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Emerging dangers: Deadly effects of an emergent parasite in a new pollinator host

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ABSTRACT

There is growing concern about the threats facing many pollinator populations. Emergent diseases are one of the major threats to biodiversity and a microsporidian parasite, *Nosema ceranae*, has recently jumped host from the Asian to the Western honeybee, spreading rapidly worldwide, and contributing to dramatic colony losses. Bumblebees are ecologically and economically important pollinators of conservation concern, which are likely exposed to *N. ceranae* by sharing flowers with honeybees. Whilst a further intergeneric jump by *N. ceranae* to infect bumblebees would be potentially serious, its capacity to do this is unknown. Here we investigate the prevalence of *N. ceranae* in wild bumblebees in the UK and determine the infectivity of the parasite under controlled conditions. We found *N. ceranae* in all seven wild bumblebee species sampled, and at multiple sites, with many of the bees having spores from this parasite in their guts. When we fed *N. ceranae* spores to bumblebees under controlled conditions, we confirmed that the parasite can infect bumblebees. Infections spread from the midgut to other tissues, reduced bumblebee survival by 48% and had sub-lethal effects on behaviour. Although spore production appeared lower in bumblebees than in honeybees, virulence was greater. The parasite *N. ceranae* therefore represents a real and emerging threat to bumblebees, with the potential to have devastating consequences for their already vulnerable populations.

1. Introduction

Pollinators are of major ecological and economic importance, being essential for the reproduction of at least two thirds of flowering plant species (Ollerton et al., 2011) and pollinating crops with an estimated value of $153 billion pa globally (Gallai et al., 2009; Potts et al., 2010). However, the populations of many pollinator species have declined substantially in recent years due to a multitude of factors, with some species going extinct and many more species now being vulnerable (Biesmeijer et al., 2006; Potts et al., 2010). In the UK, for example, 8 out of 25 species of bumblebees have decreased substantially in abundance since 1940 and another two have gone extinct, while 13 species have gone extinct in at least one European country and four across the entire continent (Goulson et al., 2008).

One of the major threats to biodiversity in general, and vulnerable species in particular, are emergent diseases (Daszak et al., 2000). Disease emergence occurs when a parasite infects a novel host population either through translocation or by chance development within hosts previously incompatible for pathogen replication (Daszak et al., 2001). The likelihood of this 'pathogen spillover' varies between host and pathogen, with closely related, sympatric hosts having a greater potential to transmit pathogens between them (Perlman and Jaenike, 2003). Recently, the microsporidian parasite *Nosema ceranae*, has emerged as an important disease of honeybees (*Apis* spp.). It is a gut parasite of adult bees, transmitted horizontally via the faecal-oral route, with infection occurring following the ingestion of spores which germinate in the midgut of the host insect and infect the epithelial cells (Gisder et al., 2011). Successful infections produce spores which are excreted in the faeces, and which are persistent, remaining viable on the bees, pollen and hive materials that they contaminate for periods in excess of a year (Fenoy et al., 2009; Higes et al., 2010). The parasite originated in the Asian honeybee, *Apis ceranae*, but following translocation of honeybees for apiculture, has successfully jumped host in recent decades to multiple other *Apis* species, most notably the Western honeybee, *Apis mellifera*, in which it has now spread worldwide to be the most common *Nosema* species found in many areas (Klee et al., 2007). The parasite can negatively affect lifespan, immunocompetence, learning and flower handling ability in its new *A. mellifera* host (Higes et al., 2007, 2008a; Antúnez et al., 2009; Mayack and Naug, 2009; Naug and Gibbs, 2009; Paxton et al., 2007). However, while in some areas, particularly Spain, the parasite appears to have a major effect on bee health and has been implicated in substantial colony losses (Higes et al., 2006, 2008a, 2010), in other areas, such as North America and Germany, the im-

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pact of the parasite appears to be less significant (Cox-Foster et al., 2007; Fries, 2010; Genersch et al., 2010; Gisder et al., 2010; Klee et al., 2007; Paxton et al., 2007; Vanengelsdorp et al., 2009). The effects of the parasite therefore appear to be strongly dependent on context, other stresses or perhaps host-parasite strains (Aufauvre et al., 2012; Chaimanee et al., 2013; Fenoy et al., 2009; Fries, 2010; Klee et al., 2007; Paxton et al., 2007; Vidau et al., 2011).

Alarmingly, two recent studies have found molecular evidence of *N. ceranae* in some bumblebee (*Bombus*) species, suggesting a spillover from honeybees to bumblebees may be occurring (Li et al., 2012; Plischuk et al., 2009). This spillover could be a recent event brought about by the rapid geographic spread of *N. ceranae*, exposing naive bumblebee species from Europe and America which will not previously have encountered the parasite. However, it is currently unclear whether the *N. ceranae* detected molecularly in bumblebees represents infections or simply vectoring of ungerminated spores. Equally, it is not known how virulent *N. ceranae* is to bumblebees if it can infect them. Bumblebees are economically and ecologically important pollinators in a variety of ecosystems and many species are of conservation concern (Goulson, 2010). Therefore the emergence of a new, virulent pathogen could have significant ramifications, particularly for populations that are already threatened by other causes such as habitat loss. Here we determine the prevalence of *N. ceranae* in wild British bumblebees and test experimentally whether *N. ceranae* from honeybees is able to infect the most widely distributed European bumblebee, *Bombus terrestris*. We also examine the lethal and sub-lethal effects of exposure to the parasite, and compare the virulence of the parasite across its host range.

2. Methods

2.1. Prevalence of Nosema ceranae in wild bumblebees

A total of 764 *Bombus* sp. were captured with sweep nets at five sites in the UK (Cambridgeshire, Merseyside, Oxfordshire, Kent and Essex), and stored immediately in 100% ethanol (see Supplementary table). The midgut, malpighian tubules and fatbody were dissected from each bee (as these are the tissues in which *Nosema bombi* and *N. ceranae* infections have been reported in bumblebees and honeybees respectively; (Chen et al., 2009; Fries et al., 2001; Gisder et al., 2011)), homogenised together, and screened for *Nosema* by PCR. DNA was extracted using 5% Chelex and all samples were amplified for the *18S* Apidae host control gene to confirm the quality of the DNA extraction (Meeus et al., 2010) using 0.4 mM dNTP, 1.5 mM $MgCl_2$, 3 μl Buffer, 1.25 U Taq, 0.2 μM each primer and 1 μl template, giving 10 μl in total. This PCR was then subject to 2 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 56 °C, 45 s at 72 °C before a final elongation stage of 3 min at 72 °C. Samples were also screened for *N. ceranae* and *N. apis* based on 16S rRNA sequences (Chen et al., 2008), with reactions containing 0.25 mM dNTP, 3.75 mM $MgCl_2$, 2 μl Buffer, 0.25 U Taq, 0.2 μM each primer and 1 μl template, giving 10 μl in total for each reaction. Reaction conditions were 2 min at 94 °C, 35 cycles of 30 s at 94 °C, 45 s at 61 °C (63 °C for *N. apis*), 120 s at 72 °C before a final elongation stage of 7 min at 72 °C. Finally, the samples were also screened for *N. bombi* (Klee et al., 2006), with reactions containing 0.3 mM dNTP, 3.75 mM $MgCl_2$, 2 μl Buffer, 0.25 U Taq, 0.2 μM each primer and 2 μl template, giving 10 μl in total for each reaction. Reaction conditions were 4 min at 94 °C, 35 cycles of 60 s at 95 °C, 60 s at 50 °C, 60 s at 72 °C before a final elongation stage of 4 min at 72 °C. Negative and positive controls were included in every assay. To ensure accuracy when determining the species of *Nosema* detected, all *Nosema* findings were double checked with additional primers targeting species specific regions of RPB1 gene (Gisder and Genersch, 2013). In addition, the number of spores present in the tissue samples was counted using a compound microscope and haemocytometer. The three *Nosema* species are similar morphologically, so this was done only for bees that tested positive for *N. ceranae* but negative for *N. apis* and *N. bombi* by PCR.

2.2. Infectivity of N. ceranae from honeybees

The abdomens of 20 *A. mellifera* honeybees from *N. ceranae* infected hives (confirmed by species-specific PCR (Chen et al., 2008)) were homogenised and the resulting lyse was slowly poured onto the surface of an isotonic solution of 95% Percoll gradient in PBS. This solution was then centrifuged for 45 min at 11,000*g* and 15 °C to separate *Nosema* spores from other particles of different sizes (e.g. viruses or other parasites (Pertoft, 2000; Seleznev et al., 1995)), with the resulting pellet of spores then extracted with a pipette. Spores were washed by first centrifuging at 14,000*g* for 15 min, removing the supernatant, and replacing with water before vortexing for 10 s. This wash process was repeated three times to remove traces of Percoll and produce a clean suspension of *Nosema* spores, with the suspension then confirmed with a compound microscope to contain only *Nosema* spores and no other observable parasites (e.g. *Crithidia*, *Apicystis*, *Ascosphaera*). The identity of the suspended *Nosema* spores was confirmed as *N. ceranae* following PCR using 3 primer pairs specific to *N. ceranae*, *N. apis* and *N. bombi*, as above (Chen et al., 2008; Klee et al., 2006).

One hundred *B. terrestris audax* were collected from three parasite-free colonies (confirmed by screening 15 bees per colony by PCR for the three *Nosema* species) and placed into cohorts of 10 bees in 10 × 6 × 6 cm plastic boxes. All bumblebees were starved for 8 h before being individually hand-fed a single 5 μl dose of either a 40% sucrose solution containing approximately 6500 Percoll-purified *N. ceranae* spores or a 40% pure sucrose control solution ($n = 50$ bees in each case). As pollen is often contaminated with *Nosema* and other parasites (Graystock et al., n.d.), this was not fed to the bees. The bees were then replaced in their groups of 10 like-treated, nest-mate bees, provided with 40% sucrose solution *ad libitum*, and their survival checked daily for 14 days. In addition, 100 *A. mellifera* workers from three *Nosema*-free colonies were also treated in the same way and their survival was monitored for 7 days to provide comparative data on parasite virulence.

The sensitivity of a bee to low sucrose concentrations has been linked to hunger and learning ability (Naug and Gibbs, 2009; Scheiner et al., 2001) making it a good indicator of non-lethal effects of parasite infection. The sensitivity of the bumblebees to differing sucrose concentrations (10–80% in increments of 10) was therefore tested for the bees every 5 days using the proboscis extension response (Riveros and Gronenberg, 2009), with the responses of the individual bees in each group of 10 being averaged.

After the experiment, all surviving bumblebees, as well as those that died during the experiment, were screened for infection by *N. ceranae*. To avoid cross-contamination of tissues, a tergite with attached fatbody was first removed carefully from the bee, prior to opening the abdomen and dissecting out a small section of midgut. This prevented entirely the fatbody sample becoming contaminated with any material from the digestive tract. The section of midgut was homogenised in 100 μl of deionised water and the number of *Nosema* spores counted using a compound microscope and haemocytometer. To confirm spores were *N. ceranae*, and also if an infection had spread to the fatbody, the samples of midgut and fatbody from each bee were screened separately by PCR as before. In addition, a subset of the samples that were positive for *Nosema* were sequenced to confirm the PCR amplicons were indeed *N. ceranae*.

2.3. Statistical analysis

Differences in bumblebee and honeybee survival were analysed using a Cox proportional hazards regression model, with colony-of-origin and cohort included to account for the structured nature of the data, and non-significant terms removed stepwise to obtain the minimum adequate model. Pairwise comparisons were made using Kaplan–Meier models with the Breslow χ^2 statistic. The sucrose sensitivity data were analysed using Mann–Whitney *U* tests to compare the sensitivity of bees fed *N. ceranae* or control solution on 0, 5, 10 and 15 days after treatment. For comparison with other studies, the relative risk of exposure to *N. ceranae* was calculated on day 7 for both the bumblebee and honeybee data, and for comparable studies (caged bees, $n \geqslant 50$ per treatment, known spore dose), as: Relative Risk $= \frac{P \text{ exposed}}{P \text{ non-exposed}}$ where *P* is the probability of death for either individuals exposed or not to *N. ceranae*.

3. Results

3.1. Prevalence of N. ceranae in wild bumblebees

Bumblebees from 7 different *Bombus* species across three of the five sites sampled had *N. ceranae* based on PCR. In total, 21% of the 764 bumblebees screened were positive for *N. ceranae* whilst also being negative for *N. bombi* and *N. apis*. The result was identical for both the Chen et al. (2008) and Gisder and Genersch (2013) protocols. Of these individuals, 19% had infections intense enough to produce observable *Nosema* spores under a microscope, with these bumblebees having on average 6628 ± 1261 spores in the small samples of their tissue (see Supplementary table).

3.2. Infectivity of N. ceranae from honeybees

Bumblebees which ingested *N. ceranae* spores had significantly lower survival over the 15 days period than bumblebees fed control solution ($\chi^2 = 15.94$, $P < 0.001$), with the greatest mortality (38% of those ingesting spores) occurring between 3 and 7 days after exposure (Fig. 1). None of the control bumblebees had *Nosema* spores in their midguts or were positive for *N. ceranae* by PCR, but many of the bumblebees which had ingested *N. ceranae* spores were found to be infected by the parasite (Fig. 1). A low proportion (0–25%) of the treated bumblebees which died up to 4 days after exposure were positive for *N. ceranae* by PCR, but this proportion was close to 100% for bumblebees which died from day 5 onwards. Approximately a third of the bumblebees which survived to the end of the 15 days experimental period were positive for *N. ceranae* in their midgut and also in their fat body, indicating that the parasite had moved between tissues in these bees (Fig. 1). Interestingly, the proportion of bumblebees which had *Nosema* spores detectable by microscopy in their midguts was much lower than the proportion positive by PCR, and spores were only seen in the bumblebees which died between days 4 and 7 after exposure (Fig. 1). These bees, which had originally been fed 6500 *N. ceranae* spores, were found to have > 11,400 spores in just the small sample of midgut at the end of the 15 days period, indicating that substantial spore production had occurred. Sucrose sensitivity was similar on day 0 for bumblebees fed *N. ceranae* or control solution ($U = 1220$, N = 99, $P = 0.969$), but was significantly lower 5 and 10 days after exposure for bees that had ingested *N. ceranae* ($U = 655$, N = 85, $P = 0.013$, and $U = 309$, N = 60, $P = 0.049$, respectively; Fig. 2). On 15 days after exposure, the sucrose sensitivity of surviving bees was again similar for bees fed *N. ceranae* or control solution ($U = 242$, N = 49, $P = 0.394$; Fig. 2). In contrast to the bumblebee results, the ingestion of *N. ceranae* spores had little effect on the survival of honeybees in our experiment at the dose tested, with survival being >95% both for bees that ingested the control solution or the solution containing *N. ceranae* spores ($\chi^2 = 0.003$, $P = 0.953$; this also confirms that our washing protocol was successful at removing traces of Percoll). Based on mortality 7 days after treatment, the relative risk from exposure to *N. ceranae* for the honeybees treated here was broadly similar to that found in previous studies, with the relative risk associated with interspecific exposure between *Apis* spp. being somewhat greater (Fig. 3). However, the relative risk from exposure to *N. ceranae* calculated from the bumblebee data was substantially higher (Fig. 3).

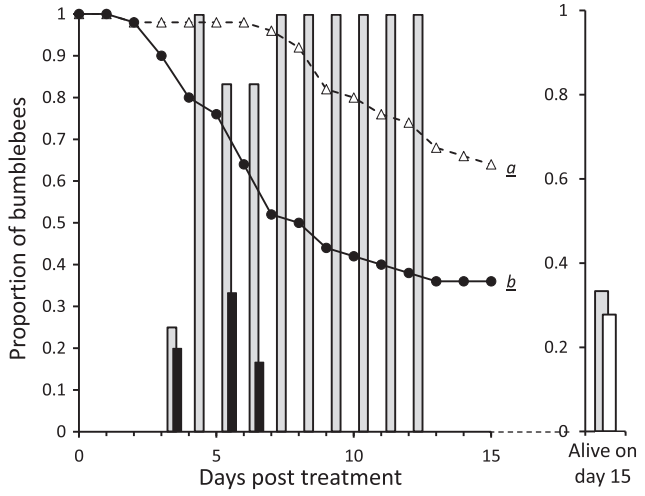

Fig. 1. The proportion of bumblebees surviving after ingestion of either a sucrose solution control (dashed line) or a sucrose solution with 6500 spores of the *N. ceranae* parasite (solid line). Bars represent the proportion of dead bumblebees from the *Nosema*-treated group that had visible spores in the midgut (black bars), PCR-detectable *N. ceranae* in their midgut (light grey bars) or fatbody (white bars; only checked for bees that survived to the end of the experimental period). The parasite was never detected in the control bees.

4. Discussion

The results show conclusively that *N. ceranae* from honeybees is capable of infecting bumblebees and is already circulating in wild populations. Between 20–47% of wild-caught bumblebees at three out of the five sites we sampled in the UK were positive for *N. ceranae*, and in many cases these bees had *N. ceranae* spores in

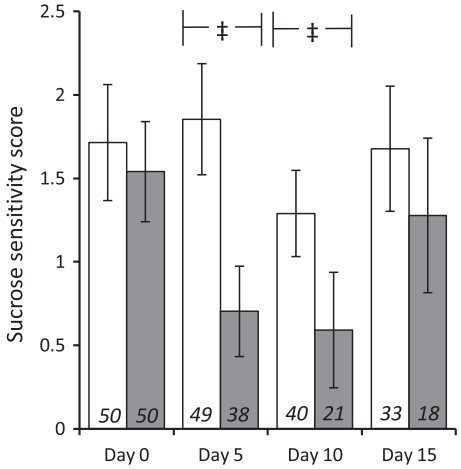

Fig. 2. Mean ± s.e. sucrose sensitivity of bumblebees on 0, 5, 10 and 15 days following ingestion of either the *N. ceranae* parasite (dark grey) or a sucrose solution (white bars). Sample size is indicated at the base of each column. Significant pairwise differences ($P < 0.05$) are indicated by the ‡ symbol.

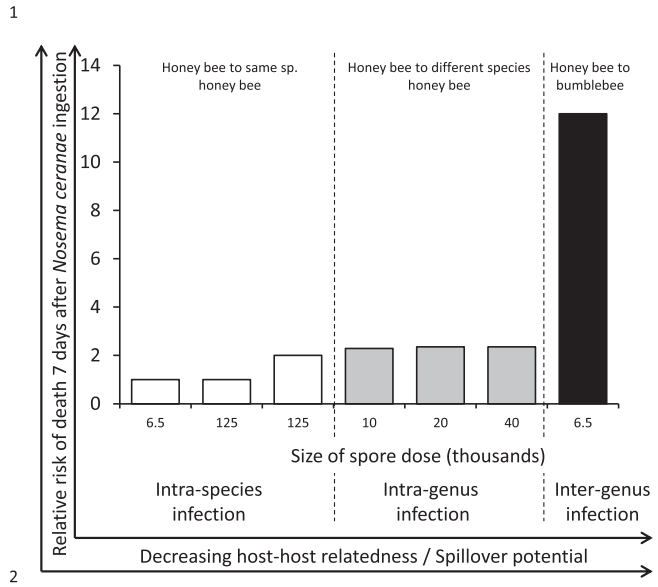

Fig. 3. The relative risk that *N. ceranae* poses to either a host of the same species (intra-species infection), a different *Apis* species (intragenus infection) or to bumblebees (intergenus infection). The relative risk is calculated on day 7 for all comparable laboratory studies where N > 50 per treatment (Aufauvre et al., 2012; Suwannapong et al., 2011; Vidau et al., 2011).

their guts. Furthermore, the presence of *N. ceranae* was not restricted to the common *B. terrestris* bumblebee but was found in a total of 7 different species (all the species tested). There have been previous reports of *N. ceranae* in wild bumblebees in Argentina and China based on PCR screening (Li et al., 2012; Plischuk et al., 2009), and our screening shows both that it is also present in a variety of UK bumblebees and that it is actually infecting them. Ingestion of *N. ceranae* spores in our experiments resulted in 62% of bumblebees becoming infected, with the dose used being less than that in many infection studies of *N. ceranae* in honeybees (Higes et al., 2007, 2008b; Vidau et al., 2011), and an order of magnitude less than that in infection studies of bumblebees by their natural *N. bombi* parasite (Rutrecht and Brown, 2009; Schmid-Hempel and Loosli, 1998). *N. ceranae* therefore appears to be at least as, and quite probably more, infective to bumblebees than to honeybees. Many bumblebee species in the UK, as well as elsewhere are declining and now highly vulnerable to new stresses (Goulson et al., 2008), so the emergence of a new, virulent disease has significant implications for their conservation.

Infections by *N. ceranae* of bumblebees were highly virulent, with 48% of exposed bees dying within 7 days of exposure compared to 4% of unexposed bees, and the risk from exposure being substantially higher than for infections of honeybees. This was in spite of the bumblebees being provided with *ad libitum* food and a constant, benign environment, and the mortality may be even greater under natural conditions (Brown et al., 2000; Mayack and Naug, 2009). It has been suggested that *N. ceranae* may be more virulent in *A. mellifera* than the natural *Nosema* species, *N. apis* (Paxton et al., 2007), and it also seems to be far more virulent in bumblebees than their natural parasite *N. bombi* (Schmid-Hempel and Loosli, 1998). Importantly, most of the mortality associated with *N. ceranae* infections occurred 3–7 days after exposure, which coincided with the period during which bees that died had spores in their midguts. *N. ceranae* spores are produced when infected epithelial cells rupture, releasing the spores into the gut, and this is a major component of the virulence expressed by the parasite (Dussaubat et al., 2012). However, many infected bees died without producing spores, suggesting that the virulence of the parasite is expressed in other ways as well. The surviving bees were either uninfected or had *N. ceranae* present in their fat body, as well as midgut. Whilst there is some debate regarding tissue tropism of *N. ceranae* in *Apis* hosts (Huang and Solter, 2013), our dissection protocol specifically excluded contamination of the fat body sample with material from the digestive tract, so it appears that *N. ceranae* can move between tissues, in a similar way to that of the closely related *N. bombi* in *Bombus* sp. (Fries et al., 2001). The results suggest that there are at least four possible outcomes of the host-parasite interaction with bumblebees either: (1) resisting the parasite and remaining uninfected; (2) succumbing to infection, producing spores and concurrently suffering high mortality; (3) tolerating infection, with the parasite moving into the fat body and not subsequently producing spores, or (4) dying due to some other effect of the infection.

In addition to the lethal effects of *N. ceranae* on bumblebees that it infected, there was also evidence of the parasite having sublethal effects on its host. The sucrose sensitivity of infected bees was substantially lower on 5 and 10 days after exposure, with these bees only extending their proboscis in response to a higher concentration of sucrose than control bees. Such reduced sucrose sensitivity has been correlated with impaired learning and flower handling ability in honeybees, which in turn reduces the efficiency, productivity and pollination services provided by the bee (Gegear et al., 2005, 2006; Iqbal and Mueller, 2007; Scheiner et al., 2001). Even a small reduction in the growth of bumblebee colonies can substantially reduce the production of new reproductives (Muller and Schmid-Hempel, 1992; Whitehorn et al., 2012), so these sublethal effects on behaviour may be significant. The effect of *N. ceranae* may well be further compounded by other sub-lethal effects, such as the reduction in immunocompetence and increased susceptibility to pesticide stressors which have been found when *N. ceranae* infects honeybees (Alaux et al., 2010; Antúnez et al., 2009).

The results demonstrate that the spillover of *N. ceranae* from honeybees to bumblebees is occurring, and that its high virulence in bumblebees means that it poses a significant risk to them. In addition to our own findings of *N. ceranae* in wild bumblebee populations in the UK, field surveys using genetic methods suggest that these infections are also taking place in Argentina and China (Li et al., 2012; Plischuk et al., 2009), meaning that there is now evidence of spillover on three different continents. Given the declines and vulnerability of many bumblebee populations, the effects of *N. ceranae* as an emergent, virulent disease may be serious. We sampled only relatively common bumblebee species, but given that all seven species were infected it seems probable that species of conservation concern will also be affected. Indeed, there is some evidence that small, isolated bumblebee populations which lack genetic diversity have higher prevalence of parasites (Whitehorn et al., 2011), so they may be affected more strongly.

The ability of *N. ceranae* to transmit between genera also raises concern about whether it may pose a threat to other genera of bees as well, many of which are also showing declines (Biesmeijer et al., 2006). We have a very poor knowledge of the natural geographic distributions and host associations of bee diseases, and hence of the risks posed by transport of honeybee and bumblebee species for pollination (Goulson, 2003). More research on the potential intergeneric spillover of parasites, and the threats they may pose, to pollinators in general is urgently needed. However, it now appears clear that *N. ceranae* represents a real threat to bumblebees, and consideration of the potential spillover of the parasite from honeybees to bumblebee populations of conservation concern is necessary.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jip.2013.06.005.

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