Beekeeping practice-related factors that impact nosemosis prevalence in honey bees in the Republic of Tatarstan, Russia

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ABSTRACT

To ensure pollination services for agriculture and implement effective management strategies to protect honey bee populations, it is necessary to understand the prevalence of pathogens and pests and the factors that impact their occurrence. The aim of this study is to investigate potential links of nosemosis prevalence in the Republic of Tatarstan, Russia. Multivariate logistic regression was used to evaluate the following factors as potential risk factors for *Nosema apis* and *N. ceranae* PCR positivity: district, wintering type, honey bee breed, hive material, varroosis, ascosferosis or nosemosis observed in the previous year, colony strength, feeding in winter, and amitraz, fluvalinate, or thymol usage. Our results show that only the variable counting for thymol usage fits the data well, where the actual observed prevalence of *N. ceranae* infection is significantly higher in honey bee populations that use thymol compared to those that do not. Honey bee populations with thymol usage in the current study with decreased, but not eliminated, *N. ceranae* infection, possibly faced preventive, uncontrolled, and excessive use of miticide in beekeeping practice.

Introduction

Honey bees are useful for managing the environment and are crucial pollinators of commercially significant crops. Biotic and abiotic factors (diseases, pesticide use, land use, and climate change) affect insect development and the quantity and quality of honey bee-related products (9, 14, 18). In recent years, there has been increased interest in the effects of *Nosema* species on honey bee colonies (the original parasites of Asian and Western honey bees are *N. ceranae* and *N. apis*, respectively) (37, 39). Sharing habitats, contaminated food sources, trophallaxis, asymptomatic and tolerant honey bees in hives, and the commerce in honey bees and their products are all factors that contribute to the spread of *Nosema* species. The primary way that foraging insects become contaminated with *Nosema* species is through environmental spores. Particularly in areas with long, harsh winters, a high incidence of infection with both *Nosema* species against variations in temperature, relative humidity, and brood rearing in mid-winter may be connected to the health of honey bees (39). The number of managed honey bee colonies has been steadily declining over time in geoclimatic regions with long, cold winters, including Russia. Various phytotherapeutics, organic acids, essential oils, polysaccharides, and metabolites are examples of organic control techniques that reduce the size of the *Nosema* parasite population; they are accessible in many countries, pose little threat to consumer safety by contaminating bee products, and are environmentally benign (14). However, as it is typical for the apiary to have

a few pathogens and diverse rearing methods (9), the lack of multifactor effect data of treatment on *Nosema* spp. in the bee operations is a disadvantage. In Russia, very few investigations on honey bee nosemosis prevalence have been carried out (28, 35, 37, 39, 40). Beekeepers can report the illness status, but this passive surveillance of honey bee pathogens must be verified because it mostly depends on their observations. Additionally, identifying the presence of a pathogen and treatment strategies that may aid in identifying colonies that are more likely to carry a pathogen is pertinent to targeted sampling in the context of pathogen monitoring. The aim of this study is to investigate potential links of nosemosis prevalence in the Republic of Tatarstan, Russia.

Materials and Methods

Sample Collection and Sample Size Estimation: Honey bee sampling was performed in the Republic of Tatarstan, Russia, in spring 2024 in the private-sector apiaries (Figure 1, Table 1, Table 2). The sample collection procedure was described by Shamaev et al (37). There are overall 43 districts in the Republic of Tatarstan. Districts are just administrative borders that have no relation to host-pathogen interaction (38). In this study 13 districts were selected, which is a proportion of the entire honey bee population in the Republic of Tatarstan. Among 13 districts, 11 were chosen for sampling because nosemosis-infected honeybees were reported there: with a high rate of infection (Almetyevsky, Aznakaevsky, Buinsky, Elabuzhsky, Laishevsky, Menzelinsky, Muslyumovsky, and Sabinsky) – 8 districts; with either no infection cases or a single positive sample (Apastovsky, Verkhneuslonsky, and Zelenodolsky) – 3 districts (39). Additionally, we included 2 districts that were not surveyed previously (Kamsko-Ustinsky and Vysokogorsky) – they border the above-mentioned districts and have different honey bee breeds. According to the latest data from the Ministry of Agriculture and Food of the Republic of Tatarstan (27), the number of apiaries in the selected districts for 2022 is 496 in Aznakaevsky district, 181 in Almetyevsky district, 395 in Apastovsky district, 225 in Buinsky district, 286 in Verkhneuslonsky district, 220 in Vysokogorsky district, 128 in Elabuzhsky district, 155 in Zelenodolsky district, 213 in Kamsko-Ustinsky district, 178 in Laishevsky district, 136 in Menzelinsky district, 354 in Muslyumovsky district, and 381 in Sabinsky district. The minimum number of apiaries to be sampled was determined through the following formula (1, 48):

$$n = \frac{Nt^2p(1-p)}{\mathrm{d}^2(N-1) + t^2p(1-p)}$$

In the formula, N is 3384, which is the total number of apiaries in all 13 districts, i.e., in the selected proportion of the entire population. According to Aguila and Gonzalez-Ramırez, the formula is acceptable to calculate a proportion (1). The prevalence value P was considered as 0.059, since the average value of nosemosis prevalence in Russia is 5.9% (28, 39), while d2 is 0.0025 (a minimum error of 5% was chosen), which is the margin of error in the sample, and t2 is 3.8416 (for 95% CI). According to this formula, the minimum number of apiaries to be selected in all 13 districts, i.e., in the selected proportion of the entire population, was determined as 21. We used this information as the border of a minimum number of apiaries. Overall, 26 apiaries were studied, which is 2 apiaries per district.

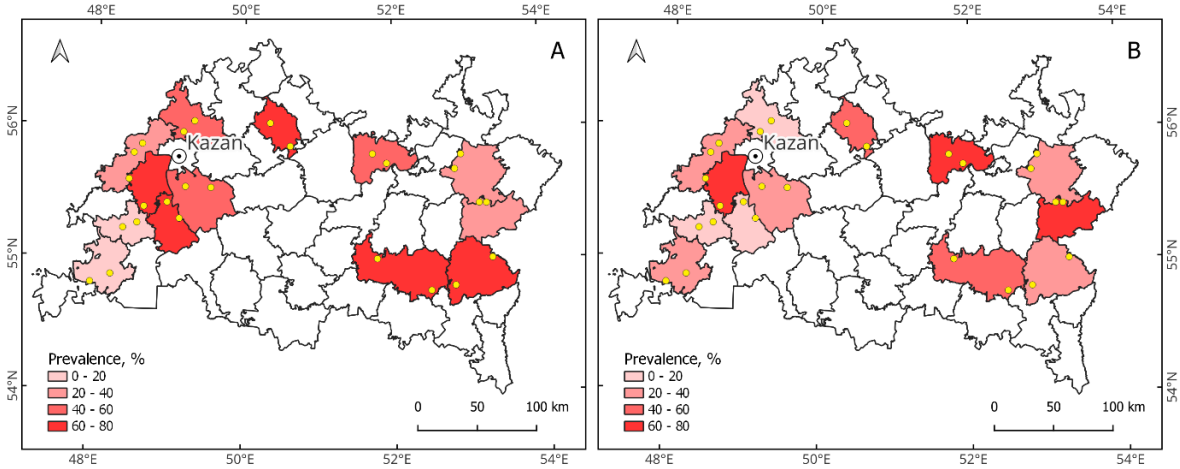

Figure 1. Cartographic data visualization on *Nosema* spp. prevalence in the private-sector apiaries in the districts of Republic of Tatarstan, Russia. (A) *N. apis*. (B) *N. ceranae*. Spatial referencing of sampling sites and data visualization was carried out using a global positioning system (GPS) and the free and open-source geographic information system QGIS 3.28 (https://qgis.org). Geodetic coordinates were projected into planar rectangular coordinates in the Universal Transverse Mercator projection on the WGS-84 ellipsoid (Universal Transverse Mercator (UTM), zone 39N, EPSG:32639). The coordinates on the maps are presented as geodetic coordinates (WGS-84, degrees north latitude and east longitude). To visualize thematic objects (administrative boundaries, regional capital), a set of vector data layers called NextGIS (https://data.nextgis.com) was used. Data license: ODbL. Prevalence data was reflected in the form of a background cartogram (choropleth map) for five equivalent classes (0-20, 20-40, 40-60, 60-80, and 80-100%).

Table 1. *N. apis* prevalence in private sector apiaries

Category		Examined	Positive	Negative	Prevalence (%)	95% CI
District	Almetyevsky	41	32	9	78.04	61.96-88.88
	Apastovsky	79	15	64	18.98	11.35-29.69
	Aznakaevsky	48	36	12	75	60.1-85.89
	Buinsky	107	21	86	19.62	12.82-28.66
	Elabuzhsky	46	21	25	45.65	31.17-60.84
	Kamsko-Ustinsky	63	41	22	65.07	51.94-76.36
	Laishevsky	76	33	43	43.42	32.25-55.25
	Menzelinsky	40	9	31	22.5	11.4-38.85
	Muslyumovsky	46	18	28	39.13	25.45-54.6
	Sabinsky	45	30	15	66.66	50.94-79.56
	Verkhneuslonsky	52	32	20	61.53	47.01-74.36
	Vysokogorsky	37	17	20	45.94	29.85-62.86
	Zelenodolsky	71	21	50	29.57	19.63-41.75
Wintering type	Winter shelter	363	139	224	38.29	33.3-43.53
	Insulated hives	388	187	201	48.19	43.13-53.28
Subspecies	*A. m. carnica*	350	162	188	46.28	41-51.66
	A. m. carpatica	102	43	59	42.15	32.57-52.34
	A. m. mellifera	94	39	55	41.49	31.56-52.12
	A. m. caucasica	42	24	18	57.14	41.07-71.92
	Not identified	163	58	105	35.58	28.35-43.5
Hive material	Wood	317	114	203	35.96	30.72-41.54
	Polystyrene	434	212	222	48.84	44.06-53.65
Varroosis reported previously	No	290	115	175	39.65	34.02-45.55
	Yes	461	211	250	45.77	41.16-50.44
Ascoferosis reported previously	No	596	143	453	24	20.65-27.66
	Yes	155	24	131	15.48	10.36-22.36
Nosemosis reported previously	No	332	105	227	31.62	26.71-36.96
	Yes	419	221	198	52.74	47.84-57.59
Colony strength	≥ 6 frames	406	143	263	35.22	30.61-40.11
	< 6 frames	345	183	162	53.04	47.62-58.38
Feeding in winter	Sugar-honey	104	69	35	66.34	56.33-75.13
	Sugar	540	211	329	39.07	34.95-43.34
	None	107	46	61	43	33.57-52.91
Amitraz used	No	609	296	313	48.6	44.57-52.65
	Yes	142	30	112	21.12	14.91-28.93
Fluvalinate used	No	645	270	375	41.86	38.03-45.78
	Yes	106	56	50	52.83	42.93-62.51
Thymol used	No	428	151	277	35.28	30.79-40.03
	Yes	323	175	148	54.17	48.57-59.68
Infected with *N. ceranae*	No	467	159	308	34.04	29.79-38.56
	Yes	284	167	117	58.8	52.82-64.54
Total		751	326	425	43.4	39.84-47.04

Table 2. *N. ceranae* prevalence in private sector apiaries

Category		Examined	Positive	Negative	Prevalence (%)	95% CI
District	Almetyevsky	41	24	17	58.53	42.19-73.29
	Apastovsky	79	10	69	12.65	6.56-22.49
	Aznakaevsky	48	19	29	39.58	26.11-54.70
	Buinsky	107	25	82	23.36	15.95-32.72
	Elabuzhsky	46	33	13	71.73	56.31-83.54
	Kamsko-Ustinsky	63	9	54	14.28	7.13-25.89
	Laishevsky	76	24	52	31.57	21.66-43.37
	Menzelinsky	40	10	30	25	13.24-41.52
	Muslyumovsky	46	33	13	71.73	56.31-83.54
	Sabinsky	45	27	18	60	44.37-73.93
	Verkhneuslonsky	52	40	12	76.92	62.82-87.01
	Vysokogorsky	37	3	34	8.1	2.11-23.02
	Zelenodolsky	71	16	55	22.53	13.8-34.28
Wintering type	Winter shelter	363	142	221	39.11	34.1-44.36
	Insulated hives	388	142	246	36.59	31.83-41.63
Subspecies	*A. m. carnica*	350	135	215	38.57	33.48-43.91
	A. m. carpatica	102	48	54	47.05	37.18-57.15
	A. m. mellifera	94	34	60	36.17	26.69-46.78
	A. m. caucasica	42	19	23	45.23	30.16-61.16
	Not identified	163	37	126	22.7	16.67-30.04
Hive material	Wood	317	105	212	33.12	28.02-38.64
	Polystyrene	434	179	255	41.24	36.59-46.04
Varroosis reported previously	No	290	102	188	35.17	29.73-41.00
	Yes	461	182	279	39.47	35.01-44.12
Ascoferosis reported previously	No	596	257	339	43.12	39.11-47.21
	Yes	155	27	128	17.41	11.98-24.51
Nosemosis reported previously	No	332	117	215	35.24	30.15-40.67
	Yes	419	167	252	39.85	35.16-44.73
Colony strength	≥ 6 frames	406	112	294	27.58	23.34-32.25
	< 6 frames	345	172	173	49.85	44.46-55.24
Feeding in winter	Sugar-honey	104	73	31	50.69	42.27-59.07
	Sugar	540	190	350	35.18	31.18-39.39
	None	107	21	86	19.62	12.82-28.66
Amitraz used	No	609	249	360	40.88	36.97-44.91
	Yes	142	35	107	24.64	17.97-32.71
Fluvalinate used	No	645	203	442	31.47	27.93-35.23
	Yes	106	81	25	76.41	67.00-83.88
Thymol used	No	428	118	310	27.57	23.44-32.11
	Yes	323	166	157	51.39	45.80-56.94
Infected with *N. apis*	No	425	117	308	27.53	23.38-32.08
	Yes	326	167	159	51.22	45.66-65.75
Total		751	284	467	36.35	32.92-39.92

Table 3. List of districts, used for the sample collection and the PCR-RFLP results

District	Number of colonies	Number of samples succeed in PCR-RFLP	Breed based on the PCR-RFLP
Almetyevsky	1	27/27	*A. m. caucasica*
	1	11/14	*A. m. caucasica*
		3/14	NA
Apastovsky	1	41/41	*A. m. carnica*
	1	3/38	*A. m. carnica*
		35/38	NA
Aznakaevsky	1	21/21	*A. m. carnica*
	1	14/27	*A. m. carpatica*
		13/27	NA
Buinsky	1	27/59	*A. m. carpatica*
		32/59	NA
	1	48/48	*A. m. carnica*
Elabuzhsky	1	31/31	*A. m. carnica*
	1	15/15	*A. m. carnica*
Kamsko-Ustinsky	1	22/22	*A. m. carnica*
	1	41/41	*A. m. carpatica*
Laishevsky	1	15/34	*A. m. mellifera*
		19/34	NA
	1	18/42	*A. m. mellifera*
		24/42	NA
Menzelinsky	1	15/22	*A. m. caucasica*
		7/22	NA
	1	7/18	*A. m. carnica*
		11/18	NA
Muslyumovsky	1	26/26	*A. m. carnica*
	1	20/20	*A. m. carpatica*
Sabinsky	1	38/38	*A. m. carnica*
	1	7/7	*A. m. carnica*
Verkhneuslonsky	1	23/23	*A. m. carnica*
	1	29/29	*A. m. mellifera*
Vysokogorsky	1	32/37	*A. m. mellifera*
	1	4/5	*A. m. mellifera*
		1/5	NA
Zelenodolsky	1	60/60	*A. m. carnica*
	1	11/11	*A. m. carnica*

NA: samples with no result obtained.

We used the following approach for the minimum sample size estimation in the selected proportion of the entire population (13 districts). According to the existing rules/guidelines of sample size, there is a ratio of 20-to-1 (10), where a study with 1 item (question) requires 20 samples. We had 20 main questions about the relationship between nosemosis prevalence and: 1. Usage of winter shelter as a wintering type; 2. Usage of insulated hives as a wintering type; 3. Usage of wood as a hive material; 4. Usage of wood as a hive material; 5. Honey bee breed *A. m. mellifera*; 6. Honey bee breed A. *m. carnica*; 7. Honey bee breed A. *m. carpatica*; 8. Honey bee breed *A. m. caucasica*; 9. Data from beekeeper regarding varroosis in the previous year; 10. Data from beekeeper regarding ascoferosis in the previous year; 11. Data from beekeeper regarding nosemosis in the previous year; 12. Usage of sugar-honey in feeding in winter; 13. Usage of sugar in feeding in winter; 14. No feeding in winter; 15. Colony strength ≥ 6 frames; 16. Colony strength < 6 frames; 17. Amitraz usage; 18. Fluvalinate usage; 19. Thymol usage; 20. Another *Nosema* species occurrence in study. In total, a minimum sample size should be 400 (20x20) in the selected proportion of the entire population (13 districts). We used this information as the border of a minimum sample size. Overall, 751 honey bees were studied (the number of honey bees collected in each apiary is shown in the Table 3). A survey of beekeepers was conducted to assess the beekeeping practices used in the apiary,

evaluate the use of pharmaceutical products or other additives, and consider recorded cases of honey bee diseases in the colonies annually.

DNA Extraction and PCR: Only worker bees were studied. Prior to DNA extraction, each individual honey bee was washed in ethanol and sterile water and then ground in 1 ml of newly added sterile water. DNA extraction was performed for each individual honeybee. DNA was extracted using the AmpliPrime kit (NextBio, USA) following the previously established protocol (42). Duplex PCR was performed to amplify the 321 bp and 218 bp fragments corresponding to the 16S ribosomal gene of *N. apis* and *N. ceranae*, respectively. PCR-RFLP was performed to amplify the cytochrome oxidase 1 gene region and evaluate the honey bee breed among the samples. PCR procedures with corresponding primer sequences are detailed in the previous report (34, 37).

Statistical Analysis: All analyses were performed using R Statistical Software (version 4.3.0) (43). Multivariate logistic regression was used to evaluate the different factors as potential risk factors for PCR positivity with *Nosema* species. Quantitative data were replaced with 0 or 1 dummy variables. Honey bee breed variables were replaced by 0, 1, 2, and 3 for *A. m. mellifera*, *A. m. carnica*, *A. m. carpatica*, and *A. m. caucasica*, respectively. Feeding in winter was replaced by 0, 1, and 2 for honey, sugar-honey, and sugar, respectively. Multicollinearity among the explanatory variables was assessed using Spearman's rank correlation coefficient. None of the Spearman's coefficients were greater than 0.6 (Figure 2). To find the best-fitting model, a backward selection procedure was used. Predictive performance analysis, model fitting, and computation of the standard errors for the predicted probabilities, as well as the list of software packages used in this study, were described previously (38). P value from the CI of estimated *N. ceranae* prevalence was calculated using a method reported previously (2). The P-values less than 0.05 were considered statistically significant.

Results

Among 751 worker honey bees from 26 colonies in 13 districts, 326/751 (43.4%, 95% confidence interval; CI: [39.84–47.04]) and 284/751 (36.35%, 95% confidence interval; CI: [32.92–39.92]) of the honey bee samples showed *N. apis* and *N. ceranae* positivity, respectively (Figure 1, Table 1, Table 2).

Hive conditions were not counted as factors for nosemosis because the beekeepers do regular inspections every 2 weeks to monitor colonies' health and progress, look for symptoms associated with established pests and diseases of honey bee colonies. Average temperature in the hive was 77-82.4 F and relative humidity 55-70%, which did not exceed the values in other regions with similar climates (25, 47). We found that 38.29% (139/363) and 48.19% (187/388) of the honey bees managed in the winter shelter and insulated hives were *N. apis* positive, and 39.11% (142/363) and 36.59% (142/388) were *N. ceranae* positive, respectively. 35.96% (114/317) and 48.84% (212/434) of the honey bees managed in the wooden and polystyrene hives were positive for *N. apis*,

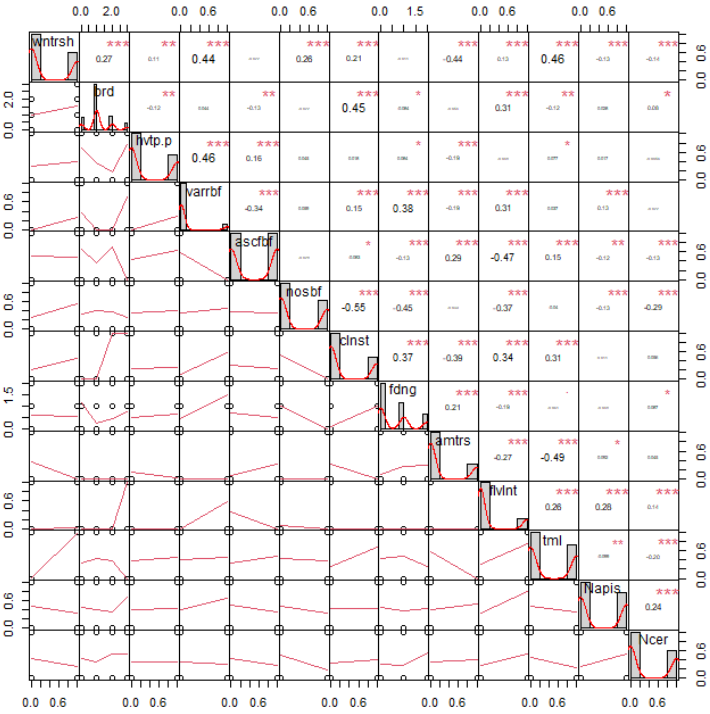

Figure 2. Multicollinearity of the explanatory variables using Spearman's coefficient. None of the Spearman's coefficients were greater than 0.6. Variable designations: Wntrsh - Wintering type, brd - Subspecies, hvtp.p - Hive material, varrbf - Varroosis reported previously, ascbf - Ascoferosis reported previously, nosbf - Nosemosis reported previously, clnst - Colony strength, fdng - Feeding in winter, amtrs - Amitraz used, flvnt - Fluvalinate used, tml - Thymol used, Napis - Infected with *N. apis*, Ncer - Infected with *N. ceranae*.

and 33.13% (105/317) and 41.24% (179/434) were positive for *N. ceranae*, respectively. *N. apis* and *N. ceranae* positivity among honey bee colonies with other pathogens reported previously were as follows: 45.77% (211/461) and 39.47% (182/461) for varroosis, 15.48% (24/155) and 17.41% (27/155) for ascosferosis, and 52.74% (221/419) and 39.85% (167/419) for nosemosis.

According to the PCR-RFLP results, four distinct subspecies were identified, although some samples exhibited multiple bands or yielded negative results. Those samples were abbreviated as "NA" and were included in the statistical analysis (Table 3). *N. apis* and *N. ceranae* positivity among honey bee subspecies were 46.28% (162/350) and 38.57% (135/350) for *A. m. carnica*, 42.15% (43/102) and 47.05% (48/102) for *A. m. carpatica*, 41.49% (39/94) and 36.17% (34/94) for *A. m. mellifera*, 57.14% (24/42) and 45.23% (19/42) for *A. m. caucasica*, and 35.58% (58/163) and 22.7% (37/163) for those honey bee samples that were not identified. *Nosema* species positivity among honey bee colonies with a colony strength of 7 frames or more and less than 7 frames were as follows: 35.22% (143/406) and 53.04% (183/345) for *N. apis*, and 27.58% (112/406) and 49.85% (172/345) for *N. ceranae*. *N. apis* and *N. ceranae* positivity among honey bee colonies with different feeding in winter was as follows: 66.34% (69/104) and 50.69% (73/104) for sugar-honey syrup, 39.07% (211/540) and 50.69% (190/540) for sugar syrup, and 43% (46/107) and 19.62% (21/107) for no feeding.

There are various registered names for thymol, fluvalinate, and amitraz available for purchase for beekeepers in Russia, and all the products have the same quantities of active ingredient that should be applied in the hive. According to information obtained from 26 beekeepers in spring, all of them used the exact portion of product according to the product instructions for use. For thymol, it was fed to honey bees together with syrup (3 g of thymol powder diluted in 25 L of 50% syrup, and 100 mL was added in the hive feeder for each frame). Such treatment was applied 4 times during 1 month with an equal interval between treatments. For fluvalinate, all the beekeepers used 2 strips per 8-12 frame hive. 1 strip was used with a lesser number of frames. Each strip contains 80 mg of fluvalinate. Because there was no difference in quantity and feeding period, thymol, fluvalinate, and amitraz were included in the statistical analysis as “used” or “not used”. *N. apis* and *N. ceranae* positivity among honey bee colonies treated with synthetic or organic chemicals and compounds was as follows: 21.12% (30/142) and 24.64% (35/142) for amitraz, 52.83% (56/106) and 76.46% (81/106) for fluvalinate, and 54.17% (175/323) and 51.39% (166/323) for thymol usage.

Multivariate logistic regression analysis was performed to separately validate risk factors for *Nosema* spp. infection. Using a backward selection procedure, three models were generated. A best-fitted model 1 to estimate the risk factors for *N. apis* infection included the following factors: *N. ceranae* infection, previously observed nosemosis, colony strength, amitraz usage, feeding in winter, and previously observed varroosis. A best-fitted model 2 to estimate the risk factors for *N. ceranae* infection included the following factors: fluvalinate usage, *N. apis* infection, feeding in winter, thymol usage, previously reported ascosferosis, colony strength, hive material, and previously observed nosemosis. A best-fitted model 3 to estimate the risk factors for infection with both *Nosema* species included the following factors: thymol usage, previously reported nosemosis, feeding in winter, and fluvalinate usage. A plot of the modified Akaike information criterion (AICc) of several models showed that model 3 minimizes AICc, and is therefore chosen as the best model out of this set (Figure 3). To assess the estimates of the actual prevalence of the honey bee population obtained from the model 3 and evaluate its goodness of fit, we plotted the model-based estimates of prevalence against the raw prevalence from the population (Figure 4). Among variables, only the variable counting for thymol usage fits the data well, where the actual observed prevalence of *N. ceranae* infection was also significantly higher ($P < 0.05$) in honey bee populations with thymol usage than in the populations without it.

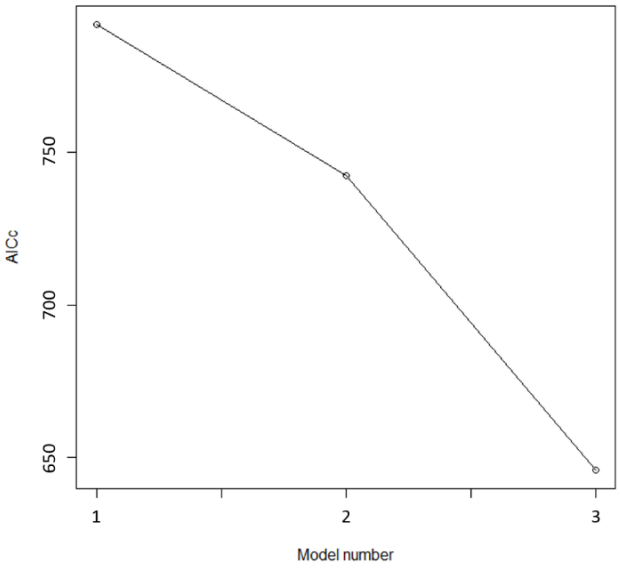

Figure 3. A plot of AICc of several models, where model 3 minimizes AICc, and is therefore chosen as the best model out of this set

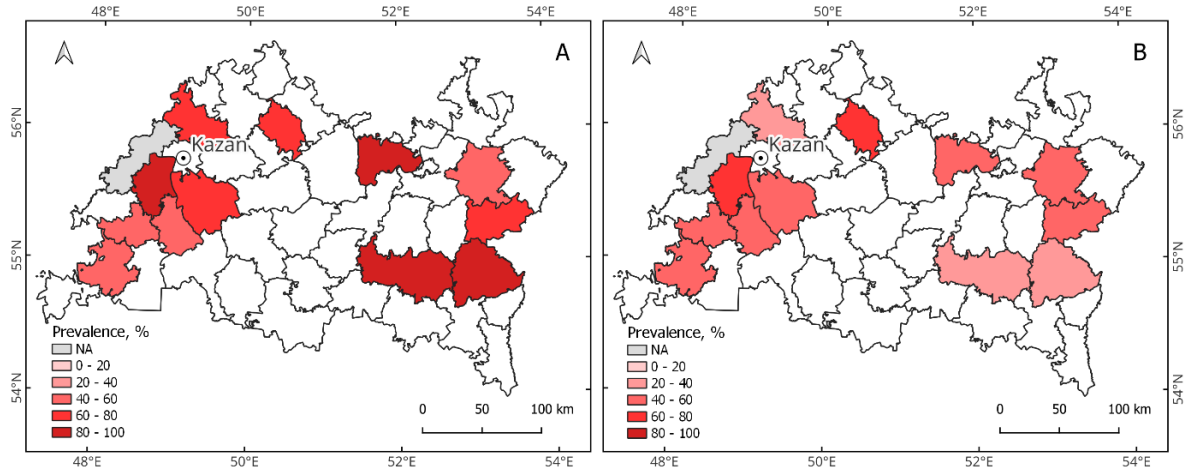

Figure 4. Map of estimated *Nosema* species prevalence in the honey bees where thymol was either used or not used. Estimated prevalences among the honey bees where (A) thymol was used and (B) was not used. The observed prevalence and 95% CIs are shown from the fitted model. Values for wintering type, honey bee breed, hive material, colony strength, feeding in winter, amitraz or fluvalinate usage, reported varroosis, ascosferosis or nosemosis were set to zero in the model.

Discussion and Conclusion

Variety of pests and pathogens, including microsporidians *N. apis* and *N. ceranae* are responsible for mass bee colony losses in Russia (28, 33, 36, 39). In this study, we surveyed the prevalence of nosemosis and the factors that impact its occurrence among 13 districts in Tatarstan, Russian Federation. Wintering type (winter shelter or insulated hive) was chosen because the wintering technique had an impact on honey bee survival; colonies that spent the winter indoors had lower mortality rates when infected with *Nosema* species and a quicker spring population build-up than colonies that spent the winter outdoors (32). Hive material reflects the internal conditions within a hive, too. According to a survey, keeping bees in wooden hives preserves ideal temperature conditions in the brood-rearing zone, which benefits queen egg production, worker bee flight activity indicators, and colony strength. As opposed to wooden hives, polyurethane foam hives are difficult to sterilize, have no vapor permeability, and water is not absorbed; instead, it flows down and stays on the bottom (46). The prevalence of nosemosis infection is naturally found at a high infection rate in *A. mellifera* populations (8), but it remains unknown within subspecies present at the same study area. Such variables as varroosis, ascosferosis, and nosemosis (observed in the apiary in the previous year or not) were included in the analysis, as the findings of studies conducted on bee colonies in different regions of Russia (Arkhangelsk, Belgorod, Voronezh, Kirov, Leningrad, Moscow, Orenburg, Penza, Tomsk, Tula, and Tyumen regions; Altai, Krasnodar, Perm, and Stavropol krai; Republics of Mari El, Tatarstan, and Udmurtia) indicate that varroosis-nosemosis and varroosis-nosemosis-ascosphaerosis are included in the list of the most prevalent infection-invasions of bees (12). Feeding in winter was included in the analysis because different winter feed types may be associated with any significant differences in nosemosis prevalence (5). Nosemosis has been associated with its negative impact on colony strength and productivity in several studies (30). Since robust colonies consistently produce more broods, there is a direct correlation between colony strength and brood raising. More worker bees can make more honey and feed and care for more broods. According to Bhusal and Thapa (2006), honey output from less than six frames is much lower than that from six, eight, and ten frame types (3). We counted frames in order to gauge the strength of the honey bee colonies under examination (≥ 6 frames or < 6 frames). Other variables included in the analysis were amitraz, fluvalinate, or thymol usage. For example, beekeepers can leave strips soaked in amitraz in the hive for longer than necessary (49). It is reported that in the honey bee family, nosemosis and exposure to the commonly used in-hive acaricide amitraz are common stressors that both result in higher mortality rates than bees exposed separately, with no difference in the development of parasites (22). It is also a typical practice in Russia, where higher dosages of amitraz, thymol, and fluvalinate active ingredients resulted in higher fatality rates or decreased reproductive performance in colonies (7, 24). The rate at which pests and pathogens are eliminated from the colony allows beekeepers to calculate its appropriate dosage. However, according to data from beekeepers in this study, they used the exact dosage according to the product instructions. Using logistical regression analysis, we found that the honey bee populations with thymol usage significantly impacted *N. ceranae* prevalence but not wintering type, honey bee breed, hive material, colony strength, feeding in winter, amitraz, fluvalinate, or thymol

usage, and varroosis, ascosferosis or nosemosis observed in the previous year. This result is reinforced by the fact that *N. ceranae* prevalence among the honey bees treated with thymol was significantly higher statistically than that without thymol. Honey bee populations in the current study may have faced preventative, uncontrolled, and excessive thymol treatment in beekeeping practices. *Thymus vulgaris* is the natural source of thymol (3-hydroxy-p-cymene), which is an essential oil constituent utilized for decades in *Varroa* control due to its anti-parasitic properties (19). Different studies in which honey bees fed on thymol report that it may be able to control *N. ceranae* to varying extents (*N. ceranae* spore load reduction or no effect) (4). Thymol itself may cause certain disorders that affect bee survival, lowering oxidative capacity, and downregulating some immune-related gene expressions in *Nosema*-free bees, but in *Nosema*-infected bees, some studies show increasing levels of immune-related genes and values of oxidative stress parameters in addition to decreasing *Nosema* spore loads (16). Other studies also show reduced survival in the honey bees and genotoxic effects of thymol (17). To understand the potential detrimental effects on brood growth after thymol treatment, its usage should be further examined in the honey bees exposed to both common stressors (varroosis and nosemosis).

The overall *N. apis* and *N. ceranae* prevalence in honey bees was 43.4% and 36.35%, respectively. There is sufficient information regarding the prevalence of *N. apis* and *N. ceranae* in the Republic of Tatarstan, Russia, from other researchers: 5.9% prevalence of nosemosis on the regional level, including the Republic of Tatarstan and one N. *apis*-infected honey bee reported in the Republic of Tatarstan (28, 39, 47). Comparing our results collected from the same apiary in Laishevsky district in February between 2023 and 2024, infection prevalences became 2.6 (16.66% vs. 43.42%) and 7-fold times higher (4.44% vs. 31.57%) for *N. apis* and *N. ceranae*, respectively (35, 39, 40). Interestingly, co-infection with both species decreased 2.6-fold times (38.88% vs. 14.47%). Also, in the same study, we found a moderate differentiation in the genetic structure of *N. apis* (na1.1 haplotype) and *N. ceranae* subpopulations (nc1.4, nc7.1, nc13.3, nc17.1, nc20.3, nc35.1, and nc1.1, nc4.1, nc4.4, nc5.1, nc6.2, nc11.1, nc24.1, and nc29.1 haplotypes) (39). In another study in the same apiary in 2024, we observed the negative effect of high infection loads on *N. apis* spore size by the depletion of resources needed for spore production (37). With an increase in spore load, more atypical *N. apis* spores were observed (including the data from honey bees co-infected with both *Nosema* species). *N. apis* in the current study was found to be more prevalent in honey bees than *N. ceranae*. However, the drastic increase of *N. ceranae* prevalence from 2023 to 2024, the presence of *N. ceranae* multiple haplotypes, and atypical *N. apis* spores as a result of resource depletion altogether can be related to the higher adaptability of *N. ceranae*, which seems that the situation in the Republic of Tatarstan reflects broader global trends in Europe, where *N. ceranae* became increasingly dominant compared to *N. apis*.

The intraspecific taxonomic affiliation of honey bee colonies determines their susceptibility to nosemosis; in temperate and northern latitudes, colonies of bees belonging to the subspecies *A. m. ligustica*, *A. m. caucasica*, and *A. m. carnica* are more likely to be infected with *Nosema* species than colonies of *A. m. mellifera* (44). Unlike the reported data, in our study, *N. apis* prevalence among *A. m. carnica*, *A. m. carpatica*, *A. m. mellifera* and *A. m. caucasica* was in the range 41.49-57.14%. *N. ceranae* prevalence among *A. m. carnica*, *A. m. carpatica*, *A. m. mellifera* and *A. m. caucasica* was in the range 36.17-47.05%. Among domestic *A. mellifera* honeybee subspecies, there are some differences in resistance to nosemosis, which are assumed from the expression of immune genes, mortality rates, events of hybridization or the prevalence of pathogens (6, 20). Kharitonov found that severity of *N. apis* and *N. ceranae* infection in *A. m. mellifera* was significantly lower than in *A. m. caucasica* (20). Petukhov et al. observed that *A. m. caucasica* and *A. m. carpathica* tend to be affected by *N. apis* in a more intensive manner than *A. m. mellifera* (31). Kaskinova et al. found that *A. m. mellifera* and *A. m. carnica* were equally infected with *N. apis*, but *A. m. mellifera* were 3-fold times more infected with *N. ceranae* than *A. m. carnica* (26). Tozkar found that the highest responses from immune genes against *N. ceranae* were in *A. m. carnica*, compared to *A. m. caucasica* (45). Prevalence of either *N. apis* or *N. ceranae* was not significantly different between subspecies in the current study (Figure 5). However, *N. ceranae* prevalence in not identified subspecies was

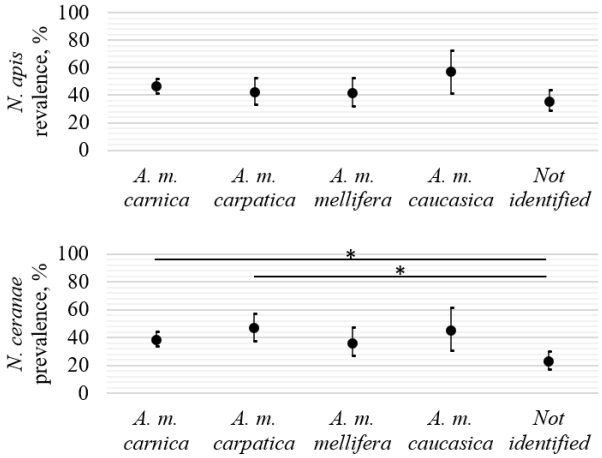

Figure 5. Difference among *N. apis* and *N. ceranae* prevalence among *A. mellifera* subspecies. Asterisk show the statistical significance with P-values less than 0.05.

significantly different from *A. m. carnica* and *A. m. carpathica* subspecies, but not *A. m. mellifera* and *A. m. caucasica*. It can be explained that when different honey bee subspecies form hybrids, new genotypes are formed and genetic imbalance arises, which leads to changes in resistance against diseases. It is not clear whether *N. ceranae*-resistant *A. mellifera* hybrids derived from *A. m. mellifera* with *A. m. caucasica* in the current study. We assume that it is unlikely because our own morphological observations of honey bees from the Tatarstan Republic revealed a positive correlation between the *A. m. mellifera* / *A. m. caucasica* hybrid and a high *Nosema* spp. spore load (40). Also, Ostroverkhova et al. reported that *N. apis* and *N. ceranae* presence was increased in naturally resistant Central Russian *A. m. mellifera* after hybridization with honey bee subspecies from southern regions (29). At last, Fontbonne et al. found that pure *A. m. carnica* and *A. m. carpathica* mortality for *N. ceranae* was up to 50%, while for *A. m. carnica* /*A. m. carpathica* / *A. m. mellifera* hybrids and for *A. m. caucasica* /*A. m. carnica* /*A. m. carpathica* hybrids it was up to 100% (13). To prove the hypothesis about genetic imbalance and know the degree to which hybridization alters resistance, further experiments with whole genome sequencing are necessary.

N. apis and *N. ceranae* can lead to Colony Collapse Disorder (CCD), a dangerous disease of the honey bee *A. mellifera* that causes the sudden death of the entire colony (11). However, in the apiaries from Spain, Switzerland, France, and Germany, almost all colonies vulnerable to CCD were infected with *N. apis* and *N. ceranae*, while in apiaries from Russia and Serbia, Bosnia and Herzegovina, and Montenegro, none of the colonies infected with *N. apis* and *N. ceranae* were susceptible to CCD (23). Although nosemosis may play a role in CCD development, other factors, including viral infection and/or honey bee intoxication from sublethal pesticide dosages and/or heavy metals, must coexist for CCD to be effective (15, 21, 23).

At last, for some explanatory variables related to beekeeping practices, it is likely that some of the colonies cannot be considered independent if they belonged to the same beekeeping operation or apiary location. For example, beekeepers might either apply thymol or not apply thymol in their apiaries. This means that if one colony belonging to the beekeeper was treated with thymol, the second colony also must have received thymol, even if the beekeeper said that only a particular colony was treated. The two colonies within the apiary may not be independent with respect to thymol treatment. However, even if the relationship between two colonies within the apiary may increase the chance of a type I error, the multicollinearity among the explanatory variables using Spearman's rank correlation coefficient didn't show values greater than 0.6. For better accuracy, we recommend using more districts, but not more than one colony per apiary, to study the beekeeping practices. In conclusion, the study indicates that honey bee populations exposed to higher levels of thymol are more likely to experience *N. ceranae* infection, possibly due to the uncontrolled use of miticides in beekeeping practices.

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Ethical Statement

Not applicable.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

NDS conceived the idea; MNM performed the field work; NDS, EAS, OVN and MNM carried out the laboratory experiments and data analysis. NDS and MNM wrote the manuscript with help from EAS.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Animal Welfare

Not applicable.

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