

Apicystis bombi (Apicomplexa: Neogregarinorida) parasitizing *Apis mellifera* and *Bombus terrestris* (Hymenoptera: Apidae) in Argentina

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Summary

The neogregarine *Apicystis bombi* is considered a low prevalence parasite of *Bombus* spp. Before our work it has only once been detected in one single specimen of the Western honeybee *Apis mellifera*. This contribution reports the presence of *A. bombi* parasitizing both *A. mellifera* and *Bombus terrestris* at a site in Northwestern Argentine Patagonia (Bariloche, close to the border with Chile) and analyses its possible absence in the Pampas region, the most important beekeeping region of the country. In Bariloche, prevalence of *A. bombi* in *A. mellifera* was 7.6% in 2009, and 13.6% in 2010, whereas in *B. terrestris* it was 12.1%. Infections were not detected in 302 bee hives periodically prospected along 3 years (almost 400 000 honeybee specimens) in the Pampas. Analysis with the probability program FreeCalc2 suggested a possible absence of *A. bombi* in this area. Because of high virulence showed in several species of *Bombus* in the Northern hemisphere, *A. bombi* should be closely monitored in *A. mellifera* and in native *Bombus* species or other Apidae.

Introduction

The neogregarine *Apicystis bombi* has been recorded in nearly 20 species of *Bombus* worldwide (Liu *et al.*, 1974; Macfarlane *et al.*, 1995; Lipa and Triggiani, 1996; Baer and Schmid-Hempel, 2001; Rutrecht and Brown, 2008). It is considered to cause serious physical (disruption of adipose tissue) and behavioural (reduced success in

colony establishment, increased mortality rates of workers, communication conflicts between queen and workers) effects in bumblebees (Schmid-Hempel, 2001; Rutrecht and Brown, 2008). Although epizootiological data are scarce, it is believed that *A. bombi* is normally of low prevalence (Lipa and Triggiani, 1996). There were no further reports in the Southern hemisphere since 2009, when it was detected infecting the non-native species *B. terrestris* in Argentina (Plischuk and Lange, 2009). *Apicystis bombi* has also been found parasitizing *Apis mellifera* [(a single specimen in Finland (Lipa and Triggiani, 1996)], what appears to be the only report in this host species.

Premature death of bumblebee queens was suggested in four different North American species that showed a higher prevalence of *A. bombi* in early-season caught queens (samples containing more nest-searching queens) compared with late-season caught queens (samples containing more foraging queens that already started colony founding) (Macfarlane *et al.*, 1995). This mortality in hibernated queens is somewhat expected because heavily infected bumblebees have reduced and disrupted adipose tissue (Liu *et al.*, 1974), necessary for colony start up (Goulson, 2010). Afterwards, a survey of workers at the end of the season showed a similar prevalence of *A. bombi* compared with spring queens (Rutrecht and Brown, 2008). *Apicystis bombi* could re-emerge from some infected queens, survivors of disease; however, transmission by vector species seems possible (I. Meeus *et al.*, unpublished).

Here we provide evidence at the molecular level on the presence of *A. bombi* in bees at a site in Northwestern Patagonia, and report its possible absence in the Pampas region.

Results and discussion

Six honeybees (7.6%) of the 79 collected in 2009 had the neogregarine. Of the 59 individuals collected during 2010, eight of them (13.6%) were positive. In bumblebees, 13 (12.1%) of the 107 insects were positive for this pathogen. In all cases, infections were only detected in adipose tissue with no obvious external signs of infections. Emergence of sporozoites from oocysts was observed in both

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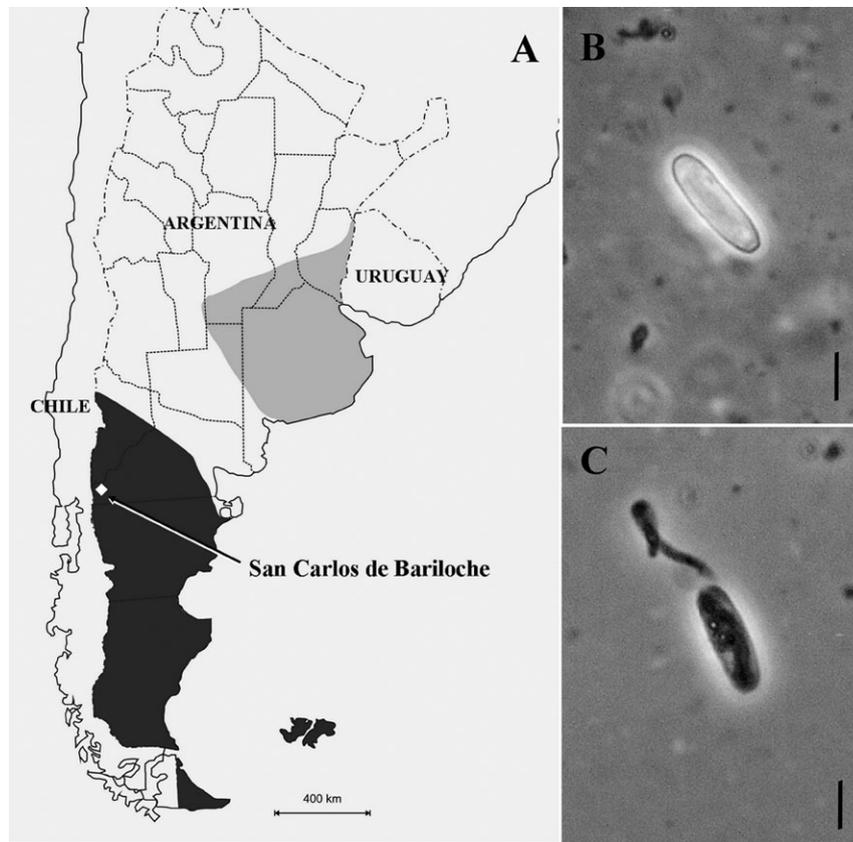


Fig. 1. A. Site of detection of *Apicystis bombi* in *Bombus terrestris* and *Apis mellifera* (San Carlos de Bariloche, Argentina) (Light shade: Pampas region; Dark shade: Patagonia region). B. Mature oocyst of *A. bombi*. C. Sporozoite of *A. bombi* emerging from oocyst. (Bars: 5 μm).

One hundred and thirty-eight workers of *Apis mellifera* were collected at a site located 8 km West of San Carlos de Bariloche, Río Negro province (41°07'33'S; 71°23'55'W), during January 2009 ($n = 79$) and January–April 2010 ($n = 59$) (Fig. 1-A). At the same site, a sample of 107 adults of *B. terrestris* (11 queens, 66 workers and 30 males) was also collected between October 2009 and April 2010. The insects were captured one by one while foraging. After collection, insects were frozen (-32°C) until their identification (Fernández and Sharkey, 2006; Abrahamovich *et al.*, 2007) and processing. On the other hand, 302 bee hives were periodically prospect (160 bees *per* hive every 30 days, in average) in 30 localities in the Pampas region between March 2006 and April 2009. A total of 394 560 honeybee adult individuals were analysed (See Table S1). Sampling was performed in commercial apiaries and samples were taken from the top of the clusters of hives disregarding the presence of disease symptoms. After collection, insects were frozen (-32°C) until analyses. Initial observations were performed under a stereoscopic microscope. Dissection and homogenization techniques were used for prospection and isolation of pathogens (Undeen and Vávra, 1997). Further observations and morphological identification of neogregarines were conducted in fresh preparations under a compound microscope ($\times 400$, $\times 1000$). Infection intensity was estimated using a haemocytometer (Undeen and Vávra, 1997). Measurements (length and width) of oocysts were taken with an ocular micrometer, are expressed as mean \pm SE, and were compared separately with a one-way ANOVA using the XLSTAT 7.5.3 program (Addinsoft). Significance level was set at $P < 0.05$ and homogeneity of variances was checked with Levene's test.

host species (Fig. 1B and C). Measurements of mature oocysts found in *A. mellifera* ($11.31 \pm 0.07 \times 3.21 \pm 0.03 \mu\text{m}$; $n = 50$) and *B. terrestris* ($12.94 \pm 0.08 \times 3.36 \pm 0.04 \mu\text{m}$; $n = 50$) were statistically different (length: $P < 0.05$; width: $P < 0.05$). In *A. mellifera*, infection intensity was $8.96 \pm 2.78 \times 10^5$ ($n = 7$) mature oocysts per insect. It has been noted that immature oocysts were more frequent than mature ones in the majority of these cases. The intensity of infections in *B. terrestris* was $6.21 \pm 4.07 \times 10^6$ ($n = 6$) mature oocysts per bumblebee. In this species, *A. bombi* was found in 10 workers (15.2%)

and three males (10.0%) only during January and March. Infections were not recorded in queens.

Sequencing of the end region of the 18S rDNA confirmed that all samples were positive for *A. bombi* and their sequences were 100% identical. From *A. mellifera* isolations, the consensus sequence was deposited at the NCBI database under accession number HQ619890. Furthermore, no mutations were found after alignment with FN546182, an *A. bombi* isolate obtained from *Bombus pratorum* of Ireland (Meeus *et al.*, 2010) (Fig. 2).



Fig. 2. To carry out the molecular diagnosis, 10 (three from *Apis mellifera*, and seven from *Bombus terrestris*) purified oocyst suspensions were prepared from *Apicystis*-like cases as determined by compound microscopy (Plischuk *et al.*, 2009). DNA extraction, PCR amplification of 18S rDNA and sequencing were performed as previously described by Meeus and colleagues (2010). Briefly, confirmation of neogregarine infection was done by universal primers NeoF and NeoR (Fig. 2 – Lanes 1–3: Infected *A. mellifera*; Lane 4: non-template control; Lane 5 negative extraction control (Non-infected *B. terrestris*); Lane 6: positive control (*Apicystis bombi* infected *B. pratorum*) Lane L: DNA ladder; Lanes 7–13: infected *B. terrestris*. All microscopic infected samples scored positive for neogregarine infection). The parasites were molecularly identified by sequencing the end of the 18S rDNA, amplified by the primers ApBF1 and ApUR2 (See Appendix S1). Finally, we applied a probability formula [FreeCalc2 (Cameron and Baldock, 1998)] to strengthen the lack of parasite detection in the Pampas region. This formula estimates the sample size needed to detect diseased insects in a whole population (assuming an expected prevalence) using different distributions (Cameron and Baldock, 1998; Venette *et al.*, 2002). We consider high levels of specificity and sensitivity concerning the test methods (100% and 90%, respectively) and statistical significance levels of 0.95. Because of a lack of previous epizootiological studies on the association *A. bombi* – *A. mellifera*, we estimated the expected infection rate (expected prevalence) very low, nearly 1% [almost 10% of the prevalence in another region of Argentina (see below)]. Because the *A. mellifera* population of the Pampas region is extremely large (SAGPyA, 2009) and samplings were not carried out simultaneously, a binomial distribution was applied in the analysis, which is appropriate for infinite populations or for sampling with replacement (Cameron and Baldock, 1998; Venette *et al.*, 2002).

The cases in *A. mellifera* seem to constitute only the second report of such association since Lipa and Triggiani (1996). The possibility that *A. bombi* can complete its life cycle in *A. mellifera* should not be ruled out. While the abundance of immature oocysts was higher than mature ones (suggesting some sort of limitation to complete its natural cycle) the observation of sporozoites emerging from mature oocysts might be indicating proper viability in this host species (Liu *et al.*, 1974; Lipa and Triggiani, 1996). There was a tendency towards a lower infection intensity in honeybees compared with bumblebees, this difference could simply be due to variances in size (and in the volume of available adipose tissue) between the two hosts. The lower intensity of infection in honeybees may also reflect the inability of the parasite to fully exploit the host in contrast with bumblebees, where both high and low oocyst countings were measured. Comparison of the genetic structures of the parasites in their different hosts and experimental infection studies are needed. Finally, oocyst differences in size reported in this contribution would agree with a previously suggested intraspecific variability (Plischuk and Lange, 2009).

Similar to the possible absence of *A. bombi* in native species of *Bombus* from elsewhere in Argentina (Plischuk and Lange, 2009), infections by this neogregarine were not detected in 302 bee hives (more than 394 000 honeybee individuals) sampled in the Pampas region. Using FreeCalc2 program (Cameron and Baldock, 1998) we have estimated a sample size of 301 hives, necessary to detect a case of *A. bombi* in an infinite population under an expected prevalence of 1.1%. The used algorithm corrects for imperfectness of the diagnostic test. Low sensitivity of the test could result in possible false negatives and the sample size would need to be increased to detect the parasite. On the other hand, low specificity may

provide false positives in a population actually free from disease. This would require an increase in sample size because then more positive cases would need to be found in order to guarantee that disease is present at a significant prevalence (Cameron and Baldock, 1998; Venette *et al.*, 2002). In our survey, we did not detect any case of *A. bombi* in the Pampas region, so we can assume the absence of false positives. A high level of sensitivity could be backed by the great amount of analysed individuals; hence the possibility of false negative samples in this stock seems really improbable. Although sampling can never provide sufficient evidence to prove that a pathogen is truly absent (Venette *et al.*, 2002), an actual freedom from disease in that region appears conceivable. However, *A. bombi* could still be present in the Pampas region in a lower prevalence, or an aggregated distribution. Further studies will be necessary to clarify these aspects.

In this study, we did not capture infected bumblebee individuals in spring, having registered a later appearance of the pathogen in workers and males, as suggested by Rutrecht and Brown (2008). After high mortalities in hibernated queens (Rutrecht and Brown, 2008), the role of honeybees as a potential vehicle or reservoir of *A. bombi* could be relevant to support its re-emergence throughout the season.

Keeping in mind the history of the Northwestern Patagonia in terms of invasion of other Hymenoptera species (Farji-Brener and Corley, 1998), the detection of infected *A. mellifera* and *B. terrestris* only in that area could represent another case of recent entry of pathogens from Chile through low-altitude passes across the Andes. In Argentina, although the entry of *A. bombi* seems to be recent, the adaptability of *B. terrestris* would facilitate the speed and geographic reach of an eventual spread of the

neogregarine. Current knowledge about the ubiquitous dispersal of *B. terrestris* in Chile (Montalva *et al.*, 2011) and Argentina (S. Plischuk and C.E. Lange, unpublished) also seems to predict its possible arrival in the Pampas region, one of the most important beekeeping regions in the world, carrying *A. bombi*. These and other possibilities must be seriously evaluated and until proven otherwise, this gregarine should be regarded as a multihost invasive parasite with potential risks and consequences for Argentine bee populations.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Summary of sampling performed in the Pampas region between March 2006 and April 2009.

Appendix S1. Experimental procedures.

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