. 2017 Oct;61(Pt A):13-19. doi: 10.1016/j.ejop.2017.07.002

Chen Y, Evans JD, Zhou L, et al. Asymmetrical coexistence of Nosema ceranae and Nosema apis in honey bees. *J Invertebr Pathol*. 2009;101(3):204-209. doi:10.1016/j.jip.2009.05.012

Chen Y, Evans JD, Smith IB, Pettis JS. Nosema ceranae is a long-present and wide-spread microsporidian infection of the European honey bee (Apis mellifera) in the United States. *J Invertebr Pathol*. 2008;97(2):186-188. doi:10.1016/j.jip.2007.07.010

Chen Y, Evans J, Feldlaufer M. Horizontal and vertical transmission of viruses in the honey bee, Apis mellifera. J Invertebr Pathol. 2006;92(3):152-159. doi:10.1016/j.jip.2006.03.010

Copley TR, Jabaji SH. Honeybee glands as possible infection reservoirs of Nosema ceranae and Nosema apis in naturally infected forager bees. *J Appl Microbiol*. 2012;112(1):15-24. doi:10.1111/j.1365-2672.2011.05192.x

Costa C. La Nosemiasi in :Carpana E, Lodesani M. Patologie e avversità dell'alveare. Springer Verlag. 2014

Cox-Foster DL, Conlan S, Holmes EC, et al. A metagenomic survey of microbes in honey bee colony collapse disorder. *Science*. 2007;318(5848):283-287. doi:10.1126/science.1146498

Cringoli G, Maurelli MP, Levecke B, et al. The Mini-FLOTAC technique for the diagnosis of helminth and protozoan infections in humans and animals. *Nat Protoc*. 2017;12(9):1723-1732. doi:10.1038/nprot.2017.067

Cringoli G, Rinaldi L, Maurelli MP, Utzinger J. FLOTAC: new multivalent techniques for qualitative and quantitative copromicroscopic diagnosis of parasites in animals and humans. *Nat Protoc*. 2010;5(3):503-515. doi:10.1038/nprot.2009.235

Cringoli G. FLOTAC, a novel apparatus for a multivalent faecal egg count technique. *Parassitologia*. 2006;48(3):381-384.

Doull KM, Eckert JE. A survey of the incidence of Nosema disease in California. J Econ Entomol. 1962;55(3):313-7. doi: https://doi.org/10.1093/jee/55.3.313

Dussaubat C, Brunet JL, Higes M, et al. Gut pathology and responses to the microsporidium Nosema ceranae in the honey bee Apis mellifera. *PLoS One*. 2012;7(5):e37017. doi:10.1371/journal.pone.0037017

Eiri DM, Suwannapong G, Endler M, Nieh JC. Nosema ceranae can infect honey bee larvae and reduces subsequent adult longevity. PLoS One. 2015;10(5). doi: https://doi.org/10.1371/journal.pone.0126330

Endler MB. Feeding larval honey bees (Apis mellifera) dead Nosema spores improves their ability to resist Nosema infections as adults. Thesis in Science. San Diego, CA, USA: university of California. 2014

FAO. 2020. Good beekeeping practices: Practical manual on how to identify and control the main diseases of the honeybee (Apis mellifera). TECA – Technologies and practices for small agricultural producers, 1. Rome. https://doi.org/10.4060/ca9182en

Fries I, Chauzat M-P, Chen Y-P, et al. Standard methods for nosema research. In: Dietemann V, Ellis JD, Neumann P (eds) The COLOSS BEEBOOK: Volume II: Standard methods for *Apis mellifera* pest and pathogen research. *J Apic Res*. 2013 doi:10.3896/IBRA.1.52.1.14

Fries I, Feng F, da Silva A, et al. Nosema ceranae n. sp.(Microspora, Nosematidae), morphological and molecular characterization of a microsporidian parasite of the Asian honey bee Apis cerana (Hymenoptera, Apidae). *Eur J Protistol.* 1996;32(3):356-65. doi: https://doi.org/10.1016/S0932-4739(96)80059-9

Fries I. Nosema apis-a parasite in the honey bee colony. *Bee World.* 1993; 74:5-19. doi: 10.1080/0005772X.1993.11099149

Fries I. Infectivity and multiplication of Nosema apis Z. in the ventriculus of the honey bee. *Apidologie*. 1988;19(3):319-28. doi: 10.1051/apido:19880310

Giersch T, Berg T, Galea F, Hornitzky M. Nosema ceranae infects honey bees (Apis mellifera) and contaminates honey in Australia. *Apidologie.* 2009;40(2):117-23. doi: 10.1051/apido/2008065

Gisder S, Hedtke K, Möckel N, et al. Five-year cohort study of Nosema spp. in Germany: does climate shape virulence and assertiveness of Nosema ceranae?. *Appl Environ Microbiol*. 2010;76(9):3032-3038. doi:10.1128/AEM.03097-09

Glinski Z, Jarosz J. Infection and immunity in the honey bee Apis mellifera. *Apiacta*. 2001;36(1):12-24.

Goblirsch M. Nosema ceranae disease of the honey bee (Apis mellifera). *Apidologie.* 2018;49(1):131-50.

Goblirsch M. Using Honey Bee Cell Lines to Improve Honey Bee Health. In:Beekeeping–From Science to Practice. pp. 91-108. Springer, Cham. 2017

Graystock P, Goulson D, Hughes WO. Parasites in bloom: flowers aid dispersal and transmission of pollinator parasites within and between bee species. *Proc Biol Sci*. 2015;282(1813):20151371. doi:10.1098/rspb.2015.1371

Gross KP, Ruttner F. Is Nosema apis developing a resistance to the antibiotic Fumidil B?. *Apidologie.* 1970;1(4):401-21.

Han B, Weiss LM. Microsporidia: Obligate Intracellular Pathogens Within the Fungal Kingdom. *Microbiol Spectr*. 2017;5(2) doi:10.1128/microbiolspec.FUNK-0018-2016.

Higes M, García-Palencia P, Urbieta A, et al. *Nosema apis* and *Nosema ceranae* Tissue Tropism in Worker Honey Bees (*Apis mellifera*). *Vet Pathol*. 2020;57(1):132-138. doi:10.1177/0300985819864302

Higes M, Martín-Hernández R, Meana A. Nosema ceranae in Europe: an emergent type C nosemosis. *Apidologie*. 2010;41(3):375-92. doi: https://doi.org/10.1051/apido/2010019

Higes M, Martín-Hernández R, Garrido-Bailón E, et al. Honeybee colony collapse due to Nosema ceranae in professional apiaries. *Environ Microbiol Rep*. 2009;1(2):110-113. doi:10.1111/j.1758-2229.2009.00014.x

Higes M, Martín-Hernández R, Botías C, et al. How natural infection by Nosema ceranae causes honeybee colony collapse. *Environ Microbiol*. 2008a;10(10):2659-2669. doi:10.1111/j.1462-2920.2008.01687.x

Higes M, Martín-Hernández R, Garrido-Bailón E, Botías C, García-Palencia P, Meana A. Regurgitated pellets of Merops apiaster as fomites of infective Nosema ceranae (Microsporidia) spores. *Environ Microbiol*. 2008b;10(5):1374-1379. doi:10.1111/j.1462-2920.2007.01548.x

Higes M, Martín-Hernández R, Garrido-Bailón E, García-Palencia P, Meana A. Detection of infective Nosema ceranae (Microsporidia) spores in corbicular pollen of forager honeybees. *J Invertebr Pathol*. 2008c;97(1):76-78. doi:10.1016/j.jip.2007.06.002

Higes M, García-Palencia P, Martín-Hernández R, Meana A. Experimental infection of Apis mellifera honeybees with Nosema ceranae (Microsporidia). *J Invertebr Pathol*. 2007;94(3):211-217. doi:10.1016/j.jip.2006.11.001

Huang Q, Li W, Chen Y, et al. Dicer regulates Nosema ceranae proliferation in honeybees. *Insect Mol Biol*. 2019;28(1):74-85. doi:10.1111/imb.12534

Huang WF, Solter LF. Comparative development and tissue tropism of Nosema apis and Nosema ceranae. *J Invertebr Pathol*. 2013;113(1):35-41. doi:10.1016/j.jip.2013.01.001

Huang WF, Solter LF, Yau PM, Imai BS. Nosema ceranae escapes fumagillin control in honey bees. *PLoS Pathog*. 2013;9(3):e1003185. doi:10.1371/journal.ppat.1003185

Huang WF, Jiang JH, Chen YW, Wang CH. A Nosema ceranae isolate from the honeybee Apis mellifera. *Apidologie*. 2007;38, 30–37. doi: 10.1051/apido:2006054

Human H, Brodschneider R, Dietemann V et al. Miscellaneous standard methods for Apis mellifera research. In V Dietemann; J D Ellis; P Neumann (Eds) The COLOSS BEEBOOK, Volume I: standard methods for Apis mellifera research. *J Apic Res*.2013; 52(4) http://dx.doi.org/10.3896/IBRA.1.52.4.10

Keeling PJ, Fast NM. Microsporidia: biology and evolution of highly reduced intracellular parasites. *Annu Rev Microbiol*. 2002;56:93-116. doi:10.1146/annurev.micro.56.012302.160854

Kralj J, Fuchs S. Nosema sp. influences flight behavior of infected honey bee (Apis mellifera) foragers. *Apidologie*. 2010;41(1):21-8.doi:https://doi.org/10.1051/apido/2009046 h

Li J, Chen W, Wu J, et al. Diversity of Nosema associated with bumblebees (Bombus spp.) from China. *Int J Parasitol*. 2012;42(1):49-61. doi:10.1016/j.ijpara.2011.10.005

Li W, Chen Y, Cook SC. Chronic Nosema ceranae infection inflicts comprehensive and persistent immunosuppression and accelerated lipid loss in host Apis mellifera honey bees. *Int J Parasitol*. 2018;48(6):433-444. doi:10.1016/j.ijpara.2017.11.004

Maiolino P, Carella F, De Leva G, et al. Histopathology as diagnostic tool for Ascospaera apis infection in apparently healthy honey bees (Apis mellifera ligustica). *J Interdiscipl Histopathol.* 2013; 1:160-162.

Maiolino P, Iafigliola L, Rinaldi L, et al. Histopathological findings of the midgut in European honey bee (Apis Mellifera L.) naturally infected by Nosema spp. *Vet Med Anim Sci*. 2014;2(4). doi: http://dx.doi.org/10.7243/2054-3425-2-4

Malysh JM, Ignatieva AN, Artokhin KS, et al. Natural infection of the beet webworm Loxostege sticticalis L. (Lepidoptera: Crambidae) with three

Microsporidia and host switching in Nosema ceranae. *Parasitol Res*. 2018;117(9):3039-3044. doi:10.1007/s00436-018-5987-3

Martín-Hernández R, Bartolomé C, Chejanovsky N, et al. Nosema ceranae in Apis mellifera: a 12 years postdetection perspective. *Environ Microbiol*. 2018;20(4):1302-1329. doi:10.1111/1462-2920.14103

Martín-Hernández R, Botías C, Barrios L, et al. Comparison of the energetic stress associated with experimental Nosema ceranae and Nosema apis infection of honeybees (Apis mellifera). *Parasitol Res*. 2011;109(3):605-612. doi:10.1007/s00436-011-2292-9

Maurelli MP, Rinaldi L, Alfano S, et al. Mini-FLOTAC, a new tool for copromicroscopic diagnosis of common intestinal nematodes in dogs. *Parasit Vectors*. 2014;7:356. doi:10.1186/1756-3305-7-356

Mayack C, Natsopoulou ME, McMahon DP. Nosema ceranae alters a highly conserved hormonal stress pathway in honeybees. *Insect Mol Biol*. 2015;24(6):662-670. doi:10.1111/imb.12190

Mayack C, Naug D. Individual energetic state can prevail over social regulation of foraging in honeybees. *Behav Ecol Sociobiol.* 2013;67:929–936. doi: 10.1007/s00265-013-1517-6.

Mayack C, Naug D. Energetic stress in the honeybee Apis mellifera from Nosema ceranae infection. *J Invertebr Pathol*. 2009;100(3):185-188. doi:10.1016/j.jip.2008.12.001

McCowen MC, Callender ME, Lawlis JF Jr. Fumagillin (H-3), a new antibiotic with amebicidal properties. *Science*. 1951;113(2930):202-203. doi:10.1126/science.113.2930.202

McGowan J, De la Mora A, Goodwin PH, et al. Viability and infectivity of fresh and cryopreserved Nosema ceranae spores. *J Microbiol Methods*. 2016;131:16-22. doi:10.1016/j.mimet.2016.09.021

Mehlhorn H. Encyclopedic Reference of Parasitology. Biology, Structure, Function. Springer-Verlag Berlin Heidelberg New York. 2001

Mura A, Pusceddu M, Theodorou P, et al. Propolis Consumption Reduces *Nosema ceranae* Infection of European Honey Bees (*Apis mellifera*). *Insects*. 2020;11(2):124. Published 2020 Feb 15. doi:10.3390/insects11020124

Nelson CM, Ihle KE, Fondrk MK, et al. The gene vitellogenin has multiple

coordinating effects on social organization. *PLoS Biol*. 2007;5(3):e62. doi:10.1371/journal.pbio.0050062

Nicolson SW. Water homeostasis in bees, with the emphasis on sociality. *J Exp Biol*. 2009;212(Pt 3):429-434. doi:10.1242/jeb.022343

OIE (World Organization for Animal health) Manual of diagnostic tests and vaccines for terrestrial animals. Chapter 2.2.4 Nosemosis of the honeybee. 2013

Paris L, El Alaoui H, Delbac F, Diogon M. Effects of the gut parasite Nosema ceranae on honey bee physiology and behavior. *Curr Opin Insect Sci*. 2018;26:149-154. doi:10.1016/j.cois.2018.02.017

Paxton RJ. Does infection by Nosema ceranae cause "Colony Collapse Disorder" in honey bees (Apis mellifera)?. Journal of Apicultural Research. 2010 Jan 1;49(1):80-4.

Porrini C, Mutinelli F, Bortolotti L, et al. The Status of Honey Bee Health in Italy: Results from the Nationwide Bee Monitoring Network. *PLoS One*. 2016;11(5):e0155411. doi:10.1371/journal.pone.0155411

Ptaszyńska AA, Trytek M, Borsuk G, et al. Porphyrins inactivate Nosema spp. microsporidia. *Sci Rep*. 2018;8(1):5523. doi:10.1038/s41598-018-23678-8

Ptaszyńska AA, Borsuk G, Zdybicka-Barabas A, et al. Are commercial probiotics and prebiotics effective in the treatment and prevention of honeybee nosemosis C?. *Parasitol Res*. 2016;115(1):397-406. doi:10.1007/s00436-015-4761-z

Ptaszyńska AA, Borsuk G, Mułenko W, Demetraki-Paleolog J. Differentiation of *Nosema apis* and *Nosema ceranae* spores under Scanning Electron Microscopy (SEM), J Apic Res, 2014; 53:5, 537-544, doi: 10.3896/IBRA.1.53.5.02

Ptaszynska AA, Borsuk G, Anusiewicz M, Mulenko, W. Location of *Nosema* spp. spores within the body of the honey bee. *Med. Weter*. 2012;68, 618–621

Remolina SC, Hafez DM, Robinson GE, Hughes KA. Senescence in the worker honey bee Apis Mellifera. *J Insect Physiol*. 2007;53(10):1027-1033. doi:10.1016/j.jinsphys.2007.05.015

Roberts KE, Evison SE, Baer B, Hughes WO. The cost of promiscuity: sexual transmission of Nosema microsporidian parasites in polyandrous honey bees. *Sci Rep*. 2015;5:10982. doi:10.1038/srep10982

Smart MD, Sheppard WS. Nosema ceranae in age cohorts of the western honey bee (Apis mellifera). *J Invertebr Pathol*. 2012;109(1):148-151. doi:10.1016/j.jip.2011.09.009

Snow JW, Ceylan Koydemir H, Karinca DK, et al. Rapid imaging, detection, and quantification of Nosema ceranae spores in honey bees using mobile phone-based fluorescence microscopy. *Lab Chip*. 2019;19(5):789-797. doi:10.1039/c8lc01342j

Sulborska A, Horecka B, Cebrat M, et al. Microsporidia Nosema spp. - obligate bee parasites are transmitted by air. *Sci Rep*. 2019;9(1):14376. Published 2019 Oct 7. doi:10.1038/s41598-019-50974-8

Toth AL, Kantarovich S, Meisel AF, Robinson GE. Nutritional status influences socially regulated foraging ontogeny in honey bees. *J Exp Biol*. 2005;208(Pt 24):4641-4649. doi:10.1242/jeb.01956

Traver BE, Fell RD. Nosema ceranae in drone honey bees (Apis mellifera). *J Invertebr Pathol*. 2011;107(3):234-236. doi:10.1016/j.jip.2011.05.016

Traver BE, Williams MR, Fell RD. Comparison of within hive sampling and seasonal activity of Nosema ceranae in honey bee colonies. *J Invertebr Pathol*. 2012;109(2):187-193. doi:10.1016/j.jip.2011.11.001

Traver BE, Fell RD. A scientific note: Survey for Nosema spp. in preserved Apis spp. *Apidologie*, 2015*;* 46(2), 194-196. https://doi.org/10.1007/s13592-014-0306-1

Urbieta-Magro A, Higes M, Meana A, et al. The levels of natural Nosema spp. infection in Apis mellifera iberiensis brood stages. *Int J Parasitol*. 2019;49(8):657-667. doi:10.1016/j.ijpara.2019.04.002

Valera F, Gómez-Moracho T, Yuan HW, et al. Any role for the dissemination of Nosema spores by the blue-tailed bee-eater Merops philippinus?. *J Apic Res.* 2017;56(3):262-9. doi: 10.1080/00218839.2017.1306375

van den Heever JP, Thompson TS, Curtis JM, et al. Fumagillin: an overview of recent scientific advances and their significance for apiculture. *J Agric Food Chem*. 2014;62(13):2728-2737. doi:10.1021/jf4055374

Vidau C, Panek J, Texier C, et al. Differential proteomic analysis of midguts from Nosema ceranae-infected honeybees reveals manipulation of key host functions. *J Invertebr Pathol*. 2014;121:89-96. doi:10.1016/j.jip.2014.07.002

Wang DI, Moeller FE. Ultrastructural changes in the hypopharyngeal glands of worker honey bees infected by Nosema apis. *J Invertebr Pathol.* 1971;17(3):308-20.

Webster TC, Pomper KW, Hunt G, et al. Nosema apisinfection in worker and queen Apis mellifera. *Apidologie* 2004;35, 49–54

Webster TC, Thacker EM, Pomper K, et alNosema apis infection in honey bee (Apis mellifera) queens. *J Api. Res.* 2008;47, 53–57.

Zander E.: Tierische Parasiten als Krankenheitserreger bei der Biene. Münch.Bienenzeit. 1909, 31, 196-204

Conclusions

During the past years, many factors have reduced the number of honeybees worldwide, with great damage to biodiversity and to agro-zootechnical economics.

Honeybee pollination represents a key element in ecosystem function; it not only ensures the reproduction of wild plants and agricultural crops but also ensures genetic variability among species, thus defending global biodiversity. Moreover, the abundance and diversity of flora ensure food for human beings and feed for animals.

Also, honeybees are the only zootechnical species which give more than one product, highly appreciated by consumers and of great interest as a therapeutic tool, especially in fighting antimicrobial resistant infections.

Although the concurrent action of different stressors (including bacteria, virus, parasites, pesticides) has been identified as the source of loss of honeybees, very little information is available about how these factors actually affect the health of honeybees.

Honeybee pathology has been for many years a subject of great interest for researchers with different backgrounds, including biologists, agronomists, entomologists, and ecologists. However, this subject has elicited little interest among veterinarians and veterinary pathologists. Yet, who could better support these researchers in defining the pathogenesis of viral, bacterial, parasitic, or fungal diseases than veterinary pathologists?

Those of us who study honeybee pathology often find ourselves explaining why a veterinarian, and especially a veterinary pathologist, could be a great researcher in this specific issue and could be of great support to researchers from other fields. We often answer by asking these simple rhetorical questions: “Aren’t honeybees animals?” “Don’t honeybees produce products that are consumed by people, and therefore mustn’t we, as veterinarians, be interested in honeybee health in order to guarantee animal food safety?” “Don’t veterinarians study animal pathology thoroughly during their courses?”

Moreover, identification of macroscopic and microscopic alterations, understanding of morphological changes that have occurred in tissues, and

classification of lesions may be best performed by those who have deeply studied these specific subjects and have deep knowledge and experience in disease processes, diagnostics, and research to better understand pathogenesis and association between pathogens and lesions.
Increased interest of pathologists in this field could help unravel the mysteries around the huge colony losses by creating effective laboratory protocols, diagnostic tools, and therapeutics.
Histopathology has been used for decades in other species and has already been suggested as an efficient tool to diagnose honeybee pathologies.
Very little is still known about basic phenomena such as what is the pathogenic mechanism behind the deformed wings caused by the deformed wing virus, how the honeybee immune system controls pathogens, and the reasons that colonies suddenly collapse.
Unfortunately, until we understand the mechanisms and pathogenesis of these diseases, we will be unable to provide answers and solutions to beekeepers and to all of those who are interested in honeybee loss.
The present research collects the results of studies on Deformed Wing Virus, male hypofertility and Nosemosis carried out during my three year PhD program. The studies propose new laboratory and diagnostic techniques and describe the pathological findings connected to the three pathologies.
The results reported in these studies are an appeal to veterinary pathologists, especially younger researchers, to broaden their knowledge and to engage in studying this new field that could generate new research opportunities, provide new discoveries, and open the world of veterinary pathology to new challenges.
I strongly believe that today is the right time to start, as honeybees have never been so present in public discourse as nowadays, and funding by private and public institutions has never been so consistent.
So why shouldn’t we put more energy in this field?
Why shouldn’t veterinary pathologists be interested in honeybee pathology?