Journal of Invertebrate Pathology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Short Communication

Within-host competition among the honey bees pathogens *Nosema ceranae* and Deformed wing virus is asymmetric and to the disadvantage of the virus

Vincent Doublet [a,*], Myrsini E. Natsopoulou [a], Luise Zschiesche [a], Robert J. Paxton [a,b,c]

[a] Institut für Biologie, Martin-Luther-Universität Halle-Wittenberg, Hoher Weg 8, 06120 Halle (Saale), Germany
[b] German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig, Deutscher Platz 5e, 04103 Leipzig, Germany
[c] School of Biological Sciences, Queen's University Belfast, UK

ARTICLE INFO

Article history:
Received 13 June 2014
Accepted 22 October 2014
Available online xxxx

Keywords:
DWV
Microsporidian
Apis mellifera
Priority effect
Interaction

ABSTRACT

Two pathogens co-infecting a common host can either interact positively (facilitation), negatively (competition) or act independently. A correlative study has suggested that two pathogens of the honey bee, *Nosema ceranae* and Deformed wing virus (DWV), interact negatively within a host (Costa et al., 2011). To test this hypothesis, we sequentially co-infected honey bees with these pathogens in a reciprocally crossed experimental design. Prior establishment in the host ventriculus by *N. ceranae* inhibited DWV while prior infection by DWV did not impact *N. ceranae*, highlighting an asymmetry in the competitive interaction between these emerging pathogens.

1. Introduction

Co-infection of a host by multiple pathogens is widespread, particularly in social insects where transmission of microbes may be facilitated by the high density of individuals in a colony, high genetic relatedness between nest mates and frequent social interactions (Schmid-Hempel, 1998). Indeed, multiple pathogen infections are widely observed in honey bees (Cox-Foster et al., 2007; Ravoet et al., 2013; Runckel et al., 2011) and interactions between emerging pathogens have been considered a major cause of global colony mortality (Cornman et al., 2012; Doublet et al., 2014; Evans and Schwarz, 2011). Co-infecting pathogens may act independently of each other in the host, or interact positively, when one proliferates due to the presence of the other, or negatively, when pathogens suppress each other (Cox, 2001). In a recent study, Costa et al. (2011) observed in host honey bee ventriculi a negative correlation in pathogen loads between the microsporidian *Nosema ceranae* and Deformed wing virus (DWV), two emerging pathogens associated with bee mortality (Fürst et al., 2014; Higes et al., 2008; Nazzi et al., 2012). To explore putative competition between these pathogens in adult honey bee midguts, we performed sequential experimental oral infections (i.e. one pathogen after the other), giving a potential advantage of prior establishment to the first inoculated pathogen over the second, and compared their performance in terms of pathogen load per bee.

2. Material and methods

Five colonies of honey bees were used as a source of pupae, and bees were mixed in cages across treatments. Worker honey bees that emerged in the laboratory were kept two days in an incubator with 50% sucrose solution before inoculation. Pathogens were fed to individual bees in 10 µl of 50% sucrose solution. For each treatment, bees were fed twice, at day 2 and day 6 post emergence, for sequential feeding of pathogens. Two competition treatments were tested: Nosema/DWV treatment (N/D), where *N. ceranae* spores were fed first (day 2) and DWV second (day 6), and DWV/Nosema (D/N), where DWV was fed first and *N. ceranae* spores second (day 2 and day 6 respectively). Following the same nomenclature, four treatments with only one pathogen were used as controls: Control/Nosema (C/N), Nosema/Control (N/C), Control/DWV (C/D), and DWV/Control (D/C). Additionally, a double control treatment (C/C) was included, where bees were fed twice with a control solution. During the experiment, bees were maintained in metal cages, placed in an incubator at 30 °C ± 1 °C and 50% relative humidity, and fed with 50% sucrose solution *ad libitum*, as recommended by Williams et al. (2013). All treatments were run in triplicate, with 16 bees per cage.

* Corresponding author.
E-mail address: vincent.doublet@zoologie.uni-halle.de (V. Doublet).

http://dx.doi.org/10.1016/j.jip.2014.10.007
0022-2011/

Spores of *N. ceranae* used for inoculations were obtained from artificial propagations in adult honey bees kept in the laboratory. Spores were isolated following a triangulation method (Fries et al., 2013) and counted using a Fuchs-Rosenthal hemocytometer. Each worker bee was inoculated with 10^5 spores. DWV was obtained from symptomatic bees crushed in cold 0.5 M PBS (pH 8), filtered through cotton wool, and subsequently centrifuged at 4 °C for 15 min at 15,000*g*, before carefully extracting the supernatant and diluting in PBS (Bailey and Ball, 1991). This extract was then injected into uninfected pupae for propagation. After 6 days, injected pupae were crushed and viral particles extracted as above. Extracts were placed into clean aliquots and checked for the presence of DWV and co-propagation of other viruses using qRT-PCR (see Supplementary Table S1). Each inoculum contained 10^7 genome equivalents of DWV, with a non-significant amount of Chronic bee paralysis virus (CBPV < 0.001%) contamination. Control inoculum was prepared from uninfected pupae and was devoid of viral contamination.

Nine days after the second pathogen feeding and before the onset of significant mortality, five bees were randomly sampled per treatment per replicate (except for the third N/C replicate with only 2 surviving hosts). Sampled bees were flash-killed in liquid nitrogen and conserved in RNAlater ICE (Ambion, USA) at −20 °C. Total RNA from individual midguts was extracted using an RNeasy Mini Kit in a Qiacube robot (Qiagen). Pathogens and reference gene RP49 were quantified by qRT-PCR, using standard 10-fold dilutions of cloned fragments for absolute quantification (see Supplementary Table S1). Relative quantification of DWV was calculated by the ratio of DWV to RP49 copy numbers. The potential presence of other co-occurring pathogens in experimental samples was examined using RT-qPCR and reverse-transcriptase multiplex-ligation probe dependent amplification (RT-MLPA; De Smet et al. (2012)). Amplified fragments from RT-MLPA were visualized on a QIAxcel (Qiagen) with an acceptance threshold of 0.1 relative fluorescence units. Six bees with unsuccessful *N. ceranae* infections (three from N/C and three from N/D treatments) and two bees with *N. ceranae* contamination (C/D treatment) were discarded from the analysis, as well as one bee from the D/N treatment with unsuccessful viral infection. To estimate *N. ceranae* spore number in bees, a linear regression between qPCR Cq values and actual spore numbers in a bee midgut was calculated using five randomly selected bees ($y = -11.773x + 109.35$; $R^2 = 0.95$).

3. Results and discussion

Inoculated bees developed infections (Figs. S1–S3). Sequential inoculation of worker honey bees by *N. ceranae* and DWV revealed an asymmetric competitive interaction between the two pathogens. Inoculation by DWV had no impact on the load of *N. ceranae* spores in a honey bee's midgut, both when the virus was inoculated before (Figs. 1 and S1; Mann–Whitney with 2-tailed Monte-Carlo correction $U = 94$; $p = 0.653$) or after the microsporidian ($U = 46.5$; $p = 0.621$). Conversely, prior establishment of *N. ceranae* had a significant negative impact on the load of DWV (Fig. 2; $U = 41$; $p = 0.047$; see Supplementary Figs. S2 and S3). Though *N. ceranae* also seemed to inhibit DWV titres when the microsporidian was inoculated after the virus, the effect was not significant ($U = 64$; $p = 0.079$). No other viral pathogen was associated with experimental treatments (see Supplementary Table S2).

Competitive suppression of DWV by *N. ceranae* may be due to direct competition of pathogens for host resources or space in the midgut. Indeed, *N. ceranae* induces a degeneration of the epithelial gut cells and reduces their capacity to self-repair (Dussaubat et al., 2012). This suggests that biological cell functions are compromised by *N. ceranae*, and microsporidian-infected cells might not be suitable for RNA virus replication, or that *N. ceranae* infection limits the number of host cells available for viral infection, thereby limiting viral load. Conversely, viral infection does not seem to reduce the susceptibility or suitability of host cells for microsporidian infection.

Alternatively, suppression of DWV by *N. ceranae* might be mediated by immune priming of the host. Although, *N. ceranae* has been shown to induce immune suppression in honey bees (Antúnez et al., 2009; Aufauvre et al., 2014; Chaimanee et al., 2012), recent transcriptomic and proteomic studies demonstrate that *N. ceranae* infection is associated with oxidative stress in the ventriculus, which may constitute the main cellular immune response of the honey bee midgut to microsporidia, and potentially responsible of the cellular damage of the gut epithelium (Dussaubat et al., 2012; Vidau et al., 2014). In the mosquito *Aedes*

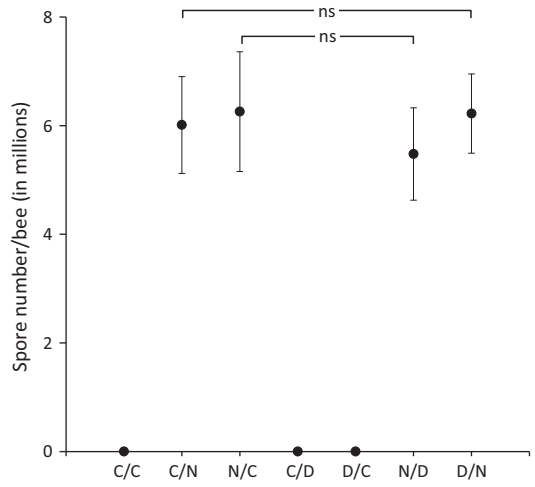

Fig. 1. Average estimated number of *N. ceranae* spores (±sem) per honey bee midgut from the seven treatments. Abbreviations of the treatments are on the *x*-axis in chronological order of infection at day 2 and day 6 post-eclosion: C = control, N = *N. ceranae*, D = DWV. As an example, treatment C/N means that bees first received a sugar solution then, four days later, a sugar solution with *N. ceranae* spores.

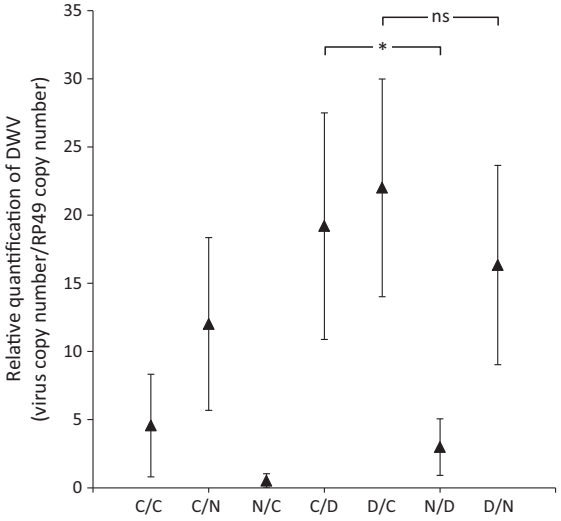

Fig. 2. Average relative quantification of DWV (±sem) per honey bee midgut from the seven treatments (reference gene: RP49). Abbreviations of the treatments are on the *x*-axis in chronological order. C = control, N = *N. ceranae*, D = DWV (see also legend to Fig. 1).

aegypti, the endosymbiotic bacteria *Wolbachia* has been shown to activate the host innate immune system by inducing the production of reactive oxygen species (ROS) following oxidative stress, thereby controlling dengue virus (Pan et al., 2012). It is therefore possible that antiviral defenses were activated in bees infected by *N. ceranae*, but this remains speculative as virus control by pathogenic fungi in insects has not yet been observed.

Here we fed two pathogens sequentially with an interval of four days, enough time for the first inoculated pathogen to establish in the host midgut. The *N. ceranae* life cycle takes four days (Gisder et al., 2011) and first intracellular spore germination starts minimally 3 days post-infection (Higes et al., 2007). Oral infection of honey bees by DWV also leads to rapid establishment of the pathogen in the midgut two days post-infection (Möckel et al., 2011). Four days seem sufficient for both virus and microsporidia to colonize the midgut epithelial cells, and benefit from a competitive advantage as observed among other host-pathogen systems (Hoverman et al., 2013; Jackson et al., 2006; Thomas et al., 2003) and described in community ecology as a priority effect (Alford and Wilbur, 1985). Here, *N. ceranae* seems to exhibit a priority effect over DWV.

Competitive suppression might be expected to have a negative impact on the dynamics of the virus. However, DWV is transmitted via multiple routes, horizontally (orally and by the parasitic mite vector *Varroa destructor*) and vertically (de Miranda and Genersch, 2010). These multiple transmission routes may reduce the impact of competition with *N. ceranae* on viral dynamics. Nevertheless, in the absence of *Varroa* mites (e.g. after winter or in isolated honey bee populations), our results suggest that the presence of *N. ceranae* in honey bee colonies may reduce viral proliferation in honey bee ventriculi.

Conflict of Interest

Q2 The authors declare no competing interests.

Acknowledgments

We acknowledge financial support of the EU 7th-Framework project BEEDOC, Grant Agreement FP7-KBBE-2009-3-244956-CP-FP and the Bundesministerium für Ernährung, Landwirtschaft und Verbraucherschutz project FITBEE, grant 511-06.01-28-1-71.007-10. The authors thank Dino McMahon, Panagiotis Theodorou, Anja Miertsch and Maureen Labarussias for technical support and discussion, and two anonymous reviewers for their useful comments that helped improve the manuscript.

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.2014.10.007.

References

Alford, R.A., Wilbur, H.M., 1985. Priority effects in experimental pond communities: competition between *Bufo* and *Rana*. Ecology 66, 1097–1105. http://dx.doi.org/10.2307/1939161.

Antúnez, K., Martín-Hernández, R., Prieto, L., Meana, A., Zunino, P., Higes, M., 2009. Immune suppression in the honey bee (*Apis mellifera*) following infection by *Nosema ceranae* (Microsporidia). Environ. Microbiol. 11, 2284–2290. http://dx.doi.org/10.1111/j.1462-2920.2009.01953.x.

Aufauvre, J., Misme-Aucouturier, B., Viguès, B., Texier, C., Delbac, F., Blot, N., 2014. Transcriptome analyses of the honeybee response to *Nosema ceranae* and insecticides. PLoS One 9, e91686. http://dx.doi.org/10.1371/journal.pone.0091686.

Q3 Bailey, L., Ball, B.V., 1991. Honey Bee Pathology, second ed. Academic Press, London.

Chaimanee, V., Chantawannakul, P., Chen, Y.-P., Evans, J.D., Pettis, J.S., 2012. Differential expression of immune genes of adult honey bee (*Apis mellifera*) after inoculated by *Nosema ceranae*. J. Insect Physiol. 58, 1090–1095. http://dx.doi.org/10.1016/j.jinsphys.2012.04.016.

Cornman, R.S., Tarpy, D.S., Chen, Y., Jeffreys, L., Lopez, D., Pettis, J.S., vanEngelsdorp, D., Evans, J.D., 2012. Pathogen webs in collapsing honey bee colonies. PLoS One 7, e43562. http://dx.doi.org/10.1371/journal.pone.0043562.

Costa, C., Tanner, G., Lodesani, M., Maistrello, L., Neumann, P., 2011. Negative correlation between *Nosema ceranae* spore loads and Deformed wing virus infection levels in adult honey bee workers. J. Invertebr. Pathol. 108, 224–225. http://dx.doi.org/10.1016/j.jip.2011.08.012.

Cox, F.E., 2001. Concomitant infections, parasites and immune responses. Parasitology 122 (Suppl.), S23–S38. http://dx.doi.org/10.1017/S003118200001698X.

Cox-Foster, D.L., Conlan, S., Holmes, E.C., Palacios, G., Evans, J.D., Moran, N.A., Quan, P.-L., Briese, T., Hornig, M., Geiser, D.M., Martinson, V.G., vanEngelsdorp, D., Kalkstein, A.L., Drysdale, A., Hui, J., Zhai, J., Cui, L., Hutchison, S.K., Simons, J.F., Egholm, M., Pettis, J.S., Lipkin, W.I., 2007. A metagenomic survey of microbes in honey bee colony collapse disorder. Science 318, 283–287. http://dx.doi.org/10.1126/science.1146498.

de Miranda, J.R., Genersch, E., 2010. Deformed wing virus. J. Invertebr. Pathol. 103 (Suppl. 1), S48–S61. http://dx.doi.org/10.1016/j.jip.2009.06.012.

De Smet, L., Ravoet, J., de Miranda, J.R., Wenseleers, T., Mueller, M.Y., Moritz, R.F.A., de Graaf, D.C., 2012. BeeDoctor, a versatile MLPA-based diagnostic tool for screening bee viruses. PLoS One 7, e47953. http://dx.doi.org/10.1371/journal.pone.0047953.

Doublet, V., Labarussias, M., de Miranda, J.R., Moritz, R.F.A., Paxton, R.J., 2014. Bees under stress: sublethal doses of a neonicotinoid pesticide and pathogens interact to elevate honey bee mortality across the life cycle. Environ. Microbiol. (in press). http://dx.doi.org/10.1111/1462-2920.12426.

Dussaubat, C., Brunet, J.L., Higes, M., Colbourne, J.K., Lopez, J., Choi, J.H., Martin-Hernandez, R., Botias, C., Cousin, M., McDonnell, C., Bonnet, M., Belzunces, L.P., Moritz, R.F., Le Conte, Y., Alaux, C., 2012. Gut pathology and responses to the microsporidium *Nosema ceranae* in the honey bee *Apis mellifera*. PLoS One 7, e37017. http://dx.doi.org/10.1371/journal.pone.0037017.

Evans, J.D., Schwarz, R.S., 2011. Bees brought to their knees: microbes affecting honey bee health. Trends Microbiol. 19, 614–620. http://dx.doi.org/10.1016/j.tim.2011.09.003.

Fries, I., Chauzat, M.-P., Chen, Y.-P., Doublet, V., Genersch, E., Gisder, S., Higes, M., McMahon, D.P., Martin-Hernández, R., Natsopoulou, M.E., Paxton, R.J., Tanner, G., Webster, T.C., Williams, G.R., 2013. Standard methods for Nosema research. In: Dietemann, V., Ellis, J.D., Neumann, P. (Eds.), The Coloss Beebook, Volume I: Standard Methods for *Apis mellifera* Research, J. Apicult. Res. 52. http://dx.doi.org/10.3896/ibra.1.52.1.14.

Fürst, M.A., McMahon, D.P., Osborne, J.L., Paxton, R.J., Brown, M.J.F., 2014. Disease associations between honeybees and bumblebees as a threat to wild pollinators. Nature 506, 364–366. http://dx.doi.org/10.1038/nature12977.

Gisder, S., Möckel, N., Linde, A., Genersch, E., 2011. A cell culture model for *Nosema ceranae* and *Nosema apis* allows new insights into the life cycle of these important honey bee-pathogenic microsporidia. Environ. Microbiol. 13, 404–413. http://dx.doi.org/10.1111/j.1462-2920.2010.02346.x.

Higes, M., García-Palencia, P., Martín-Hernández, R., Meana, A., 2007. Experimental infection of *Apis mellifera* honeybees with *Nosema ceranae* (Microsporidia). J. Invertebr. Pathol. 94, 211–217. http://dx.doi.org/10.1016/j.jip.2006.11.001.

Higes, M., Martin-Hernández, R., Botías, C., Garrido Bailón, E., González-Porto, A.M., Barrios, L., del Nozal, M.J., Bernal, J.L., Jiménez, J.J., García Palencia, P., Meana, A., 2008. How natural infection by *Nosema ceranae* causes honeybee colony collapse. Environ. Microbiol. 10, 2659–2669. http://dx.doi.org/10.1111/j.1462-2920.2008.01687.x.

Hoverman, J.T., Hoye, B.J., Johnson, P.T.J., 2013. Does timing matter? How priority effects influence the outcome of parasite interactions within hosts. Oecologia 173, 1471–1480. http://dx.doi.org/10.1007/s00442-013-2692-x.

Jackson, J.A., Pleass, R.J., Cable, J., Bradley, J.E., Tinsley, R.C., 2006. Heterogenous interspecific interactions in a host–parasite system. Int. J. Parasitol. 36, 1341–1349. http://dx.doi.org/10.1016/j.ijpara.2006.07.003.

Möckel, N., Gisder, S., Genersch, E., 2011. Horizontal transmission of Deformed wing virus: pathological consequences in adult bees (*Apis mellifera*) depend on the transmission route. J. Gen. Virol. 92, 370–377. http://dx.doi.org/10.1099/vir.0.025940-0.

Nazzi, F., Brown, S.P., Annoscia, D., Del Piccolo, F., Di Prisco, G., Varricchio, P., Della Vedova, G., Cattonaro, F., Caprio, E., Pennacchio, F., 2012. Synergistic parasite–pathogen interactions mediated by host immunity can drive the collapse of honeybee colonies. PLoS Pathog. 8, e1002735. http://dx.doi.org/10.1371/journal.ppat.1002735.

Pan, X., Zhou, G., Wu, J., Bian, G., Lu, P., Raikhel, A.S., Xi, Z., 2012. *Wolbachia* induces reactive oxygen species (ROS)-dependent activation of the Toll pathway to control dengue virus in the mosquito *Aedes aegypti*. Proc. Natl. Acad. Sci. USA 109, E23–E31. http://dx.doi.org/10.1073/pnas.1116932108.

Ravoet, J., Maharramov, J., Meeus, I., De Smet, L., Wenseleers, T., Smagghe, G., de Graaf, D.C., 2013. Comprehensive bee pathogen screening in Belgium reveals *Crithidia mellificae* as a new contributory factor to winter mortality. PLoS One 8, e72443. http://dx.doi.org/10.1371/journal.pone.0072443.

Runckel, C., Flenniken, M.L., Engel, J.C., Ruby, J.G., Ganem, D., Andino, R., DeRisi, J.L., 2011. Temporal analysis of the honey bee microbiome reveals four novel viruses and seasonal prevalence of known viruses, *Nosema*, and *Crithidia*. PLoS One 6, e20656. http://dx.doi.org/10.1371/journal.pone.0020656.

Schmid-Hempel, P., 1998. Parasites in Social Insects. Princeton University Press, Princeton, NJ.

Thomas, M.B., Watson, E.L., Valverde-Garcia, P., 2003. Mixed infections and insect–pathogen interactions. Ecol. Lett. 6, 183–188. http://dx.doi.org/10.1046/j.1461-0248.2003.00414.x.

Vidau, C., Panek, J., Texier, C., Biron, D.G., Belzunces, L.P., Le Gall, M., Broussard, C., Delbac, F., El Alaoui, H., 2014. Differential proteomic analysis of midguts from *Nosema ceranae*-infected honeybees reveals manipulation of key host functions. J. Invertebr. Pathol. 121, 89–96. http://dx.doi.org/10.1016/j.jip.2014.07.002.

Williams, G.R., Alaux, C., Costa, C., Csáki, T., Doublet, V., Eisenhardt, D., Fries, I., Kuhn, R., McMahon, D.P., Medrzycki, P., Murray, T.E., Natsopoulou, M.E., Neumann, P., Oliver, R., Paxton, R.J., Pernal, S.F., Shutler, D., Tanner, G., van der Steen, J.J.M., Brodschneider, R., 2013. Standard methods for maintaining adult Apis mellifera in cages under in vitro laboratory conditions. In: Dietemann, V., Ellis, J.D., Neumann, P. (Eds.), The Coloss Beebook, Volume I: Standard Methods for *Apis mellifera* Research, J. Apicult. Res. 52. http://dx.doi.org/10.3896/ibra.1.52.1.04.

Please cite this article in press as: Doublet, V., et al. Within-host competition among the honey bees pathogens *Nosema ceranae* and Deformed wing virus is asymmetric and to the disadvantage of the virus. J. Invertebr. Pathol. (2014), http://dx.doi.org/10.1016/j.jip.2014.10.007