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Fungal diseases of honey bees (Hymenoptera: Apidae) that induce considerable losses to colonies and protocol for treatment

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Abstract
Honeybee diseases cause considerable expenses to beekeepers for cost of maintaining apiary's inspection, colonies damaged or destroyed and drugs fed to prevent bee's infections. Therefore, current article compiled discusses and provides detailed protocol for various fungal diseases control methods to assist beekeepers and scientists entering this area of research. Two ascomycetes fungi of genera *Ascosphaera* and *Aspergillus* within Eurotiomycetes are known to cause chalkbrood (larvae turn grey or pale yellow, die and finally black) and stonebrood (larvae become black covered with powdery fungal spores and difficult to crush) diseases in honey bees. Beekeepers should remove infected larva from the colony and if this happens soon enough, beehives may survive. Selection of bees with pronounced hygienic behavior holds the most promise for control of these diseases. Appropriate management of colonies for diseases prevention must avoid accumulation of spores inside hive, periodically renew brood combs and eliminate combs severely contaminated with mummies. Best ways to prevent bee diseases is to apply Terramycin or Sulfazole and other available therapeutic medicines to bee's food during spring and fall seasons. These treatments can help to save life of huge amount of bees annually.

Keywords: Honey Bee, Fungus, Chalkbrood, Stonebrood, *Ascosphaera*, *Aspergillus*, Control Recommendations

1. Introduction

Honeybees are thriving for thousands of years and beekeepers have been reporting troubles with collapsing of colonies for more than many years. The story is almost and always the same in that a far greater losses results from reduction in honey and bees wax production, and insufficient bees for pollination. The population starts to dwindle and then there is a rapid disappearance of bees, sometimes within just some days. The growing consensus is that there are multiple causes working together to weaken the immune system of bees [1, 2]. Most fungi associated with colonies of the honeybees are not a problem for beekeepers. A large number of yeasts can usually be isolated from the intestines of healthy brood or adult bees and none of them seem to be pathogenic. Stored pollens sometimes goe moldy with the growth of *Bettsia alvei*, and a small number of bee larvae can be affected by several fungi of various genera producing a few diseases. Nevertheless, these diseases do not usually appear unless the colony is seriously weakened by other factors [3].

Insect pathogenic fungi can be found throughout the fungal kingdom all being capable of invading their hosts and overcoming their immune systems. It is apparent, therefore, that both beginning and advanced beekeepers should learn to recognize and control bee diseases [4]. Chalkbrood and stonebrood are two fungal diseases associated with honey bee brood. Chalkbrood, caused by *Ascosphaera apis*, is a common and widespread disease that can result in severe reduction of emerging worker bees and thus affects the overall colony productivity [5]. Stonebrood is caused by *Aspergillus* spp., which is rarely observed, so the impact on colony health is not very well understood. But, a major concern with the presence of *Aspergillus* in honey bees is the production of airborne conidia [6]. In the current article, it is described the common and widespread honey bee's fungal pathogens diseases that can result in severe reduction of emerging workers and thus overall colony productivity. In addition, it is provided research methodologies and protocols for isolating and culturing, *in vivo* and *in vitro* assays that are commonly used to study these host pathogen interactions. There are also give guidelines on the preferred methods used in pests control and the application of research techniques.

2. Fungal Diseases of the Honeybee

The only two fungal diseases causing serious problems in honeybee colonies are named chalkbrood or ascosphaeriosis, and stonebrood. Clinical symptoms of chalkbrood often appear for only a short time, typically under cold and damp weather conditions. Stonebrood caused by several fungi from the genus *Aspergillus* has been found worldwide and is a honey bee brood disease [7].

2.1 Chalkbrood of Honey Bee

Chalkbrood is a disease of bee's brood caused by a fungus, *Ascosphaera apis* (Massen ex Claussen) Olive and Spiltoir, a pathogen, which is the primary cause of sickness. The bee's larvae must ingest the spores of the fungus in order for the infection to occur. It only infects larvae that are three to four days old, which are most susceptible to the fungus, especially if these are chilled after ingesting spores of *A. apis*. Worker, drone and queen larvae all are susceptible to chalk brood disease. Chalkbrood disease in honeybees (*Apis mellifera* L.) is an invasive mycosis produced by the fungus *A. apis*, affecting stretched larvae. At first, dead larvae inside recently capped cells are covered by a fluffy white mold, and later on these dry and become black or white mummies, and at the peak of the disease, mummies are easily detected at the entrance to the hive as nurse bees remove them from their cell. Infected larvae become permeated with the mycelia of the fungus, thus

leading to their death. Eventually, the mycelia-filled larvae dry up to form the typical hard white mummies that are a characteristic of chalkbrood disease. Diseased larvae are pushed out in their cells and also can be mottled or completely gray or black. This color variation is due to the presence or absence of the black fruiting bodies that are formed on the outside of the larvae. The infected larvae are quickly covered by the white cotton-like mycelium of the fungus which eventually fills the entire cell. The white or grey mass soon hardens, forming a hard, shrunken mummy which is easily removed from the cell. The larva in the cell can resemble a chunk of chalk, hence, the name of the disease chalkbrood is originated [8, 9]. The fungus kills the larva after it is stretched out in the cell, turning it into a hard, white mummy. It may be covered with small black spots, which are the reproductive bodies of the fungus. Such mummies can be seen in the combs of infected colonies and on the landing boards of the hives where they are often dropped by house- cleaning bees. The disease may become severe in some colonies, but is not expected to be a serious problem for beekeepers. Chilled or starved brood may sometimes be confused with diseased brood. Such brood is usually found outside the cluster area of small colonies and lacks most of the specific symptoms of the diseases because all brood stages may be affected. When the weather warms or the colony receives a new supply of food, the bees can quickly clean out all of the dead brood [10].

2.1.1 Field Symptoms

Affected larvae die of chalkbrood after their cells have been capped and the fluffy white mold covering the stretched larvae can be seen inside the cells uncapped by the worker bees. After these dry, the mummies can retain their white color or if sporulation occurs, they appear black. They can remain inside the brood cells for some time before being removed by the bees. Nevertheless, when the infection is prevalent, some mummies may appear at the entrance and at the bottom of the hive, and when the brood combs are shaken the mummies rattle inside their cells. Clinical symptoms are the presence of mummies in these places and a progressive weakening of the colony. A mold *Bettsia alvei*, growing on stored pollen, can be mistaken for chalkbrood, but the substrate supporting the fungus is friable and does not have the appearance of a piece of chalk, as in the disease. The *Aspergillus* spp., which can infect brood (stonebrood), can be easily differentiated from chalkbrood wherein the latter has globose ascocysts. The disease can be detected by examining the combs, the entrance and bottom boards of the hives for the presence of the mummies. The mummies do not stick to the cells and are easily removed by nurse bees. If colonies have pollen traps, the mummies frequently are found in the traps and are a source of infection in trapped pollen, and the mummies have a faint yeast-like odor [11].

2.1.2 Samples for Examination

The presence of globose, dark ascocysts on the surface of mummies is a pathological symptom of the disease. A magnification of 30 X is sufficient to detect these reproductive forms. If only a small amount of the brood or a few bees are affected or if the symptoms are unusual, a definite diagnosis in the apiary is sometimes difficult. Examination by laboratory methods is then necessary because sometimes laboratory verifications of diagnoses made in the apiary also are desirable.

Diagnosis of disease in the laboratory is a service made available to beekeepers and apiary inspectors by the Department of Agriculture. A sample of brood comb for laboratory examination should be 4 or 5 inches square and may contain as much of the dead brood as possible. No honey should be present, and the comb should not be crushed. A sample of adult bees should consist of at least 200 sick or recently dead bees and the samples can be mailed in a wooden or strong cardboard box [12, 13].

2.1.2.1 Field Diagnosis

i. Chalkbrood disease affects bee larvae and is caused by the fungus *Ascosphaera apis.*
ii. Chalkbrood incidence increases in the fall and spring, and mummified larvae in front of the hive and on the bottom board are easily detected. Mummies on the bottom board may not necessarily indicate a serious problem, but confirm hygienic bee behavior.
iii. High incidence of chalkbrood mostly indicates poor hygienic behavior and stress due to weather, poor management or diseases.
iv. When there is a persistent halkbrood problem, replace the queen with one supplied by reputable bee breeder. In addition, remove heavily mummied comb and scrape bottom board in order to reduce principal source of infection.

2.1.2.2 Laboratory Diagnosis

i. Fungus *A. apis* infests the gut of the larva. The fungus will compete with the larva for food, ultimately causing it to starve. The fungus can then go on to consume the rest of the larva's body, causing it to appear white and chalky.
ii. Mummified larvae are generally white in color and the mycelium of the fungus infiltrates the larval tissue that eventually hardens. The white color is the result of asexual reproduction, while sexual reproduction can produce black or grey colored mummies.
iii. The entrance to this beehive is littered with chalkbrood mummies that have been expelled from the hive by hygienic worker bees.
iv. Chalkbrood is most commonly visible during wet springs and hives with chalkbrood can generally be recovered by increasing the ventilation through the hive.

2.1.3 Procedure for Quantifying Level of Colony Infection

Remove one brood comb from the hive containing 5th instar larvae that have not yet been sealed i.e., the larvae are close to being capped with wax. Mark on a plastic transparency the area with these unsealed larvae (this step is only important if the frame also contains capped brood). Return the brood comb to the colony and try to remove the brood comb from the hive a maximum of 20 hours later. Cut a piece of comb containing at least 100 recently capped cells (capped within the last 20 hours). These larvae can be identified using the plastic transparency and it is best to remove unsealed brood from the comb before placed in the incubator. Place one or more pieces of comb with capped cells in an incubator at 25 °C at approximately 65% humidity for 5 days. The chilling can ensure disease development and an open water bottle placed in the incubator might provide sufficient humidity. After 5 days, open all capped cells and record the results [14].

The fungus kills the larvae and the disease may become severe in some colonies, and is expected to be a serious problem for beekeepers. Most bee colonies suffer from chalkbrood at least once in their life. The spores of the fungus responsible for the disease might exist in the hive without causing too much damage if the bee family is a strong one. Weak colonies on the other hand may seriously affected by the chalkbrood fungus, which is quite powerful during the first spring days. Bee families that die should be closely examined and their honey house should be sealed so as to prevent honey robbing and spread of spores and other agents causing bee diseases. There are probably differences in resistance among strains of bees, and re-queening with a different strain may be of value in some cases. Bee stocks selected for hygienic behavior can be expected to minimize outbreaks of this disease. Hygienic queens are available from some nationally-advertised queen breeders. Hives should lean forward slightly so that rainwater runs out the entrance instead of accumulating inside; this precaution is not necessary if beekeepers use screen hive floors for Varroa mite control. If a hive interior is moist, then prop the lid to air out the interior. Some operations have recurring problems with the disease that are not easily traced to season or management practices. This suggests a genetic susceptibility in the bee stock. Because old combs harbor spores of the disease, brood combs should be replaced at least every five years to improve brood production [15].

2.1.4 Prevention and Treatment

Colonies that are severely infested with chalkbrood should be re-queened. Fumigation with acetic acid is considered to have a certain effect upon the chalkbrood causative agents. However, in certain countries, it is recommended to destroy comb that are severely infested. For the time being there is no medication that can be used in order to cure a colony from chalkbrood. Thus, prevention is advisable and the best solution to the problem. Hygiene is extremely important and therefore all hive should be in perfect condition and the beekeeping tool should be sterilized every once in a while. The brood should be inspected carefully especially in spring and autumn. The old combs in hive should be replaced on a regular basis and they have to be inspected before being moved from one hive to another. Proper ventilation and a sufficient number of bees should be assured to all hives within an apiary. It is also important to take measures against honey robbing and drifting since through these actions the bees spread various diseases including chalkbrood [16].

2.1.4.1 Treatments

A review of chemical treatments, which under laboratory (in culture), or field conditions (fed to colonies, sprayed over bees, etc.) have given some control of chalkbrood in *A. mellifera*. It has been pointed out that there is not easy to develop chemotherapy for the control of this disease whch is cheap, effective, non-toxic for bees and can be steadily released in the colony throughout he active season. However, an essential oil of Labiaceae (Savory: *Satureia montana)* has been used successfully in apiaries. This essential oil is mixed with sugar-candy (dilution 0.1%; dose 1 g per colony) and fed to the colony at the end of wintering [17].

It is very difficult to induce chalkbrood infection at a homogeneous level in a group of 10-15 hives or more, by experimental inoculation. This is one of the primary problems that has inhibited the investigation of treatments or prevention. As far as the authors are aware, there is not an effective and widely-used treatment for chalkbrood in *A. mellifera*. However, some preventative measures can help to control the disease. There is no recommended chemical control for chalkbrood ang often re-queening a colony can be effective treatment. Good hygienic behavior by a colony, that is quick removal of the mummies by workers, appears to aid in clearing up the symptoms. As chalkbrood is aggravated by chilling, practices that lead to loss of heat in infected hives, the removing of adult bees, or giving them extra brood to rear should be avoided [18].

2.1.4.2 Prevention

The use of clean combs annually is one of the most effective measures for preventing disease outbreaks. The reason for this is that the pathogen can remain infective as spores on the combs inside colonies for many years, and a single chalkbrood mummy can produce a huge amount of these spores. Selection of bees with pronounced hygienic behavior holds the most promise for the control of this disease. If exposure to the pathogen is the normal situation in hives, and outbreaks of the disease are dependent on many physiological and environmental factors, it seems very difficult for professional beekeepers to control the disease with periodical and systematic actions. Suitable management of the colonies for disease prevention must avoid the accumulation of spores inside the hive; periodically renew the brood combs and eliminate combs severely contaminated with mummies. When the brood nest temperature or the nutrition of larvae is inadequate, chalkbrood seems to appear. Worker bees clean out the infected brood and the hive may recover depending on factors such as the strength of the colony, the level of infection and hygienic habits of the strain of bees [19].

2.2 Stonebrood of Honey Bee

Stonebrood is another fungal disease that affects the honey bees. In spite of being a minor and less encountered disease, it should be not overlooked by beekeepers since it stresses and weakens the bee families making them prone to other more dangerous bee diseases. Stonebrood is a fungal disease caused by *Aspergillus fumigatus*, *Aspergillus flavus*, and *Aspergillus niger*. It is considered that the fungus named *Aspergillus flavus* is the main pathogen agent causing the stonebrood disease. It causes mummification of the brood of a honey bee colony. The spores of *A. flavus* might be present within a bee family without causing damage to it. The disease is spread outside the hive by drifting, robbing or swarming honey bees. Beekeepers also transmit the disease through their beekeeping tools or by moving combs that contain *A. flavus* in hives inhabited by healthy families. In contrast, the fungi causing stonebrood are facultative pathogens with a broad host range, they produce asexual conidia and their infection biology resemble many well-known insect pathogenic fungi, like *Beauveria* and *Metarhizium*; so several standard insect pathological methods can be directly transferred to this system. The fungi are common soil inhabitants and are also pathogenic to other insects, birds, and mammals. The spores of the different species have different colors and can also cause respiratory damage to humans and other animals. Stonebrood can be diagnosed by its gross symptoms, but positive identification requires its cultivation in the laboratory and subsequent

microscopic examination. Structures of the conidiophores (spore forming structures) are very important for identification of *Aspergillus* species. The conidiophores originate from a basal cell located on the supporting hyphae and terminate in a vesicle. The morphology, color and roughness of the conidiophores vary from species to species. Additionally, the position of the flask-shaped phialides (spore producing cells) on the vesicle is an important character [20, 21].

2.2.1 Morphological Description

Bee's larvae that died because of this disease are mummified like those that have died because of chalkbrood. However, the stonebrood disease makes the infected individuals green or yellow. The spores are more numerous in the vicinity of the head of the infested larvae. The fungus forms a sort of green ring near the larvae head. The mummies are solid, hard to crush and they do not have the sponge appearance typical for the chalkbrood disease. At the end, the pathogen fungus comes out from the integument of its host and creates a fake skin. The dead larvae are removed from the cell combs by the adult bees, so, it is not uncommon to find green mummified larvae on the hive floor or at the entrance of the hive. The disease is difficult to identify in the early stages of infection, and when a bee larva takes in spores, they may hatch in the gut, growing rapidly to form a collar-like ring near the head. After death, the larvae turn black and become difficult to crush, as the fungus erupts from the integument of the larva and forms a false skin, in this stage, the larvae are covered with powdery fungal spores. The typical symptoms observed in a colony affected by stonebrood are not very different from chalkbrood symptoms and includes irregular capping of the brood. Infected brood also called 'mummies', can be seen in the combs. Stonebrood mummies turn hard and they resemble small stones, not sponge-like as chalkbrood mummies. Stonebrood mummies are difficult to remove from the cells with forceps and removal by the worker bees is also difficult. Infected brood becomes covered with powdery yellow, brown, green or black fungal spores depending on the species. In some cases infected or deceased larvae looked dry, but they do not produce visible conidia within a 48 hrs after pathogen inoculation. Stonebrood can be diagnosed by its gross symptoms, but positive identification requires its cultivation in the laboratory and subsequent microscopic examination. Mostly, larvae infected with stonebrood show disease symptoms after the cell has been capped and they lie stretched out on the lower wall of the cell. Caps may be perforated or totally removed and infected larvae become gondola or banana shaped with the head raised towards the top of the cell opening [22, 23].

2.2.2 Isolation Techniques

Fungus *Aspergillus* grows readily on many different standard media, but Czapek-Dox medium, which contains sucrose as carbon source and nitrate as the nitrogen source should be very suitable. Czapek-Dox medium with addition of yeast extract (5.0 gm/ L) is recommended. Fungus *Aspergillus* spp., can be isolated from sporulating mummies as putting a sterile microbial loop in an area with many conidia, streak on agar plates, if the plates become contaminated with other microbes, repeat the procedure. Once a clean culture is established, proceed with single spore isolation as described below [24, 25]:-

i. Add 10 ml 0.05% Triton-X on the culture agar plate.
ii. Rub its surface gently with a sterile Drigalski spatula to loosen the conidia.
iii. Transfer the suspension conidia into a 15-50 ml sterile tube.
iv. Wash the suspension twice (to remove agar and hyphal fragments), centrifuge 3 min at 7000 g for 3 min, discharge the supernatant and add 10 ml 0.005% Triton-X.
v. Prepare a serial dilution (remember to whirl mix before pipetting).
vi. Count the spore concentration in a haemocytometer method.
vii. Transfer 100 µl of a spore solution at 5×10^2 spore per ml to a new plate.
viii. Incubate at 25 °C for two-four days.
ix. Transfer a single small colony to a new plate.

To harvest conidia for experimental purposes the above procedure can be used (minus step 6-8). It is important to use Triton-X or another detergent to avoid spore clumping [26].

2.2.3 Control and Treatment

This disease is not difficult to identify, diagnosis can be put by inspecting the frames where the brood lives and the debris find on the hive floor. If the honeybees are gently pushed aside and it is easy to spot the cell containing larva that died due to stonebrood infestation. The fungi that cause the disease might affect humans. For this reason it is advisable to destroy the heavily infected combs and honey that comes from infected hives should not be sold for human consumption. It is believed that the fungus causing stonebrood can trigger respiratory diseases in both humans and animals. There is no chemical treatment for stonebrood, thus, prevention is the only solution to have healthy, stonebrood-free colonies. The hives and all the beekeeping tools must be clean so as to prevent infestation. The dead larvae must be removed and the combs that are severely infested must be taken away. The hives have to be well ventilated and equipped with new frames that also have a new foundation. It is not advisable to let bees to pass the winter in an over-crowded hive. If bees do not produce enough honey they should be given food supplements. Hygiene is the key for having healthy bee families and the only available method to fight stonebrood and other bee diseases. Beekeepers should remove infected larvae from the colony, and if this happens soon enough, the beehives may survive. The best way to prevent bee diseases is to apply Terramycin or Sulfazole and other available therapeutic medicine to the bee's food during the spring and fall seasons. These treatments can help to save the life of huge amount of bees annually. Recently, a new medicine named Symbeeotic is invented after researching few years on bee diseases. Symbeeotic is an antibiotic that enables the bees to fight against diseases naturally. It is very simple and this medicine can make capable the bees to use its own and useful bacteria and strengthen its stomach to fight against diseases naturally [27, 28, 29].

2.3 Breeding for Resistance

Professional beekeepers are breeding honey bees for resistance to diseases and *Varroa destructor*. The most devastating fungal disease of honey bees is chalkbrood, which is a highly infectious sickness of brood (larvae). It has been demonstrated that honey bees bred for hygienic behavior, a genetic trait, also demonstrate good resistance to fungal disease chalkbrood. Bees bred for hygienic behavior are able to detect and physically remove disease-infected brood from the colony

before it becomes infectious. Hygienic bees are able to detect and remove diseased brood before the human eye can detect any sign of disease symptoms. When bees remove the disease in the non-infectious stage, it prevents the disease from spreading throughout the colony [30, 31, 32].

3. Conclusion

There are growing consensuses that the multiple causes work together to weaken the immune system of honey bees. Environmental factors may play an important role and some scientists point out to be bee's nutrition. Since honey bees are often forced to pollinate one specific crop, they may not get all the nutrients they need to be healthy. Others, like beekeepers point to be chemical exposures as many pesticides and fungicides are highly toxic to bees. But, technologists say it is likely that a combination of pathogens and environmental stressors work together to create a perfect storm for bees. There are no specific chemical treatments for these diseases; instead, it can be controlled by bees breeding and good management approaches. Another way to minimize the diseases is to maintain a warm, dry hive interior. Hives having drafty, damp, lying in low spots, or heavily overgrown with vegetation are susceptible to chalkbrood disease. Hygienic bees detect, uncap and remove a sealed 5th instar larva that is infected with stonebrood or chalkbrood disease. Hygienic bees are able to detect that the larva is diseased before it reaches the infectious stage; in this way, hygienic bees eliminate the pathogen and avoid further disease transmission through the colony. Research on the interaction with other pathogens and stressors, such as sublethal concentration of various chemicals, is also warranted.

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