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Nutritional limitation and resistance to opportunistic *Aspergillus* parasites in honey bee larvae

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ABSTRACT

Honey bees are threatened by land use changes which reduce the availability and diversity of pollen and nectar resources. There is concern that poor nutrition may be involved in recent population declines, either directly or due to indirect effects on immunocompetence. The larval stage is likely to be the most vulnerable to a poor diet, but the effects of larval nutrition on the disease susceptibility of bees are not well known. In this study we used laboratory-reared honey bee larvae to investigate the effects of diet quality on disease susceptibility to the opportunistic fungal parasites *Aspergillus flavus, Aspergillus phoenicis* and *A. fumigatus.* Larvae fed on a nutritionally poor diet were found to be significantly more susceptible to *A. fumigatus*. Larval resistance to *A. fumigatus* was enhanced by feeding with a diet supplemented with either dandelion or polyfloral pollens. This indicates that dandelion and polyfloral pollens contain elements that enhance resistance to this fungal disease, illustrating an interaction between nutrition and parasitism and emphasising the benefit of diverse floral resources in the environment to maintain honey bee health.

1. Introduction

Honey bee (*Apis mellifera*) health is a major current concern because of the substantial colony losses experienced in some countries in recent years and the important role honey bees play in pollination and food production (Breeze et al., 2011; Evans and Schwarz, 2011; vanEngelsdorp and Meixner, 2010; Williams et al., 2010). Many hypotheses have been proposed to explain these serious population declines and it has become increasingly apparent that multiple factors, possibly acting in synergy, are involved (vanEngelsdorp et al., 2009). In particular, it has been suggested that nutritional limitation due to poor diet could result in immunocompromised individuals that are more susceptible when exposed to pathogens (Naug, 2009). Modern intensive agricultural practices, characterised by monocultures and simplification in crop rotations, can provide abundant supplies of pollen and nectar when in bloom, but limited resources at other times due to a lack of continuity in the flowering phenology of crops (Decourtye et al., 2010). Moreover, habitat fragmentation and loss of buffer zones of wild and semi-wild habitats in intensively farmed areas not only leave bee colonies short of food resources during times of dearth, but also lack the natural forage diversity that may be required for optimum nutrition (Kremen et al., 2002).

Activation of the immune system is energetically costly and nutritionally limited organisms are less able to meet this cost, making them more susceptible to parasite infections (Coop and Kyriazakis, 1999; Moret and Schmid-Hempel, 2000). In addition, parasites compete directly with the host for their own nutritional requirements and nutritionally deprived hosts may be less able to tolerate this added stress (Thompson and Redak, 2008). The microsporidian parasite *Nosema ceranae* has been confirmed to impose an energetic stress on adult honey bees, increasing either hunger levels or susceptibility to disease in bees with a lower sugar intake (Mayack and Naug, 2009). Diets may also provide particular elements which may be essential for immune responses and/or the detoxification of xenobiotics (Johnson et al., 2012). Excess nutrients and energy reserves are stored in the insect fat body which are mobilized in response to the energy demands of other tissues. This storage function is essential in larval stages to ensure the survival of starvation periods and for development during metamorphosis (Arrese and Soulages, 2010). During many types of immune response, the fat body cells release proteins including antimicrobial peptides (AMP's) and lysozymes into the haemolymph (Bulet et al., 1999; Cheon et al., 2006). Phagocytosis and encapsulation are the most well known immune defenses of bees against fungal parasites (Gliñski and Buczek, 2003), but there can be upregulation of a variety of transcripts including a chitinase-like enzyme, serine protease, lysozymes and AMP's abaecin and defensin (Aronstein et al., 2010). Immune responses to fungi and

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bacteria were also associated with a downregulation of major storage proteins vitellinogen and hexamerins, suggesting a trade-off between immune responses, and the biosynthesis and accumulation of these proteins (Aronstein et al., 2010; Lourenço et al., 2009). The immune effects of poor nutrition in honey bee larvae have not been well studied yet it is this stage that may be most vulnerable. During temporary food shortages colonies tend to regulate brood rearing rather than rear malnourished pupae (Imdorf et al., 1998). However, under experimental conditions, impairments in development have been reported (Brodshneider et al., 2009; Mattila and Otis, 2006), and sub-lethal effects can persist into the adult stage (Hoover et al., 2006).

Pollen provides the main source of dietary proteins, lipids, sterols, vitamins and minerals for honey bees (Crailshem, 1990). The pollen from different floral sources has different nutritional value for bees with most pollen analysis studies focusing on the protein content (DeGroot, 1953; Roulston and Cane, 2000). The nutritive value of different pollen types has been assessed using various physiological and productivity parameters such as brood rearing capacity (DeGrandi-Hoffman et al., 2008; Keller et al., 2005; Loper and Berdel, 1980; Mattila and Otis, 2007), lifespan (Maurizio, 1954), hypopharyngeal gland development (DeGrandi-Hoffman et al., 2010; Maurizio, 1954; Standifer, 1967) and fat body growth and development (Maurizio, 1954). Using colony reared larvae, Rinderer et al. (1974) reported decreased mortality caused by American foulbrood *Paenibacillus larvae* exposure when the diet was supplemented with bee collected pollen from a variety of floral sources. More recently, pollen and protein supplement fed caged, adult bees yielded lower Deformed Wing Virus titres in comparison to sugar syrup fed bees, indicating a vital role for dietary protein in immune responses of honey bees (DeGrandi-Hoffman, 2010). Diet quality affected by pollen diversity, rather than protein content, has been shown to exert immunocompetence effects in adult honey bees (Alaux et al., 2010). Yet the significance of pollen diversity in the larval diet has not previously been studied.

Here, we investigated under controlled, laboratory conditions whether nutritional limitation affects the susceptibility of honey bee brood to the Ascomycetous fungi from the genus *Aspergillus*, the causative agents of aspergillosis or stonebrood disease in bees. As our model parasites we used *A. flavus, Aspergillus phoenicis* and *Aspergillus fumigatus*. These fungi are ubiquitous, opportunistic parasites that have been little studied in honey bees, but which in other animal host species such as dogs, horses and birds, generally require immunocompromised individuals for successful infection (Tell, 2005). We monitored the survival of exposed and unexposed larvae fed diets varying in either (1) the amount of royal jelly and sugars or (2) the origin and diversity (monofloral versus polyfloral) of pollen to test how diet quality affects the ability of larvae to resist exposure to stonebrood parasites.

2. Materials and methods

We used larvae from a total of nine healthy colonies of honey bees *Apis mellifera carnica* of similar size and headed by one year old unrelated queens. One to two day old larvae (age estimated by size) were grafted into sterile 48-well tissue culture plates using a Swiss grafting tool. During the grafting process, plates and diet were warmed, approximately to hive temperature (32–34 °C), and a moist tissue was placed between each filled plate and its lid to keep the larvae in a relatively high humid atmosphere. Plates were then transferred into an incubator at 34 °C, 90% relative humidity and a 24 h dark cycle. The larvae were fed *ad libitum* with either a standard diet which consisted of 50% of royal jelly (RJ) (v/v) (Apitherapy, Norfolk, UK), 6% D-fructose (w/v), 6% D-glucose (w/v) and distilled water (Jensen et al., 2009), or with modified diets according to the experiment (see Sections 2.3. and 2.4.).

2.1. Aspergillus species identification

The *Aspergillus* isolates used were collected from naturally-exposed adult worker bees and larvae in our experimental apiary (West Yorkshire, UK) and cultured on malt extract agar (MEA) plates at 30 °C until the fungi produced conidia. The plates were then stored at 4 °C until use. To extract the fungal DNA approximately 0.05 g of the fungal conidia were added to 200 μl 5% Chelex solution (in 10 mM Tris buffer) and 0.05 g of 0.1 mm Zircona/Silica beads and placed in a QIAGEN Tissue Lyser beadbeater for 4 min at 50 oscillations/s. Samples were then incubated in a 90 °C water bath for 20 min then centrifuged for 30 min at 8 °C. The supernatant was cleaned with OneStep-96 PCR Inhibitor Removal Kit (Zymo Research) prior to PCR. Species identification of the *A. flavus* and *A. fumigatus* isolates was carried out by sequencing 547 and 459 bp long fragments from the internal transcribed spacer regions 1 and 2 respectively (Henry et al., 2000). BLASTn searches produced a 100% maximum identity match with *A. flavus* (GenBank ID: <GU172440.1>) and 96% maximum identity with *A. fumigatus* (GenBank ID: <JN216834.1>). *A. phoenicis* was identified by sequencing a 694 bp long fragment of the calmodulin gene (O'Donnell et al., 2000) producing a 99% maximum identity match with *A. phoenicis* (GenBank ID: <JF838353.1>).

2.2. Parasite treatments

Conidia of *A. flavus, A. fumigatus* or *A. phoenicis* were harvested from MEA plates, suspended in 2 ml of sterile water and vortexed for 15 s. The conidia were counted with a 0.001 ml Nebauer hemocytometer and the conidia concentrations adjusted to 2×10^6 conidia/ml. To test for conidia viability, 40 μl of the conidia solution was added to 200 μl GLEN liquid medium which is suitable for the germination of entomopathogenic fungi (Beauvais and Latgé, 1988), and vortexed for 5 s. 15 μl of this solution was pipetted into six of the 6 mm spots on sterile Teflon coated slides and placed into sterile petri dishes with moist filter paper. One slide was prepared per species. These were incubated for 18–24 h at 30 °C after which time the proportion of germinated conidia per species was calculated and the conidia solutions, which contained >90% viable conidia, were considered as suitable for use. Once they had been adjusted to account for the percentage of non-viable conidia, *A. fumigatus* and *A. phoenicis* solutions of 5×10^6 conidia/ml and an *A. flavus* solution of 1×10^3 conidia/ml were prepared. These were concentrations which had previously been determined in a doses response procedure as LD_{50} doses (unpublished data).

Larvae were exposed to 5 μl of each parasite treatment by pipetting the solution onto the food near the mouthparts. Larvae were not fed again for another 24 h when all the food in the wells had been ingested to ensure thorough intake of the administered parasite treatment. Following exposure, larvae were maintained for 7 days and examined daily under microscope for mortality (i.e. no movement) and signs of stonebrood infection (i.e. hyphae and conidia on the cuticle).

2.3. Experiment 1 – variation in royal jelly and sugars concentrations

We used a total of 768 larvae, from four colonies. Four diets were tested: (1) standard diet: 50% royal jelly (v/v), 6% D-fructose (w/v), 6% D-glucose (w/v) and sterile water; (2) reduced RJ diet: 40% royal jelly (v/v), 6% D-fructose (w/v), 6% D-glucose (w/v) and sterile water; (3) reduced sugars diet: 50% royal jelly (v/v), 3% D-fructose (w/v), 3% D-glucose (w/v) and sterile water; (4) reduced RJ and reduced sugars diet: 40% royal jelly (v/v), 3% D-fructose

(w/v), 3% D-glucose (w/v) and sterile water. Larvae were treated with either *A. flavus*, *A. phoenicis*, *A. fumigatus* or a sterile water control (as described in Section 2.2), in a full factorial design. Forty-eight larvae per colony were tested with each diet-treatment combination.

2.4. Experiment 2 – variation in pollen origin and diversity

The three pollen species used were oilseed rape *Brassica napus*, dandelion *Taraxicum officianalis* and hawthorn *Crataegus monogyna*, which are among the species most commonly collected by honey bees in the UK during springtime (Keller et al., 2005). All pollen was collected in a local apiary from honey bee colonies using pollen traps. Supplementation was 1.25% (w/v), which approximated the quantity of pollen larvae naturally ingest during their development (Aupinel et al., 2005; Babendrier et al., 2004). Five diets were tested. As our control, pollen-free diet, we used the reduced RJ diet from Experiment 1, because of its reasonably high control survival and interaction with *A. fumigatus* exposure. The three monofloral diets consisted of this reduced RJ diet supplemented with pollen from oilseed rape, dandelion or hawthorn, and the polyfloral diet consisted of a mix of all three in equal measure. A total of 960 larvae, from five colonies, were treated with either *A. fumigatus* or sterile water control (as described in Section 2.2). Again, 48 larvae per colony were tested with each diet-treatment combination.

2.5. Statistical analysis

The survival of larvae was analysed using Cox proportional-hazards regression models, with treatment, diet and colony of origin, and interactions between these factors included in the models. Terms were removed in a stepwise manner to obtain the minimum adequate model which contained all main effects. When the diet factor was significant, the different diets were subsequently compared pair-wise using Kaplan–Meier analyses with the Breslow statistic. All analyses were carried out in SPSS 16.0.

3. Results

Daily observations showed only dead larvae had signs of infection which were either hyphae and/or conidia growing from the cuticle. No signs of infection were observed on larvae in the control treatments.

3.1. Experiment 1 – variation in royal jelly and sugars concentrations

There were no significant differences in the survival time of larvae between the experimental colonies, and no significant interactions between colony and either diet or parasite treatment ($P > 0.05$ in all cases). The survival of larvae was significantly different between parasite treatments (Wald = 39.2, $df = 3$, $P < 0.001$), being the highest for unexposed larvae (67.8%), the lowest for larvae exposed to *A. flavus* (37.0%), and intermediate for larvae exposed to *A. phoenicis* (49.5%) or *A. fumigatus* (55.2%) (Fig. 1). The survival of larvae was also significantly different between diets (Wald = 21.5, $df = 3$, $P < 0.001$), being generally higher for larvae fed the normal diet (62.0%) and lowest for larvae fed the diet with reduced RJ and reduced sugars (38.0%), and intermediate for larvae fed the reduced RJ diet (49.0%). There was a significant effect of diet on larvae exposed to *A. fumigatus* (Wald = 12.9, $df = 3$, $P = 0.005$), most larvae survived when fed the normal diet (77.1%), but survival was lower when fed either the diet with reduced royal jelly (43.8%) or with reduced royal jelly and reduced sugars (37.5%)

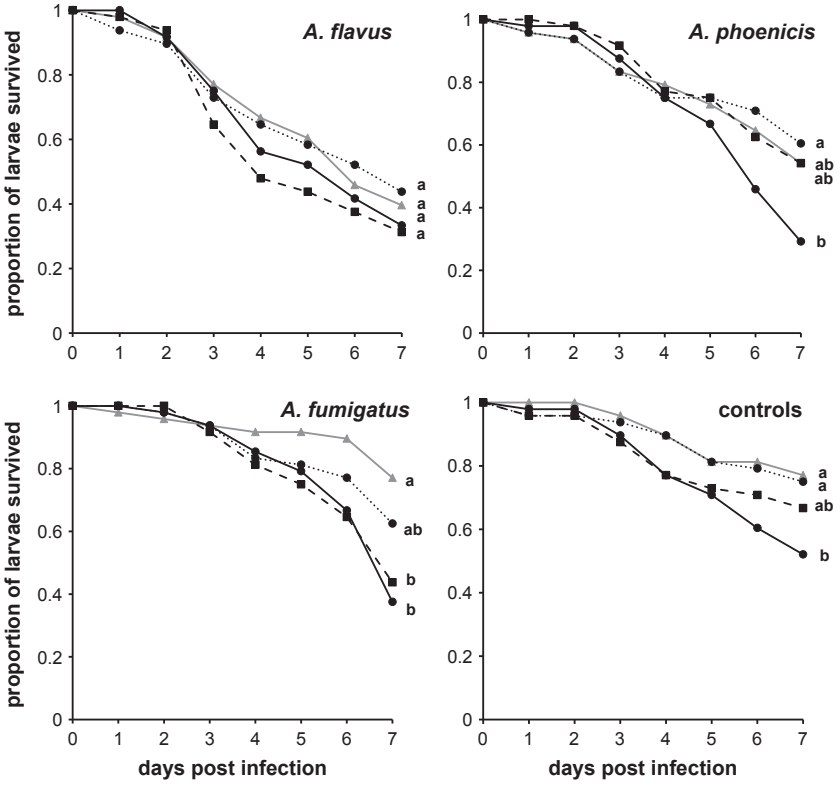

Fig. 1. Survival of laboratory-reared larvae exposed to either *A. flavus*, *A. phoenicis*, *A. fumigatus* or a control and fed with either the normal (gray triangles and solid lines), reduced royal jelly (black squares and dashed lines), reduced sugars (black circles and dotted lines) or reduced royal jelly and reduced sugars (black circles and solid lines) diets. Different letters indicate diets which resulted in significant differences in survival based on pairwise Kaplan–Meier tests ($P < 0.05$).

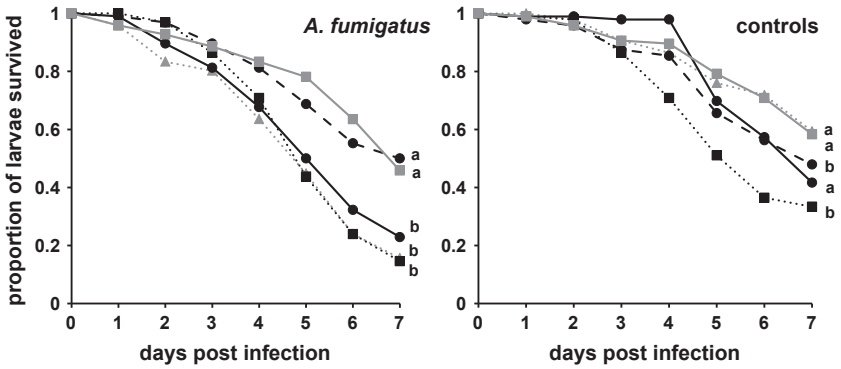

Fig. 2. Survival of laboratory-reared larvae that were exposed to either *A. fumigatus* or a control solution and fed either reduced royal jelly, pollen-free diet (gray triangles and dotted lines), or reduced royal jelly diet supplemented with pollen from oilseed rape (black squares and dotted lines), dandelion (black circles and dashed lines), hawthorn (black circles and solid lines) or all three (grey squares and solid lines). Different letters indicate diets which differed significantly from one another in pairwise Kaplan–Meir tests ($p < 0.05$).

(Fig. 1). There was in contrast no effect of diet on the survival of larvae exposed to *A. flavus* (Wald = 2.17, *df* = 3, *P* = 0.537), with survival being low in all cases (Fig. 1). The effect of diet was also not statistically significant for unexposed larvae (Wald = 7.45, *df* = 3, *P* = 0.059) or for larvae exposed to *A. phoenicis* (Wald = 6.96, *df* = 3, *P* = 0.073), although in both these cases there was a trend for low survival in larvae fed the diet with reduced royal jelly and reduced sugars (unexposed 52.1%; *A. phoenicis* 29.2%) suggesting that the diet induced increased larval mortality (Fig. 1).

3.2. Experiment 2 - variation in pollen origin and diversity

There was a significant interaction between diet and parasite treatment (Wald = 33.1, *df* = 4, *P* < 0.001; Fig. 2). The effect of *A. fumigatus* exposure on the survival of larvae varied depending on the diet, with larvae to *A. fumigatus* surviving worse than control larvae when fed the pollen-free diet (unexposed 59.4%; *A. fumigatus* 15.6%) (Wald = 33.8, *df* = 1, *P* < 0.001) and the hawthorn pollen diet (unexposed 41.7%; *A. fumigatus* 22.9%) (Wald = 31.3, *df* = 1, *P* < 0.001). Parasite treatment had no significant effect on the survival of larvae fed the dandelion (Wald = 0.6, *df* = 1, *p* = 0.440) and mixed pollen diets (Wald = 2.29, *df* = 1, *P* = 0.130). Larvae fed oilseed rape diet survived similarly poorly whether they were exposed to *A. fumigatus* or not (Wald = 2.75, *df* = 1, *P* = 0.097). There was no significant difference in survival between colonies (Wald = 5.3, *df* = 4, *P* = 0.254).

4. Discussion

Our results showed that nutritional limitation can significantly increase the susceptibility of honey bee larvae to the effects of exposure to the *A. fumigatus* fungal parasite, and that this effect was rectified if diet contained dandelion or polyfloral pollen. Pollen diversity has been shown to enhance the immunocompetence of adult bees (Alaux et al., 2010), and polyfloral pollen supplements have also been shown to confer resistance to *Bacillus larvae* infections in honey bee larvae (Rinderer et al., 1974). Our results show there are differences in the ameliorative potential of individual pollen species in nutritionally limited larvae on exposure to a ubiquitous, opportunistic fungal parasite.

The effect of diet on the survival rate of larvae depended on the parasite treatment. The most virulent parasite, *A. flavus*, caused high mortality irrespective of the diet on which larvae were fed. The apparent impact of nutritional limitation is therefore dependent upon the strength of the parasite challenge. In a wide variety of host-parasite systems a dose-dependent invasion threshold for successful parasite infection has been observed, which is related to parasite fitness and the effectiveness of the hosts defences (Ebert et al., 2000; Hughes et al., 2004; Regoes et al., 2002). *A. flavus* is the most frequent *Aspergillus* species reported to infect insects with its ability to produce pectinase and protease isoenzymes being implicated as important virulence factors (St. Leger et al., 2000). Moreover, *Aspergillus* produce a variety of mycotoxins including aflatoxins and ochratoxins which not only vary between species and strain of the fungus but also according to substrate (Medina et al., 2004). It is not known what role these mycotoxins play in the pathogenicity of stonebrood infections, but it is possible that larval mortalities were in part due to toxicity rather than fungal invasion.

Royal jelly is comprised of water (60–70%), protein (12–15%), carbohydrates (10–16%), lipids (3–7%), trace vitamins and mineral salts, and is known to contain potent antioxidants and antimicrobial compounds (Crane, 1990; Lercker et al., 1993). A reduction of 20% of these royal jelly components in the artificial larval diet made larvae more susceptible to *A. fumigatus* exposure, indicating that the nutrients are important for resisting infection or for detoxification purposes. In larvae not exposed to the parasite, a reduction of sugars as well as royal jelly produced low survival, which may suggest a threshold level for deficiencies in dietary sugars or the effects of a macronutrient imbalance (Human et al., 2007).

In Experiment 2, the survival of larvae in both the *A. fumigatus* and control treatments showed different effects of each pollen species in the diet. The positive effects of dandelion and polyfloral pollen diets are evident by the increased survival observed in larvae exposed to *A. fumigatus*. Whether the increased survival of larvae fed diet supplemented by polyfloral pollen is simply due to the presence of dandelion pollen, or due to the combination of pollens, is not clear. Roulston and Cane, (2000) reported protein concentrations for 377 species of floral pollen, yet few complete analyses are available for the composition of many bee collected pollens. Honey bees collect pollen that ranges from 12–61% in protein, 0.8–31.7% in lipid, and 21–48% in carbohydrate (corbicular pollen) content (Evans et al., 1991; Roulston and Cane, 2000; Todd and Bretherick, 1942). However, results may vary according to the extraction methods used. A total of 10 amino acids are regarded as essential to the diet of honey bees (DeGroot, 1953) and it may be the presence of these that determine the nutritive quality of the pollen species rather than the total protein content. Dandelion has been reported to contain 19.2% protein and 15.1% lipids, oilseed rape 31.9% protein and 25.4% lipids and hawthorn 26.19% protein and 2.81% lipids (Evans et al., 1991; Roulston and Cane 2000;

Zhang et al., 1999). Analyses have shown that relative to honey bee nutritional requirements, dandelion pollen possesses multiple amino acid deficiencies and is lacking tryptophan, phenylalanine and arginine (Loper and Cohen, 1987). Yet, it is also considered to be rich in lipids, with a high diversity of fatty acids, antioxidants and vitamins (Guo et al., 2009; Standifer, 1966). It has been noted that dandelion pollen is highly attractive to bees with high colony productivity observed in colonies situated in areas where dandelion bloom is abundant (Keller et al., 2005; Standifer, 1966). Nevertheless, the brood rearing capacity of colonies fed solely on dandelion pollen is severely diminished or absent in comparison to pollen from other floral sources with higher protein content (Loper and Cohen, 1987). This suggests that some other nutrient or element present in dandelion pollen is beneficial for brood rearing. Pollen fed naturally to honey bee larvae by nurse bees may be providing an important source of lipids, vitamins and antioxidants and could be providing essential components needed for general good health and disease resistance, as well as the macronutrients required for normal growth and development.

Oilseed rape is a widespread, intensively cultivated crop and provides an important pollen source for honey bees (Abrol, 2007). The increased mortality rates of larvae fed the oilseed rape pollen diet in both the *A. fumigatus* and control treatments is surprising as the pollen is of apparently high nutritional value to honey bees. One possible explanation could be that the pollen contains pesticide residues which were toxic to the larvae, with a lethal dose being present in the oilseed rape single pollen diet but not in the mixed pollen diet. Larvae fed on the hawthorn pollen showed higher mortality when exposed to *A. fumigatus* than uninfected larvae suggesting that this pollen species may lack the properties involved in supporting resistance, as observed with larvae fed the dandelion and polyfloral diets. Larvae are typically fed pollen in the form of bee bread rather than the corbicular pollen used in this study. The pollen is fermented by lactic acid bacteria produced in the honey stomach of forager bees which is thought may standardize the process (Vásquez and Olofsson, 2009). Enzymes produced by other microorganisms present e.g. molds, are involved in lipid, protein and carbohydrate metabolism which increase the digestibility and nutritive value of the pollen (Gilliam et al., 1989). However this process may not compensate for all the nutrients in which the raw pollen is lacking and honey bee colonies may not be able to compensate for an unbalanced diet in environments where foraging diversity is low leading ultimately to deficiencies in particular essential nutrients.

The results of this study show that rather than susceptibility of honey bee larvae to fungal parasites being affected by nutrition in a general way, different nutrients may be important for coping with different parasite species. It is possible that honey bee colonies may be able to compensate for food of limited quality by adjusting their brood production or by the fermentation of pollen into bee bread before feeding to larvae. However, the results nevertheless suggest that the impact of inadequate foraging resources in the environment on honey bee health can be significant and that nutritional limitation of larvae may be an important factor in colony losses. Schemes to enhance biodiversity on agricultural land include seed mixes to provide pollen resources for bees and further work would therefore be warranted to determine which pollen species will provide the best nutritional resources to help bees deal with the complexity of threats with which they are faced.

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