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SHORT COMMUNICATION

First data on the prevalence and distribution of pathogens in bumblebees (*Bombus terrestris* and *Bombus pascuorum*) from Spain

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Abstract

Bumblebees provide pollination services not only to wildflowers but also to economically important crops. In the context of the global decline of pollinators, there is an increasing interest in determining the pathogen diversity of bumblebee species. In this work, wild bumblebees of the species *Bombus terrestris* and *Bombus pascuorum* from northern and southern Spain were molecularly screened to detect and estimate prevalence of pathogens. One third of bumblebees were infected: while viruses only infected *B. pascuorum*, *B. terrestris* was infected by *Apicystis bombi*, *Crithidia bombi* and *Nosema bombi*. Ecological differences between host species might affect the success of the pathogens biological cycle and consequently infection prevalence. Furthermore, sex of the bumblebees (workers or males), sampling area (north or south) and altitude were important predictors of pathogen prevalence. Understanding how these factors affect pathogens distribution is essential for future conservation of bumblebee wild populations.

Additional key words: pollinators; pathogen dispersion; PCR; *Apicystis bombi; Crithidia bombi; Nosema bombi*.

Abbreviations used: AKI (Acute bee paralysis, Kashmir bee and Israeli acute paralysis viruses complex); BQCV (Black Queen Cell Virus); DWV (Deformed Wing Virus); LSV (Lake Sinai Virus); PCR (Polymerase Chain Reaction); RT-PCR (Real Time PCR).

Authors' contributions: Conceived and designed the experiments: CO, RMH, MH and PDLR. Performed the experiments: CJU and RMH. Performed the statistical analyses: CJU and EB. Contributed taxonomic analysis: CO. Wrote the paper: CJU, RMH, CO, MH, EB, PDLR.

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Introduction

Pollination is important for ecological processes and worldwide agricultural productivity (Potts *et al.,* 2016). Several causes of global pollinator decline have been described, including climate change, pathogens, habitat loss and pesticides (Goulson *et al.,* 2015).

Among pollinators, bumblebees (*Bombus* Latreille, 1802) provide pollination services not only to wildflowers but also to economically important crops; for this, some species are reared and commercialized for greenhouse production (Ortiz-Sánchez, 1992; Ornosa, 1996; Velthuis & van Doorn, 2006). Several pathogens (*Crithidia* Léger, 1902: Trypanosomatidae, *Apicystis* (Lipa & Triggiani, 1996): Neogregarine, *Nosema* Nägueli, 1857: Microsporidia, and viruses) are related to the decline of bumblebee species, and their prevalence has been monitored elsewhere but not in the Iberian Peninsula (Williams & Osborne, 2009; Cameron *et al*., 2011; Meeus *et al.,* 2011; Gallot-Lavallée *et al*., 2016). To address this knowledge gap,

samples of the most widely distributed species *Bombus terrestris* (Linnaeus, 1758) and *Bombus pascuorum* (Scopoli, 1763) were collected to map pathogen prevalence. We hypothesized that infection rates differ between northern and southern Spain, between species and between sexes.

Material and methods

Worker and male bumblebees were sampled from 19 locations in Spain: either from a southern mountain region (Sierra Nevada National Park, Granada) at altitudes between 1041 and 2752 m a.s.l., where conservation and protection measures of fauna and flora are implemented, or in northern human-altered landscapes (mainly edges of agricultural fields, river banks and urban gardens) between 63 and 1031 m a.s.l (Table 1).

Bombus terrestris and *B. pascuorum* species remain abundant in the study area, and are not considered threatened according to the International Union for Conservation of Nature (Rasmont *et al.,* 2015). Individuals were sampled with nets while foraging on flowers during August 2013 with dry weather (above 18 ºC) and clear weather conditions, and preserved in 100% ethanol at – 4 ºC.

Every sampled bumblebee was dissected to remove the genitalia and only the remaining abdominal tissues (gut and fat body) were used for DNA extraction following the Chelex method (Scriven *et al.,* 2013). RNA was isolated with RNase-Free DNase kit (Quiagen) and transformed in cDNA with QuantiTec reverse transcription kit (Quiagen) following manufacturer's instructions.

Bumblebee species identification was confirmed through morphological and sequence analyses (Murray *et al*., 2008).

Table 1. Sampling information of Spanish bumblebees (M = male and F = female worker). Mean temperatures during August at each location were taken from Ninyerola *et al*. (2007).

Area	Locality (municipality, location)	Altitude (m)	Latitude	Longitude	Temp (ºC)	Species	M	F
North	Vitoria, garden	543	42.8433	-2.6698	18.8	*B. terrestris*	3	0
	San Sebastián, Monte Urgull	63	43.3215	-1.9878	19.2	*B. terrestris*	1	0
						B. pascuorum	0	2
	Hontoria del Pinar, Cañón del Río Lobos	1023	41.7677	-3.0595	19.2	*B. terrestris*	1	0
	Soria, bank of river Duero*	1010	41.7678	-2.4529	19.7	*B. terrestris*	2	9
						B. pascuorum	1	12
	Santo Domingo de Silos, coomb La Yecla	1031	41.9512	-3.4397	19.3	*B. terrestris*	0	1
						B. pascuorum	0	1
	San Millán de la Cogolla, parking	733	42.3210	-2.8595	18.6	*B. pascuorum*	0	3
	Santo Domingo de la Calzada, garden	641	42.4381	-2.9542	19.9	*B. terrestris*	3	0
	Markina-Xemein, parking	77	43.2698	-2.4917	19.4	*B. terrestris*	0	1
	Hondarribia, Mount Jaizkibel	190	43.3588	-1.8206	20.2	*B. pascuorum*	0	1
South	Güejar Sierra, road A-395	1818	37.1175	-3.4427	21.7	*B. terrestris*	3	0
	Sierra Nevada, southern Alpujarra	1943	36.9591	-3.3378	19	*B. terrestris*	9	1
						B. pascuorum	0	1
	Monachil, Loma de S. Juan	2752	37.0739	-3.3738	16.2	*B. terrestris*	0	10
	Monachil, Hoya de la Mora	2507	37.0894	-3.3855	16.7	*B. terrestris*	1	9
	Monachil, Hoya de la Mora road	2646	37.0844	-3.3757	17.4	*B. terrestris*	0	10
	Güejar Sierra, Vereda de la Estrella (1)	1381	37.1230	-3.3569	21.8	*B. terrestris*	2	1
						B. pascuorum	1	2
	Güejar Sierra, Vereda de la Estrella falls	1337	37.1234	-3.3565	22	*B. terrestris*	2	1
						B. pascuorum	4	0
	Güejar Sierra, Vereda de la Estrella (2)	1355	37.1242	-3.3675	20	*B. terrestris*	5	0
	Güejar Sierra, Vereda de la Estrella (3)	1184	37.1338	-3.3880	22.6	*B. terrestris*	2	1
						B. pascuorum	1	1
	Güejar Sierra, road GR-3200	1041	37.1407	-3.4247	21.9	*B. terrestris*	4	1

* One *B. pascuorum* individual could not be sex-determined and was excluded from the statistical analyses.

To amplify the fragment of the 18S rDNA gene of *Crithidia* spp. and *Apicystis* spp., the reaction mixture consisted of a triplex PCR reaction including Apidae primers (as DNA extraction and amplification control for *Bombus* species) (Meeus *et al*., 2010). To amplify *Nosema bombi* (Fantham & Porter, 1914) the reaction mixture consisted of a duplex PCR also including the Apidae primers. For *Nosema apis* (Zander 1909) and *Nosema ceranae* (Fries *et al*., 1996) BioTools amplification plates were used (Martín-Hernández *et al*., 2007), consisting of a prepared microplate with gelled enzymes and primers to which only water and the DNA sample need to be added. To amplify viruses, the reaction mixtures were performed following Ravoet *et al*. (2013) for Lake Sinai Virus (LSV), Francis & Kryger (2012) for AKI (Acute bee paralysis, Kashmir bee and Israeli acute paralysis viruses complex) and Chantawannakul *et al*. (2006) for Deformed Wing Virus (DWV) and Black Queen Cell Virus (BQCV). For detecting BQCV and DWV virus, cDNA amplification was performed with a RT-PCR, using as internal control Apidae primers from Meeus *et al*. (2010). Detailed information about primer and probe sequences are provided in Table 2.

PCR reactions were run for 10 min at 95 ºC and followed by 35 cycles of denaturing at 95 ºC for 30 s, specific annealing temperature for 30 s, extending at 72 ºC for 45 s, and final extension of 7 min at 72 ºC. Positive (DNA of the pathogen) and negative (water instead of DNA) controls were included in each PCR reaction.

To verify that amplicons (those from the bumblebees for species confirmation, and from the pathogens) corresponded to the target organisms, positive samples were sequenced and compared with sequences uploaded in the NCBI through the BLAST tool in MEGA v6 (Tamura *et al*., 2013).

Prevalence of infection was estimated as a percentage of the number of infected divided by the total number of individuals. Proportions were compared using Yates-corrected chi-squared test, or when appropriate, Fisher´s exact test. Medians were compared with the

Table 2. Primers (F: forward; R: reverse; T: probe), oligonucleotide sequences of the primers, annealing temperature (Ta), expected size of the fragments (bp: base pairs) used for PCR and RT-PCR amplification of the genes from the different organisms.

Target DNA	Primer	Sequence (5' – 3')	Ta (ºC)	Fragment size (bp)	Reference
Bombus spp.	cox1 F	ATAATTTTTTTTATAGTTATA	45	1064	Murray *et al*., 2009
	cox1 R	GATATTAATCCTAAAAAATGTTGAGG			
Apidae spp.	Apidae F	AGATGGGGGATTCGTATTG	57	130	Meeus *et al*., 2010
	Apidae R	ATCTGATCGCCTTCGAACCT			
N. bombi	Bombi F	GGCCCATGCATGTTTTTGAAGATTATTAT	57	101	Plischuk *et al*., 2009
	Bombi R	CTACACTTTAACGTAGTTATCTGCGG			
N. ceranae	218 CER F	CGGCGACGATGTGATATGAAAATATTAA	61.8	218–219	Martín-Hernández *et al*., 2007
	218 CER R	CCCGGTCATTCTCAAACAAAAAACCG			
N. apis	321 APIS F	GGGGGCATGTCTTTGACGTACTATGTA	61.8	321	Martín-Hernández *et al*., 2007
	321 APIS R	GGGGGCATGTCTTTGACGTACTATGTA			
Crithidia spp.	SE F	CTTTTGGTCGGTGGAGTGAT	57	417	Meeus *et al*., 2010
	SE R	GGACGTAATCGGCACAGTTT			
Apicystis spp.	Neo F	CCAGCATGGAATAACATGTAAGG	57	260	Meeus *et al*., 2010
	Neo R	GACAGCTTCCAATCTCTAGTCG			
LSV	LSVdeg F	GCCWCGRYTGTTGGTYCCCCC	60	578	Ravoet *et al*., 2013
	LSVdeg R	GAGGTGGCGGCGCSAGATAAAGT			
AKI	AKI F	CTTTCATGATGTGGAAACTCC	59	101	Francis & Kryger, 2012
	AKI R	AAACTGAATAATACTGTGCGTA			
DWV	DWV958 F	CCTGGACAAGGTCTCGGTAGAA	60	400	Chantawannakul *et al*., 2006
	DWV9711 R	ATTCAGGACCCCACCCAAAT			
	DWV9627 T	CATGCTCGAGGATTGGGTCGTCGT			
BQCV	BQCV8195 F	GGTGCGGGAGATGATATGGA	60	350	Chantawannakul *et al*., 2006
	BQCV8265 R	GCCGTCTGAGATGCATGAATAC			
	BQCV8217 T	TTTCCATCTTTATCGGTACGCCGCC			

non-parametric Kruskall-Wallis test. The relationship between infection with any pathogen or with *Crithidia* spp. and *Apicystis* spp. and explanatory variables was further explored using mixed logistic regression analysis to correct for spatial autocorrelation. Infection status (infected or non-infected) was the response variable. Explanatory variables including sex (female worker or male), environmental temperature, altitude, geographical distribution (north and south) and bumblebee species (*B. terrestris* and *B. pascuorum*) were fitted as fixed effects, and location was included as a random effect. A backward selection strategy was used to select explanatory variables, starting with a model that included all variables. Due to the high correlation between explanatory variables (for example altitude and sampling area), different combinations of models with two variables were finally compared using Akaike´s Information Criteria to select the model with the lowest value. Models were estimated using the maximum likelihood estimation method and significance was set at $p<0.05$ for a double-sided test.

Results and discussion

A total of 115 bumblebees were sampled: 83 *B. terrestris* and 32 *B. pascuorum*. Bumblebee species abundance differed between regions: 75% *B. terrestris* came from the south, while 68% *B. pascuorum* were from the north ($p<0.05$). The sex ratio was similar for *B. terrestris* individuals, and 72% of *B. pascuorum* individuals were females ($p<0.05$).

Alignment of the amplicons with sequences FN546181 and FN546182 (Meeus *et al.,* 2010) confirmed the presence of *Crithidia bombi* (Lipa & Triggiani 1988) and *Apicystis bombi* (Liu *et al*., 1974). Pathogens distribution is described in Table 3. Both protozoa were present in 37% *B. terrestris* and in none *B. pascuorum*. The estimated prevalence (95% CI) for *B. terrestris* was 15.7% (7.8-23.5%), for *C. bombi* and 24.1% (14.9-33.3%) for *A. bombi,* respectively. Two individuals were infected by both pathogens. Prevalence of both protozoans was significantly higher in males than in female workers, and *A. bombi* was more frequent in the south and *C. bombi* in lower altitude areas ($p<0.05$). Logistic regression models corroborated the relationship between protozoa infection, sex and area (north and south) or altitude. The risk of infection was greatest in middle range altitude ($p<0.05$).

Of the three *Nosema* species, only one *B. terrestris* male from the north was infected with *N. bombi*. Viruses were only found in *B. pascuorum* and with low prevalence: two workers were infected with DWV (one southern and one northern), and three were infected with BQCV (one male and two workers from the north). No bumblebees were infected with LSV or AKI.

Overall, pathogens (protozoa, *N. bombi* and bumblebee viruses) were detected in 34% of sampled bumblebees; 16% of *B. pascuorum* and 37% of *B. terrestris* ($p<0.05$). Pathogen prevalence was significantly greater in males than in females ($p<0.05$), and did not differ according to environmental temperature or altitude, although it was numerically greatest in *B. terrestris* males, in low altitudes and warmer places ($p>0.05$). There was no evidence for significant variation in infection risk according to location.

This study shows that pathogen prevalence in two bumblebee species in Spain varies depending on host and pathogen species, sex and area, although in general the level of pathogens was low. Prevalence of infection

Table 3. Pathogen prevalence: percentage of PCR-positive individuals and 95% confidence interval (CI) in bumblebees from Spain per species, area (N = northern and S = southern provinces) and sex (F = female worker and M = male). BQCV refers to Black Queen Cell Virus and DWV to Deformed Wing Virus.

Bumblebee species	N	*Crithidia bombi*		*Apicystis bombi*		*Nosema bombi*		BQCV		DWV	
		%	CI	%	CI	%	CI	%	CI	%	CI
*B. pascuorum*_N_F	20	0.0	0.0-0.0	0.0	0.0-0.0	0.0	0.0-0.0	10.0	0.0-23.2	5.0	0.0-14.6
*B. pascuorum*_N_M	1	0.0	0.0-5.9	0.0	0.0-0.0	0.0	0.0-0.0	100.0	100-100	0.0	0.0-0.00
*B. pascuorum*_S_F	4	0.0	0.0-0.0	0.0	0.0-0.0	0.0	0.0-0.0	0.0	0.0-0.0	25.0	0.0-67.4
*B. pascuorum*_S_M	6	0.0	0.0-0.0	0.0	0.0-0.0	0.0	0.0-0.0	0.0	0.0-0.0	0.0	0.0-0.00
Total *B. pascuorum*	31	0.0	0.0-0.0	0.0	0.0-0.0	0.0	0.0-0.0	9.4	0.0-19.5	6.3	0.0-14.6
*B. terrestris*_N_F	11	0.0	0.0-0.0	0.0	0.0-0.0	0.0	0.0-0.0	0.0	0.0-0.0	0.0	0.0-0.0
*B. terrestris*_N_M	10	0.0	0.0-0.0	0.0	0.0-0.0	10.0	0.0-28.6	0.0	0.0-0.0	0.0	0.0-0.0
*B. terrestris*_S_F	34	5.9	0.0-13.8	20.6	7.0-34.2	0.0	0.0-0.0	0.0	0.0-0.0	0.0	0.0-0.0
*B. terrestris*_S_M	28	17.9	3.7-32.0	39.3	21.2-57.4	0.0	0.0-0.0	0.0	0.0-0.0	0.0	0.0-0.0
Total *B. terrestris*	83	15.7	7.8-23.5	24.1	14.9-33.3	1.2	0.0-3.6	0.0	0.0-0.0	0.0	0.0-0.0

was higher in *B. terrestris* than in *B. pascuorum*, however, the former had protozoa and a microsporidium infection, and the latter only carried viruses.

A. bombi showed the highest prevalence and appeared exclusively in southern *B. terrestris*. This neogregarine has been recently detected in other *Bombus* species (Gamboa *et al.,* 2015), but its dispersion has been reported mainly due to managed bumblebees such as *B. terrestris* (Graystock *et al.,* 2014). In Spain, the extensive use of this species for greenhouse tomato pollination may have contributed to the spread of this pathogen.

The second most common parasite in *B. terrestris* was *C. bombi* as in UK (Goulson *et al.,* 2012). Trypanosomatids have rapid adult-adult transmission linearly related to host density (Goulson *et al.,* 2012), occurring either in sympatric or allopatric populations (Meeus *et al.,* 2011). They can be transmitted by direct contact or indirectly by dispersal onto flowers (Graystock *et al.,* 2015). *B. terrestris* has larger nests than *B. pascuorum* (Goulson *et al.,* 2012), which could facilitate faster transmission. In this study, males showed higher infection prevalence than workers, which may be explained by biological reasons: males live mainly outside the nests, and are more exposed to pathogen spillover from other pollinator species (Goulson, 2010).

Regarding microsporidia, *N. bombi* was the only species detected in *B. terrestris*. This pathogen has also been found in the UK (Goulson *et al*., 2012), but in Spain has not yet been reported. Notably, *N. ceranae* was not present in our sample, despite its high prevalence in bumblebees from UK (Graystock *et al*., 2013b) and in honeybees in Spain (Martín-Hernández *et al*., 2007).

As in other studies (Singh *et al.,* 2010; Meeus *et al.,* 2011), LSV and AKI-complex viruses did not appear in Spanish bumblebees albeit BQCV and DWV have been found with a low prevalence only in *B. pascuorum*. DWV is highly widespread among bumblebees in Europe, and has been found in *B. terrestris* and *B. pascuorum* living nearby honeybee colonies in Germany (Genersch *et al.,* 2006). Given that we did not account for honeybee colony density in the sampling areas, a cause-effect of this low prevalence cannot be assessed.

Two individual bumblebees were infected by more than one pathogen, suggesting individual differences regarding susceptibility to parasitic infection, or that pathogens can act synergistically (Whitehorn *et al.,* 2011). The higher prevalence of pathogens in *B. terrestris* might reflect a different resistance and susceptibility to infections between species. Alternatively, protozoa virulence in *B. terrestris* may be lower compared to *B. pascuorum* (Goulson *et al.,* 2012). Moreover, *B. terrestris* emerges early in the spring, whereas *B. pascuorum* emerges later (Ornosa & Ortiz-Sánchez, 2004; Ploquin *et al*., 2013). We conclude that this ecological difference between host species might affect the success of the parasite's biological cycle and consequently infection prevalence.

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