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Heather pollen is not necessarily a healthy diet for bumble bees

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ABSTRACT

There is evidence that specialised metabolites of flowering plants occur in both vegetative parts and floral resources (i.e., pollen and nectar), exposing pollinators to their biological activities. While such metabolites may be toxic to bees, it may also help them to deal with environmental stressors. One example is heather nectar which has been shown to limit bumble bee infection by a trypanosomatid parasite, *Crithidia* sp., because of callunene activity. Besides in nectar, heather harbours high content of specialised metabolites in pollen such as flavonoids but they have been poorly investigated. In this study, we aimed to assess the impact of *Crithidia* sp., heather pollen and its flavonoids on bumble bees using non-parasitised and parasitised microcolonies fed either control pollen diet (i.e., willow pollen), heather pollen diet, or flavonoid-supplemented pollen diet. We found that heather pollen and its flavonoids significantly affected microcolonies by decreasing pollen collection as well as offspring production, and by increasing male fat body content while parasite exposure had no significant effect except for an increase in male fat body. We did not highlight any medicinal effect of heather pollen or its flavonoids on parasitised bumble bees. Our results provide insight into the impact of pollen specialised metabolites in heather-bumble bee-parasite interactions. They underline the contrasting roles for bumble bees of the two floral resources and highlight the importance of considering both nectar and pollen when addressing medicinal effects of a plant towards pollinators.

Keywords: Plant-pollinator interaction, Pollen specialised metabolite, Microcolony performance, Bumble bee health, Parasite

47 For their own subsistence and that of their offsprings, bee females mostly forage on two floral
48 resources, namely nectar as main source of carbohydrates (Nicolson & Thornburg, 2007), and pollen as
49 main source of proteins and lipids (Campos et al., 2008). Among these nutritional resources, the pollen
50 chemical composition is particularly complex and highly variable among plant species (Vaudo et al., 2020).
51 While pollen central metabolites, for instance, the protein-to-lipid ratio, play a crucial role in bee health,
52 development, and fitness (Di Pasquale et al., 2013), pollen also contains numerous specialised metabolites
53 (e.g., alkaloids, flavonoids and terpenoids, Irwin et al., 2014; Palmer-Young et al., 2019). The biological
54 activities of these metabolites are multiple so that they may be involved in protecting pollen from abiotic
55 factors, such as UVs (Li et al., 1993), but also from biotic factors, acting as antibacterial, antifungal or
56 insecticidal compounds (Pusztahelyi et al., 2015; Zaynab et al., 2018). When ingesting pollen, bees are then
57 exposed to all these biological activities that may be beneficial, for instance by reducing parasite load
58 through antimicrobial activities (Manson et al., 2010; Biller et al., 2015; Richardson et al., 2015), but also
59 detrimental, for instance by impairing resource collection (Wang et al., 2019; Brochu et al., 2020),
60 decreasing offspring size and production (Arnold et al., 2014), inducing larvae or imago death (Hendriksma
61 et al., 2011; Weber, 2004), and altering immune system (Gekièrè et al., 2022a). Given these opposite
62 effects on bees, it is essential to question how specific specialised metabolites may impact bee health,
63 especially in a changing world with multiple environmental pressures.

64
65 In the current context of biodiversity erosion (Butchart et al., 2010), bees are unfortunately no
66 exception, and many threats have been pinpointed as responsible for their negative population trends
67 (Dicks et al., 2021) such as pesticide exposure (Sánchez-Bayo & Goka, 2014), metalloid pollution (Gekièrè
68 et al., 2023), habitat loss (Baude et al., 2016), resource scarcity (Naug, 2009), competition with
69 domesticated species (Mallinger et al., 2017), and diseases (Van Engelsdorp et al., 2009). Among
70 environmental challenges, bees indeed suffer from a high diversity of pathogens and parasites (Meeus et
71 al., 2011; Goulson & Hughes, 2015) of which effects vary from small ethological alterations of minor
72 consequences (Paris et al., 2018) to large reductions in host bee fitness (McMenamin & Genersch, 2015).
73 Social bee species such as bumble bees (Apidae; *Bombus* spp.) are particularly impacted by parasites, the
74 latter benefiting from their social system to readily infect numerous individuals (Folly et al., 2017). One of
75 the most prevalent parasites in wild bumble bee populations is the gut trypanosomatid *Crithidia bombi*
76 Lipa & Triggiani, 1980 (Euglenozoa: Trypanosomatidae; Schmid-Hempel, 2001). Despite its generally
77 moderate impacts, it can decrease foraging effectiveness (Otterstatter et al., 2005), offspring production
78 (Schmid-Hempel, 1998), queen survival through hibernation (Fauser et al., 2017), and increase mortality in
79 synergy with other stresses (Brown et al., 2000). To deal with such parasite pressure, bumble bees may
80 rely on specific floral resources displaying appropriate antimicrobial properties through their specialised
81 metabolites (Manson et al., 2010; Biller et al., 2015; Richardson et al., 2015; Fitch et al., 2022).

82
83 Among potential medicinal floral resources, the heather (*Calluna vulgaris* Hull. 1808), an Ericaceae
84 commonly foraged by bumble bees (Descamps et al., 2015), produces a nectar documented to affect *C.*
85 *bombi* (Koch et al., 2019). This effect has been attributed to the presence of callunene, a terpenoid that
86 induces the loss of *C. bombi* flagellum, preventing the parasite from settling in the bumble bee digestive
87 tract (Koch et al., 2019). Such medicative properties of heather nectar make heather-rich heathlands even
88 more valuable for these bumble bees (Descamps et al., 2015; Moquet et al., 2017). However, although
89 heather is a major resource for European bees, only a handful of studies have sought for specialised
90 metabolites with biological activities in heather pollen, showing a high prevalence of flavonoids (Gekièrè
91 et al., *in prep.*). Flavonoids can have very contrasting effects on insect-plant interactions and affect them
92 in multiple ways (Simmonds 2003; Onyilagha et al., 2012). Bees are attracted to some flavonoid compounds
93 (e.g. quercetin; Liao et al. 2017a) while others repel them (e.g. kaempferol, catechin; Detzel & Wink 1993;
94 Onkokesung et al., 2014). However, despite some deleterious effects on larval development (Wang et al.,
95 2010), flavonoids are mainly not toxic molecules for insects (Detzel & Wink, 1993). Once ingested,
96 flavonoids can have antioxidant properties and are potentially beneficial for bees (e.g. quercetin; Treutter,
97 2005). They can stimulate the activation of detoxification enzymes (cytochrome P450 monooxygenase) and
98 enhance bee resistance to certain insecticides and acaricides (Scott et al., 1998; Johnson et al. 2012; Liao

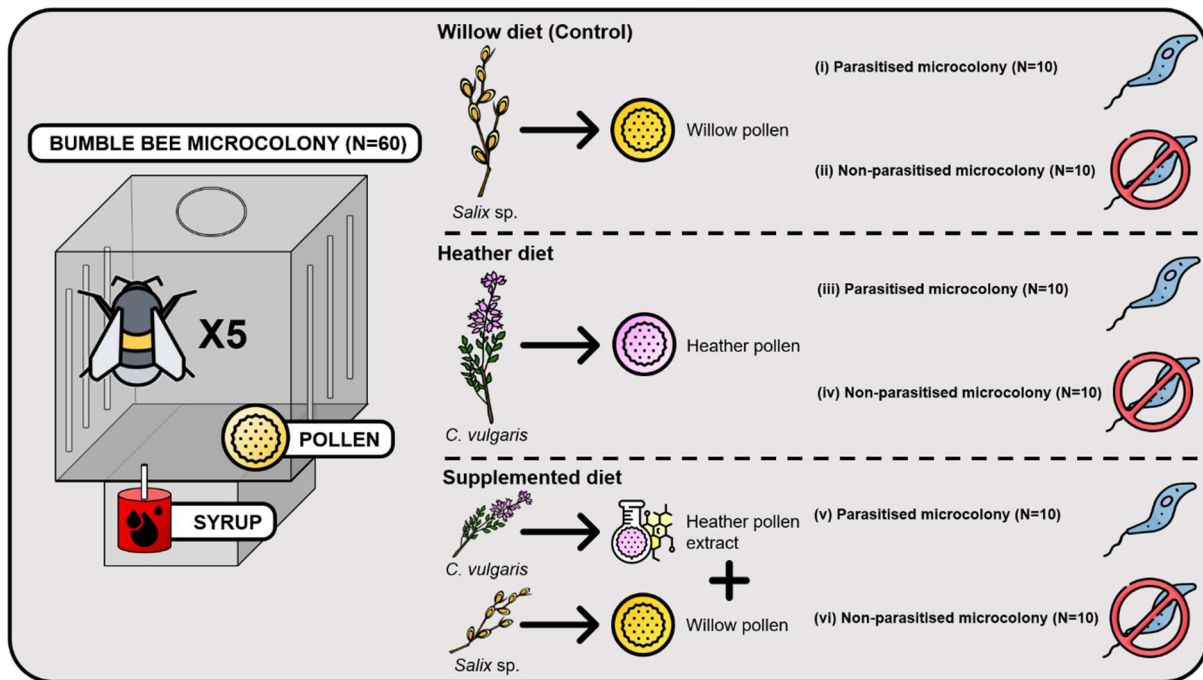
99 et al., 2017b). The case of heather pollen flavonoids remains to be addressed and this incomplete picture
100 of the pollen side does not allow for fully arguing that heather is a bumble bee health-promoting plant.
101 Therefore, bioassays to determine heather pollen effects on bumble bee brood, bumble bee health, and
102 parasite dynamics are warranted. To fill this gap, we herein present a study that aimed to assess the effects
103 of heather pollen and its flavonoids on bumble bee health, at both individual and colony levels, considering
104 the bumble bee interaction with the parasite. We specifically addressed the following questions: (i) how
105 does the parasite influence the development of bumble bee microcolonies and individual
106 immunocompetence? (ii) do heather pollen and its flavonoids challenge bumble bees, impacting their
107 resource collection and offspring production? (iii) do heather pollen and its flavonoids affect the parasite
108 dynamic in infected bumble bee workers, or help bumble bees to counteract parasite effects? We expect
109 (i) a mild effect of the parasite on bumble bees reared in optimal conditions; (ii) detrimental effects of
110 flavonoids, and potentially of heather pollen on healthy bumble bees and microcolonies; and (iii) beneficial
111 effects of heather pollen, and potentially its flavonoids, on infected bumble bees by reducing parasite load.

112

Materials and methods

113 Bumble bee bioassays

114 Queenless microcolonies of five workers were exposed to specific diet treatments (Fig.1): control pollen
115 (i.e., willow pollen is used because artificial pollen is unsuitable for bumblebee development and because
116 its flavonoid profile does not overlap with any flavonoids found in heather pollen; Gekière et al., 2022b;
117 Gekière et al., *in prep*) containing either (i) parasitised or (ii) non-parasitised bumble bees; heather pollen
118 containing either (iii) parasitised or (iv) non-parasitised bumble bees; microcolonies fed with willow pollen
119 supplemented with extracts of flavonoids from heather pollen containing either (v) parasitised or (vi) non-
120 parasitised bumble bees. Diets (i) and (ii) were used as controls as well as to assess the parasite impacts.
121 Diets (iii) and (v) were used to establish the effects of heather pollen or its flavonoids on infected
122 microcolonies. Diets (iv) and (vi) were used to establish the effects of heather pollen or its flavonoids on
123 uninfected microcolonies. For each treatment, ten microcolonies have been established using five different
124 queenright colonies (colonies from Biobest *bvba*; Westerlo, Belgium) (2 microcolonies per colony per
125 treatment). Colonies of the species *Bombus terrestris* were selected since this species is easy to rear and a
126 natural forager of heather pollen (Kleijn & Raemakers, 2008; Ballantyne et al., 2015). Faeces of queenright
127 colonies were observed under the microscope to confirm the absence of parasites (*Nosema* spp., *Apicystis*
128 spp. and *Crithidia* spp.) as guaranteed by the supplier. The microcolonies were kept in plastic boxes (10 x
129 16 x 16 cm; Regali & Rasmont, 1995) and reared at the University of Mons (Belgium, Mons, Campus de
130 Nimy, WGS84 50°27'54.9"N 3°57'24.9"E) in a dark room at 26-28°C and 65% of relative humidity. Bumble
131 bees were provided *ad libitum* with sugar syrup (water/sugar 35:65 w/w) and pollen candies (i.e., pollen
132 mixed with a 65% sugar solution) for 35 days, with pollen candies being freshly prepared and renewed
133 every two days. When workers died, they were discarded, weighed and replaced by a worker from the
134 same queenright colony, which was marked with a colour dot on the scutum. Larvae ejected from the
135 brood were also checked every day, counted and discarded from the microcolonies. Microcolonies were
136 handled under red light to minimise disturbance.



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Figure 1. Bioassay design. Microcolonies initiated with five *B. terrestris* workers were fed for 35 days with one of three diets. For each diet, ten microcolonies contained parasitised individuals (*Crithidia* sp.), ten others were non-parasitised. Icon used for the figure: <https://www.flaticon.com/>. and author conception.

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Diet preparation

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Willow pollen batch (i.e., pollen loads from *Apis mellifera* L. 1758) was supplied by the commercial company *Ruchers de Lorraine* (Nancy, France) while heather pollen batch was obtained from a private beekeeper (Dittlo François, France, Gironde, Le Nizan). Although honey bee collected pollen loads may contain parasites, analysis of faeces of uninfected microcolonies fed with this pollen diet were parasite free. We then consider that no contamination occurred from the pollen batch. Pollen loads from the heather batch were hand-sorted based on the colour after microscopical identification to ensure monoflorality (800 g in total) (Sawyer & Pickard, 1981; Dafni et al., 2005). Each pollen batch was then homogenised and crushed before being used for the experiments. Half of the sorted heather batch served directly for the bioassays; the other half served for massive extraction of flavonoids. Flavonoids were extracted using a Soxhlet extraction for approximately 40 cycles with methanol as solvent, at 100°C. Extract was then vacuum filtered and evaporated to dryness (rotavapor IKA RV8). For flavonoid purification, extract was solubilized in water with a minimal amount of methanol, and placed in a separatory funnel with dichloromethane. The funnel was shaken and left to settle overnight before recovering the aqueous phase. The purified extract was then dried using rotary evaporator and dissolved in aqueous ethanol solution (1:1, v/v) before addition to the control diet to prepare a flavonoid-supplemented diet. Control and heather pollen diets were also supplemented with a similar amount of ethanol to avoid any bias (for details see Appendix A, Table S1).

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Parasite inoculation

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Multiple morphologically identical trypanosomes affect *B. terrestris* (Bartolomé et al., 2021). Although *Crithidia bombi* is by far the most abundant in wild populations (Shykoff & Schmid-Hempel, 1991; Popp et al., 2012), parasite identification will be limited to *Crithidia* sp. in this manuscript to avoid misinterpretation. Parasite inoculation was performed using *Crithidia* sp. reservoirs maintained in the laboratory (i.e., commercial colonies regularly renewed and repeatedly inoculated with contaminated faeces in order to ensure a turnover of the available *Crithidia* sp. pool). Faeces from a total of 45 infected workers were collected and pooled together to ensure multiple-strain inoculum (Gekièrè et al., 2022a). The inoculum was homogenised, brought to 1 mL with 0.9% NaCl solution, and purified by a triangulation method (Cole, 1970) adapted by Baron et al. (2014) and Martin et al. (2018). The concentration of *Crithidia*

170 sp. cells was then estimated by counting with a Neubauer chamber, and the inoculum was diluted to 2,500
171 *Crithidia* sp. cells/ μ L with a 40% sugar solution. Workers allocated to the infected microcolonies were
172 placed in individual Nicot[®] queen rearing cages and given 10 μ L of the inoculum (i.e., 25,000 *Crithidia* sp.
173 cells; Logan et al., 2005) by letting them feed on the sugar solution in a glass microcapillary after a 5-hour
174 starvation period. Workers allocated to uninfected microcolonies also underwent the same treatment
175 (isolation, starvation) but with 10 μ L of sterile sugar solution.

176 **Parameters evaluated**

177 To investigate the impacts of pollen diet and parasite, several parameters in microcolonies were
178 measured (Tasei & Aupinel, 2008), namely resource collection, reproductive success, stress response, and
179 individual health through fat body content (i.e., immunocompetence proxy; Arrese & Soulages, 2010;
180 Rosales, 2017; Vanderplanck et al., 2021) and parasite load measurements.

181
182 Resource collection was assessed by weighing every two days in each microcolony the syrup container,
183 as well as the recovered pollen candy and the newly introduced one. These data were corrected for
184 evaporation using controls, as well as divided by the total worker mass by microcolony to avoid bias due
185 to worker activity. To evaluate the reproductive success, all microcolonies were dissected at the end of the
186 experiment (Day 35) to weigh the total hatched brood mass, as well as the individual mass of each emerged
187 male used as reference of viable offspring at the end of development (Goulson, 2010). Offspring masses
188 were divided by the total worker mass by microcolony to avoid any bias due to worker care. Regarding
189 stress response, we assessed worker mortality, larval ejection, pollen dilution (ratio between the collection
190 of syrup and pollen) as well as pollen efficiency (ratio between offspring mass and pollen collection; Tasei
191 & Aupinel, 2008) that highlights when a micro-colony needs to consume more pollen to produce offspring.

192
193 For the individual health parameters, fat body content was measured at the end of the bioassays on
194 two males and two workers per microcolony (40 individuals per treatment) following Ellers (1996). The
195 abdomens were cut and dehydrated in an incubator at 70°C for three days before being weighed. They
196 were then placed for one day in 2mL of diethyl ether to solubilise lipids constituting the fat body. The
197 abdomens were then washed twice with diethyl ether, and incubated at 70°C for seven days before being
198 weighed. Fat body content was defined as the mass difference between dry abdomen before and after
199 lipid solubilisation, divided by the dry abdomen mass prior to solubilisation. Moreover, in infected
200 treatments, we repeatedly monitored the parasite load within microcolonies using the same marked
201 worker along the bioassays. The first measurement was made three days post-inoculation (day 4) to enable
202 *Crithidia* sp. to multiply and ensure its presence in the faeces (Logan et al., 2005). A total of seven further
203 measurements were taken to establish the infection curve of *Crithidia* sp., namely on days 6, 8, 10, 12, 16,
204 20 and 35. Measurements were performed at larger intervals after day 12 because infection reached the
205 plateau phase (Schmid-Hempel & Schmid-Hempel, 1993; Otterstatter & Thomson, 2006). In practice, the
206 marked worker was held in a 50mL Falcon in the light until the faeces were expelled. Faeces were then
207 collected in a 10 μ L microcapillary tube and diluted two to ten times with distilled water to enable rational
208 cell counting. Parasite cells were then counted using a haemocytometer (Neubauer) under an inverted
209 phase contrast microscope (400X magnification, Eclipse Ts2R, Nikon). Uninfected microcolonies faeces
210 were checked to be free of parasites at the end of the experiment, and a marked worker was isolated at
211 days 4, 6, 8, 10, 12, 16, 20 and 35 in each uninfected microcolony to induce the same stress as in infected
212 treatments.

213 **Data analysis and statistics**

214 To detect a potential effect of pollen diet or parasite on resource collection, reproductive success,
215 stress response, and individual health, mixed models were fitted for each parameter using diet, parasite
216 and their interaction as fixed factors, and colony as random factor. Pollen collection, pollen efficacy, and
217 pollen dilution (log-transformed data) were analysed using a Gaussian distribution (i.e., normality of
218 residuals; *shapiro.test* function from the stats R-package v.4.1.0; R Core Team, 2021) (*lmer* function from
219 the nlme R package v.3.1.160; Pinheiro et al., 2022). Total hatched offspring mass, emerged male mass,
220 and fat body content (i.e., proportion data) were analysed using a Gamma distribution and a log link
221 function (*glmmTMB* function from the glmmTMB R-package v.1.1.4; Brooks et al., 2017). For fat body

222 content, values were square root-transformed and sex was added as crossed-fixed effect. For emerged
223 male mass and fat body content, microcolony was nested within colony in the random factor to deal with
224 pseudo-replication (i.e., several measures per microcolony).

225

226 For larval ejection, a binomial distribution (ejected larvae and total number of living offspring produced
227 as bivariate response) was used after checking for overdispersion and zero inflation (*testDispersion* and
228 *testZeroInflation* functions from DHARMA R-package v.0.4.6; Hartig, 2022). For worker mortality, a Cox
229 proportional hazard (mixed-effect) model was run with individuals alive at the end of the 35-day treatment
230 assigned as censored, and those who died as uncensored (*coxme* function from the *coxme* R-package
231 v.2.2.18.1; Therneau, 2022). For these two parameters, diet, parasite and their interaction were also used
232 as fixed factors and colony was included as a random factor.

233

234 The last parameter measured was the parasite load at different time points within infected
235 microcolonies. As infection dynamics is a discrete time series, it was analysed using a generalised additive
236 mixed-effect model (GAMM; Wood, 2006). Parasite loads were square root-transformed and fitted using
237 a Gaussian distribution with a log link. Diet and day were set as fixed factors and microcolony nested within
238 colony as random factors. The model assumptions were tested using diagnostic graphs and tests.

239

240 Contrasts were then performed on the models to determine whether the uninfected control differed
241 from the infected control, and whether effects on uninfected or infected microcolonies differed among
242 diets (*emmeans* function from the *emmeans* R-package v.1.8.2; Lenth, 2022). For fat body content, data
243 were analysed separately for workers and males as a sex-significant effect was detected. Graphs and plots
244 were all performed using the R-package *ggplot2* v.3.4.0 (Wickham, 2016) except the one referring to the
245 survival probability of the workers performed with the *ggsurvplot* function of the *survminer* R-package
246 v.0.4.9 (Kassambara et al., 2021). All the statistical analyses were done using the R software v.4.1.0 (R Core
247 Team, 2021). For all statistical analyses, $p < 0.05$ was used as a threshold for significance.

248

Results

249 Parasite impact

250 Comparison of microcolonies fed with control pollen between parasitised and non-parasitised
251 treatments showed that *Crithidia* sp. infection did not impact the parameters related to resource collection
252 (Fig 2A), reproductive success (Fig 2B-2C), or stress response (Fig 3A-C) ($p > 0.05$, Fig. 2 and 3). However,
253 fat body content in newly emerged males was significantly higher with a mean that increased by 56% in
254 infected microcolonies fed the control diet compared to uninfected ones fed the same diet ($t = -3.828$, $p =$
255 0.0012 ; Fig. 4B). The estimates (mean \pm standard error) of our variables for each treatment are available in
256 the appendices (Appendix B, Table S2)

257

258 Effect of heather pollen and its flavonoids on healthy bumble bees

259 Regarding resource collection, total pollen collection was significantly lower in microcolonies fed the
260 supplemented diet compared to those fed the other diets (control vs supplemented: 43% less pollen
261 collected, $t = -5.672$, $p < 0.001$; heather vs supplemented: 33% less pollen collected, $t = 3.924$, $p < 0.001$;
262 Fig. 2A). With regards to the reproductive success, microcolonies fed the supplemented and heather diets
263 produced a significantly lower brood mass compared to microcolonies fed the control diet (control vs
264 supplemented: brood mass 52% lower, $t = 3.890$, $p < 0.001$; control vs heather: brood mass 32% lower, $t =$
265 2.189 , $p = 0.0331$; Fig. 2B), as well as significantly smaller emerged males (control vs supplemented: $t =$
266 2.350 , $p = 0.0192$; control vs heather: $t = 2.925$, $p = 0.0036$; Fig. 2C)

267

268 In terms of stress responses, pollen dilution was significantly higher in microcolonies fed the
269 supplemented diet compared to those fed the other diets (control vs supplemented: $t = 2.282$, $p = 0.0268$;
270 heather vs supplemented: $t = -3.191$, $p = 0.0025$; Fig. 3A). Microcolonies fed the heather or supplemented
271 diets also displayed a lower pollen efficacy than the microcolonies fed the control diet (control vs
272 supplemented $t = -2.741$, $p = 0.0085$; control vs heather: $t = -3.025$, $p = 0.0039$; Fig. 3B). On the contrary,

273 no significant difference was detected for larval ejection ($p > 0.05$) or for worker mortality ($p < 0.05$, Fig.
274 3C).

275

276 Regarding individual health, while no difference was detected in worker fat body content among diet
277 treatments ($p > 0.05$; Fig. 4A), fat body content in newly emerged males was significantly higher in
278 microcolonies fed the supplemented or heather diets compared to those fed the control diet (control vs
279 supplemented: fat body content 62% higher, $t = -3.891$, $p = 0.0012$; control vs heather: fat body content
280 41% higher, $t = 2.850$, $p = 0.0223$; Fig. 4B).

281 **Effect of heather pollen and its flavonoids on parasitised bumble bees**

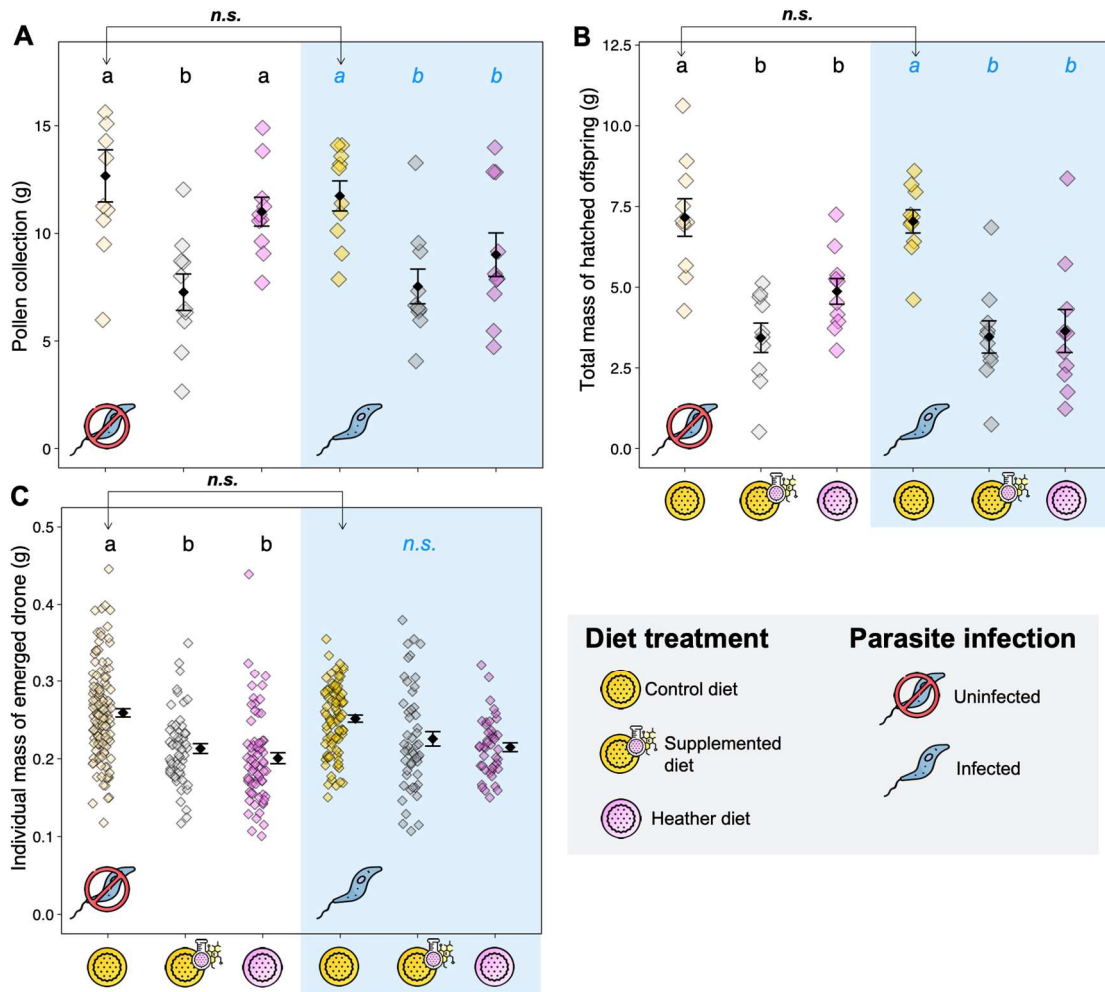
282 Similarly to previous results with uninfected microcolonies, total pollen collection was significantly
283 lower in infected microcolonies fed the supplemented diet than in microcolonies fed the control diet (36%
284 less pollen collected, $t = -4.414$, $p < 0.001$), but also in infected microcolonies fed heather diet compared
285 to those fed the control diet (16% less pollen collected, $t = -2.866$, $p = 0.0061$) (Fig. 2A). Regarding the
286 reproductive success, as observed in uninfected microcolonies, microcolonies fed the supplemented and
287 heather diets produced a significantly lower brood mass compared to microcolonies fed the control diet
288 (control vs supplemented: brood mass 51% lower, $t = 3.784$, $p < 0.001$; control vs heather: brood mass
289 41% lower, $t = 3.551$, $p < 0.001$; Fig. 2B). However, no significant difference was detected for the mass of
290 newly emerged males among diet treatments ($p < 0.05$; Fig. 2C).

291

292 Regarding stress responses, pollen dilution was significantly higher in microcolonies fed the
293 supplemented diet than in microcolonies fed the other diets (control vs supplemented: $t = 2.111$, $p =$
294 0.0398 ; heather vs supplemented: $t = -2.120$, $p = 0.0390$; Fig. 3A). Microcolonies fed the heather or
295 supplemented diets also displayed a lower pollen efficacy than those fed the control diet (control vs
296 supplemented: $t = -3.684$, $p < 0.001$; control vs heather: $t = -4.904$, $p < 0.001$; Fig. 3B). While no significant
297 difference was detected for larval ejection ($p > 0.05$), the worker survival probability was significantly
298 reduced in infected microcolonies fed the heather diet compared to those fed either the control or
299 supplemented diets (heather vs control: $t = -2.265$, $p = 0.0235$; heather vs supplemented: $t = -3.331$, $p <$
300 0.001 ; Fig. 3C).

301

302 In terms of individual health, no difference was detected in fat body content of workers or newly
303 emerged males among diet treatments ($p > 0.05$; Figs. 4A and 4B). Regarding the parasite load, the infection
304 dynamic was more gradual in infected microcolonies fed the control diet compared to those fed the other
305 diets which supported a parasite load peak around day 20 before a decrease up to the end of treatment
306 (supplemented vs control: $t = 2.893$, $p = 0.0126$; heather vs control: $t = 2.328$, $p = 0.0313$; Fig. 4C).



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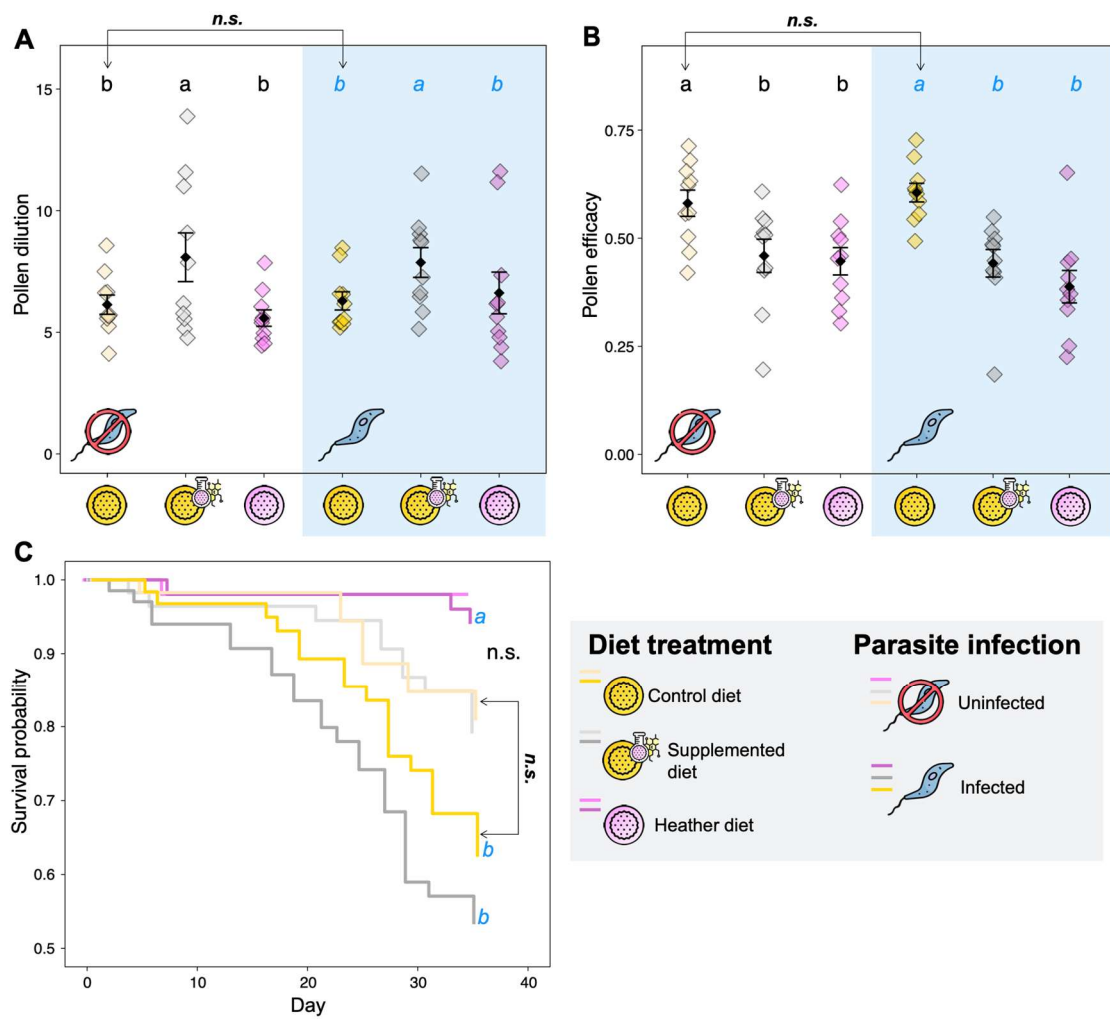
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Figure 2. Resource collection and reproductive success. (A) Total mass of collected pollen; (B) Total mass of hatched offspring produced; and (C) Individual mass of emerged males. Each coloured data point represents a microcolony (in A and B) or an individual (in C), diamonds are mean values of each treatment, and error bars indicate the standard error. For (C), means and error bars have been shifted in the graph to improve readability. Letters indicate significance at $p < 0.05$ (pairwise comparisons within uninfected treatments in black, and pairwise comparisons within infected treatments in blue); n.s., non-significant. Arrows indicate the pairwise comparisons for the control diet between infection treatments (i.e., parasite effect). Symbol caption is in the grey zone.



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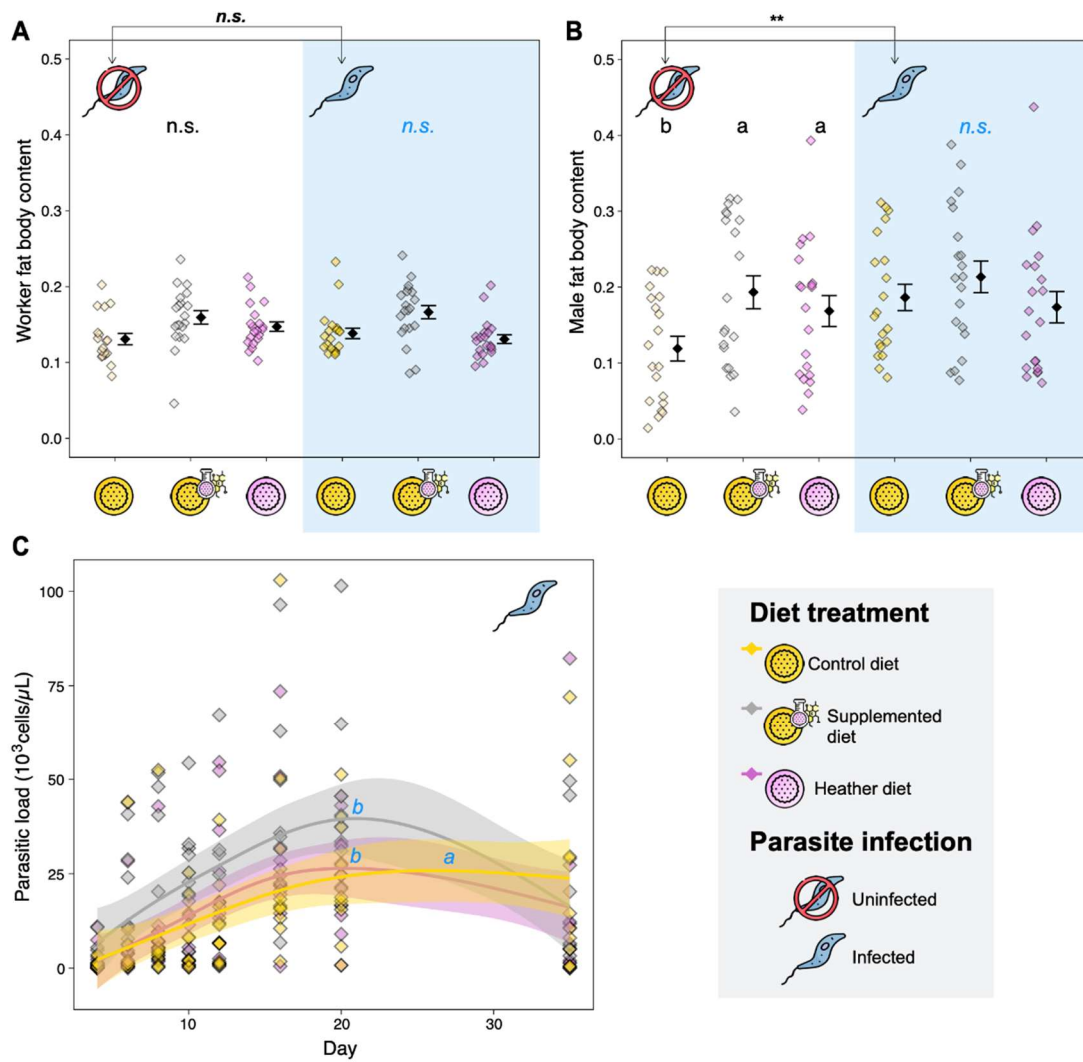
317 **Figure 3. Stress responses.** (A) Pollen dilution, defined as the ratio between syrup and pollen
 318 collection; (B) Pollen efficacy, defined as the ratio between total mass of hatched offspring and pollen
 319 collection; and (C) Worker survival probability over time. For (A) and (B), each coloured data point
 320 represents a microcolony, diamonds are mean values of each treatment, and error bars indicate the
 321 standard error. Letters indicate significance at $p < 0.05$ (pairwise comparisons within uninfected
 322 treatments in black, and pairwise comparisons within infected treatments in blue); n.s., non-
 323 significant. Arrows indicate the pairwise comparisons for the control diet between infection
 324 treatments (i.e., parasite effect). Symbol caption is in the grey zone.

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331 **Figure 4. Health parameters. (A)** Worker fat body content, and **(B)** Male fat body content; each

332 coloured data point represents a microcolony, diamonds are mean values of each treatment, and

333 error bars indicate the standard error. Means and error bars have been shifted in the graphs to

334 improve readability. **(C)** Parasite load over time. Generalized additive mixed-effect models (in C) were

335 used to fit smoothers to the data showing mean trends [$\pm 95\%$ confidence intervals, light coloured

336 bands] over time. Here, each dot represents one data point (i.e., parasite load for the monitored

337 worker for each time point and each microcolony). Letters indicate significance at $p < 0.05$ (pairwise

338 comparisons within uninfected treatments in black, and pairwise comparisons within infected

339 treatments in blue); n.s., non-significant; **, $p < 0.01$. Arrows indicate the pairwise comparisons for

340 the control diet between infection treatments (i.e., parasite effect). Symbol caption is in the grey

341 zone.

341

343 Parasite effect on bumble bee

344 The parasite had no impact neither on larval ejection, total mass of offspring produced, nor on
345 individual mass of newly emerged males. Such results suggest that infection is unlikely to reduce colony
346 and offspring fitness, or dissemination success, which are related to individual size (Greenleaf et al., 2007;
347 Amin et al., 2012). The limited effects of *Crithidia* sp. on the reproductive success of bumble bees herein
348 observed are in line with the literature (Brown et al., 2003; Goulson et al., 2018; Gekière et al., 2022a). This
349 absence of impacts on development performance and offspring fitness may stem from the fact that the
350 parasite only infects the adult stage (i.e., *Crithidia* sp. does not develop in bumble bee larvae, Folly et al.,
351 2017).

352

353 Besides our results showed that *Crithidia* sp. induced larger fat body content in males emerged in
354 infected microcolonies compared to those that emerged in uninfected ones, whereas it has no impact on
355 worker fat body content. We propose two rationales to explain such a *Crithidia*-induced difference in fat
356 body content only in newly emerged males and not in workers. First, newly emerged males and workers
357 were likely not inoculated at the same age. Indeed, workers developed in healthy colonies and were
358 inoculated at the adult stage (most likely > 2 days old) for the establishment of infected microcolonies.
359 However, males (most likely one day old) developed in infected microcolonies and ingested *Crithidia* sp.
360 cells upon emergence resulting in the infection of up to 90% of them (Gekière et al., 2021, *unpublished*
361 *results*). Second, while the difference in male fat body content between infected and uninfected
362 microcolonies is unlikely to have arisen from a difference in brood care (i.e., no significant difference in
363 pollen efficacy), we cannot rule out that infected workers displayed specific brood caring behaviour. For
364 instance, they could have added peculiar nutrients or microorganisms to larval food from their
365 hypopharyngeal and mandibular glands and/or stomach to prepare their offspring to face infection (e.g.,
366 addition of sterols, Svoboda et al., 1986). Such an increase in offspring fat body content through adapted
367 larval feeding by workers could be interpreted as a trans-generational prophylactic behaviour. Indeed,
368 enhanced fat body content has been assumed to correspond to a specific allocation of resources to
369 counteract parasites by mounting an immune response (Brown et al., 2003). It would be interesting to test
370 whether infected workers provide their larvae with specific central and specialised metabolites. We should
371 however underline that the relationship between fat body content and immunity has become controversial
372 since some results have been contradictory (e.g., Brown et al., 2000, Gekière et al., 2022a).

373

374 Although *Crithidia* sp. only showed mild effects in our experiment and in previous laboratory
375 experiments (Brown et al., 2003; Goulson et al., 2018; Gekière et al., 2022a), it is important to keep in mind
376 that interpretation of results observed in laboratory conditions must be interpreted with caution as
377 controlled conditions are often not representative of natural constraints encountered in the field such as
378 for instance, predation, flight, and foraging. For example, infection by *Crithidia* sp. has been shown to
379 impair pollen foraging (Shykoff & Schmid-Hempel, 1991; Otterstatter et al., 2005; Gegeer et al., 2006), but
380 such effects cannot be fully comprehended under laboratory conditions.

381 Heather pollen quality: the case of flavonoids

382 Heather pollen harbours kaempferol flavonoids linked to one/two coumaroyl groups which are also
383 linked to one/two hexosides (Gekière et al., *in prep*). Herein, we have shown that these heather flavonoids
384 reduced the total offspring production, as indicated by a decreased pollen collection and a lower pollen
385 efficacy, as well as a reduced mass of newly emerged males, thereby altering microcolony performance.
386 Indeed, drone mass is known to impact flight distances, but also reproductive abilities, affecting the
387 dissemination and reproductive success of bumble bee populations (Greenleaf et al., 2007; Amin et al.,
388 2012). Such poor quality of heather pollen for the maintenance of buff-tailed bumble bee microcolonies
389 has already been pinpointed (Vanderplanck et al., 2014). While it was partly attributed to its nutritional
390 content (i.e., low concentration of amino acids and abundance of δ -7-avenasterol and δ -7-stigmasterol,
391 Huang et al., 2011, Vanderplanck et al., 2014), our study demonstrated that specialised metabolites may
392 also impact heather pollen quality, regardless of its nutritional content (i.e., central metabolites).

393

394 Both heather pollen and its flavonoids showed detrimental effects (i.e. reduction of offspring
395 production, pollen efficacy). However, heather flavonoids seemed to induce a higher stress response than
396 heather pollen as dilution behaviour was significantly higher in microcolonies fed the supplemented diet
397 compared to those fed the control diet (i.e., mixing behaviour to mitigate unfavourable diet properties,
398 Berenbaum & Johnson, 2015; Vanderplanck et al., 2018) while such a difference was not observed for
399 microcolonies fed the heather diet. The reason for this discrepancy is not obvious, as both diets harbour
400 the same flavonoids and should therefore lead to similar dilution behaviour. Two hypotheses could be
401 proposed to explain this difference: (i) flavonoids were more bioavailable in the supplemented diets
402 (outside pollen grains after the chemical extraction) and then more easily absorbed by the workers, which
403 ultimately reduced the diet palatability (Wang et al., 2019); and (ii) as flavonoid extract was added to the
404 control diet (i.e., willow pollen) that already contained flavonoids, the supplemented diet was richer in
405 flavonoids than the other diets, reaching a threshold that ultimately reduced the diet palatability.
406 Unfortunately, it is not possible to unravel these hypotheses without additional experiments. Another
407 observation supporting the potential toxicity of heather flavonoids is the increase in fat body content in
408 males emerging from microcolonies fed heather and supplemented diets compared to those emerging in
409 microcolonies fed the control diet. Indeed, such an increase could be interpreted as a specific allocation of
410 resources to the fat body for performing a detoxification activity (Li et al., 2019). In that way, flavonoid
411 assimilation is known to induce the activation of defence mechanisms based on cytochrome P450
412 monooxygenase, a molecule that is highly active in the fat body (Scott et al., 1998). This increase in fat
413 body content was not observed in workers, which could be explained by the different exposure to
414 flavonoids during their life stages. Indeed, workers within microcolonies mainly fed on syrup, while males
415 fed on pollen during their whole larval development and were then more exposed to specialised
416 metabolites. Moreover, it is highly possible that sensitivity to pollen-specialised metabolites is higher in
417 larvae than in adults, as already demonstrated in honey bees (Lucchetti et al., 2018).

418 **The complex response of parasitised bumble bees to heather pollen and its flavonoids**

419 Flavonoids were associated with an increase in parasite load, which has been also observed for other
420 classes of specialised metabolites (Thorburn et al., 2015; Gekière et al., 2022a). Therefore, by contrast to
421 our expectations based on previous studies (Baracchi et al., 2015, Koch et al., 2019), the detrimental effects
422 of heather pollen flavonoids on bumble bees were not balanced by any therapeutic effect against the
423 parasite *Crithidia* sp. These results suggest a potential additive effect between phytochemical and parasite
424 stress as previously described (Thorburn et al., 2015), with the diet effect mostly overriding the parasite
425 effect in bumble bees in the case of heather as already shown for the sunflower (Gekière et al., 2022a).
426 The nutritional stress due to heather pollen feeding could then increase the effect of *Crithidia* sp. which
427 could be more effective under stressful conditions (Brown et al., 2000; Brown et al., 2003).

428
429 We found that mortality in infected microcolonies was lower in microcolonies fed the heather diet
430 compared to those fed the control diet. Although infection was not shown to have a significant impact on
431 mortality in microcolonies fed the control diet, heather pollen could then increase host tolerance to the
432 parasite but this effect is unlikely due to its flavonoid content as mortality in infected workers fed the
433 supplemented diet did not significantly differ from those fed the control diet. Regarding fat body content
434 in newly emerged males, males that emerged in microcolonies fed the heather or supplemented diets
435 displayed higher fat body content than those that emerged in microcolonies fed the control diet, but this
436 diet effect was not significant anymore in infected microcolonies, probably because, as discussed before,
437 the parasitic stress also increased fat body content in microcolonies fed the control diet.

438 **Conclusion**

439 How heather pollen and its specialised metabolites impact the buff-tailed bumble bee, and how they
440 modulate the interaction with its obligate gut parasite *Crithidia* sp. are complex questions given the
441 diversity of specialised metabolites found in the floral resources of this species. Previous studies have
442 found that heather nectar does not contain any flavonoids (Gekière et al., in prep) but protected the
443 pollinator from its parasite *Crithidia* sp. through callunene activity (Koch et al., 2019). In this study, we
444 found that the occurrence of flavonoids in heather pollen reduced its collection, as well as the bumble bee

445 fitness. Moreover, heather pollen did not help to counteract the parasite but rather appeared to induce an
446 additional stress that could potentially increase the parasite effect. Actually, our results complete the
447 understanding of the bumble bee-heather-parasite relationship by underlining that heather pollen is not
448 suitable for buff-tailed bumble bee performance and does not display any therapeutic effect. This study
449 highlights the complexity of the plant-pollinator interaction by illustrating the distinct roles and effects of
450 specialised metabolites found either in nectar or pollen. We strongly encourage the consideration of these
451 two floral resources in future studies investigating the medicinal effects of plant species, especially when
452 defining pollinator conservation strategies.

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464 **Authors’ contributions**

465 Conceptualization, M.V.; chemical analyses and extraction, I.S. and P.G.; bioassays, C.T., with help from
466 A.M. and A.G.; resources, D.M. and P.G.; writing—original draft preparation, C.T., with help from A.G. and
467 M.V.; supervision, A.G. and M.V.; funding acquisition, D.M., P.G. and M.V. All authors have read and agreed
468 to the published version of the manuscript.

469 **Conflict of interest disclosure**

470 The authors declare that they comply with the PCI rule of having no financial conflicts of interest in
471 relation to the content of the article.

472 **Data, script, and code information availability**

473 Datasets and R script are available on a Zenedo repository: <https://doi.org/10.5281/zenodo.7804841>

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Appendix A – Supplemented diet

728 Total flavonoid content of heather bee pollen, as well as its associated dried extract, were analysed in
 729 triplicates by HPLC-MS/MS (triplicates of 20 – 40 mg) for quantification (expressed as quercetin equivalent,
 730 QE). Based on these analyses, the supplementation formula was established to have a similar amount of
 731 ethanol and willow pollen in candies for all diets, as well as flavonoid concentrations in the supplemented
 732 diet mimicking natural concentration found on average in bee pollen candies, namely 12.08 mg QE/heather
 733 candy on average (14.73 ± 1.69 mg QE/g for heather bee pollen) (Table S1). We found that heather bee
 734 pollen extract contained 40.63 ± 0.72 mg QE/g (209.79 g extract).

735 **Table S1.** Diet compositions.

Diets	Ethanol ($\mu\text{L/g candy}$)	Pollen (g/g candy)	Flavonoids (mg/g candy)
Control diet	17.02	0.68	11.89
Heather pollen-supplemented diet	19.80	0.67	11.85 ¹
Heather diet	20.50	0.82	12.08

736 ¹ The concentration indicated for supplemented diets does not take into account the flavonoid
 737 concentration already present in the control diet (11.89 mg/g candy).

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Appendix B – Variable estimates

741 **Table S2.** Mean \pm standard error (SE) values of the variable used to describe parasite and diet effects.

Variable	Uninfected			Infected		
	Control	Supplemented	Heather	Control	Supplemented	Heather
Pollen collection	12.7 \pm 1.21	7.27 \pm 0.85	11.0 \pm 0.67	11.7 \pm 0.70	7.53 \pm 0.81	9.01 \pm 1.01
Total mass of hatched offspring	7.16 \pm 0.58	3.43 \pm 0.46	4.87 \pm 0.40	7.04 \pm 0.36	3.46 \pm 0.50	3.64 \pm 0.67
Individual mass of emerged drone	0.259 \pm 0.005	0.213 \pm 0.006	0.201 \pm 0.007	0.252 \pm 0.005	0.226 \pm 0.009	0.215 \pm 0.006
Pollen dilution	6.14 \pm 0.40	8.08 \pm 1.00	5.58 \pm 0.34	6.29 \pm 0.37	7.87 \pm 0.61	6.62 \pm 0.86
Pollen efficacy	0.581 \pm 0.030	0.459 \pm 0.038	0.447 \pm 0.032	0.605 \pm 0.022	0.442 \pm 0.032	0.388 \pm 0.037
Larval ejection	0.984 \pm 0.006	0.970 \pm 0.010	0.999 \pm 0.001	0.969 \pm 0.007	0.889 \pm 0.053	0.990 \pm 0.007
Worker fat body content	0.131 \pm 0.008	0.159 \pm 0.009	0.147 \pm 0.006	0.138 \pm 0.007	0.166 \pm 0.009	0.131 \pm 0.006
Male fat body content	0.119 \pm 0.016	0.193 \pm 0.022	0.168 \pm 0.020	0.186 \pm 0.017	0.213 \pm 0.021	0.173 \pm 0.021

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