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Nosema hylobii n. sp. (Nosematidae, Microsporida), a new microsporidian parasite of *Hylobius abietis* L. (Curculionidae, Coleoptera)

By K. PURRINI

Abstract

The morphology of a microsporidian parasite *Nosema hylobii* n. sp. is described by light and electron microscopy. The parasite invades the gut epithelial cells of the adult *Hylobius abietis*. Some data on the prevalence of infection by the microsporidian *N. hylobii* in relation to other parasites found in *H. abietis* are also presented.

1 Introduction

The beetle *Hylobius abietis* L., with its high biological potential (living period is 2–3 and more years) is, besides *H. piceus* De G. and *H. pinastri* Gyll, the most dangerous pest of coniferous trees in the world. Data on the damage caused by this pest have been reported by several authors. This large beetle (6–14 mm long), destroys the bark of living young trees in plantations. Flights and attacks begin in the period between March and June. Egg-laying season begins in May, lasting to August, sometimes September. The beetles lay approximately 80–120 eggs in one generation. Larvae, pupae and adults overwinter.

Our knowledge of some predators as a population limiting factor is based on textbooks (SCHWENKE 1974). Several species of parasitoid wasps of the hymenopteran families Ichneumonidae and Braconidae, some dipteran and coleopteran predators, and various species of birds attacking *H. abietis* have also been reported, but their role in limiting the populations of the pest is unimportant.

At two localities, Eversen and Ehra Lessien near Lüneburg (Lower Saxony, Federal Republic of Germany) in the outbreak zone of the pest, we found two populations of adult *H. abietis* heavily infected by three different Sporozoan parasites: *Gregarina hylobi* (FUCHS 1922) GEUS 1969, and two new species, one neogregarine *Ophryocystis hylobii* n. sp. and one microsporidian *Nosema hylobii* n. sp.

As no protozoan infections have yet been known to cause mortality of *H. abietis*, this study forms a part of a project to evaluate microbial agents for the control of this important pest. Only the microsporidian parasite is described in this paper. The results of infections by *G. hylobi* and *O. hylobii* (PURRINI and

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ORMIERES 1981) will be the subject of our next paper. Some data on the pathogenicity and prevalence of infection by microsporidians in relation to the other parasites (*O. hylobii* and *G. hylobi*) found in *H. abietis* are also presented.

2 Material and methods

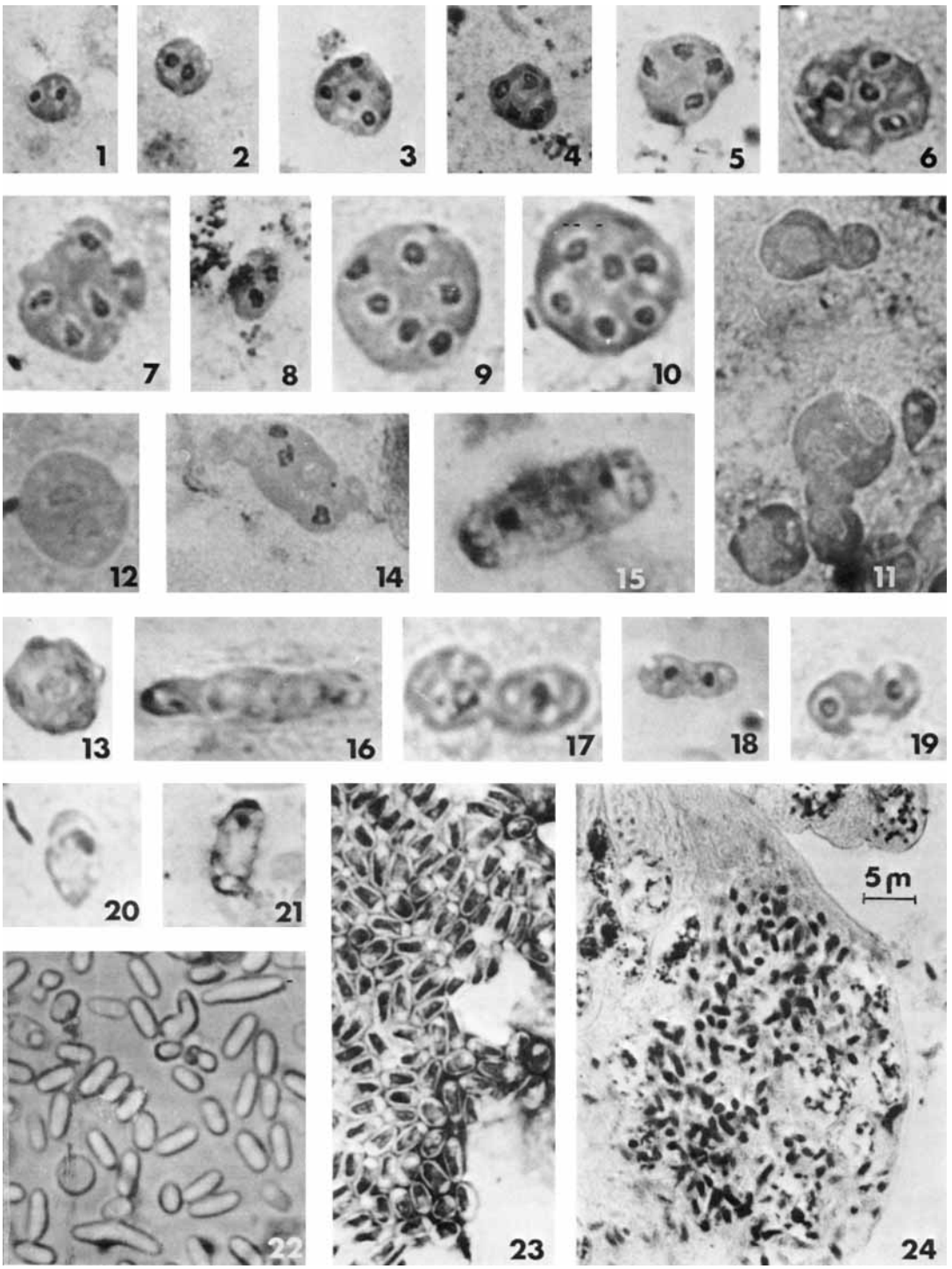
In the years 1978–1980, several samples of *H. abietis* infesting pine trees were collected and brought to the laboratories of the Exp. Stat. of Forestry in Goettingen (F. R. of Germany). They were used for experiments with insecticides, during which research workers at the Station observed approx. 20–30 % mortality of control insects (not treated with insecticides). In August 1980, Ms. Renate Hofmann and Mr. George Vacek of the Station kindly supplied me with 806 specimens of *H. abietis* collected at two localities in Lower Saxony (F. R. of Germany), in Eversen (29. 5. 1980) and Ehra Lessien (11. 6. 1980). The samples were brought to our laboratories and reared for two months (table) in special boxes at 20 °C; they were fed on young pine-trees. The food was replenished every two weeks. The beetles survived for 6 weeks under these conditions without significant mortality. Later the mortality rapidly increased (table). Living and dead beetles were examined for pathogens at six different times during the rearing (table). The gut, Malpighian tubules, gonads and parts of the fat body were dissected from the insects and observed with a light microscope. Distribution of pathogens in the organs was recorded. Dry smears fixed with methanol and stained with Giemsa were prepared for light microscopy. The intestine and Malpighian tubules, the principal sites of infection by the parasites found in *H. abietis*, were fixed in Bouin for histological sections 4 mm thick und stained with Heidenhain's iron haematoxyline. For electron microscopy, the gut was dissected and fixed in phosphate buffered glutaraldehyde (2 % glutaraldehyde in 0.1 M phosphate buffer, pH 7.4). The gut (cut into small pieces) was transferred in distilled water (10 min), following two washes in 0.1 M cacodylate and 5 % sucrose, postfixed in 2 % osmium tetroxyde in veronal acetate buffer, dehydrated several times in acetone series of 30 % to 90 %; three times in 100 % acetone and washed with propylene oxyde for 3 min. After dehydrations with the acetone series and washing with propylene oxyde the objects were embedded in SPURR's medium. Sections were stained with uranyl acetate and lead citrate. The sections were examined with EM Philips 301.

3 Results

3.1 Prevalence of infections

In the course of two months of our diagnostic studies (table) on the parasites of *H. abietis*, altogether 806 adults were individually diagnosed by light microscope either in fresh or Giemsa stained smears or in Heidenhain's iron haematoxyline serial sections. Three sporozoan parasites, nematodes and some entomogenous fungi, such as *Beauveria*, sp. *Mucor* sp. a. o. were found to parasitize the pest. They were recovered from living and dead adult beetles

Fig. 1–24. Light microscopy of *Nosema hylobii*. Stains: figs. 1–21 and 23 Giemsa stain; 22. fresh; 24. Heidenhains iron haematoxylin; magnifications: figs. 1–4, 16 and 18–21 appr. 3000 x, figs. 15 and 17 appr. 3800 x, figs. 22 and 23 appr. 1900 x, fig. 24 appr. 1500 x. – 1, 2: binucleate schizonts; 3–7: tetranucleate schizonts; 8: schizonts with 3 (unpaired) nuclei; 9 and 10: schizonts with 6 nuclei; 11 and 13: cells considered to be the stages of second schizogony, some filamentous bodies „chromosomes-like" are visible in nuclei; 12: „diplokaryon-like" stage; 14–16: stages belonging to the sporogony with ring nuclei; 17–19: dividing sporonts; 20 and 21: sporoblasts; 22 and 23: free mature spores; 24: masses of mature spores in the gut epithelium. ———— = 6 μ (fig. 1–21), ———— = 5 μ (fig. 22–24)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Occurrence of different parasites found by dissection of living and dead adults of *Hylobius abietis* in Lower Saxony (Federal Republic of Germany), 1980

Examination dates	Total number of live and dead animals examined n	infected %	Parasites G	O+G	O+G +Nos	G+ Nos	O+ Nos	G+ Nem	G+Nos +Nem	Nos	Nem	Mortality n
11.–14. 8.	97	70	15	20	13	5	1	2	4	8	2	7
16.–18. 8.	105	64	20	25	5	2	0	1	2	9	1	16
20.–22. 8.	95	63	19	21	14	2	3	0	0	21	0	23
19.–22. 9.	52	65	12	14	6	4	0	0	0	29	0	18
5.– 7. 10.	227	60	6	17	4	0	0	0	0	31	2	62
12.–15. 10	240	70	10	13	7	3	3	0	0	32	2	93

G = *Gregarina hylobi*, O = *Ophryocystis hylobii* n. sp., Nos = *Nosema hylobii* n. sp., Nem = Nematodes, n = number

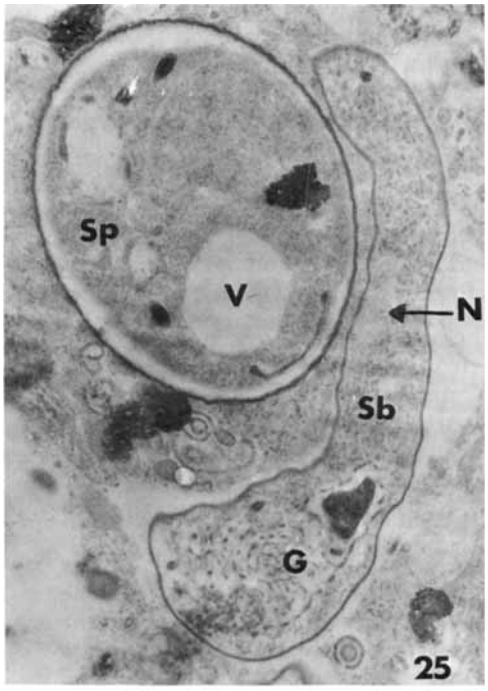
occurring as single or mixed infections. Pathogens in the larvae of the pest have not yet been examined. The microsporidian was identified with 22 % prevalence in single infections, while in mixed infections with *Ophryocystis* and *G. hylobi* the prevalence of infection was 6.5 %; 2.6 % with *G. hylobi*; 1.2 % with *Ophryocystis* and 1 % with *Ophryocystis* and nematodes (table). The microsporidian with a total prevalence of 33 % in single and mixed infections was the most important natural control factor responsible for the high mortality of the pest populations.

3.2 Pathogenicity

The disease caused by microsporidian parasite *(N. hylobii)* did not show any apparent external symptoms; the adults appeared normal. However, it could be noted that the diseased adults were not as active as healthy ones, and they stopped feeding.

White dots, masses of microsporidian spores, were observed in the midgut of dissected adults of *H. abietis*. They were composed of pseudocysts situated in the cells of epithelial layers of the gut without any spread of the infection to other parts of the insect body (fig. 24.) In young infections, vegetative stages of the microsporidia were distributed throughout the cells, which later divided and pseudocysts with several hundreds of spores (fig. 24) were formed. Various stages of the life cycle of microsporidia (schizonts, sporonts, sporoblasts, and spores) were found in epithelial cells of the midgut, but never in migrating oenocytes and lymphocytes near the gut. We also never found any

Fig. 25–29. Electron microscopy of *Nosema hylobii*. 25: longitudinal section of sporoblast (Sb) showing some vesicles of the Golgi apparatus (G) and nucleus (N), and transversal section of young spore (Sp) showing one vacuole (V) and formation of the filament (a larger dark body inside of the spore is staining artifact) (17000 x); 26: transversal and longitudinal sections of two empty spores; in a longitudinal section only the transversal plane cutting coils of the filament are clearly visible (17000 x); 27: longitudinal section of young spore; exospore (Ex), endospore (En), nuclei (N_1N_2), polaroplast (Pl), filament in longitudinal and transversal planes cutting (ff_1), posterior vacuole (Pv), (14000 x); 28: one part of the spore showing nucleus (N) and endoplasmatic reticulum (ER) (37000 x); 29: one part of the spore showing lamellar polaroplast (Pl) and manubroid part of the filament (F), (69000 x). ———— = 0,5 μ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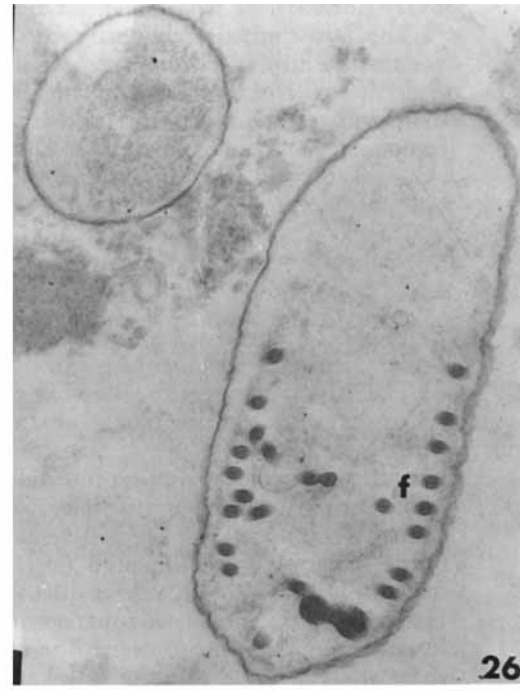
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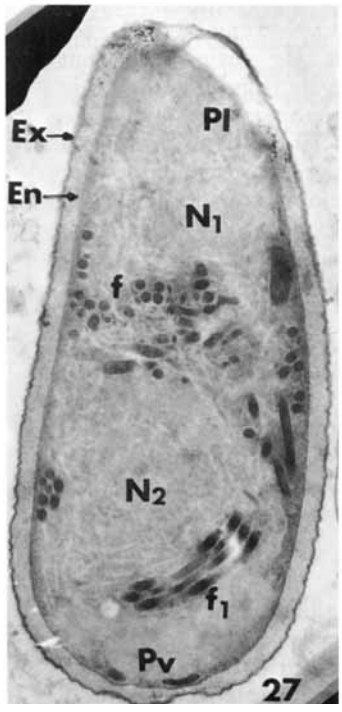
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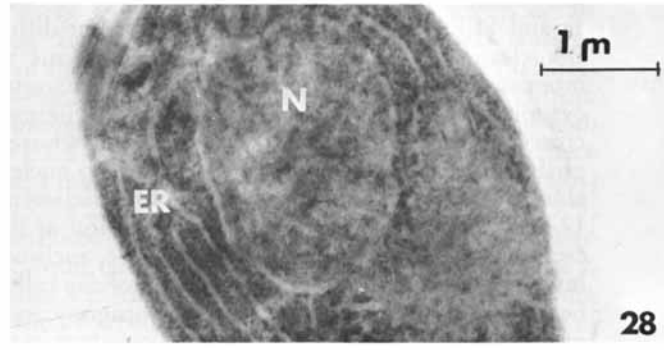
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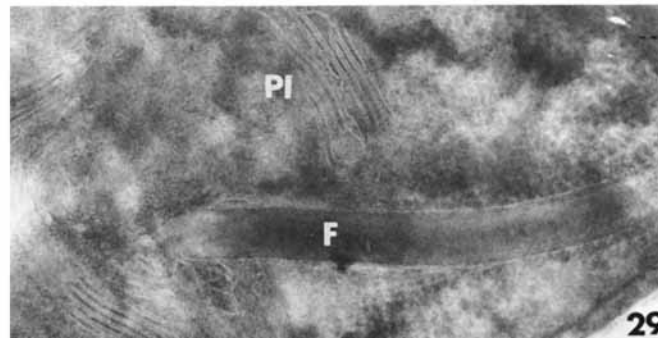
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infection by microsporidia in the fat body and other organs, either in the ovary, muscles, or Malpighian tubules.

The gut epithelial cells as principal site of infection of microsporidian eventually displayed intensive reduction of cytoplasmic substance, which degraded with the increase of infection. Finally the cells membrane became disrupted releasing cellular contents together with the spores into the gut lumen.

3.3 Description of *Nosema hylobii* n. sp.

3.3.1 Light microscopy

Figures 1–24 represent various stages in the life cycle of *N. hylobii* observed in fresh, Giemsa and Heidenhain's iron haematoxyline stained preparations.

The earliest stages of the life cycle recognized were round or oval binucleate schizonts with compact nuclei that stained deeply with Giemsa (figs. 1,2). They measured 2.5–4 µm in diameter. Their multiplication occurs by binary fission; the nuclei divide first into tetranucleate forms (figs. 3–7), which were found round or oval, measuring up to 7 µm in diameter. Their cytoplasm stained somewhat less and light areas were seen around the nuclei (figs. 6,7). Some schizonts with unpaired nuclei (fig. 8) producing schizonts with six nuclei, measuring 8–12, µm in diameter (figs. 9,10) were also observed. The nuclei in multinucleate schizonts are arranged in pairs scattered throughout the irregularly stained protoplasm. The ultimate stage of the schizogony appears to be a large, round cell, the diplokaryon (fig. 12) measuring approximately 7 µm in diameter. The stages of the second schizogony are presented in figures 11 and 13 in which an apparent chromosome differentiation can be observed. Binucleate and quadrinucleate elongated forms with ring nuclei have been interpreted as stages transitional to the sporogonic cycle (figs. 14–16). Their cytoplasm generally stained very lightly. The early sporont is a binucleate crescent-shaped body, about 6–7 µm long, whose nuclei divide to produce a piriform tetranucleate sporont (fig. 16). Two nuclei migrate to each end of the elongated sporont (figs. 15, 16) and a constriction develops at the midline (figs. 17–19). Two sporoblasts result from division of the sporont. The sporoblasts are about 5–6 µm long and 2–3 µm wide, each containing two nuclei which invariably lie parallel to the short axis of the cells (figs. 20, 21). The vacuole bearing sporoblast elongates during sporogony and produces a binucleate long oval spore. The spores were not quite constant in shape and size; for the most part they are round, or long oval in fresh preparations, or were slightly flattened posteriorly in Giemsa stained ones (figs. 22, 23). Measurements of several spores in fresh and stained preparations gave mean values of 4.5 – 6.0 µm in length and 1.5 – 3 µm in width. Some macrospores measuring 7.0 – 8.0 x 2.0 – 3.5 µm were also observed.

3.3.2 Electron microscopy

The ultrastructure of some developmental stages in the life cycle of *N. hylobii* is represented in figures 26–29.

The early sporoblast (Sb) and young spore (Sp) were the youngest stages observed with the electron microscope (fig. 25). They were covered with a

layer of unit type membrane. Accumulation of some dark vesicles belonging to the Golgi apparatus (G) was evident in the cytoplasm of the sporoblast. The surface coat of young spore was more uniform and thicker than that of the sporoblast, showing cytoplasm from which the organelles typical of the spore were absent. Only two large vacuoles (V) and cytoplasm containing granular electron dense material were distinguishable features. Two longitudinal plane cuttings of maturating spores are presented in figures 26 and 27. The spore wall consisting of a thin exospore (Ex) and a thick endospore of 120 nm (En) has a surface where the exospore is finely rugose (fig. 27). In longitudinal plane cutting throughout all the coils of a filament one section shows 9 coils on one side and 13 on the other (fig. 26). In fig. 27 are presented some longitudinal (f1) and transversal (f) plane cuttings of the filament during its formation. Other ultrastructural features of the spore were sporoplasm, two nuclei typical of microsporidian species of the genus *Nosema*, polaroplast, manubroid part of the filament, and endoplasmatic reticulum. The presence of the posterior vacuole (Pv) could only be supposed (fig. 27). The nuclei (figs. 27,28 N) lie in intrasporal sporoplasm containing electron dense material. The double-layered membrane covering the nuclei is not clearly visible. Ultrastructure of the extrusion apparatus could be distinguished as the lamellar polaroplast (Pl) and manubroid part of the filament (F) (fig. 29). The endoplasmatic reticulum could also be seen in one plane cutting of the spore (fig. 28).

4 Discussion

Very few microsporidian parasites have been described from insects of the family Curculionidae. Only five have been reported, as follows: *Nosema longifilium* Hesse 1905 from the fat body of *Otiorrhynchus fuscipes* (locality: France) with oval spores 4–5 x 3 μm; *Nosema otiorrhynchi* Weiser 1951 from Malpighian tubules of *Otiorrhynchus ligustici* (locality: Czechoslovakia) with cylindrical spores 3.8 – 4.0 x 1.8 – 2.0 μm (common spores) and macrospores 6 x 4 μm; *Nosema* sp. Issi and Lipa (1968) from *Pissodes piceae* (locality: USSR) with elongate spores 3.6 – 5.0 x 2.0 – 2.9 μm; *Nosema* sp. Street at al. (1975) from different tissues of the *Pissodes strobi* (locality: USA) with spores of two ranges: 3.5 – 4.2 x 1.8 – 2.2 μm, and 5.0 – 6.0 x 1.8 – 2.5 μm; and, finally, *Microsporidium* sp. Bell and McGovern (1975) from *Anthonomus grandis*, without data either on the life cycle, size und shape of spores, or on the site of infection. The microsporidians mentioned above without informations on vegetative and sporulation stages can not be adequately compared with the parasite found in *Hylobius abietis*. It is apparent that the microsporidian from *H. abietis* is a new species and the name *Nosema hylobii* n. sp. is herewith proposed.

5 Conclusion

Because our knowledge of the diseases of weevils (Curculionidae) is limited, the infections described here preclude accurate evaluation of their efficacy in the control of these serious pests of agriculture and forestry. Their value may be estimated after further studies on the distribution of the parasites in a larger

region, and after discovering the effects of diseases on other coleopteran hosts of the family Curculionidae. In the populations sampled at two localities in Lower Saxony, the diseases caused by *N. hylobii* and a neogregarine, *Ophrocystis hylobii* n. sp. appeared to be a significant population limiting factor.

Zusammenfassung

Nosema hylobii n. sp. (Nosematidae, Microsporida), ein neues Sporozoon von Hylobius abietis L. (Coleoptera)

Die Morphologie der Mikrosporidie, *Nosema hylobii* n. sp. werden auf Grund licht- und elektronenmikroskopischer Untersuchung beschrieben. Der Parasit befällt das Darmepithel der Käfer von *Hylobius abietis*. Über die Infektionsrate der Mikrosporidie von *N. hylobii* in Zusammenhang zu dem Befall durch andere *Hylobius*-Parasiten wird berichtet.

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