The Trojan hives: pollinator pathogens, imported and distributed in bumblebee colonies

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Summary

1. Over a million commercially produced bumblebee colonies are imported annually on a global scale for the pollination of greenhouse crops. After importation, they interact with other pollinators, with an associated risk of any parasites they carry infecting and harming native bees. National and supranational regulations are designed to prevent this, and commercially produced bumblebee colonies are accordingly now often sold and imported as being parasite-free.

2. Here, we used molecular methods to examine the occurrence of parasites in bumblebee colonies that were commercially produced in 2011 and 2012 by three producers. We then used controlled experiments to determine whether any parasites present were infectious.

3. We found that 77% of the commercially produced bumblebee colonies from the three producers, which were imported on the basis of being free of parasites, in fact carried microbial parasites, with five different parasites being detected across the total sample of bumblebees and a further three in the pollen supplied with the colonies as food.

4. Our controlled experiments demonstrated that at least three of these parasites were infectious to bumblebees with significant negative effects on their health. Furthermore, we also found that at least four of the parasites carried by commercially produced bumblebees were infectious to honeybees, indicating that they pose a risk to other pollinators as well.

5. *Synthesis and applications.* The results demonstrate that commercially produced bumblebee colonies carry multiple, infectious parasites that pose a significant risk to other native and managed pollinators. More effective disease detection and management strategies are urgently needed to reduce the pathogen spillover threat from commercially produced bumblebees.

Key-words: *Bombus*, commercial bumblebee production, pathogen spillover, pollinator conservation

Introduction

Bumblebees are amongst the most ecologically and economically important groups of pollinators in temperate regions, but many bumblebee species and other pollinators are suffering declines world-wide (Potts *et al.* 2010). Out of 25 bumblebee species in the UK, for example, two have gone extinct and eight decreased substantially in abundance since 1940, while 13 species have gone extinct in at least one European country and four across the entire region (Goulson, Lye & Darvill 2008). Other species have undergone similar declines in North America (Cameron *et al.* 2011; Szabo *et al.* 2012), and 11% of all bumblebee species world-wide are listed in a threat category on the IUCN Red list (Williams & Osborne 2009).

The importance of bumblebees for the pollination of many high-value crops has led to the commercial production and importation of over a million colonies per year in Europe, North America, South America and Asia (Velthuis & van Doorn 2006). Emergent parasites represent one of the major threats to biodiversity and spillover from introduced organisms to native species can be particularly damaging, either by introducing novel species or strains of parasite or by increasing the density of infected hosts (Daszak, Cunningham & Hyatt 2000).

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Commercially produced bumblebees interact with native bumblebees and other pollinators after importation during shared flower use (Whittington *et al.* 2004; Murray *et al.* 2013), which can cause bee parasites to be transmitted (Durrer & Schmid-Hempel 1994). The introduction of commercially produced bumblebees in North America, South America and Japan has been correlated with declines in native bumblebee species, increases in the prevalence of parasites or the introduction of foreign strains or species of parasite (Goka *et al.* 2001; Colla *et al.* 2006; Otterstatter & Thomson 2008; Meeus *et al.* 2011; Arbetman *et al.* 2012; Szabo *et al.* 2012). In addition, there have been a number of reports of bumblebees commercially produced up to 2008 having parasites (Whittington & Winston 2003; Gegear, Otterstatter & Thomson 2005; Colla *et al.* 2006; Otterstatter & Thomson 2007; Manson, Otterstatter & Thomson 2010; Singh *et al.* 2010; Meeus *et al.* 2011; Murray *et al.* 2013). As a result, the regulatory requirements for bumblebee importation have been tightened in some countries in recent years to stipulate mandatory disease screening, and the producers of bumblebee colonies now often claim that their colonies are free of parasites (European Commission 1992; HM Government 2006; Velthuis & van Doorn 2006; Winter *et al.* 2006; The Food & Environment Research Agency 2011). In England for example, the importation licences for the non-native subspecies most commonly imported are specifically limited to parasite-free colonies, and 40–50 thousand colonies are imported annually to the UK on this basis (Natural England 2009, 2012). However, concern remains about whether bumblebee colonies being produced currently may nevertheless carry parasites, and it is also unclear whether any parasites that may be present in the hives are infectious, making the pathogen spill-over risk posed currently uncertain.

Here, we examined bumblebee colonies that were commercially produced in 2011 and 2012 for the presence of three bumblebee parasites and six honeybee parasites. We then tested experimentally whether any of the parasites found were infectious to bumblebees or to honeybees. Our results show that parasites are present and infectious in bumblebee colonies that are currently being commercially produced, substantiating concerns about significant risks of pathogen spillover.

Materials and methods

COLONY SCREENING

Forty-eight commercially produced *Bombus terrestris* (Linnaeus 1758) colonies were purchased in 2011 and 2012 from three of the main producers in Europe, with 15 (five from each producer) being of the non-native *B. terrestris dalmatinus* or *B. t. terrestris*, and the remainder *B. t. audax*. The colonies of the non-native subspecies were imported into England by the producers under Natural England licences that are limited to disease-free colonies (Natural England 2009). Immediately upon arrival, 15 workers were removed from each colony, as well as

25 samples of the pollen (originally sourced from honeybees) that were supplied with the colonies as food (five samples from separate bags or bottles for each of the three producers in 2011 and from Producers A and B in 2012). A *c.* 0·2-cm^3 sample of the hind gut, fat body and malpighian tubules was dissected out from each of the workers and homogenized with a micropestle. Pollen samples (0·6 g) were homogenized in TRIS buffer for 2 min with 0·1 mm zirconia/silica beads in a Qiagen TissueLyser. DNA and RNA were extracted by boiling either the entire homogenate (in the case of worker samples) or 5 µL of the supernatant (in the case of the pollen samples) in 145 µL of 10% Chelex solution, which is effective at isolating viral RNA, as well as the DNA of the other parasites (Rekand *et al.* 2003; Rudenko *et al.* 2004; Evison *et al.* 2012). For 34 of the colonies, we pooled DNA/RNA extracts from the 15 bees such that there was a single pooled sample of bee DNA/RNA per colony, while for the other 14 colonies we ran the 15 bees separately. We screened the bees for the three main bumblebee parasites [the trypanosome *Crithidia bombi*, the microsporidian *Nosema bombi* and the neogregarine *Apicystis bombi*, all of which are faecal–orally transmitted parasites of adult bees, (Schmid-Hempel 1998)], four widespread honeybee parasites [the faecal–orally transmitted microsporidian parasites of adult bees *Nosema apis* and *N. ceranae* and the orally infecting foulbrood bacteria *Melissococcus plutonius* and *Paenibacillus larvae* of bee larvae, (Morse & Flottum 1997)], deformed wing virus (DWV), which is a common parasite in honeybees and bumblebees (Evison *et al.* 2012), and the orally infecting fungal parasite *Ascosphaera* of bee larvae (Aronstein & Murray 2010). We screened the samples for parasites using conventional, nested or hemi-nested PCR, or Taqman RT-PCR for DWV, using parasite-specific primers (see Table S1 in Supporting Information). Amplification at the host *18S Apidae* gene was used to check for quality of the DNA extractions, and positive and negative controls were included in all sets of samples.

EXPERIMENT 1: INFECTION RISK TO ADULT BUMBLEBEES

A total of 150 adult *Bombus terrestris audax* workers were collected from three commercially produced colonies that we had found to be free of parasites by PCR screening 15 adult bees per colony (our data on parasite prevalence for a subset of 14 colonies indicated that parasites, when present, infected >10% of bees; Table S2, Supporting Information). The uninfected status of these colonies was then confirmed by the fact that all control bees used in the experiment were found subsequently to be uninfected (see Results). Each bumblebee was placed in a holding harness and individually fed 5 µL of 40% sucrose solution containing either pollen (0·6 g mL^{-1}) or bumblebee faeces (diluted 3 : 1) from commercially produced bumblebee colonies that had been found by PCR to contain parasites, or sterile sucrose solution control. The pollen used was that supplied with colonies by the commercial producers, with pollen samples from the three producers being mixed in equal measure to produce a single homogenous solution. Faeces was obtained by placing bees in holding pots until they defecated, with faeces then collected from the pot with a syringe and combined to produce a single solution. The pollen and faeces used were confirmed by PCR and RT-PCR to contain *Nosema bombi*, *N. ceranae*, *Crithidia bombi*, *Apicystis bombi* and DWV, with the pollen also having *N. apis* (as well as the *Ascosphaera* parasite of bee larvae). The pollen solution con-

tained 8.4×10^4 *Nosema* spores and 24 *Apicystis* spores per μl (1.4×10^5 *Nosema* spores and 40 *Apicystis* spores per mg of pollen), while the faeces solution contained 6.1×10^2 *Nosema* spores and five *Apicystis* spores per μL (2.4×10^3 *Nosema* spores and 20 *Apicystis* spores per μL of bumblebee faeces), based on counts under a phase-contrast microscope with a haemocytometer. No *Crithidia* were observed in these counts, and levels of DWV were not quantified. The bees were then placed in $10 \times 6 \times 6$ cm plastic boxes, with each box containing 10 bees that were from the same colony and had received the same treatment. The boxes of bees were kept at 28 °C and 60% RH with 40% sucrose solution provided *ad libitum* for 15 days with mortality checked daily. The proboscis extension response was used to assess the sucrose sensitivity of the bees every 5 days, by placing bees in individual holding harnesses and presenting them with a series of sucrose solutions increasing from 10–80% in increments of 10%. Harnessed bees were initially hand-fed to satiation with 30% sucrose solution and then starved for 5 h, before testing them by touching the test sucrose solutions on to an antenna, with distilled water applied to the antenna for a 60-s period in between each test to prevent conditioning. The concentration at which the bee extended its proboscis to drink was then recorded, with bees scoring from 0 to 8 for low to high sensitivity, with eight indicating that the bee extended its proboscis in response to all concentrations of sucrose and one indicating it only extended it in response to the 80% sucrose solution. After the 15-day experimental period, all surviving bees, as well as all bees that died during the experiment, were screened for parasites of adult bees (*Apicystis bombi*, *Crithidia bombi*, *Nosema bombi*, *N. ceranae*, *N. apis*, DWV) by PCR and RT-PCR as above, and the numbers visible in the tissue samples were counted using light microscopy.

EXPERIMENT 2: INFECTION RISK TO ADULT HONEYBEES

Capped brood frames were taken from three *Apis mellifera* honeybee colonies that had been confirmed previously by PCR to be free of parasites, with the exception of the ubiquitous *Varroa* mite and asymptomatic DWV, and placed in an incubator at 34 °C and 60% RH for eclosion. A total of 180 freshly eclosed workers were collected from these brood frames and transferred to sterile boxes with *ad libitum* sucrose solution until 2 days of age. As in Experiment 1, the honeybees were then placed in a holding harness and individually fed 5 μL of the 40% sucrose solutions containing either pollen or bumblebee faeces from parasite-infected, commercially produced bumblebee colonies, or sterile sucrose solution control. The pollen solution used in this experiment contained 7.1×10^4 *Nosema* spores and 40 *Apicystis* spores per μL (1.2×10^5 *Nosema* spores and 67 *Apicystis* spores per mg of pollen), while the faeces solution contained 6.6×10^2 *Nosema* spores and eight *Apicystis* spores per μL (2.6×10^3 *Nosema* spores and 32 *Apicystis* spores per μL of bumblebee faeces). The honeybees were then placed in cohorts of 20 like-treated nestmates in a $10 \times 6 \times 6$ cm plastic box and kept at 34 °C and 60% RH with 40% sucrose solution provided *ad libitum* for 14 days with mortality checked daily. After this period, all surviving bees as well as those that had died during the period were screened by PCR as before for parasites of adult bees (*Apicystis bombi*, *Crithidia bombi*, *Nosema bombi*, *N. ceranae*, *N. apis*; DWV was excluded).

EXPERIMENT 3: INFECTION RISK TO HONEYBEE LARVAE

A total of 144 one-day-old larvae were collected from the three honeybee colonies that had been confirmed to be free of disease as above, and placed in 48-well tissue culture plates on 60-μL drops of diet (50% royal jelly, 6% D-glucose, 6% D-fructose and sterile deionized water), with the plates then placed in sealed boxes containing a pool of 0.04% K_2SO_4 to ensure high RH, at 34 °C. Two days later, the larvae were fed 20 μL of a mixture consisting of four parts diet to one part of either a solution in distilled water of the same parasite-contaminated pollen supplied with commercially produced bumblebee colonies as was used in Experiment 2, a solution of the same pollen but after it had been frozen at −20 °C for 24 h and then microwaved at 600 W for 5 s to reduce the viability of any parasites, or sterile distilled water control. In addition to the adult parasites mentioned above, the pollen solution also contained 1.7×10^6 spores per μL of the *Ascosphaera* fungal parasite. The larvae were fed the same diets on each subsequent day, increasing by 10 μL per day, until the larvae defecated (indicating the end of larval growth) on about day 6, when the faeces was cleaned from their wells and the larvae were not fed any further. The survival of larvae was checked daily with a dissecting microscope for the 6-day feeding period and a further 4-day period. All larvae that survived to the end of the 10-day experimental period, as well as those that died during the period, were rinsed in TRIS buffer and screened for parasites of bee larvae (*Ascosphaera*, *Paenibacillus larvae* and *Melissococcus plutonius*) as above.

STATISTICAL ANALYSIS

All analyses were carried out in IBM SPSS 19 (SPSS Inc., an IBM Company, Chicago, IL, USA). The parasite species richness of the commercially produced bumblebee colonies was compared between the two subspecies, three producers and 2 years using generalized linear models (GLMs) with gamma distribution and log link function on $x + 1$ data, while the numbers of colonies in which each parasite was detected were analysed using GLM with a binomial distribution and log link function. The likelihood ratio χ^2 statistic was used to test for significance and to check for model fit compared to the intercept-only model. The deviance/d.f. ratio was used to check for over- or underdispersion, with cases of overdispersion being dealt with by using the inverse of the deviance/d.f. value as a scale parameter to fit an overdispersed model. When there was a quasi-complete separation of the data, Fisher's exact tests were used to explore the data instead. We also carried out analyses using subsets of the data for which we had information from all three producers (colonies of *dalmatinus/terrestris* or pollen from 2011 only), both subspecies of bumblebees (colonies from Producers A and B in 2011 only) or both years (*B. t. audax* colonies or pollen from Producers A and B only), but these did not materially change the results (see Table S6 for the one exception, Supporting Information). The effect of treatment on the survival of bumblebees and honeybees in the three experiments was examined using Cox proportional-hazards regression models, with Kaplan–Meier tests using the Breslow statistic for pairwise comparisons, which accounted for the censored nature of the survival data. The effects of treatment on the sucrose sensitivity of bumblebees in Experiment 1 were examined using a GLM with a gamma distribution and log link function

on $x + 1$ data, followed by pairwise comparisons of treatments on each day in which the P-value was adjusted by the sequential Bonferroni method. The numbers of individuals in the three experiments in which each parasite was detected were compared between the treatments using GLM with binomial distribution and logit link function, with day of death included as a covariate. Colony of origin was included in both the Cox model and GLM of data from the three experiments. Non-significant terms were removed stepwise in all cases to obtain the minimum adequate models.

Results

COLONY SCREENING

Five of the nine parasites we screened for were present in 13–53% (depending on the parasite) of the commercially produced bumblebee colonies, with a further three parasites being present in the pollen supplied with the colonies as food (Fig. 1; Tables S2 and S3, Supporting Information). In only 11 of the 48 colonies were the bees screened negative for all of the parasites. Of the 25 pollen samples, only a single sample was free of every parasite. Prevalence of the three bumblebee parasites ranged from 15 to 56% of colonies or pollen samples (Fig. 1). The commercial bumblebees had a somewhat lower prevalence of the honeybee parasites than of the bumblebee parasites, but the *Ascosphaera* fungal parasite was present in 60% of the pollen samples and only *M. plutonius* was completely absent from the samples examined. When examined by microscopy, we did not observe *Crithidia* in the hind guts of the bumblebees, but both *Nosema* and *Apicystis* spores were visible. The richness of parasite communities found in the bumblebees did not differ significantly between the two bumblebee subspecies supplied, the three producers

or years ($\chi^2 = 0.232$, d.f. = 1, $P = 0.63$; $\chi^2 = 5.04$, d.f. = 2, $P = 0.081$; and $\chi^2 = 0.042$, d.f. = 1, $P = 0.838$, respectively), and there was also no difference between the producers or years in the richness of parasites in their pollen ($\chi^2 = 5.04$, d.f. = 2, $P = 0.081$ and $\chi^2 = 0.581$, d.f. = 1, $P = 0.446$, respectively). However, DWV was more common in bumblebee colonies from 2011 compared with 2012 (7/30 vs. 0/18 colonies; $P = 0.036$), while the reverse was true for *C. bombi* (9/30 vs. 11/18 colonies; $\chi^2 = 4.32$, d.f. = 1, $P = 0.038$). *N. bombi* was found in bumblebees from 5/5 colonies from Producer C, compared with 5/22 from Producer A and 3/13 from Producer B ($P = 0.002$), while *N. apis* was found only in pollen samples from Producer B (4/10 samples vs. 0/15 from the other two suppliers; $P = 0.048$). There were no other significant differences between bumblebee subspecies, producers or years in the number of colonies or pollen samples that carried the different parasites ($P > 0.05$ in all other cases; Table S6, Table S7, Supporting Information).

EXPERIMENT 1: INFECTION RISK TO ADULT BUMBLEBEES

Bumblebee survival was reduced significantly by ingestion of faeces or pollen from commercially produced colonies (*Wald* = 6.11, d.f. = 2, $P = 0.047$), with the negative effect being very similar for the two treatments (after 15 days, bee survival was 61%, 44% and 36% for bees that had ingested control solution, faeces or pollen, respectively; Fig. 2). No control bumblebees became infected by parasites, but bumblebees fed sucrose solution contaminated with either faeces or pollen from commercially produced bumblebee colonies became infected by *A. bombi*, *C. bombi*, *N. bombi* or *N. ceranae*, with the first and last of
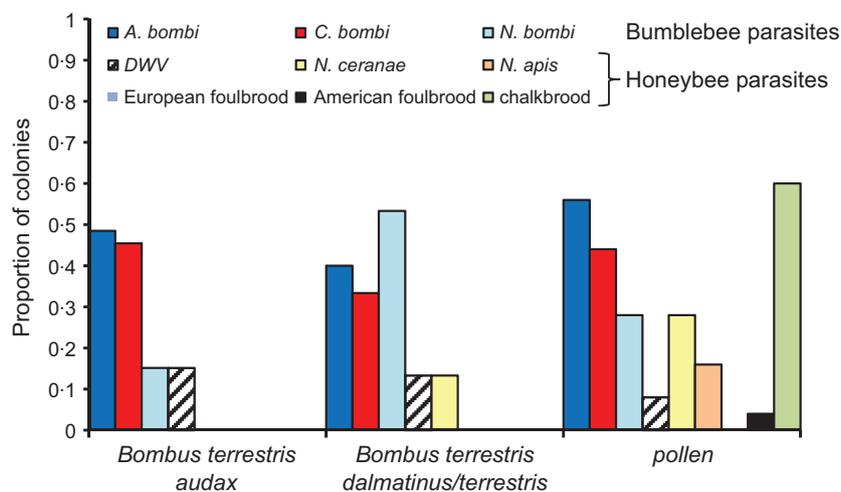

Fig. 1. Commercially produced bumblebee colonies contain a diversity of parasites. Prevalence within 48 commercially produced bumblebee colonies of three species of bumblebee parasites and six species of honeybee parasites. Data are for 33 colonies of the bumblebee subspecies native to the UK (*Bombus terrestris audax*), 15 colonies of the most common subspecies produced commercially (*B. t. dalmatinus* and *B. t. terrestris*) and 25 samples of the pollen supplied with the colonies as food. Fifteen adult bumblebee workers were screened per colony. In 34 colonies, the 15 workers were pooled to give a single presence/absence for each colony, while in the other 14 colonies the 15 workers were screened individually (see Table S2).
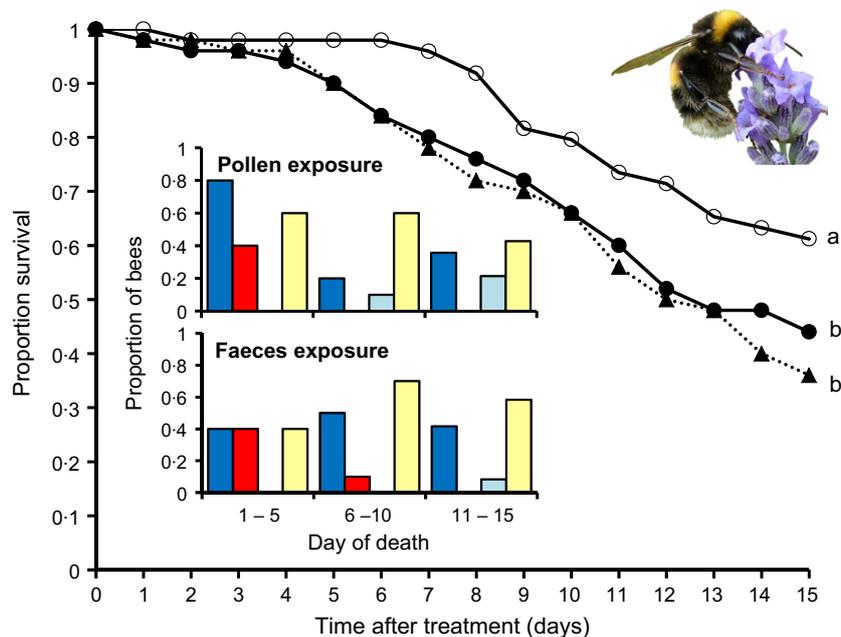
Fig. 2. Ingestion by bumblebees of pollen or faeces from commercially produced bumblebee colonies leads to parasite infections. The effect on the survival of *Bombus terrestris audax* bumblebees of ingestion of either faeces (black circles, solid line) or pollen (triangles, dashed line) from parasite-infected, commercially produced bumblebee colonies, compared to ingestion of control solution (open circles, solid line; $n = 50$ for each treatment). Different letters beside lines indicate treatments that differed significantly ($P < 0.05$) from one another in Kaplan–Meier pairwise comparisons. Inset graphs show the proportion of bumblebees that had died either 1–5, 6–10 or 11–15 days after ingesting either pollen (top graph) or faeces (bottom graph), and which were then found by PCR to be positive for either the *Apicystis bombi* (dark blue columns), *Crithidia bombi* (red columns), *Nosema bombi* (light blue columns) or *Nosema ceranae* (yellow columns) parasites. No bees contained detectable deformed wing virus or *Nosema apis*, and control bees remained free of detectable parasite infections throughout the experiment.

these parasites being most prevalent (Fig. 2). There was no difference between the faeces and pollen treatments in the prevalence of bees that developed *C. bombi* or *N. ceranae* (Table S8, Supporting Information), but significantly more of the bees that had ingested pollen developed *N. bombi* and *A. bombi* infections ($P = 0.031$ and $\chi^2 = 7.39$, d.f. = 1, $P = 0.007$, respectively). None of the bees that died within 6 days of exposure had parasite spores visible in their hind guts, and neither *A. bombi* nor *C. bombi* were visible in the bees that died later. However, 45% of the bees that died 7 or more days after exposure and had ingested pollen, and 11% of those that had ingested faeces, had *Nosema* spores visible in their guts, containing respectively 1.8×10^4 and 9×10^3 *Nosema* spores on average in the small 0.2-cm^3 tissue samples examined. There was also evidence of a sublethal effect of exposure to the pollen or faeces, with a significant interaction between the effects of treatment and day of testing on the sucrose response threshold of bumblebees ($\chi^2 = 15.3$, d.f. = 6, $P = 0.018$), due to bumblebees that ingested faeces or pollen having a lower sucrose response threshold than the control bumblebees on day 5 (Fig. S1, Supporting Information).

EXPERIMENT 2: INFECTION RISK TO ADULT HONEYBEES

The survival of honeybees was significantly affected by treatment (*Wald* = 15.6, d.f. = 2, $P < 0.001$), being significantly reduced by the ingestion of bumblebee faeces from 70% to 40% of bees surviving after 14 days, but less affected by the ingestion of pollen (Fig. 3). None of the control honeybees became infected by the various parasites, but substantial proportions of the honeybees that ingested either pollen or faeces from commercially produced bumblebee colonies became infected by *N. apis*, *N. ceranae* and *Apicystis bombi*, with 33–60% of bees becoming infected by *N. ceranae* after ingesting bumblebee faeces and 20–27% after ingesting pollen (Fig. 3). There was no significant difference between bees that ingested faeces or pollen in the numbers in which the *A. bombi* and *N. apis* parasites were subsequently detected ($\chi^2 = 0.296$, d.f. = 1, $P = 0.586$ and $\chi^2 = 0.64$, d.f. = 1, $P = 0.424$, respectively), but significantly more of the honeybees fed bumblebee faeces had *N. ceranae* than those fed pollen ($\chi^2 = 4.61$, $P = 0.032$).

EXPERIMENT 3: INFECTION RISK TO HONEYBEE LARVAE

None of the larvae tested positive for the *M. plutonius* or *P. larvae* bacteria, and none of the control larvae developed infections of the fungal parasite *Ascosphaera apis*. However, larvae fed pollen from commercially produced bumblebee colonies had significantly lower survival than control larvae or larvae fed diet containing pollen that had been frozen and microwaved before ingestion to
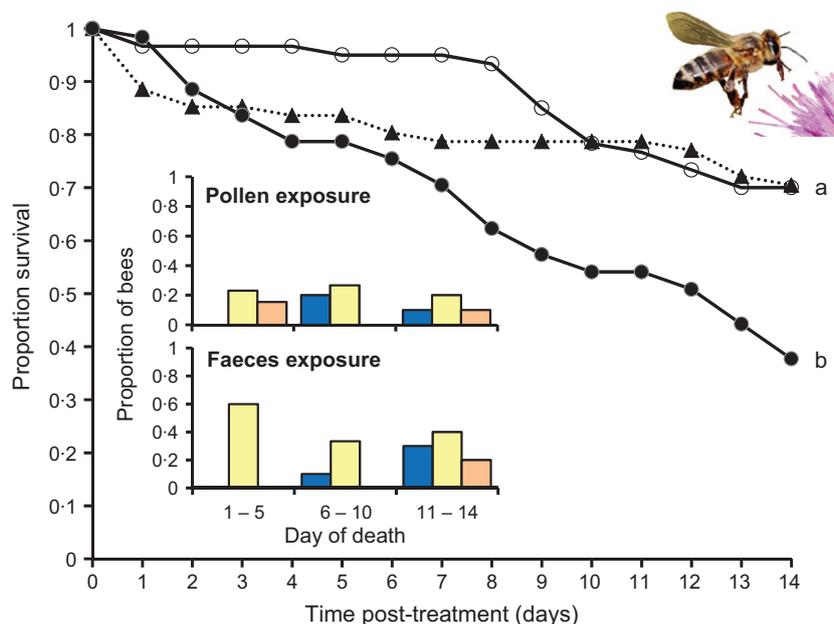
Fig. 3. Ingestion by honeybees of pollen or faeces from commercially produced bumblebee colonies leads to parasite infections. The effect on the survival of *Apis mellifera* honeybees of ingestion of either bumblebee faeces (black circles, solid line) or pollen (triangles, dashed line) from parasite-infected, commercially produced bumblebee colonies, compared to ingestion of control solution (open circles, solid line; $n = 60$ for each treatment). Different letters beside lines indicate treatments that differed significantly ($P < 0.05$) from one another in Kaplan–Meier pairwise comparisons. Inset graphs show the proportion of honeybees that had died either 1–5, 6–10 or 11–14 days after ingesting either pollen (top graph) or faeces (bottom graph), and which were then found by PCR to be positive for either the *Apicystis bombi* (blue columns), *Nosema ceranae* (yellow columns) or *Nosema apis* (orange columns) parasites. No bees contained detectable *Nosema* bombi or *Crithidia bombi*, and control bees remained free of detectable parasite infections throughout the experiment.

reduce the viability of parasites (*Wald* = 6·97, d.f. = 2, $P = 0.031$; Fig. 4). Some 55% of the pollen-fed larvae that died were found to be infected by the *Ascosphaera apis* fungal parasite, compared with 23% of those fed pollen that had been frozen and microwaved ($\chi^2 = 11.4$, d.f. = 1, $P = 0.001$, Fig. 4).

Discussion

Bumblebee colonies that were commercially produced as recently as 2011 and 2012 by all three of the producers that we investigated contained a number of faecal–orally transmitted parasites. Importantly, we sampled bees from colonies immediately upon receipt; therefore, the parasites detected must have entered the colonies during their production. The parasites included three specialist parasites of bumblebees (*Apicystis bombi*, *Crithidia bombi* and *Nosema bombi*) that can negatively affect their health (Schmid-Hempel 2001), with the colony-level prevalence of these being similar to the prevalence reported in wild bumblebee populations (Gillespie 2010; Whitehorn *et al.* 2011; Goulson, Whitehorn & Fowley 2012). There was also evidence of two parasites (DWV and *N. ceranae*), which can infect bumblebees and honeybees (Genersch *et al.* 2006; Graystock *et al.* 2013), and three other honeybee-specific parasites, including *P. larvae*. *P. larvae* causes the highly virulent American foulbrood disease in honeybee larvae which is a notifiable disease in the UK and throughout the EU (European Commission 1992), and

colonies found with the disease in the UK have to be destroyed immediately. The importation with commercially produced bumblebees of pollen carrying this parasite is thus of particular concern. The PCR and RT-PCR methods we used detected the DNA of the parasites, but spores of both *Nosema* and *Apicystis* were clearly visible in the guts of the commercially produced bumblebees, and the number of *Nosema* spores observed was comparable to that found previously for *N. bombi* or *N. ceranae* infections of wild bumblebees (Rutrecht, Klee & Brown 2007; Graystock *et al.* 2013).

The results are consistent with various reports of parasites in bumblebee colonies produced up to 2008 and demonstrate that the problem is still present, in spite of the efforts and regulations designed to ensure that imported colonies are free of disease. Detection of parasites does not necessarily mean that they are infectious parasites which pose a risk to other bees. However, our controlled experiments confirmed that at least the *Nosema bombi*, *N. ceranae* and *Apicystis bombi* parasites carried by commercially produced bumblebees and their pollen were infectious to other bumblebees, reducing survival and also having a sublethal effect on the sucrose response threshold of exposed bumblebees. Although the doses involved of *Nosema* were similar to those used in previous studies (Rutrecht, Klee & Brown 2007; Graystock *et al.* 2013), infection in the wild may well be lower or higher, and survival better or worse, than in our single inoculation laboratory experiment, but the results at a minimum
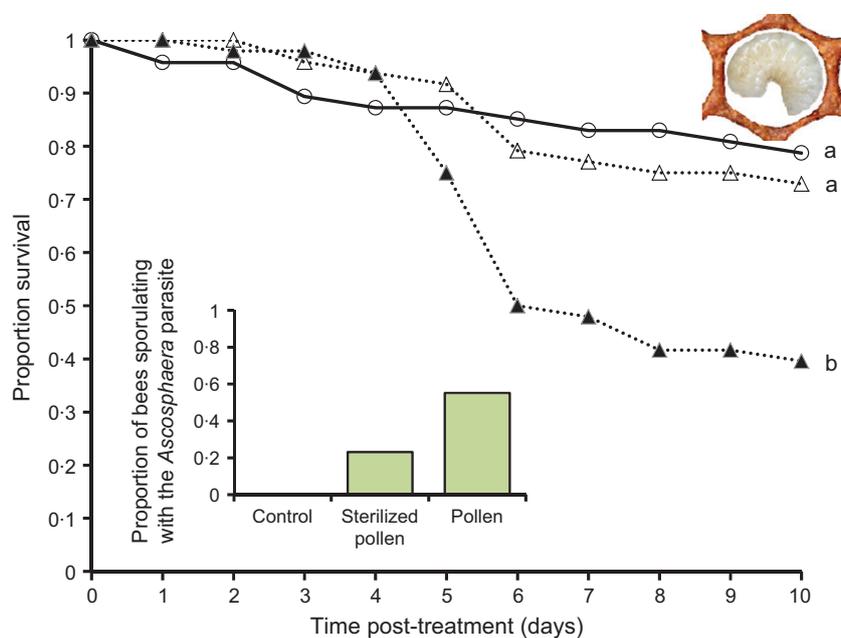
Fig. 4. Ingestion by honeybee larvae of pollen supplied with commercially produced bumblebee colonies leads to parasite infections. The effect on the survival of *Apis mellifera* honeybee larvae of ingestion of either pollen from parasite-infected, commercially produced bumblebee colonies (black triangles, dashed line) or the same pollen sterilized by freezing and microwaving (white triangles, dashed line), compared to ingestion of control solution (open circles, solid line; $n = 48$ for each treatment). Different letters beside lines indicate treatments that differed significantly ($P < 0.05$) from one another in Kaplan–Meier pairwise comparisons. Inset graph shows the proportion of honeybees that died which then sporulated with the chalkbrood fungal parasite *Ascosphaera apis* after ingesting either pollen, sterilized pollen or control solution. No European foulbrood or American foulbrood was detected in the bees.

demonstrate that some parasites carried by commercially produced bumblebees are infectious. Pollen is an important component of bee nutrition that can enhance disease resistance (Foley *et al.* 2012), but our data show that it can carry parasites and therefore be hazardous to bees as well. In contrast to the other parasites, the prevalence of *C. bombi* in the treated bees decreased over time, most probably due to the *C. bombi* having limited viability and being cleared effectively by the bees. The greater prevalence of *Nosema ceranae* and *Apicystis bombi* infections in bumblebees in Experiment 1 that had ingested pollen rather than bumblebee faeces is in keeping with the greater number of *Nosema* and *Apicystis* spores in the pollen exposure treatment, while the higher prevalence of *Nosema ceranae* in honeybees in Experiment 2 that were fed bumblebee faeces rather than pollen is not. Possibly this was because *Nosema* spores in pollen included many of the less virulent *N. apis* (Paxton, Klee & Fries 2008), whereas the apparently limited ability of *N. apis* to infect bumblebees will have meant that *Nosema* spores in bumblebee faeces will have been only *N. ceranae* or *N. bombi*.

Concern about pathogen spillover from commercially produced bumblebees has been focused on the threat to native bumblebees. However, commercially produced bumblebees intermingle with many other managed and native pollinators as well, resulting in significant potential for interspecific transmission of parasites during shared flower use (Durrer & Schmid-Hempel 1994; Singh *et al.* 2010; Evison *et al.* 2012). Our results suggest that this

danger is real, with commercially produced bumblebee colonies carrying at least five parasites of honeybees, of which *Nosema ceranae*, *N. apis* and *Apicystis bombi* were all infectious to adult honeybees. The pollen supplied with the bumblebee colonies also carried spores of the *Ascosphaera apis* fungal parasite that were infectious to honeybee larvae. Spores of this parasite are long-lasting and transmit between colonies by contaminating adult bees that then incorporate the spores accidentally in the food they feed to their larvae (Aronstein & Murray 2010).

The implications of these results are genuinely alarming. They suggest that a majority of the over a million commercially produced bumblebee colonies that are being imported globally each year still potentially contain a diversity of parasites that are viable, infectious and virulent. In some cases, these parasites are highly likely to be different strains or species to those found in native populations in the areas to which they are imported, as observed in Japan and Argentina (Goka *et al.* 2001; Arbetman *et al.* 2012). Even when the parasite strains are the same, the importation of large numbers of infected hosts will increase local parasite density and the probability of mixed parasite infections that can be particularly harmful to hosts. There is already correlational evidence of pathogen spillover from commercially produced bumblebees negatively affecting native bumblebee populations in North America and Argentina (Colla *et al.* 2006; Arbetman *et al.* 2012; Szabo *et al.* 2012). Our experimental results confirm that the parasites carried by commercially produced bum-

blebees are infectious to bumblebees and represent a threat to honeybees as well.

IMPLICATIONS FOR MANAGEMENT

Although the companies producing bumblebees have attempted in recent years to eliminate diseases from their operations, apparently with good success in the case of tracheal mites (Goka, Okabe & Yoneda 2006), the results show that far more robust measures are required. Eliminating parasites from the pollen fed to the bumblebees or replacing the pollen with a hygienic substitute is likely to be essential. In addition, the prevalence of parasites in bumblebee colonies that were sold and imported as being parasite-free demonstrates that more robust checks are also required. Many of the parasites are difficult to detect visually and currently impossible to culture *in vitro*, so these checks will have to use sensitive molecular methods in order to be effective. The prevalence and intensity of parasite infections in animals can increase during shipping from the production facilities to the end-user, a phenomenon that is well known in vertebrates and sometimes termed 'shipping fever' (Barham *et al.* 2002) and that could be one possible explanation for why colonies had heavy parasite infections upon receipt. Either extremely rigorous parasite screening at source or parasite screening on arrival, or probably both, would be needed to prevent this. A further problem relates to the regulations applied to bumblebees. In England, the importation licences requiring parasite screening are limited to the non-native subspecies of *Bombus terrestris*. Our results show that native *B. t. audax*, as well as the non-native subspecies, carries parasites, so some form of regulation to prevent the import of parasites with a commercially produced native organism will also be needed if pathogen spillover is to be prevented. Given the ecological significance and vulnerability of many wild pollinator species, the economic importance of crop pollination with commercially produced bumblebees and the substantial fitness effects of the parasites they currently carry, such measures to reduce the accidental importation of parasites with commercially produced bumblebees are urgently needed.

Acknowledgements

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Supporting Information

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

Fig. S1. Effect of ingestion by bumblebees of pollen or faeces on sucrose sensitivity.

Table S1. PCR mixes and conditions for the detection of the various parasites.

Table S2. Occurrence of parasites in adult workers from bumblebee colonies.

Table S3. Occurrence of parasites in pollen supplied with bumblebee colonies.

Table S4. Results of GLM examining parasite species richness in bumblebees.

Table S5. Results of GLM examining parasite species richness in pollen.

Table S6. Results of GLM examining parasite prevalence in bumblebees.

Table S7. Results of GLM examining parasite prevalence in pollen.

Table S8. Results of GLM examining parasite prevalence in Experiment 1.

Table S9. Results of GLM examining sucrose responsiveness in Experiment 1.

Table S10. Results of GLM examining parasite prevalence in Experiment 2.

Table S11. Results of GLM examining prevalence of the *Ascosphaera* parasite in Experiment 3.