Journal of Invertebrate Pathology 130 (2015) 165–168

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Short Communication

High prevalence and infection levels of *Nosema ceranae* in bumblebees *Bombus atratus* and *Bombus bellicosus* from Uruguay

N. Arbulo [a,*], K. Antúnez [b], S. Salvarrey [c], E. Santos [c], B. Branchiccela [b], R. Martín-Hernández [d], M. Higes [d], C. Invernizzi [c]

[a] Centro Universitario Regional del Este, Universidad de la República, Rocha, Uruguay
[b] Departamento de Microbiología, Instituto de Investigaciones Biológicas Clemente Estable, Montevideo, Uruguay
[c] Sección Etología, Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay
[d] Centro Apícola Regional, Junta de Comunidades de Castilla La Mancha, 19180 Marchamalo, Spain

ARTICLE INFO

Article history:
Received 20 January 2015
Revised 16 July 2015
Accepted 31 July 2015
Available online 3 August 2015

Keywords:
Microsporidia
Parasites
Nosemosis

ABSTRACT

Nosema ceranae is one of the most prevalent pathogens in *Apis mellifera* and has recently been found in multiple host species including several species of bumblebees. Prevalence and infection intensity of *N. ceranae* was determined in two species of native bumblebees from Uruguay. *Nosema ceranae* was the only microsporidia identified and mean prevalence was 72% in *Bombus atratus* and 63% in *Bombus bellicosus,* values much higher than those reported elsewhere. The presence of this pathogen in bumblebees may be threatening not only for bumblebee populations, but also to the rest of the native pollinator community and to honeybees.

1. Introduction

Nosema ceranae is one of the most prevalent pathogens in *Apis mellifera* worldwide (Higes et al., 2013). Originally described in *Apis cerana* (Fries et al., 1996), in 2006 was found as an emergent pathogen in the European honey bee (Higes et al., 2006) and currently it is found in multiple host species (Plischuk et al., 2009; Chaimanee et al., 2011; Li et al., 2012; Graystock et al., 2013). This wide host range is of significant epidemiological and ecological concern, since some parasite can be transmitted between host individuals that share flower resources (Durrer and Schmid-Hempel, 1994).

Bumblebees host a great variety of parasites (Schmid-Hempel, 2001). Until the work of Plischuk et al. (2009), *Nosema bombi* was the only microsporidia known to infect bumblebees (Tay et al., 2005). At present, *N. ceranae* has been detected in three South American (Plischuk et al., 2009), four Asian (Li et al., 2012) and seven European (Graystock et al., 2013) species. Recent studies confirmed that bumblebees can be genuinely infected by *N. ceranae* and may not be simply accidental carriers of its spores (Graystock et al., 2013; Fürst et al., 2014).

The aim of this study was to determine the presence, prevalence and infection intensity of *N. ceranae* in *Bombus atratus* and *Bombus bellicosus* from Uruguay.

2. Methods

2.1. Bumblebee collection

A total of 526 bumblebees, 317 *B. atratus* (167 females, 150 males) and 209 *B. bellicosus* (141 females, 68 males), were collected in April 2010 while foraging on red clover (*Trifolium pratense*) in three locations: Montevideo (34°50′S, 56°13′W), Canelones (34°40′S, 56°02′W) and Colonia (34°20′S, 57°41′W). Individual bees were collected directly from flowers and were stored at −20 °C until analysis.

2.2. Identification of Nosema species

Abdomens of bumblebees were individually macerated with 2 ml of 95° ethanol. Macerates were centrifuged and washed to remove ethanol and pellets were resuspended in 250 µl of sterile distilled water. One hundred and fifty µl of each sample were introduced into a 96-well plate (Qiagen) with glassbeads (2 mm, Sigma) and plates were shaken for 6 min at 30 Hz. Thirty µl of ATL buffer (Qiagen 19076) and 20 µl of Proteinase K (Qiagen 19131) were added to each well and plates were incubated

* Corresponding author at: Centro Universitario Regional del Este – Sede Rocha, Ruta Nacional N° 9 intersección Ruta Nacional N° 15, Rocha 27000, Uruguay.
E-mail address: arbulonatalia@gmail.com (N. Arbulo).

http://dx.doi.org/10.1016/j.jip.2015.07.018
0022-2011/

overnight at 56 °C. Subsequently, DNA was extracted using the BS96 DNA Tissue extraction protocol in a BioSprint apparatus (Qiagen).

The presence of *N. ceranae* and *N. apis* was determined using *Nosema* Biotools DNA Polymerase plates containing PCR triplex (321-APIS-F/R, 218-MITOC-F/R and COI-F/R primers; Martín-Hernández et al., 2012) master mix. Reactions were carried out in a total volume of 50 µl and the cycling program was: 94 °C (6 min); 35 cycles of 30 s denaturation at 94 °C, 45 s annealing at 61.8 °C and 60 s extension at 72 °C; and a final extension step at 72 °C for 10 min.

PCR for *N. bombi* detection was performed in a total volume of 25 µl using the Fast Start PCR Master mix (No. 04710452001 Roche Diagnostic), 0.4 mM of each primer, 0.2 mg ml-1 BSA, 0.1% Triton X-100 and 2.5 µl of DNA template. The cycling program was: 95 °C (10 min); 35 cycles of 30 s denaturation at 95 °C, 30 s annealing at 58.1 °C and 45 s extension at 72 °C; and a final extension step at 72 °C for 7 min.

Negative controls for DNA extraction and PCR were analyzed in parallel. PCR reactions were carried out in a Mastercycler® ep gradient S apparatus (Eppendorf) in Centro Apícola Regional de Marchamalo (Spain). PCR products were analyzed in a QIAxcel System (Qiagen) using a QIAxcel DNA High Resolution Kit (Qiagen, No. 929002).

Prevalence was calculated as the percentage of positive samples for each analyzed group. Chi square test with a 0.05 significance level was used to evaluate intraspecific differences between sexes and interspecific differences within sexes.

2.3. Spore counting

Infection intensity (spores per bumblebee) was determined in positive samples. Spore count was done using a haemocytometer and a light microscope at 400×. Number of spores in known volume was used to calculate spores per bumblebee. Spore detection limit was calculated at 12,500 spores per bumblebee, which corresponded to finding one spore in the total volume analyzed.

Infection intensity categories were defined for the analysis: very low (<12,500 spores), low (12,500 to 5×10^5 spores), medium ($5 \times 10^5 + 1$ to 25×10^5 spores) and high (more than 25×10^5 spores). The Mann–Whitney *U* test with a 0.05 significance level, was performed to compare infection level between the analyzed groups.

3. Results

3.1. Nosema identification and prevalence

A total of 364 samples were positive for *N. ceranae* which was the only microsporidia species detected. Prevalence was equal or higher than 50% in different locations and sexes, with two exceptions (Fig. 1).

There were intraspecific differences on prevalence between location (*B. atratus:* females $\chi^2 = 3.40$, $P = 0.182$, males $\chi^2 = 20.81$, $P < 0.001$; *B. bellicosus*: females $\chi^2 = 11.35$, $P < 0.001$, males $\chi^2 = 5.40$, $P = 0.02$) so the analysis was performed for each location separately.

Prevalence in *B. atratus* was higher in females (Montevideo: $\chi^2 = 35.26$, $P < 0.001$; Canelones: $\chi^2 = 7.86$, $P = 0.005$; Colonia: $\chi^2 = 19.32$, $P < 0.001$) while in *B. bellicosus* there was no difference between sexes (Canelones: $\chi^2 = 0.02$, $P = 0.882$; Colonia: $\chi^2 = 0.03$, $P = 0.856$) (Fig. 1).

Prevalence in females was higher in *B. atratus* than in *B. bellicosus* (Canelones: $\chi^2 = 24.80$, $P < 0.001$; Montevideo: $\chi^2 = 6.46$, $P = 0.011$). Regarding males, *N. ceranae* was more prevalent in *B. atratus* at Canelones ($\chi^2 = 5.36$, $P = 0.021$) but there was no interspecific difference at Colonia ($\chi^2 = 3.46$, $P = 0.063$).

3.2. N. ceranae infection intensity

Infection intensity was determined in 293 *N. ceranae* positive samples. Not all positive samples ($N = 364$) could be microscopically analyzed due to insufficient amount of material left in 71 samples after molecular analyses.

Spores were detectable in 246 samples (84%). Infection intensity reached loads as high as 44 million spores per individual. In 47 samples (16%) spores could not be detected microscopically, indicating that the number of spores was under the detection limit (12,500 spores per bumblebee).

Infection intensity differed between sexes, being higher in females in both species (*B. atratus:* Montevideo: $U = 133$, $P < 0.001$; Canelones: $U = 733.5$, $P < 0.001$; Colonia: $U = 164.5$, $P < 0.001$. *B. bellicosus:* Canelones: $U = 11$, $P < 0.001$; Colonia: $U = 365.5$, $P < 0.005$) (Fig. 2).

There were no interspecific differences on infection intensity between females at Canelones ($U = 314.5$, $P = 0.209$) or between males at Colonia ($U = 227.5$, $P = 0.312$). There were differences between males at Canelones, being *B. atratus* the most infected ($U = 57.5$, $P < 0.001$) but sample size of *B. bellicosus* was low. At Colonia there were differences between females with *B. atratus* also having higher infection intensity ($U = 915$, $P = 0.042$).

4. Discussion

We presented the detection of *N. ceranae* in native *B. atratus* and *B. bellicosus* from Uruguay. Since *N. ceranae* in *A. mellifera* is widely distributed in Uruguay since the nineties (Invernizzi et al., 2009; Anido et al., in press), bumblebees might have acquired spores through their close contact with honey bees like Plischuk et al. (2009) suggested for Argentina.

Mean prevalence values of *N. ceranae* found in *B. atratus* and *B. bellicosus* (72% and 63%, respectively) are much higher than those reported in other countries for the same (Plischuk et al., 2009) or other bumblebee species (Li et al., 2012; Graystock et al., 2013; Fürst et al., 2014). The high prevalence could reflect the rapid spread of a recent invasion of *N. ceranae* to Uruguayan bumblebees. Nevertheless, high prevalence does not necessarily mean that an epizootic is occurring (Cordes et al., 2012) and alternatively, *B. atratus* and *B. bellicosus* may be natural reservoirs of the pathogen supporting high populations of *N. ceranae*.

The location-specific differences found on prevalence indicate that not all populations may be equally affected as has already been suggested for other parasites in different bumblebee species (Cordes et al., 2012).

The same pathogen (e.g. *N. bombi* or *Crithidia* spp.) could generate infection with different characteristics (prevalence or intensity) in different bumblebee species (Cordes et al., 2012). Here, interspecific differences were found in prevalence of *N. ceranae* mainly in females and there were also some interspecific differences on spore loads.

On the other hand, species infection intensity was lower for males in both bumblebee species. Sex effect at field conditions have been reported for various parasites in several bumblebee species although there appears to be no general pattern (review in Ruiz-Gonzalez and Brown (2006), Murray et al. (2013)). The lower infection intensity found in males may be due to higher resistance that prevents infection growing further or to highly infected males