Prevalence of *Nosema* microsporidians in commercial bumblebees (*Bombus terrestris*) is not related to the intensity of their use at the landscape scale

Alejandro Trillo[1], Mark J. F. Brown[2] and Montserrat Vilà[1]

[1]Estación Biológica de Doñana (EBD-CSIC), Avda. Américo Vespucio 26, Isla de la Cartuja, E-41092, Sevilla, Spain

[2]School of Biological Sciences, Royal Holloway University of London, Egham TW20 0EX, UK

Corresponding author: A. Trillo

atrilloig@gmail.com

Abstract – The use of commercial bumblebees to aid crop pollination may result in overcrowding of agricultural landscapes by pollinators. Consequently, transmission of parasites between pollinators via shared flowers may be substantial. Here we assessed the initial infection status of commercial *Bombus terrestris* colonies, and then explored spatial and seasonal influences on changes in parasite prevalence across a landscape where bumblebee colonies are intensively used to pollinate berry crops in SW Spain. Colonies were placed inside strawberry greenhouse crops and in woodlands adjacent and distant to crops in winter and in spring, as representative periods of high and low use of colonies, respectively. Worker bumblebees were collected from colonies upon arrival from a producer and 30 days after being placed in the field. The abdomen of each bumblebee was morphologically inspected for a range of internal parasites. Upon arrival 71% of the colonies were infected by spores of *Nosema*. Three bumblebees from two colonies harbored *A. bombi* spores at the end of their placement in woodlands adjacent to crops. *Nosema* colony prevalence did not change significantly either among sites or between seasons. We found no evidence for the density of commercial *B. terrestris* impacting *Nosema* epidemiology in those commercial colonies, but our results highlight the potential risk for parasites to be transmitted from commercial bumblebees to native pollinators.

agricultural landscape / ***Apicystis bombi*** **/** ***Fragaria × ananassa*** **/ parasite**

1. INTRODUCTION

In the last half century there has been an increase in the expansion of pollinator-dependent crops (Aizen et al., 2008) that has required a parallel demand for commercially produced bees (Potts et al., 2016). Bumblebees (*Bombus* sp.) started to be commercially produced in Europe in the late 1980s, to replace the costly mechanical-pollination of tomatoes (*Solanum lycopersicum*) (Ravestijn and Sande, 1991; Velthuis and van Doorn, 2006). Quickly, bumblebee breeding techniques advanced and colonies were mass-produced and transported worldwide, where they currently pollinate over 20 different pollinator-dependent crops. Over two million bumblebee colonies are produced annually (Graystock, Blane, et al., 2016).

The use of commercial pollinators such as bumblebees to aid crop pollination is not free of environmental risks. For instance, queens of commercial bumblebees have become established in many parts of the world (Matsumura et al., 2004; Morales et al., 2013), and there is empirical evidence showing competition for nest sites with other native bumblebee queens in the lab (Ono, 1997) and in the field (Inoue et al., 2008). In addition, commercial bumblebees may compete for food with other native pollinators (Matsumura et al., 2004; Morales et al., 2013), as well as promote the spread of parasites via shared flowers (Colla et al., 2006; Meeus et al., 2011; Schmid-Hempel et al., 2014).

Several bee parasite species have been found in commercial bumblebee colonies. In 1999, Goka et al. (2000) found for the first time the presence of a parasite, *Locustacarus buchneri*, in commercially produced *Bombus terrestris* colonies upon arrival in Japan from an overseas supplier. The presence of this parasite has been linked to shorter lifespan (Otterstatter and Whidden, 2004) and changes in behavior of bumblebees (Otterstatter et al., 2005). Further studies have reported that commercial bumblebee colonies frequently have a range of bumblebee parasites (Graystock et al., 2013a; Murray et al., 2013) and even honeybee parasites (Graystock et al., 2013a), with the latter probably via the consumption of honeybee pollen by reared bumblebees (Goulson and Hughes, 2015). Importantly, the use of commercial pollinators in crops produces high densities of pollinators not only in the agricultural fields or greenhouses, but in adjacent natural areas as well (Ishii et al., 2008; González-Varo and Vilà, 2017; Trillo et al., 2018). Presumably, in those areas, the rate of parasite transmission among pollinators will rise, because high densities of hosts provide ideal conditions for the spread of parasites (Arneberg et al., 1998). In fact, several studies have shown, through the collection of free-flying bumblebees, high prevalence of parasites in sites adjacent to greenhouses where commercial bumblebees are used compared with sites distant to those greenhouses (Colla et al., 2006; Murray et al., 2013) or in greenhouses absent of such commercial bumblebees (Graystock et al., 2014), although there is also evidence against this (Whitehorn et al., 2013).

To partially reduce the impact of commercial bumblebees on native pollinator populations and because healthy bumblebees may perform better, as is seen with honeybees (Geslin et al., 2017), producers are under pressure to produce parasite-free bumblebee colonies. In this study, we first examined whether commercially produced *B. terrestris* colonies, used to pollinate berry crops in Huelva (SW Spain), carried parasites upon arrival from a producer. We morphologically searched for five common internal bee parasites: larvae of the family Conopidae and Braconidae, *L. buchneri*, *Apicystis bombi* and parasites of the genus *Nosema*, which all potentially affect bumblebee health.

For instance, larvae of parasitic flies lead to bee death (Schmid-Hempel and Schmid-Hempel, 1996), *L. buchneri* likely reduces lifespan of individual infected host (Otterstatter and Whidden, 2004), *A. bombi* is linked to deterioration of the fat body (Graystock, Meeus, et al., 2016; Macfarlane et al., 1995) and most *Nosema* species reduce worker survival and colony size (Otti and Schmid-Hempel, 2007; Rutrecht and Brown, 2009; Graystock et al., 2013a). We then experimentally tested spatial and seasonal influences on changes in the prevalence of these parasites across a landscape where bumblebee colonies are intensively used. Importantly, *L. buchneri*, *A. bombi* and *Nosema* are likely to be transmitted among pollinators via shared flowers (Durrer and Schmid-Hempel, 1994; Goka et al., 2006; Graystock et al., 2015). Colonies were placed inside strawberry crops and in woodlands adjacent and distant to those crops in January (winter) and again in April (spring), as representative periods of high and low use of bumblebee colonies in berry crops, respectively. We expected parasite prevalence to be highest with high densities of commercial bumblebees in the landscape, that is, 1) higher levels of prevalence at sites inside and adjacent to greenhouse crops than distant, and 2) higher levels in winter than in spring because of the greater use of colonies in winter.

2. MATERIALS AND METHODS

2.1. Study system

The study was conducted in the Guadalquivir Valley in the province of Huelva (SW Spain). In this region there are large intensively cultivated areas of berries (9,500 ha), especially strawberries (~70% of the total area devoted to berry crops) (Freshuelva, 2015). Strawberries are cultivated in semi-open polytunnel greenhouses with open sides from November to May. In order to aid crop pollination, farmers use commercial bumblebees (*Bombus terrestris*). Notably, bumblebee colonies are especially used at the beginning of the flowering period (i.e. in winter; personal observations) due to major revenues and worse weather conditions than in spring. The most common remaining natural habitat patches across berry crops are woodlands composed of a rich flora of entomophilous Mediterranean shrubs and herbs, which provide flowers throughout the strawberry cultivation period (Herrera, 1988).

2.2. Experimental design

In 2015, we purchased 48 *B. t. terrestris* colonies from Koppert Biological Systems, one of the main producers in Europe and specifically in this region. Each colony consisted of a plastic box within a cardboard container, with syrup solution provided *ad libitum*. Each colony included a queen and ~100 workers.

First, to quantify colony parasite prevalence, at the arrival of the colonies (period 'before'), we collected 10 workers from each colony. Each worker was frozen in an individual clean vial at -20 ºC for later analyses.

Second, to investigate changes in colony parasite prevalence across the landscape, we placed two colonies each in four strawberry crops ('inside') and in eight woodlands, four adjacent to the selected strawberry crops (~50 m; 'adjacent') and four without berry crops in the surrounding 2 km radius landscape ('distant') (Fig. 1). We chose a 2 km buffer radius because most bumblebee foraging flights do not exceed this distance (Osborne et al., 2008). The surrounding landscape for inside and adjacent plots had a high berry crop cover (overall mean ± SE = 48 ± 5.6%; see Table S1). Inside/adjacent plots and distant plots are representative of contrasting landscapes in terms of commercial bumblebee colony density. The density is high and absent in those landscapes, respectively. In fact, commercial bumblebees are frequently observed in landscapes with berry crop cover, rather than when berry crop cover is absent in the landscapes (Trillo et al., 2019). The average (± SE) distance between adjacent and distant woodland plots was 5903 ± 1038 m (range = 3.1–11.4 km). This distance meets independence criteria to avoid spatial pseudoreplication between non-paired plots.

Third, to investigate seasonal change effects, the experiment was conducted in January ('winter') and repeated in April ('spring'), as representative periods of high and low use of bumblebee colonies in strawberry crops, respectively. Here, the climate is typically Mediterranean with mild winters and warm springs (AEMET, 2015). These two seasons also differ in wild floral resources. The flowering peak is in spring when the floral richness and density are almost triple that in winter (Trillo et al., 2019). Wild pollinator species occur as flowering plant species thrive (Herrera, 1988).

In each season, we placed two colonies of bumblebees in the center of each plot. Bumblebees were allowed to forage for 30 days. In strawberry crops the two colonies were hung between four separate greenhouses. The distance between the two colonies within a plot was ~20 m. In woodland plots the two colonies were hidden in wooden boxes to avoid predation. At the end of the experiment, we collected 10 bumblebee workers per colony returning to it (period 'after') over two days using aerial nets. Bumblebees were kept in individual clean vials with ice until arrival at the lab where they were stored at -20 ºC.

2.3. Parasite screening

The abdomen of each bumblebee was dissected and inspected under a magnifying lens for larvae of parasitic conopid flies (Conopidae, Diptera) and braconid wasps of the genus *Syntretus* (Braconidae, Hymenoptera), and the air sacs were specifically inspected for the tracheal mite *Locustacarus buchneri* (Podapolipidae) (Yoneda et al., 2008). Then, a piece (0.2 cm × 0.2 cm, approx.) of the fat body was dissected out from each bumblebee and mounted on a slide (note that the gut was not included for these analyses). By screening only the fat body, we were able to confirm that we were detecting true infections, not just passage through the gut by vectored spores. We completely screened each slide at ×400 magnification for the presence of spores of the neogregarine *Apicystis bombi* (Lipotrophidae) and microsporidians of the genus *Nosema* (Nosematidae). We estimated parasite prevalence (presence or absence) instead of individual infection levels (abundance) because the latter is influenced by many confounding factors that drive infection intensity (Rutrecht and Brown, 2009).

2.4. Statistical analyses

Only *Nosema* infections (Table S2) were statistically analysed, because the remaining parasites showed no or very low prevalence in the colonies (see results). *Nosema* prevalence was calculated estimating the percentage of bumblebees infected taking into account the 10 individuals collected per colony. A linear mixed model (LMM; Gaussian error distribution based on homogeneity in the residuals) was used to analyse whether changes in *Nosema* prevalence were related to our experimental setting. The difference in *Nosema* prevalence in the colonies before and after being placed in the field was used as the response variable. Season (winter/spring), plot type (inside, adjacent and distant), and their interaction were included as fixed factors in the model, while study plot was included as a random factor to account for the paired design between inside and adjacent plots and the re-sampled plots in winter and in spring (see Table S3 for the R code). All statistical analyses were conducted in R (v.3.1.3, R Core Team, 2014). We used the package *lmerTest* (Kuznetsova et al., 2013) for the LMM and Satterthwaite's approximations for F- and p- values.

3. RESULTS

In total, over the two seasons we screened 919 bumblebee workers. We missed one colony and several individuals from other colonies due to low colony activity. On average (± SE) we collected 19.55 ± 0.13 (range = 16–20) bumblebees per colony. None of the bumblebees were infected by larvae of parasitic conopid flies (Conopidae, Diptera), braconid wasps of the genus *Syntretus* (Braconidae, Hymenoptera), or the tracheal mite, *Locustacarus buchneri*. The prevalence of *Apicystis bombi* was extremely low; only three bumblebees harbored spores in their fat body, and these were collected from two colonies at the end of their placement in adjacent woodlands.

In contrast, spores of *Nosema* were found in 58.3% (14 out of 24 colonies) of colonies in winter and in 83.3% (20 out of 24) in spring at the start of each experimental block, that is, upon arrival from the producer prior to their placement in the field. The average *Nosema* prevalence per colony in the before period was 14.0 ± 3.4% (mean ± SE, hereafter) in winter, and 19.7 ± 3.2% in spring. The average *Nosema* prevalence in the after period was 10.2 ± 2.3% in winter and 26.4 ± 6.6% in spring. Neither the season ($F_{1, 35} = 2.88$, $p < 0.10$) nor the distance ($F_{2, 19} = 0.25$, $p < 0.79$) or their interaction ($F_{2, 35} = 0.50$, $p < 0.61$) had a significant effect on changes in *Nosema* colony prevalence between periods (Fig. 2A and 2B).

4. DISCUSSION

The use of commercial bumblebees has been linked to the decline of several native pollinator species (Cameron et al., 2011; Morales et al., 2013; Schmid-Hempel et al., 2014). Among the mechanisms behind this decline, parasite spillover from commercial to native pollinator populations may play a substantial role (Meeus et al., 2011). Mass commercial breeding programs may facilitate the probability of parasite transmission among hosts, as companies usually handle high densities of bumblebees in their facilities. In parallel, the provision of *ad libitum* food may facilitate the reproduction of infected hosts (Brown et al., 2000). Furthermore, even in the case that commercial bumblebees are parasite-free, they may act as reservoirs for parasites in the field, through a spill-back mechanism, leading to an increase in parasite prevalence (Stout and Morales, 2009; Meeus et al., 2011).

Upon arrival, we found no evidence for the presence of larvae of parasitic conopid flies (Conopidae, Diptera) and braconid wasps of the genus *Syntretus* (Braconidae, Hymenoptera), or the tracheal mite, *Locustacarus buchneri*, in the screened *Bombus terrestris* colonies. Although the presence of larvae of parasitic insects has never been reported in commercial bumblebees, the tracheal mite, *L. buchneri*, was highly prevalent at the end of the 20th century (Goka et al., 2000) spilling over to native bumblebees (Goka et al., 2006). However, it seems that producers have largely eliminated this parasite from commercial bumblebee colonies (Goka et al., 2006; Murray et al., 2013; although see Sachman-Ruiz et al., 2015). In addition, neither these parasitoids nor the tracheal mite, *L. buchneri*, were observed in bumblebees from the colonies after being placed in the field for a month. One explanation for this is that parasitoids of bumblebees might be at low abundance in our study sites, because native bumblebees (*B. terrestris lusitanicus*) are rare (Magrach et al., 2017; Trillo et al., 2019), as they are at the limit of their distributional range (Goulson, 2010). In fact, in this region, the density of commercial bumblebees is around four times greater than that of native bumblebees (Trillo et al., 2019). Another possible and complementary explanation for this low prevalence might be that when bumblebees are parasitized, they desert their colony (Schmid-Hempel and Müller, 1991). In addition, even though *L. buchneri* may be present in native bumblebees (although we note that there is no information in Spain; Jabal-Uriel *et al*. 2017) it might be very difficult to detect parasite spillover from native to managed bumblebees because native bumblebees are at very low abundance, as described above.

Similarly, there was no evidence for the presence of the neogregarine *Apicystis bombi* (Lipotrophidae) in the screened colonies upon arrival. However, three bumblebees were found to be infected after having been placed in the field. In other regions, the parasite *A. bombi* has been detected infecting commercial bumblebee colonies, although in a low number of colonies (Graystock et al., 2013b; Murray et al., 2013; although again see Sachman-Ruiz et al., 2015). Native bumblebees can host *A. bombi* (Jabal-Uriel et al., 2017), but, as noted above, they are rare in our study region (Magrach et al., 2017; Trillo et al., 2019). In contrast, thousands of commercial colonies from at least three producers (Koppert, Biobest and Agrobio, personal observation) are used on an annual basis. Therefore, it is more likely that other commercial bumblebees infected by *A. bombi* transmitted the parasite to the bumblebee colonies we screened, rather than native bumblebees, or, more parsimoniously, our initial screen failed to detect it in arriving colonies.

In contrast, we found commercially produced bumblebee colonies to be heavily infected with parasites of the genus *Nosema* upon arrival from the producer. Other studies have also reported similar levels of prevalence with around three quarters of commercial colonies infected (Graystock et al., 2013a; Murray et al., 2013). Unfortunately, our methodology did not allow us to distinguish between the bumblebee parasite *N. bombi* and the honeybee parasite *N. ceranae*. Both can infect bumblebees (Graystock et al., 2013a; Fürst et al., 2014).

Unexpectedly, our results showed no significant variation in *Nosema* infection rate at a colony level over time, as in a previous study that monitored wild bumblebees (Goulson et al., 2018), even in landscapes where commercial bumblebees were intensively used to pollinate crops. Even in parasite-free landscapes, one would expect that if commercial colonies are infected by a parasite, it spreads within the colony across time due to the high density of hosts and low genetic variability (Schmid-Hempel, 1998). We propose two potential explanations. On the one hand, bumblebees, in line with other social insects, have evolved social immune systems that combine prophylactic and activated responses to avoid, control or eliminate parasite infections (reviewed by Cremer et al., 2007). Both colony and individual (i.e. immunocompetence, reviewed by Schmid-Hempel 2005) defense mechanisms might be involved in maintaining roughly constant *Nosema* prevalence over time. On the other hand, it has been experimentally demonstrated that *Nosema*, specifically *N. bombi*, relies more on transmission through the larval stage than through transmission among adults (Rutrecht et al., 2007). If we consider that colonies were placed in the field for a month period and that the total development of a bumblebee from larvae to adult is about 4-5 weeks (Alford, 1975), this could explain why we failed to detect an increase in prevalence. Imhoof and

Schmid-Hempel (1999) showed an average delay to *Nosema* infection in commercial colonies placed in the field of ~30 days.

Our study showed, for the first time in Spain, that commercially produced bumblebee colonies can be infected by *Nosema* parasites prior to their deployment in the field. These parasites may reduce lifespan and have detrimental effects on bumblebee behavior (Otti and Schmid-Hempel, 2007; Rutrecht and Brown, 2009; Graystock et al., 2013a). Because commercial bumblebees placed in semi-open greenhouses frequently forage in natural areas (Foulis and Goulson, 2014), they have the potential to spread the parasites into native pollinator populations (Colla et al., 2006; Murray et al., 2013). Despite the fact that there is some regulation about commercial bee colony health (e.g. for Europe see 92/65/EEC in European Commission 1992), this regulation does not cover all parasites. This implies that commercial colonies can be highly infected by parasites such as *Nosema*, as our study show. Therefore, there is a need for the enforcement of more stringent protocols to preserve the health of commercial and native pollinators.

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AUTHOR CONTRIBUTIONS

AT and MV conceived this research and designed experiments; MJFB participated in the design and interpretation of the data; AT performed experiments, analyses and wrote the first draft of the manuscript; MJFB and MV edited and contributed to the writing of the manuscript. All authors read and approved the final manuscript.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no potential conflict of interest in relation to the study in this paper.

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FIGURES CAPTIONS

Fig. 1 Geographical distribution of plots inside, adjacent and distant to berry crops located in the province of Huelva (SW Spain). Names denote towns.

Fig. 2 Mean (+SE) change in *Nosema* prevalence in commercially produced bumblebee colonies before and after being placed in plots inside, adjacent (~50 m) and distant (>2 km) to berry crops in winter (A) and in spring (B). Differences were not significant

Fig. 1

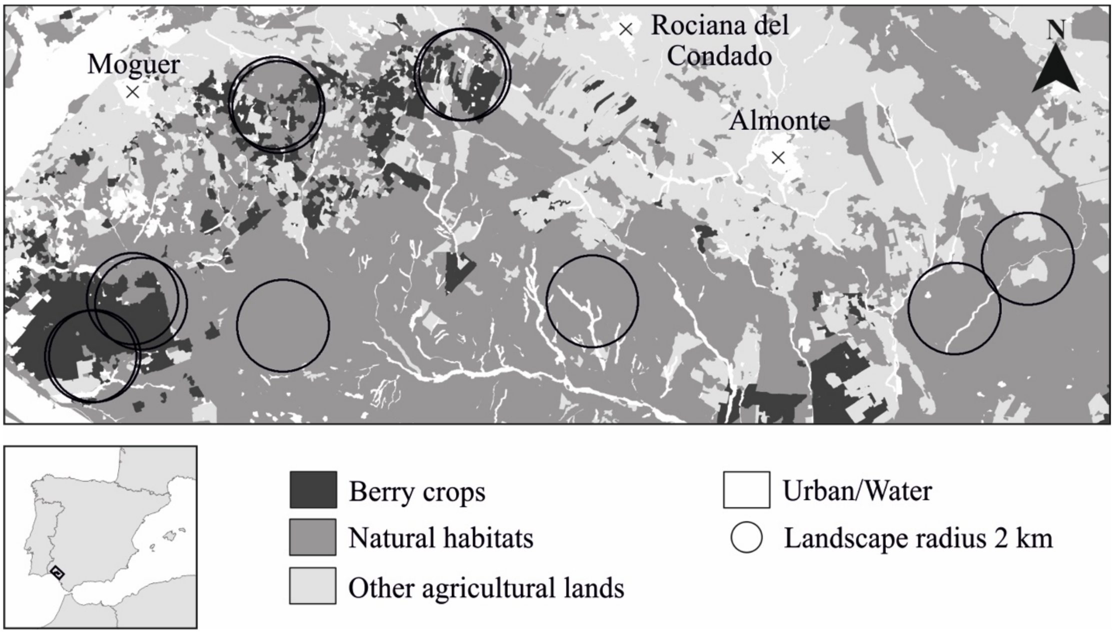
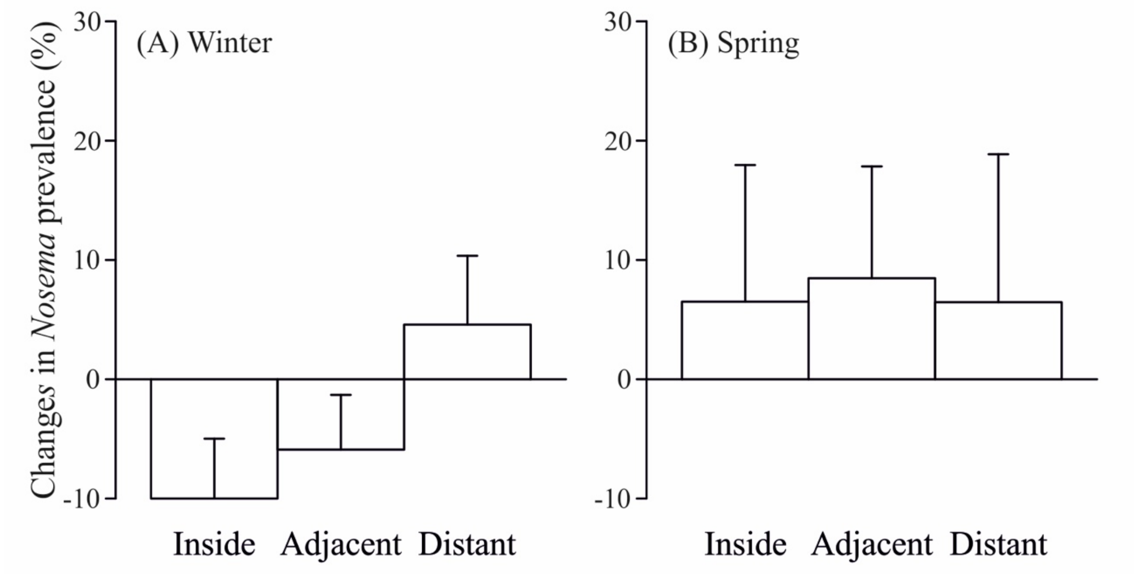


Fig. 1 Geographical distribution of plots inside, adjacent and distant to berry crops located in the province of Huelva (SW Spain). In total, 12 plots were selected and two commercial bumblebee colonies were used per plot in winter and again in spring. Names and crosses denote towns.

Fig. 2



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