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The pathological pebrine diseases of *Bombyx mori* caused by *Nosema bombycis* (m*Nb*) in South Sulawesi, Indonesia

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Abstract. *Nuraeni S, Soma AS, Sadapotto A, Mujetahid A, Baharuddin, Nur RJB, Huda N, Fattah A. 2024. The pathological pebrine diseases of* Bombyx mori *caused by* Nosema bombycis *(mNb) in South Sulawesi, Indonesia. Biodiversitas 25: 2990-2995.* South Sulawesi is the main producer of raw silk in Indonesia. However, over the past decade, raw silk production has plummeted due to an epizootic of pebrine disease. Pebrine disease is caused by the obligate parasite microsporidium *Nosema bombycis* Filatova, 1942 (m*Nb*) (Microsporidia: Nosematidae). This study aims to identify m*Nb* spore morphology and pathological characteristics, including disease symptoms, infectivity tests, and pebrine epizootic history. Spore morphology was observed using a light microscope. Infectivity test with a spore concentration of 1×10^8 mL^{-1} in 3rd, and 4th instars, inoculation using the smear method on mulberry leaves. Information on pebrine disease rates was obtained from documents of egg certification between 2002 and 2016. The research results show that the spores are oval in shape. The size of the spores can reach a length of 4.57±0.46 µm and a width of 2.24±0.493 µm. The main symptoms of this disease are the change in color of the cuticle to brownish with irregular black spots and non-uniform growth because many fail to molt, and the larval phase increases by 8-10 days. Inoculation in the 3rd instar can cause larval death starting on the second day after inoculation, with a cumulative mortality rate of 64%. Inoculation of the 4th instar, death begins on the eighth day, and a cumulative mortality rate of 52%. The pebrine epizootic in 2010 reached 72.42%, which reduced production by 57%. Understanding the pathological causes of pebrine disease and its history is useful to prevent rather than control the occurrence of the next epizootic.

Keywords: Infectivity, *Nosema bombycis* spores, prevalence pebrine, silkworm

INTRODUCTION

The most serious disease of mulberry silkworms (*Bombyx mori* Linnaeus 1758) that can thwart cocoon production is pebrine disease. Pebrine disease can reduce the weight, shell of the cocoon, and the silk fiber ratio (Suraporn and Terenius 2021; Rasool et al. 2022). Pebrine disease is caused by a group of microsporidia fungi, previously classified as protozoa (Bojko et al. 2022). An entomopathogen that mostly infects *B. mori* and other Lepidoptera (moths and butterflies) is from the genus of Nosema of the family of Nosematidae (Tokarev et al. 2020). One of the Nosema species, *Nosema bombycis* Filatova 1942, not only parasitizes silkworms but can also infect other Lepidoptera such as *Spodoptera litura* (Fabricius 1775) and *Helicoverpa armigera* (Hübner 1808) (Pei et al. 2021). The microsporidium *N. bombycis* (m*Nb*) is a significant pathogen for silkworms. The impact of m*Nb* infection on silkworms is substantial, as it can reduce cocoon production in each rearing cycle and even completely prevent production (Kampliw and Monthatong 2019; Jagadish et al. 2021).

Microsporidia have two life cycle phases, namely the intercellular phase and the extracellular phase (Zheng et al. 2020). The intracellular phase of microsporidia is in the form of spores and sporoplasm in the host body (He et al. 2020). The extracellular phase is in the form of spores with thick cell walls so that they can survive in an environment without a host (Huang et al. 2023). These two phases can be sources of the spread of the m*Nb* inoculum. The m*Nb* pathogen is transmitted through eggs from infected mothers, or transovarial transmission, and is a primary spread. Spread through contamination of mulberry leaves, tools, or rearing rooms, fecal and ingestion by larvae is a secondary spread (Bagheri et al. 2023). Infection via eggs or transovarial cells can reduce egg production and quality (Gupta et al. 2016).

The transovarial spread of m*Nb* is a major consideration, so it is recommended to examine silkworm mothers in the seed industry. Inspecting the silkworm mothers after laying eggs is a method to ensure healthy seed stock. Silkworm seeds that reach farmers in the form of certified eggs are declared free from pebrine disease. This inspection is very important for preventive control in an effort to save national sericulture (Rahul et al. 2021). This method is also

used to detect and track the source of infection (Prasobhkumar et al. 2021) and can predict the number of spores per sampling (Rahul et al. 2021).

The methods for detecting the presence of m*Nb* have been extensively developed, including serological (Li et al. 2015; Madhusudhan et al. 2016), enzyme-linked immunosorbent assay (ELISA) (Deepika et al. 2024), and more recently, molecular. In fact, molecular methods are currently more advanced and are used to complement or serve as a more accurate alternative (Rafeie et al. 2018). Due to various considerations, especially cost factors, the use of light microscopy is still applicable for the mother moth examination (Prasobhkumar et al. 2021). This obligate parasite can be directly observed under a light microscope starting at 10x magnification (Moharrami et al. 2022). Spores are clearly visible under a light microscope even without staining (Rahul et al. 2021).

The prevalence of invertebrate diseases refers to the percentage of diseases caused by pathogenic infections that impact the invertebrate population, including insects (Hajek and Shapiro-Ilan 2018). The occurrence of disease follows a cyclical pattern, starting with enzootic, pre-epizootic, epizootic, post-epizootic, and returning to enzootic (Shapiro-Ilan et al. 2012). Under normal conditions, pathogens never completely disappear from the environment, with disease intensity remaining at a low average level or in the enzootic category. However, extraordinary events can gradually or suddenly lead to an outbreak known as an epizootic. As reported in the Indonesian sericulture industry, an epizootic pebrine outbreak occurred in the 1970s. Previously, in 1972/1973, raw silk production in South Sulawesi reached a peak of 140 tons, then suddenly plummeted due to epizootic pebrine disease (Andadari et al. 2022) and in the last seven years, has only been able to produce 3 tons per year (International Sericultural Commission 2024).

There is still limited information on the characteristics of spores from different silkworm strains, pathological aspects, and fluctuations in the incidence of pebrine disease in Indonesia. Therefore, this study aims to examine the basic aspects of m*Nb* obtained from South Sulawesi Province, including morphological studies, pathological aspects, and trends in disease occurrence. This study is also expected to help predict future epizootic outbreaks of pebrine disease.

MATERIALS AND METHODS

Sampling of m*Nb* spores

The m*Nb* spore samples were obtained from four sources located in South Sulawesi Province, Indonesia: (i) larvae raised by farmers in Soppeng District, (ii) silkworms reared at the Laboratory of Forest Protection and Insects at Universitas Hasanuddin in Makassar (Makassar1), (iii) silkworms reared at the Environmental and Forestry Training Center (BPLHK) in Makassar (Makassar2), and (iv) parent stock reared at the Social Forestry and Environmental Partnership Center (BPSKL) in Gowa District. The silkworm larvae in Soppeng District use imported strains, while the Makassar and Gowa District samples use local strains.

Observation of m*Nb* spore

The thorax around the silk glands of the larvae was cut, then the hemolymph was placed on a glass object, then covered with a glass deck, and then examined under a light microscope with 400x magnification. Spore length and width were measured from each rearing location. Observations of spore size were carried out in three replicates of a larval cadaver from different sample sources, with the number of spores being n = 13. Spore size data were analyzed by one-way ANOVA, followed by Tukey's honestly significant difference post hoc test.

Isolation and purification of m*Nb* spores

Spores of m*Nb* from heavily infected larvae were crushed in distilled water, and large silkworm and mulberry debris were removed by filtration on two cheesecloth layers. The supernatant was discarded and centrifuged at 3000 rpm for 15 min, and the spore pellet was re-suspended in 10 mL of sterile distilled water, and then the suspension was re-centrifuged. This procedure was repeated three times, and the spores were washed in distilled water until the pellet was white. Finally, 10 mL of spore solution was pipetted and poured into a hemocytometer for spore counting. The spores were counted under a light microscope at magnifications of 400× for the detection of microsporidia pathogens (Yaman et al. 2014). Spore suspensions of 1×10^8 mL^{-1} were prepared by diluting with distilled water and then stored at 4°C until further use.

Infectivity test of m*Nb*

The infectivity test was carried out on the 3rd and 4th instar larvae of the silkworm strain BS09. This infectivity test was carried out in a rearing room with conditions of an average temperature of 25.9°C, a humidity of 86.5%, and 12 hours of lighting in the light and 12 hours in the dark. The larvae were fed with fresh mulberry leaves before and as a control treatment three times a day. The treatment involved infecting the larvae with a spore concentration of m*Nb* isolate at 1×10^8 mL^{-1}. Inoculation was performed by applying the isolate on the mulberry leaves, which were then air-dried before being given to the larvae. Inoculation was administered to larvae that had just completed their second molt (early 3rd instar) and third molt (early 4th instar). Each treatment unit consisted of 25 larvae, and each treatment was repeated three times. Data collected from this phase of the study included disease symptom descriptions, cumulative mortality percentage, and m*Nb* infectivity using the equation below:

$$I = a/b \times 100\%$$

Where:

I: m*Nb* Infectivity (%);

a: the number of larvae that died showing pebrine symptoms;

b: the total number of test larvae.

Pebrine disease prevalence

The data on pebrine disease prevalence were obtained from records of pebrine disease incidents, including the eggs' certification notes for each production period. The m*Nb* spore observation method involved examining the hemolymph of female moths after they had laid their eggs. The certification records were sourced from the Association of Natural Silk Entrepreneurs (KPSA) of Perum Perhutani, Donri-Donri Sub-district, Soppeng District, South Sulawesi. These certification data were collected from 2002 to 2016. The Natural Silk Center (BPA) verified the data on pebrine disease inspections, which later became the Social Forestry and Environmental Partnership Center (BPSKL), the official institution issuing distribution permits and silkworm egg certificates. Pebrine disease incident data after 2016 has not been well-recorded since BPA transitioned to BPSKL. Similarly, raw silk production data were only available from BPA until 2016, after which raw silk production data were sourced from the International Sericultural Commission (2024).

RESULTS AND DISCUSSION

Morphology of m*Nb*

Samples of larvae that die with symptoms of pebrine disease have their entire body fluid filled with mature spores. Without extraction treatment, spore masses can be observed filling the hemolymph, body tissues, and even the larvae's feces. In Figure 1, mature m*Nb* spores are shown under a light microscope at 400x magnification, resembling rice grains with an oval to elongated shape, and light-reflective properties. Table 1 shows that the sample from Soppeng District has a large size, with an m*Nb* length of 4.57±0.46 µm and a width of 2.24±0.493 µm, resulting in a length-to-width ratio of 2.24:1. On average, the m*Nb* spores from the Soppeng District sample are oval-shaped.

Symptoms of pebrine disease

Healthy larvae have normal growth and a stable life cycle as presented in Figure 2.A and marked with the letter N. Meanwhile, larvae infected with m*Nb* show symptoms of pebrine disease with smaller body size in the 5th instar and an inhibited life cycle which is marked with the letter P in Figure 2.A. Infected larvae show stunted larval growth, appearing more stunted than normal because they cannot molt. Very susceptible larvae will die on the edge of a shelf or under a pile of food scraps (Figure 2.B). Larvae that survive until the end of the 5th instar show slow movement and reduced appetite. The cuticle changes color to become opaque, and there are black spots. Another symptom is that the larvae are trying to release their exuviae, but the larvae are already weak, their movements are very slow, so the exuviae are still attached.

The m*Nb* infectivity

The pathological response of silkworms to m*Nb* infection differs based on inoculation at the onset of feeding after molting in the two tested instars. Figure 3 shows that m*Nb* inoculation in the third instar results in mortality starting on the second Day After Inoculation (DAI), whereas inoculation in the fourth instar leads to larval mortality on the eighth DAI. Larval mortality occurred almost daily, especially after entering the fifth instar until spinning in the third instar inoculation, resulting in an accumulated larval mortality rate of 64%. In the fourth instar inoculation, the incubation period was slower, and mortality began to occur just before molting. Daily larval deaths peaked after molting into the fifth instar, leading to an accumulated larval mortality rate of more than 52%.

The m*Nb* infection affects the developmental duration of silkworm larvae. Table 2 shows that the developmental duration of larvae from infection to cocoon formation (spinning) is longer for both the 3rd and 4th instar. The duration of the larval phase infected with m*Nb* is longer compared to the control, increasing by 1.5 days at the 3rd instar, 3 days at the 4th instar, 2.5 days at the 5th instar, and 2 days during the cocoon formation phase (spinning), resulting in a total increase of 10 days.

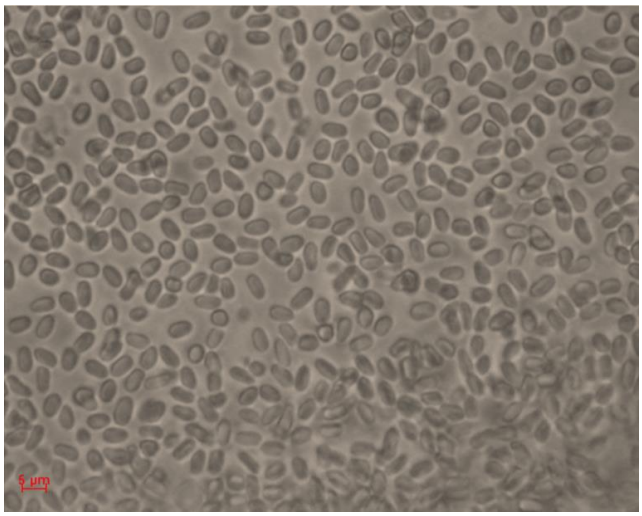

Figure 1. The shape of m*Nb* spore magnified at 400 times using a light microscope

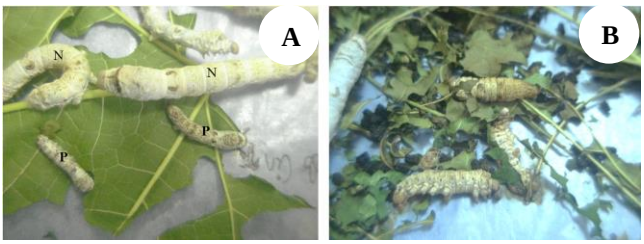

Figure 2. Symptoms of pebrine disease in the larval stage. A. Larvae show non-uniform growth at the same stage; sick larvae (marked with P) are smaller than the healthy ones (marked with N); B. Dead larvae are often found on the edge of the rearing rack

Table 1. The spore size and shape of m*Nb* obtained from South Sulawesi, Indonesia

Sample origin	Spore size (µm)		Length to width ratio	Dominant form
	Length	Width		
Soppeng	4.57±0.46 a	2.24±0.43 c	2.24 : 1	Oval
Gowa	3.68±0.41 b	1.82±0.30 a	2.02 : 1	Oval
Makassar1	3.92±0.48 b	1.91±0.36 ab	2.05 : 1	Oval
Makassar2	3.91±0.41 b	2.12±0.19 bc	1.84 : 1	Oval

Note: Different letters in the same column indicate significant changes, P<0.05

The occurrence of epizootic diseases and raw silk production

The occurrence of the pebrine disease and raw silk production in South Sulawesi can be seen in Figure 4. The percentage of pebrine disease occurrence before the year 2010 was very low, averaging below 3%, and suddenly surged to over 70% in 2010 (Figure 4.A). Raw silk production showed an inverse relationship with the incidence of epizootic pebrine disease; before 2009, production remained above 35 tons. However, starting in 2009, disease occurrences led to a decline in production, which continued to fluctuate but never exceeded 20 tons per year (Figure 4.B).

Table 2. The larval stage and spinning phase of silkworms after m*Nb* inoculation at the 3rd instar

Instars	m*Nb* infection			No. infection		
	Feeding stages (days)	Moulting (days)	Larval stages (days)	Feeding stages (days)	Moulting (days)	Larval stages (days)
3	5.0	1.5	6.5	4.0	1.0	5.0
4	7.0	2.5	9.5	4.5	2.0	6.5
5	10.5	-	10.5	7.0	-	7.0
Spinning	5.0	-	5.0	3.0	-	3.0
	Total		31.5	Total		21.5

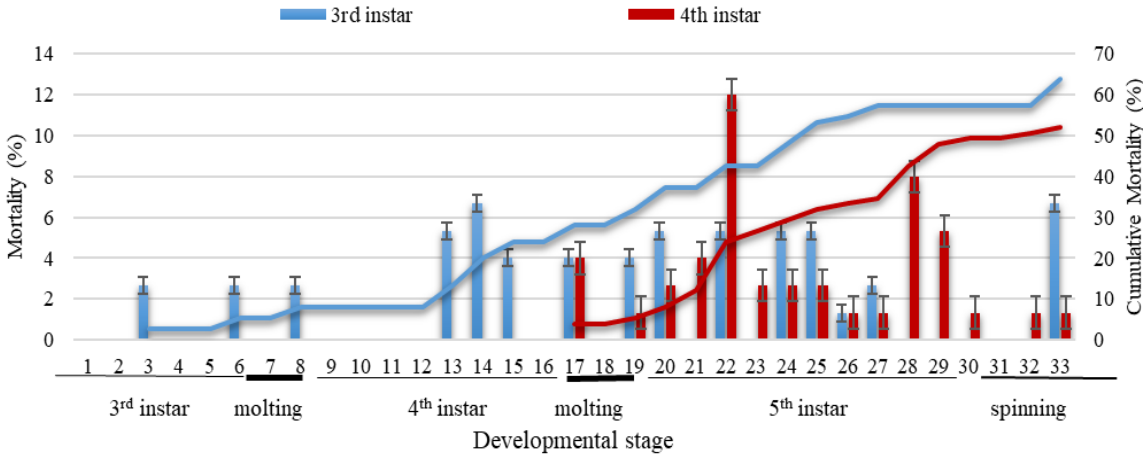

Figure 3. The rate and accumulation of larval mortality in silkworms following inoculation at the onset of the 3rd and 4th instars

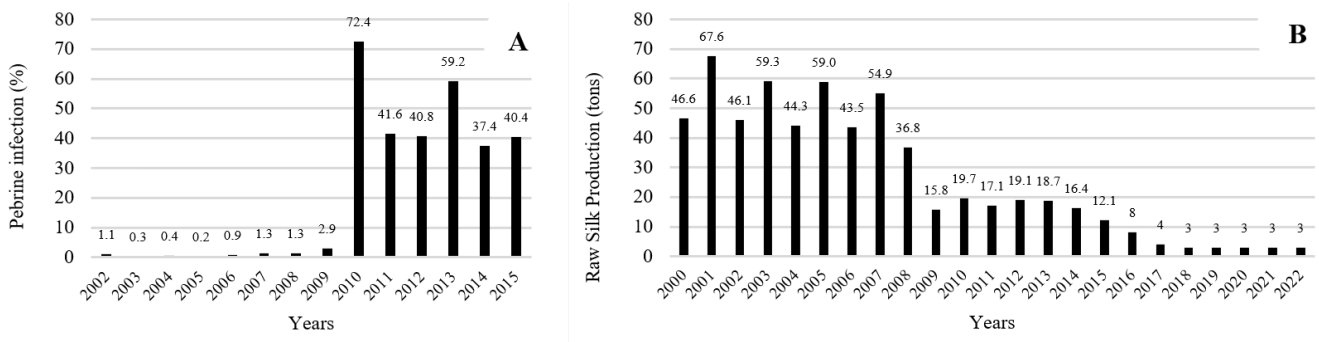

Figure 4. History of pebrine disease and raw silk production in South Sulawesi, A. Epizootic of pebrine disease occurred in 2010 (%) (BPA 2015 and BPSKL 2016); B. Production of raw silk (tones) (BPSKL 2016 and The International Sericultural Commission 2024)

Discussion

The presence of pebrine disease in silkworms can be readily identified using a light microscope. Pebrine examination is a quarantine requirement through examination of the mother silkworm using a light microscope (Chandrakanth et al. 2021). A positive pebrine diagnosis is stated when m*Nb* spores are found under a microscope from hemolymph samples of larvae or mother silkworms (McCook et al. 2021). Recognition of the shape and size of spores is important in identifying each genus and species of microsporidia (Dziuba et al. 2024). In general, m*Nb* spores are oval in shape, as found in all maintenance locations (Table 1). Meanwhile, the size of the spores from the Soppeng-reared sample is significantly larger than those from other locations. This difference is thought to be due to differences in the strains of silkworms being reared. The strains reared at the Soppeng location were imported seeds from China. According to Bojko et al. (2022), the size of microsporidia spores depends on the type of host. The spore size of the imported strain was similar to that in Chongqing, China, ranging from 3.8 µm to 4 µm in length and ±2.3 µm in width (He et al. 2020).

External symptoms of sick larvae (as marked with letter P in Figure 2.A) are typical of the cuticle color becoming dull brownish, and there are irregular dark spots. While healthy larvae grow normally (marked with letter N), the cuticle color is pure white. The brownish spot color change is also called the 'pepper' spot which is the beginning of the name of the pebrine disease (Singh et al. 2021). During observations in this study, sick larvae were seen to have lost their appetite, weak, and could not molt. According to Huang et al. (2024), silkworms with pebrine disease will experience delayed molting and stunted growth. Susceptible larvae die faster after being infected with m*Nb*. Dead larvae and their cadavers are not as soft as larvae that die from viral and bacterial diseases or as hard as when they die from fungi (Figure 2.B). Dead larvae shrink in size and solidify and odorless. The larvae die, the cadaver remains rubbery for a longer time and then turns black (Chopade et al. 2021).

The duration of larval reared is longer in larvae infected with m*Nb*. Table 2 shows that the duration of the larval phase and cocoon spinning increases by 10 days from normal. The duration of each instar increases due to late molting to enter the next instar. According to Rasool et al. (2022), pebrine disease can significantly increase the duration of the larval phase in all silkworm strains. A typical symptom in silkworm larvae infected with m*Nb* is a longer larval development period due to the delayed molting process (Solter et al. 2012). m*Nb* infection in the larval phase induces the metabolism of the juvenile hormone synthesis pathway so that the growth of the larvae is inhibited (Tang et al. 2020).

Infectivity is a major factor in assessing entomopathogenic epizootics (Solter 2014). Host mortality is one measure of pathogen infectivity (Thomas and Elkinton 2004; Shapiro-Ilan et al. 2005). In this study, larval mortality began to occur on day 3 after inoculation at the beginning of instar 3rd (Figure 3). Cumulative larval mortality in this infection was still below 10%. Larval mortality increased after entering instar 5th, with cumulative mortality reaching 64%. Compared to the study of Sharma et al. (2014), inoculation at the beginning of instar 3rd with a spore concentration of 1×10^{6} spores mL^{-1} showed cumulative larval and pupal mortality of 50.7%. While inoculation at instar 4th, mortality began to occur at the end of instar 4th, and death continued almost every day after entering instar 5th, so cumulative mortality was 52%. According to Rasool et al. (2022), larval mortality can reach 46.34%, especially after entering instar 5th, which occurs in all silkworm races.

One of the most feared scourges of sericulture worldwide is the pebrine epizootic (Hukuhara 2017). The pebrine epizootic occurred in South Sulawesi in the 1970s and had an impact on the decline in national raw silk production (Razak 2017). Figure 4.A shows that in 2002-2009 the prevalence of pebrine disease was below 3%. In 2010, the prevalence of pebrine disease suddenly increased to 72.4%, which was called the pebrine epizootic. Many silkworms raised by farmers died on the rearing rack, larvae failed to produce cocoons, if there were any that cocooned, the silk fibers were very few, thin and the pupae died before becoming moths.

Midway through 2009, raw silk output fell from 36.8 tons to 15.8 tons, or a 57% decrease in production (Figure 4.B). In the following years, production continued to decline and the last six years have stagnated at 3 tons (International Sericultural Commission 2022). This epizootic event is still difficult to control. Control efforts with preventive measures continue to be carried out. The egg group is immediately destroyed by examining the parent and strict selection if one infected mother is found. As with sericulture in Europe which was once successful, the epizootic of pebrine disease is the main cause of the difficulty in recovering sericulture (Habeanu et al. 2023).

The morphology of m*Nb* spores from all silkworm strains is the same, namely oval in shape, but what differentiates them is their size. Typical symptoms of the disease from infection are late larvae molting or failure at all so that the duration of the larvae is longer, slow movement, decreased appetite, and changes in the color of the cuticle to brownish with black spots spreading. m*Nb* infection in the earlier instar 3rd causes a higher mortality rate, reaching 64% compared to infection in instar 4th, which is 52%. The epizootic began in mid-2009 in rearing farmers, then in 2010 it occurred in egg producers, resulting in a 57% decline in raw silk production. As a preventive measure, handling pebrine disease needs to be more serious and stricter in silkworm seed producers. Improvement of sanitation and feed quality is required to break the horizontal transmission of m*Nb* spores in farmer-reared.

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