PhD THESIS

Potential of genetic resistance to *Varroa destructor* for local populations of *Apis mellifera L.*

(SUMMARY OF Ph.D. THESIS)

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INTRODUCTION

Varroa destructor is an ectoparasite that represents a major threat to *Apis mellifera* colonies (Rosenkranz et al., 2010). This parasite managed to make a host switch from its original host, *Apis cerana.* Once able to infest a new host that laked any form of Resistance, the mite managed to spread through the populations of *Apis mellifera* around the world (Rosenkranz et al., 2010).

Today the beekeepers have to apply intensive treatments with chemical substances that have an acaricide effect only to keep the mite population under control. However, at the moment, no substance or treatment plan can completely eradicate Varroa from a colony. Moreover, the mite managed to develop resistance to some of the applied compounds leaving beekeepers with a limited number of solutions; as a result, alternative ways to counter the parasite are needed (Dietemann et al., 2012).

Chapter I.

1. 1. Species Biology

1.1.1. *Apis mellifera*

Short description of *A. mellifera,* her natural areal and areas where they were introduced. We cover the caste system and the role of each caste in the hive.

1.1.2. *Apis cerana*

Short description of the natural areal for *Apis cerana*, areas where it was introduced and a short description in comparison with *A. mellifera*.

1.1.3. *Varroa destructor*

Description of the female mite's average size, colour and shape as well as a sort presentation regarding the male mite size shape and lack of pigmentation.

1.1.3.1. Apparition in Romania based on the global spread

After 1964 the mite was noticed in different areas of the U.R.S.S., and around 1967 was noticed in Bulgaria (Ograda, 1977; Sabanov & Nedialkov, 1972). However, official mentions in the scientific literature for Varroa were made in 1975 (Denmark et al., 1991).

1.1.3.2.*Varroa destructor* biology

Describes *Varroa destructor* and how it was mistaken for a long time as *V. jacobsoni* due to the phenomenon of cryptic speciation frequently encountered in Acari species; as a result, it is hard to detect with standard morphological analysis. After the publication from 2000 that used mtDNA to prove that *V. destructor* was the one able to switch hosts and mentions several varroa species (Anderson and Trueman, 2000).

1.1.4. Life cycle

The parasite's life cycle lacks a free life stage and can be divided into 2 phases. The first phase is represented by a phoretic phase. This stage takes place on adult bees.

At this stage, the female parasite uses adult bees or drones to move from one cell to another or from one colony to another. (Rosenkranz et al., 2010; Piou et al. 2016).

The parasite's reproductive cycle is synchronized with its host; the female Varroa chooses to infest the cell with larva in the 5th stage, that has only a few hours before the capping stage. A few hours after the cell is sealed, the female makes the first feed from the larva (Donzé and Guerin, 1994; Garrido et al., 2000).

1.1.4.1. Insights in the reproductive cycle with artificial infestation experiments

Describes mite dependence on the host, the narrow interval in which the mite has to feed on the larva in order to activate the reproductive phase and an experiment with the artificial infestation on larvae and pupa in different stages of development, where it was shown that the female Varroa regulates its reproductive cycle according to its host (Garrido and Rosenkranz, 2003).

1.1.4.2. Development of the parasite in the original host *Apis cerana*

Describes the behaviour of *V. destructor* on the original host. The limitation of the reproduction of the mite on drone brood as well as mechanisms of defence that allows *A. cerana* to deal with the infestation.

Chapter II

2.1 Dynamics of the parasite population on the new host

Presents how *V. destructor* infests a colony of *Apis mellifera*; how the mite reproductive phase is not limited only to drone brood on the new host, which leads to an exponential growth rate of mite population in a colony.

2.2.Negative effects of parasitation at the individual level

Describes the effect of parasitation at the individual level for drones and workers alike and how the infestation significantly impacts the individual (weight loss, suppression of immune function, reduced life span, decreased flight performance).

2.3. Negative effects at the colony level

Here we consider the hive from the superorganism perspective and see the negative effects on the growth rate and performance of the hive.

2.3.1 Synergic effect with other biotic stressors

Describes how the negative effects of varroa infestation can favour apparition of other diseases.

2.4. Beekeepers response when faced with economic losses

As soon as the mite started to do economic damage in production or with colony losses, the beekeepers used the chemical option by applying various substances with acaricide effect to reduce the mite load.

However, most of these substances have a limited effect on the mite population, and as a result, a part of them manage to survive, morover it seems that they manage to develop resistance to the treatments over time, leaving the beekeepers with a limited number of options (Pettis, 2004; Kamler, 2016; Dietemann et al., 2012).

2.4.1.Mite response to chemical treatments

The ability of the mite to develop resistance to acaricides is a fact, and there is clear evidence that the mite can develop resistance to the hard acaricides such as coumaphos, fluvalinate and amitraz (Martin 2004; Sammataro et al., 2005).

2.5. Identified resistance mechanisms

Describes the main resistance mechanisms identified on honey bees until present and describes the principle for each mechanism as follows: 2.5.1. Entombing of drone brood, 2.5.2. Grooming behaviour, 2.5.3., Hygienic behaviour and Varroa Sensitive Hygiene, 2.5.4. Reducing the reproductive success of the parasite, 2.5.5. Recapping behaviour (Uncapping/recapping).

CHAPTER III

3.1. Identified resistant populations

Presents how, despite the high virulence of the mite and the fact that it can decimate *A. mellifera* populations (without the intervention of the beekeeper), specific populations of bees have been identified that can cope with this infestation. Some of these populations were identified as resistant or obtained using specific techniques or breeding plans. In some cases, the identified population was studied, and the biological material was disseminated in some cases reaching the commercial level as it is for Varroa Sensitive Hygiene bees (VSH) or Russian honey bee (RHB) population or they are subject to ongoing research. Among the described populations, we have:

3.1.1. Gotland's bee population
3.1.2. The bee population of Avignon
3.1.3. Varroa Sensitive Hygiene bees (VSH)
3.1.4. Russian honey bee (RHB) population
3.1.5. Resistance of African bees to Varroa
3.1.6. Resistance of bees on the island of Fernando de Noronha
3.1.7. Resistance of bee population from in the Arnot Forest, Ithaca, NY, U.S.A.
3.1.8. Resistance of the bee population in the Østlandet region of Norway
3.1.9. Resistance of the bee population in Toulouse, France

3.2. Development of the breeding schemes

Presents the development of different breeding programs and strategies used over time to obtain resistant colonies or disseminate biological material from identified resistant populations.

3.3 Avenues and opportunities for breeding programs for Romanian honey bees

Presents our local bee populations of *A.m. carpatica*, how they are adapted to our local floral resources, and how they were identified and described.

More recent studies suggest that *A. m. carpatica* is relatively well preserved in Romania, and two distinct subpopulations are still present; however, there is a high risk of hybridization due to tee influx of non-native bees (Tofilski et al., 2021).

3.4. Opportunity to study host-parasite relation at the genomic and transcriptomic level

Describes the efforts for genomic sequencing for *A. mellifera*. The quick development of databases and the availability of new and more complete genome assembly *A. mellifera* Genome assembly 3.1 (www.ncbi.nlm.nih.gov). Moreover, presents the genome availability of *V. destructor* as well as *A. cerana* (original host of the parasite) genome.

Chapter IV

4. Research purpose and objectives

4.1. Motivation and importance of the research

Varroa destructor represents a global threat to the colonies of *Apis mellifera*. His abilities to spread to new colonies and make the naive colonies of *Apis mellifera* a sure target. The relatively short lifecycle allows the mite population to overwhelm the colonies of *A. mellifera* in a few years. Currently, only a few areas are free of this parasite.

4.2. Research objectives

a) Transcriptomics of the host and the parasite
b) Creating and implementing a breeding program with local populations of *A. mellifera* that will guide the new population to develop resistance to mite infestation and be applied at the apiary level.

4.3. Description of the research process

4.3.1. Description for objective a) Transcriptomics of the host and the parasite

The transcriptomics of the host and the parasite presents the experimental design, the necessary steps to prepare the apiary, the number of samples, and the relevant time interval for the study. Moreover, it presents the steps for artificial infestation steps in compliance with standardised methods.

4.3.2. Purpose and proposed objectives for the breeding plan

Implementation of a breeding program for natural resistance against *V. destructor* at the apiary level presents arguments in favour of the chosen breeding plan for our local population as well as adaptation of the breeding plan in order to optimize it for our local population and available resources.

Chapter V

5. Material and methods

5.1. Transcriptomics of the host and the parasite

Presents the process of apiary preparation for artificial infestation experiment. Describes the procedure used to obtain viable varroa mites able to enter the reproductive phase. The sampling procedure for specific time points and the artificial infestation process used for this experiment.

This chapter presents the required steps for sample processing and reactive used for RNA extraction and the optimisation of the protocol for *A mellifera* workers, drones and mites. It also presents Sample codification, RNA quality evaluation and Sample shipment for sequencing.

5.2. Overview of the protocol for RNA analysis

Describes the Equipment used, Hardware and software, the pipeline adapted for this RNA analysis, and the raw data received after the sequencing step. In order to align the transcripts, we had to use a reference genome for each organism studied. This section presents the downloaded reference genome data, the soft used to construct the reference genome, and the process of alignment to the reference genome with examples of the command lines used. It also presents the resulted output and information regarding the output for each step.

Section 5.2.2.4. presents a specific option of the HISAT2 soft, that allows storing unaligned reads separately and how these unaligned reads can be realigned to another reference genome. In our case, we used unaligned reads from varroa samples and realigned them to the *A. mellifera* genome to confirm that the mite managed to feed on the larva.

The further downstream analysis presents all the steps used to convert the output of the samples, assemble and quantify genes and transcripts for *A. mellifera* and *V. destructor* samples, as well as for the realigned genes. Moreover, presents how to examine the transcripts compared with the reference annotation and all steps performed for the data processing.

5.3 *Preparation and implementation of the breeding plan for our local bee population*

Presents how the biological material was obtained, the location of each apiary that provided biological material in compliance with the protocol requirements, as well as the location where the new population was formed.

A complete description of the protocol's steps and the hive's success rate after each step of the protocol is presented for each season. In total, in this thesis, we cover a total of three seasons.

6. Results and discussion

6.1. Results regarding breeding plan implementation

6.1.1 First season

In season 1, from the 25 hives selected at the beginning of the breeding plan, only 14 hives responded to the indicator frame and managed to develop. The colonies were selected from 4 counties in Transylvania. From Cluj County, there were 4 sources, Salaj County 2 sources, Harghita 1 source, and Alba county 2 sources.

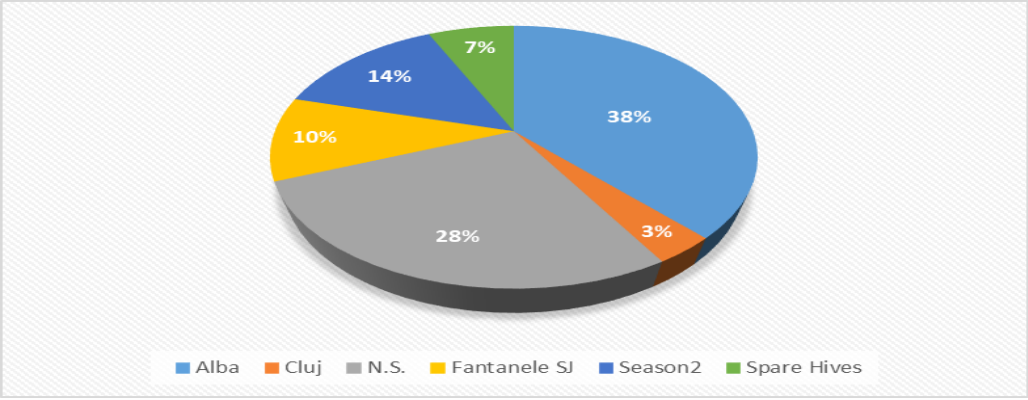
All these colonies successfully responded to the division procedure and had a viable queen, obtaining 42 hives. To these were added 2 natural swarms caught near USAMV Cluj.

6.1.2. Second season

The second season is characterised by significant losses in population in the spring. Despite the significant losses in the spring, all the remaining hives developed well and responded to the indicator frame. All hives that survived over winter were split successfully and obtained a viable queen, and most of them developed well until the end of the season.

6.1.3. Third season

At the end of the third season, we have a total of 29 hives, as presented in the following configuration: 38% represents hives from the initially selected populations from Alba county, 3% represented by the Initial population of Cluj County, 10% of the configuration represents now the new addition form Fântânele Rus28 % coded as N.S. represents the 8 swarms with queens that mated in the DBBB area. Finally, the remaining 7% is represented by the two spare hives.



6.2. Analysis of the principal axis of variation (Principal component analysis) for *Apis mellifera* and *Varroa destructor* transcriptome

6.2.1 PCA *for V. destructor*

The Principal Component Analysis (PCA) on 20 *V. destructor* samples resulted in a clear separation by time point. We also identified two outlier samples in the mites that infested worker larva at the 8H time point, represented by the samples V8H4 and

V8HW5. By removing the two outlier samples and rerunning the PCA analysis, we can see a more clear separation between the groups.

6.2.2 PCA for *A. mellifera*

We ran the PCA analysis for *A. mellifera* infested and uninfested samples. The first results already suggested that IW30H3 and IW30H5 do not group the same condition and time point as the other samples.

6.3. Differential Expression analysis

We performed a group differential expression (DE) analysis by comparing the RNA expression profiles of the mites that infested drones at 8H versus 30H and mites that infested worker larva at the same time points using DESeq2 (Love et al., 2014).

6.3.1. Difference expression analysis of *Varroa destructor*

Comparing the mites that infested drones 8H vs 30H, we obtained 1966 upregulated genes. Applying the same process to the mites that infested the workers, we obtained 1344 upregulated genes. The intersection of these two sets of upregulated genes resulted in 823 genes that are common for both groups.

For the downregulated genes, the mites that infested drones 8h vs 30H, we obtained 1911 genes. For the mites that infested workers 8H vs 30H, we obtained 1434 genes. Intersecting these lists, we obtained 899 common down-regulated genes.

In both comparisons, we observe a large number of host-specific DE genes. Interestingly, the are more DE genes for the mites that infested drones compared to the mites that infested worker brood. This difference is in line with the known preference of mites for drone brood and its adaptation to the original host, *A. cerana.*

6.3.2. Difference expression analysis of *A. mellifera*

For *A. mellifera*, we decided to compare the expression profile between groups for each caste. We pre-filtered the genes with low counts for each group comparison by removing all genes that did not sum 100 counts in the two compared groups. As a result of the total number of 12,241 genes available for *A. mellifera* for each compared group, we used for analysis only the genes that were above this threshold. For this analysis, we apply similar p-value and log fold change thresholds as in the *V. destructor* analysis.

Comparison between drones vs infested drones at 30H resulted in 382 upregulated genes and 378 downregulated genes. For workers at the same condition and time point, it resulted in 677 upregulated genes and 751 downregulated genes. The intersection between the upregulated genes for drones and workers resulted in 46 common genes

For downregulated genes at 30H, we have a total of 378 genes for drones and 751 for workers; among these, a total of 33 were common between these two groups.

Comparison between drones vs infested drones at 8H resulted in 866 upregulated genes, and for worker vs infested worker at 8H resulted in 973 genes. The intersection between these two sets of genes resulted in 265 common genes. Moreover, a total of 601 genes were specific for the drone group, and 708 were specific for the worker group

Comparison between drones vs infested drones at 8H resulted in 977 downregulated genes, and for worker vs infested worker at 8H resulted in 1106 genes.

The intersection between these two sets of genes resulted in 304 common genes. Moreover, a total of 673 genes were specific for the drone group, and 802 were specific for the worker group.

Considering that the host for the mites is *A. mellifera*, the *V. destructor* is able to activate the reproductive phase on both worker and drone brood. By analysing these genes further, we should find important insights regarding the host-parasite interaction between the *V. destructor* and *A. mellifera.*

6.4. Functional enrichment

We performed functional enrichment analysis for all groups of *A. mellifera* from table 29 using G: Profiler (Raudvere et al., 2019). To prevent spurious enrichments due to the inherent gene selection induced by the experimental setup, we adjusted the **statistical domain scope** and used as an enrichment background the complete list of genes included in this analysis. We used the default parameters (g:SCS multiple testing and 0.05 significance threshold). The number of genes for each comparison is available in the **following table**

Compared Groups	**No. genes >100 counts**	**No. of upregulated genes**	**No. of downregulated genes**
drone 0 vs 30 H	10757	2717	2154
drone 0 vs 8 H	10351	1742	1582
drone 8 vs 30 H	10884	1893	2088
worker 0 vs 30 H	10534	2677	2547
worker 0 vs 8 H	10201	1095	1165
worker 8 vs30	10606	2224	2115
worker vs infested worker 30 H	10471	677	751
drone 0H vs infested drone 8 H	9986	1189	1314
drone 0H vs infested drone 30 H	10774	2771	2180
worker 0H vs infested worker 8 H	10074	1176	1254
worker 0H vs infested worker 30 H	10208	1708	1956
worker 8H vs infested worker 30 H	10316	1442	1090
Drone 8H vs infested drone 30 H	10925	2020	2220

6.5. Realigned genes.

We investigated the *A. mellifera* transcript molecules detected in the *V. destructor* samples and reported that we identified 5908 genes and 6921 genes for the groups that infested drones and workers (on the combined 8h and 30h samples; detected with at least 100 read counts). Previous literature described the process of extraoral digestion and the ingestion of the semisolid tissue, mainly the fat body, by *V. destructor* (Ramsey et al., 2019).

7. Conclusions and recommendations

7.1. Conclusions regarding transcriptomics study for *Apis mellifera* and *Varroa destructor*

The analysis of the *V. destructor* and *A. mellifera* transcriptomes and molecular data have the potential to enable the understanding of both mite biology and of the molecular processes triggered by the infestation. This led to several results, some already well documented in the literature.

First, the analysis of principal components of variation (Figure 54) showed that time is the principal factor affecting the transcriptome with a clear separation of 30h drones and workers front the corresponding control and 8H samples. In addition, we observe a clear separation (PC2) by caste.

Second, relying on changes in the composition of non-self reads detected in *V. destructor* samples, our results suggest that the mites feed early (8h) and decrease the amount consumed from the host. The percentage of *A. melifera* reads detected in mites decreased between 8h and 30 h. We were unable to indicate the type of tissue ingested by mites but consider that by relying on additional transcriptional profiles of *A. melifera* tissues, future studies could use RNA Seq experiments to estimate the main tissues ingested by the mite.

Third, we observe that the workers are more affected than the drones and speculate that this might be elicited by the worker's lower body mass and, implicitly, the fat body. The difference in weight and size between workers and drones is well documented in the literature (at the moment of capping is between 140 and 162 mg for worker larva, while drone larva may vary between 260 to 419 mg; Hrassingg and Crailshein, 2005).

7.2. Conclusions regarding breeding plan implementation

Hives that will survive the winter will remain in the selection plan; the protocol steps will be repeated next season.

The mite alone places a high selective pressure on the hives, and in case the food resources from the environment are low artificial feeding will be required in order to keep a decent selection pressure on the colonies.

The first season of the breeding plan is crucial; however, it is necessary to ensure optimal logistics for the following seasons, especially the operations necessary in the active season.

The monitoring period until the division step of the colonies is essential.

Although this breeding program is limited at the research level and aims to guide local populations to develop resistance mechanisms, it has the potential for development in the commercial area.

In principle, this protocol should bring to the surface within the population genes that confer resistance against the parasite.

Adapting local populations to this parasite seems to be one of the most promising ways to control diseases and parasites in the long term. A protocol based on natural selection could be a long-term solution for the beekeeping sector.

7.2.1. Keeping the new population under surveillance to observe host-parasite interaction and possibly other clinical signs of other pathogen agents

All hives were evaluated during the active season. Particular attention was presented to the hive mirror, the flight board, and inside the hive for clinical signs of disease.

Among the pathogen agents identified in our population, we have *Nosema spp.,* also confirmed with microscopical diagnostic.

In the first season of the breeding plan, we did not notice other signs of clinical disease. However, in the second season, they started to be present.

Other clinical signs were observed in season two as we noticed bees and drones with DWV (deformed wing virus) and chalkbrood (*Ascosphaera apis*).

In the third season of the breeding plan, we did not notice other signs of clinical disease in the remaining hives.

7.3. Recommendations

In our population case, the most significant impact on our hives was not *Varroa destructor* alone but a synergic effect between *V. destructor* and *Nosema spp.* infestation. Despite the lack of molecular analysis for confirmation, we can point to the presence of *N. ceranae* spores based on information available in Romania and the rest of Europe.

Regarding the transcriptomic analysis, this data could be used to analyse and compare with honey bee populations that have acquired the suppressed mite reproduction trait. It could also be compared with samples on the same time points from the original host of the parasite *Apis cerana* or even with *A. m. scutellata.*

8. Originality and innovative contributions of the thesis

- We managed to adapt and implement a breeding protocol at the apiary level that can be followed by professional and hobbyist beekeepers to obtain resistant populations to *V. destructor*.
- The fact that we managed to apply the breeding protocol without the aid of a professional beekeeping firm should prove that this breeding protocol can be applied at the apiary level by professional and hobbyist beekeepers.
- The information presented for the three seasons suggests that our local bee population responds well to the breeding protocol splits. Furthermore, the resulting nukes develop well.
- The transcriptomic datasets presented in this thesis are part of a larger dataset sampled and processed during the time as a member in the GRAL project and are subject to ongoing research.
- Further data analysis could offer important insights regarding the host-parasite interaction between *A. mellifera* and *V. destructor*.

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