A first approach in the correlation of pathogens load affecting Bombus pauloensis to the land use in Buenos Aires Province

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Abstract

Bombus pauloensis is a native bumblebee species widely distributed over South America and a key pollinator for native plants and commercial crops. This species is affected by pathogens such as *Nosema ceranae*, *Crithidia bombi* and *Apis mellifera* Filamentous Virus (AmFV). This work aims to document the presence and intensity of exogenous pathogens on the native bumblebee *B. pauloensis* in different periods of the year during spring and summer. Bumblebees were sampled in four study areas with contrasting land uses to preliminary evaluate if anthropization levels can be related to the presence and intensity of pathogens. DNA was isolated from twenty individuals per sampling site and *N. ceranae, C. bombi* and AmFV pathogens load were quantified by quantitative PCR. The results showed a wide and ubiquitous prevalence of *N. ceranae* and *C. bombi* pathogens in all the sampled bumblebees throughout the year, with a pathogens load that did not differ significantly among the sampling sites. AmFV was undetected in any of the individuals analyzed in any sampling site. This suggests that human activities could equally impact all habitats populated by *B. pauloensis*. Honeybees were detected with a relevant abundance in all the sampling sites and could be one of the main anthropogenic drivers of pathogens spread and hosts switches in the analyzed sampling sites. This study analyzes the general occurrence of the pathogens prevalence and intensity in a native Argentinian bumblebee and attempts the correlation with the land use

Keywords: *Bombus pauloensis,* bumblebees, bee pathogens, *Nosema ceranae*, *Nosema ceranae, Crithidia bombi*, parasite ecology, land use

Statements and Declarations

Competing Interests: The authors declare that they have no conflict of interest in relation to the study in this paper.

Introduction

Pollinators are key elements of the agroecosystem, providing pollination services on crops and orchards, consequently supporting human food provisioning. Their ecosystem service for the entire biosphere is estimated between 16 to 54 billions US$ per year (Costanza et al., 2017). Bees represent a large percentage of these pollinators, with up to 20,000 species known worldwide (Potts et al., 2016). Currently, different bee genera with social or solitary behavior such as *Apis mellifera* (European honeybees), *Bombus* (bumblebees), *Megachile* (leafcutter bees) and *Osmia* (mason bees), are being commercialized to support pollination services of a wide number of herbaceous and arborous plants (Aizen et al., 2008; Müller, 2019; Müller et al., 2019; Sinpoo et al., 2019).

Bumblebees are considered a key pollinator group of species in diverse ecosystems (Pérez-Méndez et al., 2020; McNeil et al., 2020; Miñarro & García 2021). Despite their importance in agricultural and natural systems, bumblebees are suffering a sharp decrease in their population (McArt et al., 2017; Vray et al., 2019). This decline has been associated with several biotic and abiotic factors impacting on a multitude of aspects of their biology (Potts et al., 2010; Graystock et al., 2013; Fürst et al., 2014; McMahon et al., 2015). Abiotic factors such as intensive agriculture impact bees through the fragmentation of their habitat (Cunningham-Minnick et al., 2019; Dicks et al., 2021), but also plant protection products (agrochemicals) can severely impact them (Sanchez-Bayo et al., 2016).

Argentina harbors eight species of native bumblebees, and *Bombus pauloensis* (basonym *Bombus atratus*) is the most abundant (Abrahamovich et al., 2007), mainly thanks to its high tolerance to climatic and altitudinal changes (Abrahamovich et al., 2001; Ramello et al., 2021). As for other species of pollinating insects, *B. paulonensis* is also subject to biotic and abiotic stresses, and pathogen infections are the main biohazard. *Bombus pauloensis,* is infected by pathogens such as *Apicystis bombi*, *Crithidia bombi* (Gamboa et al., 2015), and *Nosema ceranae* (basonym *Nosema ceranae*) is the most prevalent pathogen harbored by *B. pauloensis* (Bravi et al., 2019). Among the different *Nosema* species, *N. apis, N. ceranae* and *N. neumanni* are pathogenic microsporidia isolated in the genus *Apis*. (Martín-Hernandez et al., 2018). Only *N. bombi,* was originally isolated from bumblebees (Sinpoo et al., 2019). However, *N. ceranae* had switched from *Apis cerana* to *A. mellifera* (Paxton et al., 2007) and it was found to infect many species of *Bombus* (Hicks et al., 2018; Plischuk et al., 2009) and *B. pauloensis* as well (Plischuk et al., 2009; Gamboa et al., 2015) but also wild solitary bees such as *Ancistrocerus* and *Heriades truncorum* (Cilia et al., 2023). In honeybees, *N. ceranae* is responsible for the disease “nosemosis type C” which leads to lower honey yield, adult weakness, and increased colonies mortality (Martín-Hernandez et al., 2018).

N. ceranae is mainly transmitted horizontally by fecal-oral contacts (Fries & Camazine, 2001) although some evidence also suggested a vertical transmission of this pathogen among bumblebees (Traver & Fell, 2012). *Crithidia bombi* is a protozoan parasite from the Trypanosomatidae family that infect multiple bumblebee species with a detrimental effect on their health (Salathe & Schmid-Hempel, 2011; Mockler et al., 2018; Koch et al., 2019; Schmid-Hempel, 2019). This trypanosomatid increases the mortality of bumblebees under stressful environmental conditions (Salathe & Schmid-Hempel, 2011) and is also responsible for a lower success in colony-founding of bumblebee queens and the starvation tolerance in workers (Brown et al., 2000, 2003). As for *N. ceranae, C. bombi* is mainly transmitted horizontally by fecal-oral contact with contaminated feces, but it can also be transmitted vertically when progeny acquire *C. bombi* in their natal nest (Brown et al., 2003; Yourth et al., 2008).

Recently, Quintana et al. (2019) reported a host switch of *Apis mellifera* Filamentous Virus (AmFV) from *A. mellifera* to different Argentinean native bees, including *B. pauloensis* from Argentina. Similar dynamics were detected in Italy, where free-ranging *Megachile sculpturatus* adult bees were found AmFV positive (Cilia et al., 2023). Acute infections by AmFV on honeybees lead to tissue lysis, which triggers the production of a characteristic milky-white hemolymph. Moreover, infected honeybees crawl at the colony entrance (Clark, 1977) losing efficiency in their working activities.

A recent review study of Nanetti and coauthors (2021) shows a wide perspective of the pathogen's host switch from *A. mellifera* to different insects. For *N. ceranae* the authors found that five species of *Bombus*, five species of M*elipona,* two species of *Osmia,* two species of *Vespa,* and in the species *Tetragonisca fiebrigi, Heriades truncorum, Aethina tumida* and *Scaptotrigona jujuyensis* (Cilia et al., 2018, 2023). On the other hand, something similar happened with *C. bombi* and AmFV. It was observed also by Nanetti et al., (2021) a spillover of *C. bombi* from *A. mellifera* to seven species of different insects including bees and wasps, and the spillover of AmFV affected 10 different genera of insects including wasp and wild bees.

Recent research works have shown that different land-use strategies at both local and landscape scales directly affect bee populations (Cohen et al., 2017) and that agricultural intensification and habitat fragmentation can influence the abundance and diversity of bees (Ricketts et al., 2008; Winfree et al., 2009). Land use changes can affect food availability and the natural resources for bee nesting (Goulson et al., 2010; Odanaka & Rehan, 2019), and it was found to be linked with the parasite and pathogen metrics (Becker et al., 2015). Cavigliasso and co-authors (2020) investigated the habitat selection of *Bombus pauloensis* in Argentina. Their findings revealed significant differences in the movement behavior of bumblebees based on the nest establishment

period (pre and post). For instance, Blueberry Farmlands with agricultural scenarios appeared to be more attractive during the pre-nest-establishing period, when bees covered larger areas, primarily within the orchard. In contrast, during the post-nest-establishing period, bumblebees showed a preference for edges near forest plantations and shifted their nutritional resource preference to wild floral species

While landscapes with larger proportions of natural and seminatural habitats could dilute parasite impact on bees, modified landscapes can lead to an amplification of parasitism effects, mainly caused by the intensity of host aggregation (Becker et al., 2015; Cohen et al., 2017). Despite all this information, little is known about how the land use changes might alter bumblebee-parasite interactions in *B. pauloensis*.

The primary objective of this study is to investigate the prevalence and intensity of three common pathogens (*N. ceranae, C. bombi*, and AmFV) in wild populations of *B. pauloensis* inhabiting various sites across the Buenos Aires province, characterized by different land use patterns." Sampled bumblebees were expected to show a positive correlation with the level of anthropization in pathogens prevalence and intensity.

Methodology

Study site

This study was carried out sampling in four different sites of 7,07 km^2 each, at least 10 km apart from each other. Sites were *a priori* selected based on their status of land use and anthropization level. A first sampling site was defined as a control site (or preserved site) due to its scarce human impact. This place was the Natural Reserve *Paititi* in Buenos Aires Province, P (37° 54' 47.774" S, 57° 48' 44.806" W), a mixed used reserve, with less than 30% of the land area used for agroecological activities. The rest of the reserve is a protected area intended for nature conservation. The second sampling site was a productive field located in Santa Paula, route 226, km 10, Mar del Plata, Buenos Aires (37° 56' 0.69" S; 57° 40' 40.53" W). This scenario is strongly impacted by conventional agriculture, with the prevalence of managed honeybees, and scarce diversity of native flora. A third sampling site was the commercial plant nursery, *Vivero Antoniucci* (38° 1' 42.014" S, 57° 37' 59.374" W). This scenario is characterized by a plant reservoir supplied by introduced ornamental plants, and honeybee colonies that are used for pollination purposes. The last sampling site was Mar del Plata city (38° 1' 4.745" S, 57° 33' 47.105" W) in the Chauvin neighborhood, characterized as an urban scenario.

Normalized Different Vegetation Index

The NDVI was applied with the use of the free license software QGIS, over a collection of satellite images of the General Pueyrredón District, where all the study sites are located. The satellite images dated from September to March of the season 2018-2019, and only the images with a cloud density lower than 5% were considered. The satellite information was taken from the national repository of National Space Activities Commission (CONAE) (https://www.argentina.gob.ar/ciencia/conae) and it belongs to the Satellite Landsat 8. With raster tools provided by QGIS a 3km "buffer" zone was created at each sample site to represent the flight range area of the collected bumblebees (Pardo & Jimenez, 2006) (**Fig. 1**).

The normalized difference vegetation index (NDVI) was utilized as an indicator to characterize the studied sites in their level of anthropization (i.e. land use changes). Even when this index does not represent an intrinsic physical quantity itself, it is considered as a good indicator of physical properties of the vegetation covers like leaf area index, vegetation condition and biomass (Carlson & Ripley, 1997). As such, vegetation indices are useful measurements despite their limitations.

The NDVI is obtained through

$$NDVI = \frac{(NIR - R)}{(NIR + R)}$$

where:

- NDVI - Normalized Difference Vegetation Index;
- NIR - reflectance of near infrared band;
- R - reflectance of red band.

Bombus pauloensis collection

During the sampling season, different campaigns (i.e. sampling days) were carried out in each site during the spring and summer seasons of 2018 and 2019. Every sampling day consisted of the collection of bumblebees on three transects of 70m. long in a period of 30 minutes per transect, on sunny days between 9 A.M and 1 P.M. A total of 113 *B. pauloensis* workers were captured directly from the flowers (34 of Reserva Natural Paititi, 30 of Santa Paula's Farm, 30 of Vivero Antoniucci and 19 of Mar del Plata) (**Table S1**). Bees were sampled using a homemade bee vacuum, consisting of two clear hoses of different diameters, so that one fits inside the other, and a piece of fabric in between to hold the bees inside the vacuum and prevent them from getting into the

mouth of the operator. Collected bumblebees were separately stored in plastic vials at -20ºC for further analysis. To confirm that the collected bumblebees belong to *B. pauloensis*, specimens were observed under a stereoscopic microscope (x40), relying on region-specific literature (Abrahamovich et al., 2001; 2007)

Pathogens identification and quantification

Once bumblebees were confirmed as belonging to *B. pauloensis*, total genomic DNA was individually extracted with High Pure PCR Template Preparation kit (Roche Diagnostics). quantification of *N. ceranae*, *C. bombi* and AmFV pathogens was carried out in qPCR (StepOne real-time PCR system, Applied Biosystems) using specific primer sets (**Table S2**). Standard curves for absolute quantification were constructed using PCR amplicons obtained from the pathogens DNA. The total number of PCR amplicons was determined according to Baffoni et al. (2021) and then serially diluted to obtain the desired standard concentrations. Finally, the prevalence $Prevalence = \frac{N°ofinfectedindividuals}{TotalAnalyzedindivuals} X100\%$ and intensity (n° of pathogen units in each infected bumblebee) (Margolis et al., 1982; Bush et al., 1997) of the three pathogens were calculated in the collected individuals of *B. pauloensis*.

Statistics

The prevalence of *N ceranae*, *C. bombi*, and AmFV was compared separately among sites using a General Lineal Models (GLM) with binomial error structure and "logit" link function (Crawley, 2007). The response variables were the presence-absence (i.e. presence =1, absence =0) of the pathogens and the explanatory variable was the sampling site. To compare the intensity of each pathogen when present among sites, the ANOVA analysis followed by Tuckey test were used. Pathogens load was log-transformed to fulfill the assumptions of normality and homogeneity of variance (Shapiro-Wilk and Bartlett's tests, $p > 0.05$). All statistical analyses were carried out using R software version 4.0.5 (R Development Core Team 2021). All tests were two-tailed with a significance level of $P \leq 0.05$.

Results

The NVDI analysis of the four study areas showed the following values: a) Natural Reserve (control) (NDVI mean = 0.57); b) Farmland (NDVI mean = 0.49); c) Urban Environment (NDVI mean = 0.26); and d) Exotic Meadow (NDVI mean = 0.50). While *N. ceranae* and *C. bombi* were detected in all the sites (**Table S1**), AmFV was not detectable in any sample (prevalence= 0%). No statistical differences in *N. ceranae and*

C. bombi prevalence among all the sample sites were detected (all p-values > 0.5). 97.6% bumblebees samples analyzed resulted positive to *N. ceranae* while 96.5% of the bumblebees were positive for *C. bombi.* All the samples collected in Reserva Natural *Paititi* and *Santa Paula*'s farm were positive for *N. ceranae* and both Mar del Plata city and *Vivero Antoniucci* showed the only samples negative for this same pathogen. On the other hand, all samples collected in Santa Paula's farm and Mar del Plata city were positive for *C. bombi,* but there were negative samples collected in Vivero Antonicucci and in Reserva Natural *Paititi*.

On another hand, there were no statistical differences regarding to the intensity levels of *C. bombi* and the four sampling sites (all p-values > 0.5) (**Fig. 2A**). An equal tendency was observed for the effect of sampling site on *N. ceranae* intensity levels (all p-values > 0.5) (**Fig. 2B**).

Discussion

The *Reserva Natural Paititi* was considered in this study as the less impacted area since it harbors abundant natural flora if compared with the other sampling sites. However, *Reserva Natural Paititi* shows still a small fraction that results exploited with agricultural practices although from an ecological perspective. Mar del Plata city showed the lowest vegetation cover (reflected in the NDVI values) because of its urban development, while Santa Paula´s Farms and *Vivero Antoniucci* showed similar values in their vegetation covers to the Natural Reserve (similar values of NDVI) but the vegetation structure and its configuration were different. Santa Paula farm is a traditional cultivation field destined mainly to kiwi fruit orchards, while Vivero Antoniucci consisted of a large repository of ornamental plants introduced to supply commercial nurseries within the city area.

Multiple factors such as urbanization, agrochemical use and landscape vegetation composition were found to synergically affect the prevalence and intensity of *N. ceranae* in *A. mellifera* and *Bombus impatiens* sampled in the United States or in *A. mellifera* sampled in Argentina and England (Pettis et al., 2013; Figueroa et al., 2020; Samuelson et al., 2020; Pacini et al., 2021). In a similar way, several studies reported an effect of urbanization and landscape vegetation composition on the prevalence and intensity of *C. bombi* on *Bombus terrestris* and *Bombus pascourum* from Scotland and Germany or in *Bombus vosnesenskii* from the United States (Goulson et al., 2008; Theodorou et al., 2016; Ivers et al., 2022). In this work, on the contrary, the equal prevalence and intensity of *N. ceranae* and *C. bombi* in all the sampled *B. pauloensis* lead to an undifferentiated pathogen spread in the different sampling sites. The different conditions could explain these unexpected results of equal prevalence and intensity,

such as the abundance of *A. mellifera* that often share the same habitat, pathogens amplification dynamics, and endemization of analyzed pathogens.

The Buenos Aires province results the region with the highest number of honeybee colonies and beekeeping-related activities of the whole Argentina (RENAPA 2020). Many studies have described the role of *A. mellifera* as a vector of pathogens that are easily spread to wild bees. The distribution of *N. ceranae* worldwide and to a wide number of non *Apis* bees, represents the best example of honeybees mediated pathogens spread (Furst et al., 2014; Graystock et al., 2014). Recently it was shown that *N. ceranae* can be transmitted to new hosts through infected flower nectar (Bodden et al. 2019), contributing to the fast spread of this pathogen. The high transmissibility of this pathogen can trigger continuous re-infections and hosts switches, both in honeybees and in other bee genera which in turn can become new vectors, effectively contributing to *N. ceranae* endemization.

Critihdia bombi is a pathogen imported with the invasive European buff-tailed bumblebee *Bombus terrestris*, a pollinator relatively easy to reproduce and widely exploited for pollination. *Bombus terrestris* was imported in south America since 1998, and it expanded from Chile to Argentina (Torretta et al., 2006) probably with its set of pathogens. In 2009, in an epidemiological study conducted by Plischuk and Lange (2009) in Argentina, *C. bombi* was not yet detected in *B. pauloensis* and other bumblebees' genera populating Argentina. More than a decade later, *C. bombi* is here found in only 80 samples of *B*. *pauloensis* but with high abundance, which is the most common and widely distributed bumblebee in the region. Interestingly, *C. bombi* spilled over *A. mellifera*, where it was found to proliferate (Maggi et al., 2020) in the same region (Buenos Aires) analyzed in this study for *B*. *pauloensis*. A redundant *C. bombi* host change between *B*. *pauloensis* and *A. mellifera* cannot be ruled out. In fact, *C. bombi* was found to reproduce better in social bees (Koch & Schmid-Hempel, 2011).

Moreover, *N. ceranae* and *C. bombi* might be so diffused in the local bee population to became endemic in the study area. The occurrence of these pathogens was confirmed also in solitary wild bees such *Xylocopa augusti*, *Eucera fervens* and *Lasioglossum* spp. sampled in the same region (Fernandez de Landa et al., 2023). If endemicity is in fact happening into the local bee population, it could explain the occurrence of these pathogens and the scarce influence of the landscape. However, the pathogens intensity and the longevity of infected bees, might anyway be correlated with the feed resources, climate conditions and environmental pollution (Di Pasquale et al., 2013; Viadu et al., 2011).

Finally, the distance between sampling sites could also contribute to the land use impact on pathogens spread. Although the biology of *B. pauloensis* is well known as it is currently reared for commercial purposes, its flight range has not been well documented, yet. Pardo & Jimenez (2006) reported that Colombian individuals of *B. pauloensis* could fly up to 1,5 km and return successfully to their nest in urban areas. However, Hagen et al. (2011) stated that bumblebees in less anthropized landscapes can expand their flight ranges in comparison with bumblebees populating urban areas. *Bombus pauloensis* populations were observed foraging outside the sampling sites and this detection highlight the presence of other wild populations that may contribute to pathogens spread from intensively exploited lands and natural land. Consequently, a weakened land use contribution over the pathogens intensity and prevalence can be hypothesized. In conclusion, the results obtained in the present study show that the occurrence of *N. ceranae* and *C. bombi* intensity and prevalence in the local *B. pauloensis* populations is uniform in the study sites taken into account and could be related to the ubiquitous impacts of human activities on the studied sites, which was found as determining driver for pathogens spread in previous studies. However, also the endemization of the analyzed pathogens can play an important role, since *A. mellifera* and bumblebees seem to be key contributors to pathogens spread in the region. The impacts of the spreading process of pathogens should be monitored along with the impacts on other social and solitary bees.

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Authors contribution

All authors contributed to the study conception and design, especially Martin J. Eguaras and Mantias D. Maggi. Material preparation and data collection were performed by Gregorio Fernandez de Landa, Pablo D. Revainera, Anabella R. Nicolli and Camila Corti. DNA extraction and qPCRs were performed by Diana Di Goia, Danielle Alberoni, Silvina Quintana, Francisco Reynadi and Romina Petrigh. The corresponding analysis were performed by Mateo Fernandez de Landa, Francisco Zumpano and Constanza Brasesco The first draft of the manuscript was written by Gregorio Fernandez de Landa, Matias D. Maggi and Leonardo Galleto, all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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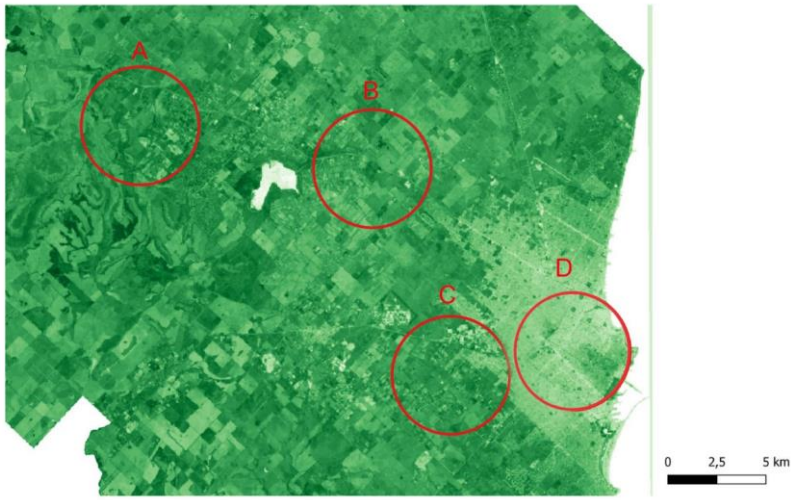
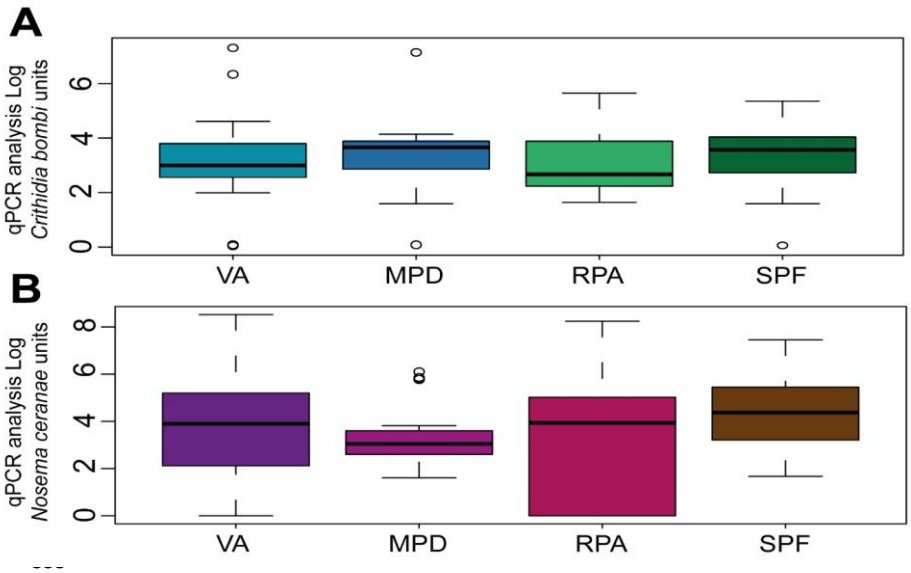


Figure 1. Spatial Characterization of the three study site. The figure represents the three sampling sites (A= Reserva Natural Reserva Natural Paititi; B= Santa Paula; C= Vivero Antoniucci; D= Mar del Plata city). The green scale shows the map constructed from the NDVI values.



555 **Figure 2.** Boxplot showing the distribution of *C. bombi* (A) and *N. ceranae* (B) intensity
556 among sites. VA, Vivero Antoniucci; MDP, Mar del Plata City; RNP, Reserva Natural
557 Paititi; SPF, Santa Paula's Farm.

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