Hygienic food to reduce pathogen risk to bumblebees

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ABSTRACT

Bumblebees are ecologically and economically important pollinators, and the value of bumblebees for crop pollination has led to the commercial production and exportation/importation of colonies on a global scale. Commercially produced bumblebee colonies can carry with them infectious parasites, which can both reduce the health of the colonies and spillover to wild bees, with potentially serious consequences. The presence of parasites in commercially produced bumblebee colonies is in part because colonies are reared on pollen collected from honey bees, which often contains a diversity of microbial parasites. In response to this threat, part of the industry has started to irradiate pollen used for bumblebee rearing. However, to date there is limited data published on the efficacy of this treatment. Here we examine the effect of gamma irradiation and an experimental ozone treatment on the presence and viability of parasites in honey bee pollen. While untreated pollen contained numerous viable parasites, we find that gamma irradiation reduced the viability of parasites in pollen, but did not eliminate parasites entirely. Ozone treatment appeared to be less effective than gamma irradiation, while an artificial pollen substitute was, as expected, entirely free of parasites. The results suggest that the irradiation of pollen before using it to rear bumblebee colonies is a sensible method which will help reduce the incidence of parasite infections in commercially produced bumblebee colonies, but that further optimisation, or the use of a nutritionally equivalent artificial pollen substitute, may be needed to fully eliminate this route of disease entry into factories.

1. Introduction

Insect pollinators are essential for sustainable food production. While most of the major human food crops are not reliant on pollinators, insect pollinators are necessary for the production of a wide diversity of other food crops that contribute important micronutrients to human diets, and there is consequently great concern about declines in the wild populations of many pollinator species (Biesmeijer et al., 2006; Potts et al., 2010; Vanbergen et al., 2013). The economic importance of pollination has led to the commercial utilisation of bees for the pollination of many crops. Although the western honey bee *Apis mellifera* is the best known managed pollinator species, bumblebees are more efficient pollinators of certain plant species and hence several species of bumblebees (*Bombus* spp.) are also produced commercially for the

pollination of a variety of fruit and vegetable crops in glasshouses, polytunnels and open fields (Velthuis and van Doorn, 2006). The bumblebee colonies are reared by a small number of companies, with over a million colonies now being produced and used on a global scale (Goulson and Hughes, 2015). Increasingly, local bumblebee species are being produced in local factories, but still a significant number of colonies are exported.

As with the production of any animal, the commercial production of bumblebees has to deal with the threat of disease. Bumblebees suffer from three main microbial parasites, the neogregarine *Apicystis bombi*, the trypanosome *Crithidia bombi*, and the microsporidian *Nosema bombi* (Schmid-Hempel, 2001). In addition, they can also be infected by the parasites *Nosema ceranae* and deformed wing virus, which are best known from honey bees but are now realised to have multi-host dynamics and to be widespread in bumblebees (Plischuk et al., 2009; Evison et al., 2012; Li et al., 2012; Fürst et al., 2014; Manley et al., 2015; McMahon et al., 2015). All of these parasites can have significant effects on

bumblebees, reducing lifespan, fat stores, learning ability, and capacity to deal with other stresses (Schmid-Hempel, 2001; Graystock et al., 2013a; Fürst et al., 2014; Graystock et al., 2016). Parasite infections are therefore very likely to reduce the pollination services that a commercially produced bumblebee colony will provide to farmers, in addition to presenting a threat of parasite spillover to wild bees. Many studies have shown that commercially produced bumblebee colonies are often infected by a diversity of parasites (Goka et al., 2000; Whittington and Winston, 2003; Gegear et al., 2005; Colla et al., 2006; Otterstatter and Thomson, 2007; Manson et al., 2010; Singh et al., 2010; Meeus et al., 2011; Graystock et al., 2013b; Murray et al., 2013; Sachman-Ruiz et al., 2015). There is correlative evidence that parasites from commercially produced bumblebees have spilled over to wild bumblebees in at least North America, South America and Japan, and of there being concordant declines of wild bumblebees in North America and Argentina (Goka et al., 2001; Colla et al., 2006; Otterstatter and Thomson, 2008; Plischuk and Lange, 2009; Plischuk et al., 2011; Szabo et al., 2012; Arbetman et al., 2013; Maharramov et al., 2013; Schmid-Hempel et al., 2014).

One of the major reasons why commercially produced bumblebee colonies continue to carry parasites is that the colonies are reared on pollen collected from honey bees (Goulson and Hughes, 2015). Honey bee pollen is often contaminated with a diversity of bee parasites, both of honey bees and bumblebees (Singh et al., 2010; Graystock et al., 2013b), which may be because the honey bees themselves were diseased or because the flowers they visited have been contaminated by previous pollinator visits (Graystock et al., 2015). Feeding commercially produced bumblebees with pollen contaminated with bumblebee parasites is problematic enough, but there is also growing evidence that some of the honey bee parasites found in pollen can infect bumblebees too, notably *N. ceranae* and deformed wing virus (Graystock et al., 2013a; Fürst et al., 2014; Meeus et al., 2014a; Manley et al., 2015; McMahon et al., 2015). There are two solutions to the problem of rearing commercially produced bumblebees on parasite-contaminated pollen food: (1) replace the pollen with a hygienic, artificial pollen substitute, or (2) sterilise the pollen in some way to kill any parasites that it contains. To date, there is no commercially available artificial pollen substitute for rearing bumblebees over multiple generations, and the challenge for sterilising pollen is developing a method which is effective at killing all parasites without negatively affecting the nutritional value of the pollen for the bees.

Irradiation and ozone (O_3) treatment are two methods commonly used to kill microbes on food for human consumption that have been considered for sterilising pollen, with gamma irradiation having been shown to reduce the viability of the Israeli acute paralysis virus in pollen (Yook et al., 1998; Meeus et al., 2014b). At least one major producer of bumblebees (Biobest) now exclusively uses irradiated pollen in its factories. However, the effectiveness of irradiation against the full diversity of bee parasites that can be present in pollen is not known. Here we examine the effectiveness of gamma irradiation, as well as an experimental method of ozone treatment and an artificial pollen substitute, for providing parasite-free food for bumblebees.

2. Materials and methods

In order to compare the effectiveness of pollen sterilisation methods, our experiment tested six treatments: (1) irradiated, fresh pollen (processed for the experiment immediately upon receipt from the pollen supplier), (2) ozone-treated, fresh pollen, (3) untreated, fresh pollen, (4) untreated pollen that had been stored frozen for >2 years, or (5) Nutri-bombus artificial pollen substitute

control, in each case made up as 40% w/v suspensions in sucrose solution, or (6) sterile 40% sucrose solution control. The pollen for Treatments 1, 2 and 3 was provided by Biobest. While Biobest only uses irradiated pollen on its premises, the pollen for these treatments was taken at delivery from a batch before it was irradiated. As a consequence, Treatments 1, 2, and 3 came from the same batch of pollen, allowing direct comparisons between the treatments. Pollen for Treatment 1 underwent gamma irradiation of 16.9 kGy in the GAMMIR irradiation cell of Sterigenics (Fleurus, Belgium), while pollen for Treatment 2 received ozone (O_3) treatment from an external contractor under a nondisclosure agreement; both methods are used to remove to kill microbes on human food and have previously been shown to have potential for sterilising pollen (Yook et al., 1998; Meeus et al., 2014b). The pollen for Treatment 4 was purchased from a major distributor of commercially produced bumblebee colonies, had been dehydrated and stored for at least two years, and was hard and grainy compared to the fresh pollen, which was soft and fluffy in texture. The Nutri-bombus pollen substitute is a new experimental diet for bumblebees that was developed and provided by Nutrifeed Canada Inc. Four samples of pollen from the same batch provided by the supplier were collected randomly for each treatment and checked by PCR or RT-PCR (see below) for the presence of 13 parasites: *C. bombi*, *A. bombi*, *N. bombi*, *N. apis*, *N. ceranae*, deformed wing virus (DWV), Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV), black queen cell virus (BQCV), sacbrood virus (SBV), *Ascosphaera* fungi, American foulbrood and European foulbrood bacteria.

The experiment used 15 *Bombus terrestris terrestris* colonies that were provided by Biobest. Colonies were queenright, each with ~120 workers, and appeared in good health. Initially, 16 workers from each colony were screened for disease using PCR (see below), representing about 13% of the colony population. This screening identified four colonies that appeared to be uninfected by any parasites and which were selected for use in the experiment. Three of these colonies were confirmed by the additional bees screened during the experiment to have been genuinely free of parasite infections (see Section 3). However, one of the four 'uninfected' colonies (Colony 3) was subsequently found to have low prevalence (<10%) infections with *A. bombi* and *N. ceranae*, and the experimental results were therefore analysed both including and excluding this colony (see Section 3). Each of the six treatments was fed to 64 bees from the four selected colonies (16 bees per colony). For this, bees were initially starved for 8 h, then placed individually into an Eppendorf tube with a small hole at the end through which they were hand-fed a 5 µl dose of the treatment. All treatment solutions were thoroughly vortexed immediately before use to ensure pollen or Nutri-bombus particles were fully in suspension. The bees were then placed in groups of 8 like-treated nestmates in 10 × 6 × 6 cm plastic boxes, provided with 40% sucrose solution *ad libitum*, and their survival checked daily for 14 days. Any bees that died during the experiment were stored at −80 °C. All of the experimental bees which survived at the end of 14 day period, as well as all bees which died during the experiment, were screened by PCR or RT-PCR for seven parasites that infect adult bees: *C. bombi*, *A. bombi*, *N. bombi*, *N. apis*, *N. ceranae*, DWV and IAPV.

2.1. Parasite screening

In order to check for the presence of parasites in the pollen used for the experiment, four samples of each of the six treatments were screened prior to the experiment for the parasites *A. bombi*, *C. bombi*, *N. bombi*, *N. ceranae*, *N. apis*, *Ascosphaera*, American foulbrood and European foulbrood using conventional PCR, and for the DWV, IAPV, Kashmir bee virus, black queen cell virus, and sacbrood virus using RT-PCR. In order to check whether the bumble-

bees used for the experiment were free of infections at the start of the experiment, a set of 16 randomly selected workers from each bumblebee colony was screened for the parasites which can affect adult bees: *A. bombi*, *C. bombi*, *N. bombi*, *N. ceranae*, *N. apis*, DWV and IAPV. All bumblebees used for the experiment were also screened for these parasites when the experiment ended. Pollen samples (0.6 g) were homogenised in TRIS buffer for 2 min with 0.1 mm zirconia/silica beads in a Qiagen TissueLyser. For each bumblebee, the 5th and 6th tergites were removed and a ca. 0.2 cm³ sample of the hind gut, fat body and malpighian tubules was dissected out and homogenised with a micropestle. DNA and RNA were extracted using TRIzol (Invitrogen), which is effective at extracting viral RNA as well as the DNA of the other parasites (Rekand et al., 2003; Rudenko et al., 2004; Evison et al., 2012; Graystock et al., 2013b). Reagent mixes and PCR conditions for *A. bombi*, *C. bombi*, *N. bombi*, *N. apis*, *N. ceranae*, *Ascosphaera*, American foulbrood and European foulbrood were as described previously (Graystock et al., 2013b, 2014; Table S1). We used general primers for *Ascosphaera* fungi because these parasites can show host species specificity and the identity of *Ascosphaera* species that infect bumblebees is unknown. To screen for RNA viruses, cDNA was synthesised using random hexamers and M-MLV reverse transcriptase (Cox-Foster et al., 2007). Amplification of cDNA was then carried out using primers specific to DWV, IAPV, BQCV and SBV (Singh et al., 2010; Table S1). PCR products were visualised on a 1% agarose gel stained with ethidium bromide. The *18S Apidae* control gene was amplified for all bumblebee samples to confirm quality of the extractions (Meeus et al., 2010). All assays included both negative and positive controls.

2.2. Fat analysis

In order to examine whether the health of bumblebees may affect their susceptibility to infection, we measured fat content of bees by calculating their lipid content relative to their body size (Brown et al., 2000). This served as a proxy measure of health, given that no clear measure of bumblebee health is available. This was done for all of the experimental bumblebees, both those that died during the experiment and those that survived to the end of the 14 day experimental period. The abdomen of each bumblebee (minus two tergites that had been removed for parasite screening) was dried at 70 °C for 5 days, weighed, and then placed in diethyl ether for 24 h to dissolve lipids. The abdomens were then rinsed in fresh diethyl ether before being dried at 70 °C for a further 5 days and then reweighed. The difference between the first and second weight measurements was taken as the fat content of the bumblebee, and divided by the dry weight of the bee to obtain the fat content relative to body size for each bumblebee.

2.3. Statistical analyses

The diversity of parasites (number of parasite types) found in the pollen samples was compared between the treatments using a generalized linear model (GLM) with Poisson distribution, log link function and the χ^2 test statistic. The survival of bees in the six treatments was examined overall using a Cox proportional-hazards regression model, with fat content of bees included as a covariate. Survival was then compared pairwise between treatments with Kaplan-Meier tests and the Breslow statistic. The number of experimental bees that were subsequently found to be infected with *A. bombi* were compared between treatments using a generalized linear mixed model (GLMM) with a binomial distribution and negative log-log link function, with colony-of-origin, and box nested within treatment, included as random factors to account for the structured nature of the data. The GLMM was carried out first with all data and then repeated excluding Colony 3,

which was discovered after the experiment began to have low level infections of *A. bombi* and *N. ceranae*. Bees that died within the first three days of the experiment, and for which parasites detected might represent the experimental dose, were excluded in both cases. The relationship between the fat content of bumblebees and their day-of-death was examined with a Spearman's Rank correlation. The fat content of bumblebees was also examined in a GLMM with a gamma distribution and log link function, with treatment as a fixed factor, colony-of-origin as a random factor, and day-of-death and infection status for each of the parasites included as covariates. In all models, non-significant interaction terms were removed stepwise based on AIC values to obtain the minimum adequate models. All analyses were carried out in SPSS 22.0 (IBM Corporation).

3. Results

No parasites were detected in the sucrose or Nutri-bombus controls, and none of the American foulbrood, European foulbrood, *N. bombi* or KBV parasites were detected in any of the pollen samples. DNA/RNA of the other parasites was detected in the pollen samples to varying extents, with *A. bombi* and *Ascosphaera* fungi being particularly common (Fig. 1). The diversity of parasites in the pollen samples did not differ significantly between the treatments ($\chi^2 = 7.32$, df = 3, $P = 0.062$), although with only four samples of
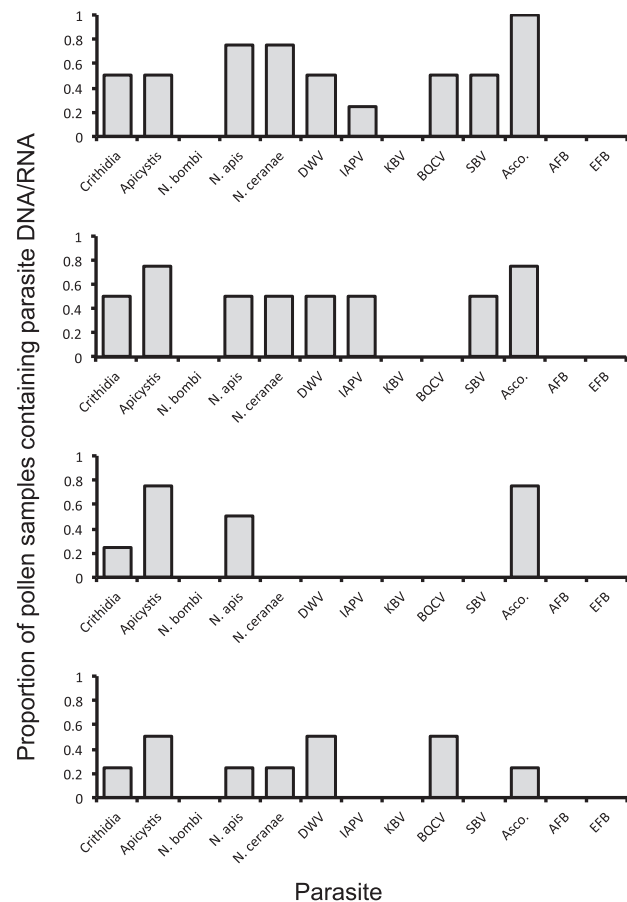

Fig. 1. Proportions of samples of fresh pollen that had been gamma irradiated, ozone-treated, or untreated, or untreated pollen that had been stored for >2 years, which were positive for the microbial parasites *C. bombi*, *A. bombi*, *N. bombi*, *N. apis*, *N. ceranae*, deformed wing virus, Israeli acute paralysis virus, Kasmir bee virus, black queen cell virus, sacbrood virus, *Ascosphaera* chalkbrood fungus, American foulbrood or European foulbrood bacteria. The irradiated, ozone-treated and untreated, fresh pollen samples all came from the same batch of pollen, while the untreated stored pollen came from a different batch.

each treatment, this test had limited statistical power. Overall, parasite DNA/RNA was detected approximately half as frequently in the irradiated or ozone-treated pollen as in the untreated pollen (Fig. 1).

There was a significant effect of treatment on the survival of bumblebees ($\chi^2 = 13.1$, $P = 0.023$), and no effect on survival of fat content or colony-of-origin ($\chi^2 = 1.91$, $P = 0.167$, and $\chi^2 = 4.26$, $P = 0.235$, respectively). Bees exposed to sucrose control or irradiated pollen survived well, those fed either Nutri-bombus pollen substitute, ozone-treated pollen, or untreated, fresh pollen survived slightly less well, and bees fed the untreated pollen that had been stored for >2 years survived worse (Fig. 2a). Bumblebees fed fresh, untreated pollen or irradiated pollen had about 10% higher fat contents than bees fed the other diets (Fig. 3a), but there was substantial variation between individuals and no significant effect of treatment on the fat content of the bumblebees when controlling for day-of-death and parasite infection status ($F_{5,324} = 2.02$, $P = 0.075$). There were also no significant effects of colony-of-origin or parasite infection status on the fat content of bumblebees ($P > 0.05$ in all cases). However, there was a significant relationship between day-of-death and the relative fat content of bumblebees, with individuals that survived longer tending to have a higher fat content ($\rho = 0.116$, P = 0.023; Fig. 3b).

Detection of parasites in bees that died within the first three days of the experiment could reflect the initial dose, so these bees were excluded from the analyses. No DNA/RNA of *C. bombi*, *N. bombi*, *N. apis*, or IAPV was detected in any bees post-exposure, and only one case was detected of DWV (in a bee fed the untreated, stored pollen). However, up to 30% of the experimental bees fed untreated or ozone-treated pollen were subsequently found to be infected with *A. bombi*, while infection rates for *A. bombi* in the other treatments were <10% (Fig. 2b). Up to 12% of bees fed untreated or ozone-treated pollen were subsequently found to be infected with *N. ceranae*, while infection rates for the other treatments were <5%. Colony 3 carried infections of these parasites before the experiment and so conclusions from these bees need to be treated with caution (the lack of infections in sucrose control and Nutri-bombus control bees from Colonies 1, 2 and 4 confirm that these colonies were genuinely free of infections). Excluding Colony 3, no infections of any parasites were found in bees fed the sucrose control or Nutri-bombus control. Low prevalence infections of *N. ceranae* were detected in bees fed the untreated, fresh pollen, irradiated pollen or ozone-treated pollen (ca. 3%), and a higher prevalence infection was detected in bees fed the untreated, stored pollen (11%). There was a significant effect of treatment on the number of bees that were found to be infected with *A. bombi*, either including or excluding Colony 3 ($F_{5,337} = 5.48$, $P < 0.001$, and $F_{5,248} = 6.16$, $P < 0.001$, respectively). There was a relatively high prevalence of *A. bombi* in bees fed the untreated pollen (stored or fresh) or ozone-treated pollen, either including or excluding Colony 3 (Fig. 2b, c). Infections in bees fed the irradiated pollen were relatively low either including or excluding Colony 3, though still greater than the equivalent figures of 0% for bees fed the Nutri-bombus and sucrose solution controls.

4. Discussion

The honey bee pollen tested here contained DNA indicating the presence of a diversity of bee parasites, at least some of which were infectious. Treatment with gamma irradiation reduced substantially the infectivity of parasites in pollen, while the artificial pollen substitute tested was confirmed to be entirely free of parasites. The fat store of bees was positively correlated with their susceptibility to infection, but was not affected by the irradiation of the pollen they were fed.
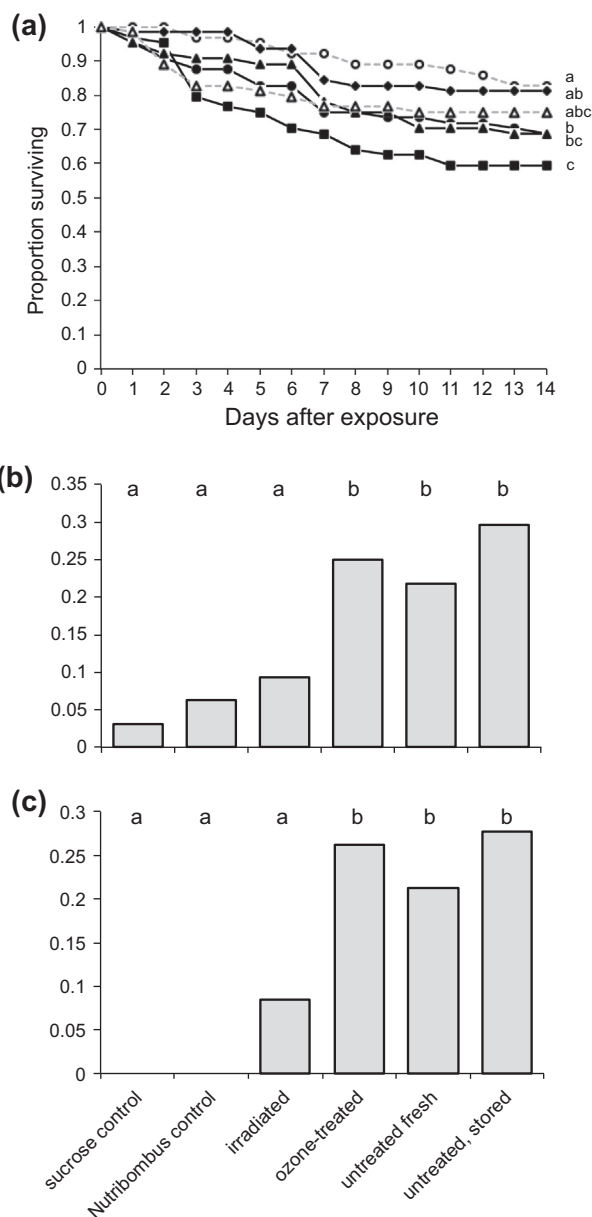

Fig. 2. (a) Survival of *Bombus terrestris* bumblebee workers after ingestion of either sucrose solution control (grey dashed line, white circles), sucrose solution containing Nutri-bombus artificial pollen substitute (grey dashed line, white triangles), or sucrose solution containing fresh honeybee pollen that had been irradiated (black diamonds), ozone-treated (black triangles), or untreated (black circles), or untreated pollen that had been stored (black squares). Different letters beside lines indicate treatments that differed from one another at $P < 0.05$. (b) The proportion of the bumblebees workers from all four colonies which were subsequently found to be infected with the *Apicystis bombi* parasite, and (c) the proportion of bees infected with *A. bombi* excluding Colony 3 (which carried infections prior to the experiment; bees which died during the first three days of the experiment are excluded from both graphs). Different letters above the columns indicate treatments that differed significantly from one another at P < 0.05. The irradiated, ozone-treated and untreated, fresh pollen samples all came from the same batch of pollen, while the untreated stored pollen came from a different batch. Bumblebees came from four Biobest colonies, with n = 64 bumblebees tested for each treatment.

The parasites detected in pollen included one that has as yet only been recorded infecting honey bees (the microsporidian *N. apis*), one which infects only bumblebees (the trypanosome *C. bombi*), and several which may be capable of infecting both honey bees and bumblebees (*A. bombi*, *N. ceranae*, DWV, IAPV, SBV, BQCV and *Ascosphaera* fungi). These results are in keeping
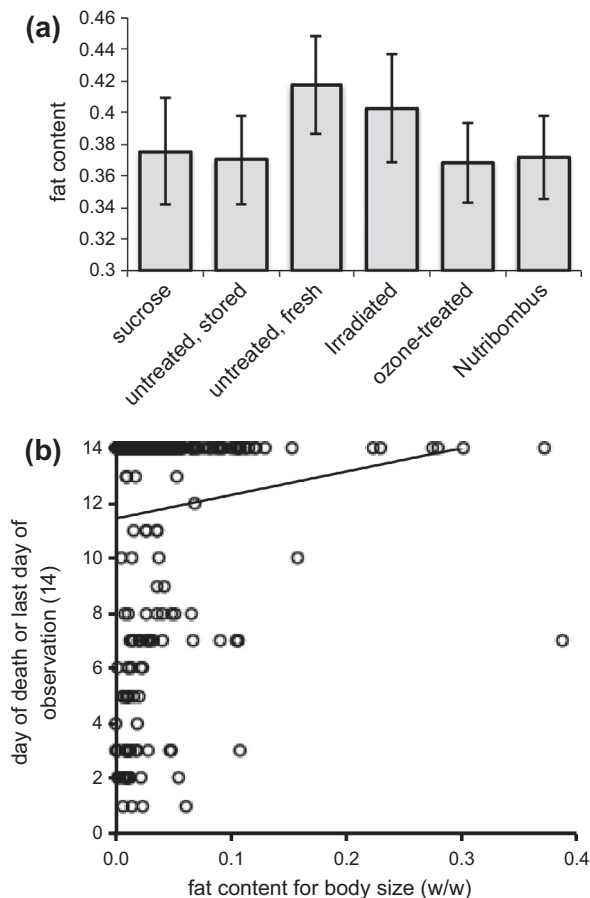
Fig. 3. (a) Mean ± s.e. fat content of bumblebees fed either sucrose solution control, or sucrose solution containing untreated pollen which had been stored, untreated pollen which was fresh, irradiated fresh pollen, ozone-treated fresh pollen, or Nutri-bombus pollen substitute. (b) The relationship between the fat content of bumblebees and their day of death or the last day of observation if they survived for the duration of the 14 day experiment. The equation of the line of best fit is $y = 8.53x + 11.5$, $r^2 = 0.011$. In both graphs, the fat content of bumblebees is relative to their body size (w/w).

with previous work that has also reported the presence of a diversity of viable bee parasites in honey bee pollen (Singh et al., 2010; Graystock et al., 2013b). The high prevalence of bee parasites in pollen is not surprising given the way pollen from multiple flowers is mixed together and incorporated with bee saliva during processing by bees. Many flowers are shared between bumblebees and honey bees, as well as other pollinating insects, and it is likely that flowers are hot spots for parasite transmission (Durrer and Schmid-Hempel, 1994; McArt et al., 2014; Graystock et al., 2015).

Given the prevalence of parasites in honey bee pollen, it is therefore imperative to either sterilise or, when an alternative becomes commercially available, replace this food in the commercial production of bumblebee colonies. Our results suggest that irradiation is of potential value for sterilisation. Gamma irradiation appeared to reduce the prevalence of parasite DNA/RNA in the pollen, with only four parasites still being detected. Deformed wing virus, *N. ceranae*, Israeli acute paralysis virus, and sacbrood virus were all removed by the irradiation treatment, presumably due to irradiation killing the parasites and degrading their DNA/RNA. Of those parasites that remained, one is not able to infect bumblebees (*N. apis*), and two others were also not infectious in our experiment (*C. bombi* and *Ascosphaera*). *A. bombi* remained infectious after irradiation, with infections of this reduced by about half by irradiation of the pollen. Ozone treatment of the pollen was less effective and did not significantly reduce the infectivity of the *A.*

bombi parasite. The sterilising benefit of irradiating pollen is in agreement with previous work reporting that it reduced infective IAPV in pollen (Meeus et al., 2014b), but extends its benefits to activity against a broad range of bee parasites. Importantly, however, the irradiation treatment was not completely effective, with the *A. bombi* parasites that irradiated pollen contained still being able to infect some bumblebees. The results therefore indicate that further optimisation of the irradiation treatment may be needed to achieve complete sterilisation. One concern about irradiation, or other sterilisation methods, is that it may reduce the nutritional value of pollen to bees. Pollen is primarily important as a food for bee larvae so future work should confirm whether there may be any negative nutritional effects of irradiating pollen fed to the larval stage, as well as whether there are any effects on adult bees when feeding is carried out over a long time period. However, the limited results here on nutrition support Biobest data over four years that there are no negative effects on bumblebee colony production of using irradiated pollen compared to unirradiated pollen (FW unpubl. data).

An unexpected result was that the pollen samples obtained from an external source (which is different from the pollen used by Biobest for bumblebee production) carried more parasites, caused higher bumblebee mortality, and more infections with the *A. bombi* and *N. ceranae* parasites, than the fresh pollen obtained directly from Biobest. This most likely reflects differences in parasite prevalence between honey bee pollen obtained in different years or from different apiculturists. The stored pollen was dehydrated, hard and grainy, whereas the fresh Biobest pollen was soft and fluffy, so the differences in bumblebee survival may also reflect stored pollen being of lower nutritional value. Pollen composition can also be important for the resistance of bees to parasites (Foley et al., 2012), so this may be another reason why bees fed the stored pollen suffered more from disease. As a result of these findings, this pollen supplier has withdrawn the stored pollen from sale and replaced it with fresh, irradiated pollen supplied to them by Biobest.

The results both highlight the risk posed to bees by feeding them with pollen sourced from honey bees, and demonstrate that gamma irradiation of pollen can be effective at reducing this risk. Although no significant benefit was seen here from ozone treatment, it may nevertheless be worth further exploration to determine if it can be optimised to deliver complementary benefits alongside irradiation. The production of parasite-free bumblebees is in the interests of all parties, including conservationists, commercial producers of bumblebees, and the farmers who use bumblebees for crop pollination. Although the results suggest it may not be a complete solution, at least in current protocols, the irradiation of pollen used to feed bumblebees should therefore be encouraged in order to enhance the quality of the bumblebee colonies produced and reduce the parasite spillover threat they may otherwise pose to wild and managed bees.

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.2016.03.007.

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