

# Microbes make the meal: oligolectic bees require microbes within their host pollen to thrive

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**Abstract.** 1. For solitary bees that specialise on select pollen types (oligoleges), larval development depends on the availability of forage pollen from appropriate host plants and the naturally occurring microbiota present therein. While access to host pollen may be critical for the development of oligolectic bees, the extent to which pollen microbiota contribute to their brood success is unknown.

2. To investigate, we used a diet manipulation experiment to rear larvae of the oligolege, *Osmia ribifloris*, under *in-vitro* conditions. Larvae were reared either on host pollen provisioned by their mother or on non-host pollen collected by honey bees, in the presence or absence of the respective pollen-associated microbiota. We assessed impacts on components of larval fitness: developmental time, biomass, and survivorship.

3. Our results revealed a significant interaction between pollen type and pollen-associated microbes. The relative effect of microbes on larval performance was substantially greater than that of pollen type. Host pollen substrate produced the fittest larvae but only when combined with its full complement of naturally occurring microbiota. In contrast, host pollen without microbes resulted in a marked decline in fitness components. Larvae consuming non-host pollen showed intermediate fitness, regardless of whether microbes were present or not.

4. These findings imply that the microbiota associated with maternally provisioned host pollen perform critical functions in larval nutrition and survival. For oligoleges in particular, the ability to develop on poorer quality host pollen likely derives from this sustained symbiosis with their microbial exosymbionts, rather than the biochemical characteristics of pollen type alone.

**Key words.** Bee fitness components, exosymbionts, microbes, oligolectic solitary bees, pollen specialization, symbiosis.

## Introduction

Managed and wild bees face increasing threats from multiple risk factors (e.g. pesticides, diseases, overexploitation, landscape fragmentation etc.), leading to unprecedented population declines among these critical pollinators (IPBES, 2016; Potts *et al.*, 2016). Habitat loss, agricultural expansion and intensification erode the amount and diversity of floral resources supporting bee health (Baude *et al.*, 2016; Kovács-Hostyánszki *et al.*, 2017), with pollen specialist bees typically being more

adversely affected (Bommarco *et al.*, 2010; Gill *et al.*, 2016). In addition to these well-investigated factors, recent research has revealed that the microorganisms associated with bees (collectively referred to as the bee microbiome) play critical roles in shaping the fitness outcomes of social and solitary bees (Lozo *et al.*, 2015; Engel *et al.*, 2016; McFrederick & Rehan, 2016; Kwong *et al.*, 2017).

Although the gut microbiome of adult honey bees performs critical nutritive and immune functions, that directly impact colony fitness (Kwong & Moran, 2016; Rubanov *et al.*, 2019), it appears to be insufficient to meet all the nutritional requirements of their larvae (Martinson *et al.*, 2012). Larval nutrition may instead depend on a lesser known aspect of bee-microbe

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symbioses, one which involves an external community of non-pathogenic microbes associated with larval diet (Gilliam *et al.*, 1990; Dharampal *et al.*, 2019). Specific microbial taxa within pollen provisions are responsible for the fermentation and preservation of raw pollen, production of vital dietary macromolecules, and defence against parasites and pathogens (Anderson *et al.*, 2011, 2014; Hoffman *et al.*, 2012). As a result of such broad-ranging services, these exosymbiotic microbes have been consistently linked to the health of adult and larval bees (Steffan *et al.*, 2017b; Donkersley *et al.*, 2018; Manirajan *et al.*, 2018). This tight partnership between bees and pollen-associated microbes may have greater implications for the larvae of solitary bees that have fewer opportunities of acquiring microbes through social interactions and brood care (Gilliam *et al.*, 1984).

Solitary bees make up the vast majority of all bee species, and are vital pollinators within commercial agricultural landscapes (Cardinal & Danforth, 2013; Garibaldi *et al.*, 2013). Many species of solitary bees are pollen specialists (oligolectic) and forage on select pollen types sourced from a narrow range of related host plants (Cane & Sipes, 2006). The nest of a solitary bee is provisioned by a single foraging female and contains several chambers that are stocked with a one-time supply of pollen, nectar, and sometimes oils (Bosch *et al.*, 2008; Danforth *et al.*, 2019). The pollen provisions also harbour a diverse and biologically important microbial community, often sourced from the environment and nest building material (Keller *et al.*, 2013). This external community includes nutritional mutualists that transform raw pollen into a nutrient-dense mixture of pre-digested pollen, nectar, and diverse microbes (Pimentel *et al.*, 2005; Cohen *et al.*, 2020). Microbes embedded within this detrital complex also serve as a major prey item for the larvae feeding on the fermented pollen provisions (Sgolastra *et al.*, 2015; Steffan *et al.*, 2017a, 2019; Steffan & Dharampal, 2018). For oligoleges that specialise on low-quality pollen, the rich profusion of pollen-borne microbes may be especially critical for the adequate nutrition of the developing larvae. Indeed, the absence of microbes from pollen provisions of oligolectic bees can severely compromise larval success and lead to brood failure (Dharampal *et al.*, 2019).

Forage pollen types utilised by oligoleges often lack key nutrients, and/or may be structurally and chemically protected, making them unfit to support the development of pollen generalist (polylectic) species (Sedivy *et al.*, 2011; Spear *et al.*, 2016). While this strategy allows oligoleges to avoid competition and natural enemies, the adaptive advantage is thought to have come at an evolutionary cost, with the absence of host pollen being directly linked to brood failure (Praz *et al.*, 2008). Thus, deficits in host plant abundance due to landscape alterations and agricultural intensification may mean reproductive failure for oligoleges (Steffan-Dewenter *et al.*, 2006; Arena & Sgolastra, 2014). Interestingly, a recent study revealed that despite being sourced from the appropriate host plants, provisions that lacked pollen microbiota were unable to provide adequate nutrition to support growth of oligolectic larvae (Dharampal *et al.*, 2019). This implies that the ability of oligolectic larvae to develop on low-quality host pollen likely stems from the sustained symbioses with the naturally occurring pollen microbiota, rather than access to host pollen alone. However, little is

known about the nature of the interaction between pollen type and pollen-borne microbes, and its implication for larval development.

In this study, we hypothesised that development of the oligoleges, *Osmia ribifloris*, would depend not only on the identity of forage pollen, but also on the presence of the natural microbiota within larval provisions. To investigate, we reared *O. ribifloris* larvae on host and non-host pollen, in the presence and absence of the respective pollen-associated microbes. We estimated the relative importance of pollen type and pollen-borne microbes by comparing the biomass, developmental time, and survivorship of oligolectic larvae across diet treatments.

## Materials and methods

### *Bees and pollen provisions*

Nesting reeds of *O. ribifloris sensu lato (s.l.)* were received in a single overnight shipment from a commercial supplier (NativeBees.com). All reeds were collected from a single location in Kaysville, Utah, in April 2017, where the nesting females foraged almost exclusively on the nectar-rich flowers of *Mahonia aquifolium* (Oregon grape) found within the region. Since *Osmia* sp. allocate smaller pollen provisions to the male offspring (Bosch, 1994), which leads to a gender bias in biomass, the eggs were sexed and only the male eggs were used in the study.

Male pollen provisions were individually weighed and then pooled into a single mass to reduce any potential bias arising from maternal provisioning and genetic relatedness. Half of this collected mass was sterilised to obtain sterile host pollen, while the remaining half represented natural (microbe-rich) host pollen. Non-host pollen was obtained by homogenizing commercially purchased organic honey bee pollen using a laboratory ball-mill. Half of the non-host pollen was sterilised to obtain sterile (microbe-deficient) non-host pollen, while the remaining untreated half represented natural non-host pollen. To prepare the sterile pollen provisions, pollen was freeze-dried, soaked in 95% ethanol, and dried under germicidal ultraviolet light in a biosafety cabinet overnight. Previous studies indicate that this method of sterilization does not alter pollen nutritional quality (Steffan *et al.*, 2017b; Dharampal *et al.*, 2019, 2020). Dry sterilised pollen was rehydrated based on the moisture content of maternally allocated provisions (~20%). sterile host pollen was rehydrated using sterile water, and sterile non-host pollen received an equal volume of 40% sterilised sugar solution to mimic the nectar content of maternally allocated provisions (Krunić *et al.*, 2005; Elliott *et al.*, 2008).

### *Pollen nutrient analysis*

Freshly harvested pollen provisions were randomly divided into two groups, placed in dram vials and one group was sterilised to serve as the control. Vials containing sterile host pollen provisions were capped tightly, whereas those with natural host pollen (control group) capped loosely. All vials were left undisturbed inside a biosafety cabinet under laboratory

conditions and sampled on day 1, 7, and 14 ( $n = 4$  for each time point for sterile host pollen,  $n = 5$  for each time point for natural host pollen) for amino acid analysis. Amino acid profiles were also determined for natural host pollen and natural non-host pollen ( $n = 4$  each). In addition, quantitative analysis of macronutrients was used to compare the nutritional profile of natural host pollen and sterile host pollen ( $n = 6$  each) and natural non-host pollen and sterile non-host pollen ( $n = 5$  each) (outsourced to University of Wisconsin, Marshfield).

**Protein extraction and alkaline hydrolysis.** Protein-bound amino acid content was compared between natural host pollen and natural non-host pollen. About 1 to 2 mg aliquots of pollen were weighed into microcentrifuge tubes and dusted with aluminium oxide to facilitate disruption of the pollen coat (Roulston *et al.*, 2000). Samples were manually ground for 1 min before 1 ml of 0.1 M NaOH was added. Pollen proteins were extracted at 4 °C for 48 h. Next, samples were centrifuged at 10 000g for 10 min and the supernatant was transferred to a new microcentrifuge tube and dried. Protein samples were then resuspended in 200 µl of 4 M NaOH, transferred to glass vials, and incubated at 90 °C for 18 h. The high pH and temperature disrupted the peptide bonds, freeing individual amino acids.

**Amino acid derivatization and gas chromatography–mass spectrometry analysis.** The protein hydrolysate was neutralised with 6 M HCl and an internal standard (DL-Norvaline) was added, before undergoing ethyl chloroformate derivatization. Anhydrous ethanol, ethyl chloroformate, and pyridine were added to the aqueous amino acid solution in a 60:32:8 ratio, producing N-(ethyloxycarbonyl) amino acid derivatives. The reaction was deemed complete when CO<sub>2</sub> emission ceased. Derivatized amino acids were then extracted in 100 µl of chromatography-grade chloroform and transferred to glass vials. Amino acid concentrations were determined by gas chromatography–mass spectrometry using a Thermo-Fisher instrument fitted with a DB-5 column. The oven was initially held at 40 °C for 2 min, before ramping to 130 °C at 15 °C min<sup>-1</sup>. The rate was decreased to 10 °C min<sup>-1</sup> between 130 and 240 °C, before being increased to 20 °C min<sup>-1</sup> until a final temperature of 320 °C was reached. The final temperature was held for 2 min. The mass spectrometer was operated in full scan mode, scanning between 50 and 600 m/z. Retention time windows associated with amino acid elution were identified by derivatizing and running a standard blend of amino acids (Sigma). A PARAFAC2 model was then applied to deconvolute these time periods, removing baseline noise and co-eluting compounds from pure amino acid spectra (Johnsen *et al.*, 2017).

#### Diet manipulation study

**Experimental design.** The experiment consisted of four diet treatments based on a fully crossed 2 × 2 factorial design ( $n = 12$  larvae/treatment). Each factor consisted of two levels; Factor 1: Pollen type (levels: Host pollen; Non-host pollen), and Factor 2: Pollen-borne microbes (levels: Present; Absent). Based on

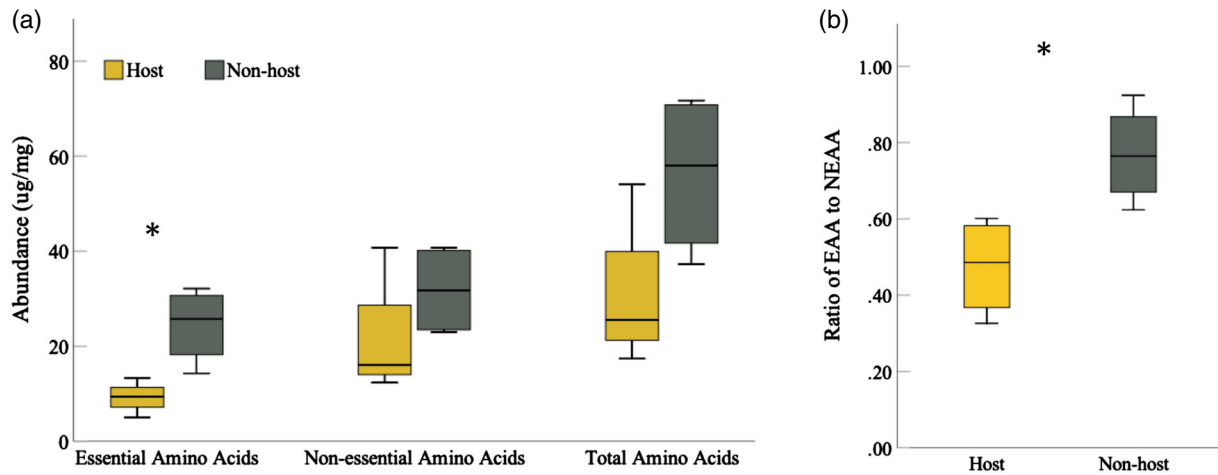
previously described methods, separate plates were used for each diet treatment to minimise the risk of cross-contamination (Dharampal *et al.*, 2018). Briefly, each pollen provision was weighed to approximate the amount originally allocated to the male provisions within the reeds (~0.35 g). The provision was then placed inside a well of a 48 well plate along with a randomly selected male egg. All procedures were carried out inside a biosafety cabinet using standard aseptic technique. The plates were loosely taped and maintained under dark conditions at 22 °C in an incubator.

**Data collection and analysis.** Larvae were observed daily until they reached the prepupal stage, characterised by the completion of a pale silken cocoon. To minimise handling stress and reduce the risk of contamination, all surviving larvae were aseptically weighed on days 1, 10, 15, and 20. Each larva was removed from the well plates using sterilised silicone forceps, placed on an aluminium weigh boat that was surface sterilised with 90% ethanol, and weighed using a standard laboratory microbalance placed inside a biosafety cabinet.

A two-way ANOVA was used to analyse the main and interactive effects of pollen source and pollen-borne microbes on prepupal biomass and larval developmental time. The percentage of the total variance observed in larval fitness components accounted for by each factor (microbe and pollen type) was measured using the partial eta squared ( $\eta_p^2$ ) as an estimate of effect size. Within each pollen type, the percent variance in larval fitness explained by microbes was calculated using the eta squared ( $\eta^2$ ) (Lakens, 2013). Survivorship across treatments was compared using Kaplan–Meier analysis and log-rank tests, pooled over strata. Growth rate across treatments was compared using repeated measures ANOVA followed by Tukey post hoc comparisons. Pearson correlation analysis was used to explore the relationship between prepupal biomass and larval developmental time. A two-way ANOVA was used to analyse the main and interactive effects of pollen type and sterilization on pollen macronutrient content. Student's *t*-test was applied to analyse initial differences in essential, nonessential, and total amino acids between natural host pollen and natural non-host pollen. The difference in amino acid content of natural host pollen and sterile host pollen over time was analysed using Pearson correlation. All statistical analyses were conducted using SPSS 23.0 (IBM, Chicago, Illinois, U.S.A.) and R (R Core Team, 2015).

## Results

Results from the two-way ANOVA indicated that pollen identity had a significant main effect on nutrient content, wherein non-host pollen had significantly higher amounts of crude protein ( $F_{1,18} = 25.87$ ,  $P < 0.0001$ ), and fat ( $F_{1,18} = 21.75$ ,  $P < 0.0001$ ) than host pollen measured as percent dry matter content (Table S1). However, the main effect of sterilization was non-significant (Table S2). Our results show that natural non-host pollen [ $24.45 \pm 3.97 \mu\text{g mg}^{-1}$ , (mean  $\pm$  SE)] contained significantly higher ( $t_6 = 3.52$ ,  $P = 0.01$ ) amounts of essential amino acids (valine, leucine, isoleucine, lysine,



**Fig. 1.** (a) Amino acid concentrations in host and non-host pollen provisions. (b) Ratio of essential amino acids (EAA) to non-essential amino acids (NEAA) across pollen type. Essential amino acids included valine, leucine, isoleucine, lysine, and phenylalanine ( $*P < 0.05$ ). [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

**Table 1.** Effects of pollen type and pollen-associated microbes on bee fitness components.

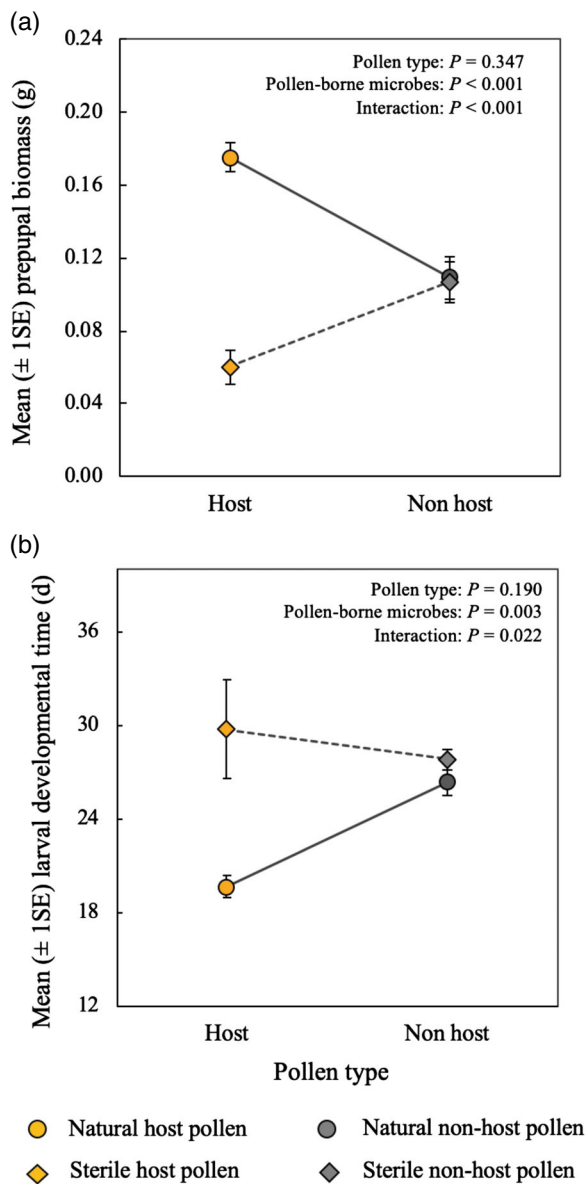
Source	Type III sum of squares	df	Mean square	<i>F</i>	<i>P</i> value	$\eta_p^2$
(a) Dependent variable: prepupal biomass						
Corrected Model	0.067 <sup>†</sup>	3	0.022	30.286	0.000	0.764
Intercept	0.376	1	0.376	508.747	0.000	0.948
Pollen type	0.001	1	0.001	0.917	0.347	0.032
Microbes	0.026	1	0.026	34.652	0.000	0.553
Pollen type $\times$ Microbes	0.024	1	0.024	31.773	0.000	0.532
Error	0.021	28	0.001			
Total	0.559	32				
Corrected total	0.088	31				
(b) Dependent variable: developmental time						
Corrected Model	580.64 <sup>‡</sup>	3	193.55	8.34	0.000	0.473
Intercept	19808.321	1	19808.321	858.122	0.000	0.968
Pollen type	41.654	1	41.654	1.804	0.190	0.061
Microbes	247.705	1	247.705	10.731	0.003	0.277
Pollen type $\times$ Microbes	136.013	1	136.013	5.892	0.022	0.174
Error	646.333	28	23.083			
Total	21177.000	32				
Corrected total	1226.969	31				

<sup>†</sup> R Squared = 0.764 (Adjusted R Squared = 0.739).

<sup>‡</sup> R Squared = 0.473 (Adjusted R Squared = 0.417).

phenylalanine) than natural host pollen ( $9.27 \pm 1.69 \mu\text{g mg}^{-1}$ ), although non-essential amino acid concentration was comparable ( $t_6 = 1.29$ ,  $P = 0.25$ ) between natural non-host pollen ( $31.81 \pm 4.82 \mu\text{g mg}^{-1}$ ) and natural host pollen ( $21.33 \pm 6.53 \mu\text{g mg}^{-1}$ ). Consequently, the proportion of essential amino acids was significantly higher ( $t_6 = 3.23$ ,  $P = 0.02$ ) in natural non-host pollen ( $0.77 \pm 0.06$ ) compared to natural host pollen ( $0.47 \pm 0.07$ ) (Fig. 1). Pearson's correlation showed that compared to sterile host pollen, there was a significant increase in the relative abundance of essential amino acids valine ( $r = 0.712$ ,  $P = 0.01$ ), leucine ( $r = 0.832$ ,  $P < 0.001$ ), and isoleucine ( $r = 0.802$ ,  $P = 0.001$ ) in natural host pollen as time progressed (Figure S1).

The interaction between pollen-borne microbes and pollen type had a significant impact on prepupal biomass (Table 1a). Fresh weight of prepupae raised on natural host pollen was significantly greater (pairwise contrast:  $F_{1,28} = 23.55$ ,  $n = 18$ ,  $P < 0.001$ ) than that of prepupae on natural non-host pollen. Conversely, prepupae reared on sterile host pollen weighed significantly less (pairwise contrast:  $F_{1,28} = 10.12$ ,  $n = 14$ ,  $P = 0.004$ ) than prepupae on sterile non-host pollen (Fig. 2a). There was a significant main effect of pollen-borne microbes, with larvae consuming natural pollen and sterile pollen attaining a mass of  $0.15 \pm 0.01 \text{ g}$  ( $n = 18$ ) and  $0.08 \pm 0.01 \text{ g}$  ( $n = 14$ ), respectively. The main effect of pollen type, however, was non-significant, in which prepupal weights from host pollen



**Fig. 2.** Effects of pollen identity and pollen-borne microbes on the (a) prepupal biomass and (b) larval developmental time (mean  $\pm$  1 SE) of *O. ribifloris* larvae. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)].

diets were  $0.13 \pm 0.06$  g ( $n = 20$ ) and on non-host pollen diets,  $0.11 \pm 0.01$  g ( $n = 12$ ) (Fig. 2a). The interaction between pollen-borne microbes and pollen type had a significant effect on larval developmental time (Table 1b). Larvae raised on natural host pollen had significantly shorter developmental time (pairwise contrast:  $F_{1,28} = 7.70$ ,  $n = 18$ ,  $P < 0.001$ ) than that of larvae on natural non-host pollen. Larval developmental time did not vary significantly (pairwise contrast:  $F_{1,28} = 5.55$ ,  $n = 14$ ,  $P = 0.45$ ) between larvae raised on sterile host pollen and sterile non-host pollen (Fig. 2b). There was a significant main effect of pollen-borne microbes, with larvae consuming

natural pollen and sterile pollen completing larval development by  $21.89 \pm 0.92$  days ( $n = 18$ ) and  $28.93 \pm 1.78$  days ( $n = 14$ ), respectively. The main effect of pollen type, however, was non-significant, in which developmental times on host pollen were  $23.70 \pm 1.70$  days ( $n = 20$ ), and on non-host pollen,  $27.08 \pm 0.54$  days ( $n = 12$ ) (Fig. 2b).

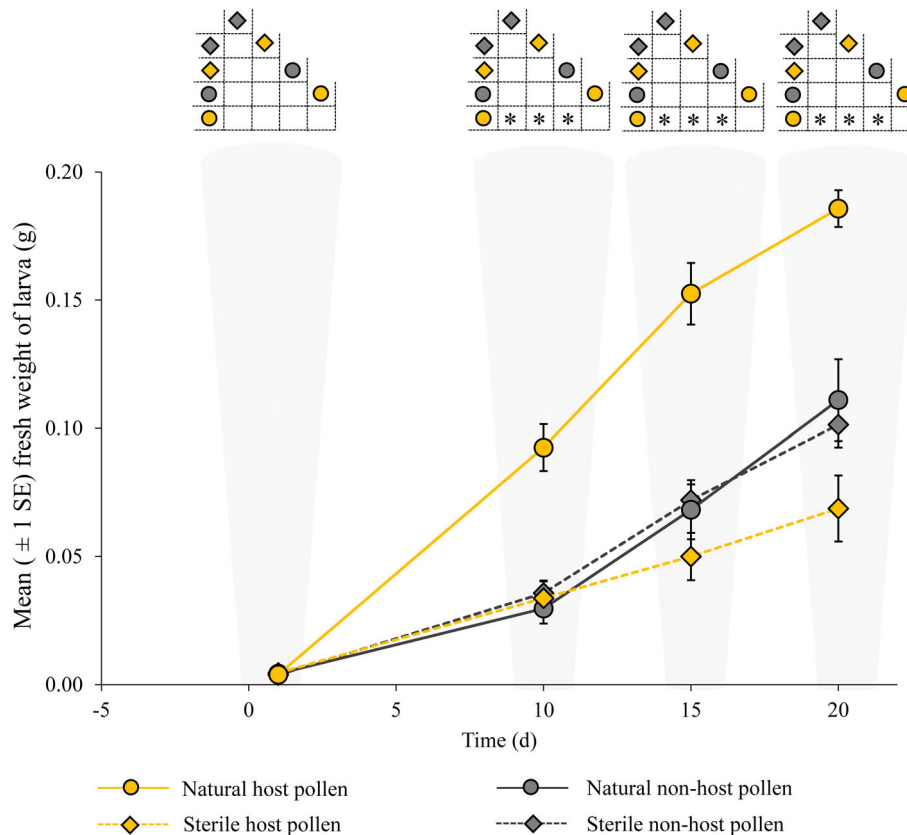
Estimates of effect size based on the eta squared ( $\eta^2$ ) indicated that for larvae reared on host pollen, the presence of microbes dramatically improved larval performance, significantly increasing prepupal biomass ( $F_{1,18} = 88.83$ ,  $P < 0.001$ ,  $\eta^2 = 0.83$ ) and decreasing larval developmental time ( $F_{1,18} = 14.30$ ,  $P < 0.001$ ,  $\eta^2 = 0.44$ ). In contrast, for larvae reared on non-host pollen, prepupal biomass ( $F_{1,10} = 0.24$ ,  $P = 0.88$ ,  $\eta^2 = 0.002$ ) and developmental times ( $F_{1,10} = 2.10$ ,  $P = 0.18$ ,  $\eta^2 = 0.17$ ) were comparable between natural non-host pollen and sterile non-host pollen diets, the effect of microbes being trivial (Table S3).

Results from the repeated measures ANOVA (Greenhouse–Geisser correction  $\epsilon = 0.52$ ,  $\chi^2(5) = 47.74$ ,  $P < 0.001$ ), indicated that the interaction between time and treatment had a significant effect on larval biomass ( $F_{4,67,35,77} = 15.10$ ,  $P < 0.0001$ ) (Fig. 3). The main effects of time ( $F_{1,55,35,77} = 272.98$ ,  $P < 0.0001$ ), and diet treatment ( $F_{3,23} = 16.01$ ,  $P < 0.0001$ ) were also significant. Pairwise post hoc tests indicated that while the initial larval biomass was comparable across treatments, for all subsequent time points, larvae reared on natural host pollen had significantly higher biomass compared to those reared on all other diets. Kaplan–Meier log rank test followed by pairwise comparisons indicated that survivorship was significantly higher among larvae reared on natural host pollen compared to those reared on all other diets (Bonferroni-corrected  $P < 0.001$ ) (Fig. 4). Pearson correlation analysis showed a significant negative correlation between prepupal biomass and larval developmental time across all diet types ( $r = -0.68$ ,  $P < 0.0001$ ,  $n = 32$ ) (Figure S2).

## Discussion

The development of oligolectic bees is considered to be critically linked to the availability of suitable pollen types sourced from specific host plants. However, our findings revealed that for the oligolectic, *O. ribifloris*, access to appropriate host pollen alone was not sufficient to ensure brood success. Instead, larval performance was strongly predicted by the presence of exosymbiotic microbes within pollen provisions. Larvae consuming host pollen developed faster, reached higher biomass, and showed greater survivorship, but only when the associated pollen microbiota were also present. However, when devoid of these microbes, host pollen was unable to support larval nutrition and resulted in a striking decline in bee fitness components. Thus, the extent to which host pollen influenced brood outcome for the specialist larvae was critically dependent on the presence of its natural microbiota, rather than its botanical identity alone.

Given the competition to harvest high quality pollen, certain oligolectic species have adapted to exploiting pollen types that are nutritionally inadequate or toxic to generalist bees (Weiner *et al.*, 2010; Lawson *et al.*, 2016). Indeed, our results indicated that host pollen had significantly lower concentrations of total



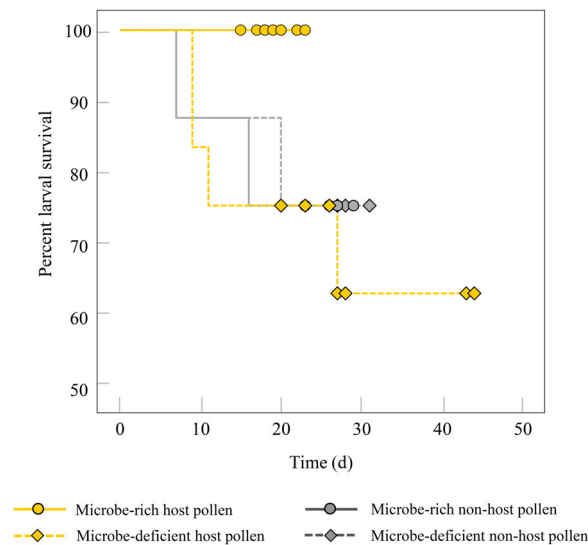
**Fig. 3.** Larval fresh weights measured over four time points across four diet treatments. Greenhouse–Geisser corrected results from repeated measures ANOVA show significant effects of Time  $\times$  Diet ( $P < 0.0001$ ), Time ( $P < 0.0001$ ), and Diet ( $P < 0.0001$ ) on larval fresh weight. Inset figures represent pairwise comparisons of larval biomass across diet treatments within a given time increment ( $*P < 0.05$ ). [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)].

essential amino acids (Fig. 1), crude protein, and fat (Table S1) and thus, represented a poorer quality substrate than non-host pollen. However, adaptation to low-quality diets does not limit an oligolege's ability to exploit other nutrient-rich pollen, even when sourced from non-host plants (Williams, 2003). Interestingly, our results show that regardless of its botanical identity (host or non-host) or nutrient content (high-nutrient or low-nutrient), pollen type had a trivial effect on larval health. While pollen type accounted for a mere 3% of the total variance in prepupal biomass and 6% in larval developmental time, pollen-associated microbes accounted for 55% and 27%, respectively (Table 1). This weak association between pollen type and larval performance suggests that the nutritional adequacy of larval diet is primarily mediated by the function of its pollen-borne microbes.

Although exosymbiotic microbes had a significantly larger impact on larval health than pollen type, the magnitude of this effect varied substantially. For larvae reared on non-host pollen, fitness components remained unchanged whether microbes were present or not, while for those reared on host pollen, the presence of microbes dramatically improved larval performance. In fact, microbes accounted for 83% of the variance in prepupal biomass and 44% in developmental time between larvae reared on natural

versus sterile host pollen (Table S3). Despite having comparable nutrient contents, natural host pollen produced the largest and fastest growing larvae, whereas sterile host pollen resulted in the smallest and slowest growing ones. These findings imply that host pollen of *O. ribifloris* represents an inadequate or inaccessible resource, its nutritive aspect only made available by the exosymbiotic microbes present within the substrate.

Larval growth and survival were strongly influenced by diet treatment. While all larvae had comparable initial weights by day 10, larvae fed natural host pollen had gained significantly more biomass than all other treatments. This pattern was consistent for all subsequent time points and the greatest difference in final weight was observed between larvae reared on natural and sterile host pollen. Despite being sourced from the appropriate host plant, removing pollen-associated microbes significantly delayed larval development. In fact, larvae fed hostpollen without microbes took even longer to reach the prepupal stage than those fed non-host pollen, suggesting that microbes play an essential role through all stages of larval development (Fig. 3). The analysis of larval survivorship mirrored these findings; all larvae that received both their host pollen and the associated microbiota reached the prepupal stage. However, when microbes were removed from host pollen, only two-thirds of the larvae



**Fig. 4.** Survival plot of *Osmia ribifloris* larvae across diet treatments. Circles and diamonds corresponding to each treatment indicate the time at which individual larva reached the prepupal stage. Log rank test indicated significant differences in survivorship across the four treatments ( $n = 12$  each,  $P < 0.001$ ). [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)].

survived. In fact, fewer larvae survived on host pollen without microbes than they did on non-host pollen diets (Fig. 4). These findings further illustrated that symbiotic microbes perform a fundamental role in host pollen adaptation by creating a nutritionally ideal provision, ultimately yielding the healthiest larvae.

The microbial community within pollen provisions of solitary bees consist of symbiotic taxa that serve as conduits for nutrient transfer from pollen to larval biomass. This includes nutritional mutualists that are involved with pollen fermentation (Cohen *et al.*, 2020; Voulgari-Kokota *et al.*, 2020), as well as other heterotrophic microbes that form a dominant source of dietary proteins and lipids for the developing bees (Dharampal *et al.*, 2019; Steffan *et al.*, 2019). Our results show that while pollen sterilization did not alter the net abundance of major dietary macromolecules, it would have eliminated the nutritional services derived from these pollen microbiota. As a result, essential nutrients locked inside the recalcitrant pollen grains would be rendered biochemically inaccessible to the larvae, eventually compromising bee nutrition. Indeed, our data showed that despite having access to ample amounts of appropriate host pollen, the absence of the associated microbiota led to lowered performance among oligolectic larvae. These findings present strong evidence that, whether serving as nutritional mutualists or a major dietary subsidy, microbes within host pollen are fundamental to the success of larval oligolectes.

The quality of forage pollen is based on its protein content, with higher protein concentrations resulting in healthier bees (Leach & Drummond, 2018). Interestingly, our data showed that larvae performed worse on the protein-rich honey bee pollen than on host pollen with microbes. Compared to honey bee pollen, host pollen had significantly lower protein and fat content. Yet, when replete with its natural microbiota, the inferior

quality host pollen was transformed into a nutritiously ideal diet, producing the healthiest larvae. Therefore, the nutritional suitability of larval diet could not be ascribed to pollen protein content alone. Instead, these findings suggest that a sustained partnership with pollen-associated microbial symbionts likely contributed to the specialist larva's ability to thrive on a low-quality substrate. Such tight symbiotic partnerships are common among insects that consume poorer quality diets (Moran *et al.*, 2008). Nutritional mutualists embedded within these insect diets function as an external rumen, pre-digesting and enhancing the quality of the recalcitrant substrate before it is consumed (Scheu & Setälä, 2002; Lavelle *et al.*, 2005; Steffan & Dharampal, 2018). In many cases, such microbe-digested substrates result in higher survival and increased growth rates among the insect consumers (Scheu & Falca, 2000; Nalepa *et al.*, 2001; Frainer *et al.*, 2016), and our data suggest that this may be true for oligolectic bees as well.

The ability of oligolectes to utilise low-quality diets has been previously linked to their physiology (Dobson & Peng, 1997; Praz *et al.*, 2008). However, this does not explain why the oligolectic larvae failed to develop on the appropriate host pollen substrate if the pollen microbiota were removed. Instead, our data suggest that adaptation to low-quality host pollen could be attributed to the nutritional function of microbial exosymbionts that are capable of extracting the sparsely dispersed nutrients within host pollen. However, this adaptive advantage can cause oligolectes to become strongly dependent on a handful of microbial taxa, and thus, more sensitive to subtle shifts within the microbiome of their pollen provisions. Given the high variability in the solitary bee microbiome (Keller *et al.*, 2013; McFrederick & Rehan, 2016; Cohen *et al.*, 2020), the pollen microbiota are more likely to experience frequent alterations to their community structure. Reshuffling of key members within this external microbial community can potentially disrupt the symbioses between microbes and oligolectic larvae, leading to unpredictable fitness outcomes for the developing bees (Voulgari-Kokota *et al.*, 2019b, 2020).

Unlike host pollen, microbes associated with non-host pollen had virtually no effect on larval growth and survival. One explanation could be that the higher nutrient content of the wild-collected non-host honey bee pollen closely matched the ideal ratio of essential amino acids needed to support bee nutrition (de Groot, 1952). Alternatively, honey bee pollen, which consists of diverse pollen types, may have been more digestible, reducing larval reliance on the microbial nutritional mutualists and/or microbial prey. Interestingly, honey bee pollen, while considered nutritious for most developing bees (Vanderplanck *et al.*, 2014), represented an inferior quality diet compared to host pollen with microbes. Given the intrinsic difference between the microbiome structure of host and non-host pollen (Lozo *et al.*, 2015; Saraiva *et al.*, 2015; Voulgari-Kokota *et al.*, 2019b), and its direct impact on brood success, we speculate that the lowered performance on honey bee pollen could be driven by the dissimilarity between the natural microbiota of the two pollen types. Although less plausible, the lack of key micronutrients could have also contributed to the nutritional inadequacy of honey bee pollen.

Prior research in social bees has demonstrated the link between specific beneficial microbial taxa and larval health (Vásquez & Olofsson, 2009; Degrandi-Hoffman *et al.*, 2015; Steffan *et al.*, 2017b; Dharampal *et al.*, 2020). Although similar studies in solitary bees are scarce, previous work indicates that the microbiota within the nests of congeners, *Osmia bicornis* (Keller *et al.*, 2013) and *Osmia cornuta* (Lozo *et al.*, 2015), are distinctly different from those seen in honey bees. The microbial community within the provisions of *Osmia* sp. consists of diverse environmentally acquired microbes, and is more likely to fluctuate based on location, foraging preferences, and nesting ecology (Keller *et al.*, 2013; Lozo *et al.*, 2015; McFrederick *et al.*, 2016; McFrederick & Rehan, 2016; Voulgari-Kokota *et al.*, 2018, 2019a; Cohen *et al.*, 2020). Despite having lower taxonomic specificity, these microbes have a major influence in shaping the larval microbiome and determining bee fitness components (Voulgari-Kokota *et al.*, 2019b, 2020). While comparing the microbial community associated with each pollen type fell beyond the scope of this study, further research is required to identify key microbial taxa responsible for ensuring the nutritional adequacy of host pollen types.

Our results provide strong evidence that for the oligolege, *O. ribifloris*, access to host pollen alone was not sufficiently predictive of larval success. The natural microbiota associated with host pollen was crucial in enhancing the nutritive value of larval diet, dramatically improving larval performance. Additionally, for larvae reared on host pollen, the presence of exosymbiotic microbes had a substantially greater impact on bee fitness components than the type of pollen itself. While oligolectic bees can develop on pollen types that are toxic, inadequate, or indigestible for other generalists, our data show that this evolutionary advantage likely stems from the function of symbiotic microbes within forage pollen. In fact, this bee-microbe symbiosis could be a possible explanation, or at least a consequence of pollen specialization among bees. As oligolectic bees face increased risk of resource limitation, preserving the partnership between wild bees and their microbial partners will be critical for conserving healthy bee populations.

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## Author contribution

PSD, and SAS conceived of the study; PSD and MH performed the experiments; PSD, MH and SAS performed data analyses and wrote the manuscript. All authors gave final approval for publication.

## Data availability statement

The data that supports the findings of this study are available in the supplementary material of this article.

## Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1.** Change in essential amino acid content between natural and sterile host pollen over time.

**Figure S2.** Pearson correlation between larval developmental time and prepupal biomass.

**Table S1.** Proximate nutritional analysis of host and non-host pollen.

**Table S2.** Effects of pollen type and sterilization treatment on pollen nutritional parameters.

**Table S3.** Estimates of effect size of pollen-associated microbes on bee fitness components.

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