Invertebrate Zoology, 2024, 21(4): 478–486

Regular occurrence of atypically small spores in *Apis mellifera carnica* (Hymenoptera: Apidae), naturally infected with *Nosema* spp. (Microsporidia)

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ABSTRACT: Reliable and accessible methods for differentiating the *Nosema* spp. microsporidia spores are still missing. However, proper identification and measuring the respective spore loads might be crucial for disease characterization, as well as for understanding the causes of emergence of natural honeybee microspopridioses and severity of their symptoms. The ultrastructure and size range of *Nosema* spp. spores and relationships between the spore size and spore load have been assessed in naturally infected *Apis mellifera carnica*. The average size of *Nosema* spp. spores was 5.7±2.1 × 3.05±0.75 μm (n=335). We discovered that in addition to normally sized spores (3.6–7.8 × 2.3–3.8 μm (n=335), atypical spores of smaller size (3.25±0.25 × 2.1±0.1 μm (n=81)), were occasionally observed. Atypical spores were separated by filtration though a 3 μm membrane filter. PCR analysis revealed that the majority of atypical spores belonged to *N. apis*, although *N. ceranae* spores were also present. With an increase in spore load, more atypical spores were observed. Ultrastructural analysis of atypical spores confirmed the PCR diagnosis and revealed the presence of a polar filament and other elements of extrusion apparatus, indicating their maturity and functionality.
How to cite this article: Shamaev N.D., Salnikov V.V., Yuzmanova L.A., Shuralev E.A., Mukminov M.N. 2024. Regular occurrence of atypically small spores in *Apis mellifera carnica* (Hymenoptera: Apidae), naturally infected with *Nosema* spp. (Microsporidia) // Invert. Zool. Vol.21. No.4. P.478–486. doi: 10.15298/invertzool.21.4.03

KEY WORDS: *Nosema apis*, *Nosema ceranae*, microsporidia, morphometric analysis, atypical spore size, spore load, *Apis mellifera carnica*.

Регулярное появление атипично мелких спор у *Apis mellifera carnica* (Hymenoptera: Apidae), естественно зараженных *Nosema* spp. (Microsporidia)

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РЕЗЮМЕ: Надежные и доступные методы дифференциации спор микроспоридий *Nosema* spp. в настоящее время отсутствуют. В то же время, правильная идентификация и измерение соответствующих споровых нагрузок имеют решающее значение для характеристики заболевания, а также для понимания причин возникновения естественных микроспоридиозов медоносных пчел и тяжести симптомов. В этой статье были изучены ультратонкое строение и диапазон размеров спор микроспоридий пчел видов *Nosema*, а также оценена взаимосвязь со споровой нагрузкой у естественно инфицированных *Apis mellifera carnica*. Средний размер спор *Nosema* spp. составил 5,7±2,1 × 3,05±0,75 мкм (n=335). Помимо спор нормального размера (3,6–7,8 × 2,3–3,8 мкм (n=335) изредка наблюдались атипичные споры меньшего размера (3,25±0,25 × 2,1±0,1 мкм (n=81)). Использование 3 мкм мембранных фильтров позволило отделить атипичные споры от остальных. ПЦР-диагностика показала, что большинство атипиных спор принадлежат *N. apis*, хотя споры *N. ceranae* также присутствовали в образцах. С увеличением споровой нагрузки у медоносных пчел наблюдалось больше нетипичных спор. Ультраструктурный анализ атипичных спор подтвердил их принадлежность к *Nosema apis* и выявил наличие сформированного аппарата экструзии, что говорит о зрелости спор и их потенциальной инфекционности.
Как цитировать эту статью: Shamaev N.D., Salnikov V.V., Yuzmanova L.A., Shuralev E.A., Mukminov M.N. 2024. Regular occurrence of atypically small spores in *Apis mellifera carnica* (Hymenoptera: Apidae), naturally infected with *Nosema* spp. (Microsporidia) // Invert. Zool. Vol.21. No.4. P.478–486. doi: 10.15298/invertzool.21.4.03

КЛЮЧЕВЫЕ СЛОВА: *Nosema apis*, *Nosema ceranae*, микроспоридии, морфометрический анализ, споры нетипичных размеров, споровая нагрузка, *Apis mellifera carnica*.

Introduction

Microsporidia, unicellular parasitic eukaryotes related to Fungi, are classified based on their ultrastructural features, including the number of coils of their polar tube, the size, shape, morphology of their spores, the characteristics of their developmental life cycle, and host-parasite relationship. *Nosema* species (*N. ceranae* and *N. apis*) are reported to infect honeybees *A. mellifera* worldwide (Fries, 2014). Microsporidiosis causes significant losses in honeybee populations and decrease in colony numbers (Fries, 2014), however relative impact of *N. ceranae* vs. *N. apis* remains unclear, and reliable and accessible methods for differentiating the spores of these two microsporidia are missing. At the same time, proper identification of the species and measuring the respective spore loads might be crucial for disease characterization, as well as for understanding the causes of emergence of natural honeybee microspopridioses and severity of their symptoms.

N. ceranae and *N. apis* spore sizes may vary during spore maturation (Huang, Solter, 2013). Standard morphometric analysis is based on estimating the average spore size of mature spores, which can be differentiated from immature spores by size (Kudo, 1920; Grobov *et al*., 1987; Fries *et al*., 1996). The spore size is an important characteristic, and accurate morphometric analysis is helpful in distinguishing *Nosema* spp. spores from spores of fungi and other pathogens (Ilyasov *et al*., 2014). It is important to examine fresh spores, because different methods of fixation may alter the spore size (Spelling, Young, 1983). The goal of this paper was to identify the species, examine the ultrastructure, and estimate the size range of *Nosema* spp. spores infecting honeybee populations in Tatarstan (Russian Federation). Additionally, the study aimed to assess the relationships between the spore size and spore loads in crude extracts of naturally infected bees *Apis mellifera carnica*.

Material and methods

SAMPLE COLLECTION, FILTRATION AND PREPARATION FOR DNA EXTRACTION AND MICROSCOPY. In the first step, to enhance the interpretation of microsporidian morphometric analysis, we investigated the spore load and size of spores in the crude extracts from honeybees. A total of 11 honey bee samples from *A. mellifera* were collected from a single hive at the local apiary in the Laishevsky district, Republic of Tatarstan, Russian Federation, in April 2024. Dead worker honeybees were randomly selected at the entrance of the hive or on frames away from the brood nest. The honeybees were placed in an empty Falcon tube and stored at room temperature prior to analysis. Each individual honeybee was washed in sterile water, a small intestine was extracted and then ground in 1 mL of freshly added sterile water following the method described previously (Fries *et al*., 2013). A homogenate was then filtered through an 8 μm membrane filter (MF-Millipore, Merck, Germany). A droplet of filtered homogenate was added to the Neubauer improved bright-line hemocytometer (Paul Marienfeld GmbH & Co. KG, Germany), and the total spore count per 5 large squares (80 small squares) was evaluated. The count was then multiplied by 50,000 (Cantwell, 1970). Spore load counts and evaluation of spore size were conducted using a compound microscope (Biomed-3, Russian Federation) equipped with phase contrast optics and a digital camera ScopeTek DCM310 (Hangzhou Scopetek Opto-Electric Co., Ltd, China). Spore loads and spore sizes were estimated at 400X and 1000 X, respectively. Spore images were captured with Scope photo software (ver. 3.1.312). Spore size ranges were estimated using ImageJ (Schneider *et al*., 2012). Prior to DNA extraction, each individual honeybee was washed in 70% ethanol and sterile water.

TRANSMISSION ELECTRON MICROSCOPY (TEM). In the second step, we examined the ultrastructure of atypical spores in the crude extracts from honeybees. Filtered spores were centrifuged at 7200 g at RT for 5 min, and the supernatant was removed. For the analysis of the spore ultrastructure, samples were fixed following the standard procedure. The samples were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 3 h, rinsed, and then postfixed for 2 h in 1% (w/v) OsO_4. After dehydration in an ethanol series, acetone, and propylene oxide, the samples were embedded in Epon (Ted Pella Inc.). Serial ultrathin sections were obtained using an ultramicrotome (LKB AB, Sweden) and stained with saturated aqueous uranyl acetate, followed by Reynolds' lead citrate. Observations were made with a transmission electron microscope Hitachi 7800 (Hitachi, Japan).

DNA EXTRACTION, PCR DETECTION AND HONEYBEE SUBSPECIES IDENTIFICATION. In the third step, we identified the species of the microsporidium and the host by polymerase chain reaction (PCR) with specific primers. Total DNA isolation from the honeybee homogenate was evaluated using the commercial EZNA Tissue DNA kit (OMEGA, USA) following the manufacturer's instructions. The purified DNA was stored at −20°C until the PCR assay. Duplex PCR was performed with reported primer

sequences to amplify the 321 and 218 bp fragments corresponding to the small subunit ribosomal region (SSUrRNA) of *N. apis* and *N. ceranae*, respectively (Ostroverkhova, 2021). The conventional PCR was performed to amplify (internal transcribed spacer 1 (*ITS1)* ribosomal gene of fungi species (Horisawa *et al*., 2009). PCR-RFLP procedure was performed to amplify cytochome oxidase subunit 1 (*COX1*) gene region of honeybee subspecies (*A. m. carnica*, *A. m. carpatica*, *A. m. mellifera* Haplotype 1, and Haplotype 2) (Syromyatnikov *et al*., 2018). The list of primer sequences used in this study is shown in the Table 1. Duplex and conventional PCRs were performed in a reaction volume of 170 μL containing 5000 U/ml of Taq DNA Polymerase (NPO SibEnzyme, Novosibirsk, Russia), 27.5 μL of 10X SE Buffer (600 mM Tris-HCl, 15 mM MgCl2, 250 mM KCl, 100 mM 2-mercapto-ethanol, 1% Triton X-100; pH 8.5), 10 mM dNTP mix, 5 μL of template DNA and 25 μM of primers. The reaction mixture was treated at 94 ºC for 3 min, followed by 35 cycles of 94 ºC for 30 s, 57 ºC for 40 sec and 72 ºC for 2 min, followed by treatment at 72 ºC for 4 min. The PCR-RFLP reaction mixture was treated at 94ºC for 3 min, followed by 35 cycles of 94 ºC for 30 sec, 51 ºC for 30 sec and 72 ºC for 1 min, followed by treatment at 72 ºC for 10 min, the amplicon was purified and treated with the HspAI restriction enzyme. PCR runs were performed using a Bio-Rad T100 thermocycler (Bio-Rad Laboratories Ltd., Canada). The PCR amplicons were stained dyed with bromophenol blue, and electrophoresis was conducted on a 1.5% agarose gel containing ethidium bromide, followed by visualized under UV light.

STATISTICAL ANALYSIS. Data analysis and visualization were conducted using R Statistical Software (version 4.3.0) and Microsoft Excel. A generalized Kruskal-Wallis test was conducted to compare K samples (groups) and obtain the means and standard deviations of total spores obtained, categorized as either average or below-average sizes, among honey bee samples. All length and width values have been ranked. The total number of ranks was equal to the number of observations in the pooled sample. The sums of the ranks assigned to each group were calculated. Then, the Kruskal-Wallis test statistic was calculated and the mean values were compared as described previously (Zar, 2010).

A logistic regression (LR) analysis was used to find the relationships between two data factors "spore size" and "spore load", and to predict the value of one of those factors based on the other. Spore sizes with an average size were counted as 0, and those with an

Table 1. List of primers.
Таблица 1. Список праймеров.

Primer name	Primer sequence (5'-3')
N. apis forward	GGGGGCATGTCTTTGACGTACTATGTA
N. apis reverse	GGGGGGCGTTTAAAATGTGAAACAACTATG
N. ceranae forward	CGGCGACGATGTGATATGAAAATATTAA
N. ceranae reverse	CCCGGTCATTCTCAAACAAAAAACCG
Fungi ITS1 forward	TCCGTAGGTGAACCTGCGG
Fungi ITS1 reverse	TCCTCCGCTTATTGATATGC
A. m. carnica *COX1* forward	ATTTCATCAATTATAGGATCATTAAATTTAC
A. m. carnica *COX1* reverse	CAGCTAATACAGGTAATGA
A. m. carpatica *COX1* forward	AGATATTGGGATCTTGTA
A. m. carpatica *COX1* reverse	CTAGTAACAATTGTATTATAAATTTGATCAGCG
A. m. mellifera H1 *COX1* forward	GGATGAACAGTATATCCACC
A. m. mellifera H1 *COX1* reverse	GTAACTATTAAGTTTAATGATCCTATAATAGC
A. m. mellifera H2 *COX1* forward	CTTTAATACTAGGATCACCTGATATAGCGAT
A. m. mellifera H2 *COX1* reverse	CTGATAATGGTGGATATA

atypical size as 1. The spore load per honeybee was a numerical variable. R packages list and details regarding LR model calculation were described previously (Shuralev *et al*., 2018).

The Shapiro-Wilk test was used to estimate the difference between the data sample and the normal distribution (Shapiro, Wilk, 1965). A Kolmogorov-Smirnov test was performed on spore area values and then for each atypical spore with different spore load to determine the cut-off value, because it was impossible to obtain either typical or atypical values or high and low spore loads values, respectively. A p-value less than 0.05 was considered as positive. Test procedure and result interpretation were described previously (Shamaev *et al*., 2020).

A multinomial logistic regression (MLR) analysis was performed, and a model was developed to assess the relationship between the atypical spore size and the identified genus-species of spores under high spore load conditions. R packages list and details regarding dataset preparation, MLR model selection, and fitness testing were described previously (Shamaev *et al*., 2021).

Results and Discussion

SPORE SIZES AND LOADS. Microsporidian spores were detected under phase-contrast light microscopy in all 11 samples. The Shapiro-Wilk test on spore area distribution showed a significant departure from normality ($W = 0.025$, $p < 0.001$). Based on the distribution of the samples (Fig. 1B), we assumed that the spore area value at the peak "8" (cut-off threshold) represented spores smaller than average (atypical), and considered the outliers ($p < 0.05$) with significantly higher spore area values to be spores with average size. The average *Nosema* spp. spore size was estimated by previous authors. It has been demonstrated previously that *N. apis* spores are oval, measuring 4.5–7.5 μm in length × 2.5–3.5 μm in width, whereas *N. ceranae* spores are straight, oval, and slightly curved, with a size range of: 3.6–5.5 μm in length and 2.3–3.0 μm in width (Kudo, 1920; Grobov *et al*., 1987; Fries *et al*., 1996; Sulborska *et al*., 2019). In accordance with published data, our studies demonstrated that most spores had an average size ranging from 3.6–7.8 μm in length and 2.3–3.8 μm in width. No connection between the spore size and spore load in the raw data was observed (Fig. 1B). In addition to conventionally sized spores, we observed atypical spores in significantly lower quantities, as determined using the Kruskal–Wallis test (significant at level alpha=0.05) (Fig. 1A, C). The load of these "atypical" spores varied from 5×10^4 to 1.496×10^6. The size of atypical spores ranged from 3.25±0.25 μm in length and 2.1±0.1 μm in width. Percentage of atypical spores increased with the spore load: 33.33% with a spore load of 1.5×10^5, 15.38% with a spore load of 3.25×10^5, 28.57% with a spore load of 3.5×10^5, 20% with a spore load of 5×10^5, 63.63% with a spore load of 5.5×10^5, 62.5% with a spore load of 1.2×10^6, and 68.18% with a spore load of 2.2×10^6.

The LR model was developed to predict the variation in spore size based on the spore load. The total number of atypical spores increased with the growth of the spore load, indicated by the LR model (with acceptable discrimination under AUC score <0.7) (Fig. 1D).

SEPARATION OF ATYPICAL SPORES BY MEMBRANE FILTERS. Homogenized honeybee samples with different spore loads were filtered through membrane filters with pore sizes 1.2, 3, 5 and 8 μm to separate atypical spores measuring 3.0–3.5 μm in length and 2.0–2.2 μm in width from larger spores ranging from 3.6–7.8 μm in length and 2.3–3.8 μm in width. Each homogenate of honeybees with a specific spore load was divided into three portions filtered independently in three repetitions with each of the four filters. No *Nosema* spp.-like spores were found when filtered through 1.2 μm membrane (Fig. 1E). The majority of spores, filtered through 3 μm membrane, were atypical and exhibited a reduced numbers in the sample after filtration (Fig. 1E, yellow bars). Spores filtered through a 5 and 8 μm membrane did not differ significantly between groups and contained both atypical and average-sized *Nosema* spp.-like spores (Fig. 1E, green and blue bars). An average number of atypical spores (3.25±0.25 × 2.1±0.1 μm) for each spore load after filtration through 3 μm membrane filter was as follows: $5 \times 10^4 – 1 \times 10^5$ atypical spores in a spore load ranging between $5 \times 10^4 – 1.5 \times 10^5$, $5 \times 10^4 – 3 \times 10^5$ atypical spores in a spore load ranging between $3 \times 10^5 – 5.5 \times 10^5$, $1.5 \times 10^5 – 4.5 \times 10^5$ atypical spores in a spore load ranging between $8 \times 10^5 – 1.2 \times 10^6$, and $3 \times 10^5 – 6 \times 10^5$ atypical spores with a spore load of 2.2×10^6. Thus, after filtration through the 3 μm filter, the number of atypical spores also increased with the spore load.

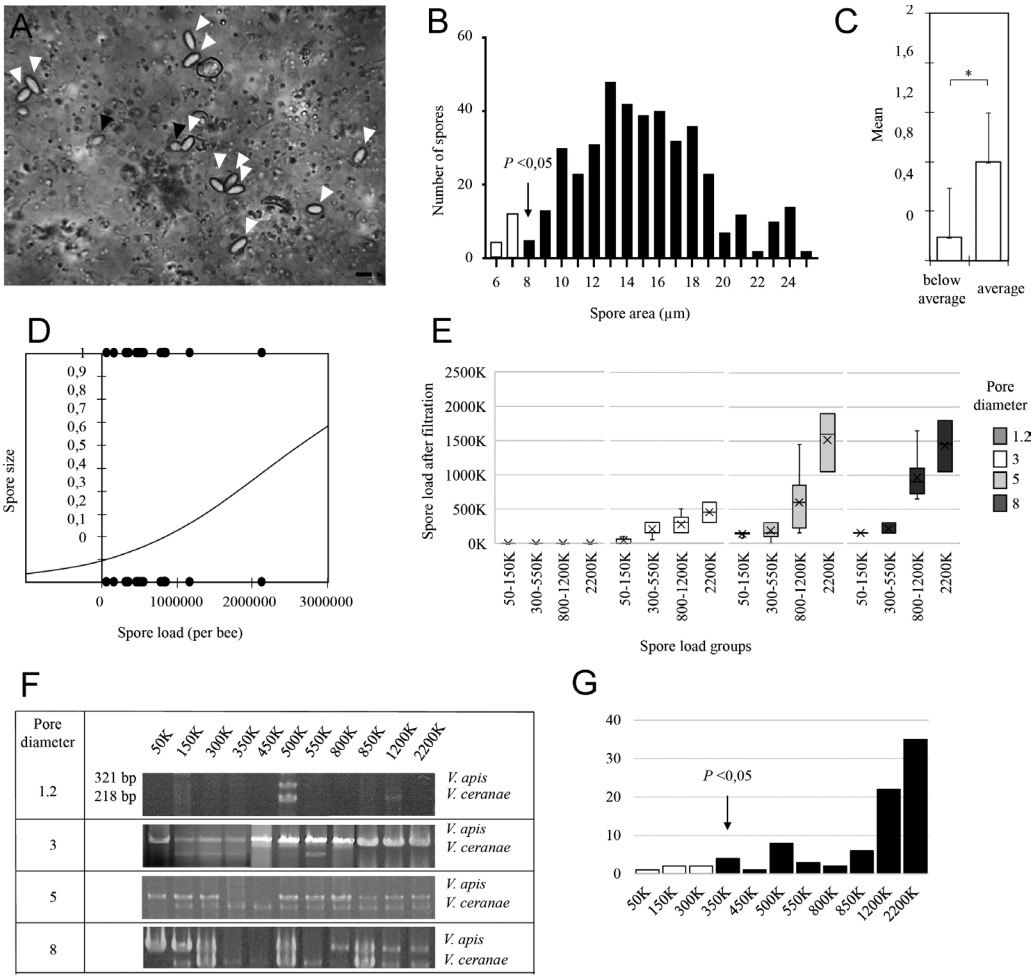

Fig. 1. Normally and atypically sized spores of *Nosema s*pp. A — white arrowheads show normally sized spores. Black arrowheads show atypically sized spores. Scale bar: 5 μm. B — estimation of borderline between average-sized spores and smaller spores (atypical) using Kolmogorov-Smirnov test. A cut-off is shown at peak "8" (significant at p <0.05). C — Kruskal-Wallis test for comparing K samples means and standard deviations of spore size among *A. mellifera* samples. * — significant at level alpha=0.05. D — LR model of spore size by spore load per bee. E — comparison between spore load counts without filtration and membrane filtered spore load (with different pore diameter). F — membrane filtered spore genus-species definition. G — estimation of borderline between low and high spore loads using Kolmogorov-Smirnov test. A cut-off is shown at 350K (significant at p <0.05).

Рис. 1. Споры *Nosema* spp. типичных и нетипичных (мелких) размеров. A — белые наконечники стрелок указывают на типичные споры. Черные наконечники стрелок указывают на мелкие споры нетипичного размера. Масштаб: 5 μm. B — оценка границы между спорами среднего размера и спорами размерами меньше среднего (атипичными) с использованием критерия Колмогорова-Смирнова. Отсечение показано на пике «8» (достоверно при p <0,05). C — тест Крускала-Уоллиса для сравнения средних значений K образцов и стандартных отклонений размера спор среди образцов *A. mellifera*. * — достоверно на уровне альфа=0,05. D — LR-модель размера спор в зависимости от нагрузки спор на пчелу. E — сравнение количества спор без фильтрации и количества спор, подвергнутых мембранной фильтрации (с различным диаметром пор). F — определение рода и вида спор, подвергнутых мембранной фильтрации. G — оценка границы между низкой и высокой споровой нагрузкой среди образцов с использованием критерия Колмогорова-Смирнова. Отсечение показано при 350K (достоверно при p <0,05).

Table 2. MLR analysis output regarding the association between the *Nosema* spp. PCR-positivity and atypical spore load.
Таблица 2. Результаты анализа MLR относительно связи между *Nosema* spp. положительностью в ПЦР и атипичной споровой нагрузкой.

Variable	*p*-value
Spore size (atypical) × *Cladosporium* spp.	0.22
Spore size (atypical) × *N. ceranae*	0.717
Spore size (atypical) × *N. apis*	0.005

ATYPICAL SPORE GENUS-SPECIES AND *APIS MELLIFERA* SUBSPECIES IDENTIFICATION. To determine if the isolated spores belong to *N. apis and N. ceranae* or to a non-microsporidian organism, we utilized PCR diagnostics. As a result, single and mixed infections with *N. apis* and *N. ceranae* were detected (Fig. 1F, pore diameter 8 μm, amplicons of 321 and 218 bp, respectively). Interestingly, after filtration through 8 μm filter, in addition to amplicons corresponding to *Nosema* spp., there were also amplicons corresponding to *Cladosporium* spp. (Fig. 1F, amplicons of 500 and 169 bp). There were almost no amplifications of *Nosema* spp. targets in the sample after filtration though 1.2 μm filter (Fig. 1F). The presence of fungal spores can be attributed to feeding behavior of honeybees, which may feed on plants infected with fungi or forage on fruiting bodies, as recently reported for *Apis cerana* (Takahashi *et al*., 2019). In contrast to 1.2 μm fractions, the 3 μm fractions showed a solid *N. apis* band, which was visually much stronger than the *N. ceranae* band, suggesting that atypical spores that passed through the 3 μm filter predominantly belonged to *N. apis* (Fig. 1F). Five and 8 μm fractions showed no differences in amplification between *N. apis* and *N. ceranae* targets (Fig. 1F), which was predictable since all spore sizes passed through.

Estimation of difference between the presence of atypical spores among samples with low and high spore loads showed a cutoff at 3.5×10^5 ($p < 0.05$) (Fig. 1G). When creating a MLR model, a spore load (intercept) below the cutoff was counted as 0, and the values above the cutoff was counted as 1. The MLR model included 3 variables: 1) atypical spore size and *Cladosporium* spp. PCR positive; 2) atypical spore size and *N. apis* PCR positive; 3) atypical spore size and *N. ceranae* PCR positive. MLR analysis showed that atypical spores belonging to *N. apis* PCR-positive honeybee samples were significantly associated with a high spore load ($p = 0.005$), but not *N. ceranae* ($p = 0.22$) and *Cladosporium* spp. ($p = 0.717$) (Table 2).

ATYPICAL SPORE ULTRASTRUCTURE ANALYSIS. Spores that passed through a 3 μm membrane filter were examined by TEM (n=9/42). Spores isolated from honeybees with high (2.2×10^6) and low spore loads (5×10^4) were oval to roundish, which is characteristic of *N. apis* (Kudo, 1920) (Fig. 2A, *N. apis*) in contrast to *N. ceranae* spores exhibiting an elliptical shape and pointed ends (Fries *et al*., 1996) (Fig. 2B, *N. ceranae*). Atypical spores similar to *N. apis* and *N. ceranae* demonstrated standard features of mature spores: spore envelopes with exo- and endospore layers, polar filament coils, nucleus, anchoring disk and polaroplast which may suggest maturity and potential functionality of atypical spores.

General considerations and Conclusions

Spores of atypical sizes were reported for several *Nosema* species. Production of atypical (smaller than average) spores were observed in *Apis florea* after experimental infection with *N. ceranae* (Suwannapong *et al*., 2010), in *Antheraea pernyi* infected with *Nosema pernyi* (Wang *et al*., 2015), *Pieris rapae* infected with *N. mesnili*, and in *Pseudoaletia correcta* infected with *Nosema* sp. in (Lom, Weiser, 1972).

In this paper we demonstrated for the first time the presence of atypical *N. apis* spores produced in heavily infected honeybees. Despite their minute size, the ultrastructure of atypical spores remained the one of regularly sized spores. The observed negative effect of high infection loads on the spore size can be explained by the depletion of resources needed for spore production. In addition to discovering atypically small *N. apis*

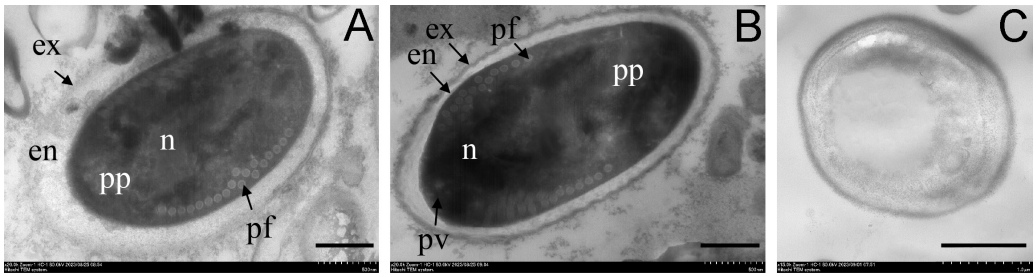

Fig. 2. Ultrastructural analysis of the fraction of *Nosema* spp.-like atypical spores. A — longitudinal section of *N. apis*-like spores from the fraction of atypical spores. B — longitudinal section of *N. ceranae*-like spores from the fraction of atypical spores. C — spore after extrusion of the sporoplasm.
Abbreviations: en — endospore; ex — exospore; n — nuclei; pf — polar filament; pp — polaroplast. Scale bars: A, B — 500 nm; C — 1 μm.

Рис. 2. Ультраструктурный анализ фракции *Nosema* spp.-подобных атипичных спор. А — продольный срез *N. apis*-подобных спор фракции атипичных спор. Б — продольный срез *N. ceranae*-подобных спор фракции атипичных спор. С — спора после экструзии спороплазмы.
Обозначения: en — эндоспора; ex —экзоспора; n — ядро; pf — полярная нить; pp — поляропласт. Масштаб: A, B — 500 nm; C — 1 μm.

spores, we outline the methodology for isolating spores from crude extracts using membrane filters of different pore sizes. We also introduce a novel method of morphometric analysis, which is unique in microsporidia research. New experiments are needed to assess the comparative viability of smaller spores. Further studies will demonstrate how widespread the "small spores phenomenon" is among honeybee populations in various geographical zones and whether it is connected with the decline of *N. apis* incidence and its replacement with *N. ceranae*, as observed worldwide (Fries, 2014; Shamaev *et al*., 2024).

CONFLICTS OF INTEREST: The authors declare that they have no conflicts of interest.

Acknowledgements. Authors are thankful to two anonymous reviewers who essentially improved the English and overall logic of the paper narrative. The study was supported by the Russian Science Foundation grant No. 24-26-00079, https://rscf.ru/project/24-26-00079/

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Responsible editor V.V. Aleoshin, E.N. Temereva