

1 **Disease associations between honeybees and bumblebees as a threat to wild pollinators**

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15 **Emerging infectious diseases (EIDs) pose a risk to human welfare, both directly<sup>1</sup> and**  
16 **indirectly, by affecting managed livestock and wildlife that provide valuable resources**  
17 **and ecosystem services, such as the pollination of crops<sup>2</sup>. Honey bees (*Apis mellifera*),**  
18 **the prevailing managed insect crop pollinator, suffer from a range of emerging and**  
19 **exotic high impact pathogens<sup>3,4</sup> and population maintenance requires active**  
20 **management by beekeepers to control them. Wild pollinators such as bumble bees**  
21 **(*Bombus* spp.) are in global decline<sup>5,6</sup>, one cause of which may be pathogen spillover**  
22 **from managed pollinators like honey bees<sup>7,8</sup> or commercial colonies of bumble bees<sup>9</sup>. In**  
23 **our study, a combination of infection experiments with landscape scale field data**  
24 **indicates that honey bee EIDs are indeed widespread infectious agents within the**

25 **pollinator assemblage. The prevalence of deformed wing virus (DWV) and the exotic**  
26 ***Nosema ceranae* is linked between honey bees and bumble bees, with honey bees having**  
27 **higher DWV prevalence, and sympatric bumble bees and honey bees sharing DWV**  
28 **strains; *Apis* is therefore the likely source of at least one major EID in wild pollinators.**  
29 **Lessons learned from vertebrates<sup>10,11</sup> highlight the need for increased pathogen control**  
30 **in managed bee species to maintain wild pollinators, as declines in native pollinators**  
31 **may be caused by interspecies pathogen transmission originating from managed**  
32 **pollinators.**

33 Trading practices in domesticated animals allow infectious diseases to spread rapidly and to  
34 encounter novel hosts in newly sympatric wildlife<sup>12</sup>. This “spillover” of infectious disease  
35 from domesticated livestock to wildlife populations is one of the main sources of Emerging  
36 Infectious Disease (EIDs)<sup>13</sup>. Small or declining populations are particularly challenged, as the  
37 source host may act as a disease reservoir<sup>14</sup>, giving rise to repeated spillover events and  
38 frequent disease outbreaks which, in the worst case, might drive already vulnerable or  
39 unmanaged populations to extinction<sup>14</sup>. Such severe impacts have been well documented over  
40 the past decades in vertebrates<sup>10</sup>, but have largely been overlooked in invertebrates<sup>15</sup>. Recent  
41 years have seen elevated losses in multiple populations of one of the major crop pollinating  
42 insects, the honey bee (*Apis mellifera*)<sup>16</sup>. EIDs have been suggested as key drivers of decline,  
43 with deformed wing virus (DWV) (especially in combination with the exotic *Varroa* mite  
44 (*Varroa destructor*)) and *Nosema ceranae* (*N. ceranae*) being two likely causes for losses of  
45 *Apis*<sup>17</sup>. As generalist pollinators, honey bees are traded and now distributed almost worldwide  
46 for crop pollination and hive products. They share their diverse foraging sites with wild  
47 pollinators and thus facilitate interspecific transmission of pathogens, as has been suggested  
48 for intraspecific disease transmission from commercial to wild bumble bee populations<sup>18</sup>. Our  
49 focus is on inter-specific transmission, as EIDs in *Apis* are a potential threat to a range of wild

50 pollinators worldwide. Whilst evidence from small scale studies suggests that wild pollinators  
51 like *Bombus* spp. may already harbour some honey bee pathogens<sup>7,8,19,20</sup>, the true infectivity  
52 and landscape scale distribution of these highly virulent EIDs in wild pollinator populations  
53 remains unknown

54 To examine the potential for *Apis* pathogens to cross host-genus boundaries, we tested the  
55 infectivity of the DWV complex (which includes the very closely related, co-occurring and  
56 recombinant *Varroa destructor* virus (VDV)<sup>21,22</sup>; we will refer to “DWV complex” as  
57 “DWV” throughout the text) and *N. ceranae*, in controlled inoculation experiments, to one of  
58 the most common *Bombus* species in Great Britain (*B. terrestris*). DWV is infective for *B.*  
59 *terrestris*; we found significantly more DWV infections 21 days after inoculating *B. terrestris*  
60 workers versus control (likelihood ratio test comparing the full model to one with only the  
61 intercept:  $X^2 = 5.73$ ,  $df = 1$ ,  $p < 0.017$ ; Fig 1) and mean survival was reduced by 6 days. As  
62 for *Apis*, DWV causes deformed wings in *Bombus* when overtly infected<sup>8</sup>, resulting in non-  
63 viable offspring and reduced longevity (Fig 1). *N. ceranae* is also infective for *B. terrestris*;  
64 infections increased in *Bombus* versus control ( $X^2 = 17.76$ ,  $df = 1$ ,  $p < 0.001$ ; Fig 1), though  
65 overt symptoms were not seen (mean survival increased by 4 days).

66 Having established both DWV and *N. ceranae* as infective for *B. terrestris*, we conducted a  
67 structured survey across 26 sites in GB and the Isle of Man, collecting 10 *Apis* samples, and  
68 20 *Bombus* samples per site to assess EID prevalence (for details on species identity across  
69 sites, see Extended Data Fig. 1). We analysed a total of 745 bees from 26 sites for DWV  
70 presence, DWV infection (replicating DWV) and *N. ceranae* presence. DWV was present in  
71 20% (95% confidence interval (CI) 17-23%) of all samples; 36% (95% CI: 30-43%) of *Apis*  
72 and 11% (95% CI: 9-15%) of *Bombus*. Of the *Apis* harbouring DWV, 88% (95% CI: 70-  
73 98%) of the samples tested had actively replicating virus, whilst 38% (95% CI: 25-53%) of  
74 *Bombus* harbouring DWV had replicating virus (see Extended Data Fig. 2 and Extended Data

75 Table 1). *N. ceranae* was less frequent, being detected in 7% (95% CI: 6-10%) of all samples;  
76 9% (95% CI: 6-13%) of *Apis* samples and 7% (95% CI: 5-9%) of *Bombus* samples.

77 We estimated the GB-wide prevalence of the two pathogens in *Apis* and *Bombus* spp. based  
78 on our field survey data (Fig. 2). We found no evidence for spatial clustering of DWV  
79 presence in *Bombus* (Moran's I = 0.023,  $p > 0.211$ ) or either of the pathogens in *Apis* (DWV  
80 presence: Moran's I = 0.03,  $p > 0.186$ ; *Nosema*: Moran's I = -0.061,  $p > 0.649$ ). There was,  
81 however, weak clustering of DWV infection in *Bombus* (Moran's I = 0.061,  $p < 0.044$ ) and  
82 very strong clustering of *N. ceranae* in *Bombus* (Moran's I = 0.25,  $p < 0.001$ ), indicating  
83 disease hotspots for DWV in *Bombus* in the south west and east of GB and for *N. ceranae* in  
84 *Bombus* in the south east of GB (Fig. 2). Because prevalence was lower in *Bombus* than *Apis*,  
85 we modelled pathogen prevalence in *Bombus* as dependent on pathogen prevalence in *Apis*,  
86 *Bombus* to *Apis* density, and *Apis* abundance, including biologically relevant interactions,  
87 whilst controlling for latitude, longitude, and sunlight hours, and adding collection site and  
88 species identity as random factors. Our full model for DWV presence was significantly better  
89 than the null model without any of the test predictors and their interactions included  
90 (likelihood ratio test:  $X^2 = 19.03$ ,  $df = 5$ ,  $p < 0.002$ ). After removal of the non-significant  
91 interactions (GLMM: *Bombus* to *Apis* density X DWV presence in *Apis*: estimate  $\pm$  SE = -  
92  $0.105 \pm 1.376$ ,  $p = 0.939$ ; *Apis* abundance X DWV presence in *Apis*:  $0.425 \pm 1.309$ ,  $p = 0.745$ ),  
93 it is clear that prevalence of DWV in *Apis* has a strong positive effect on DWV prevalence in  
94 *Bombus* (GLMM:  $2.718 \pm 0.921$ ,  $z = 2.951$ ,  $p < 0.004$ ) (Fig. 2, Extended Data Fig. 3), while  
95 none of the other predictors played a role (GLMM: *Bombus* to *Apis* density:  $0.315 \pm 0.387$ ,  $z$   
96  $= 0.814$ ,  $p < 0.416$ ; *Apis* abundance :  $-0.085 \pm 0.364$ ,  $z = -0.232$ ,  $p < 0.816$ ). In the case of *N.*  
97 *ceranae*, our full model was significantly better than the null model ( $X^2 = 15.8$ ,  $df = 5$ ,  $p <$   
98  $0.008$ ). Specifically there was an effect of *Nosema* prevalence in *Apis* on *Nosema* prevalence  
99 in *Bombus* and this varied with *Apis* abundance (interaction between *Nosema* prevalence in

100 *Apis* and *Apis* abundance:  $X^2 = 7.835$ ,  $df = 2$ ,  $p < 0.02$ ), while *Bombus* to *Apis* density did not  
101 explain *Nosema* prevalence in *Bombus* (GLMM:  $8.386 \pm 6.793$ ,  $z = 1.235$ ,  $p = 0.217$ )(Fig. 2,  
102 Extended Data Fig. 3).

103 The prevalence data implied local transmission of DWV between *Apis* and *Bombus*. To test  
104 this, we sequenced up to 5 isolates per DWV infected *Bombus* sample from 5 sites matched  
105 by up to 5 isolates of sympatric DWV infected *Apis* samples. If a pathogen is transmitted  
106 between these two hosts, we would expect *Apis* and *Bombus* to share the same DWV strain  
107 variants within a site. Marginal log likelihoods estimated by stepping stone sampling<sup>23</sup>  
108 decisively support clades constrained by site as opposed to host, indicating pathogen  
109 transmission within site (Fig. 3, Extended Data Table 2).

110 Our results provide evidence for an emerging pathogen problem in wild pollinators that may  
111 be driven by *Apis*. Our data cannot demonstrate directionality in the interspecific  
112 transmission of DWV. However, the high prevalence of DWV in honey bees, which is a  
113 consequence of the exotic vector *Varroa destructor*<sup>24</sup>, is consistent with their acting as the  
114 major source of infection for the pollinator community. Similar results have been found for  
115 intraspecific transmission of *Bombus*-specific pathogens from high prevalence commercial  
116 *Bombus* colonies to low prevalence wild *Bombus* populations<sup>18</sup>. Our field estimates of  
117 prevalence are conservative for DWV, as highly infected individuals have deformed wings,  
118 are incapable of flight, and thus would not be captured by our sampling protocol.  
119 Consequently, DWV prevalence and, as a result, impact are likely to be higher in managed  
120 and wild populations than our data suggest. Interestingly, *N. ceranae* prevalence in *Bombus*  
121 depends positively on *Apis* abundance, but only when *N. ceranae* prevalence in *Apis* is low,  
122 suggesting a possible environmental saturation effect of *N. ceranae* spores. In contrast to the  
123 low impact of *N. ceranae* on the survival of *B. terrestris* in our study, Graystock et al.<sup>25</sup> found  
124 very high virulence. This might be explained by our use of young bees vs Graystock et al.'s<sup>25</sup>

125 non-age-controlled design, indicating age dependent differential susceptibility in *B. terrestris*,  
126 as has been shown to be the case in honey bees<sup>26</sup>.

127 Ongoing spillover of EIDs could represent a major cause of mortality of wild pollinators  
128 wherever managed bees are maintained. While our data are only drawn from GB, the  
129 prerequisites for honey bees to be a source or reservoir for these EIDs – high colony densities  
130 and high parasite loads – are present at a global scale. In addition, global trade in both honey  
131 bees and commercial *Bombus* may exacerbate this impact<sup>6,27</sup>. Reducing the pathogen burden  
132 in managed honey bees so as to reduce the risk of transmission to wild pollinators is not  
133 straightforward. Tighter control of importation and hygiene levels of transported colonies  
134 could be imposed with regulation, but policies developed in this direction must learn from the  
135 past; such regulation is difficult to implement and hard to evaluate<sup>9,28</sup>. Clearly, it is essential  
136 to ensure that those managing bees (including commercial producers, growers and  
137 beekeepers) have access to the methods and skills to monitor, manage and control EIDs for  
138 the benefit of their managed colonies, and the wider pollinator community. A consensus on  
139 the threat of EIDs for wild pollinators can only be reached with greater knowledge of their  
140 epidemiology, global extent and impact, and it will be crucial to involve key stakeholders  
141 (e.g. the beekeeping community, *Bombus* exporters) in any decision process, as any progress  
142 made will largely be driven by their actions.

143

#### 144 **Methods summary**

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#### 146 ***Bombus* inoculation experiment**

147 Two day old workers of *Bombus terrestris audax* colonies (Biobest) were individually  
148 inoculated with either  $10^5$  spores/bee purified *N. ceranae* or  $10^9$  genome equivalents/bee

149 purified DWV in 10 µl sucrose solution. Bees surviving for 21 days were freeze killed and  
150 molecularly tested for pathogen presence.

151

## 152 **Sampling scheme**

153 Sampling took place at 24 mainland sites and two currently *Varroa destructor* (the main  
154 vector for DWV in *Apis mellifera*) free islands: Colonsay and the Isle of Man (see Extended  
155 Data Fig. 1 for site distribution). Cryptic *Bombus* species were identified by PCR-RFLP-  
156 analysis<sup>29</sup>. *Apis* and *Bombus* densities were estimated for each site by timing the collection  
157 effort for 20 samples from each genus simultaneously. Samples collected were freeze-killed  
158 at -20 °C and transferred to -80 °C as soon as possible thereafter. RNA and DNA preparation  
159 followed standard protocols. Virus strand specific RT-PCR was carried out following Craggs,  
160 et al.<sup>30</sup>.

161

## 162 **Statistics**

163 True prevalences with 95% confidence intervals were computed based on Stevenson, et al.<sup>31</sup>  
164 (R library epiR, version 0.9-45, function epi.prev).

165 Overall prevalence for each of our parasites was calculated using Gaussian kernel estimators  
166 with an adaptive bandwidth of equal number of observations (set to 3x the maximum  
167 observations per site)<sup>32</sup> (R library prevR, version 2.1, function kde).

168 Moran's I was calculated as implemented in Paradis, et al.<sup>33</sup> (R library ape, version 3.0-7,  
169 function Moran.I).

170 We ran Generalized Linear Mixed Models (GLMM)<sup>34</sup> to investigate both effects on disease  
171 status of individuals 21 days after pathogen challenge and also pathogen prevalence in  
172 *Bombus* using the function lmer of the R package lme4<sup>35</sup>. All analyses were run in R<sup>36</sup>.

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 251 Experiments were designed by M.A.F. and M.J.F.B.; M.A.F prepared the manuscript;  
 252 M.J.F.B., D.P.M., R.J.P. and J.O. edited the manuscript. M.A.F. carried out the experimental  
 253 work, molecular work and analyses apart from the phylogenetic analysis carried out by  
 254 D.P.M..

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261 **Figures:**

262 **1 Infectivity:** Prevalence of infections in treated *Bombus terrestris* workers 21 days after  
263 inoculation (in percent). Bars indicate 95% confidence intervals. Colours indicate treatment,  
264 with *Nosema* treated samples in green and DWV treated samples in black. Sample sizes are  
265 given inside the mean data point. The survival graph over the 21 day test period shows  
266 uninfected control treatments in grey compared to infected DWV treatments in blue (Cox  
267 mixed effects model fitted with penalized partial likelihood:  $X^2 = 11.93$ ,  $df = 4.17$ ;  $p < 0.021$ ,  
268 see Supplementary Information).

269 **2 Prevalence:** Estimated pathogen prevalence in *Apis* and *Bombus* across Great Britain and  
270 the Isle of Man. Colour gradient (based on Gaussian kernel estimators with an adaptive  
271 bandwidth of equal number of observations over 26 sites, see Methods) corresponds to  
272 percent prevalence (note different scales). DWV prevalence is displayed in blue and *Nosema*  
273 prevalence in green.

274 **3 Viral strain relations:** RNA-dependent RNA polymerase (RdRp) partial gene phylogeny  
275 of pollinator viruses (see main text). Gene trees were estimated using PhyML v.3.0  
276 maximum-likelihood (ML) bootstrapping (500 replicates) and MrBayes v3.1.2 (see  
277 Methods). Coloured boxes correspond to sites H, L, Q, R and X (as shown on the map) while

278 text colours correspond to host (Red: *Bombus*; Black: *Apis*). Symbols represent node support  
279 values: posterior probability (left), bootstrap support (right). Filled circle: >90%, Target  
280 symbol: >70%, Empty circle: >50%. Branches (//) one third of true length.

## 281 **Methods**

### 282 ***Bombus* inoculation experiment**

283 Each of the 7 experimental *Bombus terrestris* colonies (Biobest) was tested for presence of  
284 the two treatment pathogens DWV and *N. ceranae*. Daily, callows (newly emerged workers)  
285 were removed from the colony, assigned sequentially to random treatment blocks and housed  
286 individually in small Perspex boxes on an *ad libitum* diet of 50% sucrose solution and  
287 artificial pollen (Nektapoll), as natural pollen has been shown to contain viable *N. ceranae*  
288 spores and DWV virions<sup>19,37</sup>. Two day old bumble bee workers were individually inoculated  
289 with a treatment dependent inoculum in 10 µl sucrose. Crude hindgut extracts of 5 *Apis*  
290 workers propagating *N. ceranae* were purified by the triangulation method<sup>38</sup> with slight  
291 adaptations.

292 We used small cages with 30 *N. ceranae* infected honey bees to propagate *N. ceranae* spores  
293 for the inoculum. Every second day we collected 5 honey bees from these cages, and  
294 removed and ground the hindguts. The resulting extract was filtered through cotton and  
295 washed with 0.9% insect ringer (Sigma Aldrich). We triangulated extracts using Eppendorf  
296 tubes and spin speeds of 0.5g for 3 minutes, purifying *N. ceranae* spores over 7 tubes. Spore  
297 numbers were quantified in a Neubauer counting chamber. In parallel, we extracted and  
298 purified *N. ceranae* free bees to use for control inoculations.

299 DWV virus inoculum was prepared according to Bailey & Ball<sup>39</sup> with modifications.  
300 Honeybees with DWV symptoms (crippled wings and body deformities) were crushed in  
301 0.5M potassium phosphate buffer (pH 8.0), filtered and clarified by slow speed centrifugation

302 (8000g for 10 minutes) before being diluted and injected (1µl) into white-eyed pupae for bulk  
303 propagation of virus. After 5 days, up to 100 pupae were harvested, and after a further screen  
304 by qRT-PCR, virus was purified as follows. Virus extraction buffer consisted of 0.5M  
305 potassium phosphate pH 8.0, 0.2% DEICA, 10% diethyl ether. Purification consisted of two  
306 slow speed clarifications (8000g for 10 minutes), one high speed clarification (75000g for 3  
307 hours) followed by re-suspension in 0.5M potassium phosphate buffer (pH8.0) and a final  
308 slow speed clarification. Virus preparations were aliquoted and stored at -80°C until use in  
309 inoculation experiments.

310 The purified virus was checked by quantitative Reverse Transcription (qRT) PCR for the  
311 presence of DWV and the absence of other common honey bee RNA viruses: BQCV, IAPV,  
312 SBV, CBPV, ABPV, and SBPV by PCR.

313 A duplicate dilution series of external DNA standards covering  $10^2$  to  $10^8$  molecules (reaction  
314 efficiencies: 90-110%,  $r^2$ : 0.95-0.99) were included in qRT-PCR runs to quantify DWV  
315 genome equivalents present in the inoculum. For absolute quantification of virus dose, an  
316 external DNA standard was generated by amplifying a genomic fragment of 241bp using the  
317 primers F8668*std* (5'-GAT GGG TTT GAT TCG ATA TCT TGG-3') and B8757*std* (5'-  
318 GGC AAA CAA GTA TCT TTC AAA CAA TC-3') via RT-PCR that contained the 136bp  
319 fragment amplified by the DWV-specific qRT-PCR primers F8668/B8757<sup>40</sup>.

320 Shortly before administering, inocula were prepared to a total concentration of  $10^5$  spores/bee  
321 in 10 µl ( $10^4$  spores/µl sucrose solution). Inocula were administered individually in a small  
322 Petri dish after 30-60 minutes starvation. Only workers ingesting the full 10 µl within 1h  
323 were used in the experiment.

324

325 **Sampling scheme**

326 The mainland sampling sites were chosen across Great Britain along a north-south transect  
327 (12 sampling points with fixed latitude, but free in longitude) and across two east-west  
328 transects (12 sampling points with fixed longitude, but free within a narrow latitudinal  
329 corridor). Each of the mainland sites were at least 30 km apart (mean  $\pm$  SD of nearest  
330 neighbour =  $69.21 \pm 26.39$ ). The island sites were chosen deliberately to gain background  
331 data for both *Apis* and *Bombus* disease prevalence in the absence of *Varroa*, the main  
332 transmission route for DWV in *Apis*. At each sampling site we collected approximately 30  
333 workers for each of the following species: *Apis mellifera*, *Bombus terrestris* (verified by  
334 RFLP-analysis<sup>29</sup>), and the next most common bumble bee on site. We collected free flying  
335 bees from flowers rather than bees from colonies as this is the most likely point of contact in  
336 the field. By collecting from flowers we lowered the likelihood of collecting bumblebees  
337 from different colonies. While we ran the risk of collecting multiple honeybees from the  
338 same hive, this nevertheless represents the potential force of infection for both genera in the  
339 field.

340 Each collection took place along a continuous transect, where maximally ten bees per ten  
341 metre stretch were collected before moving on to the next ten metre stretch. At each site, the  
342 collection area covered at least 1000 m<sup>2</sup> (e.g., 10 x 100m, 20 x 50m). Each sampling point  
343 was within one of the following landcover types: urban areas (gardens and parks), farmland  
344 (hedgerows, border strips, crops, and wildflower meadows), coastal cliffs, sand dunes and  
345 heather moorland.

346 If possible, we collected all bees within a single day. In the case of adverse weather, we  
347 returned as soon as possible to finish the collection at the exact same site. To estimate *Apis*  
348 and *Bombus* densities at each site we timed the collection effort simultaneously. Time taken

349 to collect 20 *Bombus* workers (of any *Bombus* species) and 20 *Apis* workers was recorded,  
350 respectively. Timed collecting efforts took place on a single day only.

351 Samples collected were put in sampling tubes, transferred straight onto ice, then freeze-killed  
352 at -20°C and transferred to -80°C as soon as possible thereafter to ensure optimal RNA  
353 (DWV) preservation.

354

### 355 **RNA work**

356 RNA extraction followed the standard RNeasy plant mini kit (Qiagen) protocol with the final  
357 eluate (in RNase free ddH<sub>2</sub>O) of 30 µl being run over the column twice (for optimal RNA  
358 concentration). For reverse transcription of RNA to cDNA we followed the standard protocol  
359 of the Nanoscript Kit (Primerdesign). Our priming was target specific in separate reactions  
360 for *N. ceranae* (primer pair *N. ceranae*<sup>41</sup>), DWV (primer pair F15/B23<sup>42</sup>) and a housekeeping  
361 gene (primer pair ACTB<sup>43</sup>) as a positive control for RNA extraction efficiency. Bees were  
362 transferred to liquid N<sub>2</sub> prior to dissection. Each bee's abdomen was cut with a sterile scalpel  
363 dorsoventrally along the sagittal plane. One half was submerged in RLT buffer (Qiagen) for  
364 RNA extraction, and the second half was archived at -80C. Tissue disruption and  
365 homogenisation of individual half-abdomens was performed on a tissue lyser II (Qiagen) at  
366 30Hz for 2 minutes followed by 20Hz for 2 minutes. RNA quality and quantity were checked  
367 on a Spectrometer (Nanodrop, Thermo Scientific). cDNA preparation was conducted at 65°C  
368 for 5 minutes for the initial priming immediately before the addition of the reverse  
369 transcriptase. For the extension, samples were incubated at 25°C for 5 minutes followed by  
370 55 °C for 20 minutes and then heat inactivated for 15 minutes at 75°C. cDNA was used as  
371 template in a standard PCR with 57°C, 54°C, and 57°C annealing temperatures, respectively.  
372 Results were visualized on a 2% agarose gel with EtBr under UV light. Agarose gels were

373 scored without knowledge of sample ID. To verify the specificity of the amplicon, one  
374 purified PCR product taken from *Apis* and one taken from *B. lapidarius* were sequenced  
375 (Macrogen Inc.).

376

### 377 **Detection of negative strand DWV**

378 Detection of pathogens in pollinators in the field does not provide proof of infection, as  
379 pathogens are likely being ingested on shared, contaminated food resources and therefore are  
380 inevitably present in the gut-lumen as passive contaminants without necessarily infecting the  
381 host. To minimize these cases, we tested all our DWV positive *Bombus* samples and a subset  
382 of DWV positive *Apis* samples for virus replication, a strong indicator for infection<sup>44</sup>. DWV  
383 is a positive strand virus whose negative strand is only present in a host once the virus is  
384 actively replicating<sup>39</sup>. Reverse transcription was conducted using a tagged primer tagB23<sup>45</sup>  
385 for the initial priming to target exclusively the negative strand. The resulting cDNA was used  
386 in a PCR with the tag sequence and F15 as primers<sup>30,45</sup>. We tested all *Bombus* samples that  
387 were positive for DWV presence and, where possible, 2 DWV-positive *Apis* samples from  
388 each site where we found DWV in *Bombus*.

389

### 390 **Sequencing**

391 DWV sequence diversity was analysed by sequencing up to 5 independent clones per DWV  
392 negative-strand infected *Bombus* sample from 5 sites (H, L, Q, R, X; chosen for their high  
393 DWV infected prevalence in *Bombus*) and 5 clones of DWV infected *Apis* samples from the  
394 same sites (we checked extra *Apis* samples for DWV infection if necessary to match *Bombus*  
395 DWV infections). All *Bombus* samples were *B. lapidarius* with the exception of one sample  
396 from site L (clone05), which was *B. pascuorum* (this sample is not included in any of the  
397 other analyses, but revealed a DWV infection in an initial screening and was hence included

398 in the virus variant analysis). We sequenced a region of the DWV genome: the RNA-  
399 dependent RNA polymerase (RdRp) gene (F15/B23 primer pair<sup>42</sup> used throughout the study).  
400 RdRp is thought to be a conserved region of the virus genome where non-synonymous  
401 substitutions may have significant implications for the epidemiology of the virus<sup>24</sup>. RT-PCRs  
402 and PCR were run as described before. DWV PCR products were verified by gel  
403 electrophoresis as described above; if a clear, clean single band was visible, we proceeded  
404 directly to the cloning protocol. If not, we purified products from the agarose gel following a  
405 standard protocol (Qiaquick Gel Extraction Kit, Qiagen) and used the purified fragment in an  
406 additional PCR. PCR products were cloned using the Invitrogen TA cloning kit (Invitrogen),  
407 according to the manufacturer's instructions. Plasmid DNA was isolated using the Spin  
408 Miniprep kit (Qiagen) and the successful insertion of target sequence was tested by restriction  
409 analysis (digested with EcoR I). Up to 5 clones per sample were sequenced in forward and  
410 reverse orientation (Source BioSciences, Cambridge).

411

### 412 **Analysis of DWV sequences**

413 The 75 *Apis* and *Bombus* clones from sites H, L, Q, R and X were supplemented with DWV  
414 and VDV reference RdRp sequences (accession nos. NC004830 and NC006494  
415 respectively), resulting in a final alignment of 420bp from 77 sequences. Forward and reverse  
416 sequences of each clone were assembled and the consensus sequence was used for further  
417 analysis. Sequences were aligned using Geneious (R 6.1.6) with standard settings. Ends were  
418 trimmed by hand. For the tree building we conducted two independent (MC)<sup>3</sup> algorithms  
419 running for 2 million generations, each with four chains (3 hot, 1 cold), sampling one tree in  
420 1000, under the GTR+ $\Gamma$  (nst = 6) substitution model. Gene trees were estimated using PhyML  
421 v.3.0<sup>46</sup> maximum-likelihood (ML) bootstrapping (500 replicates) and MrBayes v3.1.2<sup>47</sup>,  
422 under a GTR model of sequence evolution and a gamma ( $\Gamma$ ) model, using 4 categories to

423 accommodate rate variation across sites. Burn-in cutoffs were inspected manually for each  
424 parameter file in Tracer v1.4<sup>48</sup>. Inspection of the standard deviation of split frequencies  
425 confirmed that runs had converged (0.0093). To test alternative *a priori* hypotheses of virus  
426 diversification, for each virus (DWV and VDV) we constrained clades according to site (H,  
427 L, Q, R and X) or host genus (*Apis* and *Bombus*), and performed stepping stone sampling<sup>23</sup> as  
428 implemented in MrBayes v3.1.2 to accurately estimate marginal log likelihoods. MCMC  
429 sampling was conducted for 50 steps of 39000 generations each, with the first 9000  
430 generations of every step discarded as burn-in. The model with the highest likelihood score  
431 was used as the null hypothesis. We compared Bayes Factors (BF) for both models and used  
432 a threshold of  $2 \ln(\text{BF}) > 10$  as decisive support for the null against the alternative  
433 hypothesis<sup>49</sup> (Supplementary Table 2). We repeated stepping stone sampling to confirm run  
434 stability (data not shown).

435

## 436 **Statistics**

437 Mean survival of control treatments, free of the two test pathogens, was  $14.2 \pm 4.2$  (mean  $\pm$   
438 sd) days, while DWV treated bees survived for  $8.1 \pm 5.8$  (mean  $\pm$  sd) days. To assess the  
439 effect of infection on survival we fitted a Cox mixed effects model with treatment as a fixed  
440 factor and colony origin as random factor and compared it to the null model<sup>50</sup> (R library  
441 `coxme`, version 2.2-3, function `coxme`). The model was fitted with the penalized partial  
442 likelihood (PPL) and showed a significant negative impact of infection on longevity ( $X^2 =$   
443  $11.93$ ,  $df = 4.17$ ;  $p < 0.021$ ).

444 *N. ceranae* treated bees survived for  $18 \pm 1$  (mean  $\pm$  sd) days. A model with treatment as  
445 fixed factor and colony origin as random factor showed no improvement over the null model  
446 (PPL:  $X^2 = 0.12$ ,  $df = 1$ ;  $p > 0.735$ ).

447 True prevalences with 95% confidence intervals were computed to correct for varying sample  
448 sizes (due to the different species of bumble bee at the sampling sites) and test sensitivity was  
449 set to a conservative 95%<sup>51</sup>. Confidence interval estimates are based on Blaker's (2000)  
450 method for exact two sided confidence intervals<sup>52</sup> for each sampling site and for each species  
451 sampled<sup>31</sup>(R library epiR, version 0.9-45, function epi.prev).

452 To investigate our spatially distributed dataset we undertook an exploratory data analysis  
453 (EDA)<sup>53</sup> in which we calculated a prevalence surface for each of our parasites using Gaussian  
454 kernel estimators with an adaptive bandwidth of equal number of observations. This is a  
455 variant of the nearest neighbour technique, with bandwidth size being determined by a  
456 minimum number of observations in the neighbourhood (set to 3 times the maximum  
457 observations per site)<sup>32</sup> (R library prevR, version 2.1, function kde). Estimated surfaces were  
458 used for visual inspection only (Fig. 2); all the remaining analyses are based on the raw data  
459 only.

460 To investigate spatial structure and disease hotspots we used spatial autocorrelation statistics  
461 of the true prevalence of each of the pathogens in the different host genera from the 26  
462 collection sites. To identify whether or not the pathogens we found were spatially clustered,  
463 we computed the spatial autocorrelation coefficient Moran's  $I$ <sup>54</sup> with an inverse spatial  
464 distance weights matrix, as implemented in Gittleman and Kot<sup>55</sup> (R library ape, version 3.0-  
465 7, function Moran.I). Moran's  $I$  is a weighted measure describing the relationship of the  
466 prevalence values associated with spatial points. The coefficient ranges from -1 (perfect  
467 dispersion) through 0 (no spatial autocorrelation (random distribution)) to 1 (perfect  
468 clustering).

469 To investigate whether pathogen prevalence (*Nosema* and DWV were tested in separate  
470 models) in *Apis*, *Bombus* to *Apis* relative density, or *Apis* absolute abundance had an effect on  
471 pathogen prevalence in *Bombus*, we ran a Generalized Linear Mixed Model (GLMM)<sup>34</sup> with

472 binomial error structure and logit link function using the function lmer of the R package  
473 lme4<sup>35</sup>. Latitude, longitude, sunlight hours (a proxy for favourable foraging weather that  
474 would enable disease transmission; calculated cumulatively from March until the month of  
475 collection [data were collected from the MET office webpage:  
476 <http://www.metoffice.gov.uk/climate/uk/anomacts/>, averaging over area sunlight hour  
477 ranges]) and landcover type were included in the model as fixed control effects (present in  
478 the full as well as the null model) while site and species were included in the model as  
479 random effects (present in the full as well as the null model). Before running the model we  
480 inspected all predictors for their distribution, as a consequence of which we log transformed  
481 “*Bombus to Apis* density” and “*Apis* abundance” to provide more symmetrical distributions.  
482 Thereafter we z-transformed all quantitative predictors to a mean of zero and a standard  
483 deviation of one to derive more comparable estimates and to aid interpretation of  
484 interactions<sup>56</sup>. Since changes in “*Bombus to Apis* density” and “*Apis* abundance” could lead  
485 to changes in pathogen prevalence in *Bombus* because of a change in pathogen prevalence in  
486 *Apis*, we included the interactions between “*Bombus to Apis* density” and pathogen  
487 prevalence in *Apis*, and “*Apis* abundance” and pathogen prevalence in *Apis*. To test the  
488 overall effect of our three test predictors, we compared the full model with a reduced model  
489 (null model) using a likelihood ratio test comprising latitude, longitude, sunlight hours and  
490 landcover type with the same random effects structure. Model stability was assessed by  
491 excluding data points one by one and comparing the estimates derived from these reduced  
492 models with estimates from the full model (revealing a stable model). Site G had to be  
493 excluded from this analysis as no *Apis* samples were found on site.

494 We fitted linear models to assess the relationships of parasite prevalence among *Apis* and  
495 *Bombus*.

496 We investigated the effect of pathogen treatment on disease status of an individual with a  
 497 Generalized Linear Mixed Model (GLMM)<sup>34</sup>) with binomial error structure and logit link  
 498 function using the function lmer of the R package lme4<sup>35</sup>. Colony of origin was entered into  
 499 the model as a random effect. As described before, we checked model stability (the model  
 500 with interaction terms included was unstable; however it stabilised once the non-significant  
 501 interaction terms were removed), before testing the full model against the null model using a  
 502 likelihood ratio test. All analyses were run in R<sup>36</sup>.

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571

572 **Extended Data Table 1 Pathogen prevalence per species:** Pathogen prevalence is given in  
 573 percent with 95% confidence intervals (% prevalence [95% CI]). Sample numbers (N) are  
 574 shown in brackets.

575 Footnote to Extended Data Table 1: \* out of the 31 DWV present *Apis* samples tested

576

577 **Extended Data Table 2** Alternative hypotheses for the diversification of DWV and VDV  
 578 viruses in UK pollinators

579 **Extended Data Figure 1 Species and site distribution:** Distribution of sampling sites  
 580 across Great Britain and the Isle of Man. The most common *Bombus* species on site is  
 581 represented by coloured letters while the 2nd most common *Bombus* species is represented by  
 582 differently coloured dots. Total sample sizes for each site are given in the table.

583 **Extended Data Figure 2 Prevalence per site and species:** Pathogen prevalence in *Bombus*  
 584 spp. in percent per site (a. for DWV; b. for *N. ceranae*) and per species (c. for DWV; d. for *N.*  
 585 *ceranae*). Bars indicate 95% confidence intervals. Note different scales

586 **Extended Data Figure 3 Prevalence raw data:** the linear models shown only illustrate the  
 587 relationships but do not drive the conclusions in the main text. a) DWV presence in *Apis* and  
 588 *Bombus* (adj  $R^2 = 0.34$ ,  $p < 0.001$ ); b) DWV replicating in *Bombus* and DWV presence in  
 589 *Bombus* (adj  $R^2 = 0.46$ ,  $p < 0.001$ ); c) *N. ceranae* presence in *Apis* and *Bombus* (adj  $R^2 = -$   
 590  $0.04$ ,  $p > 0.728$ ). The line shows the best fit, and the dark grey region shows 95%CI of fit.

591

## 592 Extended Data Table 1

species (N)	<i>Apis</i> (250)	<i>B.ter</i> (170)	<i>B.luc</i> (60)	<i>B.lap</i> (175)	<i>B.pas</i> (60)	<i>B.hor</i> (20)	<i>B.mon</i> (10)
DWV present	36 [30, 43]	9 [5, 14]	18 [9, 29]	16 [11, 23]	4 [1, 12]	0 [0, 17]	11 [1, 47]
DWV replicating	88 [70, 98]*	1 [0, 3]	4 [1, 12]	10 [6, 15]	0 [0, 6]	0 [0, 17]	11 [1, 47]
<i>N. ceranae</i>	9 [6, 13]	2 [1, 6]	0 [0, 6]	16 [11, 23]	0 [0, 6]	5 [0, 25]	0 [0, 29]
single infection	18 [14, 23]	3 [1, 7]	3 [1, 12]	20 [14, 27]	0 [0, 6]	5 [0, 25]	11 [1, 47]
co-infection	1 [0, 3]	0 [0, 2]	0 [0, 6]	3 [1, 7]	0 [0, 6]	0 [0, 17]	0 [0, 29]

593

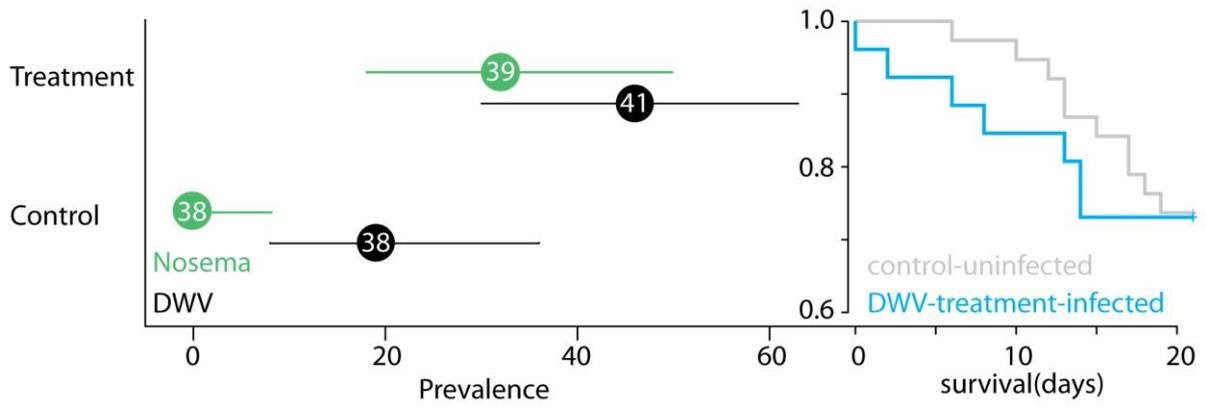
## 594 Extended Data Table 2

Model		Marginal likelihood (ln)	Difference	BF	2 ln (BF)	Preferred model
Site (S)	Null	-1512.71				
Host (H)		-1607.63	-94.92	$>10^{41}$	189.84	S

595

596

597 Figure 1.

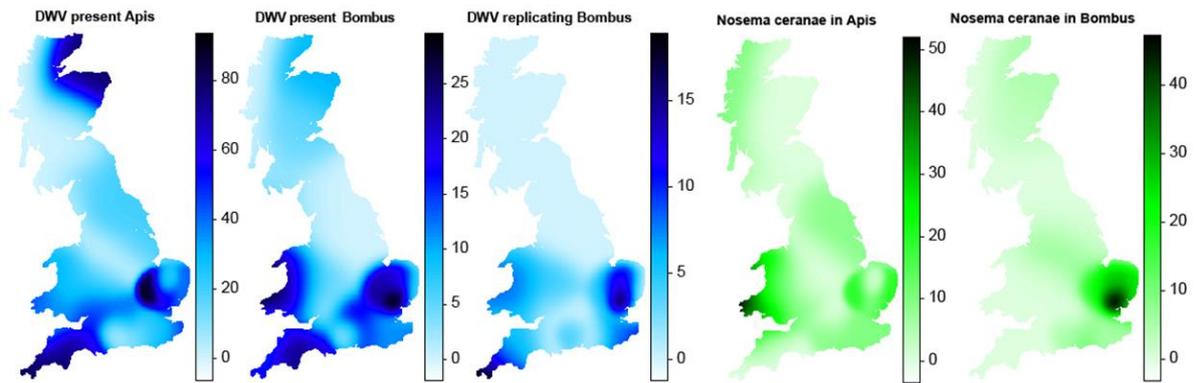


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601 Figure 2

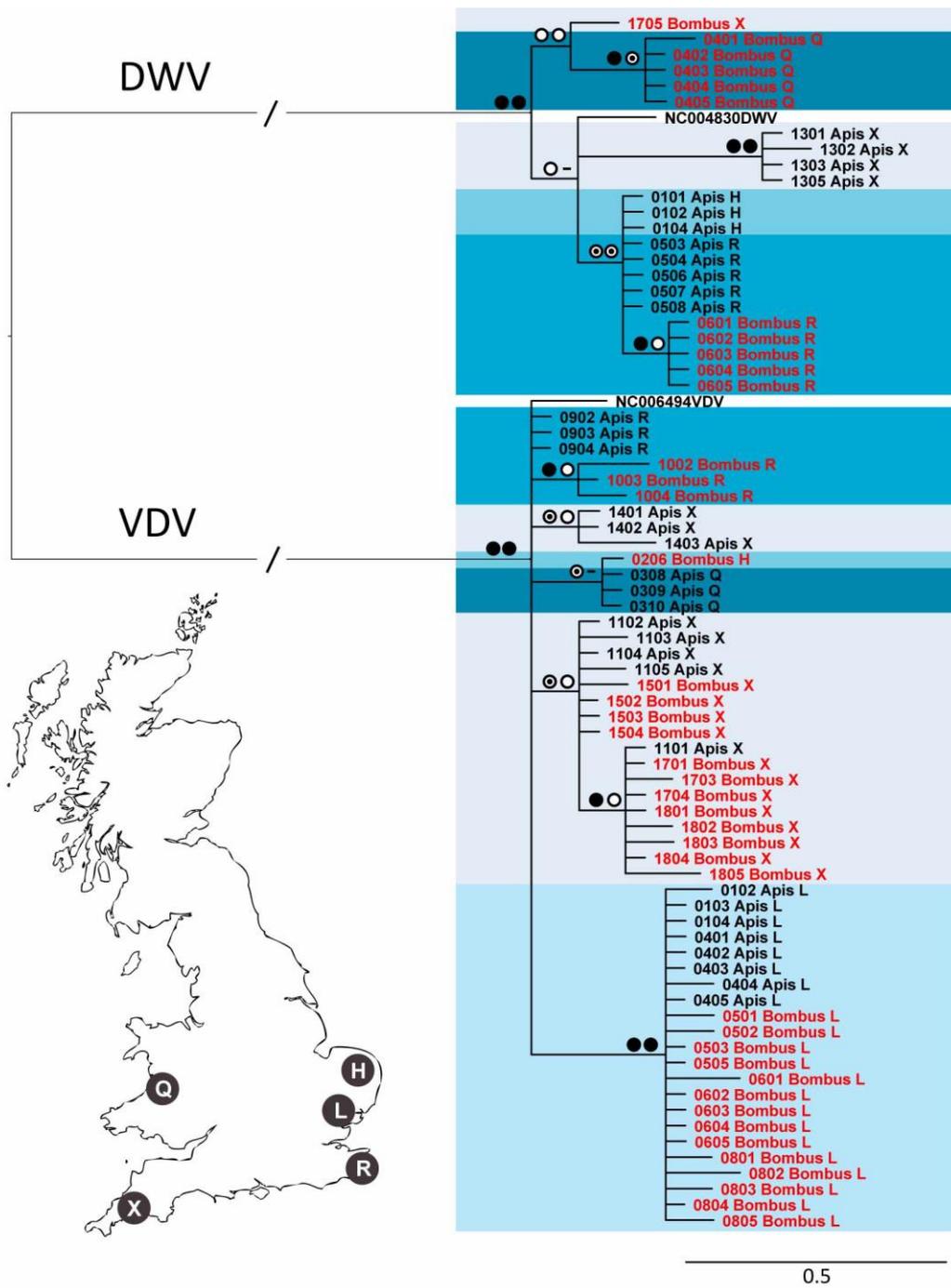


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603

604

605 Figure 3



606