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Determination of the Survival of Bees with Deformed Wing Virus and Nosemosis using a New Oxalate- Based Compound (p20) in 20 Hives Located in El Garraf, Barcelona, Spain. Proof of Concept

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Abstract

Introduction: There is a worldwide bee colony collapse, a syndrome characterized mainly by the disappearance of worker bees. It is caused by parasites such as those belonging to the genus Varroa- vector of Deformed Wing Virus (DWV) and other pathogens that affect bees, such as *Nosema ceranae*. The objective of this research was to evaluate the efficacy and safety of a new mixture of substances administered as feed to bees called p20, with the purpose of increasing the survival of bees affected by DWV and *Nosema* spp and to verify how p20 affected the presence of these pathogens in the hives.

Materials and methods: A prospective descriptive longitudinal observational study of bees with deformed wing virus and *Nosema* spp in hives in Garraf, Barcelona, Spain from July 2022 to July 2023 was carried out. The research was carried out on *A. mellifera* species, aged between 20 and 40 days of life, of female sex, in the subspecies Iberiensis, with physiological state of adult foragers, in 20 bees per hive, examining a total of 200 bees, 20 per group. Solution p20 was administered as feed, which is a mixture of sugars with syrup of glycosylated sugars and 4.6% (0.25ml) oxalic acid and sodium chlorite 28% (0.25ml) in 350 cc bags per hive. Three cycles of one bag per week were administered and consumed by the hives.

Results: Our findings suggest that hives with *Nosema* and deformed wings that were treated with a blend of oxalic acid in activated glycolytic sodium chlorite solution called p20 showed an improvement in survival rate and a significant decrease in the presence of *Nosema spp*. and deformed wings compared to previous winter seasons when we did not apply the substance under study. The efficacy of the p20 solution against *Nosema* spp. for four (4) weeks with three (3) treatments was 90.4% in total and stopped completely the new hatchings of bees with deformed wings by 100%.

Keywords: (MeSH, DeCS) • Deformed Wing Virus (DWV) • *Nosema* • Oxalic acid • Varroa

Introduction

Bees are essential to ensure two-thirds of the human food supply and therefore the survival of mankind, animals, plants and ultimately the entire planet.

As expert pollinators, bees are the largest contributors to agricultural production worldwide and do so far more efficiently than any other practice or technique in the field of agricultural management. They support, for example, the production of 87% of the world's major food crops. This highlights the dual importance of bees in sustaining life; one ecological and the other commercial, both of which are being affected in recent years by hive deaths due to various pathogens [1,2].

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However, over the last decade, bees and colonies have declined alarmingly worldwide in an environment of extensive farming and the use of pesticides that weaken the immune system of bees and promote a variety of viral, bacterial, fungal and parasitic infections [3], all of this coupled with what is known as hive depopulation syndrome.

Hive Depopulation Syndrome (HPS) occurs in different parts of the world, causing substantial economic losses for beekeepers, who on an average lose 30% of their colonies per season. The SDC, as such, is the massive disappearance of bees, observing empty hives, with or without the queen and a few young workers accompanying her. However, there is no really effective treatment to control the immense mortality of bees caused by the different diseases they suffer. So far, the only therapeutic option available are acaricide treatments to maintain control of the varroa mite and some protein and vitamin supplements to try to keep the bee's immune system in optimal condition. But none of these options are sufficient to reduce the high levels of mortality that continue to occur. Among the various existing treatments for varroa control is oxalic acid [1,2,4,5], which is one of the substances used in our blend under study.

If bees were to disappear from our planet, half of the plant species and 75% of the products consumed by humans would disappear. Today we observe a worldwide "bee colony collapse", a syndrome characterized mainly by the disappearance of worker bees [1,2,6-8]. Over the past few decades, honey bee colonies have been dying at a rapid and increasing rate, leaving many parts of the world with fewer pollinators than ever before.

German scientists have discovered that not only bees but also insect

populations in general are rapidly declining in protected areas as well. The severity of beehives losses is reaching such an alarming level that several countries are already openly talking about a "hive depopulation syndrome" to refer to the situation that has also been occurring for some years now in places like Asia.

For example, shortly after the introduction of glyphosate to reduce the growth of weeds and grasses, massive honey bee die-off rates were observed for all consecutive years. Other adverse factors include air pollution, the presence of electric and electromagnetic fields and other polluting environmental factors [9].

Among the best-studied pathogens involved in honey bee mortality is the external parasite Varroa destructor, which can be seen in honey bees.

Bees at a glance

Varroa mite: Where the problem starts: Varroa mites, specifically Varroa destructor species, are recognized as the largest pest of honey bees worldwide due to their ability to transmit diseases such as deformed wing virus to larval or pupal bees, resulting in death or severe deformity and can infest up to 100% of hives. Varroa mites feed on the fat bodies of adult and developing bees, weakening all growth stages (Figure 1). In addition, Varroa mite infestation was found to correlate significantly with the transmission of other types of viruses. In addition, it acts as a vector for virus transmission between diseased and healthy bees.

The endemic disease cannot affect all bees in a Varroa-free colony. However, in accordance with the fact that the presence of V. destructor increases viral transmission, analytical results indicate that the entire bee population can become infected when this disease vector is present in the hive [3].

DWV was found in 92% of mites from highly varroa-parasitized American colonies, 100% of workers with deformed wings, 75% of apparently normal workers, 47% of adult drones, 92% of worker pupae and 80% of larvae.

If DWV is present in Varroa parasitized hives, the impaired immune response of the bees increases their vulnerability [4]. Developmental features of affected pupae that become evident in adult animals (mainly drones) are deformed bodies and nonfunctional appendages including reduced body weight, deformed or atrophied wings that disable their navigational abilities, reduced drone reproduction, and severely decreased lifespan (on average < 48 hours). The overall clinical consequences are a decrease in the hive's bee population and, consequently, reduced honey production, eventually resulting in colony collapse during winter.

Overall, the alarming infection rate of honey bees with various pathogens reduces their productivity and, consequently, pollination worldwide [10-19].

Death from deformed wing virus is one of the most serious problems in beekeeping today, as it is one of the leading causes of death in bees. The major difficulty in establishing the real economic loss in relation to DWV-related hive losses is the lack of data collection and the difficult access of beekeepers to DWV analysis to specifically determine DWV as a cause of hive death.

Deformed Wing Virus (DWV)

The presence of DWV in Spain was high in apiaries in autumn during six seasons, being present in 99% of the apiaries and 83% of the investigated bee colonies systematically in the fall of 2012, [20].

In relation to the levels of infestation, an average of 25.9% presented moderate parasitism. very severe, higher than the annual average of 20.3%. From the typification studies

The molecular results show that 92.9% of the positive colonies were exclusively for DWV; the remaining percentage was due to mixed infections (Figure 2).

The pathological picture present in bees with DWV produces mortality, leaving the hive almost without population and with little chance of survival. The bees present characteristics such as: The bees present a shiny appearance,

Figure 1. a) Varroa mite on the upper part abdome of a bee and **b)** Varroa destructor. 10X

Figure 2. Bee affected by deformed wing virus.

general weakness and inability to fly, as a consequence of compression of the abdominal air sacs. Tremors and paralysis are manifested by weakness. It is common to find groups of dying or dead bees on the ground in front of the hive. The degenerative process of the ovaries in queens leads to multiple queen replacements in a single season.

The virus was first isolated from a sample of symptomatic honey bees in Japan in the early 1980s and is now distributed worldwide, wherever Varroa mites are found [2]. A recent survey of adult bee populations detected DWV in more than 90% of French apiaries [21] and in 100% of mite samples. The incidence was slightly reduced when pupae samples were analyzed, especially in spring [21].

DWV has also been detected by serology in the dwarf honey bee A. florae Fabr. (FR Hunter-Fujita, MS Mossadegh and BV Ball, Abstr. 36th "Apimondia" Int. Apic. Congr., abstr.) and in the Asian honey bee A. cerana Fabr [2] and by Reverse Transcriptase (RT) PCR in bumblebees (14). It is serologically related to Egyptian bee virus [18,19,22], first isolated in 1977 from infected adults in Egypt [13]. Typical symptoms of deformed wing disease are vestigial and shriveled wings, swollen abdomen, paralysis, and greatly reduced adult life span for worker bees and emerging drones [18].

For a long time it was believed that these symptoms were due to mite feeding activity [11,17,23] until it was shown that, in diseased colonies, deformed bees could emerge from cells not parasitized by Varroa mites [23-25] and that symptoms can persist in the absence of mites [6]. Symptoms are well correlated with the presence of large numbers of DWV, as well as with the reduced virus titers and lower prevalence found in asymptomatic bees from the same colonies [7,9,10,26-28]. The combination of mites and virus causes immunosuppression in bees and increases susceptibility to other opportunistic pathogens [23], leading to a progressive reduction in colony performance and a complex disease profile at the colony level that often also includes other pathogens [5,21,25,29].

Occasionally, a colony may have symptomatic bees in early spring and recover during the summer, only to see symptoms reappear later in the year. As with Acute Bee Paralysis Virus (ABPV) and Kashmir Bee Virus (KBV), parasitism of pupae by Varroa mites is the most important factor in both symptom development and virus titer in individual bees [7,26,30]. An alternative route of infection is contact of larvae with nurse bees, as the virus can be detected in oropharyngeal secretions (hypo), in food (royal jelly) of brood, in eggs and early larval stages that are not parasitized by mites [9,10], and in uninfested pupae fed in infected colonies [26,30]. High levels of virus in mites are generally associated with high levels of virus in the corresponding pupae [7,26], and there is evidence that the virus can replicate in the mite [7,26,29]. DWV is normally a weak and insignificant virus that develops slowly, allowing the offspring to develop through the pupal stage to adulthood [2,6]. It is this low virulence that has made DWV the main virus associated with Varroa mite infestations, as well as other more virulent viruses such as bee chronic paralysis virus, ABPV, KBV, and Black Queen Cell (BQCV),

At the molecular biology level the DWV virion is a 30 nm icosahedral particle consisting of a single positive-stranded RNA genome and three major structural proteins [2], features that are common to many insect viruses. Originally classified as picornavirus-like insect viruses [31,32], these viruses are now largely classified into two groups, the genus Crypavirus, family Dicistroviridae [33], with separate open reading frames for structural and nonstructural polyproteins (in the 3' and 5' halves of the genome, respectively), and the genus Iflavirus, with a genomic organization typical of picornaviruses consisting of a single open reading frame in which the structural proteins are N- terminal to the nonstructural proteins. In both genera, the viral RNA is polyadenylated and the open reading frame is flanked by untranslated regions (NTRs) containing replication and translation control elements [17-19,28]. Among honey bee viruses, BQCV, ABPV, and KBV are crypaviruses, whereas SBV, Kakugo Virus (KV), and Varroa Destructor Virus 1 (VDV-1) are iflaviruses [12,13,15,16,19,25].

The presence of DWV is strongly associated with typical pathologic symptoms and provides information on the molecular characteristics of the virus. DWV has an icosahedral particle structure, with a considerable proportion (25%) of empty particle layers visible after purification, possibly due to particle instability. Western transfer analysis with a monospecific antiserum against the VP1 protein of DWV shows that the virus is concentrated in the head and abdomen of infected adult bees, with significantly reduced titers in the thorax. DWV RNA was detected by RT-PCR in the head, thorax, abdomen and wings of infected bees, and only the legs were virus-free. DWV was also detected in infested and uninfested pupae of mites from severely diseased colonies. The Western blot technique could not detect the virus in fifth instar larvae from diseased colonies, nor in any bees (adults of various ages and functions, pupae and larvae, both sexes) from healthy colonies. More sensitive techniques, such as RT-PCR, have been successfully used to detect DWV in larvae and eggs from diseased colonies (as well as in adults and pupae), but not in those from healthy colonies [9,10,28].

Nosemosis

Among the main diseases associated with V. destructor is nosemosis (Hinojosa and González, Markovick, et al.), caused by the fungus *Nosema Apis* (Zander) Ritter, Fries and *N. Ceranae* [34].

Nosemoses are a group of bee diseases caused by microsporidia of the genus *Nosema*. They are unicellular and eukaryotic parasites that depend on a host cell to develop and complete their biological cycle (Wittner and Weiss). The most frequent in bees are *N. Apis* (*Nosema* A) and *N. Ceranae* (*Nosema* C).

As shown in Figure 3, in a photograph taken in our laboratory during the analysis of bees for spore counting and the number of positive hives, a spore of *N. Ceranae* and a second form compatible with *N. Apis* can be seen; to ensure that it is *N. Apis*, a PCR test would have to be performed since it is very similar to a type of amoeba that bees also suffer from. The presence and coexistence of these pathogens indicate a major threat to beekeeping in all countries and are directly related to the decline in beekeeping activity detected in the last ten years.

Nosemosis and DWV

Some authors have proposed a synergistic effect between *Nosema* (A,C) and other bee pathogens such as viruses. In some studies there seems to be such an effect between *N. ceranae* and Deformed Wings Virus (DWV) (Zheng, et al.). On the contrary, Costa, et al. propose a possible competition between the two pathogens for resources within the host cell. The results of studies by Doublet, et al. confirmed that *N. ceranae* inhibits DWV development, provided that *N. Ceranae* is the first to establish in the host, whereas if DWV establishes first, it does not inhibit *N. Ceranae* development.

Nosemosis generates a decrease in colony activity due to high mortality and constant loss of bees, particularly when the oldest bees die, which are usually the most parasitized [35]. As a result, the hive receives less food, with the consequent lower honey production, which generates huge economic losses [36].

Given the current scenario of global depopulation of bees and the initial test observations made by us through the use of a new mixture that we call p20 presented to the bees in the form of a food bag in the hives, we were able to observe, visually for DWV and by analytical tests for *Nosema spp*. a significant improvement of the hive. Therefore, we proposed to carry out this research to answer the question of whether this mixture could have a virucidal and fungicidal effect, thus producing an increase in the survival of the hives.

Background

There are many places where the use of pesticides causes mortality in hives and consequently, a greater proliferation of diseases in bees, and in the region of El Garraf, near Barcelona in Spain, is one of them. The hives transhumed by us from another apiary in Spain to this place nine years ago, began to present a high mortality due to the use of glyphosate, subsequently an increase of *Nosema* and an increase of bees with Deformed Wings by the virus (DWV), which highlighted the fact of pesticide exposure, the occurrence of viruses and fungi in bees with a significant decrease in the immune system of the same, which led us to very high losses of hives (Figure 3).

In the season from December to February each year we spray intensively with glyphosate in the vineyard and immediately afterwards we began to observe a high mortality among the bees. We then observed a second wave of mortality and to study its cause in the laboratory we found a considerable increase in *Nosema* spore counts coupled with an increase of Deformed Wing Bees (DWV) in the hive checks. For three years approximately seventy percent of the hives were dying; faced with this situation, seven years ago we decided to look for solutions to these diseases and after trying the usual methods of protein and vitamin reinforcement without success, we began to develop what we now call p20 solution.

For two years we developed the formulation so that it would be stable and could be easily and effectively administered to the bees, so we decided to administer it in a bag to better preserve the substance. Administered as feed, we observed that the high mortality, which occurred over four years during the period when glyphosate was sprayed, was considerably reduced (Figures 4 and 5).

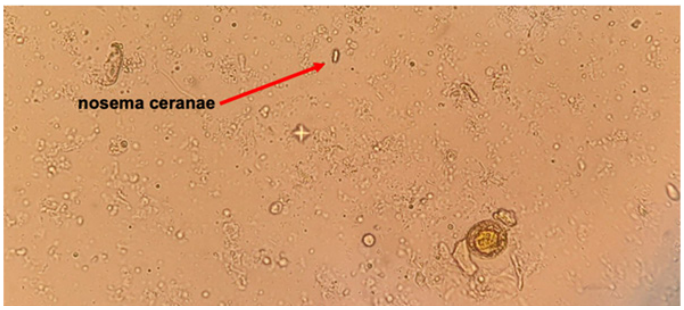

Figure 3. Sample of *Nosema ceranae* under microscope at 400X.

Figure 4. Bees killed by the use of glyphosate in the vineyard.

Figure 5. Bees killed by the use of glyphosate in the vineyard.

Nosema, an opportunistic intestinal microsporidium, appeared due to the weakness of the immune system produced by glyphosate, killing the surviving hives. For five (5) years we worked on the development of the mixture of substances until we were able to drastically reduce mortality. We made preliminary analytical observations on *Nosema* and the results obtained with this mixture of substances revealed an improvement of approximately seventy-six percent (76%) reduction in spore count. At the same time, the hives that had presence of bees with deformed wings seemed, observationally, to cease the birth of new bees with this deformity in a very important way, which generated the need to carry out the present study, which is the first of three steps or phases of the process that we have proposed to investigate. This first phase is a descriptive longitudinal prospective observational study, in the second phase we will carry out a study of direct inoculation of the virus in the laboratory and then in the third phase, a case-control blind multicenter study in hives in different countries with the accompaniment of a study group in a European university.

Oxalic acid: A common treatment for varroa

Oxalic acid is a bicarboxylic acid with the formula $H_2C_2O_4$. Its name derives from the plant genus Oxalis, due to its natural occurrence in plants, and even in honey.

Effect of oxalic acid on the bee digestive tract

When we started to do the analysis to study *Nosema* spp. we did tests with different substances, among them we analyzed bees from hives treated with oxalic acid in syrup to see the difference in the presence of *Nosema* spp. spores, the presence of bees with deformed wings and if this treatment had any incidence or efficacy. We observed how in the microscopic visualization of the bee's "digestive preparation" large oxalic crystals appeared, which can affect the bee's health. See Figure 6.

Because they are very large crystals, besides acidifying their digestive system, they could cause micro wounds at tissue level and affect their immune system. This finding concerned us because of the possible damage that oxalic acid does to bees at the usual doses used for varroa treatment (Figure 6) as noted by some authors, although Nanetti and another group of authors propose that the bee assimilates it well.

Previous studies show that the presence of DWV in bee colonies is not affected by the application of varroa treatments [37]. If DWV is present at high levels in a bee colony, they cannot be eliminated by effective varroa treatments. It is likely that alternative transmission routes within the colony, other than varroa mites, are responsible for the persistence of high DWV prevalence in honey bee populations [38,39]. Pupal cannibalism by worker bees has been reported to also contribute to the spread of deformed wing virus [40], and this cannibalism is due to the hygienic behavior of worker bees called Varroa Sensitive Hygienic (VSH).

DWV is the major problem for honey bees in Europe (R. Paxton), a virus that has long been present in honey bees and other insects; its presence has increased to worrying levels since varroa mites (Varroa destructor) have become widespread in *A. mellifera* colonies [41].

There is no specific treatment for Nosemosis and DWV. Based on the relationship between varroa, *Nosema* spp and DWV, varroa control is used as a strategy using different substances including oxalic acid and the use of protein supplements focused on improving the physiological health indicators of the hive; this is the only therapeutic approach used so far, and despite this, the mortality rate of hives continues to be around 30%. The annual loss of 48% recorded in 2022 is higher than the 39% loss recorded the previous year and the 12-year average of 39.6%, but it is not as high as the 2020-2021 mortality rate of 50.8%, according to the survey funded by the nonprofit research group Bee Informed Partnership [42].

The most widely used and most effective substance is amitraz (formamidine) at 12%, but it has a higher toxicity than oxalic acid and leaves residues in honey. In addition, it has been reported that the varroa mite has developed resistance to some pyrethroids such as fluvalinate and flumethrin, and there are studies that reveal the appearance of varroa resistance to amitraz [43,44]. In a study to verify toxicity, all samples tested were contaminated with amitraz metabolites. 2,4-DMA and DMPF were the most frequently determined compounds. The mean concentration of amitraz marker residues in honey from groups where a smoke tablet was placed directly in the hives was significantly higher than that of residues in honey from groups with indirect smoke generation [44].

The efficacy of 4.6% oxalic acid in the treatment of varroa varies according to different studies. In a comparative study with three other acaricides, it is greater than that of amitraz and does not report toxicity and contaminants in honey for human consumption [45].

Faced with this difficult scenario for bees and beekeepers, we explored new therapeutic possibilities in nosemosis and DWV treatment. We decided to use oxalic acid (R. Marti), reducing its concentration, in different mixtures to

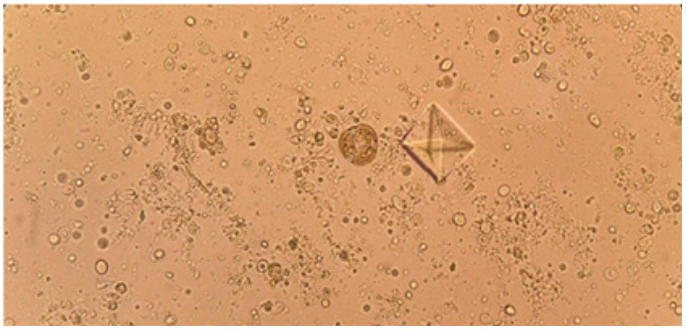

Figure 6. Presence of oxalic crystals in the bee digestive tract.

increase its efficacy in blend preparations until developing the so-called p20. Other researchers have proposed to address studies using new blends for treatments, as they have proposed to address studies using new blends for treatments, in the search for new and better therapeutic options for *Nosema* spp and DWV, while suggesting that co-formulants other than sucrose should be investigated in sugar- based foods [46].

After performing the *Nosema* analysis and macroscopic diagnosis for DWV in our preliminary observations, we started the administration of the p20 solution to the hives; we observed that no new bees with deformed wings were being hatched and at that time, the hives did not have routine protocol treatment against varroa.

That is, even though there was a high level of varroa, the hatching of new bees with deformed wings decreased drastically.

This was the main cause that made us think that the differential factor of what we had done in the hives with respect to other years was p20. Therefore, it made us hypothesize that p20 is effective in reducing the number of bees with deformed wings in the hive, while also significantly reducing the presence of *Nosema* in the hives.

As the hives presented such a high level of nosemosis, together with the presence of deformed wing viruses in them, we decided to carry out a parallel observation of the two pathologies and we focused on counting spores of N. ceranae, as well as frame by frame checks counting bees with deformed wings in all the hives under study.

Materials and Methods

The literature search was done based on MeSH and DeCS criteria, in Google Scholars, LILACS, Pubmed, Science Direct using the following terms: [Mesh]"Deformed Wing Virus, *Nosema*, OXalic" Pubmed:55 results; [LILACS] 0; [ScienceDirect]::0; Google Scholars, 379 results, [Mesh]"Deformed Wing Virus AND treatment", Pubmed: 693 results, [LILACS] 1; [ScienceDirect]: 0; Google Scholars, 18.200 results, [Mesh]"*Nosema* AND treatment, Pubmed:1726 results, [LILACS] 1; [ScienceDirect]: 1390; Google Scholars, 15.200 results; "Oxalic acid AND treatment AND *Nosema*", Pubmed: 104; [LILACS] 0; [ScienceDirect] 39; Google Scholars, 840 results; "Oxalic Acid AND treatment AND deformed wing virus", Pubmed: 870 [ScienceDirect]: 78; [LILACS] 0; Google Scholars, 1200 results "Oxalic Acid AND treatment AND deformed wing virus AND natrium Chlorite" : [Mesh] Pubmed:270; [LILACS] 0; [ScienceDirect]:0. Google Scholars 463 results; "Oxalic Acid AND treatment AND varroa", Pubmed: 1920, [ScienceDirect]: 91; [LILACS] 0; Google Scholars, 2640 results; Oxalic Acid AND treatment AND varroa AND DWV: Pubmed: 322 [ScienceDirect]: 7; [LILACS] 0; Google Scholars, 364 results; Oxalic Acid AND treatment AND *Nosema* AND DWV: Pubmed: 173 [ScienceDirect]: 5; [LILACS] 0; Google Scholars, 247 results.

Regarding the design, it is a prospective longitudinal descriptive observational descriptive study; the trial is composed of an intervention group which received the 'treatment' and was designed to determine the efficacy of the treatment with the mixture called p20. The objective of the study was to determine the efficacy of the p20 preparation on the survival of hives with deformed wing virus and nosemosis in view of the coexistence of these in the hives and to provide data on which to base reasonable hypotheses in the next phase of the study.

The study was carried out in *A. mellifera* species, subspecies iberiensis, with ages ranging from a few days old to 40 days old, males and females. The sample was carried out in adult females, between 20 and 40 days old, for *Nosema* spp. and for DWV in bees a few days old, both males and females, in hives located in El Garraf, Catalonia, Spain; The usual management of traditional beekeeping was carried out in vertical hives, with Langstroth type vertical hives as animal housing, thermoregulation and ventilation of the natural housing by the bees themselves, examining a total of ten (10) hives (N=10).

Solution p20 was administered as feed, which is a mixture of sugars with syrup of glycosylated sugars and 4.6% (0.25ml) oxalic acid post thermal and homogenized processing, and sodium chlorite 28% (0.25ml) in 350 cc bags per hive. Three cycles of one bag per week were administered and consumed by the hives.

The sample does not refer to individual bees but to the hive as a unit or superorganism and their survival was studied to determine the health of the hive.

We detected *Nosema* spp in adult bees, which are at the center of the virus transmission pathway (DWV) as well as of the external spread of these epizootics due to their high mobility, contact rate and diverse network of contacts. This makes adult bees the most suitable bee stage to detect *Nosema* spp. For the case of DWV we counted by frames the number of bees hatched with deformed wings.

The study inclusion criteria for DWV within the hive and its bees were the presence of bees with deformed wings in at least three [3] frames of the hive and for *Nosema* spp the ten [10] hives under study. We sampled them from the same age/task group throughout the experiment as much as possible to minimize the influence of such effects on the data (Van der Steen, et al.). For *Nosema* spp, bees were sampled only in the honey frames at the same time of the day (11:30 am) and at the same time observations were made for DWV in each frame [10] of each hive for a total of one hundred (100) frames examined. In each sampling, a beekeeping technician inspected the colonies; determined the number of honey bees, brood, pollen and combs using subjective method described (Delaplane, van der Steen and Guzman-Novoa); and noted the presence of symptoms, mortality and/or depopulation.

The coexistence of DWV and *Nosema* spp. occurs due to the confluence of seasonality and the increase of varroa in the hives. This, together with the use of glyphosate in neighboring vineyards, causes the bee's immune system to weaken considerably, so it is common to find hives affected by both diseases simultaneously.

We recorded bee mortality, feed consumption, temperature, relative humidity every day for 28 days. Dead bees were removed each day using dead bee collection containers at the foot of the comb.

Sample collection

In the months of November and December 2022, we proceeded to prepare our experimental apiary to carry out our study in the first days of February 2023 in the region of El Garraf, province of Barcelona, Spain. In the area where the apiary is located at the end of November and first days of December, preventive treatments for varroa are carried out following established protocols. We decided not to perform the routine preventive treatment against varroa in order to promote varroa infestation and additionally facilitate a possible increase of *Nosema* microsporidium spores; as a result, varroa and *Nosema* increased in the following months. In addition, the fields next to the apiary were sprayed with glyphosate at the same time. This spraying seriously affects the hives, causing high mortality and weakening the digestive system of the bees. Therefore, the Varroa- Glyphosate binomial creates the perfect terrain for infection by the microsporidium *Nosema*, which is an opportunistic microorganism when the bee's immune system is diminished, developing the nosemosis disease in the hive in a considerable way, as we expected. Likewise, we observed a considerable increase in the birth of bees with Deformed Wings (DWV).

The first stage of the study consisted of collecting samples of adult bees from each of the ten hives available for this study. The samples were collected from the end frames of the honey. For this purpose, a urine collection container was used, collecting slightly more than twenty bees per hive. The samples were then placed in a zip bag and the hive code was placed on each bag. Then they were placed inside a plastic container (Figure 7), repeating the same in the ten hives (Figure 7).

Once all the bags with live bee samples for *Nosema* spp analysis were placed in the plastic container, they were taken to the freezers of the laboratory at -200 °C. The observation of the hives with DWV was done visually by counting the number of hatched bees with deformed wings.

It should be noted that the Beebook, the reference book for bee research

Figure. 7 Sample of live bees before freezing.

processes, recommends the collection of samples from adult bees in the comb (Fries, et al.). The problem is that when collecting the samples in the entrance, in addition to the adults, young bees that are starting their first flights can also fall, and it is true that they usually do it in the late afternoon; but sometimes if needed, due to a large influx of nectar, they can also do it at other times. Therefore, we adapt the sample collection procedure according to Beebook techniques following recommendations described by (R. Martin) During the day the honey frames are populated by the forager bees, which are adult bees and are the ones working those frames. Therefore, the probability of collecting twenty adult bees there is much higher than collecting them in the comb.

Sample processing and analysis for *Nosema* spp

After collecting the samples and transporting them to the laboratory freezers, the bees were thawed by ordered groups so that they could be progressively thawed while the preparations were being made for analysis.

The preparations were made by dissecting the ventricle of the bee; which we did by taking the dorsal and ventral abdominal segments A7 with the forceps and holding the abdomen with the other hand (Fries, et al.). We then slowly separated the posterior portion of the alimentary canal and by stretching it, we pulled out the digestive of the bee, sometimes with the honey sac and sometimes not.

The samples from each hive were more than twenty bees. If any dissection was incorrect, the behavior was to discard it and take a new bee so that in total there would be twenty bees analyzed correctly per hive (Figure 8)

Once the digestive was extracted from the bee, it was placed in 150 microliters of Ringer's solution in an Eppendorf tube and ground in a mortar with a micropistyl for enough time so that the digestive was well dissolved in the solution. Subsequently, the Eppendorf was closed and passed for one minute in Vortex to create a homogeneous solution. This procedure was performed in a standardized manner for all samples. We proceeded to take with the pipette of two hundred microliters part of the amount of solution in the Eppendorf to put a drop on the slide and place a 12-millimeter coverslip (Figure 9).

Figure 8. Materials in the bee dissection process.

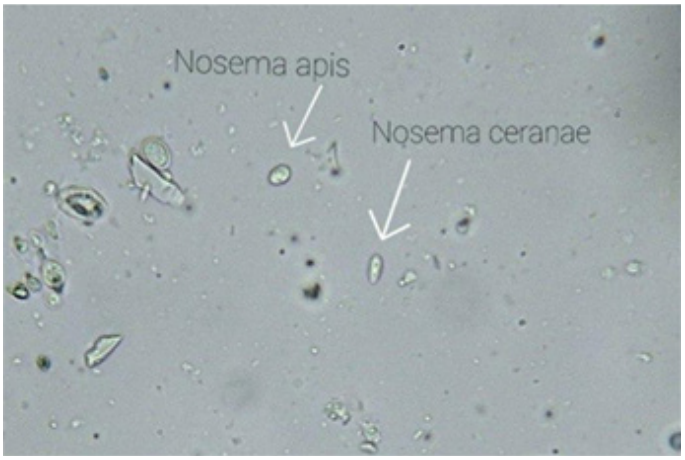

Figure 9. Microscopic observation of nosema ceranae.

Afterwards, the sample was placed in the phase contrast microscope. Upon achieving accurate focus at 400 X, a one-minute time limit was imposed for visualizing the spore count of each sample through the eyepieces. If any *N. Ceranae* spores were detected within that minute, the sample was designated as positive, indicating the presence of *N. Ceranae* in the bee. Conversely, if no spores resembling *N. Ceranae* were observed during the visualization, the sample was deemed negative. Subsequently, the same procedure was repeated with the subsequent bee, which had already been thawed, thus continuing the laboratory process in sequence. In the event that a spore resembling N. Apis was observed during the visualization period, it was merely noted, but did not hold relevance to the final result as it required confirmation through PCR due to its morphological resemblance to a type of amoeba under the microscope. This observation serves as a diagnostic measure for DWV.

Observational diagnosis of DWV

The process of examining for wing deformities was conducted through macroscopic assessment, carefully inspecting each frame of the hive on both sides, and tallying the instances of bees with deformed wings in each hive until all one hundred frames of the ten hives were examined. To enhance our observations, we supplemented the macroscopic assessment with an examination using optical microscopy at 4X magnification and stereoscopy (Figures 10 and 11).

Figure 10. Microscopic and macroscopic observation of the deformation of the wing assembly.

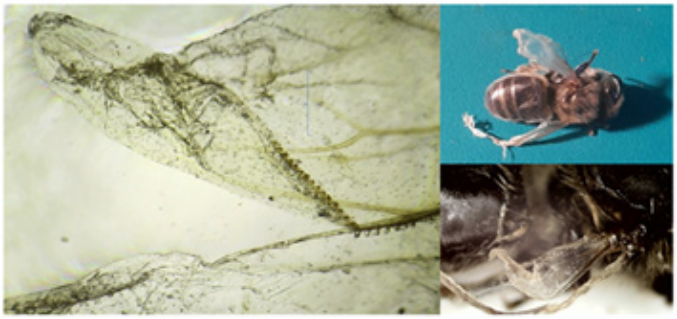

Figure 11. Detail of the bent hind wing separated from the hamuli and microscopic view of the deformed wings.

Procedure for administration of p20

On the day of the initial sample collection, bags of p20 were placed in the hives. The first bag of p20 that was placed in each hive was meticulously labeled with the hive number, study number, lot, expiration date, and the date of placement. After a week, the bags were removed from all the hives and observations were made to determine if the contents had been completely consumed or if any remnants remained. It was discovered that only two hives, specifically hive #3 and #7, had leftover amounts of 20 CC and 40 CC respectively. The remaining hives did not have any remnants in any of the treatments. Over a span of three weeks, one treatment (consisting of a 350 CC bag) was administered each week, resulting in a total of three treatments. Following the completion of the final treatment, an additional week was given before collecting the last bag. On the same day, bee samples were collected from all the hives. The live samples were once again taken from adult bees in the honey frames and transferred to zip bags for freezing in the laboratory. The same process was then conducted to analyze and determine spore count in twenty bees per hive, as well as perform macroscopic and stereoscopic observations for DWV. These new analyses were then compared to the initial results to evaluate the efficacy of p20 against the parasite *Nosema* spp and DWV (Figure 12).

Each hive was assigned a consecutive hive number and corresponding study preparation, in the order of admission to the study. The assignment of the preparation was determined prior to the commencement of the study using a computer-generated list. The hives were administered substance p20 in accordance with the generated list. The substance was administered orally using lightweight plastic bags that allowed the bees to puncture the bag for consumption. Additionally, two holes were punctured at the top of the bag

Figure 12. First day of treatment of p20.

using a needle to ensure consumption by the bees. The bags contained 350 cc of p20, which is a solution consisting of a mixture of oxalic acid in activated glycolytic sodium chlorite solution (Figure 13).

The total consumption of the preparation in the hives was determined by counting the bags that were consumed. During each visit, the beekeeper collected and recorded the empty or partially used solution containers that were kept in the hives. At the end of the study, it was found that all of the test solution samples were used completely, with no returns except for the two that were previously mentioned. These samples were disposed of in accordance with local regulations. No other substances were administered concurrently with the study, and there were no adverse effects observed with the use of the substances, indicating excellent tolerance. It is important to mention that at the end of the last sampling, protocol treatments were conducted against varroa to comply with current regulations (Royal Decree 608/2006 of May 19) [47] and protect the hives. The quantitative data obtained from the test measurements were analyzed using a measure of central tendency, specifically the mean.

Results

During the winter season, a customary protocol is implemented to prepare the hives, with the intention of preventing and/or reducing diseases and

mortality among them, particularly varroa. This protocol involves placing strips of oxalic acid inside the hives. However, in the present study, we intentionally chose not to apply this protocol to the hives under investigation. Our objective was to generate a sufficient viral load in order to conduct our research and determine the prevalence of Deformed Wing Virus (DWV) and *Nosema* among the bees. Consequently, it was anticipated that varroa infestation would increase during the winter months, leading to a corresponding increase in DWV. We made a deliberate decision not to treat the bees in November 2022 or December 2022, allowing the hives to follow their natural course. We waited until the end of January, when favorable weather conditions and temperatures allowed us to open the hives for inspection. Our purpose was to assess the number of hives that had bees with deformed wings. Out of the ten hives examined, eight exhibited numerous frames of bees with deformed wings. The presence of deformed wings was clearly visible in various frames. The incidence of bees with deformed wings was remarkably high. Specifically, in the brood chamber comprising ten frames, between six and eight frames contained bees with deformed wings. Upon conducting a count, we found up to eight bees with deformed wings on one side of a single frame. Normally, when varroa preventive treatment is administered, hives exhibit minimal occurrence of bees with deformed wings. In most cases, at most one to seven bees with deformed wings may be observed throughout the entire hive. Furthermore, a diagnosis of varroa phoretica and internal varroa (present within the pupa) was conducted. The results revealed a substantial prevalence, with eleven varroas detected in the analysis of varroa phoretica and twenty-seven in the analysis of varroa interna (Figure 14).

Figure 13. Empty bag.

Figure 14. Healthy bees after treatment with p20.

The average temperature of the hives was 34.0 degrees Celsius, with a range between 32.0 and 36.0 degrees Celsius. The average relative humidity was 57%, with a range between 44% and 59%. We identified and treated a total of 10 hives with bees exhibiting deformed wings using a blend of substances called p20. After completing the treatment, we conducted a comprehensive frame-by-frame inspection of all the hives. We observed that the presence of bees with deformed wings had completely disappeared in all frames, resulting in a 100% elimination rate. The majority of varroa mites were found inside the

Table 1. Test results for *Nosema* spp.

Before Treatment with p20						After Treatment with p20					
Bee No.	Hive No.	Treat. p20	N. C.	N. A.	Comments	Bee No.	Hive No.	Treat. p20	N. C.	N. A.	Comments
1	7	No	+	+?	2*	1	7	Yes	-	-	
2	7	No	+	-		2	7	Yes	-	-	
3	7	No	-	+?	2*	3	7	Yes	-	-	
4	7	No	+	+?	2*	4	7	Yes	-	-	
5	7	No	+	-		5	7	Yes	-	-	
6	7	No	+	+?	2*	6	7	Yes	-	-	
7	7	No	+	-		7	7	Yes	-	-	
8	7	No	+	-	2*	8	7	Yes	-	-	
9	7	No	+	-		9	7	Yes	+	-	
10	7	No	+	+?	2*	10	7	Yes	-	-	
11	7	No	+	+?	2*	11	7	Yes	-	-	
12	7	No	-	-		12	7	Yes	-	-	
13	7	No	-	-		13	7	Yes	-	-	
14	7	No	+	-		14	7	Yes	-	+?	2*
15	7	No	-	-		15	7	Yes	-	-	
16	7	No	+	+?	2*	16	7	Yes	+	-	
17	7	No	+	-		17	7	Yes	-	-	
18	7	No	+	-		18	7	Yes	-	+?	2*
19	7	No	+	+?	2*	19	7	Yes	-	+?	2*
20	7	No	+	-		20	7	Yes	-	-	
			16		80%				2		10%

2*: Compatible with *Nosema apis* only confirmable by PCR

Table 2. Analytical results of 10 hives before and after p20 against *Nosema* spp.

Hive no.	Pre. p20	Post. p20	Hive no.	After	Before
Hive 1	11	1	Hive 1	5%	55%
Hive 2	13	0	Hive 2	0%	65%
Hive 3	0	0	Hive 3	0%	0%
Hive 4	13	3	Hive 4	15%	65%
Hive 5	9	1	Hive 5	5%	45%
Hive 6	4	0	Hive 6	0%	20%
Hive 7	16	2	Hive 7	10%	80%
Hive 8	0	0	Hive 8	0%	0%
Hive 9	7	0	Hive 9	0%	35%
Hive 10	0	0	Hive 10	0%	0%
Average	73	7	Average	3,5%	36,5%
Efficiency 90,4%					

cells. In summary, by the fourth week, upon withdrawal of the final treatment, the occurrence of bees with deformed wings had completely vanished in all the hives (Table 1).

In the following tables, we present the results of the analysis for *Nosema* spp in the study hive number 7. The first table displays the findings before treatment with p20, where 16 out of 20 bees exhibited spores compatible with *N. Ceranae*, resulting in a positivity rate of 80%. This high infection rate suggests a significant risk of colony loss. Conversely, the adjacent table represents the second round of sampling after three treatments and a four-week period. It demonstrates a notable improvement in the rate of *N. Ceranae* positive bees, with only 2 out of 20 bees testing positive, equating to a 10% infection rate. This represents a significant improvement of 70%. Table 2 encompasses the results of the analysis for each hive in the study, both before and after treatment with p20. Through this analysis, we have determined that the p20 solution effectively combats *Nosema* spp for a period of four weeks, with three treatments resulting in a remarkable 90.4% reduction in spore count between the two sampling rounds (Table 2).

Substance p20 consumed as food completely stopped new hatchings of bees with deformed wings by 100% (Figure 15).

Table 3. Results of 10 hives before and after p20 against DWV.

Hive No.	Pre. p20	Post. p20	Hive No.	After	Before
Hive 1	33	0	Hive 1	33	0,0
Hive 2	26	0	Hive 2	26	0,0
Hive 3	18	0	Hive 3	18	0,0
Hive 4	0	0	Hive 4	0	0,0
Hive 5	28	0	Hive 5	28	0,0
Hive 6	36	0	Hive 6	36	0,0
Hive 7	54	0	Hive 7	54	0,0
Hive 8	11	0	Hive 8	11	0,0
Hive 9	0	0	Hive 9	0	0,0
Hive 10	19	0	Hive 10	19	0,0
Average	22,5	0	Average	22,5	0,0
Efficiency 100,0%					

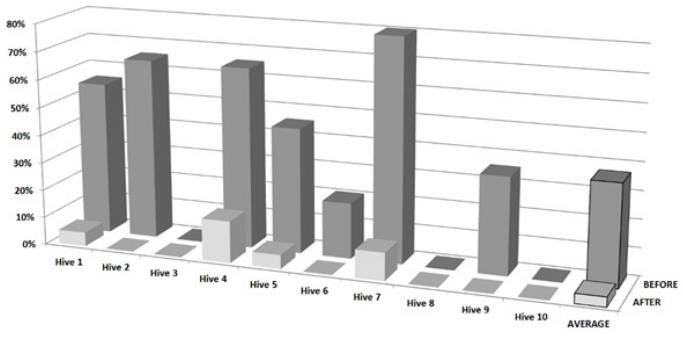

Figure 15. Analytical results of 10 hives before and after p20 against *Nosema* spp.

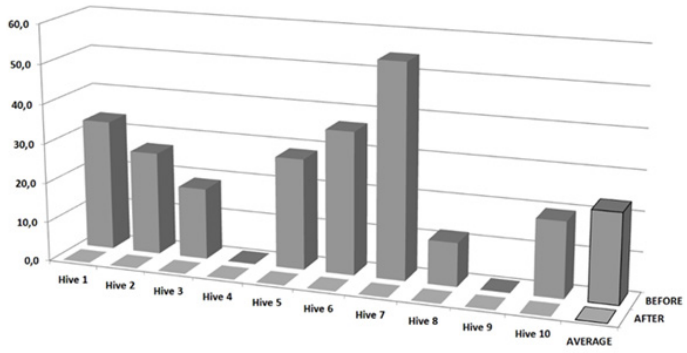

Figure 16. Results of 10 hives before and after p20 against DWV.

The review was conducted on the day when the last treatment with p20 was withdrawn. It was observed that there was a complete elimination of bees with deformed wings, as compared to their presence at the beginning of the study (day 1). This information is clearly presented in (Table 3).

Based on our meticulous assessment of every hive, we conducted a thorough examination of each frame to determine the precise count of bees born with deformed wings. As evidenced in Table 3, the mean quantity of bees displaying this apparent characteristic across the ten (10) hives under investigation on the first day was 22.5 (Figure 16).

According to the findings depicted in Figure 15, it is noteworthy that after a duration of four weeks, no instances of bees exhibiting this viral-induced malformation were observed. The sole distinguishing factor and our exclusive intervention in the beehives was the application of three (3) treatments of p20, administered once per week.

Discussion and Conclusion

Our findings indicate that the application of a blend of oxalic acid in activated glycolytic solution called p20 resulted in excellent outcomes for hives affected by nosemosis and deformed wings. The hives that received the p20 treatment demonstrated an improvement in survival rate and a significant reduction in the presence of nosemosis and deformed wings compared to previous winter seasons when the substance under investigation was not utilized. It is worth noting that in previous years, our apiary experienced hive losses ranging from 50% to 70% due to the aforementioned factors. However, in the year of the present study, despite the presence of DWV, *Nosema* spp., and the use of glyphosate in the vineyard, we chose not to implement protocol treatments against varroa. Despite these circumstances, the survival rate of the hives reached 100%. Moreover, all hives exhibited normal development despite the lower than usual rainfall during this spring season. The observations and data obtained offer no alternative interpretation other than the explanation provided here. It is important to acknowledge that a potential bias exists due to spring seasonality, whereby hives naturally strengthen and overcome winter weaknesses by moving towards growth, resulting in improved vital indicators. This bias necessitates the need for new case-control studies to further investigate. Additionally, it is important to recognize that the diagnostic observations on DWV may possess a confounding bias compared to observations regarding *Nosema* spp., as the absence of PCR tests prevents determination of the specific presence and viral load of the virus, thereby hindering evaluation of therapeutic efficacy against the virus. It is crucial to remember that the absence of wing deformity does not guarantee freedom from the virus. In future studies, we recommend analyzing the efficacy of p20 through precise techniques such as controlled space inoculation of DWV virus and the use of accurate analysis techniques like RT-PCR.

There have been multiple studies evaluating the effectiveness of oxalic acid for controlling varroa mites. These studies used methods such as dripping with sugar syrup or vaporization. Authors such as Marinelli, Arculeo and Eguaras have reported efficacy percentages ranging from 80 to 90%. Studies on the treatment of nosemosis have shown that contact substances like ammonia, bleach, and Mycoplasma OFFTM resulted in significantly higher *Nosema* mortality compared to the negative control (water) [38]. This indicates their sporicidal capacity. Oxalic acid has also been found effective in controlling *Nosema* in bee colonies, as shown by Nanetti, et al. Unfortunately, there is no specific treatment for Deformed Wing Virus (DWV). Only Israel and the USA have approved therapeutic treatments for DWV based on RNA interference (RNAi). This technique involves administering a nucleic acid with feed (sugar syrup) that introduces small RNA sequences complementary to the virus, hindering its replication. To date, no studies have evaluated the treatment of varroa, *Nosema*, and deformed wings simultaneously from an integrated perspective of the hive as a living unit. Furthermore, none of the consulted literature reports the use of the same substance for treating the presence of these three variables together. These findings are highly encouraging. Therefore, upon completing this initial stage, we propose conducting a

prospective randomized experimental study in a second phase. This study will involve direct inoculation of the DWV virus, determination of its genotypy, and treatment with the p20 solution in a comparative and controlled manner. Subsequently, in the third phase, we will conduct a blind, multicenter, case-control study in hives from different countries treated with the p20 solution.

The current stage of the study has limitations in terms of variable control due to its observational design. Therefore, it is not feasible to fully verify the accuracy of the final results. Additionally, the design used in this study carries a higher risk of potential bias compared to experimentation. As such, we propose conducting a second stage of research utilizing an experimental approach. Our findings highlight the need for future research to explore the potential impact of mitigating these diseases in bee hives, as they play a significant role in global colony depopulation syndrome.

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Conflict of Interest

The authors declare no conflict of interest or no competing economic interests. Authors have completed the ICMJE conflict of interest declaration form, translated into Spanish by Medwave, and declare that they have not received funding for the article/research; have no financial relationships with organizations that could have an interest in the published article in the last three years; and have no other relationships or activities that could influence the published article. Forms can be requested by contacting the responsible author. This does not alter the authors' adherence to all policies on the exchange of data and materials.

Observation

The authors followed the guidelines of the STROBE Statement-Checklist of items.

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