The effects of single and mixed infections of *Apicystis bombi* and deformed wing virus in *Bombus terrestris*

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(*Received 12 July 2015; revised 30 October 2015; accepted 3 November 2015; first published online 8 December 2015*)

SUMMARY

Many pollinators are currently suffering from declines, diminishing their gene pool and increasing their vulnerability to parasites. Recently, an increasing diversity of parasites has been recorded in bumblebees, yet for many, knowledge of their virulence and hence the risk their presence poses, is lacking. The deformed wing virus (DWV), known to be ubiquitous in honey bees, has now been detected in bumblebees. In addition, the neogregarine *Apicystis bombi* has been discovered to be more prevalent than previously thought. Here, we assess for the first time the lethal and sublethal effects of these parasites during single and mixed infections of worker bumblebees (*Bombus terrestris*). Fifteen days after experimental exposure, 22% of bees exposed to *A. bombi*, 50% of bees exposed to DWV and 86% of bees exposed to both parasites had died. Bumblebees that had ingested *A. bombi* had increased sucrose sensitivity (SS) and a lower lipid:body size ratio than control bees. While dual infected bumblebees showed no increase in SS. Overall, we find that *A. bombi* exhibits both lethal and sublethal effects. DWV causes lethal effect and may reduce the sub lethal effects imposed by *A. bombi*. The results show that both parasites have significant, negative effects on bumblebee health, making them potentially of conservation concern.

Key words: pollinator, emerging disease, pathogen, neogregarine, DWV.

INTRODUCTION

Bumblebees are responsible for the pollination of a variety of wild flowers and economically important crops throughout the world (Goulson, 2010). Recent evidence has highlighted declines of important pollinators in many areas worldwide and the potentially devastating consequences of its continuation (Potts *et al*. 2010). Bumblebee declines have so far been reported across Europe, North America, South America and Asia (Kosior *et al*. 2007; Xie *et al*. 2008; Martins and Melo, 2010; Cameron *et al*. 2011). Parasites are strongly implicated in the reduction of several bumblebee species (Williams and Osborne, 2009; Cameron *et al*. 2011; Meeus *et al*. 2011). In addition, as bumblebee populations decline, the threat faced from parasites will increase due to bumblebee populations becoming fragmented and losing their genetic diversity (Whitehorn *et al*. 2011).

The development of bumblebees as a model system in host–parasite evolutionary ecology has allowed us to gain a very good understanding of 2 bumblebee parasites, *Crithidia bombi* and *Nosema bombi* (Schmid-Hempel, 1998). Our knowledge of the biology of other bumblebee parasites, though, remains comparatively poor (Schmid-Hempel, 1998; Meeus *et al*. 2011). Recent advances in the molecular detection of parasites have highlighted that bumblebees are in contact with a greater diversity of parasites than previously recognized (Singh *et al*. 2010; Evison *et al*. 2012; Graystock *et al*. 2013*a*; Plischuk *et al*. 2015).

Apicystis bombi is a neogregarine parasite found infecting bumblebees and honey bees in Europe, North America and more recently, South America and Asia (Lipa and Triggiani, 1992; Colla *et al*. 2006; Plischuk and Lange, 2009; Morimoto *et al*. 2013; Graystock *et al*. 2014). *Apicystis bombi* oocysts reside primarily in the hosts fatbody and are intermittently found in faeces and flowers suggesting an oral-faecal route of transmission, similar to that of other bee infecting protists though an additional transmission route via decomposing corpses has been suggested (Schmid-Hempel, 1998; Graystock *et al*. 2013*a*, 2015*b*). Potential vertical transmission via eggs has also been suggested but not shown (Lipa and Triggiani, 1996). The prevalence of bumblebees with visually detectable *A. bombi* oocysts is often low, <7% of native UK bumblebees (Jones and Brown, 2014), however sensitive molecular screening of this parasite suggests it is often much higher (>25%), with many infections being covert (Plischuk *et al*. 2009; Graystock *et al*.

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doi:10.1017/S0031182015001614

2013*a*, 2014; Murray *et al*. 2013). A small number of infected queens have been shown to have a reduced likelihood to survive hibernation, possibly due to parasite effects on their stored fatbody (Rutrecht and Brown, 2008; Jones and Brown, 2014). It has been argued that the emergence of this parasite is due to the spillover of non-native strains from introduced bumblebees and honey bees (Plischuk and Lange, 2009; Maharramov *et al*. 2013; Ravoet *et al*. 2013; Graystock *et al*. 2014, 2015*a*). Recently there have been reports that *A*. *bombi* may be playing a role in declines in Argentinean bumblebees (Plischuk and Lange, 2009; Plischuk *et al*. 2011), but currently there is insufficient knowledge of the biology or virulence of the parasite to assess what threat it poses to bumblebees.

Another parasite with an increased incidence of detection in bumblebees is the picornavirus, deformed wing virus (DWV) (Fürst *et al*. 2014). This virus is ubiquitous in some honey bee populations, with little effect on the host unless in the presence of an additional parasite, *Varroa destructor*, which acts as a vector for viruses such as DWV, increasing their virulence and in the case of DWV, often leading to symptomatic honey bee wing deformities during their development as larvae to pupa (Ryabov *et al*. 2014). Wild bumblebees with DWV have been seen to exhibit deformed wings, suggesting the virus can impose the same deformities to bumblebees as seen in honey bee hosts, even in the absence of *V*. *destructor*, with transmission likely to occur via shared flower use. The virulence of this virus in bumblebees is currently understudied though a laboratory based study showed single infection can cause mortality in bumblebees after just 6 days (Fürst *et al*. 2014).

The detection of both DWV and *A*. *bombi* in wild and commercially reared bumblebee populations in combination with a shortage of knowledge regarding their virulence makes it imperative to understand the virulence of these parasites on bumblebee hosts. Studies often focus purely on a single parasite–host interaction, yet naturally, parasites often occur as mixed infections, having profound effects on the outcome of the interactions (Alizon *et al*. 2013). Here, we investigate lethal and sublethal effects of single and mixed infections of these 2 little-studied parasites of bumblebees.

MATERIALS AND METHODS

Apicystis bombi extraction for ingestion treatments

The fatbody of 40 *Bombus terrestris* bumblebees from 2 colonies with single infections of *A*. *bombi* (screened as in Graystock *et al*. 2013*a* for 5 parasites known to infect adult bumblebees: DWV, *Nosema ceranae, N*. *bombi, C*. *bombi, A*. *bombi* plus *Nosema apis* which has been found in bumblebees but with no evidence of active infection) were homogenized in 1000 μL of 30% sucrose solution and the resulting homogenate was slowly passed through a syringe filter to remove large tissue fragments. This solution was then centrifuged for 30 min at 9000 ***g*** and 15 °C and the resulting pellet of oocysts extracted with a pipette. Oocysts were washed by first centrifuging at 10 000 ***g*** for 20 min, removing the supernatant, and replacing with 30% sucrose solution before vortexing for 10 s. This wash process was repeated 3 times to eliminate any small particles. The resulting solution was confirmed with a compound microscope to be a suspension of *A*. *bombi* oocysts, free from bumblebee tissue membrane and other parasite infective forms. The suspended oocysts were visually identified as being *A*. *bombi*; this was then confirmed by polymerase chain reaction (PCR) amplification of the suspension with specific primers for *A*. *bombi*, and for the 2 *Nosema* species known to be found in bumblebee fatbody, *N*. *bombi* and *N*. *ceranae* (Macfarlane *et al*. 1995; Klee *et al*. 2006; Graystock *et al*. 2013*b*). The spore solution was then diluted in 30% sucrose solution to obtain a concentration of 5×10^5 oocysts mL^{-1}. The control treatment was 30% sucrose solution without any oocysts added.

DWV extraction for injection treatments

The route of DWV transmission is currently undetermined in bumblebees but in honeybees multiple routes are known and the chosen route can have profound effects on the virulence of DWV (Ryabov *et al*. 2014). Though once a bumblebee is infected, DWV is found in all bumblebee tissues except the eye (Li *et al*. 2011). To avoid any inefficiencies in the study due to an inappropriately chosen transmission method, and to ensure successful inoculations throughout, virus was administered via injection into the haemolymph as performed in other viral studies on honey bee and bumblebees by Iqbal and Mueller (2007) and Meeus *et al*. (2014). The extraction protocol for DWV was adapted from Iqbal and Mueller (2007). The fatbody of 50 *B*. *terrestris* bumblebees from a hive, singly infected with DWV (screened as above DWV, *N*. *ceranae, N*. *bombi, C*. *bombi, A*. *bombi* plus *N*. *apis*) were frozen in liquid nitrogen, and homogenized with 2·5 mL phosphate-buffered saline (PBS, pH 7·4), then centrifuged at 986 ***g*** for 30 min at 10 °C. The resulting solution was confirmed to be positive for DWV by reverse transcription polymerase chain reaction (RT–PCR), before being diluted down 1:1000 in PBS. Thus, the inoculant has been diluted from what occurs in naturally infected bumblebees down to a concentration of approximately 5%. Recent work has identified bumblebees can have multiple viruses (though their replication within bumblebees or presence in the fat body is still unclear) and while our inoculant was

https://doi.org/10.1017/S0031182015001614 Published online by Cambridge University Press

confirmed to contain DWV, it cannot be ruled out that other, unknown viruses may have been present (McMahon *et al*. 2015). Control treatments were injections of just PBS (pH 7·4) which would prevent control bees from receiving any secondary, unknown viruses which may also be present in bumblebee fatbody.

Experimental infection

A total of 350 *B. terrestris audax* workers were collected from 5 colonies that had been obtained from Biobest NV in Westerlo (Belgium) and were confirmed to be parasite-free by screening 15 bees per colony by PCR and RT–PCR for *C. bombi, A. bombi, N. bombi, N. ceranae* and DWV by PCR and RT–PCR (see below). The 350 bees were placed as groups of 5 nestmate bees in $10 \times 6 \times 6$ cm^3 plastic boxes. Seven ingestion|injection treatment combinations were tested: *A. bombi*|DWV, *A. bombi*|control, control|DWV, control|control, *A.bombi*|no injection, no ingestion|DWV and no ingestion|no injection. Two groups of bees from each of the 5 colonies (50 bees in total) were tested with each of the 7 treatment combinations. For the *A. bombi* treatment, each bee was placed into a holding harness and individually fed a 5 μL dose of the parasite suspension, which contained 2500 *A. bombi* oocysts. This amount was chosen following oocyst quantification from 9 infected wild bumblebees from England found up to 2500 oocysts in a single bee gut (the presumed transmission route). In an entire bee, intensities of around 5×10^6 oocysts are common. Bees receiving the control ingestion treatment were treated similarly, but fed 5 μL of pure 30% sucrose solution, while bees in no ingestion combinations were not hand-fed at all. For the DWV treatment, a 5 μL dose of the DWV solution was injected into the ventral side of the abdomen, between the 2nd and 3rd sternites. Bees receiving the control injection were injected with 5 μL of PBS, while those in the no injection combinations were not injected with anything. The bees received their injection treatments first and were subsequently starved for 5 h before receiving their ingestion treatments. Following treatment the bees were replaced in their cohorts of 5 like-treated nestmates, provided with 50% sucrose solution *ad libitum*, and their survival checked daily for 15 days.

Sucrose sensitivity (SS)

The sensitivity of a bee to low sucrose concentrations has been linked to hunger and learning ability (Scheiner *et al*. 2001; Naug and Gibbs, 2009) making it a good measure of sub-lethal effects of parasite infection. The SS of bumblebees to differing concentrations was therefore tested for every bee in the experiment using the proboscis extension response (PER). Every 5 days a PER experiment was performed, in which each bee was harnessed in a modified Eppendorf tube with moist cotton wool, under red-light conditions. While harnessed, bees were hand-fed to satiation with 30% sucrose solution before being left for a starvation period of 5 h. After starvation, each bee had its antenna touched with a drop of sucrose solution, the concentration of which was increased in 10% increments from 50 to 80%. Between each concentration trial, antennae were touched with H_20 after 60 s to prevent bees becoming conditioned; the next sucrose concentration was then applied following a further 60 s interval. Individuals that were responsive to a particular concentration extended their proboscis, resulting in a SS score of 1, and, as each bee was individually presented with 5 different concentrations, each bee could therefore score a maximum SS of 5 (Riveros and Gronenberg, 2009). The responses of each bee were measured, with high SS scores indicating bees responding to high and low sugar concentrations, and a low SS score indicating bees responding only to high sugar concentrations.

Lipid extraction

The leanness of each of the 350 bees was calculated by determining their lipid content relative to their body size (Brown *et al*. 2000). For this, each abdomen (minus 2 tergites which were removed for molecular screening) was dried at 70 °C for 5 days, weighed and then immersed in ether for 24 h to dissolve the lipids. After rinsing with fresh ether, the remaining abdominal tissues were dried for a further 5 days at 70 °C before being reweighed. Based on the resulting weight loss (mg) and taking the length of the left hind tibia (mm) as an index of body size, the worker lipid:body size ratio was calculated.

Molecular screening

Any bees that died during the experiment, and all those surviving to the end of the 15 days experimental period were placed in 100% ethanol. All 350 bumblebees were then removed from ethanol and each had their 5th and 6th tergites removed. These tergites are more posterior, and on the opposite side of the abdomen, to the site at which the injection treatments were administered. The fatbody attached to these 2 tergites was homogenized in 100 μL of 5% Chelex and incubated at 100 °C for 15 min to extract DNA and RNA. The fatbody extracts were then briefly vortexed before centrifuging at 2399 ***g*** for 15 min and collecting the supernatant.

Samples were first screened for 18S rDNA specific to Apidae as a host control to confirm DNA quality. A 10 μL reaction consisted of 0·4 mM dNTP, 1·5 mM $MgCl_2$, 1·25 U Taq, 0·2 μM each primer 3 μL Buffer and 1 μL template (Meeus *et al*. 2010). The PCR was

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then subjected 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 screened for *A. bombi* in 10 μL reactions consisting of 0·4 mM dNTP, 1·5 mM $MgCl_2$, 1·25 U Taq, 0·5 μM of each primer, 2 μL Buffer and 1 μL template. This was then subjected to 2 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 60 °C, 45 s at 72 °C before a final elongation stage of 3 min at 72 °C (Meeus *et al*. 2010). Samples were screened for DWV using reverse transcription PCR whereby 2 μL of sample was added to 5 μL TaqMan® Fast Virus 1-Step Master Mix, 650 nM of each primer and molecular grade water giving a total volume of 10 μL. The sample then underwent thermal cycling of 5 min at 50 °C, 20 s at 95 °C, 40 cycles of 3 s at 95 °C, 180 s at 60 °C before a final elongation stage of 10 min at 72 °C (Chen *et al*. 2005). PCR conditions for the other parasites that were screened for when preparing parasite suspensions or when confirming the parasite-free status of the experimental bees were as in Graystock *et al* (2013*a*). PCR products were run on a 1% agarose gel stained with ethidium bromide to confirm amplicon size. Every assay included negative and positive controls.

Statistical analysis

Differences in bumblebee survival were analysed using a Cox proportional hazards regression model, with ingestion treatment, injection treatment and their interaction included in the model. Pairwise comparisons were made between individual treatments using Kaplan–Meier models with the Breslow χ^2 statistic. The interactive and singular effect of injection and ingestion treatments on lipid:body size ratio was compared using generalized linear models (GLM) with gamma distribution, log link function and the likelihood ratio χ^2 statistic. The interactive and singular effect of injection and ingestion treatments on SS was compared using a generalized linear mixed model (GLMM) with gamma distribution and log link function. Colony-of-origin and cohort were included in both the GLM and GLMM models. Non-significant interaction terms were removed stepwise in all models to obtain the minimum adequate models. Pairwise comparisons were made using Estimated Marginal Means. All analyses were carried out in SPSS 21 (IBM, Armonk, NY, USA).

RESULTS

Infectivity

Treatment groups of *A. bombi* with either PBS, DWV or no injection had *A. bombi* infectivity of 80, 92 and 78%, respectively (Fig. 1). Treatment groups of DWV with either *A. bombi*, sucrose or no ingestion had DWV infectivity of 96, 98 and 96%, respectively (Fig. 1). All the following results include only those bees that were found to have systemic infections at the cessation of the experiment or upon death.

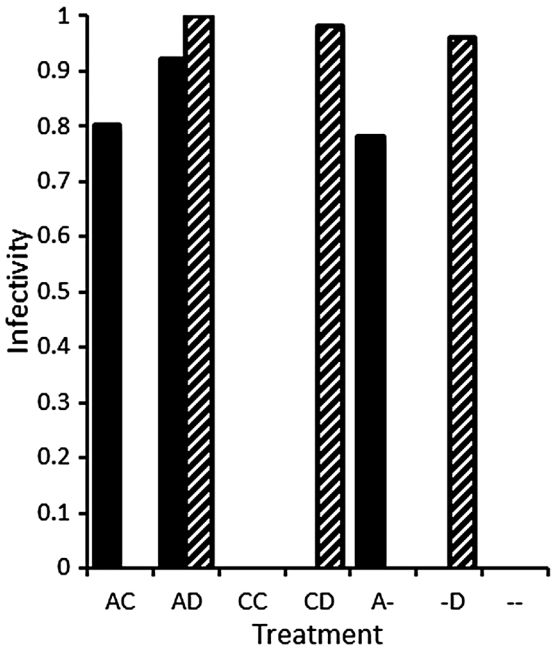

Fig. 1. Infectivity of *Apicystis bombi* (black bars, solid) and DWV (diagonal lines) in treated bees following death or the cessation of the experiment. Shown grouped by treatment combination of either fed Apicystis with PBS injection (AC), fed Apicystis and DWV injection(AD), fed sucrose control with PBS injection (CC), fed sucrose control and DWV injection (CD), fed Apicystis only (A), DWV injection only (-D), or no treatment at all (--).

Survival

There was a significant interaction between the effects of the ingestion and injection treatments on bumblebee survival ($Wald = 16{\cdot}3$, D.F. = 2, $P < 0{\cdot}001$). The bees which received either the control (sucrose)|control (PBS injection) or no ingestion|no injection treatment combinations had very high survival over the 15-day period, and the survival of bees was reduced significantly when they had received the *A. bombi*|no injection combination (Fig. 2a, Supplementary Table S1). Survival was significantly lower for bees which received either the control|DWV or, no ingestion|DWV combinations, and was very low for bees which received the *A. bombi*|control injection or *A. bombi*|DWV combinations (Fig. 2a, Supplementary Table S1). There was no interaction between injection event (weather or not they were injected, regardless of treatment) and ingestion treatment ($Wald = 1{\cdot}95$, D.F. = 1, $P = 0{\cdot}16$).

Leanness

The lipid:body size ratio was affected by the ingestion treatments ($Wald = 16{\cdot}5$, D.F. = 2, $P = 0{\cdot}001$),

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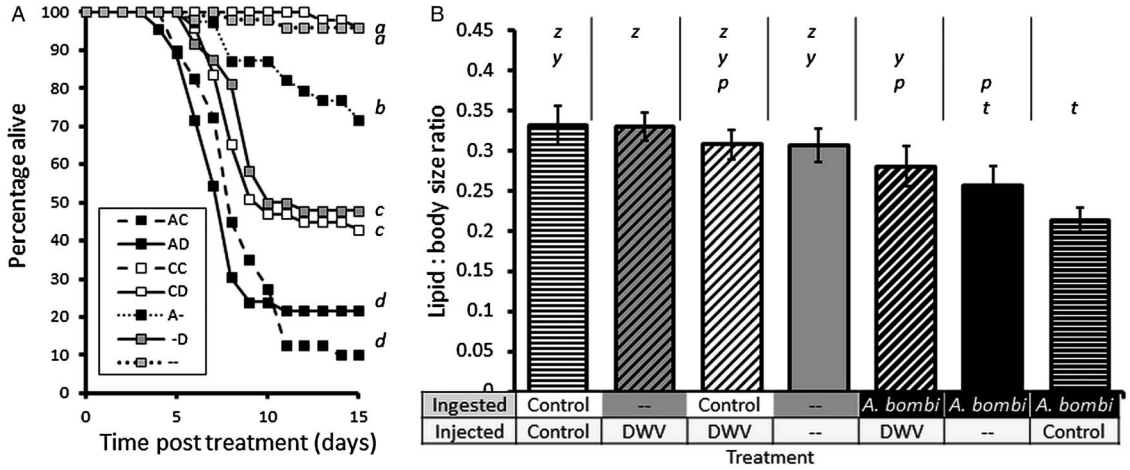

Fig. 2. Survival of bees over 15 days (a) and subsequent lipid: body ratio (b) following hand feeding with either *Apicystis bombi* oocysts (black points/bars), sucrose control (white points/bars) or no hand feeding (grey points/bars) while also being injected with DWV (solid lines/diagonal bar pattern), PBS control (dashed lines/horizontal pattern) or no injection (dotted lines/no pattern). Treatment combinations were either fed *Apicystis* with PBS injection (AC), fed *Apicystis* and DWV injection(AD), fed sucrose control with PBS injection (CC), fed sucrose control and DWV injection (CD), fed *Apicystis* only (A), DWV injection only (-D), or no treatment at all (--). Whilst different italicized letters indicate treatments which differed significantly from one another, the same italicized letters indicate non-significant in pairwise comparisons.

but not by the injection treatments and there was also no interaction between ingestion and injection treatments ($Wald = 1{\cdot}3$, D.F. = 2, $P = 0{\cdot}5$; $Wald = 5{\cdot}6$, D.F. = 2, $P = 0{\cdot}06$, respectively). Pairwise comparisons between the treatment groups find that bees in the control|control treatment had the highest average lipid:body size ratio and none of the treatments that included DWV differed significantly from this (Fig. 2b, Supplementary Table S2). The 3 treatments that included *A. bombi* ingestion had the lowest mean lipid:body ratios. The *A. bombi*|Control group was significantly lower than any of the treatments where *A. bombi* was not ingested unless DWV was also present (Fig. 2b; Supplementary Table S2).

Sucrose sensitivity

On day 0, 322 bumblebees were tested for their responsiveness to sucrose and no differences were observed between the treatment groups ($\chi^2 = 2{\cdot}56$, D.F. = 6, $P = 0{\cdot}9$; Fig. 3). On day 5, however, the remaining 313 bumblebees displayed a significant response to treatment ($\chi^2 = 26{\cdot}4$, D.F. = 6, $P < 0{\cdot}001$; Fig. 3). Pairwise analysis between all 7 treatment types on day 5 show bees infected with DWV tended to have higher SS (Fig. 3, Supplementary Table S3). On day 10 and 15, the remaining 202 and 182 bumblebees still displayed a significant response to treatment ($\chi^2 = 21{\cdot}6$, D.F. = 6, $P = 0{\cdot}001$; $\chi^2 = 14{\cdot}5$, D.F. = 6, $P = 0{\cdot}024$, respectively) while maintaining the general trend of DWV infected bees being more sensitive for sucrose except on the 15th day when dual infected bees have reduced sensitivity than DWV infected bees (Fig. 3, Supplementary Tables S4 and S5). By the 15th day, however, the experiment had become unbalanced with bees in different treatment groups dying (and so being unable to be part of the sucrose assay) at different rates in later days. Treatment group sizes ranged from 36 to 50 on day 5, 11 to 50 on day 10 and 4 to 48 on day 15 (Supplementary Table S6).

DISCUSSION

Apicystis bombi and DWV infections were virulent to bumblebees, with infections causing mortality to increase by 18 and 50%, respectively. Both *A. bombi* and DWV infections altered bumblebee sensitivity to sucrose, while *A. bombi* also significantly reduced the amounts of stored fat in infected bumblebees.

It had previously been noted anecdotally that the fatbody of bumblebees infected with *A. bombi* appeared 'much reduced' (Liu *et al.* 1974). The results here provide the first empirical evidence of this, with fat/lipid content being reduced by 17% on average by *A. bombi* infections. The fatbody is essential for overwintering queen bumblebees and any reduction in their fatbody would lower their chances of founding successful colonies. Workers would also be negatively affected by reduced fatbody as it is the site for many biochemical reactions that are important for their immunity and metabolism (Arrese and Soulages, 2010). After 5 days, the SS of *A. bombi* infected bumblebees was elevated, presumably due to an increased demand for carbohydrates to compensate for the reduced fatbody. In the wild this would likely increase the workers need to forage for nectar, reducing their pollen foraging efficiency for the colony and developing larvae. *Apicystis bombi* infections caused high mortality over

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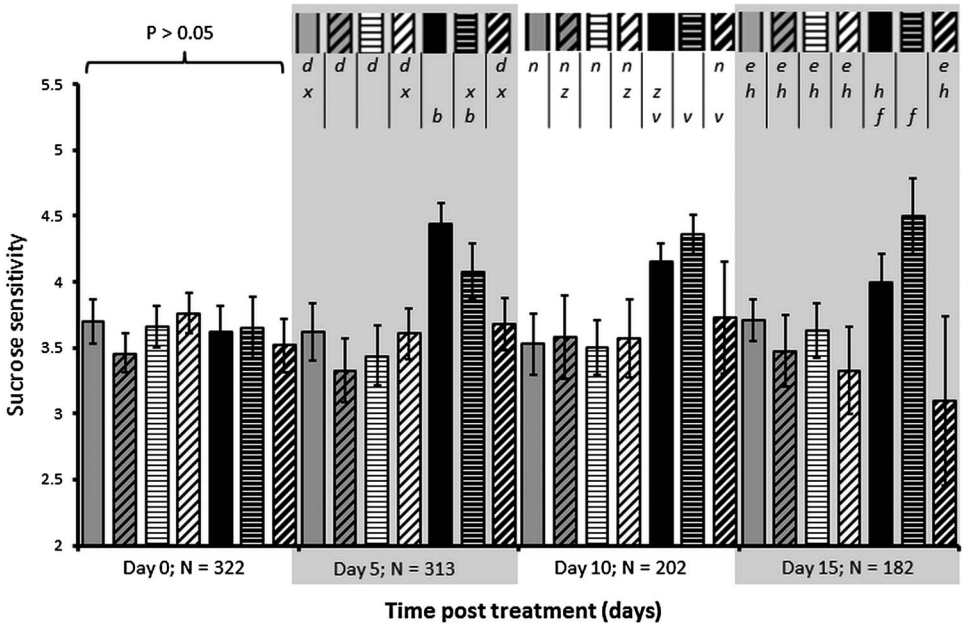

Fig. 3. Bumblebee responsiveness to sucrose over 15 days following either hand feeding with *Apicystis bombi* oocysts (black bars), sucrose control (white bars) or no hand feeding (grey bars) whilst also being injected with DWV (bars patterned with diagonal lines), PBS control (bars patterned with horizontal lines) or no injection (bars not patterned). The same italicized letters indicate non-significant in pairwise comparisons. Error bars represent ± standard error.

15 days. This mortality increased 3-fold if bees had been injected in their abdomen with either PBS or DWV prior to spore ingestion, suggesting that *A. bombi* infected bees also have reduced ability to cope with the effects of wounding. While DWV was detectable in fatbody tissue, we found no change in the lipid mass of DWV-infected bumblebees, with bumblebees co-infected by DWV and *A. bombi* having an intermediate lipid:body mass ratio. In honey bees, DWV infection increases the hosts SS (Iqbal and Mueller, 2007), but we found here the opposite effect in bumblebees with infected workers having decreased SS. Interestingly, co-infected bees maintain SS similar to that of control bees suggesting co-infected bees do not increase sensitivity in response to *A. bombi*. These results suggest there may be a negative interaction between DWV and *A. bombi* within the host whereby DWV infection reduces the sub lethal impacts of *A. bombi* infection. This would be a reverse of what is found in honeybees infected with *N. ceranae* which can inhibit DWV infection, though weather DWV infected honeybees would also benefit from reduced *A. bombi* virulence remains to be shown (Doublet *et al*. 2015).

The results demonstrate that both *A. bombi* and DWV can have significant negative effects on infected bumblebees. While single infections of *A. bombi* cause only moderate mortality, its sublethal effects may be more important, particularly for hibernating queens whose survival depends on their fat reserves. This supports recent correlative evidence that the emergence of *A. bombi* in South America may be responsible for rapid declines in some native bumblebee species (Arbetman *et al*. 2013). In addition, the high mortality of bumblebees infected with DWV makes it clear that both these parasites should be monitored more frequently in bumblebee populations. Both *A. bombi* and DWV are significantly understudied given the virulence they exhibit in bumblebees. In addition, the rates of transmission within bumblebee populations, and between honey bees and bumblebees, are still unknown. DWV is almost ubiquitous in honey bees and is being increasingly found in bumblebees (Tentcheva *et al*. 2004; Manley *et al*. 2015; McMahon *et al*. 2015), while *A. bombi* is found mostly in bumblebees (Plischuk *et al*. 2011). A recent study has also found more 'honey bee viruses' in screened bumblebees but as yet, these have not been shown to be active infections (McMahon *et al*. 2015). The negative fitness effects from viral and *A. bombi* infections shown here in *B. terrestris* highlights the pressing need to understand more about the effects of these parasites, in bumblebees and their potential role in bumblebee declines.

SUPPLEMENTARY MATERIAL

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0031182015001614.

ACKNOWLEDGEMENTS

We wish to thank Adam Smith and Sophie Evison for laboratory assistance, Katherine Roberts, Rowena Mitchell, Eamonn Mallon and Alison Dunn for

constructive comments and the Super-B COST Action for facilitating fruitful discussion of the work. We also wish to thank two anonymous reviewers for their helpful and constructive comments on the manuscript.

FINANCIAL SUPPORT

This work was funded by NERC (Natural Environment Research Council, UK; grant no. NE/G012113/1), the Bumblebee Conservation Trust and a Philip Leverhulme Prize, all awarded to WOHH.

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