

1 **TITLE:** The outcome of the colonization of *Apis mellifera* by *Nosema ceranae*

2 **RUNNING TITLE:** Colonization of honeybees by *Nosema ceranae*

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24 **Abstract**

25 A Multiplex PCR based method, in which two SSUrRNA regions are simultaneously amplified
26 in a single reaction, was designed for the in-parallel detection of honeybee microsporidia (*Nosema apis*
27 and *Nosema ceranae*). Each of the two pairs of primers exclusively amplified the 16S targeted gene of
28 the specific microsporidia. The multiplex PCR assay was useful for the specific detection of the two
29 species of microsporidia related to bee nosemosis, not only in purified spores but also in honeybee
30 homogenates and in naturally infected bees. Multiplex PCR assay was also able to detect co-infections
31 by the two species. A screening of bee samples from Spain, Switzerland, France and Germany using
32 the PCR technique revealed a higher presence of *N.ceranae* than *N. apis* in Europe, although both are
33 widely distributed. From the year 2000 onwards, statistically significant differences have been found in
34 the proportion of *Nosema* spp. spore positive samples collected, inter and intra-annually. In the first
35 period (1999-2002), the smallest number of samples diagnosed as *Nosema* positive was recorded
36 during the summer months, showing a clear seasonality in the diagnosis which is characteristic of *N.*
37 *apis*. From 2003 onwards a change in the tendency showed an increase of *Nosema* positive samples in
38 all months until 2005 when a total absence of seasonality was detected. A significant causative
39 association between the presence of *N. ceranae* and hive depopulation clearly indicates that the
40 colonization of *Apis mellifera* by *N. ceranae* is related with bee losses.

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42 **Keywords:** *Nosema apis*, *Nosema ceranae*, Microsporidia, honeybee, colonization, multiplex-PCR,
43 SSUrRNA, diagnostic.

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INTRODUCTION

Colonization is a transmission process involving the spread of a parasite into new geographical areas and leads to the establishment of that species in a host population in which none were previously present (5). Over the past few decades, many free-living animals (hosts) and their parasites have invaded new recipient areas and have come to be identified as alien or exotic species. After a parasite's invasion of a new area, it is unlikely that it will remain focally localized. Following the establishment of a viable, self-sustaining population, the subsequent stage of a species' colonization is usually its spread or dispersal throughout the area (host) or within both the distributional range of a susceptible native host and the novel range of a recently invaded exotic host (parasites) (29).

When *Apis mellifera*, a superior producer of honey, was introduced to Asia some 50 years ago, it came in contact with native bees (*Apis cerana*, for example) and therefore with their parasites (*Varroa jacobsoni*). Consequent adaptation to this new and suitable host could have led to genetic changes that caused even more severe effects in the new host; such was the description of the new species (*Varroa destructor*) in *Apis mellifera* (2). In a similar way, contact between both host species could have had repercussions on microsporidian infections. The transportation of *A. mellifera* throughout the world is associated with the transportation of the microsporidium *Nosema apis*, a parasite of the bee's digestive tract considered not highly virulent and also described as a parasite of *A. cerana* (11, 27, 28). In this Asian host, a new species, *Nosema ceranae*, was described in 1996 on the basis of gene sequences and ultrastructural features and was thought to be restricted to this host and geographically limited to Asia (12). Quite recently, this new parasite has also been detected infecting *Apis mellifera* both in Asia (20) and in Europe (17), which implies that either a great spatial leap has occurred or a great deal of ignorance exists about the real situation. In any case, the situation is worrisome as experimental infection by *N. ceranae* of this new host species has recently proved to be highly pathogenic (15).

93 The diversity of *Nosema* species was studied in a total of 290 honeybee samples sent to the
94 CAR Laboratory from different European countries (Spain, France, Switzerland and Germany), as may
95 be seen in Table 1. Data from the Spanish samples were also used to establish a relationship between
96 the clinical signs and *Nosema* detection.

97 **Spore detection and DNA extraction.** The abdomens of at least 10-20 old honeybees from
98 each sample were macerated in 5 ml of distilled water. A further 5ml were added and the suspension
99 was filtered and centrifuged for 6 min at 800 x g. Pellets were analysed by phase contrast microscopy
100 (400x magnification) to verify the presence of spores (26). This methodology was employed for the
101 determination of *Nosema* spores in all the samples used in this study.

102 For DNA extraction, spore germination was induced with 200 µl of freshly prepared
103 germination buffer (6) and incubated at 37°C for 15 minutes (27). DNA extraction was conducted using
104 the High Pure PCR Template Preparation Kit (No. 1796828 Roche Diagnostic), as previously described
105 (15). Negative controls were processed in parallel to detect possible contaminations.

106 *Nosema* samples previously characterized and sequenced (17) from different sources were used
107 for testing the new molecular tools.

108 **Duplex PCR designing methodology.** rRNA 16S locus was selected to perform the *N. apis-N.*
109 *ceranae* duplex PCR. Published sequences in GenBank (<http://www.ncbi.nlm.nih.gov/Genbank/>) from
110 *N.apis* (accession numbers DQ235446, U76706, U97150, U26534, X73894, X74112), *N. ceranae*
111 (accession numbers DQ329034, U26533, DQ078785, DQ286728), *N. bombi* (accession numbers
112 AY008373, AY741108, AY741110), *N. portugal* (accession numbers AF033316) and *N.*
113 *trichoplusiae* (accession numbers U09282) were compiled. Sequences from each species were aligned
114 using ClustalW (<http://www.ebi.ac.uk/clustalw/>) in order to identify individual polymorphic nucleotide
115 positions and to avoid their becoming primer binding zones. A consensus sequence (with variable sites,
116 named by using IUB code) was obtained for each species.

117 Specific primers for both species (*N. apis* and *N. ceranae*) were visually selected taking into
118 account that the primer sequences were specific to each of the two species. The second requirement
119 was to obtain two amplicons of different length in order to separate them using agarose gel
120 electrophoresis. The selected primers are shown in Table 2 (321-APIS for *N. apis* and 218-MITOC for
121 *N. ceranae*). The expected number of amplified bases in *N. ceranae* using the 218-MITOC primers can
122 vary between 218 or 219 depending on the sequences for *N. ceranae* available in GenBank
123 (<http://www.ncbi.nlm.nih.gov/Genbank/>). In the case of *N. apis*, the expected size of the amplicon
124 using 321-APIS was 321bp.

125 Primer suitability (G/C content and melting temperatures) was checked using the IDT
126 OligoAnalyzer programme (<http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/>). A G or
127 G/C tails were added to the 5' end of all primers in order to make the melting temperatures of the
128 primer set equal. Potential primer interactions (hairpin, self-dimer and hetero-dimer structures among
129 the four primers) were tested using AutoDimer programme
130 (<http://www.cstl.nist.gov/div831/strbase//AutoDimerHomepage/AutoDimerProgramHomepage.htm>).
131 Species specificity was performed by conducting a nearly-exact match search with BLAST
132 (<http://www.ncbi.nlm.nih.gov/BLAST/>).

133 Once the specific annealing of primers was verified, singleplex PCR and sequencing of six
134 samples was performed for each fragment prior to combining the primers for duplex assay testing, to
135 ensure that the correct sequence and size products were generated. A gradient PCR ($60^{\circ}\text{C} \pm 5^{\circ}\text{C}$) was
136 performed to empirically determine the annealing temperatures of both primer pairs. Best amplicons
137 were obtained when the annealing temperature was set at 61.8°C .

138 In order to verify whether the resulting amplicons corresponded in fact to the expected
139 sequences, subsequent single PCR reactions were carried out at this annealing temperature on six *N.*
140 *ceranae* and five *N. apis* extracted samples, which were previously characterized by sequencing
141 following the methodology described by Higes et al. (2006). The PCR products were purified with

142 Qiaquick PCR Purification Kit (No. 28104 Qiagen) as previously described (17) and fully sequenced in
143 both directions (3730 DNA Analyzer, Applied Biosystem). These sequences were aligned and
144 compared to the *N. ceranae* and *N.apis* consensus reference sequences using Sequencher (Version
145 4.1.4Fb4, GeneCodes, Ann Arbor, MI, USA).

146 Once the effectiveness and accuracy of the single PCR reactions were verified, the duplex PCR
147 was carried out by using equimolar amounts of the two primer pairs. This initial equimolar primer mix
148 was tested and the PCR products obtained showed that empirical balance primer mixes were not
149 necessary.

150 **PCR conditions.** All PCR reactions were carried out in a Mastercycler[®] ep gradient S
151 (Eppendorf), and PCR conditions were as follows: reaction cocktail 50 μ L containing 25 μ L of High
152 Fidelity PCR Master (No. 12140314001 Roche Diagnostic), 0.4 μ M of each primer, 0.4 mM each
153 dNTP, 3 mM Cl_2Mg , 0.2 mg/ml BSA, 0.1% Triton X-100 and 5 μ L of *N. apis* or *N. ceranae* DNA
154 template. Thermocycler program: 94°C (2 min), and then put through 10 cycles of 15 sec at 94°C, 30
155 sec at 61.8°C and 45 sec at 72°C, and 20 cycles of 15 sec at 94°C, 30 sec at 61.8°C and 50 sec at 72°C
156 plus 5 sec cycle elongation for each successive cycle and a final extension step at 72°C for 7 min.
157 Negative controls (from DNA extraction) were included in all PCR processes.

158 In order to evaluate the amount and quality of amplicons, monoplex and duplex PCR amplicons
159 were visualized by electrophoresis in 2% agarose gel (E-gels[®], Invitrogen) in parallel to a standard size
160 electrophoresis.

161 **Validation of duplex PCR. Reproducibility, sensitivity and specificity.** Reproducibility: 20
162 samples from bees known to be infected either with *N. apis*, *N. ceranae*, or both, as well as from non-
163 infected bees, were prepared and analysed to ensure that the specimens exhibited accurate, interpretable
164 and reproducible DNA types.

165 Sensitivity: These studies were performed with serial dilutions of *N. apis* or *N. ceranae* DNA
166 templates. A first DNA template, extracted from a sample with $3 \cdot 10^6$ spores of each microsporidia
167 counted in a haemocytometer, diluted either in 100 μ L of ddH₂O or in uninfected honeybee macerate
168 were serially diluted to DNA templates equivalent to 10^6 , 10^5 , $8 \cdot 10^4$, $6 \cdot 10^4$, $4 \cdot 10^4$, $2 \cdot 10^4$, $1.5 \cdot 10^4$, $1 \cdot 10^4$,
169 $5 \cdot 10^3$, $2 \cdot 10^3$, $2 \cdot 10^2$ and 20 spores in 100 μ L. Sensitivity was studied by testing *N. apis* or *N. ceranae*
170 templates individually and then testing a mixture of both species in order to investigate the ability of
171 the system to detect the components of mixed specimens and define the limitations of the duplex-PCR.

172 Specificity: The primers pairs were tested for their specificity by using them in PCR reactions
173 with *Nosema* DNA isolated from different sources. We analysed different European and Taiwanese *N.*
174 *ceranae* (previously characterized and sequenced) isolates (supplied by W.F. Huang), *N. bombi*
175 (supplied by Dr. Paxton), *N. trichoplusia* strain (ATCC 30702) and from three different *N. apis*
176 isolated in our laboratory.

177 All the validations were carried out with three PCR reactions performed on three different days
178 using the parameters described above (annealing temperature at 61.8°C and 0.4 μ M of each primer).
179 The amplicon products of each PCR were analyzed by electrophoresis twice. Negative controls were
180 tested in parallel to detect possible contaminations.

181 **PCR analysis for diversity of *Nosema* species.** Once the duplex PCR had been validated, a
182 study of the presence of both *Nosema* species in samples of different origin was conducted.

183 After processing 290 samples from different European countries (Spain, France, Switzerland
184 and Germany), the 16S was amplified by using 218-MITOC-FOR / 218-MITOC-REV and 321APIS-
185 FOR / 321APIS-REV primer pairs. Duplex PCR and electrophoresis conditions were the same as those
186 described above in paragraph 2.3. Extraction and PCR negative controls were included in all PCR
187 processes.

188 Additionally, samples from France, Switzerland and Germany (three different samples from
189 each country) were selected, and 16S was amplified using MICRO-F / MICROCE-R and INTER-FOR
190 / INTER-REV pair of primers and sequenced as previously described (Higes et al., 2007).

191 **Causative association between colony signs and *Nosema* spp. presence.** The same 149
192 Spanish samples used to determine *Nosema* species diversity (Table 1) were used to establish the
193 causative association between colony signs and the presence or absence of *N. ceranae* and/or *N. apis*.
194 Each one of these samples came from a different beekeeper. A total of 53% were professional
195 beekeepers (150 - 2000 hives per beekeeper), 42.3% came from non-professional beekeepers (16 – 149
196 hives) and finally 4.7% came from self-consumption beekeepers (less than 16 hives). All these
197 beekeepers add up to 50,091 hives.

198 The term “Depopulation” was applied when samples came from dead colonies with
199 depopulation or from apiaries with large numbers of dead colonies. “Weakness” was used for colonies
200 whose beekeepers had observed low production without bee mortality, and the asymptomatic group
201 included colonies without any clinical signs in routine or controlled surveys.

202 In order to avoid bias owing to possible geographically related differences, only Spanish
203 samples were included in the causative association.

204 **Statistical analysis.** In the retrospective study, different statistical analyses have been carried
205 out (all at 95% confidence level). The Pearson Chi-square test with Monte Carlo correction (to
206 determine the exact probability) was used to evaluate the differences in frequency of *Nosema* positive
207 diagnosis in the different years studied (according to the distribution of the number of analysed samples
208 per month in each year or per year and the results of the *Nosema* diagnosis, positive vs. negative
209 samples). The Fisher exact test was used to compare differences in the distribution of positives vs.
210 negatives, presenting the total number of analysed samples in the previously determined periods.
211 Finally, differences in the number of *Nosema* positive samples in the different studied years were
212 compared using the non-parametric Kruskal-Wallis and Median tests.

213 Causative association between presence or absence of *N. ceranae* and/or *N. apis*, and the signs
214 observed in the samples (depopulation, weakness or asymptomatic), were estimated by the calculation
215 of the relative risk and its confidence interval (95% confidence level).

216 All calculations were performed with SPSS v. 13.

217 **RESULTS**

218 **Duplex PCR: checking and optimizing.** In the single gradient PCR test of both primer pairs,
219 the best PCR products were obtained when the annealing temperature was set between 55.2°C and
220 62.7°C. The temperature selected for the subsequent assays was 61.8°C because it was the highest
221 temperature at which amplicons could clearly be seen and this, consequently, reduced the unspecific
222 amplification risk. After checking different primer concentrations in the PCR mixture (0.3-0.6 µM, data
223 not shown), an equimolar concentration of 0.4 µM each was fixed to reduce primer dimerization.

224 Singleplex PCR and sequencing analyses of both strands by using the designed primers
225 (321APIS and 218MITOC), yielded the expected amplicon sizes (321 for *N. apis* and 218 for *N.*
226 *ceranae*) and sequences. Neither primer interaction (hairpin, self-dimer and hetero-dimer structures
227 among the four primers) nor undesirable amplicons were detected in agarose gels. Subsequent single
228 PCRs and sequencing performed in the 6 *N. ceranae* and 5 *N. apis* known DNA extracted samples
229 amplified the expected products.

230 **Duplex PCR: Reproducibility, Sensitivity and Reliability.** All 20 samples and controls tested
231 six times (3 PCRs and 2 electrophoresis each) showed the same results, which demonstrates the high
232 reliability of the method.

233 The sensitivity of the duplex PCR was tested individually or in a mixture with DNA extracted
234 from both microsporidia and the minimum detection limit is given in Table 3. *N. apis* specific primers
235 showed the higher sensitivity and it was always detected in DNA extracted samples (macerated diluted)
236 equivalent to 750 *N. apis* spores per reaction. In some cases, as few as 500 *N. ceranae* spores or 100 *N.*

237 *apis* spores per reaction yielded visible amplicons. *N. ceranae* spores diluted in water (PCR degree)
238 yielded similar results to those diluted in macerates of naïve honeybees. On the contrary, better results
239 were obtained when *N. apis* spores were diluted in macerates of uninfected honeybees than when they
240 were diluted in water. No differences were detected in the sensibility of the duplex PCR when the two
241 *Nosema* species were amplified either in a mixture or individually.

242 To determine the primer specificity, a number of different species or isolates of *Nosema* (*N.*
243 *ceranae* from Europe and Taiwan, *N. bombi*, *N. trichoplusiae* and *N. apis*) were tested by duplex-PCR
244 using the 218MITOC and 321APIS primer pairs. All reactions were found to be specific. The
245 218MITOC pair of primers only amplified *N. ceranae*, irrespective of the geographical area from
246 which they originated (whether Taiwan or Europe). The specific primers for *N. apis* also showed a high
247 degree of specificity and were positive only in the previously characterized samples from different
248 European countries. No amplification was obtained with *N. bombi* or *N. trichoplusiae* microsporidia.

249 **Retrospective analysis.** In the last three years (2003 to 2005) there has been a surprising
250 increase in *Nosema* spores detection in the CAR laboratory. Annually positive rates (Table 4) show the
251 increase in positive samples sent to the laboratory from 13% to 24.7% in the first three years to 95.6%
252 in the last year (Figure 1).

253 From the year 2000 onwards, statistically significant differences have been found in the
254 proportion of *Nosema* spp. spore positive samples collected, inter and intra-annually. No
255 Microsporidian species identification could be performed in these samples. Two different periods in the
256 frequency of *Nosema* positive samples were established: 1999-2002 and 2003-2005. In the first period
257 (1999-2002), the smallest number of samples diagnosed as *Nosema* positives was recorded during the
258 summer months, showing the clear seasonality characteristic of *N. apis*. However, 2003 marked a
259 change in the tendency, with an increase in the proportion of *Nosema*-positive samples in all months
260 independent of the increment in the number of samples received in the laboratory (as indicated by the
261 sorting of positive mean range and their position around the median). Finally, in 2005 no statistically

262 significant differences were found in the frequency of *Nosema*-positive samples in the different months
263 or seasons, indicating the total lack of seasonality in the diagnosis of microsporidiosis (Figure 2).

264 **Nosema species diversity.** A total of 290 samples from different European countries were
265 investigated by multiplex PCR for the presence of *N. apis* and *N. ceranae* microsporidia. Eighty-eight
266 samples (30.3 %) were negative for both *Nosema* spp. *N. ceranae* was found to be the most frequent,
267 present alone in 53.8 % of samples and detected in samples from every country studied (Table 5). *N.*
268 *apis* alone was detected in 9.3 % of the samples studied. Mixed infections were detected in 6.6 % of the
269 samples.

270 Both species were found in all the countries studied so far with different prevalence (Table 5).
271 *N. ceranae* was the most prevalent species. Most French and German samples were positive for
272 *Nosema*; no mixed infection was detected in Swiss and in French samples and *N. apis* was not detected
273 alone. *N. ceranae* was present in 75% of positive Spanish samples (mixed infection with *N.apis* 13.1%)
274 while *N.apis* was present in 38,1%.

275 The consensus sequences of isolates from Switzerland, Germany and France were deposited in
276 the GenBank database under accession numbers DQ673615, DQ374656 and DQ374655.

277 **Causative association between colony signs and *Nosema* spp. presence.** The 149 Spanish
278 samples were used to study the association between the presence of microsporidian infection and the
279 symptoms observed in hives. These samples were classified as shown in Table 6. Sixty-six samples
280 were classified as “depopulation” group and seventy-nine samples were classified as asymptomatic
281 group. “Weak colonies” were excluded from the causative association study due to the small number
282 detected (four colonies).

283 Relative risks obtained are also shown in Table 6. Those colonies in which either both species
284 or only *N. ceranae* was detected, presented a risk almost six times greater than those with negative PCR
285 results. Confidence intervals (95% level of confidence) and *p value* (significance level) indicate

286 statistical significance in the causative association. In contrast, those colonies in which only *N. apis*
287 was detected did not show any increased risk in comparison with the asymptomatic group ($p>0.05$).

288 Data also showed that in 15% of hives belonging to the “depopulation” group *N. ceranae* was
289 not detected and that 11.3% of hives positive for *N. ceranae* were asymptomatic.

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DISCUSSION

292 In this study, a change in the epidemiological pattern of nosemosis and a relationship with the
293 detection of *N. ceranae* and bee depopulation have been established. To make possible a differential
294 diagnosis possible, it has been necessary to develop a new rapid technique. The duplex PCR method
295 developed in this study has been shown to be a sensitive and specific test for the detection of honeybee
296 microsporidia. Among the many molecular techniques, PCR has been the most widely used in the
297 diagnosis of microsporidian infection and epidemiological studies. The SSUrRNA (16S) gene of many
298 microsporidia have been sequenced and have been found to diverge greatly from other eukaryotes; the
299 sequence is shorter and shares little homology with other eukaryotes (30, 35). Thus, the SSUrRNA
300 genes of the microsporidia possess suitable characteristics for molecular detection (32).

301 Until recently, detection of honeybee microsporidia has been limited to microscopic
302 examination and to differentiate between both *Nosema* spp. sequencing of PCR products is required
303 (17). Microscopic detection of these microsporidia is expensive, laborious and limited to known stages
304 of development. The multiplex PCR method to detect *N. ceranae* and *N. apis* offers a number of
305 advantages over traditional microscopy, including increased sensitivity, specificity and the ability to
306 identify all developmental stages of the parasite (31). Thus, multiplex PCR decreases the risk of
307 misidentification and will facilitate epizootiological studies related to these pathogens. Moreover, other
308 molecular methods, such as sequencing or restriction analysis of PCR products, are expensive, time-
309 consuming and involve a great deal of manipulation (which will likely increase the risk of error). The
310 duplex-PCR presented here is a reliable, rapid, cost-effective and simple method to detect *N. apis*

311 and/or *N. ceranae* infections in a single step since PCR products can be easily separated and identified
312 by agarose gel electrophoresis (with no additional treatment).

313 Multiplex PCR primer design and optimization is a greater challenge than designing singleplex
314 PCR primer pairs because multiple primer annealing events need to occur in the same annealing
315 conditions without interfering with one another. So, since compatible primers are the key to successful
316 multiplex PCR, careful primer design and appropriate quality control measurements are essential in
317 order to ensure that the PCR primers will work under uniform PCR conditions and will not adversely
318 interact with one another. Initially, singleplex reactions were tested using the primer sets (218-MITOC
319 and 321-APIS) for each *Nosema* specie (*N. ceranae* and *N. apis*, respectively) to ensure locus-specific
320 amplification. Afterwards, duplex PCR studies were carried out by means of annealing temperature
321 adjustments and empirical performance testing in an attempt to generate sensitive, balanced, and
322 specific signals for both *Nosema* species in a single PCR reaction.

323 Subsequent PCR assays confirmed the suitability of our design. The *N. apis* and *N. ceranae*
324 SSUrRNA gene-specific primers developed here successfully amplified a 218 bp fragment from *N.*
325 *ceranae* and/or 321 bp fragment from *N. apis* spores, even in such distant *N. ceranae* isolates as those
326 from Taiwan. Conversely, these primer pairs did not amplify DNA preparations from uninfected
327 honeybees or other microsporidia species such as *N. trichoplusiae* or *N. bombi*, indicating that both
328 primer pairs, 218-MITOC and 321-APIS, are specific to *N. ceranae* or *N. apis*, respectively. High
329 specificity is frequently associated with low sensitivity and to avoid this, unspecific primers have been
330 previously designed, as in the case of *N. bombi* (21). In our case, the degree of sensitivity achieved by
331 using specific primers was satisfactory as the limits of detection for both *Nosema* were consistent with
332 other studies on invertebrate microsporidia (32, 36).

333 Although previous *N. apis* specific primers have been previously described (34) this is the first
334 study of the specific and sensitive molecular diagnosis of *N. ceranae*, and the first differential diagnosis
335 of both honeybee microsporidians in just one reaction.

336 No notable differences were observed when the spores were diluted in water or in uninfected
337 honeybee macerate, which differs from the results observed with *N. bombi* (21). In our case, and with
338 regard to *N. apis* specific primers (321-APIS), the degree of sensitivity was slightly higher when spores
339 were diluted in honeybee macerate. The addition of BSA and Triton X-100 to the PCR reaction
340 probably prevents PCR inhibitory substances (that might be present in honeybee macerates) from
341 having any effect during the PCR (1, 22).

342 Data from the first studied period (1999-2002) seems to follow the seasonal pattern previously
343 described for *Nosema* infection (19). In the absence of molecular diagnosis, most studies dated in the
344 last century considered *N. apis* as the aetiological agent of the disease while describing a similar
345 epidemiology and pathology of the disease all over the world. Reports of nosemosis were related with
346 low levels of infection during summer, a generally small peak in autumn and a usually slow rise during
347 winter. These seasonal trends related to *N. apis* also included a rapid and large increase in spring as
348 brood-rearing started while flight possibilities due to climatic conditions were still limited. It is
349 generally agreed that colonies in Northern climates are more seriously affected than colonies in the
350 southern regions because of the increased amount of time that bees are confined to the hive.

351 Our data are consistent with this pattern only during the first years of the study (1999-2002).
352 However, in the period 2003-2005 the increased percentage of positive samples in summer was
353 statistically different compared with the previous period. In 2005 the absence of differences in the
354 number of positive samples between months shows an evident lack of seasonality. Recent reports (8)
355 indicate that the clinical and epidemiological pattern of nosemosis is changing.

356 *Nosema* seasonality has been related with precipitation (7), and in recent years high
357 precipitation regions have been thought to represent disease reservoirs from which *Nosema* can radiate
358 each year, so that the epizootic years are closely related with higher precipitation in these areas (3). The

359 relationship between precipitation and nosemosis is not clear in the present situation. Even when the
360 established pattern for nosemosis is detected (the first years), mean rainfall can be very different from
361 one year to another. The high rainfall peak of 2000 had no direct effect on *Nosema* prevalence, and
362 after 2004 was the driest year in sixty years, 2005 recorded the highest prevalence of *Nosema* to date
363 and showed no seasonal pattern (25). Our data strongly suggest that currently the prevalence of *Nosema*
364 is not related with precipitation in Spain.

365 *Nosema* infection levels have also been related with stress due to management systems (4).
366 Spanish manipulation of hives varies greatly according to region but in the last period of the study there
367 is no reason to consider that any significant changes have been registered on a global level.

368 The lack of seasonality detected and the increasing number of pathological samples sent to the
369 laboratory from 2003 to the present without any compatible *Nosema* signs of infection can be related
370 with the higher *N. ceranae* prevalence in our country in 2005. Some differences in the epidemiology
371 and pathology caused by both microsporidians may explain the situation.

372 The introduction of this species from Southeast Asia into Spain in recent years may explain the
373 loss of seasonality in the second period of our study. The dispersion of a parasite into a new habitat or
374 geographical area is usually a chance event and does not guarantee success in colonizing a new host or
375 in becoming established reproductively. The presence of *N. ceranae* in most European countries
376 suggests that colonization has been successful but there is still a lack of equilibrium between the new
377 parasite and the new host. *Nosema ceranae* seems to be highly pathogenic to *Apis mellifera*. High rates
378 of mortality are tied to the presence of autoinfective spores that facilitate the rapid division and
379 invasion of digestive tissue and affect the regenerative digestive cells (15). These differences can be
380 related with the recently-described higher colony losses cited during the last few years, especially at the
381 end of winter (9).

382 Direct evidence of the influence of the parasite on the colony is related to changes in the activity
383 and longevity of the worker honeybee and queen (10, 14). Infection of workers by *N.apis* retards or
384 inhibits development of the pharyngeal salivary glands (33) compromising the feeding of young larvae.
385 A fairly high proportion of the eggs laid by the queen of a colony suffering from nosemosis fail to
386 produce pupae, while artificially infected pupae are resistant to infection (14). The bee population
387 begins to decrease as *Nosema* infection spreads. Due to changes in their physiological state, infected
388 bees seem to become behaviourally older than healthy bees of the same age (33), starting foraging
389 activities at an early stage (10) and appear to have a shorter life span. But somehow *N. apis* has reached
390 an epidemiological equilibrium with the host that minimizes its effects on total bee population. This
391 equilibrium probably depends on climatic conditions and reduced transmission inside the hive in
392 summer months. *N. ceranae* do not seem to follow the seasonal pattern of *N.apis*, presenting similar
393 rates of infection throughout the whole year, which indicates that the decreasing effect on transmission
394 of the hottest months is not being effective in controlling this infection by *Apis mellifera*.

395 The relative risk of bee depopulation observed in colonies with either both species or only *N.*
396 *ceranae* (almost six times greater than those with negative PCR results), indicates a significant
397 causative association between the presence of *N. ceranae* and the development of hive depopulation.
398 The presence of *N. ceranae* in asymptomatic hives can be related with the incubation period, not
399 known at present for this species, while the existence of a few negative hives in the depopulation group
400 may be due to some other aetiology or hypothesis associated with this symptom and not included in this
401 study.

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497

498

499 **LEGENDS TO FIGURES**

500

501 Figure 1: Percentage of positives samples to *Nosema* spores since 1999 up to 2005 year in the Bee
502 Pathology laboratory.

503 Figure 2: Monthly *Nosema* positives samples distribution in every year (1999-2005) by calculus of
504 *Nosema* spores positive frequency respect the total number of samples received in the Bee
505 Pathology laboratory.

Table 1: Samples used to study the diversity of *Nosema* species.

Country	Source	Number of samples	Date	Observations
	Veterinary			Sent to CAR laboratory for different pathological problems
Spain	Services and Beekeepers,	149	June 2005 to December 2006	(depopulation, weakness or asymptomatic). *These samples were also used for the causative study
France	Dr. Borneck	6	February to May 2006	Apiaries from Jura region Collected on dead hives
	Dr Fortini & Dr. Odoux	30	April to June 2006	Apiary of Experimental Centre in Surgères High bee colonies mortality and weakness Seven controlled apiaries.
Switzerland	Dr. Imdorf	36	March 2006	Collected on dead hives and colonies without abnormal findings
Germany	Dr. Ritter	69	2003, 2005 and 2006	Different German regions
TOTAL		290		

Table 2: Primers selected for detection of *Nosema ceranae* and *Nosema apis* in multiplex PCR. C / G tails added to primers are underlined. *There is a base pair (bp) difference in the *N. ceranae* amplicon size depending on the sequences for *N. ceranae* available at GenBank (<http://www.ncbi.nlm.nih.gov>).

Name		Sequence	PCR-product size	Specificity level
218MITOC	FOR	5'- <u>CGGCG</u> ACGATGTGATATGAAAATATTAA-3'	218-219* bp	<i>N. ceranae</i>
	REV	5'- <u>CCCGGT</u> CATTCTCAAACAAAAAACCG-3'		
321APIS	FOR	5'- <u>GGGGG</u> CATGTCTTTGACGTACTATGTA-3'	321 bp	<i>N. apis</i>
	REV	5'- <u>GGGGGG</u> CGTTTAAAATGTGAAACA ACTATG-3'		

Table 3: Sensitivity of the polymerase chain reaction with 218MITOC (FOR/REV) and 321APIS (FOR/REV) primers from spores diluted in water or uninfected honeybee (UHB) macerated. All samples were analysed by means of three PCR reactions performed on three different days and the amplicon products of each PCR were separated by electrophoresis twice.

Spores per 100 μ L		$3 \cdot 10^6$	10^6	10^5	$8 \cdot 10^4$	$6 \cdot 10^4$	$4 \cdot 10^4$	$2 \cdot 10^4$	$1.5 \cdot 10^4$	10^4	$5 \cdot 10^3$	$2 \cdot 10^3$	$2 \cdot 10^2$	20	UHB macerated	Water
Spores per reaction		$15 \cdot 10^4$	$5 \cdot 10^4$	$5 \cdot 10^3$	$4 \cdot 10^3$	$3 \cdot 10^3$	$2 \cdot 10^3$	10^3	750	500	250	100	10	1	-	-
218 MITOC	Water diluted*	6/6	6/6	6/6	6/6	6/6	6/6	5/6	5/6	3/6	0/6	0/6	0/6	0/6	0/6	0/6
	UHB diluted*	6/6	6/6	6/6	6/6	6/6	6/6	3/6	1/6	1/6	0/6	0/6	0/6	0/6	0/6	0/6
321APIS	Water diluted*	6/6	6/6	6/6	6/6	6/6	2/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
	UHB diluted*	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	5/6	4/6	2/6	0/6	0/6	0/6	0/6

*Number positive/Number tested

Table 4: Number of samples positives to *Nosema* spores

Year	1999	2000	2001	2002	2003	2004	2005
% positives	13.0	9.7	24.7	23.5	54.5	89.0	95.6
N	154	124	146	443	484	3002	1423

Table 5: *Nosema* species screening. Percentage is indicated in brackets.

	Total	Number of samples screened			
		Spain	France	Switzerland	Germany
Negative	88 (30.3%)	65 (43.9 %)	3 (8.3 %)	12 (33.3 %)	8 (11.6%)
<i>N. ceranae</i> positive alone	156 (53.8%)	52 (34.9 %)	27 (75 %)	23 (63.9%)	54 (78.3%)
<i>N. apis</i> positive alone	27 (9.3%)	21 (14.1 %)	0 (0 %)	1 (2.8 %)	5 (7.2%)
<i>N. apis</i> and <i>N.ceranae</i>	19 (6.6%)	11 (7.4 %)	6 (16.7 %)	0 (0 %)	2 (2.9%)
Number of samples	290	149	36	36	69

Table 6: Association between the detection of Microsporidian infection in Spanish honey bee samples and the signs observed in hives. Relative Risk (RR), Confidence Interval (IC), Probability (p).

PCR Result	Depopulation Group	Asymptomatic Group	Total	RR	IC (95%)	p
<i>N. apis</i> + <i>N. ceranae</i>	10	1	11	5,82	3,20 - 10,59	0,0000
<i>N. ceranae</i>	44	8	52	5,42	3,03 - 9,68	0,0000
<i>N. apis</i>	2	16	18	0,71	0,17 - 2,92	0,4869
Negative	10	54	64	-	-	-
Total	66	79	145			

