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PARASITES DISEASES DETERMINATION IN AN UNTREATED LOCAL POPULATION OF *APIS MELLIFERA* FOR IT'S NATURAL RESISTANCE DEVELOPMENT

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Abstract

Over the last few years, the honeybees faced a significant decline worldwide. Despite many biotic and abiotic stressors, one of the leading causes of honeybee colony loses is Varroa destructor, followed by Nosema spp.. Given the importance of honeybee in agriculture and a significantly increased resistance to treatments identified in Varroa destructor, a more sustainable method to counter this mite is needed. One of these sustainable methods is breeding for resistance to Varroa destructor. Due to the rising number of honeybee populations with potential resistance to Varroa destructor, a new promising breeding plan for natural selection was proposed. This breeding plan was adapted and implemented on a local population of honey bees in Transilvania, with the primary objective of obtaining resistant colonies to Varroa destructor. Development of these colonies was observed, and analysis of Varroa infestation level and Nosema spp. was performed to assess the health status of the population or the cause of mortality.

Key words*: honeybees, Nosema spp., Varroa destructor.*

INTRODUCTION

Given the importance of the honey bee in the ecosystem, the number of stressors becomes concerning (Goulson et al., 2015; Havard et al., 2020). For *Apis mellifera,* among the biotic stressors, there is present a wide range of natural pathogen agents as well as newly introduced pathogen agents such as *V. destructor* and *N. cerane* as an effect of the lack of many restrictive measures during the transport of biologic material over long distances (Oldroid, 1999; Paxton et al., 2007; Goulson et al., 2015).

For the biotic stressor, *V. destructor,* it was observed an increasing resistance to the treatments (Thomson et al., 2003; Pettis, 2004). Currently, there is no available treatment able to remove the parasite from the hive; thus, the mite is already selected for resistance to treatments (Pettis, 2004; Dieteman et al., 2012; Kamler et al., 2016). Furthermore, without additional ways to counter the mite, it will be only a matter of time until many of the remaining treatments will have reduced effectiveness (Dieteman et al., 2012). Over one active season, the reproductive cycle of *V. destructor* allows multiple generations of mites and allows the mite population to fixate alleles for resistance to acaricides inside one colony (Beaurepaire et al., 2017)

Moreover, if beekeepers count only on chemical treatments, in time, this will lead to an increase in the dosage and number of treatments despite alternating the treatment (Dieteman et al., 2012; Rinkevich, 2020).

In the case of *Nosema* spp. we have now present in Europe two pathogen agents, one represented by *N. apis* present on *A. mellifera* and the second one represented by *N. ceranae.* Given the difference in symptomatology, the exact time of arrival in Europe for *N. ceranae* in Europe is unknown (Paxton et al., 2007; Higes et al., 2010). Moreover, it is suggested a synergic effect between *Varroa* infested colonies and the level of infestation with *Nosema spp.* (van Dooremalen et al., 2018).

Among the alternative ways to fight against *V destructor* among the best long-term solutions is breeding for *V. destructor* resistance (Dieteman et al., 2012).

Among the reasons that favour the honey bees we have the *A. mellifera* genome witch according to literature, seems to have a high

recombination rate (Beye et al., 2006) and is suggested as an adaptative measure to increase the variation inside the colony in order to slow the spread of pathogen agents and increase colony performance and fitness (Gadau et al., 2000; Beye et al., 2006). Moreover, adding polyandry as the queen mates with 8 to 10 drones leads to a higher diversity between offspring (Fuchs & Moritz, 1999).

The emergence of the different populations across the globe that manage to resistant despite the lack of treatments presents another reason to support the idea of a breeding plan. Here we include VSH (Varroa Sensitive Hygiene) (Harbo & Harris, 1997) and Primorsky Russian honeybee (Rinderer et al., 2001; Rinderer et al., 2010) in the USA which were selected for resistance and the feral population of honey bees from Arnot forest (Seeley 2007; Seeley et al., 2015).

In Africa, it seems that populations of *A. mellifera capensis* and *A. mellifera scutellata* posed resistance to varroa infestation (Martin & Kryger, 2002). Moreover, it is suggested that *A. mellifera scutellata* seems to resist even more pathogen agents are present simultaneously (Strauss et al., 2013). And this trait seems to be passed on to the Africanized honey bees to. (Martin & Medina, 2004).

In Europe, we have Gotland population obtained from apiaries placed on Gotland Island and left untreated (Fries et al., 2006; Loke and Fries, 2011). Avignon's population was composed of bees that survived without treatment and untreated bees from different beekeepers (Le Conte et al., 2007; Le Conte et al., 2020). Toulouse population obtained from queens of *Apis mellifera intermissa* brought from Tunisia (Kefuss et al., 2004). Similarly, in the Østlandet region, Norway a population of untreated bees of mixed origin (Buckfast) was used to obtain bees that manage to survive despite lack of treatments (Oddie et al., 2017).

Over time breeding plans become available; in the case of Russian honey bee and Varroa Sensitive Hygiene, the programs reached a commercial level (Rinderer et al., 2010). In Europe, we have significant progress was made with the AGT program (The Arbeitsgemeinschaft Toleranzzucht) that began in 2003 and the BLUP (best linear unbiased prediction model that was adapted for *A. mellifera* specific reproductive particularities (Bienefeld et al., 2006; Büchler et al., 2010).

However, more recently, it was proposed a new breeding protocol. One that has as the main focus of obtaining resistant populations based on principles of natural selection and was adapted to the particularities of reproductive biology. Another important advantage is the equipment required for implementation, as in this case is represented by standard equipment that should be available in a standard apiary (Blacquière et al., 2019).

MATERIALS AND METHODS

Obtaining the population

Based on Blacquière et al. protocol, we managed to obtain 25 colonies with unrelated queens from different areas of the Transylvania region in the spring of 2019 (Blacquière et al., 2019). With these colonies, we tried to establish a new population of honey bees.

These colonies were left untreated, and we monitored their development.

From a total of 25 colonies, 14 colonies developed better and responded to the presence of the indicator frame. As a result, these colonies were selected, and we proceeded with the breeding plan. Each colony was split into 3 new colonies, and for each hive, we adjusted an equal portion of the population, brood and food resources.

Newly formed colonies were taken to a new location prepared in advance. The new apiary is located on the coordinates (46°44'15.31"N 23°37'10.45"E); the land surrounding this location belongs to the Research and Development Station for Fruit Growing within USAMV Cluj-Napoca. Since the new site was in a more isolated area and plenty of drones were available in each colony, we expect that most of the mating took place in close proximity of the hive (Moritz et al., 2007; Jaffé et al., 2010). We also added two natural swarms caught in Cluj-Napoca in this period.

The other 11 colonies were returned to the bio-base. To confirm the mating's success for a new colony was confirmed mated only after identifying the queen and the presence of eggs and fresh larva.

Over winter, we proceeded to make regular checks based on standard beekeeping practices.

From the new population of honey bees, we took samples at the beginning of February.

***Varroa* mite analysis**

In order to identify the level of infestation with *V. destructor*, we used the 75% ethanol wash method (Dietemann et al., 2013).

For each hive, we took around 300 bees and placed them in a jar. We added sufficient ethanol to cover the bees. Once the jar was closed, we shake for approximatively 90 seconds to dislodge the mites. The next step was to separate the mites and ethanol from the bees using two layers of mesh. The first layer had large gaps that allowed mites and alcohol to goo thought but separated the bees, while the second layer kept only the mites.

For better precision, after we counted the mites, we counted the total number of bees and checked the bee abdomen for mite presence.

The total number of mites was divided by the total number of bees to determine the exact proportion of infested individuals. This value was then multiplied by 100 to obtain the % of mite infestation /100 bees presented in Table 1.

***Nosema* spp. microscopic analysis**

Identification and analysis for *Nosema* took place at APHIS-DIA laboratory from USAMV Cluj-Napoca. Sample processing was made using the method recommended by the OIE manual (World Organisation for Animal Health, 2018). We used 60 worker abdomens/ hive. The abdomens were crushed using a mortar and a pestle using ultrapure 60 ml H_2O until we obtained a homogenous suspension. The suspension was then filtered through two layers of muslin and centrifuged for 6 minutes at 2700 rpm in order to remove debris. Pellets are resuspended in ultrapure H_2O to restore the initial dilution.

Sample analysis for *Nosema* spp. spores was made using a Bürker-Türk Counting Chamber and a microscope Nikon Eclipse 50i, Ob. 40x available at the laboratory.

The total number of spores/bee was using the standard formula:

$$Z = \alpha/\beta \times \delta \times 250{,}000$$

Z = represents the number of spores/bee;

α = is the total number of counted spores;

β = is the number of squares counted;

δ = represents the dilution factor;

250,000 = represents the volume for each counted square and is usually present on the counting chamber.

Table 1. Hives that reached the winter season and values of *Varroa* and *Nosema* infestation

Hive Code	Status	Sampled bees	*Varroa* infestatio/ 100 bees	Total number of spores/bee
H50	ok	yes	0.000%	11,000,000
H46	jan-feb	yes	4.762%	2,875,000
H59	feb-mar	yes	2.239%	52,750,000
H48	jan-feb	yes	2.913%	42,375,000
H49	ok	yes	0.699%	15,500,000
H43	feb-mar	yes	0.769%	46,625,000
H44	jan-feb	yes	3.000%	32,875,000
H63	jan-feb	yes	5.172%	3,125,000
H45	feb-mar	yes	3.175%	11,625,000
H47	jan-feb	yes	6.667%	4,000,000
H10	feb-mar	yes	0.730%	11,250,000
H65	jan-feb	yes	1.961%	49,500,000
H40	jan-feb	yes	5.660%	41,500,000
H42	jan-feb	yes	2.727%	29,625,000
H58	jan-feb	yes	0.901%	11,000,000
H24	jan-feb	yes	12.150%	18,875,000
H22	jan-feb	yes	5.882%	7,875,000
H26	feb-mar	yes	3.333%	10,875,000
H35	jan-feb	yes	1.563%	7,875,000
H38	feb-mar	yes	1.198%	64,500,000
H60	jan-feb	no	na	na
H25	jan-feb	yes	6.107%	2,875,000
H7	jan-feb	no	na	na
H39	ok	yes	2.667%	12,375,000
H32	**ok**	**yes**	1.000%	**42,250,000**
H67	ok	yes	0.000%	2,625,000
H34	ok	yes	2.500%	8,125,000
H53	**ok**	**yes**	0.000%	**101,375,000**
H37	jan-feb	yes	1.626%	2,000,000
H17	jan-feb	no	na	na
H20	feb-mar	yes	0.667%	15,375,000
H54	feb-mar	yes	1.754%	16,625,000
H62	jan-feb	no	0.000%	na
H57	jan-feb	no	3.030%	na
H55	ok	yes	0.000%	3,625,000
H56	feb-mar	yes	na	12,125,000

*na = no data aviable

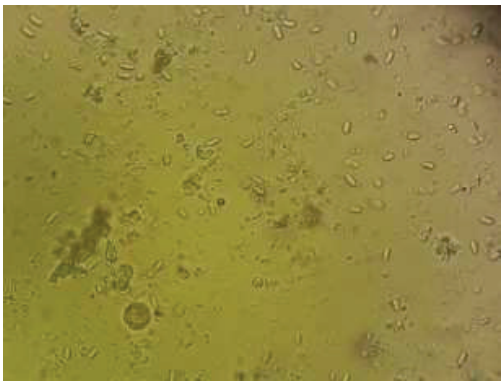

Figure 1. Preliminary test under the microscope before proceeding to the counting chamber step

All data was centralised and presented in Table 1. One colony was considered positive for *Nosema* spp. if the total number of spores exceeded 9 million spores/bees when the formula was applied (Chioveanu et al., 2009; Dumitru et al., 2020).

RESULTS AND DISCUSSIONS

Since the split was done on the natural mating season, we had available between 3 and 5 queen cells/new hive. Between 25-27 of May was the first check to confirm the queen presence and reproduction success in the interval. At this date, we confirmed the presence of brood and queen for 24 colonies. (marked with green in the supplementary table, queen presence and eggs column).

At the second inspection after one week, we confirmed mating success for the other 19 colonies (marked with purple in the supplementary table). The remaining two colonies were inspected at the third inspection one week later and confirmed the reproduction's success (marked with blue).

After the third inspection for queen presence, we confirmed a 100% success rate for queen mating with this protocol as all our colonies managed to have a newly mated queen.

Over summer, we monitored the development of these colonies, as presented in the summary table. We increased or decreased the number of frames based on each colony's available population, as shown in Supplementary Table 1.

At the end of the winter preparations, we ended up with 36 colonies. For the other 8 colonies, we noticed massive depopulation, and they had to be removed from the breeding program. (these colonies are marked as removed on the supplementary table). For the removed colonies, it can be noticed that colonies which developed well or manage to maintain over the active season suddenly collapsed in the population size and less than two frames with bees were available.

As a result, in winter, we entered with 36 colonies and the average size based on the mean between the number of frames from all hives was 5.25 frames/colony.

Over winter, we proceeded with external observations for the hives.

At the first major inspection for 2020, we confirmed the death of 19 colonies. For 6 of these colonies, we cannot confirm the brood's presence as presented in the supplementary table. However, for the rest, we could clearly identify the presence of brood in different stages.

At a second major inspection, we confirmed another mortality for one colony. They were followed by the other 4 at the end of February. In all cases, we had brood present. (Supplementary figure 1).

Despite being the first season without treatment, such high mortality levels lead us to analyse nosemosis as only varroosis infestation could not explain this mortality level.

Since both parasites reduce the worker's lifespan and contribute to some degree to immune suppression, the mortality rate for the population will be increased (Kurze et al., 2016; van Dooremalen, 2018), resulting in a decrease in the total population size.

This could explain the colony loss between January and February, with a small cluster and brood present on the frame. If the size of the bees' cluster was too small and encountered low temperature, it was not possible to operate properly.

As presented in Table 1 for 6 hives, it was impossible to take the bee sample for analysis. The reason was that inside the colony, there was almost no bee present. For the rest of the colonies, we analysed the infestation level with *Varroa* and the degree of infestation with *Nosema* spp. Based on the results, the level of infestation. Moreover, for all these hives, the Varroa mite infestation level was below 3%.

In Table 1, we have the hives with not enough bees for analysis marked in red. In yellow, we have all hives that were lost between January and February.
All hives lost between February and March were marked with purple. And in green, we have the hives that managed to enter the new season.

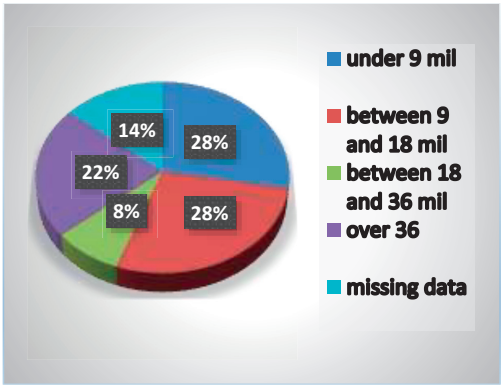

Figure 2. Spores distribution inside the sampled population

From the total population that remained in the winter, only 28% was under 9 million spores/bee. 58% of the population was over 9 million, and for the rest of 14%, we were unable to make the analysis. These values were interpreted based on the total number of hives presented in Table 1.

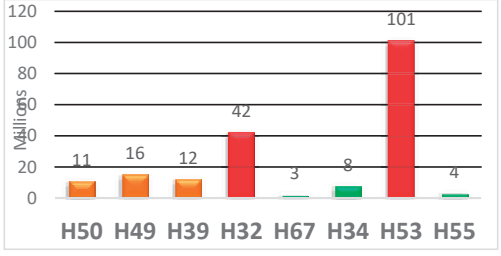

Figure 3. Number of spores/bee for the remaining hives

As shown in Figure 3, we have three colonies below nine million spores/bee marked in green. Three colonies that exceed 9 million spores but are under 20 million spores/bee marked with orange and two hives with an increased load of spores marked in red.
Despite the high *Nosema* spp. infestation level, Hive 32 and Hive 53 managed to go through the winter.

CONCLUSIONS

The breeding plan presents great potential. It can be adapted and applied at the apiary level as all the necessary equipment is usually available in an apiary focused on reproduction.
Based on our experience at the start of the breeding plan, all colonies should be inspected closely. Nosemosis can be triggered by different stress factors, including *V. destructor* infestation, prophylactic measures against *Nosema* spp. should be put in place.
Due to the hidden symptomatology of *Nosema ceranae* presence in Europe was confirmed; however, the exact arrival time is yet to be determined. Its presence was confirmed in more European countries, including Romania.
Due to its negative effects, active prophylactic measures should be put in place when breeding for resistance to *V. destructor.*
Trying to adapt against one non-native parasite puts a severe strain on the colony; fighting two at once will definitely lead to the colony's loss before it can adapt to the parasite.
Since over the active season and in the autumn, there were no clear signs of *Nosema* infestation and the level of infestation with *V. destructor* was relatively low, we suspect the presence of *Nosema crane*., further molecular analysis should confirm this.
Despite having a high mortality rate, all colonies that survived developed and responded to all the steps presented in the second season of the breeding plan protocol.

ACKNOWLEDGEMENTS

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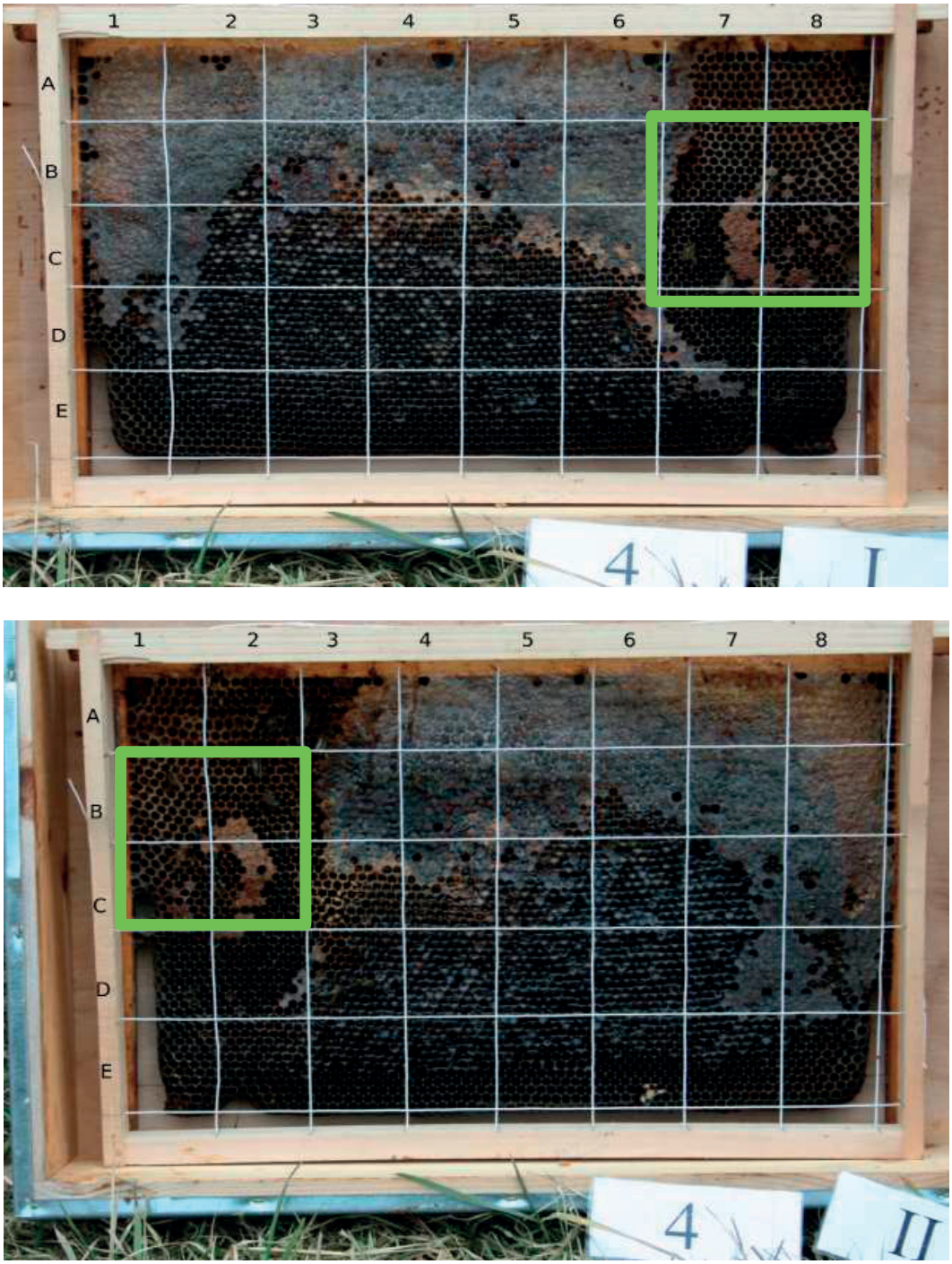
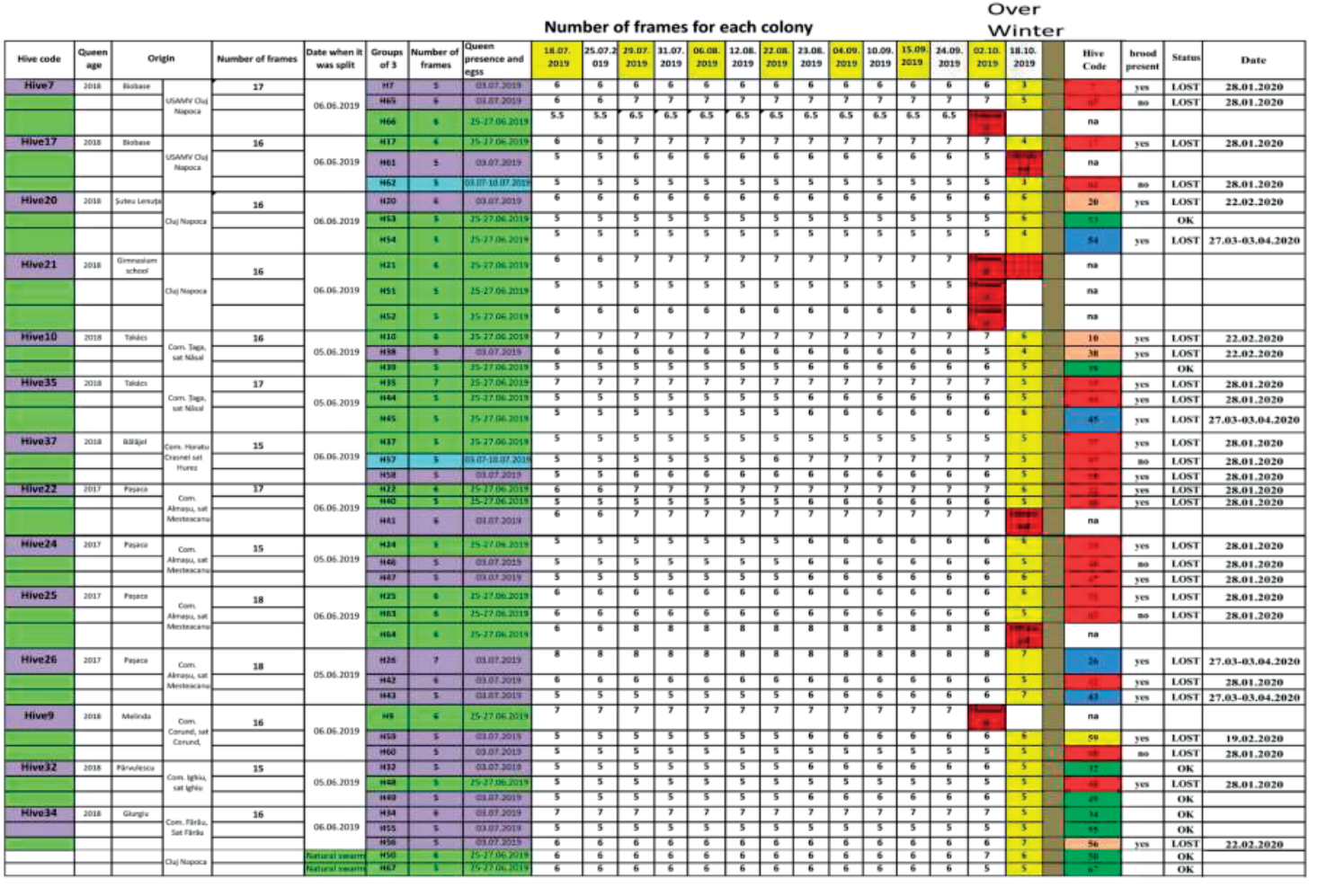
Initiation of this protocol was possible due to the help of a few interested beekeepers who wished to participate and contributed with biologic material.
Special thanks to T. Blacquiere for early access to the breeding plan.
We do not take any credit for the development of this breeding plan. All the adaptations of this protocol were made only to be more suited to our local species of *Apis mellifera.* For more details about the breeding plan, please view Blacquiere et al. paper (2019).



Supplementary Figure 1.
Frame 4 side I and side II in 18.02.2020. Confirmation of brood presence in different stages.
Side I - capped and uncapped brood in section C7 and C8, and uncapped brood in section B7and 8.
Side II- Capped brood in section B2, C1 and 2. Uncapped brood in section B2 and C2. The presence of the brood is expected for this period. Food resources are present; however, the colony did not manage to survive.



Hive code	Queen age	Origin		Number of frames	Date when it was split	Groups of 3	Number of frames	Queen presence and eggs	18.07.2019	25.07.2019	29.07.2019	31.07.2019	06.08.2019	12.08.2019	22.08.2019	23.08.2019	04.09.2019	10.09.2019	15.09.2019	24.09.2019	02.10.2019	18.10.2019	Hive Code	brood present	Status	Date
Hive7	2018	Biobase	USAMV Cluj Napoca	17	06.06.2019	H7	5	03.07.2019	6	6	6	6	6	6	6	6	6	6	6	6	6	3	[illegible]	yes	LOST	28.01.2020
						H65	6	03.07.2019	6	6	7	7	7	7	7	7	7	7	7	7	7	5	[illegible]	no	LOST	28.01.2020
						H66	6	25-27.06.2019	5.5	5.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5	[illegible]		na			
Hive17	2018	Biobase	USAMV Cluj Napoca	16	06.06.2019	H17	6	25-27.06.2019	6	6	7	7	7	7	7	7	7	7	7	7	7	4	[illegible]	yes	LOST	28.01.2020
						H61	5	03.07.2019	5	5	6	6	6	6	6	6	6	6	6	6	5	[illegible]	na			
						H62	5	03.07-10.07.2019	5	5	5	5	5	5	5	5	5	5	5	5	5	3	[illegible]	no	LOST	28.01.2020
Hive20	2018	Suteu Lenuta	Cluj Napoca	16	06.06.2019	H20	6	03.07.2019	6	6	6	6	6	6	6	6	6	6	6	6	6	6	20	yes	LOST	22.02.2020
						H53	5	25-27.06.2019	5	5	5	5	5	5	5	5	5	5	5	5	5	6	[illegible]		OK	
						H54	6	25-27.06.2019	5	5	5	5	5	5	5	5	5	5	5	5	5	4	54	yes	LOST	27.03-03.04.2020
Hive21	2018	Gimnasium school	Cluj Napoca	16	06.06.2019	H21	6	25-27.06.2019	6	6	7	7	7	7	7	7	7	7	7	7	[illegible]	[illegible]	na			
						H51	5	25-27.06.2019	5	5	5	5	5	5	5	5	5	5	5	5	[illegible]		na			
						H52	5	25-27.06.2019	6	6	6	6	6	6	6	6	6	6	6	6	[illegible]		na			
Hive10	2018	Takács	Com. Taga, sat Năsal	16	05.06.2019	H10	6	25-27.06.2019	7	7	7	7	7	7	7	7	7	7	7	7	7	6	10	yes	LOST	22.02.2020
						H38	5	03.07.2019	6	6	6	6	6	6	6	6	6	6	6	5	4	[illegible]	38	yes	LOST	22.02.2020
						H39	5	25-27.06.2019	5	5	5	5	5	5	5	6	6	6	6	6	6	5	[illegible]		OK	
Hive35	2018	Takács	Com. Taga, sat Năsal	17	05.06.2019	H35	7	25-27.06.2019	7	7	7	7	7	7	7	7	7	7	7	7	7	5	[illegible]	yes	LOST	28.01.2020
						H44	5	25-27.06.2019	5	5	5	5	5	5	5	6	6	6	6	6	6	5	[illegible]	yes	LOST	28.01.2020
						H45	5	25-27.06.2019	5	5	5	5	5	5	5	6	6	6	6	6	6	6	45	yes	LOST	27.03-03.04.2020
Hive37	2018	Bălăjel	Com. Horatu Crasnei sat Hurez	15	06.06.2019	H37	5	25-27.06.2019	5	5	5	5	5	5	5	5	5	5	5	5	5	5	[illegible]	yes	LOST	28.01.2020
						H57	5	03.07-10.07.2019	5	5	5	5	5	5	6	7	7	7	7	7	7	5	[illegible]	no	LOST	28.01.2020
						H58	5	03.07.2019	5	5	6	6	6	6	6	6	6	6	6	6	6	5	[illegible]	yes	LOST	28.01.2020
Hive22	2017	Pașaca	Com. Almașu, sat Mesteacănu	17	06.06.2019	H22	6	25-27.06.2019	6	6	7	7	7	7	7	7	7	7	7	7	7	6	[illegible]	yes	LOST	28.01.2020
						H40	5	25-27.06.2019	5	5	5	5	5	5	6	6	6	6	6	6	6	5	[illegible]	yes	LOST	28.01.2020
						H41	6	03.07.2019	6	6	7	7	7	7	7	7	7	7	7	7	[illegible]	[illegible]	na			
Hive24	2017	Pașaca	Com. Almașu, sat Mesteacănu	15	05.06.2019	H24	5	25-27.06.2019	5	5	5	5	5	5	5	6	6	6	6	6	6	6	[illegible]	yes	LOST	28.01.2020
						H46	5	03.07.2019	5	5	5	5	5	5	5	6	6	6	6	6	6	5	[illegible]	no	LOST	28.01.2020
						H47	5	03.07.2019	5	5	5	5	5	5	5	6	6	6	6	6	6	6	[illegible]	yes	LOST	28.01.2020
Hive25	2017	Pașaca	Com. Almașu, sat Mesteacănu	18	06.06.2019	H25	6	25-27.06.2019	6	6	6	6	6	6	6	6	6	6	6	6	6	6	[illegible]	yes	LOST	28.01.2020
						H63	6	25-27.06.2019	6	6	6	6	6	6	6	6	6	6	6	6	6	5	[illegible]	no	LOST	28.01.2020
						H64	6	25-27.06.2019	6	6	8	8	8	8	8	8	8	8	8	8	[illegible]	[illegible]	na			
Hive26	2017	Pașaca	Com. Almașu, sat Mesteacănu	18	05.06.2019	H26	7	03.07.2019	8	8	8	8	8	8	8	8	8	8	8	8	8	7	26	yes	LOST	27.03-03.04.2020
						H42	6	03.07.2019	6	6	6	6	6	6	6	6	6	6	6	6	6	5	[illegible]	yes	LOST	28.01.2020
						H43	5	03.07.2019	5	5	5	5	5	5	5	6	6	6	6	6	6	7	43	yes	LOST	27.03-03.04.2020
Hive9	2018	Melinda	Com. Corund, sat Corund,	16	06.06.2019	H9	6	25-27.06.2019	7	7	7	7	7	7	7	7	7	7	7	7	[illegible]	[illegible]	na			
						H59	5	03.07.2019	5	5	5	5	5	5	5	6	6	6	6	6	6	6	59	yes	LOST	19.02.2020
						H60	5	03.07.2019	5	5	5	5	5	5	5	5	5	5	5	5	5	5	[illegible]	no	LOST	28.01.2020
Hive32	2018	Pârvulesca	Com. Iglița, sat Iglița	15	05.06.2019	H32	5	03.07.2019	5	5	5	5	5	5	5	6	6	6	6	6	6	5	32		OK	
						H48	5	25-27.06.2019	5	5	5	5	5	5	5	5	5	5	5	5	5	5	[illegible]	yes	LOST	28.01.2020
						H49	5	03.07.2019	5	5	5	5	5	5	5	6	6	6	6	6	6	5	49		OK	
Hive34	2018	Giurgiu	Com. Fărău, Sat Fărău	16	06.06.2019	H34	6	03.07.2019	7	7	7	7	7	7	7	7	7	7	7	7	7	5	34		OK	
						H55	5	03.07.2019	5	5	5	5	5	5	5	5	5	5	5	5	5	5	55		OK	
						H56	5	03.07.2019	6	6	6	6	6	6	6	6	6	6	6	6	6	7	56	yes	LOST	22.02.2020
			Cluj Napoca		Natural swarm	H50	6	25-27.06.2019	6	6	6	6	6	6	6	6	6	6	6	6	7	6	50		OK	
					Natural swarm	H67	5	25-27.06.2019	6	6	6	6	6	6	6	6	6	6	6	6	5	5	[illegible]		OK	

Supplementary Figure 2. Synthesis with development for selected hives

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