Belgian Journal of Zoology
www.belgianjournalzoology.be

ISSN 2295-0451

Research article

https://doi.org/10.26496/bjz.2023.111

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.

Tourbez C., Semay I., Michel A., Michez D., Gerbaux P., Gekière A. & Vanderplanck M. (2023). Heather pollen is not necessarily a healthy diet for bumble bees. *Belgian Journal of Zoology* 153: 105–124. https://doi.org/10.26496/bjz.2023.111

Introduction

For their own subsistence and that of their offsprings, 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 (Hendriksma *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 (Sánchez-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; Treutter 2005). They can stimulate the activation of detoxification enzymes

(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)

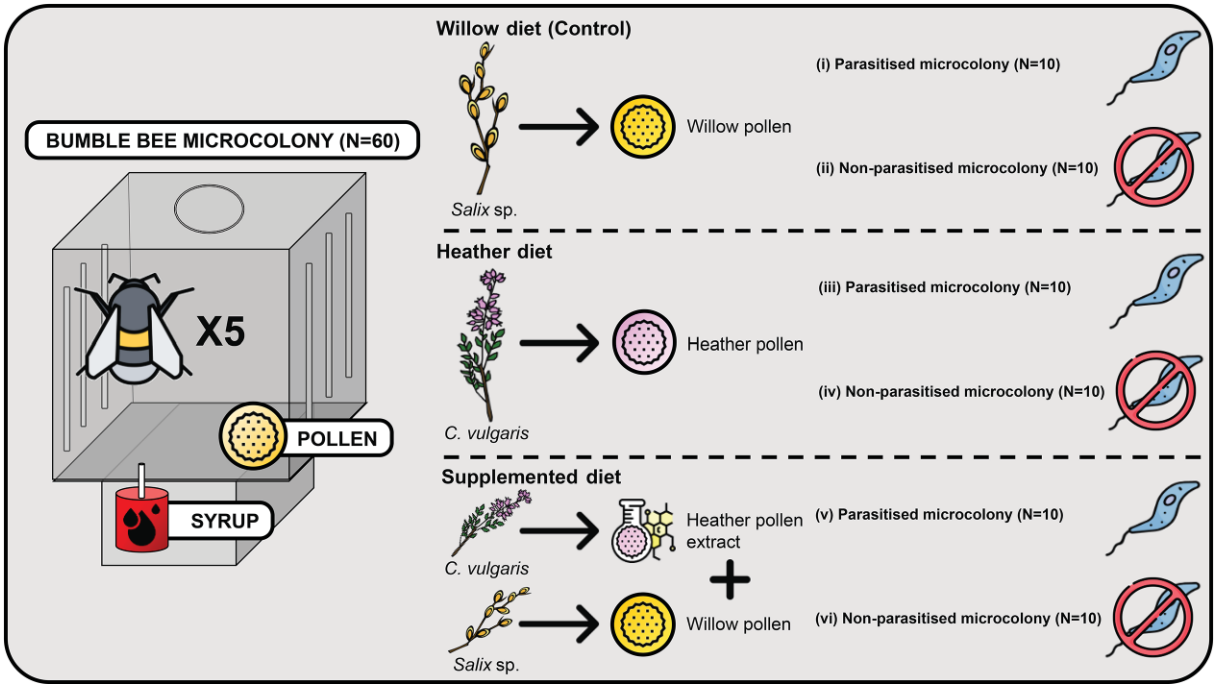

Figure 1 – Bioassay design. Microcolonies initiated with five *B. terrestris* workers were fed for 35 days with one of three diets. For each diet, ten microcolonies contained parasitised individuals (*Crithidia* sp.), ten others were non-parasitised. Icon used for the figure: https://www.flaticon.com/ 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 (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 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 *Crithidia* sp. infection did not impact the parameters related to resource collection (Fig. 2A), reproductive success (Fig. 2B–C), or stress response (Fig. 3A–C) ($p > 0.05$, 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 ($t = -3.828$, $p = 0.0012$; 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

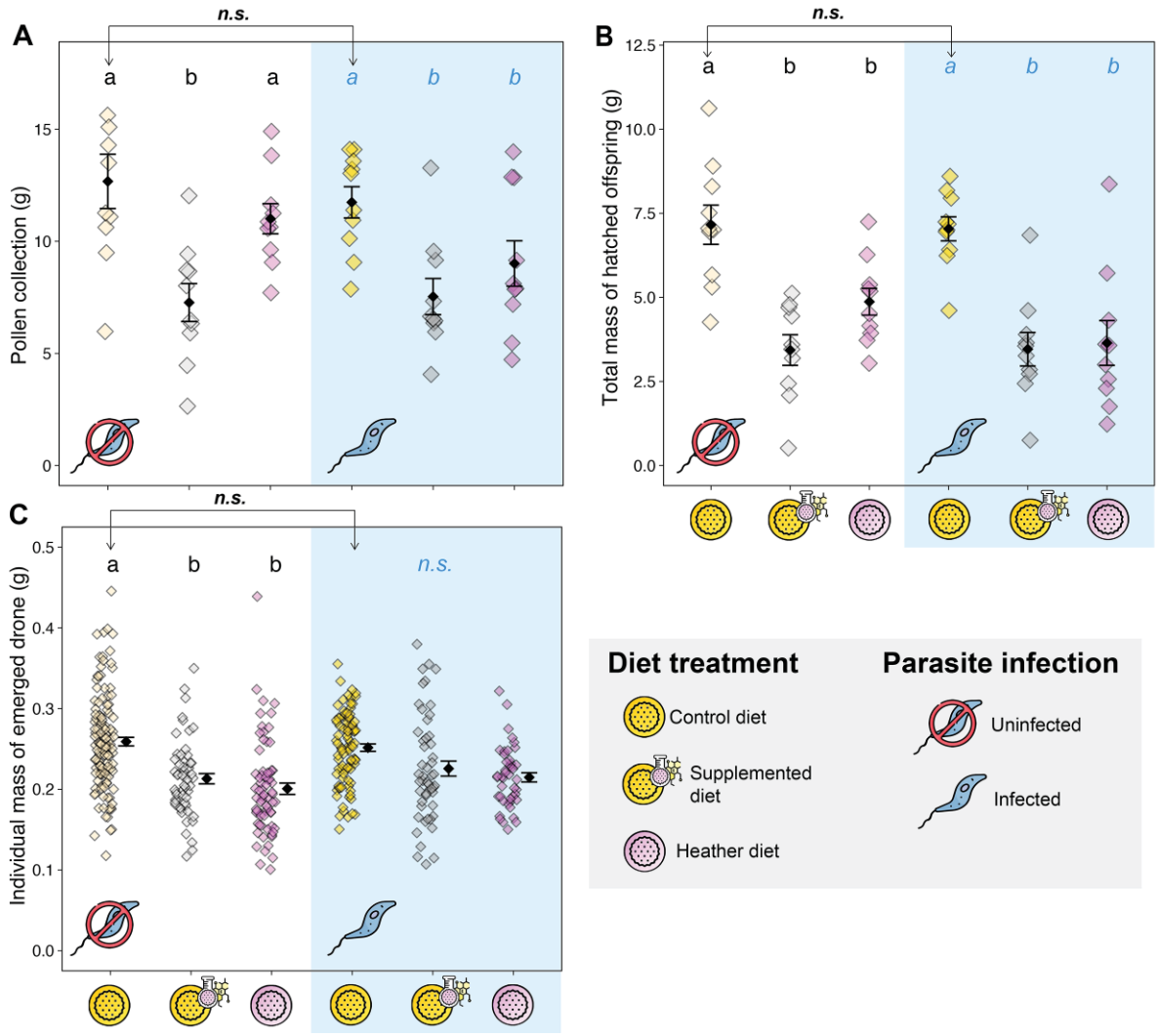

Figure 2 – Resource collection and reproductive success. **A**. Total mass of collected pollen. **B**. Total mass of hatched produced offspring. **C**. Individual mass of emerged males. Each coloured data point represents a microcolony (in A and B) or an individual (in C), diamonds are mean values of 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.

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).

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).

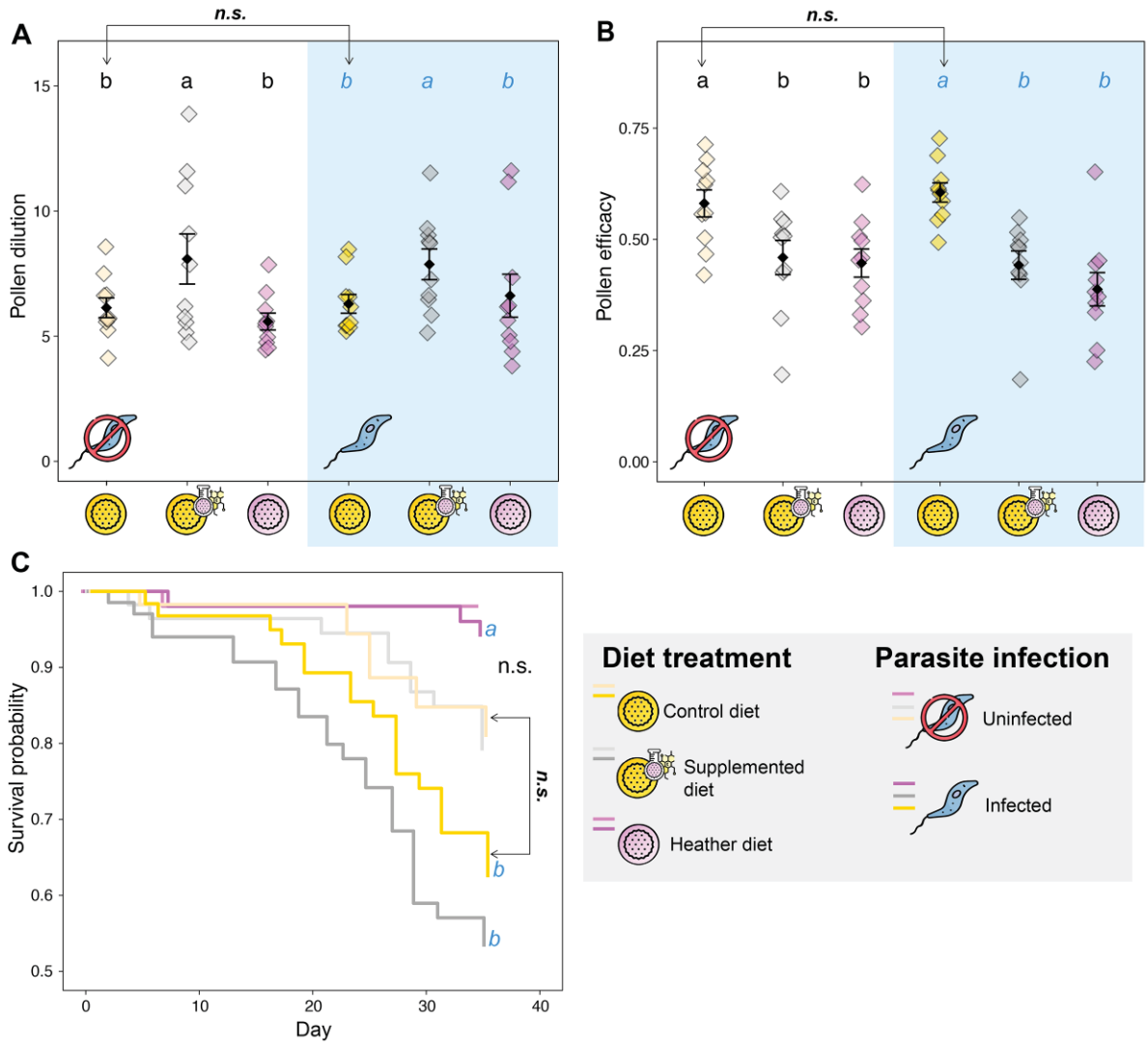

Figure 3 – Stress responses. **A**. Pollen dilution, defined as the ratio between syrup and pollen collection. **B**. Pollen efficacy, defined as the ratio between total mass of hatched offspring and pollen collection. **C**. Worker survival probability over time. For (A) and (B), each coloured data 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).

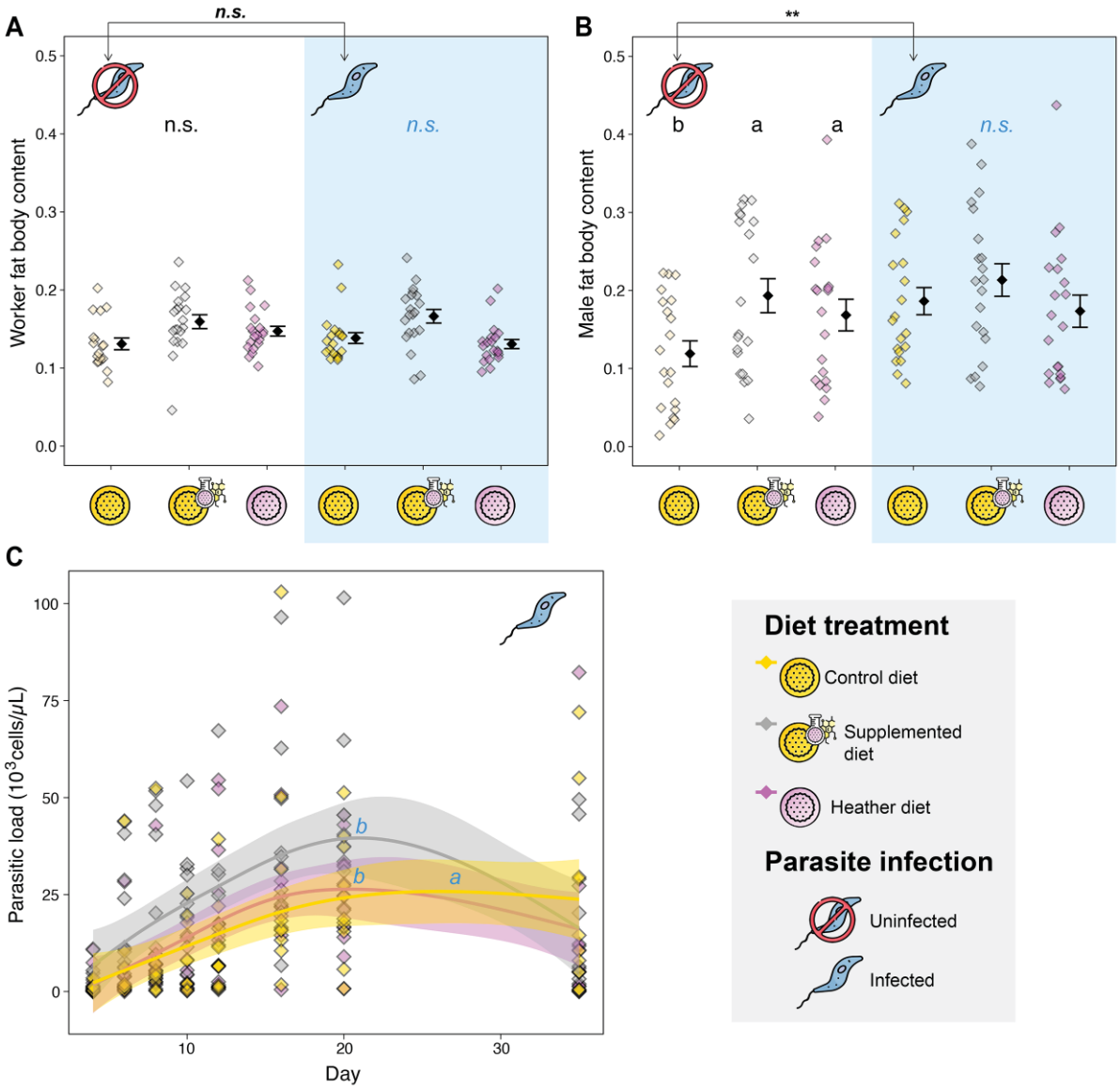

Figure 4 – Health parameters. **A**. Worker fat body content. **B**. Male fat body content. Each coloured data point represents a microcolony, diamonds are mean values of each treatment, and error bars indicate the standard error. Means and error bars have been shifted in the graphs to improve readability. **C**. Parasite load over time. Generalized additive mixed-effect models (in C) were used to fit smoothers to the data showing mean trends [± 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 s*p. 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 exposurs 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 (Lucchetti *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.

Acknowledgements

We would like to thank D. Evrard and L. Marin for their help in colony and microcolony maintenance, as well as the numerous people that helped with microcolony dissection. We are very grateful to

the Laboratory of Cellular Biology (UMons) and the Laboratory of Therapeutic Chemistry and Pharmacognosy (UMons) for their advice and the access to the device needed for the microscope analyses and the diet preparation. We also thank François Dittlo for providing us with heather pollen, as well as the family of the first author (I., C., C., and C. Tourbez) for their help. A preprint version of this article has been peer-reviewed and recommended by PCIEcology (https://doi.org/10.24072/pci.ecology.100534).

Funding

This study was funded by the 'METAFLORE,2019–2023′ project, one ARC 'Actions de Recherche Concertées' project. C.T. PhD grant is funded by the University of Mons (UMONS). A.G. is supported by a F.R.S.-FNRS PhD grant 'Aspirant'. The PhD grant of I.S. is supported by the ARC project METAFLORE.

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.

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Manuscript received: 13 September 2023
Manuscript accepted: 25 September 2023
Published on: 25 October 2023
Branch editor: Isa Schön

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

Diet compositions.

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 [1]
Heather diet	20.50	0.82	12.08

Appendix B – Variable estimates

TABLE S2

Mean ± 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 ± 1.21	7.27 ± 0.85	11.0 ± 0.67	11.7 ± 0.70	7.53 ± 0.81	9.01 ± 1.01
Total mass of hatched offspring	7.16 ± 0.58	3.43 ± 0.46	4.87 ± 0.40	7.04 ± 0.36	3.46 ± 0.50	3.64 ± X0.67
Individual mass of emerged drone	0.259 ± 0.005	0.213 ± 0.006	0.201 ± 0.007	0.252 ± 0.005	0.226 ± 0.009	0.215 ± 0.006
Pollen dilution	6.14 ± 0.40	8.08 ± 1.00	5.58 ± 0.34	6.29 ± 0.37	7.87 ± 0.61	6.62 ± 0.86
Pollen efficacy	0.581 ± 0.030	0.459 ± 0.038	0.447 ± 0.032	0.605 ± 0.022	0.442 ± 0.032	0.388 ± 0.037
Larval ejection	0.984 ± 0.006	0.970 ± 0.010	0.999 ± 0.001	0.969 ± 0.007	0.889 ± 0.053	0.990 ± 0.007
Worker fat body content	0.131 ± 0.008	0.159 ± 0.009	0.147 ± 0.006	0.138 ± 0.007	0.166 ± 0.009	0.131 ± 0.006
Male fat body content	0.119 ± 0.016	0.193 ± 0.022	0.168 ± 0.020	0.186 ± 0.017	0.213 ± 0.021	0.173 ± 0.021