Journal of Invertebrate Pathology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

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

Gamma irradiation of pollen and eradication of Israeli acute paralysis virus

Ivan Meeus [a,*], Hadi Mosallanejad [a], Jinzhi Niu [a], Dirk C. de Graaf [b], Felix Wäckers [c], Guy Smagghe [a]

[a] Department of Crop Protection, Faculty of Bioscience Engineering, Ghent University, Coupure 653, 9000 Ghent, Belgium
[b] Laboratory of Zoophysiology, Department of Physiology, Faculty of Sciences, Ghent University, Krijgslaan 281 S2, 9000 Ghent, Belgium
[c] Biobest, Ilse Velden 18, 2260 Westerlo, Belgium

ARTICLE INFO

Article history:
Received 31 March 2014
Accepted 30 June 2014
Available online xxxx

Keywords:
Gamma irradiation
Israeli acute paralysis virus
Pollen
Sterilization
Bumblebee rearing
Dicistroviridae

ABSTRACT

Honeybees and bumblebees are the most important pollinators of agricultural crops. For this purpose honeybees and bumblebees are reared and transported. A pathogen-free status of bees in general, is crucial. Indeed anthropogenic transports of hosts carrying parasites could alter the natural host/pathogen association, inducing an extra pathogenic stress. Therefore the creation of a pathogen-free rearing environment is needed. For bumblebees this is possible, as these species are reared in a closed environment. Although, a link remains between reared bumblebees and the outside bee community, as honeybee-collected pollen is essential food for bumblebee mass rearing. Here we evaluated if gamma irradiation can minimize the risk of this potential route of exposure and can inactivate viral particles present in honeybee-collected pollen. We show that 16.9 kGy gamma irradiation induced a 100–1000 fold reduction on the ability of IAPV to cause mortality after injections. This result opens avenues toward rearing pathogen-free bumblebees and towards eliminating the risks of pathogen spillover to native wild bee species.

1. Introduction

The *Dicistroviridae* is a family picornavirus-like RNA viruses, their members infect arthropods, and primarily insects (Bonning and Miller, 2010). Also bumblebees can be infected with dicistroviruses (Bailey and Gibbs, 1964; Singh et al., 2010; Ward et al., 2007). These include Black queen cell virus (BQCV), and the viruses from the acute bee paralysis virus complex (i.e. Acute bee paralysis virus (ABPV), Kashmir bee virus (KBV) and Israeli acute paralysis virus (IAPV)). Here we focus on IAPV, originally identified in honeybees (de Miranda et al., 2010), and lethal after injection in honeybees (Maori et al., 2007) and bumblebees (Niu et al., 2014). Recent data, using a single oral dose of 0.5×10^7 viral particles, also showed that this virus impaired the fitness in bumblebees, as the drone production in microcolonies was impaired (Meeus et al. submitted). IAPV can infect a wide variety of bees, and also wasps (Singh et al., 2010), making it a multi-host pathogen.

Bumblebees are the most important pollinator for greenhouse crops (Velthuis and van Doorn, 2006), but also breeders of open-field crops gain interest in bumblebee pollination, due to the rapid loss of landscapes supporting wild bees and the fact that the availability of honeybees is declining (Garibaldi et al., 2013). For this pollination purpose bumblebees are produced in closed environments. Anthropogenic transport of bees, be it commercial bumblebees, domesticated honeybees or other bees, and the multi-host character of the pathogens they may carry could potentially alter the natural host-pathogens association (Meeus et al., 2011; Murray et al., 2013). This could add pathogenic stress on wild bumblebee populations (Meeus et al., 2011; Woodroffe, 1999). Unlike honeybees, commercial bumblebees are generally produced in closed and controlled climate rooms, which opens opportunities for a pathogen-free environment. Key here is a thorough sanitary control. As bees in nature are known to be widely infected by a broad range of pathogens (Evison et al., 2012; Singh et al., 2010), any contact with outside bees needs to be strictly avoided. Indeed the tracheal mite *Locustacarus buchneri*, identifiable by binocular screening of the trachea, has been eradicated from the larger rearing facilities through sufficient quality control (Meeus et al., 2011; Murray et al., 2013). For micro-parasites, like the protozoa (Klee et al., 2006; Meeus et al., 2010a), spiroplasmas (Meeus et al., 2012) and viruses (De Smet et al., 2012; Meeus et al., 2010b; Ravoet et al., 2013) different molecular detection tools have been developed or been optimized. Hence also here quarantine measures in combination with thorough screening can be used to eliminate these pathogens from the rearing facilities. However,

* Corresponding author. Address: Laboratory of Agrozoology, Department of Crop Protection, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium. Fax: +32 9 2646239.
E-mail address: ivan.meeus@ugent.be (I. Meeus).

http://dx.doi.org/10.1016/j.jip.2014.06.012
0022-2011/

until recently, one weak point in the rearing systems remained. Since bumblebees are fed with honeybee-collected pollen, a known source for viruses (Singh et al., 2010) but also of *Nosema* (Higes et al., 2008), influx of pathogens remained possible. It is evident that pathogen sterilization techniques could greatly reduce infection risks associated with feeding on pollen and thereby enabling a pathogen-free rearing environment.

Here we investigated whether gamma irradiation of honeybee-collected pollen, as currently practiced by a commercial bumblebee breeding company (Biobest), could be used to inactivate viruses present in pollen. Specifically, we first inoculated pollen with IAPV, before irradiating the pollen. Subsequently we separated the virus from the pollen, and injected this into bumblebees to induce mortality (Niu et al., 2014). The difference in survival rate between gamma irradiated and non-irradiated viruses informs us about the efficiency of the irradiation procedure. It has to be kept in mind that this method of concentrating the virus and subsequently injecting it, provides no indication of infection risk through oral exposure. Injections are used as a proxy as oral exposure is far less likely to result in infections.

2. Materials and methods

2.1. Bumblebees

All bumblebee workers (*Bombus terrestris*) were obtained from a continuous mass rearing program (Biobest, Westerlo, Belgium) and fed on commercial sugar water (BIOGLUC®, Biobest), and honeybee-collected pollen (Soc. Coop. Apihurdes, Pinofranqueado-C'aceres, Spain) as energy and protein source, respectively. The insects were kept under standardized laboratory conditions with 28–30 °C, 60–65% relative humidity, and continuous darkness.

2.2. Gamma irradiation of IAPV inoculated pollen and separation of IAPV from pollen

The IAPV stock is a chloroform-clarified extract in 10 mM phosphate buffer (pH 7.0) 0.02% diethyl dithiocarbamate of 50 IAPV infected pupae (de Miranda et al., 2013). It had <0.1% contamination with other common honeybee viruses, as determined by RT-qPCR using previously published assays for IAPV, KBV, ABPV, Chronic bee paralysis virus, Deformed wing virus (DWV), Varroa destructor virus-1, Slow bee paralysis virus, Sacbrood virus and Black queen cell virus (Locke et al., 2012). Sequencing proved the presence of IAPV and replication in IAPV-injected bumblebees (Niu et al., 2014). Two IAPV-infected pollen samples were made, each containing 30 µl of the IAPV stock plus 0.1 g of pollen in a 1.5-ml centrifuge tube. One sample underwent the commercially used gamma irradiation of 16.9 kGy in the GAMMIR irradiation cell of Sterigenics (Fleurus, Belgium) after placing the 1.5-ml centrifuge tube (Novolab, Geraardsbergen, Belgium) in a 50-ml falcon tube (Novolab), while the control sample had a cold chain interruption to mimic possible loss of viral activity because of freezing and thawing.

Irradiation proved to be effective in destroying the microbial content. This was confirmed by a single survey of two pollen samples (100 g), comparing honeybee collected pollen irradiated by 18.1–20.5 kGy with non-irradiated pollen. We saw a clear drop in number of bacteria from an initial (1600 cfu/g), yeast (20 cfu/g) and fungi (0.9 cfu/g) in the non-irradiated samples, toward undetectable numbers in irradiated pollen (SGS Belgium nv, Antwerp, Belgium). This is in agreement with the data reported by (Yook et al., 1998).

We added 370 µl ultrapure water Type I (milliQ, Millipore) to both samples and separated pollen from the viral solutions by centrifugation at 12,000*g*. In order to ensure that the sample did not contain any viral RNA, which could results in viral replication after injection, we added RNase A (Thermo Scientific, Aalst, Belgium) at a final recommended maximum concentration of 0.10 mg/ml to a volume of 200 µl of the supernatant. The RNase A is separated from the virus by Amicon ultra-0.5 centrifugal filter devices 30 K (Millipore).

2.3. Virus injection experiments 1 and 2

A logarithmic dilution was made of both viral preparations, control versus irradiated, ranging from 10^{-1} until 10^{-5}. In a first experiment we injected 1 µl in 4 day old bumblebees ($n = 11$) per treatment per dilution. In a second experiment we injected 2 µl in 4 day old bumblebees ($n = 20$) per treatment per dilution, except for the dilutions 10^{-4} and 10^{-5} for the irradiated samples, that were skipped. Injection was used over oral feeding as injection results in infection at much lower inoculum concentrations, with only a few viral particles being sufficient to cause infection and mortality within 12 days (Niu et al., 2014). If a bee died after one day it was excluded from the analyses (3.6% and 1.9% in injection experiments 1 and 2, respectively).

In order to evaluated if irradiation influenced survival we analyzed Kaplan–Meier curves conducted in SPSS v21.0 (SPSS Inc., Chicago, Il.). The following comparative statistic tests were performed: Log Rank (Mantel-Cox) and Breslow (Generalized Wilcoxon).

3. Results

In the first experiment 10 and 100 fold dilution of the (non-irradiated) IAPV stock resulted in a high mortality rate (Fig. 1a sample: −1 and −2 (red dotted line)), with all bees being dead after 8 days. A 1000 fold dilution had an intermediate mortality. Indeed the 1000 fold dilution (Fig. 1a sample: −3 (blue dotted line)) showed a significantly higher survival at the end of the recording period compared with the 10 and 100 fold diluted samples ((Log Rank (Mantel-Cox) = 8.015; df = 2; $P = 0.018$) and (Breslow (Generalized Wilcoxon) = 3.035; df = 2; $P = 0.219$)). This intermediate mortality is justified, as it is also significantly different from the sample with no mortality at the 100,000 fold dilution (Fig. 1a sample: −5 (green dotted line)) ((Log Rank (Mantel-Cox) = 4.319; df = 1; $P = 0.038$) and (Breslow (Generalized Wilcoxon) = 4.277; df = 1; $P = 0.039$)).

Analyzing the irradiated samples we see a clear separation between the 10-fold dilution and the higher dilutions (Fig. 1a sample: Ir-1 (red line) and Ir-2, Ir-3, Ir-4 and Ir-5 (green line)), ((Log Rank (Mantel-Cox) = 64.707; df = 4; $P < 0.001$) and (Breslow (Generalized Wilcoxon) = 60.776; df = 4; $P < 0.001$)). To summarize, the 100 fold dilution of the irradiated samples already gave no mortality, while for the non-irradiated samples a 10,000 fold dilution or higher was needed to achieve this. The 10,000 fold non-irradiated sample had a slightly higher mortality (mean mortality day 10.3 CI 95% 8.3–12.4) compared to the 100 fold irradiated sample (mean mortality day 11.4 CI 95% 10.3–12.5); but this was not statically different ((Log Rank (Mantel-Cox) = 0.608; df = 1; $P = 0.436$) and (Breslow (Generalized Wilcoxon) = 0.704; df = 1; $P = 0.401$)). Taken all together this means that a 100 fold reduction in virus activity could be concluded.

In a second experiment we wanted to confirm these results by increasing our power ($n = 20$ per treatment). We also wanted to establish that the irradiated 100 dilution resulted in no mortality, while the 10,000 fold diluted non-radiated virus possibly had some active viruses left. Therefore we injected 2 µl in this second experiment instead of 1 µl. Here we saw that the 1000 fold dilution of

Please cite this article in press as: Meeus, I., et al. Gamma irradiation of pollen and eradication of Israeli acute paralysis virus. J. Invertebr. Pathol. (2014), http://dx.doi.org/10.1016/j.jip.2014.06.012

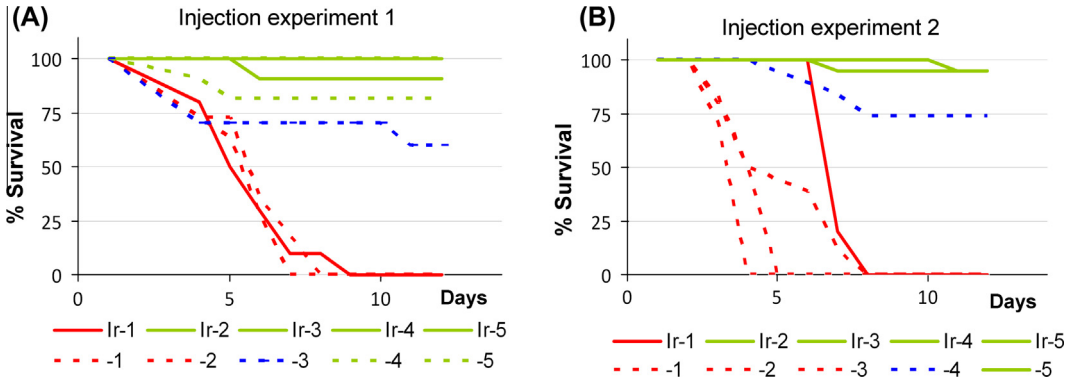

Fig. 1. The percentage of survival in function of time (days) after Israeli acute paralysis virus injection (−1 until −5 represent 10-fold until 100,000 fold dilution of the viral stock; Ir indicates that the sample is gamma irradiated (full line) the non-irradiated samples are represented by a dotted line). The colors represent three different groups of treatments with another survival based on pairwise comparisons with Kapan-Meier statistics (green line: no mortality; blue line: intermediate mortality, and red line: high mortality). (A) In injection experiment 1 we injected 1 μl in 11 bumblebees per treatment, (B) represent experiment 2 with 2 μl injection in 20 bumblebees. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the non-irradiated IAPV stock resulted in a high mortality rate (Fig. 1b sample: −1, −2 and −3 (red dotted line)), with all bees being dead after 8 days. Now a 10,000 fold dilution had an intermediate mortality. Indeed the 10,000 fold dilution (Fig. 1b sample: −4 (blue dotted line)) shows a significantly higher survival compared with the lower diluted samples ((Log Rank (Mantel-Cox) = 66.908; df = 3; $P = 0.000$) and (Breslow (Generalized Wilcoxon) = 50.847; df = 3; $P = 0.000$)). But it was also distinguishable from the non-mortal injection, being a 100,000 fold diluted sample (Fig. 1b sample: −5 (green dotted line)) ((Log Rank (Mantel-Cox) = 5.572; df = 1; $P = 0.018$) and (Breslow (Generalized Wilcoxon) = 5.552; df = 1; $P = 0.018$)).

For the irradiated samples, with 2 μl injections, the 100 fold diluted samples showed almost no mortality at all (Fig. 1b sample: Ir-2 (green line)); having a higher survival (mean mortality day 11.8 CI 95% 11.3–12.2) than the non-irradiated 10,000 fold dilution (mean mortality day 10.7 CI 95% 9.7–11.7) (Fig. 1b sample: −4 (blue dotted line)) ((Log Rank (Mantel-Cox) = 3.062; df = 1; $P = 0.080$) and (Breslow (Generalized Wilcoxon) = 3.036; df = 1; $P = 0.081$)), and a comparable survival with the non-irradiated 100,000 fold dilution, where no bumblebees died ((Log Rank (Mantel-Cox) = 1.000; df = 1; $P = 0.317$) and (Breslow (Generalized Wilcoxon) = 1.000; df = 1; $P = 0.317$)). Thus by injection more virus, we could increase the mortality in the non-irradiated samples, while it stayed constant in the irradiated ones. Therefore, in this more powerful test we confirmed a 1000 fold reduction in the activity of the virus in the radiated samples.

4. Discussion

Here we investigated the impact of gamma radiation on IAPV in a pollen matrix. This pollen matrix is essential: one, it is the virus contaminated pollen which is a possible virus influx route in bumblebee breeding facilities, and two the efficacy of gamma radiation depends on the matrix in which the virus is present (Sullivan et al., 1971, 1973; Thomas et al., 1981). We found that the gamma-radiation dose of 16.9 kGy, resulted in a 100 fold (in experiment 1) and a 1000 fold (in experiment 2) reduction in active viral particles in this pollen matrix. This difference could be a result of the experiments having been performed on two different points in time. But it is more likely explained by the difference in methodology, with, experiment 2 using more replicates and injecting double the amount of viral particles. Consequently, experiment 2 should have been more accurate and results more robust. Nevertheless, a conservative conclusion would be that the currently practiced radiation dose results in a reduction of viable IAPV levels by at least a factor 100.

Typically for dicistroviruses, low numbers of viral particles are needed to induce mortality after injection, while high numbers are needed to establish mortality after oral administration (Ribière et al., 2008). Oral administration of the IAPV stock, which represents a highly concentrated virus titer, resulted in IAPV infection, while a 10-fold reduction did not (Meeus et al. unpublished). Therefore the applied irradiation procedure did inactivate the IAPV stock enough to prevent oral infection. However, it remains to be determined at which concentration viral particles are present in honeybee-collected pollen to guarantee that the reduction in viral activity is enough to prevent infection after oral administering of highly contaminated honeybee collected pollen. Honeybee collected pollen is delivered to breeding facilities as small clump of pollen from the corbicula of the honeybee. The infectiousness of this pollen depends on the viral titer within these small clumps. However, in order to feed bumblebees these clumps are mixed, partially diluting the viral particles over the total pollen batch, lowering the viral titer. This combined with the at least 100x times reduction of infectiousness after irradiation makes oral infection by irradiated pollen less likely.

Bumblebees are also exposed to non-viral pathogens present in honeybee-collected pollen. For instance, *Nosema ceranae* described as a honeybee pathogen, has been identified in bumblebees, reducing its survival and with sub-lethal effects on behavior (Graystock et al., 2013). Therefore pollen irradiation will not only lower viral incidences in rearing facilities, but also of other pathogens. Indeed if we look at typical honeybee pathogens: for example, for *Nosema apis* a dosages of minimally 2 kGy could kill the parasite, while 10 kGy was able to inactivate the etiological agents *Paenibacillus larvae* and *Ascophaera apis* for American foulbrood and chalkbrood disease, respectively (Melathopoulos et al., 2004; Williams et al., 2013). Therefore the 16.9 kGy gamma irradiation would not only reduce viral incidences, but also eradicate other pathogens potentially infecting bumblebees or crossing the host species barrier after continuous exposure. The impact of gamma irradiation on the food quality of pollen is also an important factor to consider. No differences were found in the physiochemical properties of pollen after 7.5 kGy gamma irradiation, suggesting that the food value remains constant (Yook et al., 1998). However, a strong impact on the microbiology of pollen has been described (Yook et al., 1998). Currently, we know that the gut microbiota of bees are not just commensals as they rather live in mutualism with their bee host.

Please cite this article in press as: Meeus, I., et al. Gamma irradiation of pollen and eradication of Israeli acute paralysis virus. J. Invertebr. Pathol. (2014), http://dx.doi.org/10.1016/j.jip.2014.06.012

Still, the experience at Biobest, where for the last three years only irradiated pollen has been used, shows no negative impact on bumblebee rearing, or performance.

Our results depict an essential step toward bumblebee rearing in an environment free of pathogens. This is essential in a world with an extra demand for pollination, and a decreasing trend in pollination services provided by the wild bees (Breeze et al., 2014; Garibaldi et al., 2013; Potts et al., 2010). Currently commercial or domesticated bees are needed to ensure the pollination. However, it is essential to ensure that they do not impose a threat toward wild bees and their essential free pollination services.

Acknowledgments

This study was supported by the Fund for Scientific Research Flanders (FWO-Vlaanderen) and the Institute for the Promotion of Innovation by Science and Technology in Flanders (IWT-O&O 100899, Brussels, Belgium). H. Mosallanejad is now staff of Iranian Research Institute of Plant protection which is acknowledged.

References

Bailey, L., Gibbs, A.J., 1964. Acute infection of bees with paralysis virus. J. Insect. Pathol. 6, 395–407.
Bonning, B.C., Miller, W.A., 2010. Dicistroviruses. Annu. Rev. Entomol. 55, 129–150.
Breeze, T.D. et al., 2014. Agricultural policies exacerbate honeybee pollination service supply–demand mismatches across europe. PLoS ONE 9, e82996. http://dx.doi.org/10.1371/journal.pone.0082996.
de Miranda, J.R. et al., 2010. The Acute bee paralysis virus-Kashmir bee virus-Israeli acute paralysis virus complex. J. Invertebr. Pathol. 103, S30–S47.
de Miranda, J.R., et al., 2013. Standard methods for virus research in Apis mellifera. In: Dietemann, V., et al. (Eds.), The COLOSS BEEBOOK, Volume II: standard methods for Apis mellifera pest and pathogen research. J. Apic. Res.
De Smet, L. et al., 2012. BeeDoctor, a versatile MLPA-based diagnostic tool for screening bee viruses. PLoS ONE 7, e47953. http://dx.doi.org/10.1371/journal.pone.
Evison, S.E. et al., 2012. Pervasiveness of parasites in pollinators. PLoS ONE 7, e30641. http://dx.doi.org/10.1371/journal.pone.0030641.
Garibaldi, L.A. et al., 2013. Wild pollinators enhance fruit set of crops regardless of honey bee abundance. Science 339, 1608–1611.
Graystock, P. et al., 2013. Emerging dangers: deadly effects of an emergent parasite in a new pollinator host. J. Invertebr. Pathol. 114, 114–119.
Higes, M. et al., 2008. Detection of infective *Nosema ceranae* (Microsporidia) spores in corbicular pollen of forager honeybees. J. Invertebr. Pathol. 97, 76–78.
Klee, J. et al., 2006. Specific and sensitive detection of *Nosema bombi* (Microsporidia: Nosematidae) in bumble bees (*Bombus* spp.; Hymenoptera: Apidae) by PCR of partial rRNA gene sequences. J. Invertebr. Pathol. 91, 98–104.
Locke, B. et al., 2012. Acaricide treatment affects viral dynamics in *Varroa destructor*-infested honey bee colonies via both host physiology and mite control. Appl. Environ. Microbiol. 78, 227–235.
Maori, E. et al., 2007. Isolation and characterization of Israeli acute paralysis virus, a dicistrovirus affecting honeybees in Israel: evidence for diversity due to intra- and inter-species recombination. J. Gen. Virol. 88, 3428–3438.
Meeus, I. et al., 2010a. Multiplex PCR detection of slowly-evolving trypanosomatids and neogregarines in bumblebees using broad-range primers. J. Appl. Microbiol. 109, 107–115.
Meeus, I. et al., 2010b. Multiplex RT-PCR with broad-range primers and an exogenous internal amplification control for the detection of honeybee viruses in bumblebees. J. Invertebr. Pathol. 105, 200–203.
Meeus, I. et al., 2011. Effects of invasive parasites on bumble bee declines. Conserv. Biol. 25, 662–671.
Meeus, I. et al., 2012. Molecular detection of *Spiroplasma apis* and *Spiroplasma melliferum* in bees. J. Invertebr. Pathol. 109, 172–174.
Melathopoulos, A.P. et al., 2004. High velocity electron-beam radiation of pollen and comb for the control of *Paenibacillus larvae* subspecies larvae and *Ascosphaera* apis. Am. Bee J. 144, 714–720.
Murray, T.E. et al., 2013. Pathogen prevalence in commercially reared bumble bees and evidence of spillover in conspecific populations. Biol. Conserv. 159, 269–276.
Niu, J. et al., 2014. Analysis of reference gene stability after Israeli acute paralysis virus infection in bumblebees *Bombus terrestris*. J. Invertebr. Pathol. 115, 76–79.
Potts, S.G. et al., 2010. Global pollinator declines: trends, impacts and drivers. Trends Ecol. Evol. 25, 345–353.
Ravoet, J. et al., 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.
Ribière, M. et al., 2008. Natural history and geographical distribution of honeybee viruses. In: Aubert, M.F.A. et al. (Eds.), Virology and the Honey Bee. publications.european.eu., Luxembourg, pp. 15–84.
Singh, R. et al., 2010. RNA viruses in hymenopteran pollinators: evidence of inter-taxa virus transmission via pollen and potential impact on non-*Apis* hymenopteran species. PLoS ONE 5, e14357. http://dx.doi.org/10.1371/journal.pone.0014357.
Sullivan, R. et al., 1971. Inactivation of 30 viruses by gamma radiation. Appl. Microbiol. 22, 61–65. Q3
Sullivan, R. et al., 1973. Gamma-radiation inactivation of coxsackie-virus-B-2. Appl. Microbiol. 26, 14–17.
Thomas, F.C. et al., 1981. Gamma-ray inactivation of some animal viruses. Can. J. Comp. Med. 45, 397–399.
Velthuis, H.H.W., van Doorn, A., 2006. A century of advances in bumblebee domestication and the economic and environmental aspects of its commercialization for pollination. Apidologie 37, 421–451.
Ward, L. et al., 2007. First detection of *Kashinir bee virus* in the UK using real-time PCR. Apidologie 38, 181–190.
Williams, G.R. et al., 2013. Standard methods for maintaining adult *Apis mellifera* in cages under in vitro laboratory conditions. J. Agric. Res. 52.
Woodroffe, R., 1999. Managing disease threats to wild mammals. Anim. Conserv. 2, 185–193.
Yook, H.S. et al., 1998. Changes in microbiological and physicochemical properties of bee pollen by application of gamma irradiation and ozone treatment. J. Food Protect. 61, 217–220.