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Alien parasite hitchhikes to Patagonia on invasive bumblebee

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Abstract The worldwide trade in bumblebees can lead to the spread of diseases, which in turn has been claimed as a factor in bumblebee decline. Populations of the introduced *Bombus terrestris*, which invaded NW Patagonia, Argentina, in 2006, harbor the highly pathogenic protozoan *Apicystis bombi*. We asked whether *A. bombi* had been co-introduced with *B. terrestris*, and if so, whether spillover occurred to the two resident bumblebee species in the region: the introduced European *Bombus ruderatus* and the native *Bombus dahlbomii*. We searched for *A. bombi* by means of PCR in samples of *B. ruderatus* and *B. dahlbomii* collected before and after the invasion

of *B. terrestris* and in samples of the latter. We found no *A. bombi* in samples of *B. ruderatus* and *B. dahlbomii* collected before *B. terrestris* invasion, whereas post invasion, *A. bombi* was present in all 3 species. The identity of the parasite was established by sequencing the 18S region, which was identical for the three bumblebee species and also matched the European sequence, confirming it to be *A. bombi*. This is the first report of *A. bombi* in *B. ruderatus* and *B. dahlbomii*. Moreover, our results suggest that Patagonia had been free of *A. bombi* until this parasite was co-introduced with *B. terrestris*, and spilled over in situ to these two previously resident species. Finally, our findings provide indirect circumstantial evidence of a potential link between the population collapse and geographic retraction of *B. dahlbomii* and the introduction of this novel parasite.

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parasites · *Bombus terrestris*

Introduction

Emergent infectious diseases are increasingly recognized as a potential driver of bumblebee decline (Goulson et al. 2008 and references therein; Meeus et al. 2011; Williams and Osborne 2009). As *Bombus terrestris* rearing and trade for commercial pollination of crops has become a worldwide industry (Hayo and

van Adriaan 2006), pathogen spillover (Colla et al. 2006) from commercially reared bumblebees to wild congeners or conspecifics (Meeus et al. 2011) may become a threat to bumblebee fauna on all continents. Mass-reared bumblebees often harbor high levels of pathogens, which can be transmitted to wild congeners when they escape from greenhouses (Colla et al. 2006). Therefore, a positive correlation between the presence of commercial bumblebees and the increase in the amount of parasites in natural populations has been claimed as evidence of pathogen spillover. Despite difficulties in proving this phenomenon (Meeus et al. 2011), the most reliable conclusions to date are the result of the combination of both the increase of incidence and the genetic identities of parasites (Cameron et al. 2011; Colla et al. 2006; Goka et al. 2006).

Among emergent bumblebee diseases, the potential spread of the neogregarine *Apicystis bombi* (Liu, McFarlane and Pengelly; Neogregarinida: Ophrocystidae) is a cause for great concern, for several reasons: Firstly, its high virulence (it inhibits colony founding, increasing workers' mortality and causing other serious physical and behavioral effects, Rutrecht and Brown 2008; Schmid-Hempel 2001); secondly, its generalism (it has been recorded in wild populations of over 20 bumblebee species in Europe and North America and more recently also in *A. mellifera*, Lipa and Triggiani 1996; Macfarlane et al. 1995; Plischuk et al. 2011); and thirdly, its ability to infect commercial colonies (it has been detected in commercial colonies of *B. terrestris* imported into Ireland and Turkey, Cankaya and Kaftanoglu 2006; Meeus et al. 2011).

In NW Argentine Patagonia invasive *B. terrestris* populations derived from commercial colonies imported to Chile, are known to harbor *A. bombi* (Plischuk et al. 2011). However, as the distribution of this parasite seems to be widespread, it still remains uncertain whether *A. bombi* was co-introduced with *B. terrestris* or was already present in previously resident species, and eventually transmitted in situ to *B. terrestris*. So far, *A. bombi* has not been detected in samples of five out of eight native bumblebee species occurring north of the current distribution of *B. terrestris* in Argentina (Plischuk et al. 2011). However, no previous screening of this parasite has been carried out in bumblebee species overlapping with *B. terrestris* distribution within the invaded

region in NW Patagonia, namely *B. dahlbomii* and *B. ruderatus*. In this study, we searched for *A. bombi* in these three co-occurring bumblebee species.

Bombus dahlbomii (Guérin-Méneville 1835) is the southern-most bumblebee species worldwide, occurring from about 30° southwards to the southern tip of the South American mainland, and is the only native species of southern Argentina and Chile (Abrahamovich et al. 2001). Nowadays, *B. dahlbomii*'s geographic range partially overlaps with those of two introduced invasive European bumblebee species, *B. ruderatus* (Fabricius 1775) and *B. terrestris* (Linnaeus 1758) (Abrahamovich et al. 2001; Montalva et al. 2011). This species has shown a strong decline in coincidence with the invasion and spread of both introduced species (Montalva et al. 2011; Morales 2007; Ruz 2002).

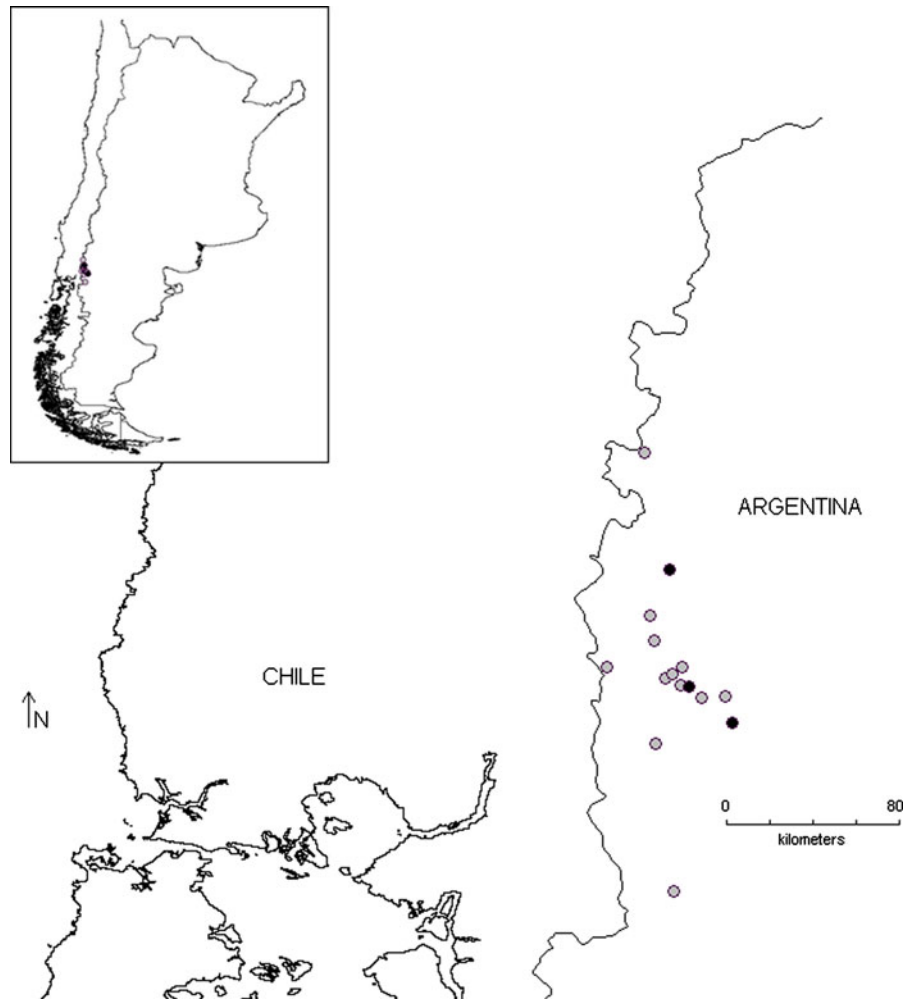
Bombus ruderatus was introduced into Chile in the early 1980s from populations previously naturalized in New Zealand (Arretz and Macfarlane 1986). It was first recorded in the wild in NW Argentine Patagonia near the border with Chile in 1994 (Roig-Alsina and Aizen 1996), and in a few years it became more abundant than *B. dahlbomii* (Madjidian et al. 2008; Morales 2007). In the case of *B. terrestris*, since 1997 colonies have been imported from Belgium and Israel to Chile for crop pollination (Ruz 2002). It was first recorded in the wild in NW Argentine Patagonia in 2006 (Torretta et al. 2006); it spread rapidly and became more abundant than both previous resident species (Morales, pers. obs.). In this study, we have screened for *A. bombi* in both native and invasive co-occurring bumblebee species collected before and after the invasion of *B. terrestris*, to assess the hypotheses that this pathogen was co-introduced with *B. terrestris* and spilled over to *B. ruderatus* and *B. dahlbomii*.

Methods

Sample collection

We screened for *A. bombi* in bumblebee specimens collected between 1994 and 2012. Sample sites covered the gradient of rainfall and vegetation types that characterizes NW Patagonia, including a variety of suburban and wild sites (Fig. 1). Dry or alcohol stored bumblebees from our own collections, as well as from recent samplings carried out in NW Patagonia,

Fig. 1 Map showing the sampling sites. *Gray circles* indicate all samples negative for *Apicystis bombi*. *Black circles* indicate at least one positive sample for *A. bombi* after *B. terrestris* invasion



were grouped according to two different periods: before and after *B. terrestris* invasion. The first period (1994–2005) spans the time when only *B. dahlbomii* and *B. ruderatus* coexisted. The second period begins with the invasion of *B. terrestris*. Sample sizes varied between periods and species, partly reflecting variations in sampling efforts and changes in species abundances (Appendix S1). Specimens were captured in the field while visiting flowers, with nets or killing jars.

Molecular methods

We extracted whole DNA using the commercial kit EZNA from Omega Bio-Tek, following manufacturer's recommendations. We performed polymerase chain reactions (PCR) in order to screen for

neogregarine infection with NeoF and NeoR primers, detecting the 18S rDNA according to Meeus et al. (2010). In addition, we amplified an Apidae region as a control (primers ApidaeF and ApidaeR) (Meeus et al. 2010). In order to identify positive samples at species level a larger fragment was obtained by PCR with the primer pair ApBF1–ApUR2 (Meeus et al. 2010). Positive bands were enzymatically cleaned using exo-sap and sent to LGC Genomics (Berlin, Germany) for direct sequencing. Low quality samples, or those that failed to amplify, were re-sequenced with an ABI3100*avant* at the Ecotono Laboratory genetics facilities, Universidad Nacional del Comahue (Argentina). Sequencing reactions were performed by means of BigDye v3.1 chemistry. Sequences were visualized using Sequencing Analysis[®] software 5.1 and BLAST in GenBank.

Results

In total we screened 130 specimens of the three bumblebee species for the neogregarine infection together with a DNA control for bumblebee (Apidae). A new PCR with species specific primers was conducted on all positive samples. All genetic identities obtained were 100 % identical and deposited for each host at the NCBI-database under accession numbers JX268789, JX268790 and JX536492. Blast analysis confirmed species identity and revealed 100 % identity with *A. bombi* already found in a European sample (FN546182). Before *B. terrestris* invasion we found all samples of *B. dahlbomii* (n = 52) and *B. ruderatus* (n = 30) to be negative for *A. bombi* (Appendix S1). In contrast, after *B. terrestris* invasion, we found 14 *B. terrestris* (47 % incidence, CI = [27.7, 55.6 %], n = 30), five *B. ruderatus* (56 %, CI = [23.1, 88.0 %], n = 9) and one *B. dahlbomii* (11 %, CI = [9.4, 31.6 %], n = 9) to be positive for *A. bombi*. Infested bumblebees were collected in three different sites (Challhuaco, Lago Moreno and Villa Traful, Fig. 1, Appendix S1). Taking the preexistent bumblebee fauna together (i.e. *B. ruderatus* and *B. dahlbomii*) Fisher exact tests revealed a significant overall increase in the percentage of infested bumblebees from the pre- to the post-*B. terrestris* periods (0–50 % incidence, $P < 0.0001$) This increase in *A. bombi* incidence was also significant for *B. ruderatus* (0–56 %, $P = 0.0021$) but not for *B. dahlbomii* (0–11 %, $P = 0.147$), which was expected, given the low sample size available for the post-*B. terrestris* period.

Discussion

Our results suggest that the NW Patagonian region was free from *A. bombi*, as we did not find infected *B. dahlbomii* or *B. ruderatus* prior to the invasion of *B. terrestris*. The possible absence of *A. bombi* in native bumblebee species and *A. mellifera* collected north of the current *B. terrestris* distribution in Argentina (Plischuk et al. 2009, 2011), although based on limited sampling, is consistent with our results, and with the hypothesis of *A. bombi* co-introduction with *B. terrestris*.

We report, for the first time, the presence of the pathogen *A. bombi* in the South American *B. dahlbomii*

and the European *B. ruderatus*. Previous studies did not detect this pathogen in five of the eight native *Bombus* species present in Argentina (Plischuk and Lange 2009) nor in *B. ruderatus* populations naturalized in New Zealand (Macfarlane 2005) where the South American populations originally came from. Thus, our results suggest that both bumblebee species acquired this pathogen in situ after the invasion of *B. terrestris*. This temporal matching between *B. terrestris* invasion and the first detection of *A. bombi* in *B. ruderatus* and *B. dahlbomii* is consistent with the hypothesis of a pathogen spillover.

The high prevalence of *A. bombi* in *B. terrestris* (47 %) is striking when compared to the prevalence found in Europe in this species of 1–8 % (see Allen et al. 2007 and references therein; Baer and Schmid-Hempel 2001; Liersch and Schmid-Hempel 1998) as well as in *Bombus pratorum* (ca. 5.5 %, Rutrecht and Brown 2008). Although ecological and/or climatological factors cannot be ruled out, this local high prevalence could be a consequence of artificial selection in commercial rearing facilities, as reviewed by Meeus et al. (2011). The rearing conditions could have selected for a parasite which has evolved to maximally exploit *B. terrestris*, or this artificial selection could have selected *B. terrestris* populations resistant to the morbidity effects of the parasite. Furthermore, our overall prevalence in *B. ruderatus* and *B. terrestris* (ca. 50 %) is three times higher than that reported by Plischuk et al. (2011) for the same region, which could reflect differences in sensitivity between histological and molecular screening methods.

The lack of *A. bombi* in *B. dahlbomii* and *B. ruderatus* samples prior to invasion may pose some methodological caveats. The 16-year-old samples of *B. dahlbomii* stored in alcohol or pinned could reflect more “loss of evidence or signal” due to DNA degradation, rather than a real absence of the parasites before the invasion. Even though we got positive PCR controls that amplify Apidae in all samples, amounts of parasite DNA are expected to be various orders of magnitude lower than amounts of host DNA. Usually the problems associated with DNA extraction from harsh conditions are of quality and not quantity as PCR can overcome this difficulty. However, there is evidence of good quality preservation of both ethanol-stored and dried pinned museum specimens for insect DNA older than 16 years (Frampton et al. 2008; Junqueira et al. 2002; Phillips and Simon 1995). In addition, we succeeded in amplifying, by means of PCR

reactions, the DNA of internal parasite *Crithidia bombi* from 12-year-old specimens (Arbetman, personal observation). Those bumblebee samples were preserved in exactly the same manner as the samples currently under analysis (both dry and ethanol-stored individuals). Given that *C. bombi* is smaller than *A. bombi* and that a recent study (Plischuk and Lange 2009) reports lower loads of *C. bombi* than *A. bombi* for the Patagonian region, we would expect that if present, there would be larger amounts of the neogregrine DNA than of *C. bombi*, and therefore detectable by PCR.

The small sample size of *B. dahlbomii* for the post-*B. terrestris* invasion period, compared to those of *B. ruderatus* and *B. terrestris*, reflects the decline of this species in the region (Montalva et al. 2011) after the invasion. However, the presence of the parasite alone does not allow us to conclude that *A. bombi* is involved in its population collapse. Experimental infections and studies in transmission pathways would provide more insight into the potential link between the population collapse of both the native and the first invasive species and the presence of this novel parasite in the region. More generally, as *B. terrestris* keeps on expanding its range, the evidence for an interspecific pathogen spillover raises concern about the risk of infection not only to *B. dahlbomii*, but also to the remaining native bumblebee species inhabiting regions to the north of current *B. terrestris* distribution. Thus, we need information about this parasite's morbidity in *B. ruderatus* and *B. dahlbomii*, and the other native species in Argentina.

The DNA sequences of the small subunit ribosomal RNA of the European and Argentinean *A. bombi* were identical. However, more variable sites need to be analyzed in order to ensure that the same haplotypes, originally from Europe, are present in Patagonia. To understand the mechanisms of transmission, we need to keep on working on the genetic identity of the parasite, as well as the inter and intra species transmission pathways. In the meantime, thorough sanitary controls to detect *A. bombi* (as carried out by large rearing facilities) are urgently needed to prevent further dissemination of pathogens by the commercialization of bumblebee colonies. More generally, our study exemplifies that pathogen introduction from bumblebee-rich northern regions into poor southern regions is a real problem, with potentially important conservation consequences, and the introduction of

non-native bumblebee species should therefore be discouraged in regions hosting native bumblebees.

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