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 find any medicinal effect of heather pollen or its flavonoids on parasitised bumble bees. Our results provide insights into the impact of pollen specialised metabolites on heather-bumble bee-parasite interactions. They underline the contrasting roles of the two floral resources for bumble bees and emphasize the importance of considering both nectar and pollen when addressing medicinal effects of a plant for pollinators.

Keywords. Plant-pollinator interaction, pollen specialised metabolite, microcolony performance, bumble bee health, parasite.

Introduction

For their own subsistence and that of their offspring, bee females mostly forage on two floral resources, namely nectar as main source of carbohydrates (Nicolson & Thornburg 2007), and pollen as main source of proteins and lipids (Campos et al. 2008). Among these nutritional resources, the chemical
composition of pollen is particularly complex and highly variable among plant species (VAUDO et al. 2020). While pollen central metabolites, for instance, the protein-to-lipid ratio, play a crucial role in bee health, development, and fitness (Di PASQUALE et al. 2013), pollen also contains numerous specialised metabolites (e.g., alkaloids, flavonoids and terpenoids, IRWIN et al. 2014; PALMER-YOUNG et al. 2019). The biological activities of these metabolites are multiple so that they may be involved in protecting pollen from abiotic factors, such as UVs (Li et al. 1993), but also from biotic factors, acting as antibacterial, antifungal or insecticidal compounds (PUSZTAHELYI et al. 2015; ZAYNAB et al. 2018). When ingesting pollen, bees are then exposed to all these biological activities that may be beneficial, for instance by reducing parasite load through antimicrobial activities (MANSON et al. 2010; BILLER et al. 2015; RICHARDSON et al. 2015), but also detrimental, for instance by impairing resource collection (WANG et al. 2019; BROCHU et al. 2020), decreasing offspring size and production (ARNOLD et al. 2014), inducing larvae or imago death (HENDRIKSMAN et al. 2011; WEBER 2004), and altering the immune system (GEKIÈRE et al. 2022a). Given these opposite effects on bees, it is essential to question how specific specialised metabolites may impact bee health, especially in a changing world with multiple environmental pressures.

In the current context of biodiversity erosion (BUTCHART et al. 2010), bees are unfortunately no exception, and many threats have been pinpointed as responsible for their negative population trends (DICKS et al. 2021) such as pesticide exposure (SANÇHEZ-BAYO & GOKA 2014), metalloid pollution (GEKIÈRE et al. 2023), habitat loss (BAUDE et al. 2016), resource scarcity (NAUG 2009), competition with domesticated species (MALLINGER et al. 2017), and diseases (VAN ENGELSDORP et al. 2009). Among environmental challenges, bees indeed suffer from a high diversity of pathogens and parasites (MEEUS et al. 2011; GOULSON & HUGHES 2015) of which effects vary from small ethological alterations of minor consequences (PARIS et al. 2018) to large reductions in host bee fitness (MCMENAMIN & GENERSCH 2015). Social bee species such as bumble bees (Apidae; Bombus spp.) are particularly impacted by parasites, the latter benefiting from their social system to readily infect numerous individuals (FOLLY et al. 2017). One of the most prevalent parasites in wild bumble bee populations is the gut trypanosomatid Crithidia bombi Lipa & Triggiani, 1980 (Euglenozoa: Trypanosomatidae; SCHMID-HEMPEL 2001). Despite its generally moderate impacts, it can decrease foraging effectiveness (OTTERSTATTER et al. 2005), offspring production (SCHMID-HEMPEL 1998), queen survival through hibernation (FAUSER et al. 2017), and increase mortality in synergy with other stresses (BROWN et al. 2000). To deal with such parasite pressure, bumble bees may rely on specific floral resources displaying appropriate antimicrobial properties through their specialised metabolites (MANSON et al. 2010; BILLER et al. 2015; RICHARDSON et al. 2015; FITCH et al. 2022).

Among potential medicinal floral resources, the heather (Calluna vulgaris Hull. 1808), an Ericaceae commonly foraged by bumble bees (DESCAMPS et al. 2015), produces a nectar documented to affect C. bombi (KOCH et al. 2019). This effect has been attributed to the presence of callunene, a terpenoid that induces the loss of C. bombi flagellum, preventing the parasite from settling in the bumble bee digestive tract (KOCH et al. 2019). Such medicative properties of heather nectar make heather-rich heathlands even more valuable for these bumble bees (DESCAMPS et al. 2015; MOQUET et al. 2017). However, although heather is a major resource for European bees, only a handful of studies have sought for specialised metabolites with biological activities in heather pollen, which show a high prevalence of flavonoids (GEKIÈRE et al. in prep.). Flavonoids can have very contrasting effects on insect-plant interactions and affect them in multiple ways (SIMMONDS 2003; ONYILAGHA et al. 2012). Bees are attracted to some flavonoid compounds (e.g., quercetin; LIAO et al. 2017a) while others repel them (e.g., kaempferol, catechin; DETZEL & WINK 1993; ONKOKESUNG et al. 2014). However, despite some deleterious effects on larval development (WANG et al. 2010), flavonoids are mainly not toxic for insects (DETZEL & WINK 1993). Once ingested, flavonoids can have antioxidant properties and are potentially beneficial for bees (e.g., quercetin; TREUUTTER 2005). They can stimulate the activation of detoxification enzymes...
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(cytochrome P450 monooxygenase) and enhance bee resistance to certain insecticides and acaricides (SCOTT et al. 1998; JOHNSON et al. 2012; LIAO et al. 2017b). The case of heather pollen flavonoids remains to be addressed and this incomplete picture of the pollen side does not allow for fully arguing that heather is a bumble bee health-promoting plant. Therefore, bioassays to determine heather pollen effects on bumble bee brood, bumble bee health, and parasite dynamics are warranted. To fill this gap, we herein present a study that aimed to assess the effects of heather pollen and its flavonoids on bumble bee health, at both individual and colony levels, considering the bumble bee interaction with the parasite *Crithidia* sp. We specifically addressed the following questions: (i) how does the parasite influence the development of bumble bee microcolonies and individual immunocompetence? (ii) do heather pollen and its flavonoids have an effect on bumble bees, impacting their resource collection and offspring production? (iii) do heather pollen and its flavonoids affect the parasite dynamics in infected bumble bee workers, or help bumble bees to counteract parasite effects? We expect (i) a mild effect of the parasite on bumble bees reared in optimal conditions; (ii) detrimental effects of flavonoids, and potentially of heather pollen on healthy bumble bees and microcolonies; and (iii) beneficial effects of heather pollen, and potentially its flavonoids, on infected bumble bees by reducing the parasite load.

**Material and methods**

**Bumble bee bioassays**

Queenless microcolonies of five workers were exposed to specific diet treatments (Fig. 1): control pollen (i.e., willow pollen is used because artificial pollen is unsuitable for bumblebee development and because its flavonoid profile does not overlap with any flavonoids found in heather pollen; GEKIÈRE et al. 2022b, in prep.) with bumble bees either (i) parasitised or (ii) non-parasitised; heather pollen with bumble bees either (iii) parasitised or (iv) non-parasitised; willow pollen supplemented with extracts of flavonoids from heather pollen with bumble bees either (v) parasitised or (vi) non-parasitised. Diets (i) (j) Parasitised microcolony (N=10)

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.
and (ii) were used as controls as well as to assess the parasite impacts. Diets (iii) and (v) were used to establish the effects of heather pollen or its flavonoids on infected microcolonies. Diets (iv) and (vi) were used to establish the effects of heather pollen or its flavonoids on uninfected microcolonies. For each treatment, ten queenless microcolonies have been established using five different queenright colonies (i.e., colonies with an active queen laying eggs; from Biobest bvba; Westerlo, Belgium) (2 microcolonies per colony per treatment). Colonies of the species Bombus terrestris (Linnaeus, 1758) were selected since this species is easy to rear and a natural forager of heather pollen (KLEIJN & RAEMAKERS 2008; BALLANTYNE et al. 2015). Faeces of queenright colonies were observed under the microscope to confirm the absence of parasites (Nosema spp., Apicystis spp. and Crithidia spp.) as guaranteed by the supplier. The microcolonies were kept in plastic boxes (10 × 16 × 16 cm; REGALI & RASMONT 1995) and reared at the University of Mons (Belgium, Mons, Campus de 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 bees were provided ad libitum with sugar syrup (water/sugar 35:65 w/w) and pollen candies (i.e., pollen mixed with a 65% sugar solution) for 35 days, with pollen candies being freshly prepared and renewed every two days. When workers died, they were discarded, weighed and replaced by a worker from the same queenright colony, which was marked with a colour dot on the scutum. Larvae ejected from the brood were also checked every day, counted and discarded from the microcolonies. Microcolonies were handled under red light to minimise disturbance.

Diet preparation
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 (Ditlo 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 therefore assume 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 batches served directly for the bioassays; the other half were used for massive extraction of flavonoids. Flavonoids were extracted using a Soxhlet extraction for approximately 40 cycles with methanol as solvent at 100°C. The extract was then vacuum filtered and evaporated to dryness (rotavapor IKA RV8). For flavonoid purification, the 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 a rotary evaporator and dissolved in aqueous ethanol solution (1:1, v/v) before being added 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).

Parasite inoculation
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 (GEKIÈRE 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 sp. cells was then estimated by counting with a Neubauer chamber, and the
inoculum was diluted to 2500 *Crithidia* sp. cells/μL with a 40% sugar solution. Workers allocated to the infected microcolonies were placed in individual Nicot® queen rearing cages and given 10 μL of the inoculum (i.e., 25,000 *Crithidia* sp. cells; LOGAN *et al.* 2005) by letting them feed on the sugar solution in a glass microcapillary after a 5-hour starvation period. Workers allocated to uninfected microcolonies also underwent the same treatment (isolation, starvation) but with 10 μL of sterile sugar solution.

### Parameters evaluated

To investigate the impacts of pollen diet and parasite, several parameters in microcolonies were measured (TASEI & AUPINEL 2008), namely resource collection, reproductive success, stress response, individual health through fat body content (i.e., immunocompetence proxy; ARRESE & SOULAGES 2010; ROSALES 2017; VANDERPLANCK *et al.* 2021) and measurements of parasite load.

Resource collection was assessed by weighing the syrup container every two days in each microcolony as well as the recovered pollen candy and the newly introduced one. These data were corrected for evaporation using controls, as well as divided by the total worker mass per microcolony to avoid bias due to worker activity. To evaluate the reproductive success, all microcolonies were dissected at the end of the experiment (day 35) to weigh the total hatched brood mass, as well as the individual mass of each emerged male used as reference for viable offspring at the end of development (GOULSON 2010). Offspring masses were divided by the total worker mass per microcolony to avoid any bias due to worker care. Regarding stress response, we assessed worker mortality, larval ejection, pollen dilution (ratio between the collection of syrup and pollen) as well as pollen efficiency (ratio between offspring mass and pollen collection; TASEI & AUPINEL 2008), the latter used as a proxy of pollen nutritional quality that indicates when a micro-colony needs to consume more pollen to produce offspring.

For the individual health parameters, fat body content was measured at the end of the bioassays for two males and two workers per microcolony (40 individuals per treatment) following ELLERS (1996). The abdomens were cut and dehydrated in an incubator at 70°C for three days before being weighed. They were then placed for one day in 2 mL of diethyl ether to solubilise lipids constituting the fat body. The abdomens were then washed twice with diethyl ether, and incubated at 70°C for seven days before being weighed. Fat body content was defined as the mass difference between dry abdomen before and after lipid solubilisation, divided by the dry abdomen mass prior to solubilisation.

In infected treatments, we repeatedly monitored the parasite load within microcolonies using the same marked worker along the bioassays. The first measurement was made three days post-inoculation (day 4) to enable *Crithidia* sp. to multiply and ensure its presence in the faeces (LOGAN *et al.* 2005). A total of seven further measurements were taken to establish the infection curve of *Crithidia* sp., namely on days 6, 8, 10, 12, 16, 20 and 35. Measurements were performed at larger intervals after day 12 because infection reached the plateau phase (SCHMID-HEMPEL & SCHMID-HEMPEL 1993; OTTERSTATTER & THOMSON 2006). In practice, the marked worker was held in a 50 mL Falcon tube in the light until the faeces were expelled. Faeces were then collected in a 10 μL microcapillary tube and diluted two to ten times with distilled water to enable efficient cell counting. Parasite cells were then counted using a haemocytometer (Neubauer) under an inverted phase contrast microscope (400 × magnification, Eclipse Ts2R, Nikon). Uninfected microcolonies faeces were checked to be free of parasites at the end of the experiment, and a marked worker was isolated at days 4, 6, 8, 10, 12, 16, 20 and 35 in each uninfected microcolony to induce the same stress as in infected treatments.

### Data analysis and statistics

To detect a potential effect of pollen diet or parasite on resource collection, reproductive success, stress response, and individual health, mixed models were fitted for each parameter using diet, parasite and
their interaction as fixed factors, and colony as random factor. Pollen collection, pollen efficacy, and pollen dilution (log-transformed data) were analysed using a Gaussian distribution (i.e., normality of residuals; shapiro.test function from the stats R-package ver. 4.1.0; R CORE TEAM 2021) (lmer function from the nlme R package ver. 3.1.160; PINHEIRO et al. 2022). Total hatched offspring mass, emerged male mass, and fat body content (i.e., proportion data) were analysed using a Gamma distribution and a log link function (glmmTMB function from the glmmTMB R-package ver. 1.1.4; BROOKS et al. 2017). For fat body content, values were square root-transformed and sex was added as crossed-fixed effect. For emerged male mass and fat body content, the variable microcolony nested within colony was used as a random factor to deal with pseudo-replication (i.e., several measures per microcolony).

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

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

Contrasting analyses were then performed on the models to determine whether the uninfected control differed from the infected control, and whether effects on uninfected or infected microcolonies differed among diets (emmeans function from the emmeans R-package ver. 1.8.2; LENTH 2022). For fat body content, data were analysed separately for workers and males as a sex-significant effect was detected. All graphs and plots were performed using the R-package ggplot2 ver. 3.4.0 (WICKHAM 2016), except the one referring to the survival probability of the workers performed with the ggsurvplot function of the survminer R-package ver. 0.4.9 (KASSAMBARA et al. 2021). All the statistical analyses were done using the R software ver. 4.1.0 (R CORE TEAM 2021). For all statistical analyses, \( p < 0.05 \) was used as a threshold for significance.

**Results**

**Parasite impact**

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

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

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

Figure 2 – Resource collection and reproductive success. A. Total mass of collected pollen. B. Total mass of hatched produced offspring. 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.
Concerning stress responses, pollen dilution was significantly higher in microcolonies supplied with the supplemented diet compared to those receiving the other diets (control vs supplemented: $t = 2.282$, $p = 0.0268$; heather vs supplemented: $t = -3.191$, $p = 0.0025$; Fig. 3A). Microcolonies feeding on the heather or supplemented diets also displayed a lower pollen efficacy than the microcolonies supplied with the control diet (control vs supplemented $t = -2.741$, $p = 0.0085$; control vs heather: $t = -3.025$, $p = 0.0039$; Fig. 3B). On the contrary, no significant difference was detected regarding larval ejection ($p > 0.05$) and worker mortality ($p > 0.05$, Fig. 3C).

Figure 3 – Stress responses. A. Pollen dilution, defined as the ratio between syrup and pollen collection. B. Pollen efficacy, defined as the ratio between total mass of hatched offspring and pollen collection. C. Worker survival probability over time. For (A) and (B), each coloured data point represents a microcolony, diamonds are mean values of each treatment, and error bars indicate the standard error. 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.
Regarding individual health, while no difference was found in worker fat body content among diet treatments ($p > 0.05$; Fig. 4A), fat body content in newly emerged males was significantly higher in microcolonies fed with the supplemented or heather diets compared to those fed with the control diet (control vs supplemented: fat body content 62% higher, $t = -3.891$, $p = 0.0012$; control vs heather: fat body content 41% higher, $t = 2.850$, $p = 0.0223$; Fig. 4B).

Figure 4 – Health parameters. **A.** Worker fat body content. **B.** Male fat body content. Each coloured data point represents a microcolony, diamonds are mean values of each treatment, and error bars indicate the standard error. Means and error bars have been shifted in the graphs to improve readability. **C.** Parasite load over time. Generalized additive mixed-effect models (in C) were used to fit smoothers to the data showing mean trends [$\pm$ 95% confidence intervals, light coloured bands] over time. Here, each dot represents one data point (i.e., parasite load for the monitored worker for each time point and each microcolony). 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; **, $p < 0.01$. Arrows indicate the pairwise comparisons for the control diet between infection treatments (i.e., parasite effect). Symbol caption is in the grey zone.
Effect of heather pollen and its flavonoids on parasitised bumble bees

Similarly to previous results with uninfected microcolonies, total pollen collection was significantly lower in infected microcolonies provided with the supplemented diet than in microcolonies provided with either the control diet (36% less pollen collected, \( t = -4.414, p < 0.001 \)), or the heather diet compared to infected microcolonies receiving the control diet (16% less pollen collected, \( t = -2.866, p = 0.0061 \)) (Fig. 2A). When investigating reproductive success, as observed in uninfected microcolonies, microcolonies supplied with the supplemented and heather diets produced a significantly lower brood mass compared to microcolonies supplied with the control diet (control vs supplemented: brood mass 51% lower, \( t = 3.784, p < 0.001 \); control vs heather: brood mass 41% lower, \( t = 3.551, p < 0.001 \); Fig. 2B). However, no significant difference was detected for the mass of newly emerged males among diet treatments (\( p < 0.05 \); Fig. 2C).

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

When analysing the effects of diet on individual health, no difference was detected in fat body content of workers or newly emerged males among diet treatments (\( p > 0.05 \); Fig. 4A–B). Regarding the parasite load, the infection dynamic was more gradual in infected microcolonies supplied with the control diet compared to those provided with the other diets which supported a parasite load peak around day 20 before a decrease continuing to the end of treatment (supplemented vs control: \( t = 2.893, p = 0.0126 \); heather vs control: \( t = 2.328, p = 0.0313 \); Fig. 4C).

Discussion

Parasite effect on bumble bee

The parasite *Crithidia sp.* (Euglenozoa: Trypanosomatidae) had no impact on larval ejection, total mass of offspring produced, nor on individual mass of newly emerged males. Such results suggest that infection is unlikely to reduce colony and offspring fitness, or reproductive success, all factors which are related to individual size (Greenleaf et al. 2007; Amin et al. 2012). The limited effects of *Crithidia* sp. on the reproductive success of bumble bees observed here are in line with the literature (Brown et al. 2003; Goulson et al. 2018; Gekière et al. 2022a). This absence of impact on development performance and offspring fitness may come from the fact that the parasite only infects the adult stage (i.e., *Crithidia* sp. does not develop in bumble bee larvae, Folly et al. 2017).

Furthermore, our results showed that *Crithidia* sp. induced larger fat body content in males emerging from infected microcolonies compared to uninfected ones, whereas this parasite had no impact on the fat body content of workers. We propose two hypotheses to explain such a *Crithidia*-induced difference in fat body content only in newly emerged males and not in workers. First, newly emerged males and workers were likely not infected at the same age. Indeed, workers developed in healthy colonies and were inoculated at the adult stage (most likely > 2 days old) to establish infected microcolonies. However, males (most likely one day old) developed in infected microcolonies and ingested *Crithidia* sp. cells upon emergence resulting in an infection rate of up to 90% (Gekière et al. 2021, unpublished results).
Second, while the difference in male fat body content between infected and uninfected microcolonies is unlikely to have arisen from a difference in brood care (i.e., no significant difference in pollen efficacy used as proxy of pollen nutritional quality), we cannot rule out the possibility that infected workers displayed specific brood caring behaviour. For instance, they could have added peculiar nutrients or microorganisms to larval food from their hypopharyngeal and mandibular glands and/or stomach to prepare their offspring for parasite infection (e.g., addition of sterols, SVOBODA et al. 1986). Such an increase in offspring fat body content through adapted larval feeding by workers could be interpreted as a trans-generational prophylactic behaviour. Indeed, enhanced fat body content has been assumed to correspond to a specific allocation of resources to counteract parasites by producing an immune response (BROWN et al. 2003). It would be interesting to test whether infected workers provide their larvae with specific specialised metabolites.

Although Crithidia sp. only showed mild effects in our experiment and in previous laboratory experiments (BROWN et al. 2003; GOULSON et al. 2018; GEKIÈRE et al. 2022a), it is important to keep in mind that results observed under laboratory conditions must be interpreted with caution as such controlled conditions are often not representative for natural constraints encountered in the field such as predation, flight, and foraging. For example, infection by Crithidia sp. has been shown to impair pollen foraging in wild populations (SHYKOFF & SCHMID-HEMPEL 1991; OTTERSTATTER et al. 2005; GEGEAR et al. 2006), but such effects cannot be fully studied under laboratory conditions.

Heather pollen quality: the case of flavonoids

Heather pollen contains kaempferol flavonoids chemically linked to one/two coumaroyl groups which are also linked to one/two hexosides (GEKIÈRE et al. in prep.). In the current study, we have shown that these heather flavonoids decreased the total offspring production, and pollen collection, and caused lower pollen efficacy as well as reduced the mass of newly emerged males, thereby altering the performance of microcolonies. Indeed, male mass is known to impact flight distances, but also reproductive abilities, affecting the dissemination and reproductive success of bumble bee populations (GREENLEAF et al. 2007; AMIN et al. 2012). Such poor quality of heather pollen for the maintenance of buff-tailed bumble bee microcolonies has already been indicated (VANDERPLANCK et al. 2014). While this was partly attributed to its nutritional content (i.e., low concentration of amino acids and abundance of δ-7-avenasterol and δ-7-stigmasterol, HUANG et al. 2011; VANDERPLANCK et al. 2014), our study demonstrated that specialised metabolites may also impact the pollen quality of heather, regardless of its nutritional content (i.e., central metabolites).

Both heather pollen and its flavonoids showed detrimental effects (i.e., reduction of offspring production, pollen efficacy). However, heather flavonoids seemed to induce a higher stress response than heather pollen as dilution behaviour was significantly higher in microcolonies fed with the supplemented diet compared to those receiving the control diet (i.e., higher dilution behaviour of the unfavourable diet, BERENBAUM & JOHNSON 2015; VANDERPLANCK et al. 2018) while such a difference was not observed for microcolonies fed with the heather diet. The reason for this discrepancy is not obvious, as both diets harbour the same flavonoids and should therefore lead to similar dilution behaviour. Two hypotheses could be proposed to explain this difference: (i) flavonoids were more bioavailable in the supplemented diets (outside pollen grains after the chemical extraction) and therefore more easily absorbed by the workers, which ultimately reduced the diet palatability (WANG et al. 2019); and (ii) as flavonoid extract was added to the control diet (i.e., willow pollen) that already contained flavonoids, the supplemented diet was richer in flavonoids than the other diets, reaching a threshold that ultimately reduced the diet palatability. Unfortunately, it is not possible to unravel these hypotheses without additional experiments.
Another result of the current study supporting the potential toxicity of heather flavonoids is the increase in fat body content in males emerging from microcolonies fed with either heather and supplemented diets as compared to those emerging from microcolonies fed with the control diet. Indeed, such an increase could be interpreted as a specific allocation of resources to the fat body for performing detoxification (Li et al. 2019). Flavonoid assimilation is known to induce the activation of defence mechanisms based on cytochrome P450 monooxygenase, a molecule that is highly active in the fat body (Scott et al. 1998). This increase in fat body content was not observed in workers, which could be explained by the different exposures to flavonoids during their life stages. Indeed, workers within microcolonies mainly fed on syrup, while males fed on pollen during their whole larval development and were then subsequently exposed to more specialised metabolites. Moreover, it is highly likely that sensitivity to pollen-specialised metabolites is higher in larvae than in adults, as already demonstrated in honey bees (Luchetti et al. 2018).

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

Flavonoids were associated with an increase in parasite load, which has also been observed for other classes of specialised metabolites (Thorburn et al. 2015; Gekière et al. 2022a). Therefore, in contrast to our expectations based on previous studies (Baracchi et al. 2015; Koch et al. 2019), the detrimental effects of heather pollen flavonoids on bumble bees were not balanced by any therapeutic effect against the parasite Crithidia sp. These results suggest a potential additive effect between phytochemical and parasite stress as previously described (Thorburn et al. 2015), with the diet effect for heather mostly overriding the effect of the parasite in bumble bees as already shown for sunflower pollen (Gekière et al. 2022a). The nutritional stress caused by heather pollen feeding could then increase the effect of Crithidia sp. which could be more virulent under stressful conditions (Brown et al. 2000, 2003). However, we found that mortality in infected microcolonies was lower in microcolonies fed with the heather diet as compared to the control diet. Therefore, heather pollen could increase bumble bee survival probability, but this effect is unlikely due to the flavonoid content of heather pollen as mortality in infected workers did not significantly differ between receiving the supplemented or the control diet.

Conclusion

How heather pollen and its specialised metabolites impact the buff-tailed bumble bee, and how they modulate the interaction with its obligate gut parasite Crithidia sp. are complex questions given the diversity of specialised metabolites found in the floral resources of this species. Previous studies have found that heather nectar does not contain any flavonoids (Gekière et al. in prep.) but protected the pollinator from its parasite Crithidia sp. through callunene activity (Koch et al. 2019). In this study, we found that the occurrence of flavonoids in heather pollen reduced its collection as well as bumble bee fitness. Moreover, heather pollen did not help to counteract the parasite but rather appeared to induce an additional stress that could potentially increase the parasitic effect. Our results contribute to the understanding of the bumble bee-heather-parasite relationship by indicating that heather pollen is not suitable to increase fitness of buff-tailed bumble bees and does not show any therapeutic effect. This study also highlights the complexity of the plant-pollinator interaction by illustrating the distinct roles and effects of specialised metabolites found either in nectar or pollen. We strongly encourage the consideration of both floral resources in future studies investigating the medicinal effects of plant species, especially when defining pollinator conservation strategies.

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**Authors’ contributions**

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

**Conflict of interest disclosure**

The authors declare that they have no financial conflicts of interest in relation to the content of the article.

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

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

**References**


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Appendix A – Supplemented diet

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

| TABLE S1 |
|---|---|---|
| Diets | Ethanol (μL/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 |
| Heather diet | 20.50 | 0.82 | 12.08 |

Appendix B – Variable estimates

Mean ± standard error (SE) values of the variable used to describe parasite and diet effects.

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