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Supplementary material: Tree-dimensional resources: hedgerow and orchard pollen for bumble bees

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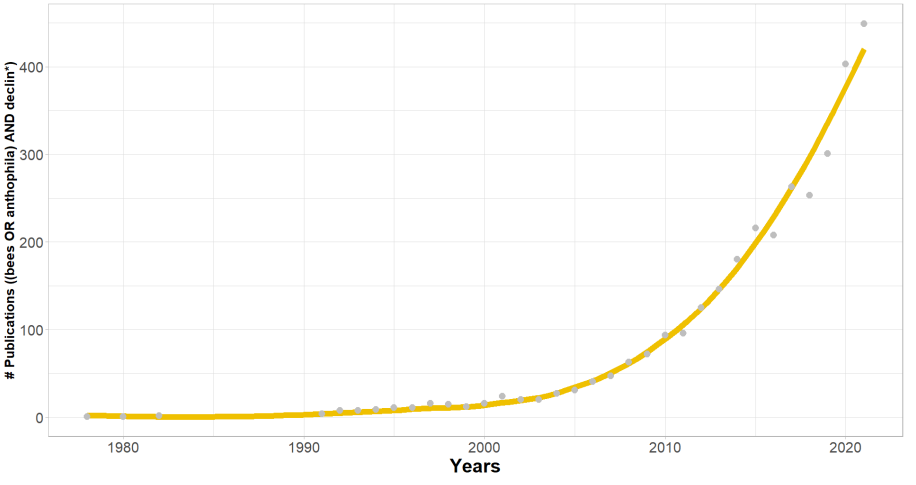

Supplementary Figure S1. Trends in research on bee decline. There has been an increasing concern over the loss of bee populations. Data extracted from Web of Science ("(bees OR anthophila) AND declin*").

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1. Pollen diet preparation

Flavonoid extract from hedgerow and orchard pollen were obtained from ground pollen pellets by Soxhlet extraction, this method allowing a continuous extraction without saturating the solvent in contact with the solid material. In each extractor, the pollen was placed in a cellulose thimble (Whatman) in the extraction chamber, beneath a reflux condenser, and on top of a collecting flask containing a magnetic stirrer. Methanol (technical grade, Chem-Lab) was added to the flask and the hot plate was set at 100 °C to ensure a high temperature all the way up to the condenser. The set-ups were wrapped in aluminium foil to prevent any light-induced degradation. The methanolic extracts were filtrated and evaporated to dryness using a rotavapor (IKA RV8), and then subjected to a partitioning using water (generated with a PureLab flex MANU38981 version 02 purifier (Elga labwater, High Wycombe, UK) and dichloromethane (technical grade, Chem-Lab) (50:50 v/v) in a separatory funnel. The aqueous phase was evaporated to dryness. For analytical purposes, the dried extracts were dissolved in 1 mL of methanol/water (HPLC grade, Chem-Lab) (70:30 v/v) solvent and diluted appropriately for LC-MS and LC-MS/MS analysis. Characterisation experiments were carried out with a WatersTM Alliance 2695 HPLC separation module, hyphenated to a WatersTM Q-ToF API US mass spectrometer. The HPLC column was a Phenomenex Kinetex C18 EVO (150 × 2.1 mm i.d., 100 Å particle size, ref 00F-4633-AN). The autosampler temperature was set at 20 °C and the column oven at 40 °C. The injection volume was 2.5 µL. The samples were all eluted with a methanol (solvent A) and water +0.01% formic acid (solvent B) gradient as follows: A = 10%, B = 90% at t = 0 min; A = 30%, B = 70% at t = 6 min; A = 35%, B = 65% at t = 11 min; A = 50%, B = 50% at t = 18 min; A = 90%, B = 10% at t = 23 min; A = 100%, B = 0% at t = 25 min; A = 100%, B = 0% at t = 27 min, A = 10%, B = 90% at t = 30 min. The solvent flow was 0.25 mL/min.

The mass spectrometer operated in electrospray (ESI) positive and negative ionization mode over a mass range of 50–2000 Da. Typical MS conditions were: capillary voltage ±3.1 kV, cone voltage ±30 V, source temperature 120 °C, desolvation gas temperature and flow 300 °C and 500 L/h, respectively, and scan time 0.5 s. Flavonoids were identified by studying collision-induced dissociation (CID) spectra in positive and negative ionization mode and comparing the data obtained with literature. Quantifications were performed using quercetin (Sigma-Aldrich, Belgium, Overijse) as internal standard (concentrations expressed as quercetin mg equivalent/sample g) in triplicates to account for analytical variability (assuming the same response factor between the extracted flavonoids and quercetin).

The extract was finally dissolved in aqueous ethanol (Ethanol absolute ≥ 99.9%, Emsure) solution (1:1 v/v) before addition to the control willow pollen in proportions that mimic flavonoid concentrations of hedgerow or orchard pollen diet. All treatment diets contained aqueous ethanol (1:1 v/v; 17–34 µL/diet g) to control for potential effects of the solvent (see Supplementary Tables S1 and S2 for diet formula). The total flavonoid content of willow pollen pellets for the hedgerow and orchard experiments, hedgerow pollen pellets, orchard pollen pellets, hedgerow flavonoid extract and orchard flavonoid extract were analysed in triplicates by HPLC-MS (triplicates of 20–40 mg) for quantification (expressed as quercetin equivalent, QE). We found that willow pollen in the hedgerow experiment contained 12.1 ± 4.4 mg QE/g, willow pollen in the orchard experiment contained 17.4 ± 0.2 mg QE/g, hedgerow pollen 17.5 ± 0.8 mg QE/g, orchard pollen 16.9 ± 2.0 mg QE/g, hedgerow flavonoid extract 36.5 ± 0.4 mg QE/g, and orchard flavonoid extract 34.8 ± 0.3 mg QE/g (mean ± SD). Flavonoid profiles in willow, hedgerow and orchard pollen differed between each other and will be released in another paper (Gekière *et al.* in preparation).

Supplementary Table S1. Diet formula for the hedgerow experiment

	Diet treatments		
	Control diet (willow)	Natural diet (hawthorn)	Flavonoid diet (willow added with flavonoid extract)
Pollen (g)	15 (willow)	15 (hawthorn)	15 (willow)
Sucrose syrup 65% (number of drops)	8	8	0
Aqueous ethanol (v:v 1:1) (mL)	1.5	1.5	0
Distilled water (mL)	5.5	8	0
Flavonoid extract (mL)	0	0	7
Final candy mass (g)	22.73	24.73	23.57
Ethanol in final candy (µL/g)	34	30	34
Pollen in final candy (g/g)	0.67	0.61	0.64
Flavonoid in final candy (mg/g)	7.99	10.64	10.39*

In every treatment, the quantities shown here enabled to feed 15 microcolonies at the onset of the experiment (i.e., when each microcolony was provided with 1 g of pollen candy). * Does not include flavonoids from willow pollen.

Supplementary Table S2. Diet formula for the orchard experiment

	Diet treatments		
	Control diet (willow)	Natural diet (orchard mix)	Flavonoid diet (willow and flavonoid extract)
Pollen (g)	10 (willow)	10 (orchard mix)	10 (willow)
Sugar syrup 65% (number of drops)	5	5	0
Aqueous ethanol (v:v 1:1) (mL)	0.5	0.5	0
Distilled water (mL)	4	3.5	0
Flavonoid extract (mL)	0	0	4.5
Final candy mass (g)	14.69	14.19	14.96
Ethanol in final candy (µL/g)	17.02	17.62	20.29
Pollen in final candy (g/g)	0.68	0.70	0.67
Flavonoid in final candy (mg/g)	11.84	11.87	10.8*

The orchard mix consisted of *Prunus avium, Malus domestica* and *Pyrus communis* pollen. In every treatment, the quantities shown here enabled to feed 10 microcolonies at the onset of the experiment (i.e., when each microcolony was provided with 1 g of pollen candy). * Does not include flavonoids from willow pollen.

Supplementary Table S3. Statistical outputs from the models

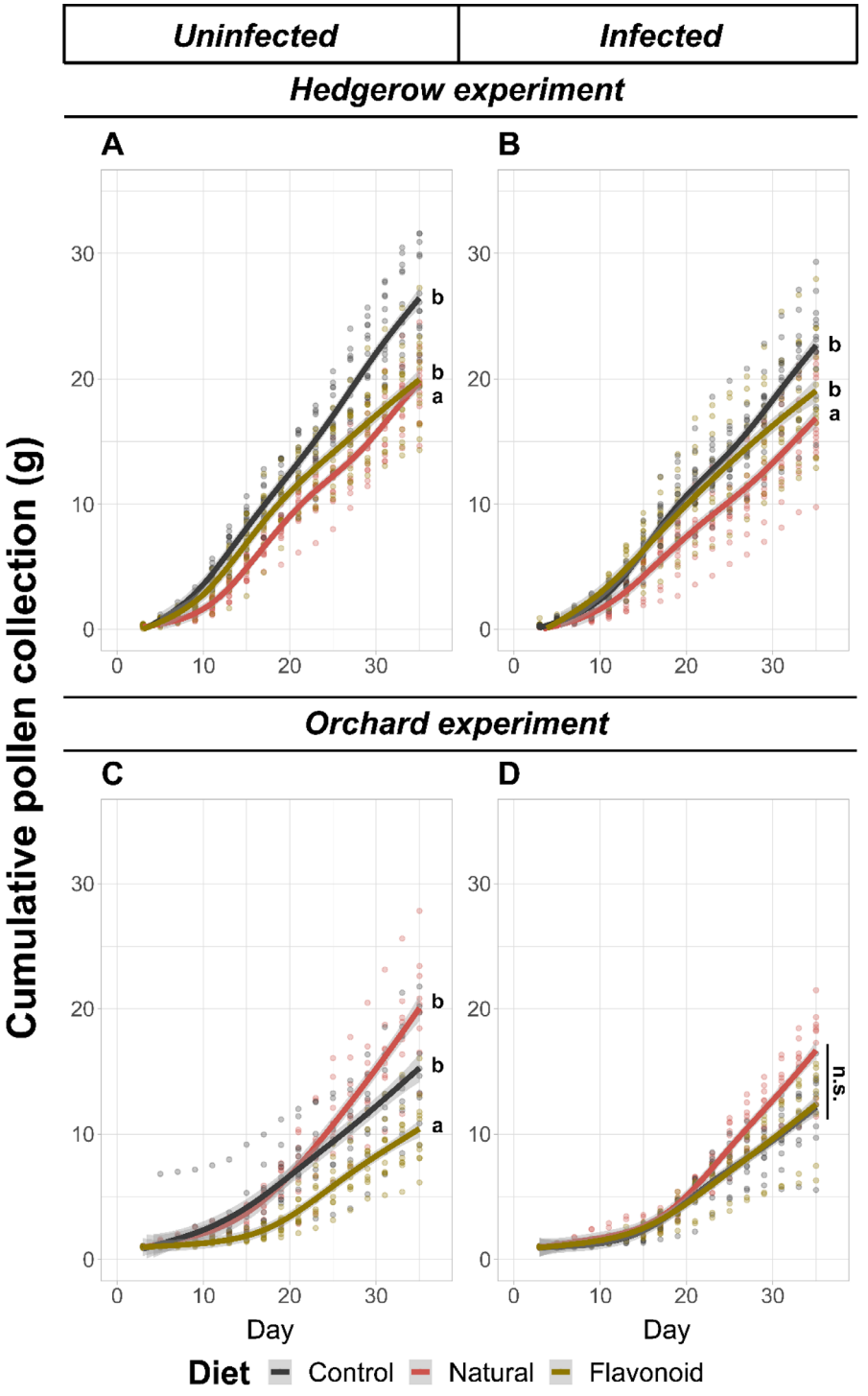
Experiment	Metric	Model (family - link)	Parameter	Degree of freedom	F or $\chi 2$	p-value
HEDGEROW						
	Pollen collection	GAMM (Gamma – log)	Diet	2	22.134	**<0.001**
			Parasite	1	6.884	**0.009**
			Diet:Parasite	2	2.621	0.073
	Syrup collection	GAMM (Gamma – log)	Diet	2	0.916	0.400
			Parasite	1	2.534	0.112
			Diet:Parasite	2	0.503	0.605
	Mass of alive hatched offspring	LMM (Gaussian – identity)	Diet	2	21.868	**<0.001**
			Parasite	1	12.837	**<0.001**
			Diet:Parasite	2	4.544	0.103
	Larval ejection	GLMM (Binomial – logit)	Diet	2	30.211	**<0.001**
			Parasite	1	2.679	0.102
			Diet:Parasite	2	8.675	**0.013**
	Pollen efficacy	GLMM (Betabinomial – logit)	Diet	2	195.302	**<0.001**
			Parasite	1	0.529	0.467
			Diet:Parasite	2	6.996	**0.030**
	Pollen dilution	LMM (Gaussian – identity)	Diet	2	178.379	**<0.001**
			Parasite	1	38.599	**<0.001**
			Diet:Parasite	2	44.102	**<0.001**
	Fat body content	GLMM (Betabinomial – logit)	Diet	2	109.873	**<0.001**
			Parasite	1	24.496	**<0.001**
			Diet:Parasite	2	7.748	**0.021**
	Mortality	Mixed effect Cox model	Diet	2	1.205	0.547
			Parasite	1	0.021	0.886
			Diet:Parasite	2	1.370	0.504
	Parasite load	GAMM (Gaulss – log, logb)	Diet	2	1.049	0.592

(continued on next page)

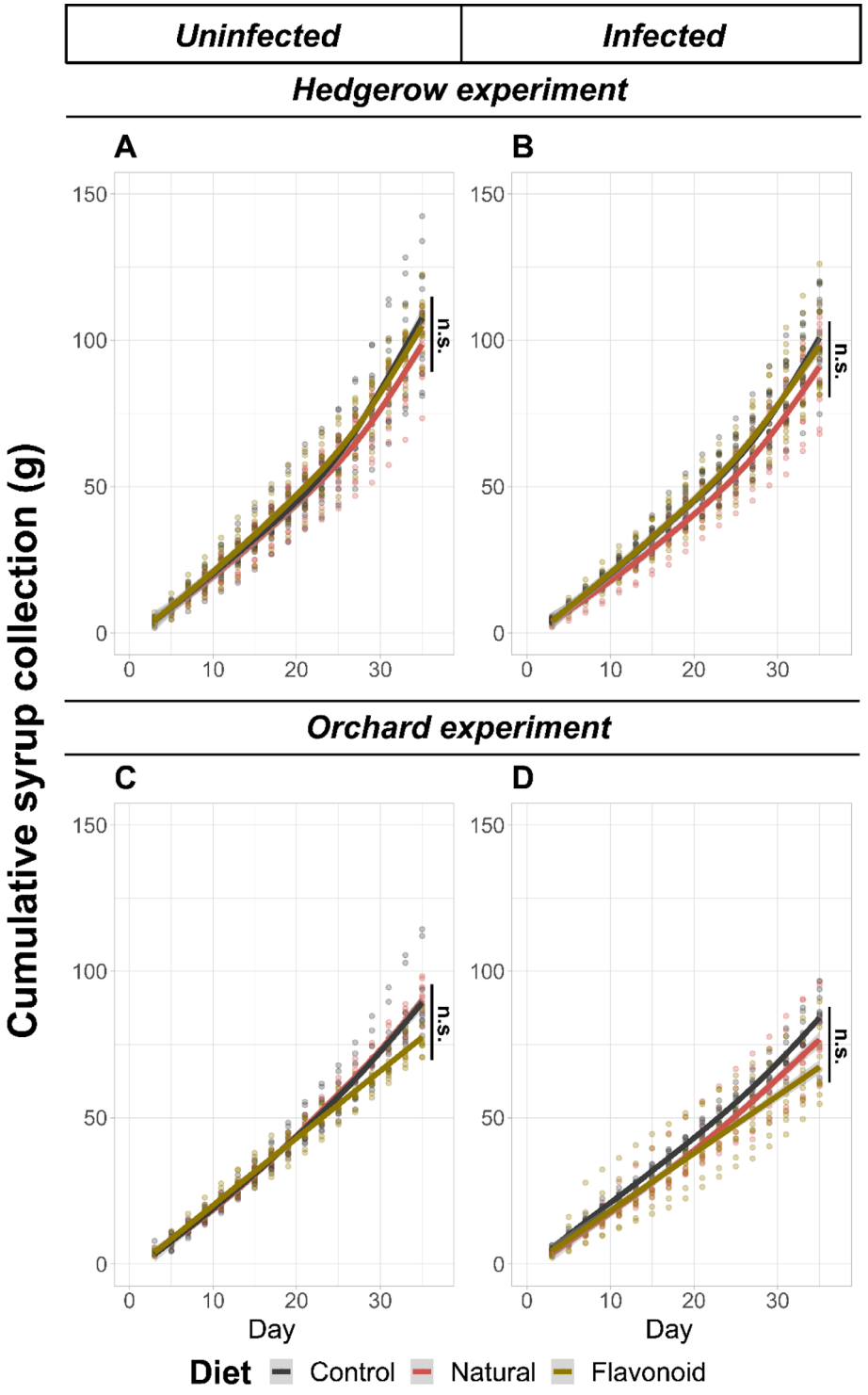
Supplementary Table S3. (continued)

Experiment	Metric	Model (family - link)	Parameter	Degree of freedom	F or $\chi 2$	p-value
ORCHARD						
	Pollen collection	GAMM (Gamma – log)	Diet	2	19.236	**<0.001**
			Parasite	1	4.303	**0.038**
			Diet:Parasite	2	7.814	**<0.001**
	Syrup collection	GAMM (Gamma – log)	Diet	2	0.029	0.971
			Parasite	1	2.900	0.089
			Diet:Parasite	2	2.442	0.088
	Mass of alive hatched offspring	LMM (Gaussian – identity)	Diet	2	108.124	**<0.001**
			Parasite	1	0.092	0.761
			Diet:Parasite	2	12.790	**0.002**
	Larval ejection	GLMM (Binomial – logit)	Diet	2	0.768	0.681
			Parasite	1	3.432	0.064
			Diet:Parasite	2	0.789	0.674
	Pollen efficacy	GLMM (Betabinomial – logit)	Diet	2	6.910	**0.032**
			Parasite	1	0.981	0.322
			Diet:Parasite	2	14.582	**<0.001**
	Pollen dilution	LMM (Gaussian – identity)	Diet	2	45.443	**<0.001**
			Parasite	1	0.676	0.411
			Diet:Parasite	2	16.813	**<0.001**
	Fat body content	GLMM (Betabinomial – logit)	Diet	2	10.620	**0.005**
			Parasite	1	9.848	**0.002**
			Diet:Parasite	2	3.587	0.166
	Mortality	Mixed effect Cox model	Diet	2	12.667	**0.002**
			Parasite	1	6.306	**0.012**
			Diet:Parasite	2	1.243	0.537
	Parasite load	GAMM (Gaulss – log, logb)	Diet	2	10.74	**0.005**

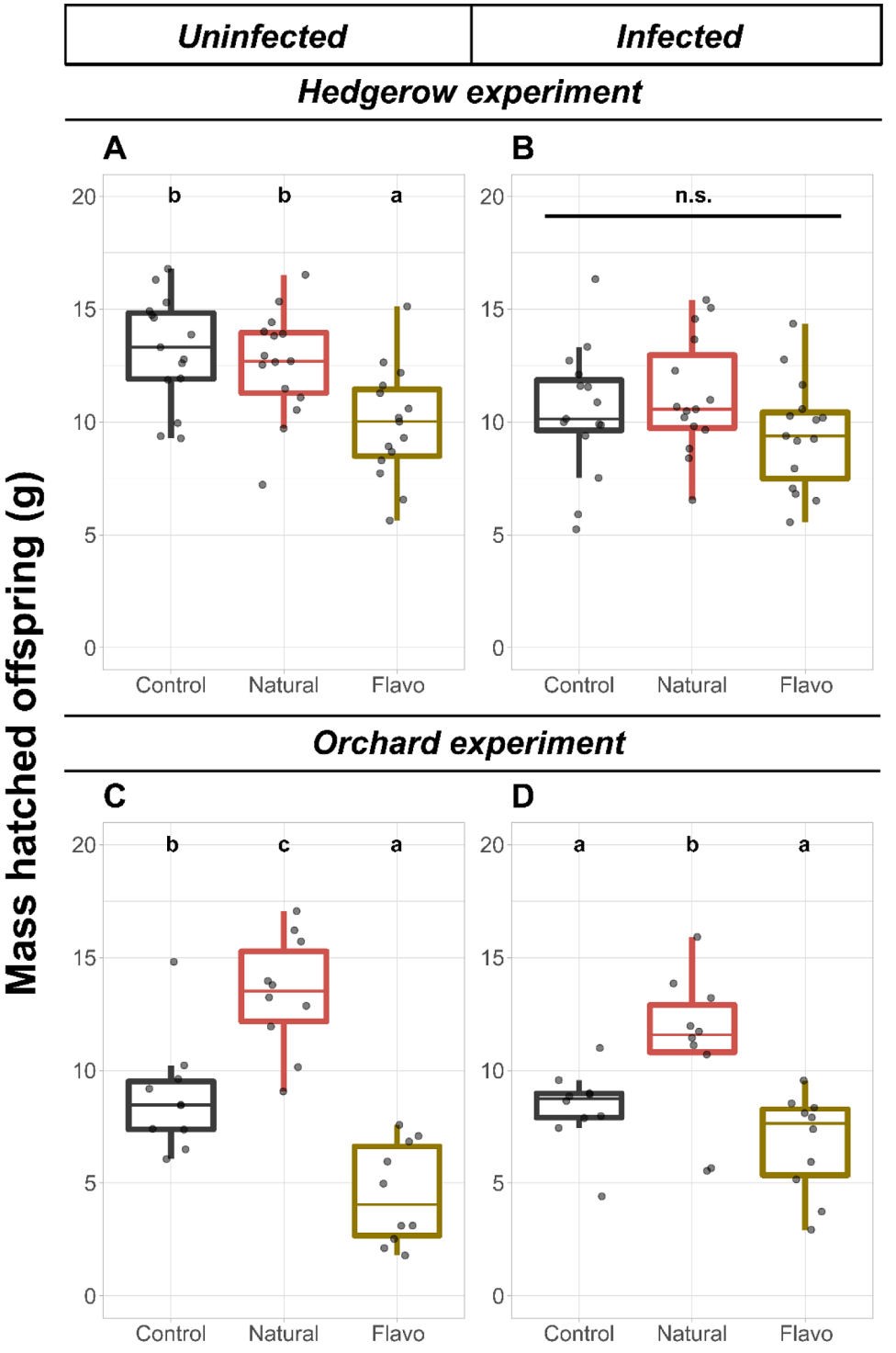
Outputs come from type-II analyses of variance. p-values in bold indicate significance at a threshold of $\alpha = 0.05$.



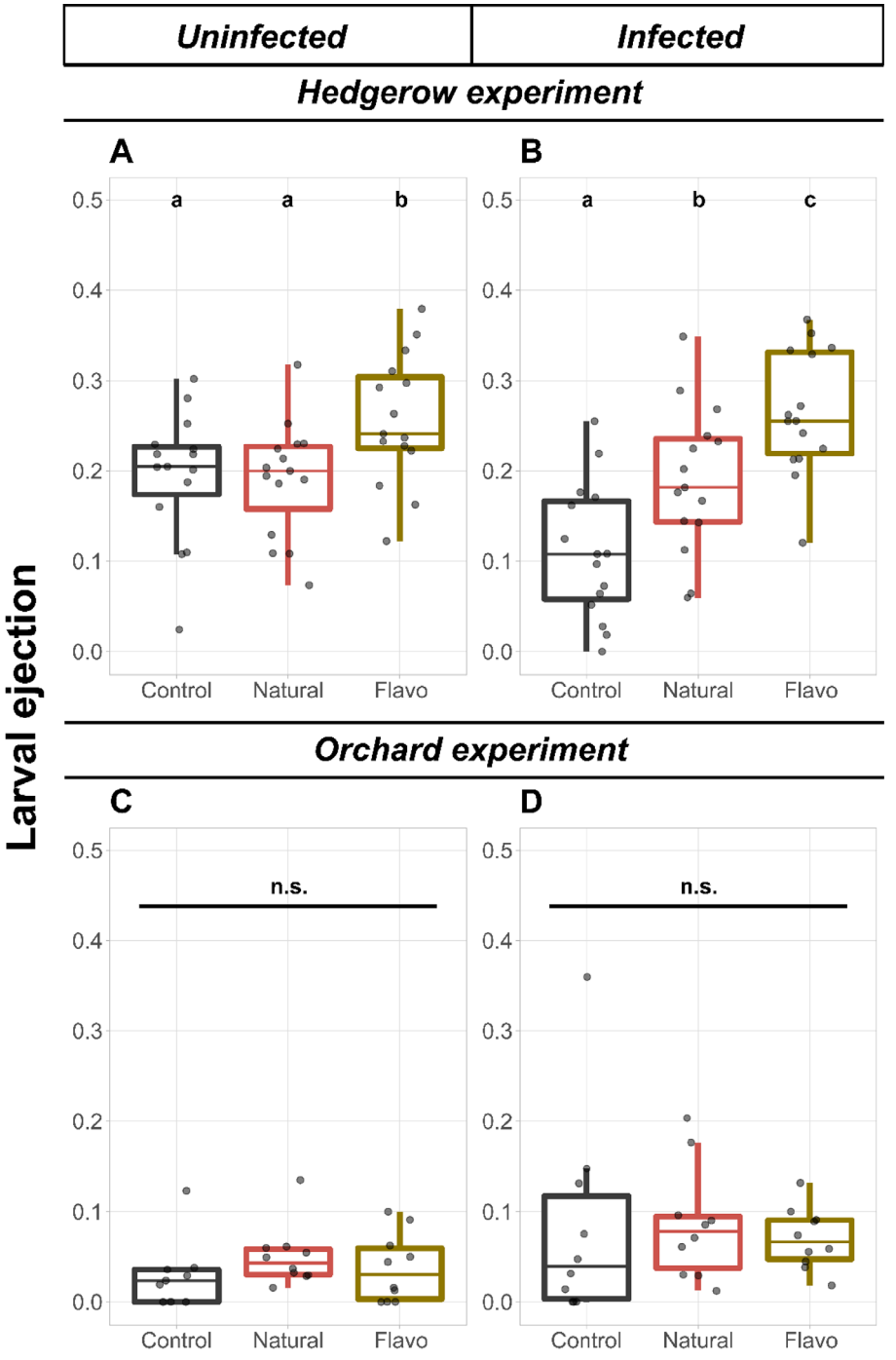
Supplementary Figure S2. Cumulative pollen collection recorded in the hedgerow (A,B) and orchard (C,D) experiments from uninfected (A,C) and infected (B,D) microcolonies. Two treatments sharing a letter are not significantly different (GAMM). n.s. Not significant.



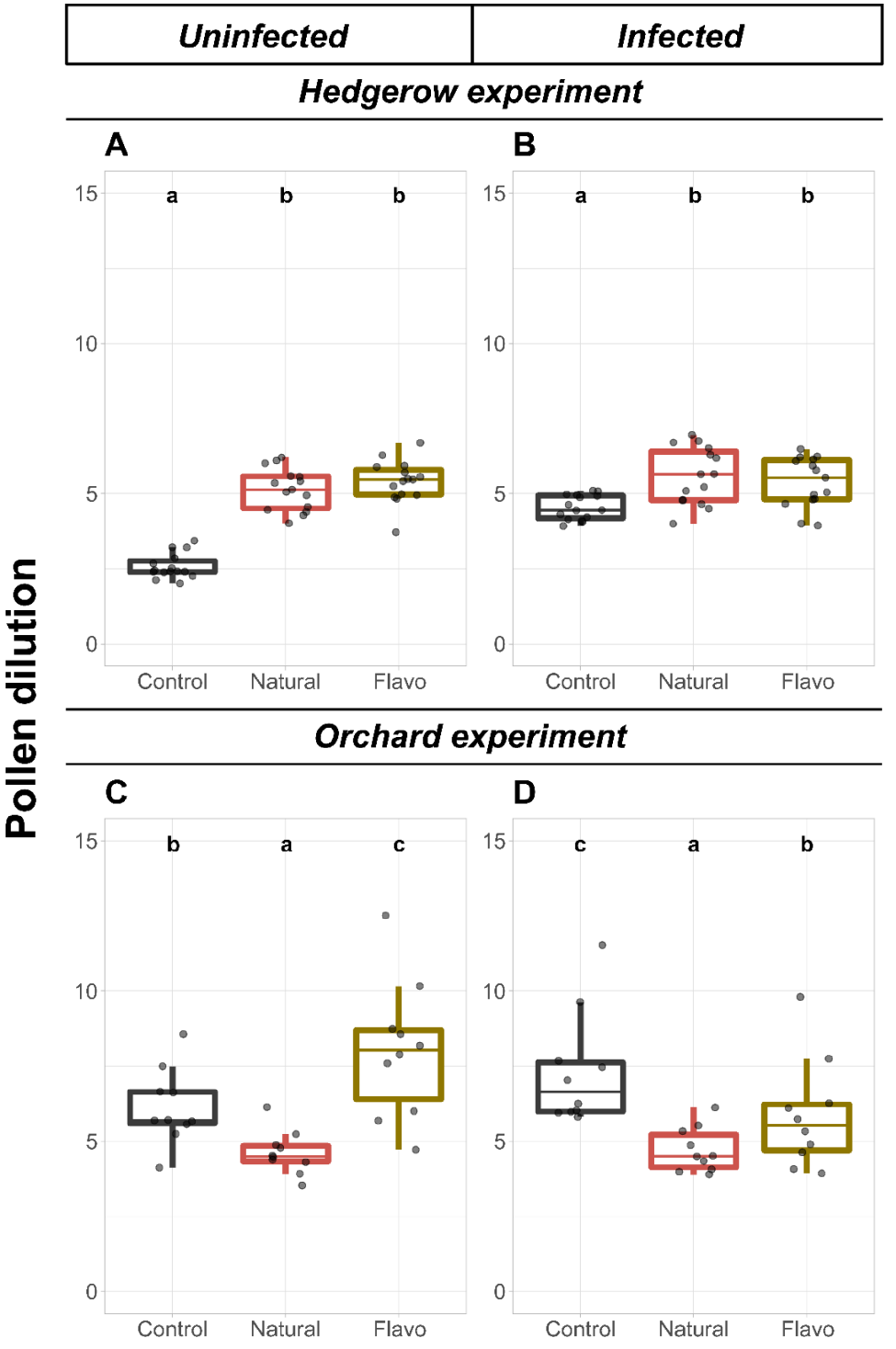
Supplementary Figure S3. Cumulative syrup collection recorded in the hedgerow (A,B) and orchard (C,D) experiments from uninfected (A,C) and infected (B,D) microcolonies. n.s. Not significant (GAMM).



Supplementary Figure S4. Total mass of hatched offspring recorded in the hedgerow (A,B) and orchard (C,D) experiments from uninfected (A,C) and infected (B,D) microcolonies. Two treatments sharing a letter are not significantly different (LMM). n.s. Not significant.

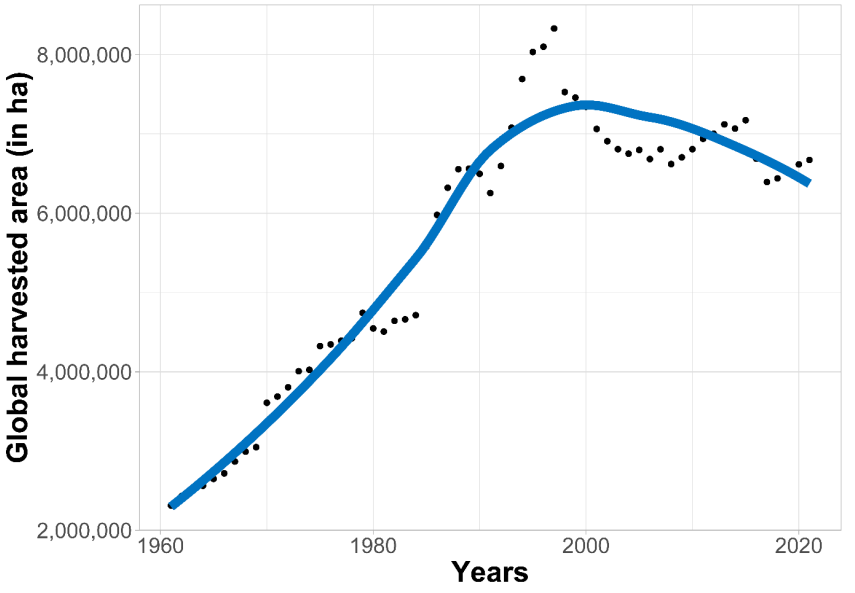


Supplementary Figure S5. Larval ejection recorded in the hedgerow (A,B) and orchard (C,D) experiments from uninfected (A,C) and infected (B,D) microcolonies. Larval ejection is a stress response defined as the number of ejected larvae divided by the sum of the number of hatched offspring and the number of ejected larvae per microcolony. Two treatments sharing a letter are not significantly different (GLMM). n.s. Not significant.



Supplementary Figure S6. Pollen dilution calculated in the hedgerow (A,B) and orchard (C,D) experiments from uninfected (A,C) and infected (B,D) microcolonies. Pollen dilution is defined as the total mass of collected syrup divided by total mass of collected pollen per microcolony. Two treatments sharing a letter are not significantly different (LMM).

Supplementary Figure S7. Flowering periods of trees considered in this study. Willow = *Salix caprea* L. Hawthorn = *Crataegus monogyna* Jacq. Sweet cherry = *Prunus avium* L. Apple = *Malus domestica* Borkh. Pear = *Pyrus communis* L. Data from https://www.ebben.nl/en/ (Accessed on 06/01/2023).



Supplementary Figure S8. Combined worldwide harvested area of sweet cherry, apple and pear trees. Data extracted from https://www.fao.org/faostat/en/#home (Accessed on 06/01/2023).