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FROM POISON TO POTION:
Effects of sunflower phenolamides on bumblebee microcolonies and individuals

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Abstract

The flowering plants substantially rely on insect pollinators for their reproduction. Bees, their major pollinators, are totally reliant on floral resources (i.e., mainly pollen and nectar) for their survival and larval development. However, plant-bee interactions hide a silent conflict, called the 'pollen dilemma', since flowering plants have to attract pollinators for their reproduction while limiting excessive resource harvesting by floral visitors. For this purpose, they have evolved a vast array of specialised metabolites, but whether their occurrence in pollen and nectar is due to adaptative (i.e., allocation) or non-adaptative (i.e., pleiotropy) mechanisms remains unclear. From a bee perspective, specialised metabolites may have pre- or post-ingestive effects, the latter being either lethal or sublethal (e.g., reduced reproductive success, altered larval development, induced malaise behaviour). Nevertheless, bees can used specialised metabolites to deal with parasite infection either prophylactically (i.e., pre-infection) or therapeutically (i.e., post-infection), and self-medicative behaviours have thus been suggested. In self-medication, an inherent condition is that consuming medicative resources should reduce the fitness of uninfected individuals while it should alleviate the fitness loss imposed by the parasite in infected ones. Herein, we investigated a plant-bee-parasite tripartite interaction using the sunflower *Helianthus annuus*, the bumblebee *Bombus terrestris*, and the trypanosomatid parasite *Crithidia bombi*. First, we assessed whether *H. annuus* could allocate its specialised metabolites among its tissues by analysing their phenolamide (HCAA) profiles via HPLC-MS/MS. We found a clear difference in HCAA profiles in floral resources (i.e., pollen and nectar) *vs.* petals and leaves. Yet, the closely related profiles among pollen and nectar warrant further studies. Next, we tested whether *H. annuus* pollen and/or its HCAA extracts could impact *B. terrestris* at the microcolonial (i.e., reproductive success and stress responses) and individual (i.e., fat body content and phenotypic variation) levels. Sunflower pollen and HCAA extracts had negative effects at both levels, but these effects were slighter for HCAA extracts. Finally, we investigated whether *H. annuus* pollen and/or its HCAA extracts could alleviate the fitness loss induced by *C. bombi* infection. The infection led to slight stress responses at both levels, but HCAA extracts mitigated a stress response at the microcolonial level, while *H. annuus* pollen did not offset *C. bombi* impacts. Consequently, we suggest that HCAAs could be a potential medicative resource for *Crithidia*-infected bumblebee colonies, but we emphasise that clarifications are required to define the mechanisms, the benefits and the costs of self- and social-medication. Further plant-bee-parasite ecological concerns are discussed, as well as the limitations of the study with suggestions of perspectives.

Keywords: *Bombus terrestris*; *Crithidia bombi*; *Helianthus annuus*; medication; phenolamide; tripartite interaction

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'If you get up in the morning and think the future is going to be better, it is a bright day. Otherwise, it's not.'

Elon Musk, 2017

'If you have to choose, be the one who does things, instead of the one who is seen to do things.'

Jordan Bernt Peterson, 2018

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1. INTRODUCTION

1.1. The angiosperms and their success

1.1.1. THE EMERGENCE OF ANGIOSPERMS

Angiosperms, or flowering plants (Eukarya: Plantae), are one amazingly diverse of the major clades of extant seed plants (Spermatophyta) with at least 290,000 species classified in 416 families (APGIV, 2016). The fossil record of the angiosperms suggests that they emerged during the early Mesozoic but mainly diversified during the late Jurassic and early Cretaceous, conservatively 130Ma ago (Morris *et al.*, 2018). In spite of their diversity, flowering plants are united by a suite of synapomorphies (i.e., shared derives features), such as double fertilization, triploid albumen, stamens with two pairs of pollen sacs, 8-cell embryo sac, apparition of flowers and carpels as well as companion cells along the phloem (Reiser & Fischer, 1993; Soltis & Soltis, 2004; Meyer *et al.*, 2008). All these traits, along with symplesiomorphies (i.e., shared ancestral features) acquired in prior land plants (e.g., vascular system, developed root system, non-disseminated megasporangium, seed), have promoted the success and diversification of this pervasive clade (Crepet & Niklas, 2009; Simpson, 2010). Today, angiosperms are considered the most abundant group of the terrestrial environment in terms of biomass and number of individuals, and their autotrophic nutritional pathway makes them a keystone in terrestrial food webs (Evert & Eichhorn, 2013).

1.1.2. THE REPRODUCTION OF ANGIOSPERMS

By contrast to cryptogam embryophytes, namely bryophytes and pteridophytes, which require some water for their sexual reproduction, phanerogam embryophytes, namely gymnosperms and angiosperms, display water-independent modes of reproduction (Campbell *et al.*, 2012). In these both clades, the male gametophyte is a pollen grain that is transported from its site of production (i.e., male cones or stamens) to the female landing site (i.e., female cones or carpels) through (a)biotic vectors in a process called ‘pollination’ (Pacini, 2015). While 98% of the gymnosperm species are abiotically wind-pollinated (anemophily; Lu *et al.*, 2011), less than 20% of the angiosperm species rely on abiotic vector for their pollination (Ackerman, 2000). Instead, more than 80% of angiosperm species depend on biotic vectors (zoophily) for their pollination, such as insects (entomophily), birds (ornithophily), or mammals (therophily), in particular bats (chiropterophily) and rodents (sminthophily; Abrol, 2012). This

animal-dependant pollination is thought to be the ancestral character of all angiosperms which implies a more-than-hundred-year period of coevolution between flowering plants and their pollinating animals (Hu *et al.*, 2008). This coevolution has enabled specific zoophilous plant traits (i.e., pollination syndromes) to arise from convergent selective pressures exerted by functional groups of pollinators, irrespective of their phylogenetic distance (Fenster *et al.*, 2004). Therefore, flowering plants vary not only in their morphology and colour but also in their phenology as well as biochemical and volatile organic compound composition to attract and utilise a specific group of animals as pollinators (Faheem *et al.*, 2004; Fenster *et al.*, 2004; Dudareva *et al.*, 2013; Ramos–Jiliberto *et al.*, 2018). Ollerton *et al.* (2011) estimated that 87.5% of all flowering plants are adapted to animal pollination. In zoophilous pollination, insect-mediated pollination is doubtlessly the most prevalent one since 70% of zoophilous flowering plants are actually entomophilous (Abrol, 2012).

1.2. Bees as main pollinators

Bees (Hymenoptera: Apocrita: Acuelata: Apoidea), also named Anthophila, represent a monophyletic group which comprises ~20,000 species allocated into seven families (i.e., Andrenidae, Apidae, Colletidae, Halictidae, Megachilidae, Melittidae and Stenotritidae; Danforth *et al.*, 2013). They represent the single most dominant pollinating taxon, because they are the only group of animals more or less totally reliant on floral resources as both adults and larvae (Ollerton, 2017). Globally, they visit more than 90% of the leading crop types (Klein *et al.*, 2007), providing around $200 billion annually (Hristov *et al.*, 2020) and thereby also providing essential services for human welfare (Eilers *et al.*, 2011). While they rely on nectar as main source of carbohydrates, bees use pollen as main source of proteins, amino acids, vitamins, lipids and other micronutrients (Roulston & Cane, 2000; Vaudo *et al.*, 2015). These both resources are vital for proper larval rearing, adult reproduction, and survival (Nicolson, 2011). Cardinal & Danforth (2013) estimated the divergence time of bees to coincide with the increasing dominance of eudicots in the middle Cretaceous (*ca*. 120Ma), which explains the strong interdependence of the two clades. It is now widely acknowledged that bees display species-specific foraging preferences (i.e., not all the bee species forage on all the flowering plant species; Dötterl & Vereecken, 2010). Preferential foraging is influenced by flower morphology, colour and scent (Faheem *et al.*, 2004), but recent studies suggest that non-random foraging is also shaped by protein, amino acid and lipid contents of floral resources as well as their respective ratios (Vanderplanck *et al.*, 2014a, 2019c; Somme *et al.*, 2015; Vaudo *et al.*, 2017, 2020; Ruedenauer *et al.*, 2020). For instance, it seems that bumblebees tend to collect

pollen richer in proteins and amino acids than honeybees (Leonhardt & Blüthgen, 2012), but that bumblebees prioritise perception of lipids in protein-to-lipid ratios (Ruedenauer *et al.*, 2020). Importantly, these biochemical profiles greatly influence larval development (Genissel *et al.*, 2002; Tasei & Aupinel, 2008a; Sedivy *et al.*, 2011) and adult health, including their resilience to stress (Di Pasquale *et al.*, 2013; Roger *et al.*, 2017; Vanderplanck *et al.*, 2019b; Barraud *et al.*, 2020). Thus, categories have been proposed to describe the continuum in bee foraging strategies, from extreme specialisation (i.e., monolecty) to extreme generalisation (i.e., polylecty; Table 1). However, it must be stressed that polylecty, a later-acquired synapomorphic foraging strategy in bees (Michez *et al.*, 2008), does not imply that generalists forage randomly on all available plants, since pollinators must be ecologically and metabolically adapted to the flowering plants they get pollen from (Vanderplanck *et al.*, 2019c), and since they forage on multiple host plants that altogether fill their physiological requirements (Hanley *et al.*, 2008; Eckhardt *et al.*, 2014; Ruedenauer *et al.*, 2016).

Table 1: Categories of bee host ranges and their definitions.

CATEGORY	SUB-CATEGORY	CLADISTIC OF THE POLLINATED PLANT(S)	EXAMPLES OF BEE SPECIES	REFERENCE
MONOLECTY	/	One and only one plant species (while different species from the same genus are available)	*Anthemurgus passiflorae* *Flavipanurgus venustus*	Neff & Rozen, 1995; González-Varo *et al.*, 2016
OLIGOLECTY	Strict	Two or more plant species belonging to the same genus	*Colletes sierrensis* *Colletes ligatus* *Andrena lonicerae* *Dasypoda pyrotrichia*	Michez *et al.*, 2008; Müller & Kuhlmann, 2008; Shimizu *et al.*, 2015
	Large	Two or more plant species belonging to the same tribe, sub-family or family		
	Eclectic	Two to four plant genera belonging to two or three families		
POLYLECTY ***SENSU LATO***	Strong preference	Several plant families but one taxon (family, sub-family, tribe, genus or species) is predominant	*Colletes creticus* *Colletes gallicus* *Dasypoda albimana* *Dasypoda cingulate* *Bombus terrestris*	Michez *et al.*, 2008; Müller & Kuhlmann, 2008; Rasmont *et al.*, 2008
	Mesolecty	More than four plant genera belonging to two or three families		
	Polylecty *sensu stricto*	Several plant genera belonging to at least four families		

1.3. The herbivore – Pollinator duality and the pollen dilemma

Plant-bee interactions hide a silent conflict that arises because bees act concurrently as effective pollinators and herbivores. This conflict is even more pronounced when bees have developed morphological features to enhance pollen storage while foraging (e.g., corbiculate bees agglutinate pollen with regurgitated nectar in their corbicula; Thorp, 2000), which makes pollen hardly accessible for pollination, sometimes to such an extent that bees do not deposit any pollen grain on conspecific stigmas thereby acting as genuine pollen thieves (Hargreaves *et al.*, 2009). Even in legitimate pollination, bee foraging represents considerable costs for the plants as foragers take enormous quantities of pollen to feed their broods and nestmates (Müller *et al.*, 2006), which can compromise the reproductive success of the plants (i.e., less pollen deposited on stigmas per visit; Thomson, 2003). Thus, bees could be more accurately described as highly specialised and extremely efficient herbivores that also act as pollinators. Hence, the conflict of interest between a plant's production of pollen for the purpose of reproduction and efficient pollen collection by bees for their survival, a trade-off called the 'pollen dilemma', occurs in most plant-bee interactions (Westerkamp, 1996). To limit excessive pollen harvesting and optimise their reproduction, plants are thus expected to have developed specific mechanisms (i) to minimise pollen withdrawal, and (ii) to narrow the spectrum of pollen feeding visitors (i.e., to narrow the number of legitimate bee species pollinators). Reducing the size of the community of possible floral visitors can also benefit plants provided that it increases the fidelity (i.e., short-term pollinator foraging specialisation on particular plant species; Brosi & Briggs, 2013) and effectiveness (i.e., stigmatic pollen deposition per visit; Brosi & Briggs, 2013) of the remaining pollinators (Harder & Thomson, 1989). Accordingly, plants have evolved various morphological adaptations in their flowers such as hidden or poricidal anthers (i.e., requiring buzz pollination), heteranthery, nototribic or stenotribic flowers, narrow floral tube and progressive pollen release (reviewed in Westerkamp & Claßen-Bockhoff, 2007). Moreover, plants have also built chemical filters using volatile organic compounds (i.e., floral scents) to attract legitimate and repel inefficient floral visitors (Dötterl & Vereecken, 2010). Besides, plants have evolved defences through pollen characteristics to deter excessive pollen collection and to appeal a small range of bee species morphologically, physiologically and metabolically adapted to these characteristics. Pollen defences have relied on mechanical features such as thick multilayer pollen wall (i.e., rich in sporopollenin) with low digestibility

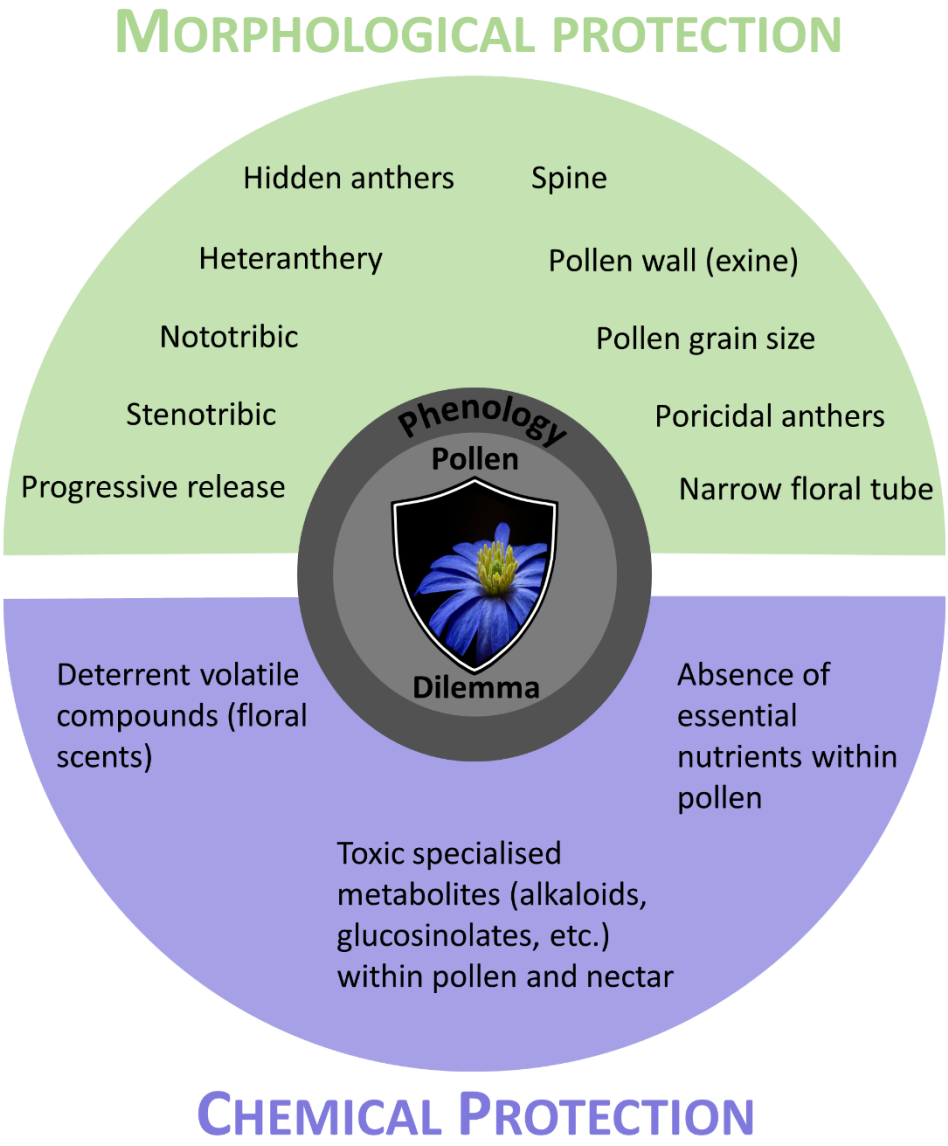

Figure 1: The pollen dilemma. This trade-off could also be described as an 'attraction-versus-protection dilemma'. For the sake of limiting pollen loss to bee herbivory and optimise their reproduction, flowering plants have evolved a wide range of traits that concern both flowers and pollen. These traits rely on morphological and chemical properties whereby plants minimise pollen withdrawal and narrow the spectrum of pollen feeding visitors. Phenological features also play a role in reproduction optimisation.

(Roulston & Cane, 2000) or long spine and pollenkit stickiness (Konzmann *et al.*, 2019). They have also leaned on chemical properties including poor nutritional quality (i.e., lack or wrong ratio of essential nutrients; Tasei & Aupinel, 2008; Vaudo *et al.*, 2017) or specialised metabolites acting as deterrent toxins (Detzel & Wink, 1993; London-Shafir *et al.*, 2003; Sedivy *et al.*, 2012; Arnold *et al.*, 2014; Wang *et al.*, 2019). Finally, flowering time along with pollinator seasonal emergence (i.e., phenology) have also played a strong role in narrowing the spectrum of pollen feeding visitors (Gallagher & Campbell, 2020). Altogether, these traits

(Figure 1) have tended to maximise pollen availability for stigmatic deposition and thus to optimise flowering plant reproduction.

1.4. Plant specialised metabolites

1.4.1. DIVERSITY AND ROLES

Plant specialised metabolites are phytochemicals that have been selected throughout evolution and that are found in different plant lineages and/or tissues to fulfil specific needs. Particularly, they fulfil major roles in the 'pollen dilemma'. Historically, they were thought to be inessential for plants and were therefore referred to as 'secondary metabolites', sometimes even considered as waste products. Now, their importance has been widely acknowledged among the scientific community and a growing number of studies have tried to elucidate their multiple roles in plant survival, communication and reproduction. Hence, one would rather refer to them as 'specialised metabolites', by contrast to 'central metabolites' (historically called 'primary metabolites'), the latter encompassing non-specific molecules involved in plant growth and development (i.e., carbohydrates, amino acids, proteins, lipids and nucleic acids; Evert & Eichhorn, 2013). Specialised metabolites include more than 200,000 compounds, which are defined as molecules that (Tissier *et al.*, 2015):

- Are associated with specific taxonomic groups, specific tissues and specific times;
- Do not intervene in plant growth and development;
- Are synthesised from a few biosynthetic pathways and key primary metabolites and;
- Have effects on other organisms (i.e., chemical communication).

The roles of plant specialised metabolites are diverse and intimately linked to their fixed mode of life. Indeed, in comparison mobile animals synthesise fewer specialised metabolites because they can move to escape predators and engage communication. In flowering plants, specialised metabolites can be (i) toxic to ward off pathogens and herbivores or to suppress the growth of neighbouring plants (i.e., supplant antagonists; Bennett & Wallsgrove, 1994); (ii) attractive to appeal pollinators, herbivore predators or seed dispersers and thus enhance fertilisation or dissemination rates (Cipollini & Levey, 1997; Dudareva & Pichersky, 2000); (iii) protective against UV radiation and oxidative stress (Li *et al.*, 1993; Rice-Evans, 2012); (iv) intermediates in the synthesis of structural cell components (e.g., lignin; Douglas, 1996); and (v) messengers by acting as chemical substrates in common or taxon-specific cellular pathways (Abou Dahab *et al.*, 1987; Isah, 2019). Thus, particularly in floral parts, specialised

metabolites play a crucial role in maintaining a compromise between attracting mutualists (i.e., efficient pollinators) and fending off antagonists (i.e., pathogens, nectar and pollen thieves, florivores and seed parasites; Adler, 2007). From a genetic perspective, it is estimated that 15-25% of the plant genomes encode enzymes for the specialised metabolism, and that all the specialised metabolism enzyme-coding genes have arisen from genes involved in primary metabolism pathways (Pichersky & Gang, 2000). The plethora of specialised metabolites results from the interconnected network of reactions involving enzymes from both central and specialised metabolisms. Such non-single linear pathways, a concept also known as 'metabolic grids', give rise to a pathway architecture enabling the production of various sets of phytochemical compounds with specific functions (Tissier *et al.*, 2015). As far as their classification is concerned, specialised metabolites extracted from plants can be subdivided in three major (i.e., alkaloids, terpenes/terpenoids and phenolics) and other smaller groups (e.g., glucosinolates, cyanogenic glycosides), which may differ according to the references (Table 2; Bennett & Wallsgrove, 1994; Kabera *et al.*, 2014; Tissier *et al.*, 2015; Nwokeji *et al.*, 2016; Ahmed *et al.*, 2017).

Table 2: Major and minor classes of plant specialised metabolites. To date, no fewer than 200,000 phytochemicals have been described. Yet, this number is likely to be a gross underestimation since (i) only a fraction of all plant species has been discovered and studied, (ii) extractions have often been conducted from dry material and (iii) extractions have often focused on specific tissues. The classification may vary according to references. The major classes are the most compound-diversified ones. **HCAA.** Hydroxycinnamic acid amides.

	SPECIALISED METABOLITE CLASS	DEFINITION
MAJOR CLASSES	Alkaloid	Nitrogen-containing compounds with basic properties usually synthesised from amino acids
	Terpene / Terpenoid	Isoprenoid compounds made of variable numbers of the C5 isoprene unit (usually lower than eight units)
		Terpenes refer to olefinic compounds
		Terpenoids refer to oxidised compounds
	Phenolic (HCAA, flavonoid and others)	Aromatic ring-containing compounds attached to one or more hydroxyl groups
		HCAA refer to hydroxycinnamic acid-conjugated polyamines
		Flavonoids refer to C15 polyphenolic compounds with two aromatic rings connected through a C3 bridge
MINOR CLASSES	Glucosinolate	Sulphur-containing compounds including a β-thioglucose unit, a sulphonated oxime unit and a variable side chain derived from an amino acid
	Cyanogenic glycoside	Amino-acid derived compounds attached to one or more carbohydrates
	Other	

1.4.2. OCCURRENCE IN POLLEN AND NECTAR

While specialised metabolites were originally studied in vegetative tissues (e.g., leaves) for their roles against foliar herbivores, a mounting number of analyses have described their occurrence in floral tissues such as pollen and nectar (Adler, 2000; Irwin *et al.*, 2014; Stevenson *et al.*, 2017; Palmer-Young *et al.*, 2019). Similarities and differences between pollen and nectar specialised metabolites can be introduced from qualitative and quantitative perspectives.

- **Qualitatively, pollen and nectar specialised metabolite profiles can be similar or distinct.** For example, among the 102 identified compounds extracted from the 31 flowering plants they studied, Palmer-Young and collegues (2019) showed that overall 66% of the compounds were not shared between pollen and nectar. Besides, they found that pollen had 63% higher chemical richness than nectar, suggesting that pollen typically had the most diversified profile. Such trends were also found in Cook *et al.* (2013) for *Delphinium* spp. alkaloids.
- **Quantitatively, for similar profiles, pollen and nectar specialised metabolites can be found either in similar or in distinct concentrations/ratios.** Once again, Palmer-Young and collegues (2019) measured that pollen had 23.8- to 235-fold higher specialised chemical concentrations. Moreover, they often found consistent specialised chemical ratios between pollen and nectar tissues. Higher concentrations of alkaloids were also found in pollen in Gosselin *et al.* (2013) and Cook *et al.* (2013) in comparison with nectar. By contrast, similar concentrations of two iridoid glycosides were found in pollen and nectar of *Chelone glabra* in Richardson *et al.* (2016), whereas Detzel & Wink (1993) found a higher alkaloid concentration in *Brugmansia aurea* nectar than pollen.

These previous studies suggest that while some plants may selectively allocate specialised metabolites both quantitively and qualitatively, some others may not be able to do so. Even more intriguingly than that, it seems that specialised chemistry not only varies between plant species but also between cultivars and sites of a given species (Palmer-Young *et al.*, 2019), thereby making the occurrence of specialised metabolites in floral parts and their regulation a challenging enigma in pollination ecology. In order to account for the presence of *toxic* specialised compounds in pollen, Rivest & Forrest (2020) have recently proposed three main hypotheses, which can be extrapolated to explain the occurrence of *toxic* specialised metabolites in nectar (Table 3; Adler, 2000; Heil, 2011).

Table 3: Hypotheses to explain the occurrence of pollen and nectar defence compounds. Several hypotheses have been proposed to explain the occurrence of *toxic* specialised metabolites in nectar and pollen of flowering plants. These hypotheses can be either non-adaptative (i.e., pleiotropy) or adaptative (i.e., defence against pollen collection or antimicrobial). Such occurrences could be species- and context-dependant, and to date, no 'golden rules' has stood out.

HYPOTHESIS	EXPLANATION	EXAMPLE IN POLLEN	EXAMPLE IN NECTAR
PLEIOTROPY (NON-ADAPTIVE)	Physiological spill-over, which makes the occurrence of specialised metabolites in pollen and nectar an indirect consequence of the production of these compounds in other plant tissues. This non-adaptive trait may stem from passive leakage via vascular transport as well as genetic correlation between tissues.	Kessler & Halitschke (2009) found that flavonoid concentrations were correlated between pollen and leaves of *Solanum peruvianum*.	Adler *et al.* (2006) found a foliar herbivory-induced alkaloid increase in nectar in *Nicotiana tabacum*. Adler *et al.* (2012) showed that nicotine concentrations were correlated between nectar and other tissues of *Nicotiana* spp.
DEFENCE AGAINST POLLEN COLLECTION (ADAPTIVE)	Defence compounds that are perceivable by floral visitors and minimise pollen collection by species which do not, or only poorly, contribute to pollen transfer (i.e., pollen thieves). These compounds could also minimise pollen collection by legitimate floral visitors, which remove a substantial proportion of pollen that will not be available for plant sexual reproduction (i.e., overexploitation) and favour efficient pollinators.	Wang *et al.* (2019) demonstrated that greater saponin concentrations in *Dipsacus* sp. pollen induced behavioural changes in bumblebee individuals (i.e., less pollen groomed from the body to the pollen baskets) which led to greater deposition of pollen grains on stigmas.	Barlow *et al.* (2017) showed that two *Aconitum* spp. produced nectar alkaloids that were more distasteful to the nectar robber *Bombus terrestris* than the legitimate pollinating bee *Bombus hortorum*.
ANTIMICROBIAL (ADAPTIVE)	Antimicrobial compounds which prevent colonisation by pollen- and nectar-adapted microbes that may act as antagonists and impair plant reproduction (e.g., by affecting pollinator-attracting pollen and nectar composition).	Fatrcová-Šramková *et al.* (2013) demonstrated that phytochemical extracts from different flowering plant species prevent some bacterial strains from growing *in vitro*.	Aizenberg-Gershtein *et al.* (2015) observed that pyridine alkaloids in *Nicotiana* spp. nectar affect the richness and composition of its bacterial communities.

Whether the occurrence of specialised metabolites in pollen and nectar is simply a pleiotropic consequence of plant defences in vegetative tissues or whether they serve an adaptive function has been scarcely tested. However, for pollen chemical defences, since plants may be able to control the production and allocation of specialised metabolites between anthers and pollen (Detzel & Wink, 1993; Cook *et al.*, 2013; Stegemann *et al.*, 2019a), and even between pollen coat and pollen cytoplasm (Kempf *et al.*, 2010), the pleiotropy hypothesis does not seem a sufficient explanation for the occurrence of specialised metabolites in pollen. By contrast, in nectar, the non-adaptive pleiotropy hypothesis cannot be ruled out so easily, as some studies assumed that specialised compounds in nectar may even have more costs than benefits for plants (Adler & Irwin, 2005, 2012).

From a defence-against-pollen-collection perspective, the Optimal Defence Theory, proposed by McKey (1974), predicts that defensive chemicals are preferentially allocated to plants' most valuable tissues. This tissue-related theory lies on three parameters, namely (i) the rate at which a tissue is attacked in absence of any defensive chemical, (ii) the ecological and physiological cost of using these chemicals in that tissue, and (iii) the fitness value of that tissue (i.e., the cost when that tissue is removed; McKey, 1974). On the one hand, given that pollen contains the male gamete (i.e., most valuable in plant sexual reproduction), the presence of chemical toxins in bee-harvested pollen makes intuitive sense. In addition, as this tissue is frequently 'attacked' by pollinators, it implies that it is likely to have high levels of constitutive chemical defence and low inducibility (Zangerl & Rutledge, 1996). On the other hand, the occurrence in nectar presents an ecological paradox since this tissue is a reward for flower visitors. However, increasing evidences suggest that floral nectar, in addition to pollen, has driven the co-evolution between plants and their pollinators, and that floral nectar may have tremendous beneficial effects for both plants and pollinators (Stevenson *et al.*, 2017; Stevenson, 2020). Given the crucial role of nectar in plant sexual reproduction along with pollen, one may understand why these tissues satisfy the Optimal Defence Theory. While mitigating visits by poorly efficient pollinators, phytochemicals in pollen and nectar could favour specialisation as well (i.e., dietary niche initially free of competition; Heil, 2011; Rivest & Forrest, 2020; Stevenson, 2020). However, a proper demonstration[1] of how pollinators drive pollen and nectar chemical evolution is lacking so far.

[1] Even though many experiments showed that pollen and nectar specialised metabolites could optimise plant sexual reproduction (Stevenson *et al.*, 2017; Stevenson, 2020), evidences supporting a primary role of bees in driving pollen chemical defences are lacking (Santangelo *et al.*, 2019).

The outer layer of the pollen grain (i.e., the pollenkit) and nectar are both suitable habitat for many microbial colonists (Herrera *et al.*, 2009; Manirajan *et al.*, 2018), but the impacts of such communities on plant fitness are rather unknown. Yet, inconsistently with the third hypothesis, it has been shown that some bacterial and fungal communities could be unaffected by specialised metabolites and, by contrast, could even modify central and specialised metabolite compositions thereby reshaping plant-pollinator interactions (Vannette & Fukami, 2016; Rering *et al.*, 2018).

Overall, despite the crucial roles that pollen and nectar specialised metabolites play, proper empirical evidences for any of the three previous hypotheses are so far limited. Even more challenging, the occurrence of specialised metabolites in these tissues is likely to be species- and context-dependant (Kessler & Kalske, 2018).

1.4.3. IMPACTS ON POLLINATORS

Overall, our knowledge concerning plant-pollinator interactions via specialised metabolites found in nectar and pollen is rather limited. It has been shown that bees may have the abilities to assess their food quality (i.e., including specialised metabolites; Wright *et al.*, 2010) via pre-ingestive (i.e., the resource is not consumed) and post-ingestive (i.e., the resource is consumed) mechanisms. While pre-ingestive mechanisms concern olfactory and chemotactile cues, post-ingestive ones can be either pre-digestive (e.g., reduction of digestibility) or post-digestive (e.g., toxin accumulation), both post-ingestive mechanisms having physiological and/or colonial consequences (Brochu *et al.*, 2020). For instance, from a pre-ingestive perspective, Detzel & Wink (1993) demonstrated that 39 out of the 63 compounds they provided to honeybees were deterrent. Besides, a very recent study demonstrated that pre-ingestive effects in bumblebees greatly rely on doses since a specialised metabolite may elicit a given behaviour for a certain dose range while eliciting the opposite behaviour for doses under/above this range (Sculfort *et al.*, 2021). From a post-ingestive perspective, Vanderplanck *et al.* (2020) observed a reduction in food collection after bees were fed with phytosterol extracted from *Taraxacum* pollen, likely because of the digestive damages this diet triggered. Despite that phytosterols cannot be considered as specialised metabolites, the latter may cause digestive damage as well (Brochu *et al.*, 2020) thereby suggesting they could reduce food consumption in this way as well. Significantly, ‘time-laspe post-ingestive mechanisms’ can occur in social species when foragers rely on behavioural or chemical feedback from relatives to adjust their foraging patterns (i.e., nurse bees and brood; Grüter *et al.*, 2013; Ruedenauer *et al.*, 2016). Particularly, a noteworthy post-ingestive effect of specialised metabolite

consumption in bees concern the phenotypic variations of the newly emerged individuals for which wing morphology can be used as a proxy (Palmer & Strobeck, 1986; Gérard *et al.*, 2018). This phenotypical trait is vital for bee selective value since it influences their foraging range, survival rate, fecundity and reproductive success (Cartar, 1992; Wootton, 1992; Beukeboom, 2018).

Pollen and nectar specialised metabolites thus seem to function as a double-edged sword by playing dual roles in plant-pollinator interactions, and such roles could concern either pre-ingestive or post-ingestive mechanisms (Table 4; reviewed in Adler, 2000; Irwin *et al.*, 2014; Stevenson *et al.*, 2017; Rivest & Forrest, 2020; Stevenson, 2020). To cope with the adverse effects induced by specific specialised metabolite consumption, bees have developed a vast array of biochemical and physiological mechanisms including detoxification, conjugation, target-site insensitivity, sequestration and rapid excretion (Irwin *et al.*, 2014). Microbial endosymbionts also play a tremendous role after the consumption of specialised metabolites (Kešnerová *et al.*, 2017), while microbial hive-associated symbionts in social bees may degrade hazardous specialised metabolites in food stores before they are consumed by larvae and adults (Anderson *et al.*, 2011). In generalist species, foragers may display pollen mixing behaviours to regulate their intake of specialised metabolites, regardless of their ability to detect them (Eckhardt *et al.*, 2014). In social species, a 'social detoxification system' may occur when adult bees mix foraged pollen altogether and with nectar to dilute toxins, and when nurse bees provide larvae with specialised metabolite-poor processed food (Berenbaum & Johnson, 2015; Vanderplanck *et al.*, 2018). It is however important to emphasise that pollen and nectar phytochemicals not only have adverse effects but also positive consequences when ingested by bees. Hence, some specialised metabolites have been found to reduce infection in parasite-challenged bees (Richardson *et al.*, 2015; Palmer-Young *et al.*, 2016; Stevenson *et al.*, 2017; Stevenson, 2020) which opens up very crucial questions in the field of pollination ecology.

Table 4: Dual roles of pollen and nectar specialised metabolites towards bee pollinators. The effects of these specialised metabolites could be either pre-ingestive or post-ingestive, or both. The interactions concern the relationships between a plant and its floral visitors. **VOC.** Volatile organic compound.

	PRE-INGESTIVE EFFECT	POST-INGESTIVE EFFECT
↗ INTERACTION	✦ Attraction (VOC) ✦ Phagostimulant ✦ Enhanced foraging	✦ Enhanced memory for high-quality pollen ✦ Reduced parasite load
↘ INTERACTION	✦ Repellence (VOC) ✦ Deterrence ✦ Reduced foraging	✦ Oocyte inhibition ✦ Decreased size and number of brood ✦ Reduced survival of larvae ✦ Reduced survival of adults ✦ Induced 'malaise' behaviour ✦ Decreased activity ✦ Male:Female ratio alteration

1.5. Bee enemies and defences

1.5.1. BEE PARASITES, PARASITOIDS AND PATHOGENS

In the wild, bees are challenged with a vast array of parasites, parasitoids and pathogens[2] that belong to taxonomically diverse groups ranging from metazoans (e.g., nematodes) to microbes (e.g., viruses; Table 5). While most studies concern social honeybees (Genersch, 2010b; Simone-Finstrom, 2017) and bumblebees (Shykoff & Schmid-Hempel, 1991a; Schmid-Hempel, 2001; Rutrecht & Brown, 2008; Meeus *et al.*, 2011; Jones & Brown, 2014), many solitary species have also been found to be infected by parasites, parasitoids and pathogens (McMenamin & Flenniken, 2018; Tian *et al.*, 2018; Moure-Oliveira *et al.*, 2019; Strobl *et al.*, 2019; Figueroa *et al.*, 2021). All these bee enemies vary in their host range, transmission route (i.e., conveyance of disease from one individual host to another) and virulence (i.e., ability to injure the host and alter its fitness). The theory of virulence-transmission trade-off predicts that virulence and transmission rate are tightly linked, wherein transmission rate increases with

[2] *Parasites* are defined as to organisms living at their host's expense by depriving the latter of useful substance or having other harmful influences. *Parasitoids* are defined as to insects with a larval stage that feeds on a host's body while the adult parasitoid is free-living. *Pathogens* are defined as to microorganisms producing disease under normal conditions of host resistance (Onstad *et al.*, 1968).

increasing virulence (Acevedo *et al.*, 2019). In bee enemies, these parameters may greatly fluctuate, as some infections can be considered as benign while other can have tremendous impacts on their bee host fitness (Schmid-Hempel, 2001; Meeus *et al.*, 2011; Jones & Brown, 2014). Such antagonists can be transmitted either horizontally between related or non-related individuals of the same generation (e.g., via trophallaxis or shared flowers; Adler *et al.*, 2019) or vertically into the next generation (e.g., transovarian transmission; Schmid-Hempel, 1998). Bee infection by biological antagonists can result in a wide diversity of outcomes regarding bee physiology (e.g., lesion, metabolic change, immune system challenge; Schmid-Hempel, 2013) and behaviour (e.g., reduced foraging efficiency; Gómez-moracho *et al.*, 2017). Nevertheless, the lifecycle and virulence of many bee enemies remain hitherto virtually unknown, rendering this area of study a major concern in a context of worldwide bee decline driven by combined factors, including their biological enemies (Potts *et al.*, 2010; Goulson *et al.*, 2015; Siviter *et al.*, 2021).

Table 5: Non-exhaustive list of bee parasites, parasitoids and pathogens. While some bee antagonists are restricted to a single host species, some can be inter-specifically transmitted. Host species list might also be non-exhaustive as solitary bee antagonists are still poorly described. Sometimes, effects on host behaviour and fitness are not supported across all studies, which suggests strong context-dependant virulence.

PARASITE SPECIES (TAXON)	HOST SPECIES	TRANSMISSION ROUTE	EFFECT ON HOST BEHAVIOUR AND FITNESS	REFERENCE
Conopid (Diptera)	Bumblebee Centridini	Gravid parasitoid adult lays eggs in the host	Stay outside the nest at night Altered floral choice Altered circadian rhythm Soil digging Premature death	Schmid-Hempel & Schmid-Hempel, 1990; Müller, 1994; Schmid-Hempel, 2001; Moure-Oliveira *et al.*, 2019
Sphaerularia bombi **(Nematoda)**	Bumblebee	Third-stage larvae come out the bumblebee anus and penetrate new hibernating queens	Queen digs small depression in soil and crawls under the leaves Castrated queens endlessly flying close to the ground	Alford, 1969; Poinar & Van der Laan, 1972
Apicystis bombi **(Apicomplexa)**	Honeybee Bumblebee Osmiini	Sporozoites reside in the fat body and oocysts are released in faeces which implies an oral-faecal route	Queen death prior to colony founding Fat body destruction	Lipa & Triggiani, 1996; Rutrecht & Brown, 2008; Tian *et al.*, 2018
Nosema bombi **(Microsporidia)**	Bumblebee	Sporoblasts develop in several host tissues and spores are shed in faeces which implies an oral-faecal route Larvae can be infected (transovarian)	Reduced colony size No sexual offspring production Queen's abdomen paralysis	Mcivor & Malone, 1995; Otti & Schmid-Hempel, 2007, 2008; van der Steen, 2008
Locustacarus buchneri **(Acari)**	Bumblebee	Immobile adult female mites live in the tracheae of the adult bees Larviform female and adult male move towards bee pupae	Lethargic behaviour Premature death Decreased foraging	Otterstatter & Whidden, 2004; Yoneda *et al.*, 2008

Deformed wing virus **(Picornavirus)**	Honeybee Bumblebee Osmiini Xylocopini	Vectorised by the mite *Varroa destructor* Oral-faecal route, larval infection and cannibalism	Deformed wings Premature death Shorter duration and distance flights Reduced homing abilities	Iqbal & Mueller, 2007; Graystock *et al.*, 2016b; Tehel *et al.*, 2016; Wells *et al.*, 2016
Crithidia mellificae **(Euglenozoa)**	Honeybee Bumblebee Osmiini Halictini	Flagellum attachment in the bee ileum and different life stages shed in faeces which implies an oral-faecal route	Intestinal epithelium damage Increased colony mortality in social hosts Reduced male survival in solitary hosts	Ravoet *et al.*, 2013; Strobl *et al.*, 2019; Ngor *et al.*, 2020
Paenibacillus larvae **(Firmicutes)**	Honeybee	Vegetative bacteria colonize the bee larval midgut and massively proliferate before turning into highly resistant and infective spores	Bee midgut epithelium disruption Increased colony mortality (American Foulbrood)	Genersch, 2010a

1.5.2. NON-IMMUNOLOGICAL DEFENCES AND SELF-MEDICATION

To cope with enemy attacks, bees have evolved several defence mechanisms that may greatly diverge between bee species. On the one hand, enemy-challenged bees can fight infection through evolutionary greatly conserved immune system-related cellular (e.g., phagocytosis and encapsulation) and humoral defences (e.g., antimicrobial peptide secretion and melanisation), whether the response is specific (i.e., immune priming) or nonspecific (reviewed in Fowler *et al.*, 2020). In bees, humoral defences are enabled by the fat body which is an immunoprotein-producing organ serving as a suitable proxy for the measurement of induced humoral immunocompetence (i.e., capacity to mount an immune response; Ellers, 1996; Wilson-Rich *et al.*, 2008; Rosales, 2017). Such immune system-related mechanisms crucially rely on preferential foraging behaviour in order to reach specific nutrient intake to develop strong immune responses (Di Pasquale *et al.*, 2013; Roger *et al.*, 2017; Poissonnier *et al.*, 2018). Yet, on the other hand, non-traditional non-immunological defences, discussed for insects in Parker *et al.* (2011), should also be considered, since they are likely to influence host-parasite evolution just as much as do classical immune defences. Such traits can lead to a reduction of the likelihood to get infected (i.e., pre-infection) or to a decrease of antagonist

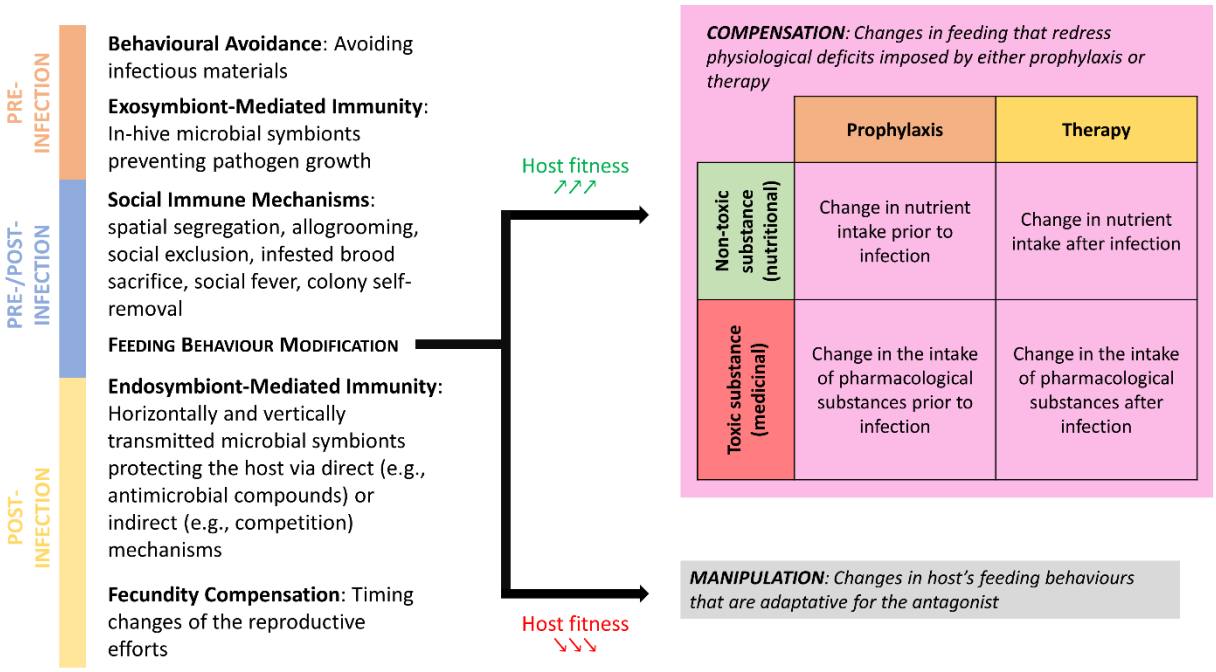

Figure 2: Non-immunological mechanisms against enemies and adaptative plasticity in insects. Non-immunological defences greatly influence host-antagonist co-evolution on condition that these defences (i) protect the host against its antagonists, (ii) are heritable, (iii) have an energetic cost for their activation/maintenance and (iv) are influenced by antagonist genotypes. Non-immunological and immune system-based defences influence the evolution of each other. While many studies suggested the occurrence of all these behaviours in bees, proper empirical demonstrations are still lacking. Such demonstrations will have to establish that non-immunological responses are adaptive for the host and not a consequence of antagonist manipulation to increase its own fitness. Diagram adapted from Anderson *et al.* (2011), Parker *et al.* (2011), Abbott (2014) and Bernardo & Singer (2017).

burden (i.e., post-infection; Figure 2). Among these mechanisms, some may be linked to the modification of host feeding behaviours, even though it should be noted that such alterations may be actually caused by parasite manipulation to its own advantage (Figure 2; Bernardo & Singer, 2017). Generally, these host fitness-increasing parasite-altered feeding behaviours are referred as to 'self-medication behaviours' which are defined as the use of organic compounds specifically for the purpose of helping to clear a biological infection or reduce its symptoms (Lozano, 1998). Self-medication patterns, as described above for other non-immunological defences, can either occur before infection (i.e., prophylactic) or after infection (i.e., therapeutic; Figure 2) at individual or collective levels (De Roode *et al.*, 2013; Abbott, 2014; De Roode & Hunter, 2018). When consumed therapeutically, these substances may influence two axes, namely 'resistance' (i.e., the ability to limit parasite infection) and 'tolerance' (i.e., the ability to limit the damage caused at a given infection load; Raberg *et al.*, 2007). Several criteria have been outlined to properly define self-medication behaviours (Figure 3),

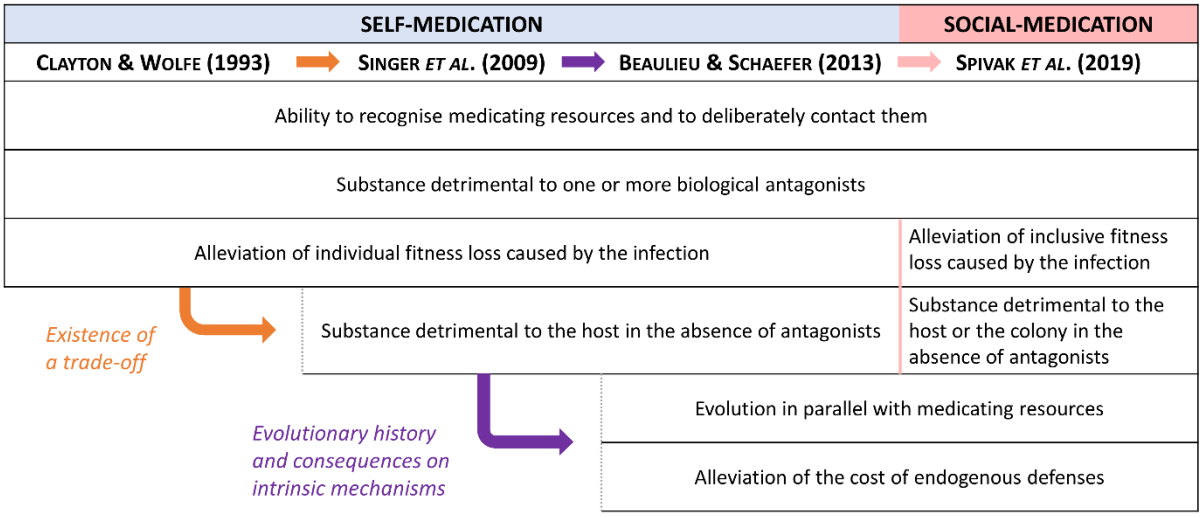

Figure 3: Conditions for self- and social-medication against antagonist attacks. Clayton & Wolfe (1993) established the three first criteria. Singer *et al.* (2009) argued that the existence of a trade-off was essential otherwise the infected host would continuously feed from the medicating resource. Beaulieu & Schaefer (2013), when talking about self-medication in the case of oxidative stress, also discussed the host-medicating resource co-evolutionary history and the host's intrinsic mechanisms to deal with antagonists. Spivak *et al.* (2019) compared self- and social-medications in social species.

sensu Clayton & Wolfe (1993), Singer *et al.* (2009), Beaulieu & Schaefer (2013) and also *sensu* Spivak *et al.* (2019) for social species (i.e., 'social-medication'). The fact that medication must compulsorily be detrimental to the antagonist is under debate in the scientific community, because medication can be beneficial for the host (i.e., increases host's tolerance) without being harmful for the antagonist (i.e., does not increase host's resistance; De Roode *et al.*, 2013; De Roode & Hunter, 2018).

As far as parasite-altered behaviours are concerned, there is mounting evidence that infected bees alter their nursing and foraging strategies in order to fight antagonists. In the social honeybee, *Nosema*-infected nurse workers showed preference for antimicrobial-rich honey (i.e., pharmacology; Gherman *et al.*, 2014) while foragers from colonies infected with the fungus *Ascophaera apis* increased their resin foraging rate to produce propolis (i.e., pharmacophory; Simone-Finstrom & Spivak, 2012). In the primitively social bumblebee, *Crithidia*-infected workers preferred nicotine-laced solution when foraging (Baracchi *et al.*, 2015). Besides, numerous non-behavioural studies showed that phytochemical consumption may alleviate parasite load in infected bumblebees (Manson *et al.*, 2010; Anthony *et al.*, 2015; Biller *et al.*, 2015; Richardson *et al.*, 2015; Thorburn *et al.*, 2015; Palmer-Young *et al.*, 2016; Koch *et al.*, 2019). However, genuine demonstrations of self-medication behaviours according to the criteria listed in Figure 3 are still missing. For instance, while Baracchi *et al.* (2015) found parasite-reducing feeding behaviours in bumblebees, they did not observe any fitness increase

in parasitised host, which is an important condition to define such behaviours. Moreover, in social species, self- and social-medication patterns are challenging to demonstrate because phytochemical effects should be discussed at individual and colonial levels. For instance, while in social-medication the phytochemical consumption must be beneficial to infected colonies, the cost in the absence of antagonist infection could concern individual and/or colonial levels, which makes this criterium a matter of attention (Spivak *et al.*, 2019).

1.6. Objectives of the study

This study is based on four main concepts that are, on the one hand, the concepts of 'Pollen Dilemma' and 'Optimal Defence Theory' in an entomophilous angiosperm, and, on the other hand, the concepts of 'Non-immunological Defence' and 'Self-medication' in a social bee species. To test these concepts, we first analysed the phenolamide-related specialised metabolite profiles in the leaves and the petals as well as in pollen and nectar of the common sunflower *Helianthus annuus* L. (Asterids: Asteraceae). We then conducted laboratory bioassays on the buff-tailed bumblebee *Bombus terrestris* (L.) inoculated with the obligate intestinal parasite *Crithidia bombi* Lipa & Triggiani and provided with sunflower pollen or sunflower pollen phenolamide extracts. We focused on parameters at the microcolonial (i.e., microcolony development, parasite load and stress responses) and individual (i.e., fat body content and phenotypic variation) levels. This experimental design allowed for the assessment of three hypotheses that could be stated as follows:

- ✓ **First hypothesis**: The entomophilous plant *H. annuus* should be able to selectively allocate its specialised metabolites, hereafter limited to phenolamide compounds, both qualitatively and quantitatively in order to protect its most valuable tissues, in particular pollen since it contains the male gametes. To test this hypothesis, we determined the phenolamide profiles in sunflower pollen, nectar, leaves and petals;
- ✓ **Second hypothesis**: Non-infected bumblebee *B. terrestris* individuals continuously provided with sunflower pollen or field-realistic concentrations of phenolamide compounds should have their individual and/or colonial fitness lowered. To test this hypothesis, we conducted bioassays on non-infected *B. terrestris* microcolonies provided either with control pollen, sunflower pollen or sunflower phenolamide-supplemented control pollen;
- ✓ **Third hypothesis**: Providing bumblebee *B. terrestris* individuals infected with the protozoan gut parasite *C. bombi* with sunflower pollen or field-realistic concentrations

of phenolamide compounds should alleviate their parasite load and/or offset their individual and/or colonial fitness loss caused by the parasite infection. To test this hypothesis, we conducted bioassays on infected *B. terrestris* microcolonies provided with the same diets as in the second hypothesis description and compared them to their respective non-infected microcolonies.

The fulfilment of these hypotheses is discussed and a thorough discussion around plant-bee-parasite concerns is also proposed. Caveats and limitations are listed, and further perspectives are suggested.

This study is part of the METAFLORE project (Figure 4; Duez, Gerbaux & Michez, 2018), which is a collaborative research action (*Action de Recherche Concertée, ARC*). The goal of this project is to determine a list of flowering plants, commonly found in Belgium, that could be favoured in flower strips in order to enhance bee health in a context of pollinator decline. This project also includes gut microbial analyses, which were not conducted in this master's thesis. All the experiments have been distributed among the Laboratory of Zoology (Prof. P. Rasmont and Prof. D. Michez; UMONS), the Laboratory of Organic Synthesis and Mass Spectrometry (Prof. P. Gerbaux; UMONS) and the Laboratory of Therapeutic Chemistry and Pharmacology (Prof. Pierre Duez; UMONS).

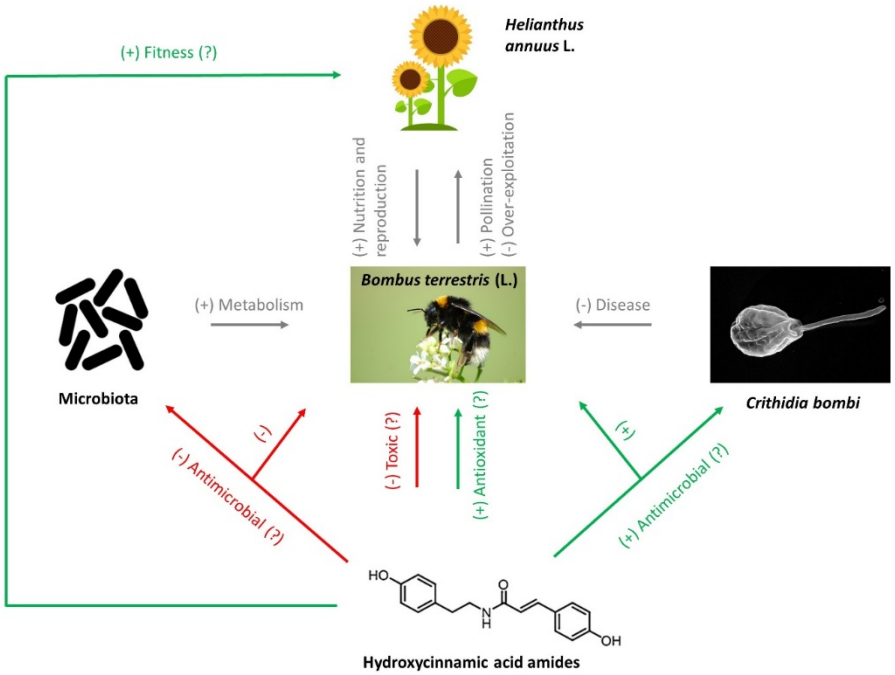

Figure 4: Graphical summary of a specific case in the METAFLORE project. This diagram depicts the complex and multidimensional plant-bee-microorganism-metabolite interactions for the plant, bee and parasite species studied here. The METAFLORE project includes other bee species such as *Osmia cornuta* (Latreille) and *Osmia bicornis* (L.), other plant species such as *Crataegus monogyna* Jacq. as well as other specialised metabolite families such as the alkaloids and flavonoids. **Hydroxycinnamic acid amides.** Synonym for 'phenolamides'. **Green arrow.** Positive impact of phenolamides on bee fitness. **Red arrow.** Negative impact of phenolamides on bee fitness.

2. MATERIALS AND METHODS

2.1. Biological models and studied molecules

2.1.1. *BOMBUS TERRESTRIS* (L., 1758)

Bumblebees (Hymenoptera: Apidae: *Bombus* spp.) are annual primitively social insects in which young overwintering queens emerge in spring to found a colony on their own. After several weeks, the colony produces gynes (i.e., daughter queens) and drones (i.e., males) that leave the nest and mate. At the end of the colony development, the queen loses its dominance and workers also start laying haploid eggs (i.e., leading to male individuals). The new mated queens go into hibernation to start the next-generation colonies in the following spring (Duchateau & Velthuis, 1988; Goulson, 2010). The bumblebee *Bombus terrestris* L. (Hymenoptera: Apidae) was selected as model because of its easy rearing and its wide geographic range in the west Paleartic. Since this social species is a highly polylectic bumblebee foraging on hundreds of different plant species (i.e., generalist species; Kleijn & Raemakers, 2008; Rasmont *et al.*, 2008; Leonhardt & Blüthgen, 2012), it plays a relevant role as a pollinator in wild and cultivated plant communities thereby ensuring significant ecosystem services (Velthuis & Doorn, 2006). *B. terrestris* is a pollen-storing species which means that workers accumulate pollen and nectar in different containers, and that they feed larvae progressively during their entire development by regurgitating a mix of pollen and nectar in their wax cell (Michener, 1974; Pendrel & Plowright, 1981; Pereboom, 2000). Bumblebees have been declining worldwide (Goulson *et al.*, 2008; Williams & Osborne, 2009; Nieto *et al.*, 2014) mainly because of habitat loss, pesticide use, climate change and parasite spread (Potts *et al.*, 2010; Vanbergen *et al.*, 2013; Goulson *et al.*, 2015), even though the opposite trend has been found for *B. terrestris* (Ghisbain *et al.*, 2021; Herbertsson *et al.*, 2021).

2.1.2. *CRITHIDIA BOMBI* LIPA & TRIAGGINI, 1980

Crithidia bombi (Euglenozoa: Kinetoplastea: Trypanosomatidae) is an obligatory gut parasite of *Bombus* spp. (Lipa & Triggiani, 1988; Schmid-Hempel, 2001). This parasite is often found at prevalence of 10–30% in bumblebee populations but has been recorded at prevalence up to 80% in some areas (Shykoff & Schmid-Hempel, 1991a). *C. bombi* infection can be relatively benign when bees have sufficient food resources, but under food-limited conditions *Crithidia*-infected bees can have a mortality rate increase of 50% (Brown *et al.*, 2000; Conroy *et al.*, 2016). *C. bombi* infection can also impair associative learning, flower handling and

foraging efficiency, as well as decrease queen survival to diapause by 15% and successful colony initiation by 40% (Brown *et al.*, 2003b; Otterstatter *et al.*, 2005; Gegear *et al.*, 2006; Otterstatter & Thomson, 2006; Fauser *et al.*, 2017). Infection is also associated with decreased likelihood of reproduction in the wild (Goulson *et al.*, 2017). In the bumblebee-*Crithidia* system, infection has been shown to increase time spent at individual flowers, which likely increases the probability of parasite transmission (Otterstatter & Thomson, 2006). As far as within-colony development is concerned, *C. bombi* infection does not alter the production of eggs and larvae (Richardson *et al.*, 2015) in queenless microcolonies or sexuals (i.e., males and young queens; Imhoof & Schmid-Hempel, 1998; Rutrecht & Brown, 2009; Fauser-Misslin *et al.*, 2014) in queenright colonies, on condition that the foundress queen did not get infected prior to hibernation (Brown *et al.*, 2003b). Nonetheless, *Crithidia*-infected workers have been shown to have smaller ovarioles (Brown *et al.*, 2000; Schmid-Hempel, 2001; but see Brown *et al.*, 2003a), which affects their timing of oviposition, their reproductive potential, and their influence on social harmony in a queenright colony (Shykoff & Schmid-hempel, 1991). *C. bombi* resides in the hindgut of bumblebees (Koch *et al.*, 2019) and is transmitted vertically by overwintering queens and horizontally through bumblebee faeces deposited on nest material (Schmid-Hempel, 2001) or on bee-pollinated flowers (Durrer & Schmid-Hempel, 1994; Adler *et al.*, 2019).

A remarkable observation in the *Bombus*-*Crithidia* system is the occurrence of strong host-parasite genotype-genotype (GxG) interactions (Schmid-Hempel & Schmid-Hempel, 1993). A single colony of *B. terrestris* represents a full sister group (Schmid-Hempel & Schmid-Hempel, 2000) with poor genotypic divergence, and bumblebee colonies are usually infected by a plethora of *Crithidia* strains (Schmid-Hempel & Reber Funk, 2004; Ulrich *et al.*, 2011; Tognazzo *et al.*, 2012) between which genetic exchanges are known to occur (Schmid-Hempel *et al.*, 2011). Molecular work has even identified different infecting types now classified as distinct species (i.e., *C. bombi* and *C. expoeki*; Schmid-Hempel & Tognazzo, 2010). Moreover, Koch & Schmid-Hempel (2012) showed that the gut microbiome, which is virtually identical among individuals from the same colony, also drives the specificity of interactions in the *Bombus*-*Crithidia* system. Discrepancy in infection outcomes among colonies is then likely to occur. For example, it has been shown that interactions between parasite strains and host colonies could affect parasite transmission (Shykoff & Schmid-Hempel, 1991b), as well as prevalence and intensity of infections (Baer & Schmid-Hempel, 2003; but see Logan *et al.*, 2005). Impacts of infection on ovariole size and fat body content have also been found to differ among host colonies (Brown *et al.*, 2000). Resistance against *C. bombi* has been found to be

compromised in small inbred population (Whitehorn *et al.*, 2011), thereby making *C. bombi* infection an accentuating threat in the current bumblebee decline (Goulson *et al.*, 2008) and ecosystem service losses (Vanbergen *et al.*, 2013; Potts *et al.*, 2016).

2.1.3. *HELIANTHUS ANNUUS* L., 1753

Sunflower *Helianthus annuus* L. (Asterids: Asteraceae) is a major oilseed crop worldwide, native to central North America (Reagon & Snow, 2006), with varieties relying on self-pollination and varieties relying on cross-pollination by floral visitors (Parker, 1981). Asteraceae pollen appears to be unfavourable for bees that are not specialised on this pollen type, including generalist bee species (Genissel *et al.*, 2002; Müller & Kuhlmann, 2008; Sedivy *et al.*, 2011; Vanderplanck *et al.*, 2020a). Especially, sunflower pollen is relatively low in protein and amino acid contents (e.g., the two essential amino acids methionine and tryptophan) compared to other important bee-pollinated plants (Nicolson & Human, 2013; Yang *et al.*, 2013), which may leads to poor performance in bees that feed on it (Regali & Rasmont, 1995; Tasei & Aupinel, 2008a; McAulay & Forrest, 2019). Nevertheless, sunflower pollen displays high content of fatty acids (Kostić *et al.*, 2017) that are essential for bee reproduction and development (Manning, 2001), as well as for the development of their fat body playing a role in energy storage and immune functions (Arrese & Soulages, 2010). Despite its poor nutritional composition, sunflower pollination is supported by numerous oligolectic bee species (Hurd *et al.*, 1980), while polylectic species mostly avoid it, as Asteraceae in general (i.e., Asteraceae paradox; Müller & Kuhlmann, 2008). As far as bumblebees are concerned, it has been demonstrated that they occasionally forage on Asteraceae (Kleijn & Raemakers, 2008) and especially on sunflower (Aslan & Yavuksuz, 2010). Moreover, *B. terrestris* has been shown to be the most efficient pollinator of *H. annuus* in comparison with other bee species (Meynié & Bernard, 1997). Recently, Giacomini *et al.* (2018) discovered that sunflower pollen led to an up-to-50-fold reduction in *C. bombi* infection in the common eastern bumblebee *Bombus impatiens* and such effects were consistent across *H. annuus* cultivars (LoCascio *et al.*, 2019b). Furthermore, LoCascio *et al.* (2019a) found that both duration and timing of exposure to sunflower pollen may affect pathogen load. Finally, Adler *et al.* (2020) demonstrated that neither the fatty acids they investigated in sunflower pollen neither the specialised metabolites rutin and tricoumaroyl spermidine derivatives were responsible for *C. bombi* reduction in *B. impatiens* gut.

2.1.4. HYDROXYCINNAMIC ACID AMIDES

Hydroxycinnamic acid amides (HCAAs) are one of the major classes of phenylpropanoid metabolites found in nature and consist in hydroxycinnamic acids mono- or poly-conjugated with polyamines (Figure 5). These metabolites are evolutionary conserved across angiosperms, including *H. annuus* (Kyselka *et al.*, 2018), and have been found both in floral and vegetative tissues. They have been shown to play crucial roles in flower development, fertilisation, senescence, stress adaptation, oxidative stress resilience and pathogen protection (Macoy *et al.*, 2015). Briefly, HCAAs are specialised metabolites derived, on one side, from the phenylalanine and tyrosine pathways to get their hydroxycinnamoyl moiety and, on other side, from polyamine and amino acid decarboxylation pathways to get their amine moiety. The conjugation of these two moieties is catalysed by a panel of N-hydrocinnamoyl transferases and the resulting conjugates are then hydroxylated by various cytochrome P450 enzymes belonging to the CYP98 family (reviewed in Bassard *et al.*, 2010). HCAAs are synthesised in the cytosol but can be transported to the cell wall and within the vacuole (Liu, 2010).

Although the importance of phenolamides throughout the plant kingdom to face herbivores and pathogens has been demonstrated, the roles of their accumulation in the pollen coat are still under debates (e.g., pollen–stigma recognition, pollen adhesion to pollinators, UV protection; Roumani *et al.*, 2021) and the consequences of their occurrence in pollen on pollinators remain unknown (Vogt, 2018). Induction of phenolamide synthesis in response to chewing and sucking insects has already been found (reviewed in Roumani *et al.*, 2021) but physiological impacts on insects are still unclear. Recently, Anyanga *et al.* (2021) showed that hydroxycinnamic acid esters had an antifeedant effect, reduced egg laying and caused higher larval mortality in sweet potato weevils. Yet, these biomolecules could have distinct bioactivities than hydroxycinnamic acid amides (HCAAs) and such effects are likely insect species specific. As intriguing as it may sound, similarities have been found between HCAAs and polyamine conjugates in venoms of predaceous spiders and wasps (e.g., Blagbrough *et al.*, 1992), but Williams *et al.* (2003) showed that they were not toxic when ingested by insects, leaving HCAA impacts on insect metabolism unexplained.

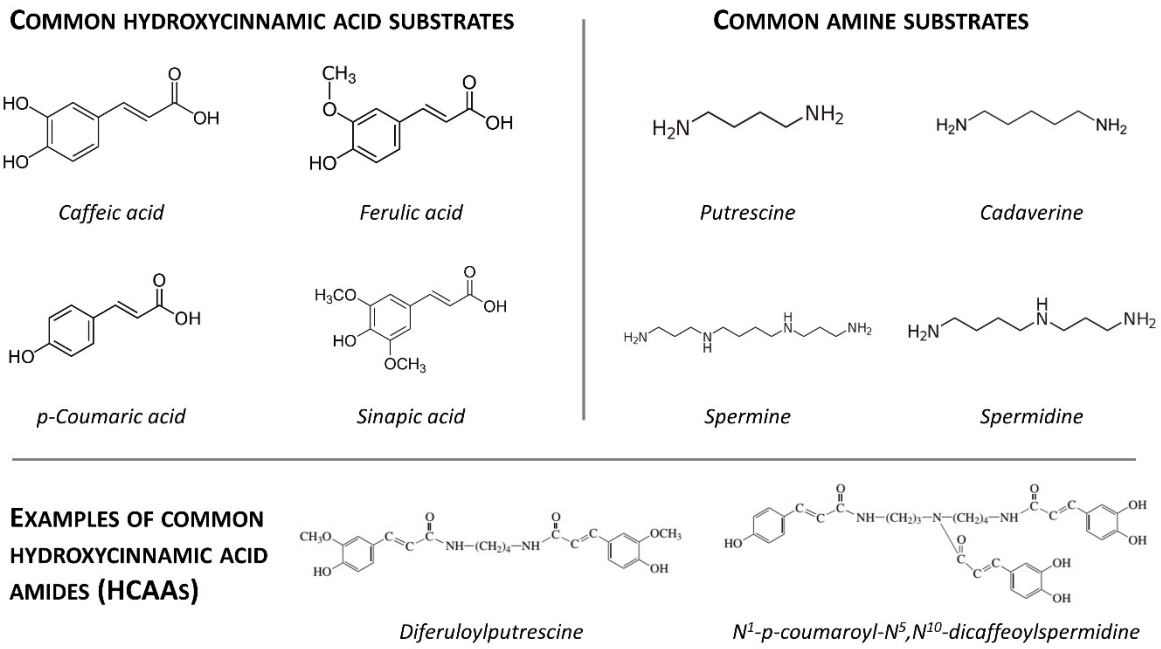

Figure 5: Chemical structures of common hydroxycinnamic acids, amines and hydroxycinnamic acid amides (HCAAs) found in plants. Several saturation degrees of polyamine amino groups occur with mono- or poly- substitution carrying the same or different hydroxycinnamic acids. All these combinations lead to a vast diversity of HCAAs.

2.2. Method details

2.2.1. PLANT TISSUE COLLECTION

In order to determine the phenolamide profiles in distinct *H. annuus* tissues, pollen, nectar, petals and leaves were sampled from five specimens (Ecoflora; Halle, Belgium) within the same location (Bee Garden, UMONS; Mons, Belgium) during August 2019 to take into account biological variation among individuals without changing the abiotic conditions (i.e., soil, exposure to light, moisture, etc.). On each individual, one or two inflorescences were covered by a net to exclude insect visits and left three days to allow massive pollen and nectar production. The first sampling session was non-invasive (i.e., no plant damage) to avoid chemical modification through the plant by activation of defensive metabolic pathways. Nectar was collected first from each inflorescence by using microcapillaries, and then pollen was collected by touching the flower with a vibrating tip. Given the massive production, one sampling session was sufficient. Samples were pooled per specimen to have sufficient amount for analyses (n = 5) and stored at -20°C until extraction. The second sampling session was invasive (i.e., plant damage) and then quickly performed to avoid biases due to activation of defensive metabolic pathways. On each individual, leaves and corolla were sampled in aluminium foil and immediately frozen in liquid nitrogen and stored at -80°C until lyophilisation (CHRIST® Alpha 1-2LDplus). Lyophilised samples were kept at room temperature, in a dark dry place.

2.2.2. EXTRACTION AND CHEMICAL ANALYSES

While pollen samples had already a powder aspect, dried sunflower leaves and petals were hammer grinded (6,000 rpm; Polymix® PX-MFC 90 D) prior to the extraction process. Samples (50 mg) were then suspended in 1 mL of a methanol/water (70:30 v/v) extraction solvent and vigorously bead beaten at 30 Hz for 2 min to disrupt the cell structure and to extract the phenolamides (five glass beads of 2 mm; Retsch® Mixer Mill MM 400). Following centrifugation at 4,500 rpm for 10 min (Sigma 2-16P), the supernatants were filtered using a 0.2 µm syringe filter (Pall Acrodisc Syringe Filter with Nylon Membrane, 13 mm) and 500 µL of the resulting solutions were accurately collected, dried at 55°C for three hours and weighed. Then the dried extracts were dissolved in 1 mL of methanol/water (70:30) solvent. Regarding nectar, samples were centrifuged at 1,000 rpm for 1 min (Sigma 2-16P) and 0.1 – 0.8 mg of the supernatants were then suspended in 200 µL of a methanol/water (70:30 v/v) extraction solvent. This solution was directly ready for injection.

Phenolamide profiles of the different samples were characterised using HPLC-MS/MS. A binary gradient was performed at a flow rate of 0.25 mL min^{-1}. The mobile phase consisted of methanol (solvent A) and water + 0.01% formic acid (solvent B). The gradient program was as follows: A = 10%, B = 90% at t = 0 min; A = 30%, B = 70% at t = 6 min; A = 35%, B = 65% at t = 11 min; A = 50%, B = 50% at t = 18 min; A = 90%, B = 10% at t = 23 min; A = 100%, B = 0% at t = 25 min; A = 100%, B = 0% at t = 27 min, A = 10%, B = 90% at t = 30 min. The temperature of the column was maintained at 40°C and the autosampler at 20°C. Injection volume was 5 µL. The mass spectrometer operated in electrospray (ESI) negative mode over a mass range of 50-2,000 Da. MS conditions were: capillary voltage 3.1 kV (2.5 kV for nectar), cone voltage +30 V (+40V for nectar), source temperature 120°C, desolvation gas temperature and flow 300°C and 500 L/h, respectively, and scan time 0.5 sec. Phenolamides from pollen and vegetative parts were separated via a Phenomenex® Kinetex C18 EVO column (150 × 2.1 mm i.d., 100 Å particle size) using a Waters™ Alliance 2695 system and then analysed via a Waters™ Q-ToF US mass spectrometer, while phenolamides from nectar were analysed using a Waters™ Acquity UPLC H-Class system (HPLC mode) and a Waters™ Synapt G2-S*i* mass spectrometer for greater sensitivity. Quantifications were performed using triferuloyl spermidine as internal standard (concentrations expressed as triferuloyl spermidine mg equivalent / sample g) in triplicates to account for analytical variability (assuming the same response factor between the extracted phenolamides and the triferuloyl spermidine). The standard of triferuloyl spermidine was synthesised in the laboratory and purified by flash chromatography (Biotage SP; Irène Semay).

2.2.3. BIOASSAYS

2.2.3.1. EXPERIMENTAL DESIGN

How *Helianthus* pollen and HCAAs from *Helianthus* pollen can impact bumblebees and their parasite load was investigated in a fully crossed design by the use of bumblebees microcolonies distributed among six different treatments: (i) uninfected microcolonies fed with control diet of *Salix*; (ii) infected microcolonies fed with control diet of *Salix*; (iii) uninfected microcolonies fed with natural diet of *Helianthus*; (iv) infected microcolonies fed with natural diet of *Helianthus*; (v) uninfected microcolonies fed with HCAA-supplemented *Salix* (i.e., HCAAs from *Helianthus* pollen added to the control diet); and (vi) infected microcolonies fed with HCAA-supplemented *Salix* (i.e., HCAAs from *Helianthus* pollen added to the control diet). The use of queenless *B. terrestris* microcolonies for testing nutritive and chemical composition of pollen diets has been successfully used in previous studies to assess the effects

of different diets on *Crithidia*-free (Genissel *et al.*, 2002; Tasei & Aupinel, 2008b; Vanderplanck *et al.*, 2014a, 2018, 2019b, 2020a; Moerman *et al.*, 2015; McAulay & Forrest, 2019; Brochu *et al.*, 2020) and *Crithidia*-infected bee colony development (Richardson *et al.*, 2015). The treatments (iii) and (v) allowed to test whether uninfected bumblebee *B. terrestris* continuously provided with field-realistic concentrations of phenolamide compounds have their individual and/or colonial fitness lowered (i.e., second biological hypothesis), while the treatments (iv) and (vi) allowed to test whether providing bumblebee *B. terrestris* infected with the protozoan gut parasite *C. bombi* with field-realistic concentrations of phenolamide compounds alleviate their parasite load and/or offset their individual and/or colonial fitness loss caused by the parasite infection.

B. terrestris workers were collected from five different colonies (i.e., A, B, C, D, and E) provided by Biobest *bvba* (Westerlo, Belgium) and allocated to 90 microcolonies (i.e., 18 microcolonies per foundress colony). Each microcolony consisted of five workers placed in different plastic boxes (10 × 16 × 16 cm), following a method adapted from Regali & Rasmont (1995). The microcolonies were distributed among the different treatments to ensure homogeneity of origins (i.e., two microcolonies from each foundress colony per treatment for a total of 15 microcolonies per treatment). Microcolonies were reared in a dark room (26-28°C; 60 ± 10% humidity), fed *ad libitum* with pollen and syrup (water:sugar 65:35 w/w), and manipulated under red light to minimise disturbance. Before the experiment, a three-day initiation period was set during which each microcolony was provided with 1 g of *Salix* pollen (pollen:water:syrup, w/w/w, 37.5:18.75:1). This initiation period allowed for workers to get used to the box, for effective parasite infection (Imhoof & Schmid-Hempel, 1998a; Logan *et al.*, 2005), and for all the microcolonies to initiate their nest. A hierarchical system occurred quickly in microcolonies and one bee typically developed into a pseudo-queen capable of laying eggs (Genissel *et al.*, 2002). After this initiation phase, microcolonies were then fed with the selected diet treatments for a 35-day period (i.e., experiment period). During this experiment period, new pollen candies were provided every two days (1-3 g depending on the size of the microcolony) to avoid nutritional and phytochemical alterations. An extra box without bees was implemented and managed in the same way as the other microcolonies to control for evaporation in every pollen diet and in sugar syrup. When a dead worker was reported inside a microcolony, it was removed, weighed and replaced with a new one originating from the same foundress colony. Replacing workers were marked to ensure a constant replacement follow-up (coloured dot). When a dead worker belonged to infected microcolony, the replacing one was inoculated with 25,000 *C. bombi* cells (see section 2.2.4.2) prior its introduction.

2.2.3.2. DIET TREATMENTS

To select a control diet that would allow for avoiding chemical interferences between *Helianthus* HCAA extracts and HCAA naturally occurring in any natural pollen diet, we designed a pilot research (Appendix A) to test different phytochemical-free commercial/artificial bee diets (i.e., Megabee, Feedbee, Nutri-bombus medium and Nutri-bombus high). Regrettably, we could not find any appropriate artificial diet and decided to use *Salix* pollen, which is described as an excellent resource for *B. terrestris* microcolonies (Tasei & Aupinel, 2008a; Vanderplanck *et al.*, 2018). The control diet consisted then in pollen loads with a dominance of *Salix* sp. purchased from the company 'Ruchers de Lorraine' (France) that were homogenised, grinded and mixed with 65% sugar solution (w/w). The natural diet of *Helianthus* consisted in hand-sorted *Helianthus* pollen loads provided by the INRAE (France) that were homogenised, grinded and mixed with 65% sugar solution (w/w). Both control diet of *Salix* and natural diet of *Helianthus* were analysed in triplicates using the extraction and HPLC-MS/MS procedure described in section 2.2.2 (results in Appendix B). The HCAA-supplemented diet consisted in HCAAs from *Helianthus* pollen added to the control diet in proportions that mimic their ratios in the *Helianthus* pollen diet. The HCAA extract used for the preparation of the HCAA-supplemented diet was prepared using a large-scale extraction of phenolamides on hand-sorted *Helianthus* pollen loads (same batch than the one used for the natural diet of *Helianthus*). In brief, around 600 g of pollen pellets were grinded and extracted using a Soxhlet apparatus with methanol at 100°C for 30 h (i.e., roughly 30 cycles). The extract was then filtrated through a Büchner vacuum filtration funnel and evaporated to dryness using a rotavapor (IKA RV8). Small samples of extract (20-40 mg) were dissolved in 1 mL of methanol in triplicates and quantified via HPLC-MS/MS as described previously. This final crude methanol extract was then dissolved into water:ethanol 1:1 (v/v) and distilled water (Appendix B). The supplementation recipe was established based on the quantification led on the natural diet of *Helianthus.* To control for the potential negative effects of solvent used for the supplementation HCAA extract, all treatments contained water:ethanol (1:1, v/v) in same proportion. The exact compositions of the different diets are provided in Appendix B.

2.2.4. INFECTION WITH *CRITHIDIA BOMBI*

2.2.4.1. IMPLEMENTATION OF PARASITE RESERVOIRS

Nine wild *B. terrestris* queens were collected from the Mont Panisel (Mons, Belgium) on the 12th of March 2021 and placed in individual plastic boxes (10 × 16 × 16 cm) with *Salix* pollen and 65% sugar solution (w/w) provided *ad libitum*. Their faeces were separately mounted on a microscope slide and screened using a light microscope (BA210, Motic; Hong Kong, China) at 400-fold magnification for the presence of the common parasites *Crithdia bombi* (Euglenozoa: Kinetoplastea: Trypanosomatidae), *Nosema bombi* (Microsporidia: Nosematidae), *Apicystis bombi* (Apicomplexa: Neogregarinida) and *Sphaerularia bombi* (Nematoda: Tylenchoidea: Allantonematidae). Six queens harboured the targeted parasite *C. bombi*. All the queens were free of *N. bombi* and *A. bombi* but three *Crithidia*-infected queens were also infected with the nematoda *S. bombi* (eggs and third-stage juveniles observed in the faeces; Appendix C). Even if *S. bombi* has never been observed in bumblebee workers (Rutrecht & Brown, 2008), which is unsurprising given its life history (Poinar & Van der Laan, 1972), we decided to discard these queens and not to use them to inoculate the colonies (i.e., three *Crithidia*-infected queens left). Five commercial colonies were imported from Biobest *bvba* (Westerlo, Belgium) to be used as *Crithidia* stock colonies. Faeces from the three infected queens were collected on an every-two-day basis for 24 days, mixed with 65% sugar solution and poured in bottle caps that were provided inside the colonies (i.e., each colony was inoculated 12 times). The five colonies developed an infection but three were more severely infected and therefore used for further microcolony inoculations.

2.2.4.2. PARASITE PURIFICATION AND INOCULATION

For microcolony inoculation, faeces were collected from 45 workers from the three most infected stock colonies as well as from the three infected queens used to implement the parasite reservoirs. Since it is likely that different *C. bombi* strains have developed in each colony and infected queens (Schmid-Hempel *et al.*, 1999; Schmid-Hempel & Reber Funk, 2004), we sampled faeces from different colonies to get multiple-strain *inocula* and thus to minimise the impact of any specific host-parasite interaction that could interfere with microcolony inoculations. Faeces were pooled, diluted with 0.9% NaCl solution to make a 1 ml solution and purified following a 'triangulation' method (Appendix D) developed by Cole (1970), and adapted by Baron *et al.* (2014) and Martin *et al.* (2018). A few microliters of the resulting solution (tube 6 in Appendix D) were placed in a Neubauer chamber, allowing for the

concentration of *C. bombi* cells in the solution to be counted. The resulting solution was adjusted to 2,500 cells μL^{-1} with 40% sugar solution (w/w). In individual Nicot® cages, bees from 'Parasite' treatments (see section 2.2.3.1) were starved for 5 h and then presented with a 10 µl drop of *inoculum* containing 25,000 cells, which lies within the range of *C. bombi* cells shed by infected workers (Logan *et al.*, 2005). Only bees that consumed the whole *inoculum* were placed in their respective microcolonies.

2.2.5. EVALUATED PARAMETERS

In this experiment, we studied parameters at the microcolonial and individual levels. At the microcolonial level, we focused on resource collection, microcolony development and parasite load. At the individual level, we measured the fat body content and the phenotypic variation.

2.2.5.1. RESOURCE COLLECTION, MICROCOLONY DEVELOPMENT AND STRESS RESPONSES

Several parameters were used to estimate the performance of the microcolonies. Thus, (i) pollen collection and (ii) syrup collection were measured every two days to account for resource collection by weighing pollen candies and syrup containers before their introduction into the microcolony and after their removal, after correction for evaporation loss. Besides, (iii) composition and fresh mass of brood (i.e., eggs, non-isolated larvae, isolated and pre-defecating larvae, isolated and post-defecating larvae, pupae, non-emerged and emerged males) were examined to account for microcolony development. Finally, (iv) percentage of ejected larvae (i.e., adult bees removed larvae from their brood and discard them when they are stressed), (v) pollen efficacy (i.e., ratio between the mass of brood and the pollen collection), (vi) pollen dilution (i.e., ratio between syrup collection and pollen collection) and (vii) worker mortality throughout the experiment (i.e., every two days) were studied as stress responses (parameters adapted from Tasei & Aupinel, 2008b and Vanderplanck *et al.*, 2020). All mass parameters (i.e., brood mass, pollen collection, and syrup collection) were standardised by the total mass of workers in the microcolonies to avoid potential bias from worker activities (i.e., consumption and brood care).

2.2.5.2. PARASITE LOAD

Parasite load was another parameter monitored at the microcolonial level. For three days after inoculation, parasite loads were not monitored to allow for the infection to develop and the number of *C. bombi* cells to increase in the faeces (Imhoof & Schmid-Hempel, 1998a;

Logan *et al.*, 2005). Then, at the beginning of the experiment period and on an every-three-day basis, microcolonies were monitored for parasite load. Three workers per microcolony were selected randomly, individually placed in 50 mL Falcon® tubes and their faeces were collected using a 10 µL microcapillary. Faeces were pooled by microcolony (i.e., 15 faecal samples per treatment) and diluted to one fifth or to one tenth to allow for counting the *C. bombi* cells by using an improved Neubauer haemocytometer at 400-fold magnification under an inverted phase contrast microscope (Eclipse Ts2R, Nikon; Tokyo, Japan). Bees that were tagged with a coloured dot (i.e., replacing workers) were not included because they did not have the same time for their infection to develop. Furthermore, since workers were selected randomly in every microcolony for parasite counting, we wanted to examine if parasite load was consistent within microcolony. For this purpose, at the end of the experiment, in every 'Parasite' treatment (section 2.2.3.1), parasite loads were counted individually in the faeces of the five workers from three randomly selected microcolonies to assess intra-microcolony parasite load distribution. Besides, in every 'Parasite' treatment (section 2.2.3.1), one non-callow (i.e., non-newly emerged) male per microcolony was selected and its faeces observed under an optical microscope (BA210, Motic; Hong Kong, China) to examine the occurrence of infection. Similarly, the faeces from one worker per microcolony were observed in every 'No parasite' treatment (section 2.2.3.1) to make sure that unwanted contaminations did not occur.

2.2.5.3. FAT BODY CONTENT

A first parameter measured at the individual level was the fat body content. In insects, an adaptative immune system is lacking but they can fight infections through inducible humoral (i.e., production of antimicrobial peptides and reactive oxygen species) and constitutive cellular (i.e., melanisation, encapsulation and phagocytosis) responses (Strand, 2008; Rosales, 2017). While constitutive responses provide a first line of defence against a broad spectrum of non-specific antagonists, inducible responses provide a second, more specific defence against a narrow range of antagonists (Brey & Hultmark, 1998). Regarding humoral responses, the fat body – an insect equivalent of the liver – is a major organ involved in insect immunity as it plays crucial roles in energy storage and haemolymph protein synthesis (Arrese & Soulages, 2010). Particularly, the fat body is the site of production of antimicrobial peptides (Brey & Hultmark, 1998; Rosales, 2017 and references therein), and is a good proxy to estimate the trade-off between reproduction and survival as the number of eggs in the ovarioles and the fat content are negatively correlated in insects (Ellers, 1996). In bumblebees, Brown *et al.* (2000) and Brown *et al.* (2003a) demonstrated that a re-allocation of fatty resources from the ovarioles

to the fat body occurred in *Crithidia*-infected workers, but that the fat body content was not higher in infected individuals than in uninfected individuals for all that. Furthermore, previous studies failed to demonstrate that poor-quality diets could impair fat body development in bumblebees (Brown *et al.*, 2003a; Roger *et al.*, 2017; Vanderplanck *et al.*, 2018), but not in honeybees as Alaux *et al.* (2010) showed that diets low in proteins led to a reduced fat body content. Yet, the fat body content is still used as an indicator of immunocompetence in bumblebees (Vanderplanck *et al.*, 2021).

At the end of the assays, the abdominal fat body content of 30 workers and 30 drones per treatment (i.e., two workers and two drones per microcolony) was measured according to Ellers (1996). If no male or only one male emerged in a microcolony, it was offset by measuring the abdominal fat body content of males from other microcolonies derived from the same foundress colony in the same treatment. Briefly, isolated abdomens were weighed before and after drying at 70 °C for 3 days. They were then placed into 2mL of diethyl ether for 24h to extract fat, rinsed twice and weighed again after drying at 70 °C for 7 days. The fat mass proportion of an individual was defined as the abdominal mass loss during this process divided by the abdomen mass of the individual before extraction.

2.2.5.4. PHENOTYPIC VARIATION

A second parameter measured at the individual level was the phenotypic variation. Shape and size are key biological traits that may enable the evaluation of the phenotypic variation of an individual undergoing different environmental pressures (e.g., Klepsatel *et al.*, 2014; Gérard *et al.*, 2021). In insects, body size has been extensively studied and linked to flight efficiency as well as many other fitness-related performances (Birch *et al.*, 2004; Beukeboom, 2018). By contrast, the impact of stressful conditions on wing shape has received little attention but this metric was suggested as an indicator of stress (Hoffmann *et al.*, 2005; Gérard *et al.*, 2018). It is assumed that drastic changes in wing shape must affect the flight ability of insects (e.g., Ortega Ancel *et al.*, 2017) even if the extent of the consequences of stress-induced wing shape variations is unknown. Hence, variations in these traits have often been tested as potential indicators of stressful conditions, especially in bees (e.g., Vanderplanck *et al.*, 2021).

In bees, body size is particularly important as larger individuals have been shown to forage farther, live longer, have better abilities to thermoregulate and have higher reproductive success (Cartar, 1992; Wootton, 1992; Bishop & Armbruster, 1999; Beukeboom, 2018). In addition, body size is positively correlated with a greater phenoloxidase level in the haemolymph (i.e., greater immune response) when bees are challenged with *C. bombi* infection

(Otterstatter & Thomson, 2006). Wings are also crucial for foraging and dispersal capacities (Wootton, 1992; Vanderplanck *et al.*, 2021). The next crucial step would be to precisely assess the impacts of phenotypic changes on foraging performance, flight efficiency, and pollination efficiency (Gérard *et al.*, 2020). For example, in butterflies it was shown that long and slender wings are well adapted for long distance flights and dispersal while shorter and broader wings can enable more efficient slow flight, with more manoeuvrability (Betts & Wootton, 1988; DeVries *et al.*, 2010). Even if this hypothesis remains to be tested, one may also propose that wing morphology alteration in bumblebee males could reduce their reproductive success, as already demonstrated in *Drosophilia melanogaster* (Menezes *et al.*, 2013).

Here, wing size – a proxy for body size (Gérard *et al.*, 2018) – and wing shape analyses were conducted following Gérard *et al.* (2018), Vanderplanck *et al.* (2021) and Gérard *et al.* (In press). Our total dataset contained 250 males (i.e., 50 males per treatment). The right and left[3] forewings of each specimen were removed, placed on a glass slide and photographed (n = 500 pictures) using an Olympus SZH10 microscope with an AF-S NIKKOR 18–105mm (Shinjuku, Japan) and GWH10X-CD oculars coupled with a Nikon D610 camera (Shinjuku, Japan; Appendix E). Five individuals were discarded because their wings were damaged or considered as outliers (see Appendix E for details). Pictures were uploaded in the tpsUTIL 1.81 software (Rohlf, 2013b) and digitised with a set of 18 two-dimensional landmarks (tps-DIG 2.31; Figure 6; Rohlf, 2013a). Each landmark coordinate was then multiplied by its scale factor provided for each specimen ('readland.tps' command, R-package geomorph; Adams *et al.*, 2021). Next, we used the Generalised Procrustes Analysis superimposition method to remove all the non-shape components by translating specimens to the origin, scaling and rotating each landmark configuration to minimise the distance between each corresponding landmark of each landmark configuration ('gpagen' command, R-package geomorph; Bookstein, 1991; Adams *et al.*, 2021). Centroid size (i.e., the square root of the sum of squared distance between all landmarks and their centroid) of the right wings was used as a wing size – and body size – estimator.

[3] Left wings were photographed for further analyses that are not included in this manuscript.

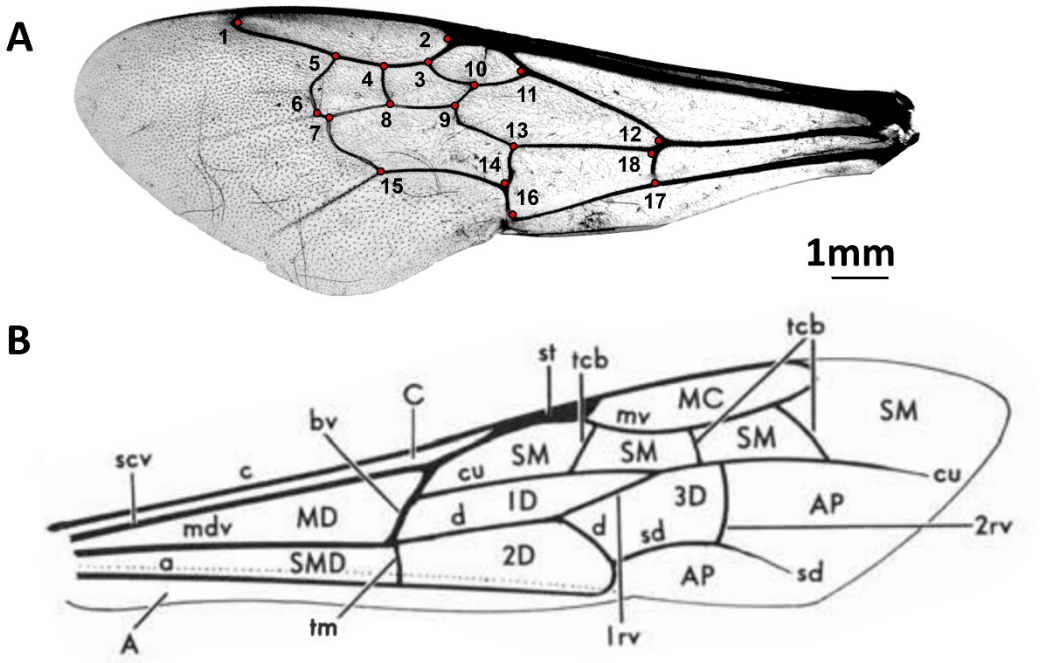

Figure 6: Hymenopteran (Apocrita) forewing. A. Left forewing picture of a bumblebee with the 18 landmarks indicated to describe the shape (retrieved from Vanderplanck et al., 2021). **B.** Right forewing diagram of a bee (Apocrita) with venational terminology (retrieved from Borror & White, 1970). VEINS: **a.** anal; **bv.** basal; **c.** costal; **cu.** cubital; **d.** discoidal; **mdv.** median; **mv.** marginal; **rv.** recurrent; **scv.** subcostal; **sd.** subdiscoidal; **st.** stigma; **tcb.** transverse cubital; **tm.** transverse median. CELLS: **A.** anal; **AP.** apical; **C.** costal; **D.** discoidal; **MC.** marginal; **MD.** median; **SM.** submarginal; **SMD.** submedian.

2.3. Data analyses

All analyses were conducted in R version 4.0.3 (R Core Team, 2020) and all the figures were generated with ggplot2 (Wickham *et al.*, 2020).

2.3.1. PHENOLAMIDE CONTENT AND PROFILE

We compared the total phenolamide content between tissues using a non-parametric Kruskal-Wallis test ('kruskal.test' command, R-package stats). When the test returned significant results, we further conducted multiple pairwise comparisons ('pairwise.wilcox.test' command, R-package stats) with Bonferroni's correction to avoid increases in type error I due to multiple testing. A total phenolamide content below the limit of detection threshold of the mass spectrometer was considered as null.

Phenolamide profiles (relative abundances) in the different plant tissues were visually assessed using a principal component analysis (PCA; 'PCA' command, R-package FactoMineR; Husson *et al.*, 2017). To determine if *H. annuus* tissues differed qualitatively and quantitatively in their phenolamide composition (relative abundances), we ran a permutational multivariate analysis of variance (perMANOVA) using the Euclidean distance and 9999

permutations (‘adonis’ command, R-package vegan; Oksanen *et al.*, 2019). When perMANOVA analyses were significant ($p < 0.05$), multiple pairwise comparisons were conducted between tissues to detect precisely the differences (‘pairwise.adonis’ command, formula from Martinez Arbizu, 2020) and *p*-values were also adjusted using Bonferroni's correction. Next, we performed an indicator species analyses to identify the phenolamides that were indicators between pollen and nectar (‘indval’ command, R-package labdsv; Roberts, 2019). This time, *p*-values were adjusted using Holm's correction to avoid increases of type error I due to multiple testing.

2.3.2. AT THE MICROCOLONIAL LEVEL

Resource collection (i.e., syrup and pollen), fresh mass of brood stages and total mass of hatched offspring were assessed using Laplace generalised linear mixed-effect models (GLMMs) with a gamma distribution and log link function (‘glmer’ command, R-package mlmRev; Bates *et al.*, 2020), which is adapted for continuous and non-normal data, after checking for overdispersion (‘testDispersion’ command, R-package DHARMa; Hartig, 2021). We could not fit linear mixed-effect models (LMMs) either because homogeneities of variances (‘leveneTest’ command, R-package car; Fox *et al.*, 2019) or normalities of residuals (‘shapiro.test’ command, R-package stats) were not met, and that no data transformation achieved to meet these assumptions. Models included Diet, Parasite and their interaction as fixed effects and Colony as a random effect. For resource collection, Day and its interactions with the other factors were added as fixed effects and Microcolony nested within Colony as a random effect to account for repeated measures. A microcolony-level random effect was also added to the models (i.e., each data point received a unique level of random effect that modelled the extra-parametric variation present in the data; Harrison, 2014) in which overdispersion occurred. Response variables that included null observations were added 0.0012 (the minimum value) to allow to use the gamma distribution.

Number of individuals per developmental stage was compared using Laplace GLMMs (‘glmer’ command, R-package mlmRev; Bates *et al.*, 2020) with a Poisson distribution and log link function with Diet, Parasite and their interaction as fixed effects and Colony as a random effect, and Microcolony nested within Colony as a random effect when overdispersion occurred. When zero inflation was detected (‘testZeroInflation’ command, R-package DHARMa; Hartig, 2021), we fitted a corresponding zero-inflated model (‘glmmTMB’ command, R-package glmmTMB; Brooks *et al.*, 2017).

Effects of Diet and Parasite on worker survival were analysed via a Cox proportional hazard (mixed-effect) model ('coxph' command, R-package survival; Therneau, 2021; 'coxme' command, R-package coxme; Therneau, 2020) which uses a hazard function evaluating simultaneously how specified factors influence the rate of a particular event happening (i.e., death) at a particular moment (Bradburn *et al.*, 2003). We treated survival data as right censored, with individuals that survived after 35 days and individuals that were accidentally lost (e.g., squashed, escaped) considered as 'censored', and dead individuals as 'uncensored'. Models included Diet, Parasite and their interaction as fixed effects, and Microcolony nested within Colony as a random effect. However, Akaike's Information Criterion (AIC) comparison between models including or dropping the random effect suggested that the random effect was not significant. We hence decided to drop it in the final model. Proportionality of hazards ('cox.zph' and 'ggcoxzph' commands, R-package survival; Therneau, 2021) and absence of influential observations ('ggcoxdiagnostics' command, R-package survminer; Kassambara *et al.*, 2021) were checked to validate the Cox model assumptions.

Larval ejection was examined by fitting a Laplace GLMM with a binomial distribution and logit link function ('glmer' command, R-package mlmRev; Bates *et al.*, 2020), with the number of ejected larvae and the total number of living offspring produced per microcolony as a bivariate response, with Diet, Parasite and their interaction as fixed effects, and Colony as a random effect. Since overdispersion occurred ('testDispersion' command, R-package DHARMa; Hartig, 2021), a microcolony-level random effect was added to the model.

To test for differences in pollen efficacy and pollen dilution, we used LMMs ('lmer' command, R-package lme4; Bates *et al.*, 2021) with Diet, Parasite and their interaction as fixed effects and Colony as a random effect. While the assumption of normality of the residuals ('shapiro.test' command, R-package stats) was met, the variance of the residuals was not constant among treatments (i.e., heteroscedasticity; 'leveneTest' command, R-package car; Fox *et al.*, 2019). We thus applied a Box-Cox transformation on the response variables (i.e., iterative process to calculate an exponential transformation variable lambda; $\lambda = 1.247$ for pollen efficacy and $\lambda = -0.896$ for pollen dilution; 'powerTransform' command, R-package car; Osborne, 2010; Bates *et al.*, 2021).

Parasite load between treatments was analysed using a Laplace GLMM with a gamma distribution, with Diet, Day and their interaction as fixed effects. We also fitted three generalised linear models (GLM; i.e., one per diet) with a gamma distribution and log link function ('glm' command, R-package stats) with Microcolony nested within Colony as a fixed effect. At the end of the follow-up period (i.e., 35 days), we measured parasite load from all

workers (n = 5) in three microcolonies per infected treatment (n = 3). We checked the normality of counting observations grouped by microcolonies ('shapiro.test' command, R-package stats). As normality was not achieved in some microcolonies ($p < 0.05$), we opted for a Fligner-Killeen test ('fligner.test' command, R-package stats) instead of Bartlett's or Levene's tests – because it was more robust against departures from normality – to assess the variance within and between microcolonies. We expected to observe lower intra-microcolony than inter-microcolony variances. We also fitted a linear model (LM; 'lm' command, R-package stats) with Diet and Microcolony nested within Diet as fixed effects. In this latter model, parasite load was square root transformed to achieve normality of residuals and homogeneity of variances.

When models returned significant *p*-values (< 0.05), we did not report and discuss the statistical outputs for explanatory variables alone when significant interactions were detected between them (see Appendix F for all the statistical outputs). In that case, we only reported and discussed the statistical outputs of the significant interactions. Indeed, there is no sense in discussing explanatory variables alone when interactions occur (Berrington de González & Cox, 2007). We computed and compared least-square means based on main explanatory variables or their interactions to detect precisely which treatments differed between each other ('lsmeans' command, R-package lsmeans; Lenth, 2018).

2.3.3. AT THE INDIVIDUAL LEVEL

Fat body content was analysed using a Laplace GLMM ('glmer' command, R-package mlmRev; Bates *et al.*, 2020) with a gamma distribution and log link function with Diet as well as Parasite as fixed effects and Microcolony nested within Colony as a random effect. Fat body ratios were arcsine-transformed prior to analysis. Over 360 observations, five were discarded because of aberrant values (i.e., negative ratio or ratio close to one, likely due to weighing errors).

Differences in right wing centroid size were tested using a Laplace GLMM with a gamma distribution and log link function ('glmer' command, R-package mlmRev; Bates *et al.*, 2020) with Diet, Parasite and their interaction as fixed effects and Colony as a random effect.

To understand the effects of Diet, Parasite and their interaction on right wing shape, we analysed the landmark configurations by fitting a linear model with randomised residuals in a permutation procedure ('lm.rrpp' command, R-package RRPP; Collyer & Adams, 2021) – using an ordinary least squares (OLS) estimation of coefficients on multidimensional data and a randomised residual permutation method. Fixed and random effects were the same as for the centroid size model. When significant differences in wing shape occurred ($p < 0.05$), we further

conducted multiple pairwise comparisons ('pairwise' function, R-package RRPP; Collyer & Adams, 2021) and we plotted a PCA ('gm.prcomp' function, R-package geomorph; Adams *et al.*, 2021) to illustrate visually the differences in wing shape depending on the conditions.

As previously described, when a significant interaction occurred, main single effects were not interpreted and multiple pairwise comparisons were performed on the interaction only.

3. RESULTS

3.1. Sunflower phenolamide content and profile

We determined the total phenolamide content (expressed as triferuloyl spermidine equivalent mg, TSE; Table 6) and the phenolamide profiles (expressed as fractions of the total phenolamide content) in *Helianthus* pollen, leaves, corolla and nectar via HPLC-MS/MS (Table 7).

Table 6: Total phenolamide content in *Helianthus* tissues. For the five specimens, results indicate the mean of the three analytical replicates per tissue. The last column indicates the mean, standard deviation, median, minimum and maximum of the five specimens per tissue. Superscript letters indicate the outputs of the Kruskal-Wallis test between tissues ($p < 0.001$). Results are expressed as triferuloyl spermidine equivalent (TSE) since this phenolamide was used as internal standard. Pollen and nectar were fresh but leaves and corolla were dried materials. **LOD.** Limit of detection of triferuloyl spermidine in a Waters™ Q-ToF US: $2.5*10^{-6}$ mg/mL.

TISSUE	TOTAL PHENOLAMIDE CONTENT (MG TSE / G TISSUE)					
	1	**2**	**3**	**4**	**5**	**Mean ± SD** ***Median (Min - Max)***
Leaf	0 (<LOD)	0 (<LOD)	0 (<LOD)	0 (<LOD)	0 (<LOD)	0^{a}
Corolla	0 (<LOD)	0 (<LOD)	0 (<LOD)	0 (<LOD)	0 (<LOD)	0^{a}
Nectar	9.19	24.8	0.83	21.07	4.77	12.12 ± 10.38^{b} *9.19 (0.83 – 24.8)*
Pollen	10.39	10.94	25.38	11.92	12.62	14.25 ± 6.18^{b} *11.92 (10.39 – 25.38)*

We found that the total phenolamide content varied between tissues (Kruskal-Wallis, $\chi 2 = 16.573$, df = 3, $p < 0.001$). Multiple pairwise comparisons clearly showed that sunflower leaves and corolla had a fewer total phenolamide content than sunflower pollen and nectar, but that no statistically significant difference was found between the two latter tissues (Table 6). Statistical outputs are also available in Appendix F.

Table 7: Characterization of phenolamide profiles in *Helianthus* nectar and pollen. No phenolamides were found in sunflower leaves and petals. Analyses were run on aqueous methanolic (70%) extracts.

TISSUE	COMPOUND	MOLECULAR FORMULA $[M–H]^-$	CALCULATED MASS $[M–H]^-$	Δ PPM
Nectar				
	N,N'-diferuloyl spermidine	$C_{27}H_{35}N_3O_6$	496,2448	0
	N,N',N''-tricoumaroyl spermidine	$C_{34}H_{37}N_3O_6$	582,2606	0,3
	N,N',N''-dicoumaroyl feruloyl spermidine	$C_{35}H_{39}N_3O_7$	612,2704	1
	N,N',N'',N'''-tetracoumaroyl spermine	$C_{46}H_{50}N_4O_8$	785,3549	0,1
	N,N',N'',N'''-tricoumaroyl feruloyl spermine	$C_{47}H_{52}N_4O_9$	815,3654	0,2
Pollen				
	N,N',N''-tricoumaroyl spermidine	$C_{34}H_{37}N_3O_6$	582,2625	3,6
	N,N',N''-dicoumaroyl feruloyl spermidine	$C_{35}H_{39}N_3O_7$	612,2734	3,9
	N,N',N'',N'''-tetracoumaroyl spermine	$C_{46}H_{50}N_4O_8$	785,3571	2,7
	N,N',N'',N'''-tricoumaroyl feruloyl spermine	$C_{47}H_{52}N_4O_9$	815,3744	10,8

The analyses showed than sunflower tissues differed in their HCAA profiles (perMANOVA, $\chi 2 = 33.158$, df = 3, $p < 0.001$). Further pairwise comparisons arranged the different tissues in three groups: (1) one with pollen, (2) one with nectar, and (3) one with leaf and corolla (Figure 7). All phenolamide molecules are clear indicators of the difference between pollen/nectar (group 1 and 2) and leaf/corolla (group 3), since sunflower leaf and corolla were totally free of phenolamides. Besides, the N,N'-diferuloyl spermidine ($p = 0.045$, indicator value = 1), the N,N',N'',N'''-tetracoumaroyl spermine ($p = 0.045$, indicator value = 0.71) and the N,N',N''-tricoumaroyl spermidine ($p = 0.045$, indicator value = 0.67) were indicative as differences between pollen (group 1) and nectar (group 2) HCAA profiles (Figure 7). Statistical outputs are also available in Appendix F.

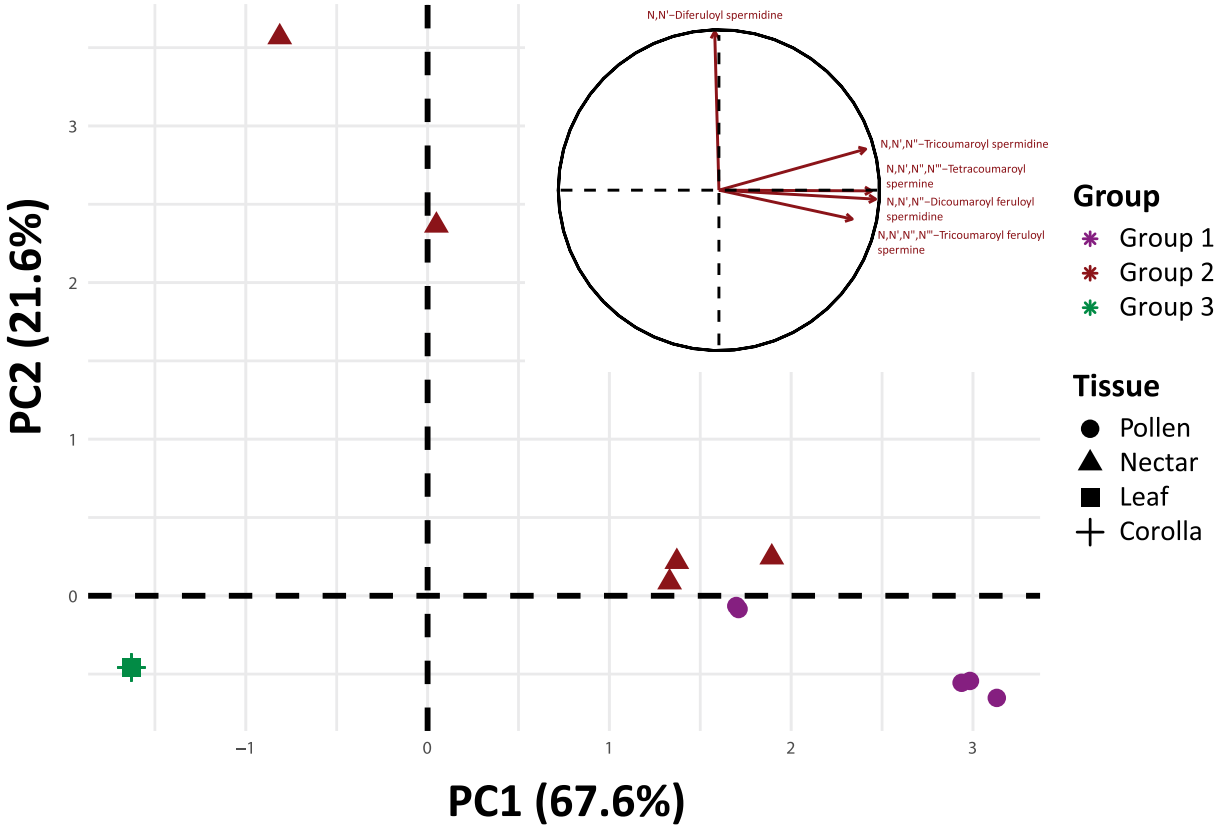

Figure 7: Ordination of the phenolamide profile in four *H. annuus* tissues along the first two axes of a principal component analysis (PCA). The correlation circle indicates the correlation between the variables and the principal components (PC) as well as the correlation between the variables themselves. The first axis (PC1) with 67.6% of the variability separates tissues according to their N,N',N''-tricoumaroyl spermidine, N,N',N'',N'''-tetracoumaroyl spermine, N,N',N''-dicoumaroyl feruloyl spermidine and N,N',N'',N'''-tricoumaroyl feruloyl spermine contents. The second axis (PC2) with 21.6% of the variability separates tissues according to their N,N'-diferuloyl spermidine contents. The first two axes of the PCA (PC1 and PC2) thus express 89.2% of the total variance.

3.2. Impact at the microcolonial level

3.2.1. Resource collection

Resource collection by bumblebee microcolonies was assessed by weighing syrup and pollen collection by workers over the course of the experiment. Statistical outputs for every explanatory variable and their respective interactions are available in Appendix F.

With regards to syrup collection, we found a significant effect of Diet, Day, Diet by Day and Parasite by Day. We also found a significant effect of the interaction between the three explanatory variables (Diet by Parasite by Day; GLMM, $\chi2 = 52.851$, df = 32, $p = 0.012$; Figure 8A). Post-hoc analyses revealed that syrup collection did not differ among diets before Day 15

but from then on, microcolonies provided with pure *Salix* pollen collected slightly more syrup than microcolonies provided with HCAA-supplemented *Salix* pollen that collected more pollen than microcolonies provided with *Helianthus* pollen. They also indicated that syrup collection peaked in the vicinity of Day 25 and Day 27 (uninfected and infected microcolonies, respectively) for pure *Salix* and HCAA-supplemented *Salix* pollen, while it remained constant in microcolonies fed with *Helianthus* pollen. Sporadically along the experiment, uninfected microcolonies collected slightly more syrup than infected ones.

With regards to pollen collection, we found a significant effect of Diet, Day, Diet by Day and Parasite by Day. We also found a significant effect of the interaction between the three explanatory variables (Diet by Parasite by Day; GLMM, $\chi 2 = 71.643$, df = 2, $p < 0.001$; Figure 8B). Further analyses suggested that microcolonies fed with pure *Salix* pollen collected more pollen than microcolonies fed with HCAA-supplemented *Salix* and with *Helianthus* pollen over the course of the experiment, while microcolonies fed with HCAA-supplemented *Salix* pollen collected more pollen than microcolonies fed with *Helianthus* pollen at the end of the experiment (i.e., after Day 25). Overall, microcolonies raised their pollen collection until Day 11 – Day 15 (uninfected and infected, respectively), which then decreased a bit and levelled off, except in microcolonies provided with pure *Salix* pollen for which another peak was observed around Day 29.

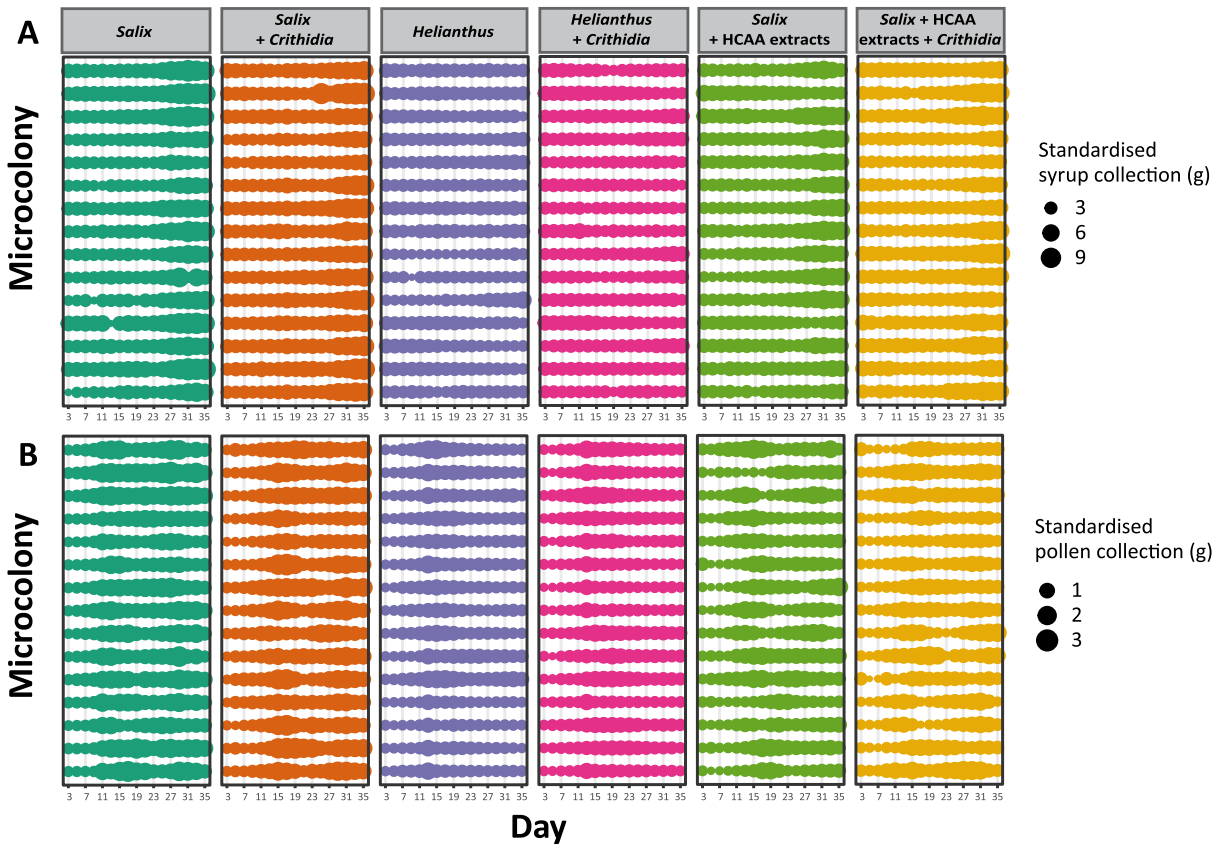

Figure 8: Standardised resources collection by bumblebees in microcolonies over time. Each horizontal line represents the consumption values for a single microcolony over time (i.e., every two days). The thickness of the line indicates the amount of resource collected. Resource collection was standardised by the total mass of workers in the microcolony. **A.** Standardised syrup collection (g). **B.** Standardised pollen collection (g).

3.2.2. MICROCOLONY DEVELOPMENT

Bumblebee microcolony development was examined through the composition and fresh mass of brood (standardised by the mass of workers within the microcolonies). Statistical outputs for every explanatory variable and their respective interactions are available in Appendix F.

We found a slightly significant effect of infection status on the number of eggs per microcolony (GLMM, $\chi2 = 5.960$, df = 2, $p = 0.015$) with post-hoc analyses showing that infected microcolonies laid fewer eggs (Figure 9A).

No difference was detected between treatments concerning the mass of non-isolated larvae but a significant difference in the number of non-isolated larvae was observed between diets (GLMM, $\chi2 = 10.429$, df = 2, $p = 0.005$), post-hoc comparisons showing that more non-isolated larvae were counted in microcolonies fed with sunflower pollen than in microcolonies fed with HCAA-supplemented *Salix* pollen (Figure 9A).

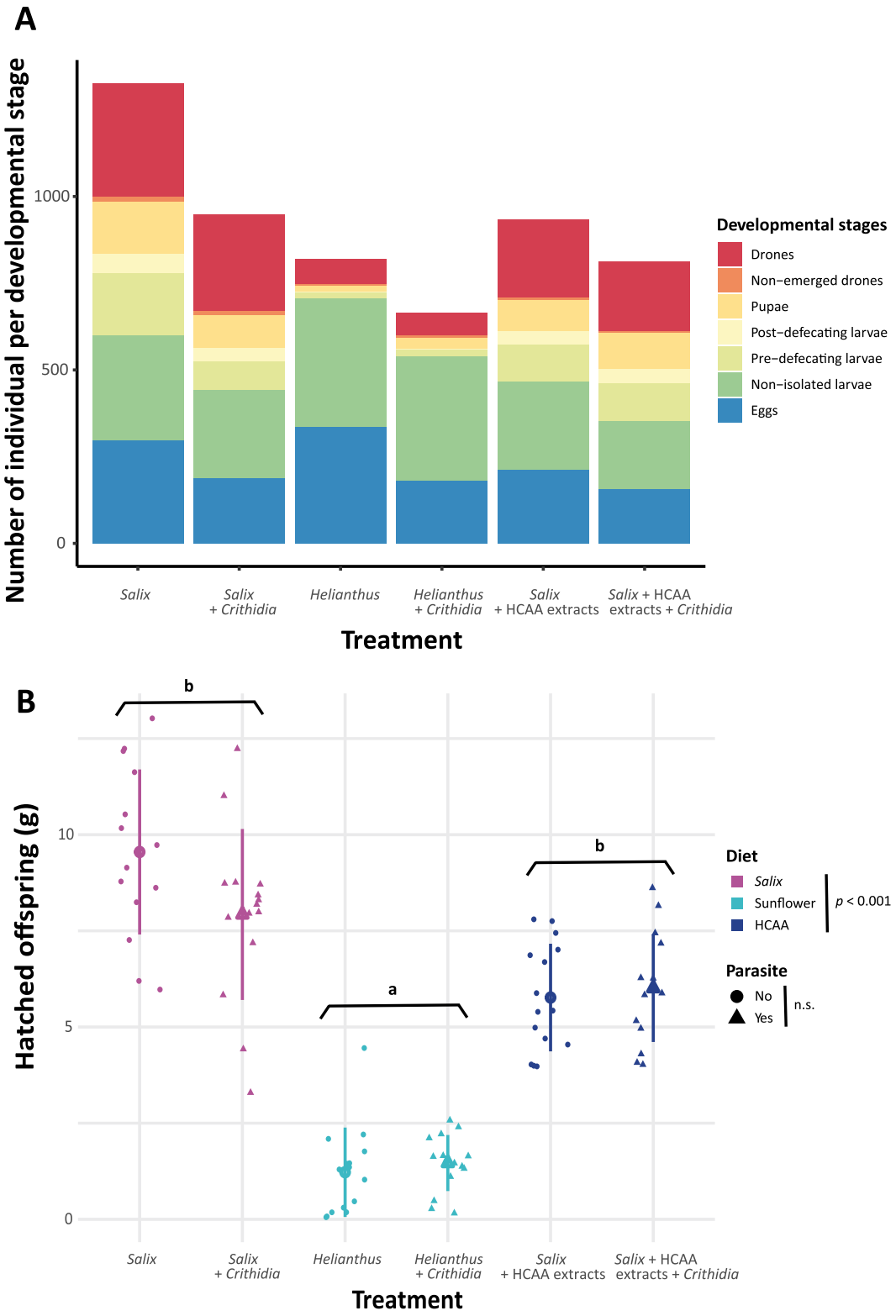

Figure 9: Effect of pollen diets and *Crithidia* infection on offspring production in bumblebee microcolonies. **A.** Number of individuals per developmental stage among treatments. **B.** Standardised mass of hatched offspring among treatments. Different letters indicate significant differences ($p < 0.05$) between treatments (GLMM and pairwise comparisons). Close points represent the microcolonies. Open points represent the mean values of each treatment and error bars indicate the standard deviations (mean ± SD). **n.s.** Non significant.

We found that Diet as well as the interaction factor Diet by Parasite had a significant effect on the mass (GLMM, $\chi2 = 9.309$, df = 2, $p = 0.010$) and the number (GLMM, $\chi2 = 8.186$, df = 2, $p = 0.017$) of pre-defecating larvae. Post-hoc pairwise comparisons showed that microcolonies fed with *Salix* pollen and HCAA-supplemented *Salix* pollen produced more pre-defecating larvae and a greater biomass of pre-defecating larvae than *Helianthus*-fed microcolonies, irrespective of their infection status. In addition, while most uninfected *Helianthus*-fed microcolonies did not produce pre-defecating larvae, many infected ones did (Figure 9A).

We found a significant effect of Diet on the mass (GLMM, $\chi2 = 105.081$, df = 2, $p < 0.001$) and the number (GLMM, $\chi2 = 36.510$, df = 2, $p < 0.001$) of post-defecating larvae produced in the microcolonies. Post-hoc analyses indicated a higher mass and a higher number of post-defecating larvae in microcolonies provided with HCAA-supplemented and pure *Salix* pollen than in microcolonies provided with *Helianthus* pollen, whether they were infected or not (Figure 9A).

Diet has a significant effect on the mass of pupae produced in the microcolonies (GLMM, $\chi2 = 105.081$, df = 2, $p < 0.001$) with post-hoc analyses showing that, in the same way as for pre- and post-defecating larvae, microcolonies fed HCAA-supplemented and pure *Salix* pollen produced more pupae than microcolonies provided with *Helianthus* pollen, irrespective of their infection status. Diet as well as the interaction factor Diet by Parasite had a significant effect on the number of pupae (GLMM, $\chi2 = 7.904$, df = 2, $p = 0.019$). Post-hoc analyses showed that microcolonies fed HCAA-supplemented and pure *Salix* pollen had a higher number of pupae than microcolonies provided with *Helianthus* pollen, irrespective of their infection status, but uninfected microcolonies provided with pure *Salix* pollen had a greater number of pupae than uninfected microcolonies fed with HCAA-supplemented *Salix* pollen (Figure 9A).

We found a significant effect of Diet on the mass (GLMM, $\chi2 = 64.862$, df = 2, $p < 0.001$) and number (GLMM, $\chi2 = 70.500$, df = 2, $p < 0.001$) of emerged males in the microcolonies. Post-hoc analyses showed that microcolonies fed HCAA-supplemented and pure *Salix* pollen had a higher mass of emerged males than microcolonies provided with *Helianthus* pollen, irrespective of their infection status. Post-hoc analyses on the number of emerged males gave the same results, but also showed that microcolonies provided with pure *Salix* pollen had more emerged males than microcolonies provided with HCAA-supplemented *Salix* pollen (Figure 9A).

Overall, we found a significant effect of Diet (GLMM, $\chi2 = 202.688$, df = 2, $p < 0.001$) on the total mass of hatched offspring (i.e., all developmental stages except eggs) with post-hoc comparisons showing a greater offspring mass production in microcolonies provided with HCAA-supplemented and pure *Salix* pollen than in microcolonies fed with *Helianthus* pollen (Figure 9B).

3.2.3. STRESS RESPONSES

Bumblebee microcolony stress responses were evaluated via larval ejection (i.e., stressed adult bees eject larvae from their brood cells and discard them), pollen efficiency (i.e., mass of hatched offspring divided by the mass of collected pollen per microcolony; it highlights when a microcolony needs more pollen to produce offspring), pollen dilution (i.e., amount of collected gram of syrup per collected gram of pollen) and worker mortality over the experiment. Statistical outputs for every explanatory variable and their respective interactions are available in Appendix F.

3.2.3.1. LARVAL EJECTION

We found a significant effect of Diet (GLMM, $\chi2 = 71.996$, df = 2, $p < 0.001$) and Parasite (GLMM, $\chi2 = 4.250$, df = 1, $p = 0.039$) on the proportion of ejected larvae in microcolonies. Post-hoc analyses revealed that this proportion was higher in microcolonies fed with *Helianthus* pollen than in microcolonies fed with pure or HCAA-supplemented *Salix* pollen, whether they were healthy or *Crithidia*-infected. Infection status was also important as healthy microcolonies displayed higher larval ejection than infected ones, even if this trend was much more pronounced in microcolonies fed with pure *Salix* pollen than in any other diets (Figure 10).

3.2.3.2. POLLEN EFFICACY

We found a significant effect of Diet and Diet by Parasite (LMM_{λ}, $\chi2 = 8.219$, df = 2, $p = 0.016$) on pollen efficacy. Post-hoc analyses showed that microcolonies provided with pure and HCAA-supplemented *Salix* pollen displayed a greater pollen efficacy than microcolonies provided with *Helianthus* pollen, irrespective of their infection status. Yet, while uninfected microcolonies fed with pure or HCAA-supplemented *Salix* pollen did not differ in their pollen efficacy, infected microcolonies fed with HCAA-supplemented *Salix* pollen showed a greater pollen efficacy than the ones fed with pure *Salix* pollen. The infection status also had significant effects in microcolonies provided with pure *Salix* pollen, as the healthy ones showed a greater pollen efficacy than the infected ones (Figure 11).

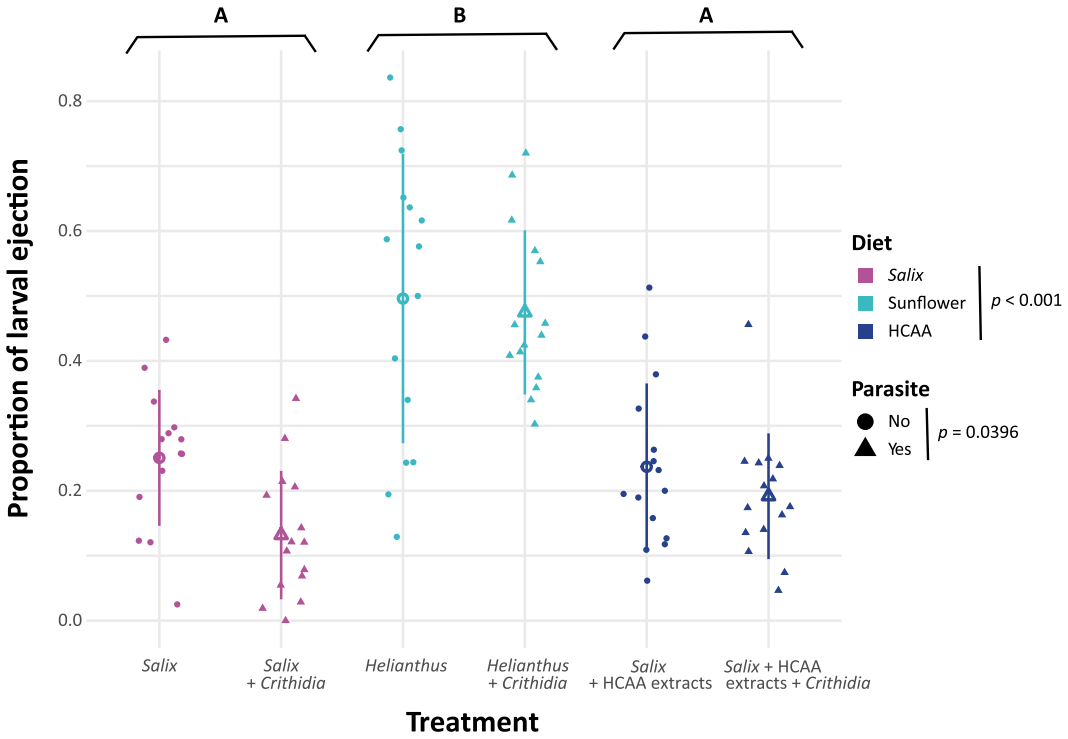

Figure 10: Effect of pollen diets and *Crithidia* infection on larval ejection in bumblebee microcolonies. The proportion of larval ejection in a microcolony was calculated as the number of ejected larvae over the total number of hatched offspring. Different letters indicate significant differences ($p < 0.05$) between treatments (GLMM and pairwise comparisons). Close points represent the microcolonies. Open points represent the mean values of each treatment and error bars indicate the standard deviations (mean ± SD).

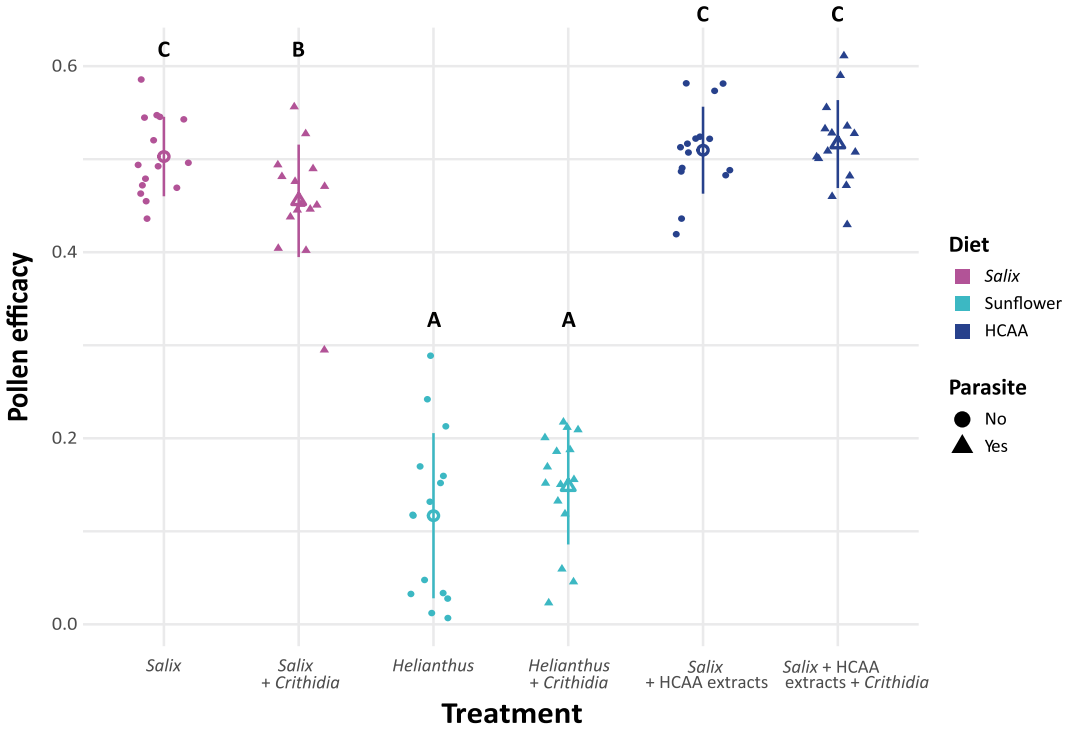

Figure 11: Effect of pollen diets and *Crithidia* infection on pollen efficacy in bumblebee microcolonies. Pollen efficacy of a microcolony was calculated as the weight of eclosed offspring divided by the total pollen consumed. Different letters indicate significant differences ($p < 0.05$) between treatments (LMM_{λ} and pairwise comparisons). Close points represent the microcolonies. Open points represent the mean values of each treatment and error bars indicate the standard deviations (mean ± SD). **n.s.** Non significant.

3.2.3.3. POLLEN DILUTION

We found a significant effect of Diet (LMM_λ, $\chi2 = 78.997$, df = 2, $p < 0.001$) on pollen dilution. Post-hoc tests indicated that pollen dilution was higher in microcolonies fed with *Helianthus* pollen and HCAA-supplemented *Salix* pollen than in microcolonies fed with pure *Salix* pollen, whether they were healthy or *Crithidia*-infected (Figure 12).

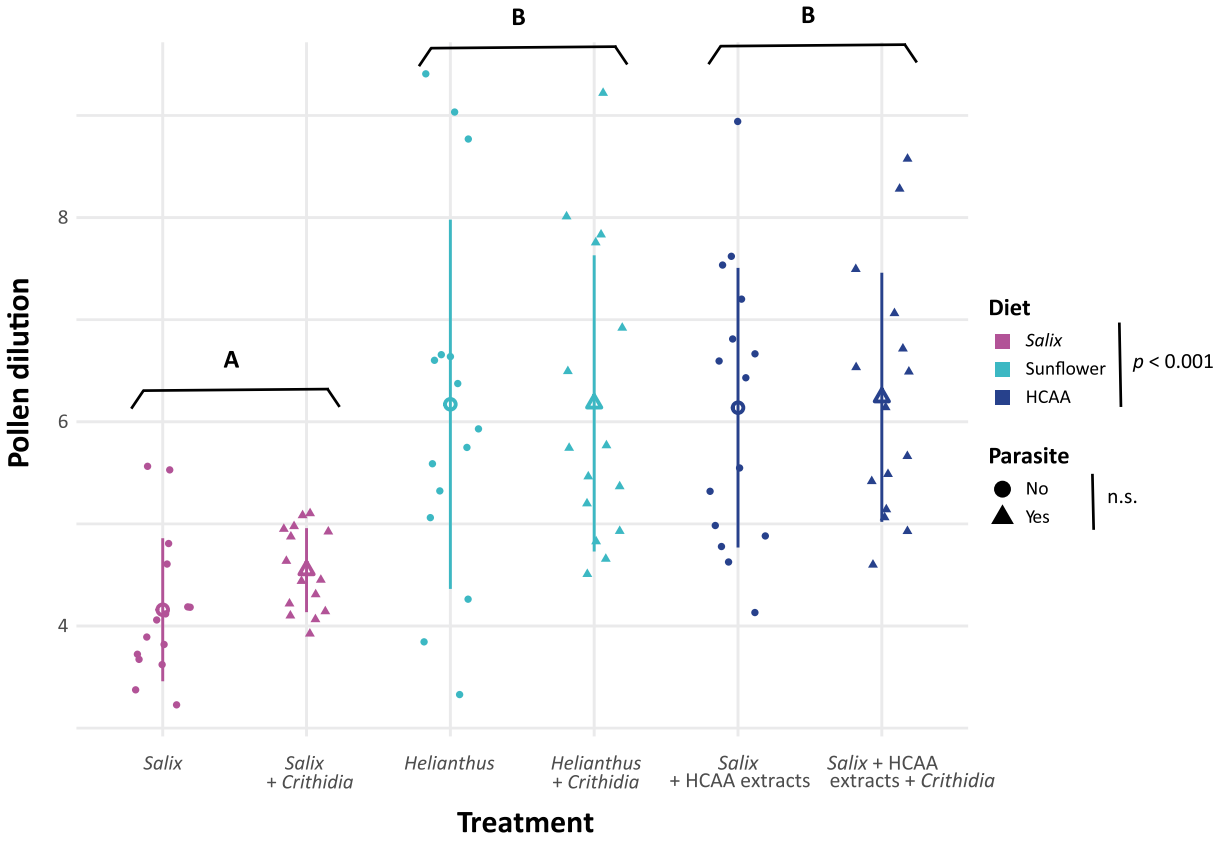

Figure 12: Effect of pollen diets and *Crithidia* infection on pollen dilution in bumblebee microcolonies. Pollen dilution of a microcolony was calculated as the amount of collected syrup divided by the amount of collected pollen. Different letters indicate significant differences ($p < 0.05$) between treatments (LMM_λ and pairwise comparisons). Close points represent the microcolonies. Open points represent the mean values of each treatment and error bars indicate the standard deviations (mean ± SD). **n.s.** Non significant.

3.2.3.4. MORTALITY

There was no difference in worker mortality rate among Diet (Cox proportional hazard model, $\chi2 = 4.330$, df = 2, $p = 0.115$), Parasite (Cox proportional hazard model, $\chi2 = 1.995$, df = 1, $p = 0.158$) and their interaction (Cox proportional hazard model, $\chi2 = 3.282$, df = 2, $p = 0.194$) despite slightly different curves in the Kaplan-Meier plot (Figure 13).

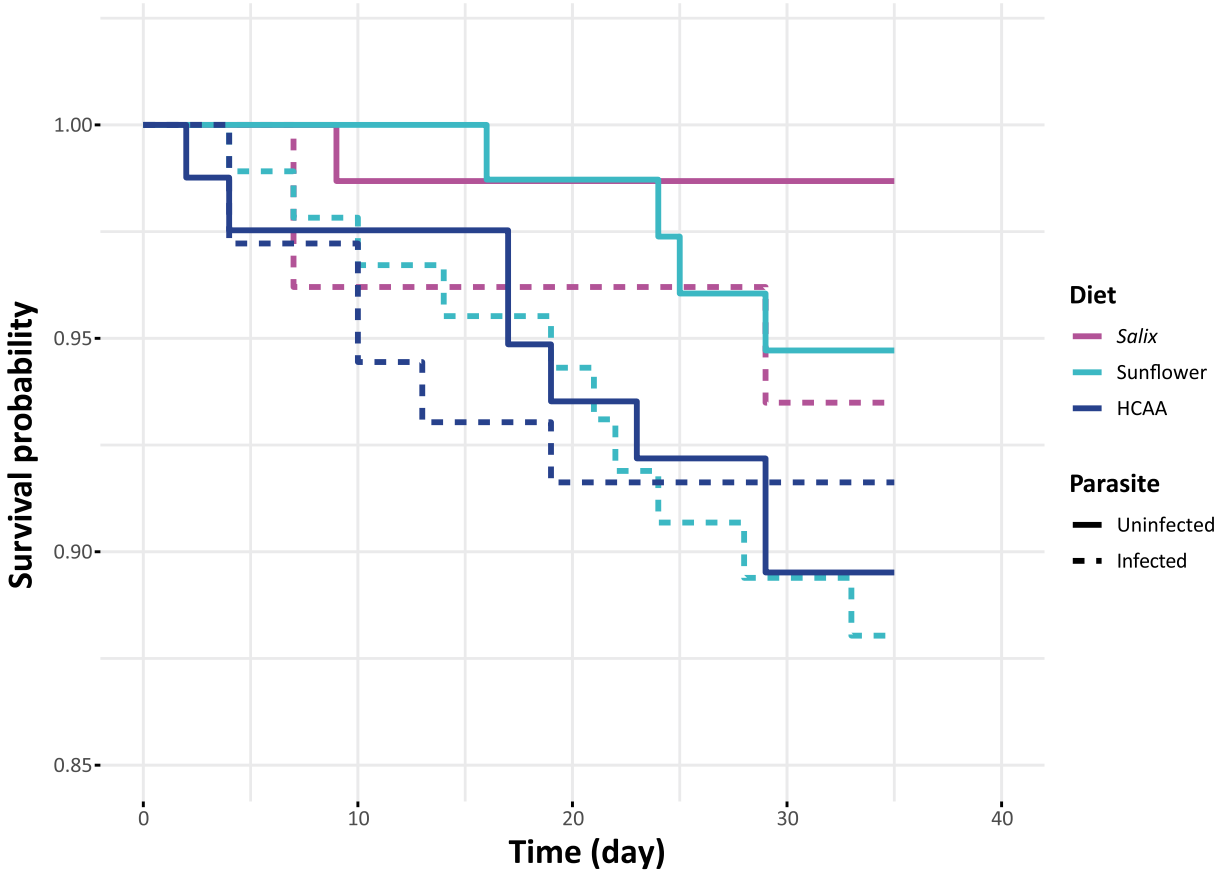

Figure 13: Kaplan-Meier survival curves of bumblebee workers. Individuals that survived after Day 35 or individuals that were accidentally lost during the experiment (e.g., squashed, escaped) were considered as right-censored data. Notice that scale limits on the y-axis (i.e., survival probability) were changed (0.85 – 1).

3.2.4. PARASITE LOAD

3.2.4.1. BETWEEN TREATMENTS

In order to assess if specific pollen diets could alter the intensity of *Crithidia* infection in bumblebee guts, we examined the parasite load in the worker faeces by microcolonies over the course of the experiment (i.e., every-three-day basis, 11 repeated measures). Statistical outputs for every explanatory variable and their respective interactions are available in Appendix F.

We found that Diet (GLMM, $\chi2 = 19.908$, df = 2, $p < 0.001$) as well as Day (GLMM, $\chi2 = 429.275$, df = 10, $p < 0.001$) had significant effects on infection intensity in worker faeces. Post-hoc analyses indicated that microcolonies provided with HCAA-supplemented *Salix* pollen had a greater infection intensity than microcolonies fed with pure *Helianthus* or *Salix* pollen, while parasite load did not differ between the two latter diets. Besides, it showed that infection intensity significantly increased until Day 10 and then started to unevenly level off, irrespective of the pollen diet. Infection never significantly decreased over time in any diet (Figure 14).

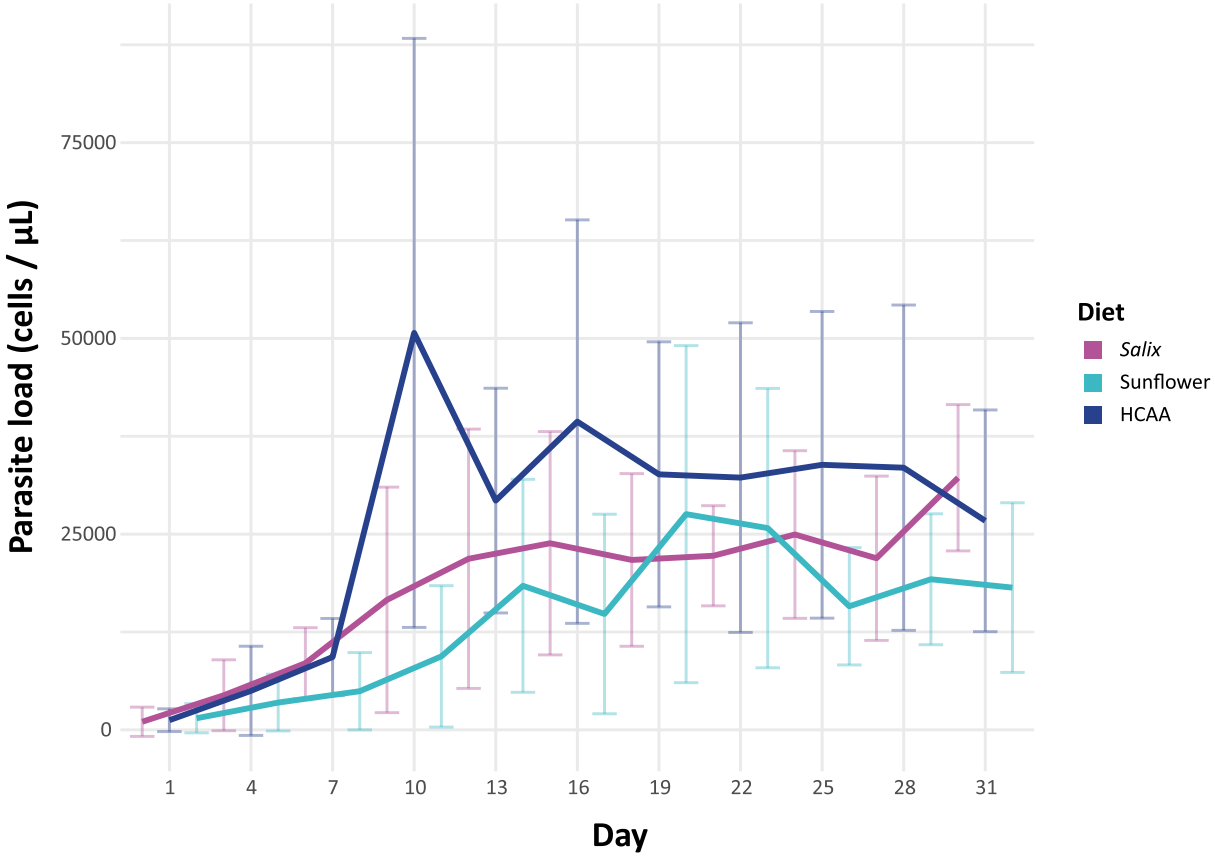

Figure 14: Dynamics of *Crithidia bombi* infection intensity over 31 days in bumblebee microcolonies. Parasite load monitoring started at Day 1, four days post inoculation (i.e., *C. bombi* infection had had four days to develop when it was counted at Day 1). Parasite load means by day and by diet were calculated to plot the lines and error bars indicate the standard deviations (mean ± SD). By-microcolony observations were not plotted to avoid graph overloading.

3.2.4.2. WITHIN TREATMENTS

We decided to compare infection intensity by diet between colonies and between microcolonies within a colony, irrespective of the day. We found that both colonies and microcolonies within a colony could displayed significant differences in parasite load (Table 8). Statistical outputs are also available in Appendix F.

Table 8: GLMs testing for differences in infection intensity between colonies and microcolonies within a colony for every diet. Significant *p*-value (<0.05) are in bold.

	SALIX	*χ2*	*df*	*p-value*
Colony		2.648	4	0.618
Colony:Microcolony		8.427	10	0.587
	HELIANTHUS	*χ2*	*df*	*p-value*
Colony		22.131	4	**< 0.001**
Colony:Microcolony		12.258	10	0.268
	***SALIX* + HCAA**	*χ2*	*df*	*p-value*
Colony		4.2904	4	0.368
Colony:Microcolony		22.164	10	**0.014**

3.2.4.3. WITHIN MICROCOLONIES

At the end of the experiment, we measured parasite loads in all workers from three microcolonies per infected treatment to assess heterogeneity of variance between microcolonies (i.e., expected heteroscedasticity). There was no evidence to suggest that variance in infection intensity was significantly different between microcolonies (Fligner-Killeen test, $\chi2 = 6.851$, df = 9, $p = 0.553$; Figure 15) which contrasts with our prior assumption (i.e., we expected to find great differences in variances between microcolonies but small differences in variances within microcolonies). Besides, a linear model showed that there was a difference in infection intensity between Diet (LM, $\chi2 = 7.370$, df = 2, $p = 0.002$) but not between microcolonies within diets (LM, $\chi2 = 2.058$, df = 6, $p = 0.085$). Post-hoc comparisons showed that infection intensity was greater in workers fed with HCAA-supplemented *Salix* pollen than in workers fed with pure *Salix* pollen (Figure 15), which is in line with results in section 3.2.4.1. Statistical outputs are also available in Appendix F.

Moreover, we isolated one emerged male per microcolony in all 'Parasite' treatments. Among 45 isolated males, 40 shed faeces (88.9%) and 36 harboured *Crithidia* cells (90%). The four males that did not show any sign of infection (10%) all belonged to the *Salix* diet. We also checked that unwanted contamination did not occur in the 'Healthy' treatments by collecting

one worker per microcolony. All workers shed faeces (n = 45) and we found that no worker was unexpectedly infected.

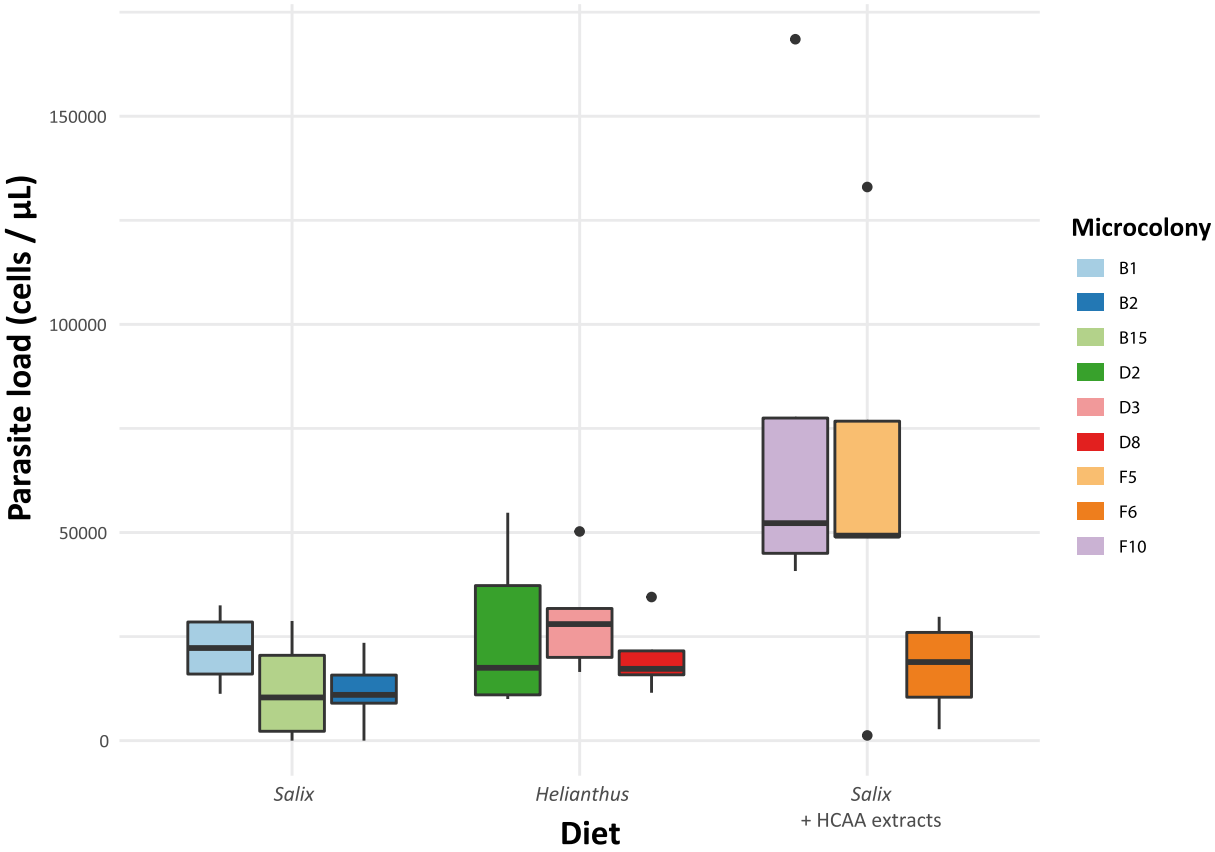

Figure 15: Comparison of *Crithidia* infections between microcolonies at the end of the follow-up period. Each microcolony consisted of five observations (i.e., five workers). We expected to observe different intra-microcolony variances but a Fligner-Killeen test countered this prediction (p = 0.553). Infection intensity was different between diets (p = 0.002) but not between microcolonies within diets (p = 0.085). The boxplots show medians (solid line, 50th percentile) and inter-quartile ranges (colored box, 25th and 75th percentile). The whiskers give the range except for "outliers" (circles) that are more than ±1.5 times the inter-quartile range larger or smaller than the median.

3.3. Impact at the individual level

3.3.1. Fat body content

We found that Caste, Diet, Parasite (GLMM, $\chi2 = 25.336$, df = 1, $p < 0.001$) and Diet by Caste (GLMM, $\chi2 = 15.219$, df = 2, $p < 0.001$) had significant effects on fat body content. Post-hoc comparisons showed that both infected workers and males had a lower fat body content irrespective of their diet. In workers, individuals fed with pure *Salix* pollen had a greater fat body content than individuals fed with HCAA-supplemented *Salix* and *Helianthus* pollen, but no difference was found between the two latter diets. In males, this difference was only found between individuals fed with pure and HCAA-supplemented *Salix* pollen. Besides, workers had a greater fat body content than males when provided with pure or HCAA-supplemented *Salix* pollen, but such a difference was not identified for the *Helianthus* diet. It is also interesting to note that the variance seemed to be higher among male individuals than among female individuals (Figure 16).

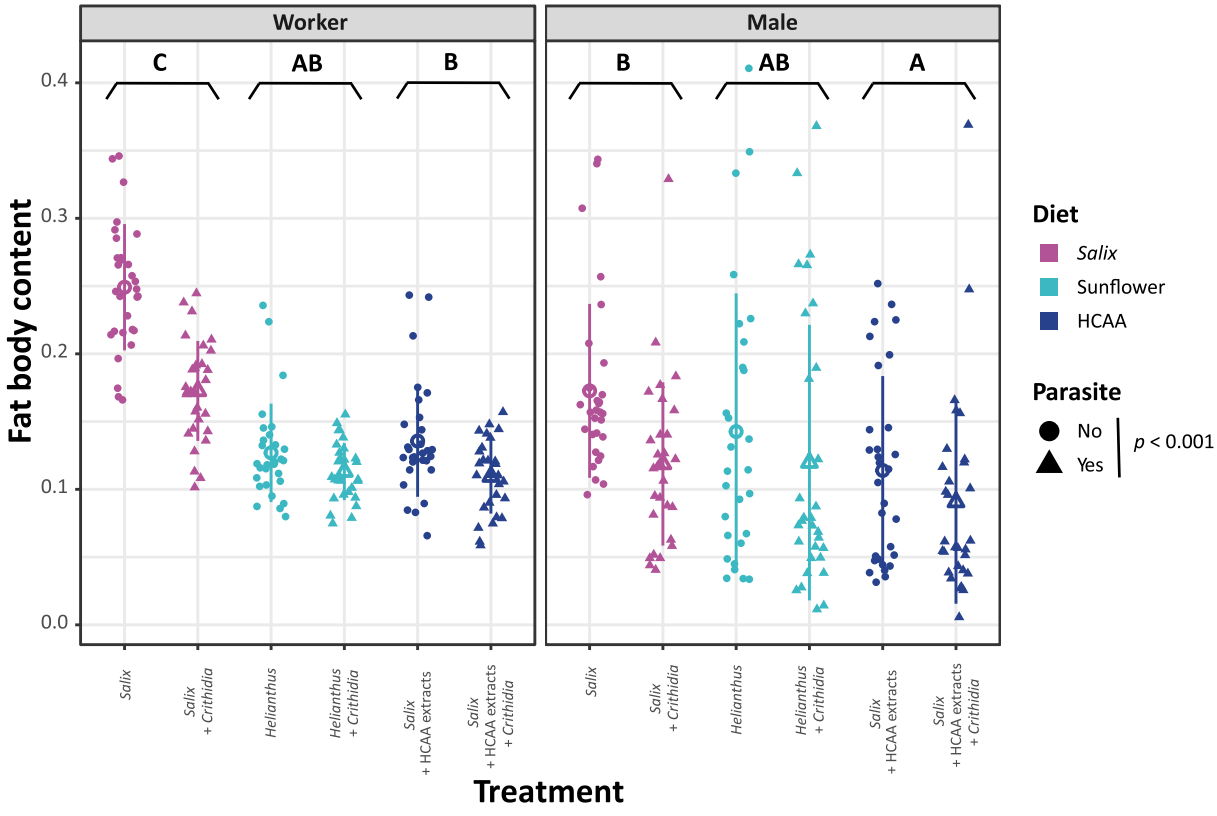

Figure 16: Effect of pollen diets and *Crithidia* infection on fat body content in bumblebee microcolonies. The fat body content was determined as the ratio between the fat body mass in the abdomen and the total abdominal mass in bumblebee individuals. Different letters indicate significant differences ($p < 0.05$) between treatments (GLMM and pairwise comparisons). Close points represent the microcolonies. Open points represent the mean values of each treatment and error bars indicate the standard deviations (mean ± SD).

3.3.2. PHENOTYPIC VARIATION

We observed a significant effect of Diet (GLMM, $\chi2 = 1030.071$, df = 2, $p < 0.001$) and Parasite (GLMM, $\chi2 = 15.093$, df = 1, $p < 0.001$) on right wing centroid size. Post-hoc analyses revealed that males in microcolonies fed with pure *Salix* pollen had larger wings than males in microcolonies fed with HCAA-supplemented *Salix* pollen that had larger wings than males in microcolonies fed with *Helianthus* pollen, irrespective of their infection status. Overall, males from infected microcolonies had larger wings than uninfected ones (Figure 17).

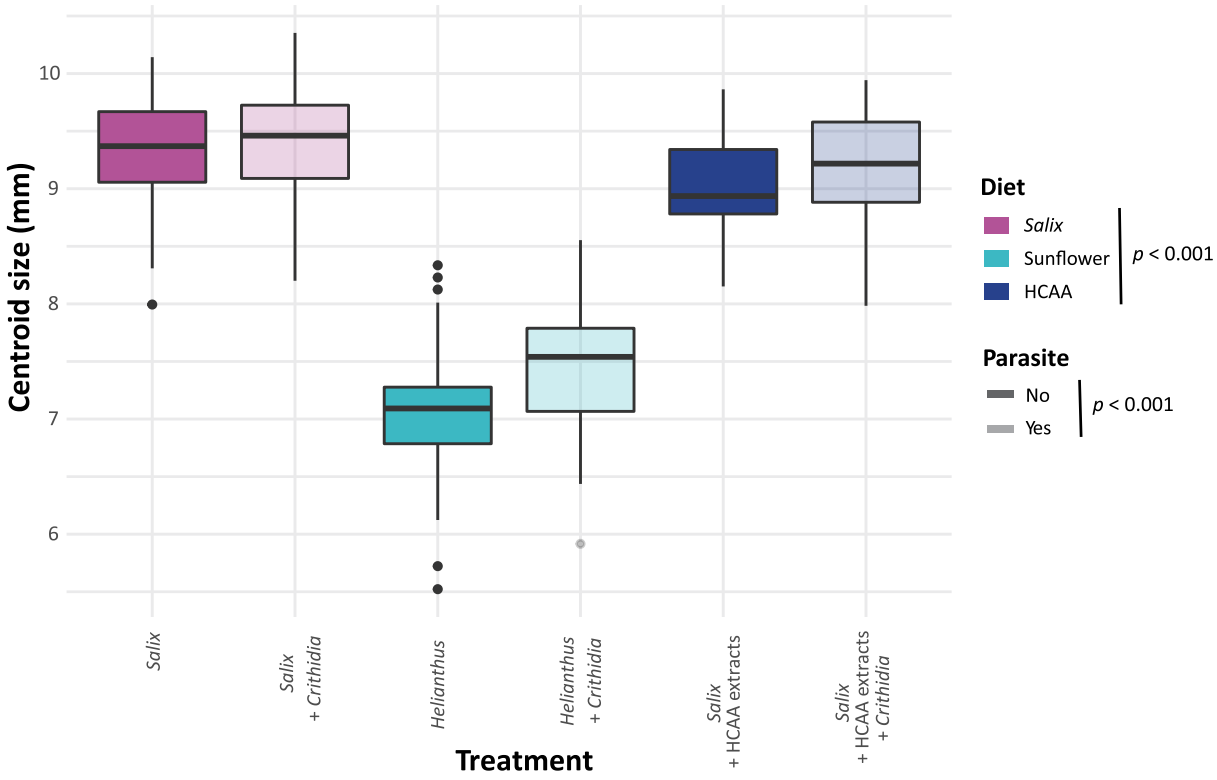

Figure 17: Wing centroid size variation in male individuals among treatments. Significant differences ($p < 0.05$) between treatments are indicated in the legend (GLMM and pairwise comparisons). The boxplots show medians (solid line, 50th percentile) and inter-quartile ranges (colored box, 25th and 75th percentile). The whiskers give the range except for "outliers" (circles) that are more than ±1.5 times the inter-quartile range larger or smaller than the median.

We found that Diet (LM_{RRPP}, $\chi2 = 22.252$, df = 2, $p < 0.001$) and Parasite (LM_{RRPP}, $\chi2 = 1.906$, df = 1, $p = 0.039$) had significant effects on right wing shape. Further analyses showed that every diet differed between each other, but that the greatest differences in wing shape were observed between the *Helianthus vs.* the two other diets (Figure 18).

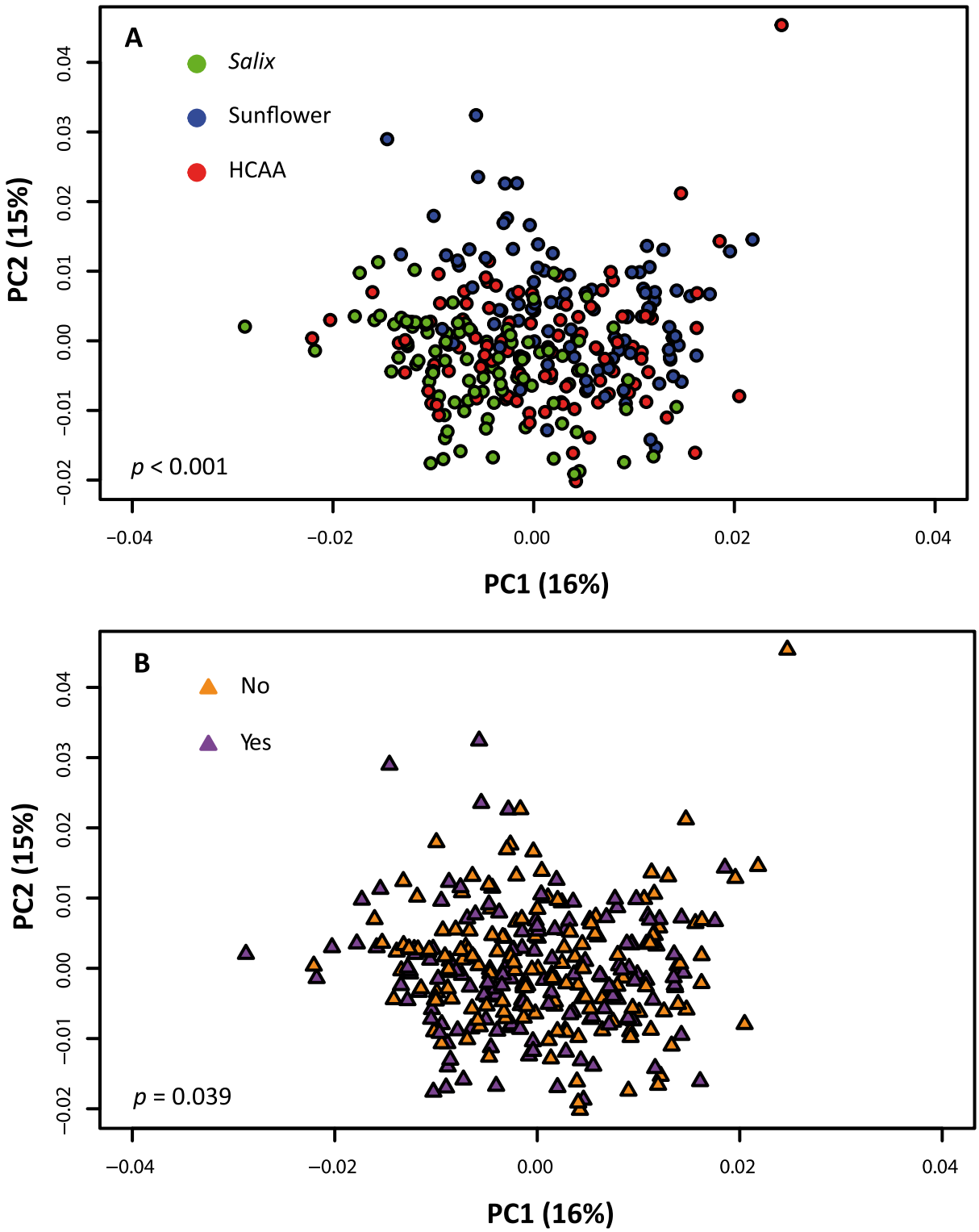

Figure 18: Ordination of the male right forewing shape according to pollen diets and infection status along the first two axes of a principal component analysis (PCA). The first two axes of the PCA (PC1 and PC2) express 31% of the total variance (16% and 15%, respectively). *P*-values from the respective linear model evaluation with a randomised residual permutation procedure (LM_{RRPP}) are indicated in the lower-left of the figures. **A.** PCA discriminating pollen diets; the greatest difference in wing shape was found between the sunflower diet *vs.* the two other diets. **B.** PCA discriminating infection status.

4. DISCUSSION

4.1. Sunflower phenolamides: from leaf to pollen

Our phenolamide-centred chemical analyses showed that sunflower tissues varied in their phytochemical compositions. No HCAAs were found in sunflower leaves and petals, by contrast to pollen and nectar. From a qualitative perspective, the four same HCAA compounds were found in pollen and nectar, but nectar also contained one HCAA compound that was not found in pollen. From a quantitative perspective, even though this difference was not statistically significant, pollen had a slightly higher total phenolamide content than nectar. To our knowledge, this is the first study that assessed the HCAA profiles through all sunflower tissues, and that demonstrated the absence of HCAAs in sunflower leaf and corolla. Actually, studies on sunflower HCAAs are quite scarce and the focus is rather on flavonoid composition of sunflower tissues (Table 9).

Table 9: Non-exhaustive phytochemical screening from sunflower tissues. Only the major specialised metabolite (SM) groups and only the tissues concerned in this study are represented. **+.** Presence. **–.** Absence **n.d.** No data.

SM GROUP	TISSUE				REFERENCE
	Pollen	Nectar	Leaf	Corolla	
Alkaloid	+	+	+	n.d.	Kamal, 2011; Ngibad, 2019; Palmer-Young *et al.*, 2019
Terpene / Terpenoid	+ (carotenoid)	n.d.	+ (sesqui-terpenoid, steroid)	+ (saponin)	Bader *et al.*, 1991; Fatrcová-Šramková *et al.*, 2016; Ngibad, 2019
Flavonoid	+	+ (in honey)	-	+	Sabatier *et al.*, 1992; Lin & Mullin, 1999; Fatrcová-Šramková *et al.*, 2016; Kostić *et al.*, 2019; Ngibad, 2019; Rocchetti *et al.*, 2019; Sharma, 2019; Adler *et al.*, 2020
HCAA	+	+	-	-	Lin & Mullin, 1999; Kyselka *et al.*, 2018; Palmer-Young *et al.*, 2019; Adler *et al.*, 2020, This study

Our findings in pollen are partially in line with the literature (Lin & Mullin, 1999; Kyselka *et al.*, 2018; Palmer-Young *et al.*, 2019; Adler *et al.*, 2020) with (i) some HCAAs already reported in previous studies (i.e., N,N',N''-tricoumaroyl spermidine), (ii) other reported

for the first time in our study (i.e., N,N',N''-dicoumaroyl feruloyl spermidine; N,N',N'',N'''-tetracoumaroyl spermine; N,N',N'',N'''-tricoumaroyl feruloyl spermine), while (iii) some HCAAs previously reported in our plant model were herein undetected (i.e., N,N'-dicoumaroyl spermidine; putrescine derivatives). The same prevails for nectar even though data in the literature are very limited (Palmer-Young *et al.*, 2019) with (i) some HCAAs already reported in previous studies (i.e., N,N',N''-tricoumaroyl spermidine) and (ii) other reported for the first time in our study (i.e., N,N'-diferuloyl spermidine; N,N',N''-dicoumaroyl feruloyl spermidine; N,N',N'',N'''-tetracoumaroyl spermine; N,N',N'',N'''-tricoumaroyl feruloyl spermine). A major meta-analysis conducted by Palmer-Young and colleagues (2019) showed that in *H. annuus*, most of the specialised metabolites were shared between pollen and nectar, which does not seem to be the rule among the plant species they studied (i.e., all species considered, pollen contained on average 63% more compounds than nectar). Moreover, in their study Adler and others (2020) found a N,N',N''-tricoumaroyl spermidine concentration in pollen that lied within the range of concentrations we observed here.

Our results indicated that HCAAs found in nectar and pollen are not due to a physiological leakage from other floral (i.e., petals) or vegetative tissues (i.e., leaves) but yet, we cannot rule out a potential pleiotropic effect between pollen and nectar since their phenolamide compositions were rather similar. In *H. annuus*, the nectaries (i.e., the nectar-producing glands) are situated at the base of the style (i.e., a tube-like structure connecting the ovary and the pollen-receiving stigma) and histological works found that sunflower nectar is a phloem derivative (Frei, 1955; Sammataro *et al.*, 1985). This suggests that pre-nectar from the vascular system is free of HCAAs and that these molecules are likely synthesised in the nectariferous tissue before being secreted via exocytosis, even though non-carbohydrate molecule synthesis and excretion in nectar remain open questions (Heil, 2011). As far as pollen is concerned, the grains arise from the differentiation of sporogenous cells in the tapetum of the anthers (Gómez *et al.*, 2015). Studies have proposed that phytochemicals in pollen grains can originate both from leakage from the anthers (Detzel & Wink, 1993; Cook *et al.*, 2013) or from biosynthesis in the pollen cytoplasm (Stegemann *et al.*, 2019b). As we did not determine the HCAA profile in sunflower anthers, we cannot postulate the HCAA origin in pollen. We would like to note that given the morphological structure of sunflower florets (Sammataro *et al.*, 1985), it is possible that cross-contamination between nectar and pollen occurred during sample collection.

We did not find significant differences in the total phenolamide content between pollen and nectar despite that for a given specimen, these two tissues harbour greatly different total

phenolamide content (e.g., in one specimen, pollen had 25 times more phenolamides than nectar). It is due to the inconsistency of the relative total phenolamide contents in pollen and nectar among the specimens (n = 5) since both tissues showed the highest total phenolamide content depending on the observed specimen. We postulate two hypotheses to explain this discrepancy:

(i) Flowers undergo rapid chemical changes during their maturation and chemical ratios in samples could thus differ slightly along with developmental stages (e.g., Majak *et al.*, 1992; Clearwater *et al.*, 2018). Even though the five specimens were planted simultaneously, they may have grown with different rates. Such a temporal variation in phytochemical compositions remains to be tested in *H. annuus* tissues;

(ii) Sunflower crops have been harvested by human populations for thousands of years and nowadays more than 100 cultivars have been selected (Hanks & Mason, 2018). Analytic studies demonstrated that sunflower pollen and nectar chemical compositions varied in their central (Nicolson & Human, 2013) and specialised (Palmer-Young *et al.*, 2019) metabolite profiles according to the tested cultivars. Here, unfortunately, we were not able to determine whether the five specimens could stem from different cultivars (Ecoflora, Pers. Comm.).

4.2. Effects of sunflower pollen and its phenolamides on bumblebee microcolonies

Our results showed that microcolonies fed with pure and HCAA-supplemented *Salix* pollen had a higher offspring production than microcolonies fed with *Helianthus* pollen, indicating that *Salix* pollen is more suitable for brood rearing than *Helianthus* pollen. This difference was not only due to a greater pollen collection in microcolonies fed with *Salix* pollen, as indicated by a bigger pollen efficacy (i.e., total mass of hatched offspring / total mass of pollen collection) in these microcolonies. Such a poor suitability of sunflower pollen for bumblebee has already been observed by Regali & Rasmont (1995), Tasei & Aupinel (2008a,b), McAulay & Forrest (2019) and Giacomini *et al.* (2021). This is unsurprising as *Helianthus* pollen is of relatively low nutritional quality in comparison with *Salix* pollen (see Nicolson & Human (2013) for a chemical analysis of sunflower pollen). For instance, sunflower pollen has two times less sterol content than willow pollen (Vanderplanck *et al.*, 2020b) and also contains fewer polypeptides (Vanderplanck *et al.*, 2014b), both families of molecules representing crucial nutrients involved in bee selective value (Vanderplanck *et al.*, 2014a). Besides,

sunflower pollen contains a huge proportion of alkaloids (Palmer-Young *et al.*, 2019) that could be toxic to bees (Detzel & Wink, 1993; Arnold *et al.*, 2014), while these compounds were not detected in willow pollen (Vanderplanck *et al.*, 2018), and sunflower pollen grains display conspicuous spines on the outer pollen wall that may act as a physical defence by damaging the bee digestive tube (Blackmore *et al.*, 2007). The low quality of sunflower pollen was also pictured by a greater rate of larval ejection, which is a behaviour displayed by nurse workers under food stress in order to feed adequately the remaining individuals (Tasei & Aupinel, 2008a). Moreover, it has been suggested that bumblebees facing such toxic diet could add a higher quantity of syrup to dilute the toxins (Vanderplanck *et al.*, 2018), which is in accordance with our findings since microcolonies fed with *Helianthus* pollen also showed a greater pollen dilution.

Both poor nutritional quality and toxicity could explain why pollen collection was reduced in microcolonies fed with *Helianthus* pollen. Indeed, several studies showed that bumblebees are able to discriminate between foods varying in their nutrient concentrations and toxicity either via pre-ingestive (e.g., olfactory and chemotactile cues; Ruedenauer *et al.*, 2015, 2016, 2020; Sculfort *et al.*, 2021) or post-ingestive cues (e.g., digestive damage, induced malaise behaviour, nutrient intake; Vaudo *et al.*, 2016, 2017; Ruedenauer *et al.*, 2020; Vanderplanck *et al.*, 2020a), although it may not always be the case (Konzmann & Lunau, 2014; Tiedeken *et al.*, 2014; Sculfort *et al.*, 2021). Despite the low quality and potential toxicity of sunflower pollen, the worker mortality was not predominant in the sunflower treatment. Actually, even if workers require pollen for their reproduction and immunology, they are much more dependent on carbohydrate resources for their short-time survival (Brodschneider & Crailsheim, 2010). As all treatments were fed *ad libitum* with the same untreated sugar syrup, it may explain why survival did not differ among diets. Later, it would be interesting to assess the concurrent impact of sunflower pollen diet and restricted sugar availability on bumblebees. This observation also suggests that specialised metabolites contained in sunflower pollen were not lethal in the concentration ranges they were consumed here. However, it contrasts with previous experiments that found that *B. impatiens* workers provided with sunflower pollen had a reduced lifespan in comparison with workers provided with better-quality diets (McAulay & Forrest, 2019). This divergence may be explained by the differing behavioural and genetic components between these two bee species (Cnaani *et al.*, 2002; Sadd *et al.*, 2015), as well as the by varying pollen chemical compositions among sunflower cultivars (Nicolson & Human, 2013; Palmer-Young *et al.*, 2019).

The poor suitability of sunflower pollen for bumblebee development may look controversial since it could lead to poor flower visitation and thus low sexual reproduction for the plant. Yet, in the field, *H. annuus* is visited by a wide range of pollinators including bumblebees, honeybees and wild solitary bees (Meynié & Bernard, 1997; Greenleaf & Kremen, 2006). Three rationales explain this paradox:

(i) Generalist species foraging on *H. annuus* (e.g., *B. terrestris*) could mix pollen from different botanical origins to complement nutrient deficiencies and improve diet suitability (Eckhardt *et al.*, 2014; Vanderplanck *et al.*, 2018). McAulay & Forrest (2019) showed that sunflower pollen mixed in 50:50 proportion with other more suitable pollen was as great as non-sunflower pollen, meaning that other pollen were able to compensate for the low nutritive quality of sunflower pollen;

(ii) In bee-plant interactions, nectar-related traits (e.g., nectar volume and composition) may be more important than pollen-related traits, to such an extent that bees may only forage for nectar and not for pollen (e.g., MacKenzie, 1994; Roldán-Serrano & Guerra-Sanz, 2005). In *H. annuus*, bee visitation rate increases with nectar amount and accessibility, and bees also forage on male-sterile flowers (i.e., pollen-free; Mallinger & Prasifka, 2017), which means that bees can pollinate sunflowers without consuming their pollen;

(iii) A strong co-evolution has occurred between sunflowers and wild bees to such an extent that some bees (e.g., *Melissodes agilis*) are oligolectic on sunflowers and therefore adapted to its pollen chemical composition (Parker, 1981; Parker *et al.*, 1981).

Pollen collection was more pronounced in pure than in HCAA-supplemented *Salix* pollen treatments but interestingly, both healthy and infected microcolonies provided with HCAA-supplemented *Salix* pollen had a superior pollen efficacy than infected microcolonies fed wit pure *Salix* pollen *(discussed below)*. Additionally, despite that microcolonies fed with pure *Salix* pollen collected slightly more syrup than microcolonies fed with HCAA-supplemented *Salix* pollen in the second half of the experiment, the latter showed a greater pollen dilution. Otherwise, no difference was observed between pure *Salix* and HCAA-supplemented *Salix* pollen treatments with regards to microcolony development. To our knowledge, this is the first time that effects of a HCAA-enriched diet are studied in bees.

Because HCAAs in pollen are ubiquitous among core eudicots (Elejalde-Palmett *et al.*, 2015), and thus among bee-pollinated plants, it is reasonable to postulate they may not exhibit any adverse effect towards bees, or at least towards generalist species such as *B. terrestris*. Nevertheless, reduced pollen collection and increased pollen dilution in comparison with the

pure *Salix* treatment showed that the HCAA-laced *Salix* treatment must have induced a stress response in bumblebee workers. It is worth noting that here, workers were likely provided with field-realistic concentrations of HCAAs because, although their diet contained HCAAs both from willow pollen (±23 TSE mg / candy g) and from sunflower pollen extracts (±42 TSE mg / candy g), it may fall within the concentrations usually consumed by bumblebees since workers are known to mix pollen from different flower species (Goulson, 2003). In addition to qualitatively determining the HCAA profiles in pollen loads (e.g., Elejalde-Palmett *et al.*, 2015; Kyselka *et al.*, 2018), it would be worthwhile to focus on their quantitative profile to assess what concentration range of HCAAs bees are used to consume.

The consequences of HCAA consumption on pollinator metabolism remain hitherto unknown. Nonetheless, it has been found that some HCAAs exhibit antifungal and antibacterial activities (Newman *et al.*, 2001; Kyselka *et al.*, 2018), but that Firm-5 bacteria in the bumblebee hindgut use phenolamides as substrates (Kešnerová *et al.*, 2017; Kwong *et al.*, 2017; Bonilla-Rosso & Engel, 2018). A phenolamide-rich diet may hence interfere with the so-important bumblebee gut microbiota (e.g., by boosting some phylotypes and depleting others; Kwong & Moran, 2016), but this hypothesis has still to be tested. If it turned out to be the case, it could have led to a considerable post-ingestive effect that could *in fine* have given rise to the reduction in pollen collection observed in the HCAA-supplemented *Salix* treatment, as microbiota-eradicated bumblebee workers in Meeus *et al.* (2013) exhibited a loss of appetite.

4.3. Interplay between sunflower pollen, its phenolamides and *Crithidia bombi*

4.3.1. Effects of sunflower pollen and its phenolamides on parasite load

We found that bumblebees provided with HCAA-supplemented *Salix* pollen had a greater concentration of *Crithidia* cells in their faeces than bumblebees provided with pure *Salix* pollen. This observation contrasts with our *a priori* hypothesis. Indeed, we were aware that specialised metabolites contained in pollen of bee-pollinated plants could have no effect on *C. bombi* (e.g., thymol and nicotine in Biller *et al.*, 2015; N,N',N''-tricoumaroyl spermidine, rutin and fatty acids in Adler *et al.*, 2020), but we did not expect that it could intensify the infection, even though a previous study found that some pollen specialised metabolites intensified *Crithidia* load in bumblebee guts (Thorburn *et al.*, 2015). Even more interestingly, this effect was not

noticed in microcolonies provided with sunflower pollen, whereas both treatments contained sunflower HCAAs. Two reasons could explain this dissimilarity:

(i) Asteraceae pollen grains have a tick wall rich in sporopollenin and pollenkitt, which makes them mechanistically resistant and renders digestion difficult (Roulston & Cane, 2000; Meier-Melikyan *et al.*, 2003; Pacini & Hesse, 2005). For instance, Vanderplanck *et al.* (2018) found that *Cirsium* (Asteraceae) pollen had around 20% digestibility for *B. terrestris* while Nicolson *et al.* (2018) found that sunflower pollen had a digestibility of 70% for *A. mellifera*. Therefore, sunflower HCAAs may not have been released in bee digestive tracts in microcolonies provided with raw sunflower pollen. However, such digestibility may greatly vary between bee species (Dobson & Peng, 1997; Praz *et al.*, 2008) and further analyses are then required to assess sunflower pollen digestibility in bumblebees;

(ii) Because we did not succeed in finding a phytochemical-free artificial diet (see Appendix A), microcolonies provided with HCAA-supplemented *Salix* pollen not only received HCAAs extracted from *Helianthus* pollen but also HCAAs occurring in *Salix* pollen, and were thus provided with roughly 40% more HCAAs than the microcolonies allocated to the *Helianthus* pollen treatment. If HCAA-induced infection boosting is concentration dependant, it may explain why this effect was observed in microcolonies fed with *Helianthus* HCAA-supplemented *Salix* pollen and not in microcolonies fed with pure *Helianthus* pollen.

The reason why an HCAA-enriched diet raised infection intensity in bumblebee workers remains enigmatic. We propose three non-mutually exclusive hypotheses:

(i) As described previously, HCAAs could disrupt the core microbiota in the bumblebee worker gut (Newman *et al.*, 2001; Kešnerová *et al.*, 2017; Kyselka *et al.*, 2018). Yet, many studies indicated that gut microbial symbionts protect bumblebees against the trypanosomatid *C. bombi* via the production of antimicrobial compounds, the activation of the host innate immune system, the digestion of pollen grains containing parasite-altering phytochemicals, the modification of the gut environment pH and/or the competition for resources (Koch & Schmid-Hempel, 2011; Cariveau *et al.*, 2014; Deshwal & Mallon, 2014; Mockler *et al.*, 2018; Näpflin & Schmid-Hempel, 2018; Palmer-Young *et al.*, 2018a; b; Praet *et al.*, 2018). Disrupting the gut microbiota could therefore have led to a reduction in parasite inhibition;

(ii) In relation to the preceding hypothesis, disrupting the gut microbiota may impair pollen digestion (Kwong & Moran, 2016) and result in gut obstruction (Meeus *et al.*, 2013). Yet, previous works showed that lack of pollen reduced *Crithidia* cell counts in bumblebee faeces (Logan *et al.*, 2005; Conroy *et al.*, 2016). These studies suggested that this trypanosomatid absorbs nutrients directly from the gut content and from the host itself. Thus, we could postulate that an HCAA-induced increase in pollen content in the gut because of an obstruction may have created a favourable environment for *Crithidia* development;

(iii) A previous study conducted by Thorburn and its team (2015) showed that both nicotine- and anabasine-enriched diets increased parasite load in *Bombus impatiens*. They suggested that these phytochemicals may act as nAChR agonists because they are chemically related to the neonicotinoids – a class of insecticides – that are known to be immunosuppressant in bees (Jeschke *et al.*, 2011). No such effects have ever been described for HCAA compounds. Yet, phenolamides are also known for their antioxidant and radical scavenging activities (Bassard *et al.*, 2010 and references therein). Because low levels of reactive oxygen species have been linked to immunosuppression (Mittler, 2017 and references therein), one may postulate that phenolamides could have immunosuppressant effects on bumblebees, which *in fine* leads to greater parasite loads. This statement remains strongly hypothetical and warrants further investigations.

In our study, we did not detect any *Crithidia* load reduction in workers provided with *Helianthus* pollen, which contrasts with previous research. Indeed, Giacomini and colleagues (2018) demonstrated that *H. annuus* pollen reduced *C. bombi* load in *B. impatiens* workers both housed individually or in microcolonies. They proposed five hypotheses that could explain the medicinal properties of sunflower pollen: (i) pollen chemical composition; (ii) conspicuous spines on the outer pollen coat; (iii) potential laxative properties; (iv) immune function boosting; and (v) changes in the gut microbiome. Importantly, as sunflower pollen increased *Crithidia* growth *in vitro* (Palmer-Young & Thursfield, 2017), they also stressed that effects of sunflower pollen on *Crithidia* must be mediated by the bee host environment (Giacomini *et al.*, 2018). Fowler *et al.* (2020b) demonstrated that sunflower pollen consumption reduced parasite load in worker and queen, but not in male bumblebees. The same year, Adler *et al.* (2020) attempted to assess the pollen chemical mechanisms underlying the effects of sunflower diet on *Crithidia* by testing diets laced with fatty acids, rutin (flavonoid) or N,N',N''-tricoumaroyl

spermidine (HCAA) but did not observe any significant infection reduction in any of these treatments, while Giacomini *et al.* (2018) had already demonstrated that sunflower pollen crude proteins were not responsible for infection reduction, hence leaving the mechanisms underlying the medicinal effect of *H. annuus* pollen unknown. It has also been proposed that poor host nutrition may affect the availability of resources for the pathogen (Logan *et al.*, 2005; Conroy *et al.*, 2016). Yet, very recently, Giacomini *et al.* (2021) further demonstrated that pure sunflower pollen as well as sunflower mixed with wildflower pollen (1:1 ratio) reduced *C. bombi* prevalence and intensity at the colony level in *B. impatiens*, suggesting that poor nutritional quality of sunflower pollen was unlikely the cause of hindered *C. bombi* growth. The incongruity between our observations and the literature is unlikely explained by difference in sunflower cultivars since pollen from nine *H. annuus* cultivars and four wild *H. annuus* populations have been showed to mitigate *Crithidia* infection in *B. impatiens*, even though it is worth noting that parasite reduction slightly varied among cultivars (LoCascio *et al.*, 2019b). The explanation of the non-reduction in infection in workers provided with sunflower observed here could lie within the timing of exposure to sunflower pollen. Indeed, LoCascio *et al.* (2019a) showed that sunflower pollen did not reduce *C. bombi* infection if it was administrated 3.5 days after inoculation, but that infection was lowered in bumblebees immediately provided with sunflower pollen after inoculation (but see Giacomini *et al.*, 2018). In our experimental design, microcolonies had been fed with *Salix* pollen for three days after inoculation (i.e., initiation period) before they were provided with *Helianthus* pollen. It would therefore be relevant to redo the experiment by administrating the treatments to microcolonies right after the inoculation to assess how the timing of exposure could impact the success of infection. It could also lead to new outcomes in parasite infection in microcolonies provided with HCAA-supplemented *Salix* pollen.

Parasites were found in the faeces of the workers upon the first screening of the experiment four days after inoculation in every diet. Next, parasite load steadily increased for 10 days and then appeared to level out, although there were considerable variations. Such a pattern of infection intensity has already been found in studies focusing on the dynamic of *Crithidia* infection in bumblebees (Schmid-Hempel & Schmid-Hempel, 1993; Logan *et al.*, 2005; Otterstatter & Thomson, 2006; Ruiz-González & Brown, 2006). As *C. bombi* cells attach themselves in the bee ileum epithelium (Koch *et al.*, 2019), it seems rational to postulate that parasite load reached a peak when crowding became an important constraint. Several non-mutually exclusive explanations have been proposed regarding the subsequent oscillations in infection intensity (Otterstatter & Thomson, 2006): (i) parasite cells are destroyed by the host's

immune system and then recolonise the gut; (ii) gut epithelial cells are damaged and then replenish which affects spatial niche availability for parasite cells; (iii) increasing chyme in the ileum dilute parasite cells; and (iv) bees sporadically wash parasite cells from their intestine when they defecate. Furthermore, in our experimental design, workers were housed together (n = 5) in microcolonies. Workers then faced constant repeated exposures to *C. bombi* cells through food contaminated by themselves and their nestmates which could also have led to parasite load fluctuation over the course of the experiment (Otterstatter & Thomson, 2006). These repeated exposures were also due to brood caring since larval feeding activities act as a *Crithidia* transient transmission hub (Folly *et al.*, 2017).

The *Bombus*-*Crithidia* system is known for its strong host-parasite genotype-genotype (GxG) interactions (Schmid-Hempel & Schmid-Hempel, 1993) leading to discrepancies in infection outcomes depending on colonies and *Crithidia* strains (e.g., Shykoff & Schmid-Hempel, 1991b; Brown *et al.*, 2000; Baer & Schmid-Hempel, 2003). Herein, we observed a difference in infection intensity between colonies of origin, but only in the sunflower treatment. By contrast to this expectation of inter-colony variability, we did not expect any intra-colony discrepancy in parasite load. We however found that microcolonies from a same foundress colony and fed with HCAA-supplemented *Salix* pollen differed in their infection intensity. It is intriguing that these inter-colony and intra-colony differences were not observed for every treatment and hence suggests the importance of environmental factors (i.e., different pollen diets). It is now assumed that host-parasite studies should consider environmental components since they could explain a wide heterogeneity in infection outcomes (Sadd & Barribeau, 2013). Furthermore, it is worth noting that the intra-colony discrepancies occurred in the treatment that showed an increased parasite infection.

We observed great intra-microcolony variances in parasite load. Contrary to our expectation, workers from a same microcolony did not have closer infection intensities between each other than when compared to workers from another microcolony. Such variances must be due to the same above-mentioned reasons that should explain the oscillations in infection intensity along the course of the experiment (Otterstatter & Thomson, 2006).

In addition, we observed that 10% of the sampled males from infected microcolonies at the end of the experiment (n = 40) did not have *Crithidia* cells in their faeces, which suggests that within-colony inoculation likely occurred after the emergence of the individuals and needed time to establish (Otterstatter & Thomson, 2007; Folly *et al.*, 2017).

4.3.2. DIET-DEPENDANT EFFECTS OF *CRITHIDIA* INFECTION ON BUMBLEBEE MICROCOLONIES

Many parasites manipulate their host behaviours to facilitate parasite development and survival. Since *C. bombi* development seems to be correlated with pollen load in the bee digestive tract (Logan *et al.*, 2005; Conroy *et al.*, 2016), it has further been demonstrated that *Crithidia* infection increased pollen collection in the bumblebee *B. impatiens* (Richardson *et al.*, 2015). This study contrasts with our findings, as we did not observe any increase in pollen collection in infected microcolonies. This difference could be explained by five dissimilarities between the experimental designs: (i) we used *B. terrestris* while they used *B. impatiens*; (ii) we used a *Salix*-dominant pollen diet while they used a pollen mix; (iii) we used a 65% syrup while they used a 30% syrup; (iv) they dipped the pollen candies in melted bees' wax; and (v) we likely used different *C. bombi* strains.

Crithidia bombi is known to induce adverse effects in bumblebee populations (e.g., Brown *et al.*, 2003b; Otterstatter *et al.*, 2005; Gegear *et al.*, 2006; Goulson *et al.*, 2017), but does not alter the production of eggs and larvae (Richardson *et al.*, 2015) in queenless microcolonies or sexuals (i.e., males and young queens; Imhoof & Schmid-Hempel, 1998; Rutrecht & Brown, 2009; Fauser-Misslin *et al.*, 2014) in queenright colonies, on condition that the foundress queen did not get infected prior to hibernation (Brown *et al.*, 2003b). Here, we did not observe any influence of *C. bombi* on the number or mass of individuals in every developmental stage which is in accordance with the previous studies, apart from the egg numbers that were reduced in our infected microcolonies. As we did not record the time of the onset of egg laying, we cannot discuss about the impact of infection on worker oviposition. Besides, *C. bombi* infection reduced pollen efficacy (i.e., mass of brood / pollen collection) in microcolonies fed with pure *Salix* pollen. Since *Crithidia* infection did not alter the offspring mass nor the pollen collection in these microcolonies, we postulate that infection-induced alterations in those two parameters were too negligible to be detected, but that those minimal alterations had a significant impact on pollen efficacy when considered together. Most interestingly, this *Crithidia*-induced decrease in pollen efficacy was not observed in microcolonies fed with *Salix* pollen supplemented with sunflower HCAA extracts. HCAAs thus alleviated the cost of parasite infection at the microcolonial level on pollen efficacy. In other words, HCAA extracts increased the tolerance of bumblebees against *C. bombi* (i.e., greater pollen efficacy) but decreased their resistance (i.e., greater parasite load; Raberg *et al.*, 2007; De Roode & Hunter, 2018).

Moreover, with regards to sunflower pollen, it was found that a 1:1 ratio of sunflower to wildflower pollen improved drone and queen production in infected colonies when compared to uninfected colonies fed with the same diet (Giacomini *et al.*, 2021). However, our experimental design (i.e., the use of queenless microcolonies fed with 100% sunflower pollen) does not allow for making proper comparisons with this study.

4.3.3. EFFECTS OF SUNFLOWER POLLEN, PHENOLAMIDES AND *CRITHIDIA BOMBI* ON INDIVIDUAL IMMUNOCOMPETENCE

At the end of our experiment, infected individuals displayed a lower fat body content than uninfected ones irrespective of their diet. As far as we know, this is the first time that such a trend is observed in the *Bombus*-*Crithidia* system. Prior research addressing the fat body content in *Crithidia*-infected bumblebees housed the bees individually (Brown *et al.*, 2000, 2003a), which differs from the housing method used here (i.e., microcolonies of five workers). As described previously, infected workers housed together consistently re-infect themselves via repeated exposure to the parasite, which could prevent the total eradication of *C. bombi* cells. Indeed, while isolated workers were eventually able to clear a *Crithidia* infection (Imhoof & Schmid-Hempel, 1998b), workers housed in groups were not (Otterstatter & Thomson, 2006). Hence, because lipid reserves are mobilised to the haemolymph in response to immune challenge (Arrese & Soulages, 2010), we postulate that repeated exposures to *C. bombi* within the microcolony were highly energetically costly for bees that *in fine* showed a reduced fat body content after 35 days of experiments.

This is the first time that the fat body content is measured in male bumblebees. Overall, workers had a greater fat body content than males except when provided with sunflower pollen. This result is unsurprising since workers have been shown to have higher levels of constitutive immune defences than drones (Moret & Schmid-Hempel, 2001; Gerloff *et al.*, 2003). Besides, here, workers were (at least ~25 days) older than males which is also associated with a greater fat body development (Doums *et al.*, 2002).

We observed that *Helianthus* pollen-fed workers had a lower fat body content than the ones provided with pure *Salix* pollen. It could be due to the poor nutritional values of this diet, as it was suggested that diet quality may influence fat body development (Alaux *et al.*, 2010; Roger *et al.*, 2017; Vanderplanck *et al.*, 2018). This could explain why no significant difference in fat body content has been observed between workers and males in the *Helianthus* treatment. Another intriguing observation concerns the lowered fat body content measured in workers fed with HCAA-supplemented *Salix* pollen in comparison with pure *Salix* pollen-fed workers. This

is interestingly in line with what was discussed above in this study and leads us to an egg-and-chicken issue. Indeed, on the one hand, one may postulate that HCAA consumption could have immunosuppressant effects via HCAA radical scavenging activities (see section 4.3.1), which could have led to a lower fat body content which caused a greater parasite load. On the other hand, one may propose that a lower fat body content was due to a higher parasite load. From this perspective, a higher parasite load in microcolonies from the HCAA treatment suggests that workers in these microcolonies likely bearded a larger immune challenge (i.e., more fat reserves must have been mobilised in the haemolymph), which could explain why individuals in the HCAA treatment showed a decreased fat body content. This tricky cause-to-consequence question deserves further attention.

4.3.4. EFFECTS OF SUNFLOWER POLLEN, PHENOLAMIDES AND *CRITHIDIA BOMBI* ON INDIVIDUAL PHENOTYPIC VARIATION

Using the right-forewing centroid size as a proxy for body size, we found that newly emerged male body size differed according to the diet and the infection status of their respective microcolony. Based on the literature, this is the first evidence that *C. bombi* infection increased body size in newly emerged males. By contrast, *Nosema bombi* and *Apicystis bombi* infections did not have any impact on male body size in previous research (Rutrecht & Brown, 2009; Gérard *et al.*, 2018). The reason why males that emerged in infected microcolonies had greater body sizes remains enigmatic. From an evolutionary perspective, one could postulate that such an infection-driven trait could be advantageous for the parasite. Indeed, when they leave the nest a few days after their emergence, males actively patrol an area of hundreds meters from spot to spot around their nest to seek out for unmated queens, leaving scent markings along the way (Bergman & Bergstrom, 1997; Goulson, 2010). Further, males actively forage on flowers and larger males likely forage farther (Ostevik *et al.*, 2010). Therefore, males with greater body sizes increase the range and the transmission potential of *C. bombi* (e.g., via shared use of flowers with other bees; Durrer & Schmid-Hempel, 1994). In addition, bigger males have been shown to copulate more rapidly for shorter durations (Amin *et al.*, 2012). Despite that so far no transmission from males to females during mating has been recorded (Schmid-Hempel *et al.*, 2019), large-sized males would improve *Crithidia* transmission if it turned out to be the case.

Unsurprisingly, we found that males from *Helianthus* pollen-fed microcolonies were smaller than males that emerged in microcolonies fed with pure or HCAA-laced *Salix* pollen. Pollen of poor quality leads to smaller larvae (Tasei & Aupinel, 2008b; a; Vanderplanck *et al.*, 2014a; Moerman *et al.*, 2017) which leads to smaller offspring (Sutcliffe & Plowright, 1988,

1990). It was especially demonstrated for *H. annuus* pollen by Regali & Rasmont (1995). Conversely, the reason why males from microcolonies provided with HCAA-supplemented *Salix* pollen were slightly smaller than males from microcolonies provided with pure *Salix* pollen is more cryptic. As described earlier, pollen dilution and fat body content indicated a potential slight toxic effect induced by HCAA supplementation. This effect may have showed through the body size of the offspring.

Considering the wing shape of males from pure *Salix*-fed microcolonies as the baseline, we found that males that emerged in *Helianthus* pollen-fed microcolonies had a greater wing shape alteration than males that emerged in microcolonies provided with HCAA-supplemented *Salix* pollen, but that males in the latter treatment also significantly differed from males of the pure *Salix* pollen treatment. It is in accordance with what was previously discussed in this study (i.e., poor nutritional quality of sunflower pollen and slight toxic effect of the HCAA-enriched diet). Besides, despite that it was slightly significant, *C. bombi* infection also induced wing shape changes in male offspring. This is the first time that wing shape variation is studied with regards to *Crithidia* infection, and our results reflect Gérard *et al.* (2018), wherein an *Apicystis bombi*-induced wing shape variation was observed. As *Crithidia* does not infect bumblebee larvae (Schmid-Hempel, 1998; Folly *et al.*, 2017), developmental perturbation did not occur because of an energy-allocated-towards-development hijacking by the parasite. Rather, stress must have occurred because of a perturbated brood care by workers, but this supposition remains to be assessed for parasite infection – as it has already been suggested for pesticide exposure (Baron *et al.*, 2014; Fauser-Misslin *et al.*, 2014).

4.4. Caveats and limitations

Despite the great efforts that were made in designing and conducting the experiments as well as in analysing the results to draw robust and consistent discussions, we would like to address a number of limitations and caveats that are important to consider with regards to this study. From these, we would like to propose a set of suggestions for further experiments in this growing area of research (e.g., next master's theses in 2021 – 2022).

4.4.1. FROM MODELS TO UNIVERSAL RULES

This study is part of the METAFLORE project (Duez, Gerbaux & Michez, 2018) and, as a previous master's thesis in this project (i.e., Gilles, 2016), it does not investigate all the components of this interdisciplinary research. In addition to not tackling bacterial concepts, we restricted this study to one bee, parasite and plant specimen while the project introduces other

bee (e.g., *Osmia cornuta* Latr.), parasite (e.g., *Apicystis bombi* Liu, Macfarlane & Pengelly) and plant (e.g., *Crataegus monogyna* Jacq.) species. Moreover, we focused on one family of specialised metabolites while many others are found in plants (e.g., alkaloids, flavonoids). It is now undeniable that no universal conclusion could be drawn from such complex interactions by only examining one specific case (Abbott, 2014; Fowler *et al.*, 2020a). Next research should integrate other model species to put forward, if possible, broader conclusions.

4.4.2. HOSTS AS MATRYOSHKA DOLLS

There is mounting evidence that microbial symbionts maintain major interactions with their host, their host's diets and their host's parasites, leading to a quadripartite network. Thus, bee hosts could be seen as Matryoshka dolls, with the smallest innermost dolls being the symbionts' metabolomes that greatly influence the outward bee phenotypes. Challengingly, for instance, if bee fitness is reduced after specialised metabolite ingestion, one does not know if these metabolites directly alter bee physiology (e.g., Vanderplanck *et al.*, 2020) or lead to gut microbial alteration (e.g., Jones *et al.*, 2018) which could further result in metabolic deficiencies (e.g., Anderson & Ricigliano, 2017). It is crucial that next studies consider microbial symbionts and their interactions with parasites and plant specialised metabolites in the bee digestive tract. Moreover, here, uninfected bees were not administrated a control sham *inoculum* at the onset of the experiment (i.e., *Crithidia*-free mix of sucrose and faeces). Since bee faeces contain a vast array of microbial symbionts, inoculated bees may have acquired a new microbial community which differed from the one of their respective sisters in the uninfected treatments (e.g., Koch & Schmid-Hempel, 2011, 2012; Mockler *et al.*, 2018). Ideally, upon arrival, workers in future *Crithidia* stock colonies should be isolated before stock colony inoculation so that later, workers allocated to uninfected treatments can be provided with *Crithidia*-free faeces from individuals from the *Crithidia* stock colonies.

4.4.3. FROM THE LAB TO THE FIELD

Under controlled laboratory conditions, environments of experiments are kept constant and enable the elucidations of many hypotheses concerning plant-bee-microbiota-parasite interactions. Although, natural interactions take place in a changeable world with spatial and temporal variations. For instance, by sheer accident (i.e., a building steam leak), Thorburn *et al.* (2015) found that some alkaloids reduced parasite infection but anyway increased worker mortality when the temperature varied during the experiment (10 – 35°C), while they observed

no effect when the temperature remained stable (27°C). In further experiments, one would therefore like to design other treatment blocks to take into account such environmental factors.

4.4.4. A PSEUDO-CONTROL TREATMENT

Despite the implementation of a two-month pilot experiment, we were not able to find a phytochemical-free diet to serve as control and decided to employ commonly used *Salix* pollen. Yet, *Salix* pollen contains specialised metabolites (Ahmed *et al.*, 2011) and especially flavonoids as well as HCAAs (Irène Semay, Pers. Comm.; see Appendix B). A significant limitation is therefore that we were not able to draw conclusions about the effects of sunflower HCAAs alone since they were provided with other *Salix* specialised metabolites (i.e., we had to consider *Salix* + HCAAs as a whole). Pursuing efforts to find a suitable artificial pollen diet is crucial for the one who wants to mimic natural conditions and accurately test pollen specialised metabolites.

4.4.5. HANDLING-INDUCED STRESSFUL CONDITIONS

Our experimental design implied an often-neglected confounding effect. Indeed, as only bees in the parasite treatment were housed individually until starvation prior to inoculation and as faeces were only sampled from infected bees, handling-induced stress may have a confounding influence with parasite infection. The impact of this kind of stress has already been demonstrated. For instance, sucrose responsiveness in honeybees was influenced by handling (i.e., whether bees were anesthetised or not while handled; Pankiw & Page, 2003). Furthermore, a study conducted on stingless bees pointed that different colony handling led to differences in fluctuating asymmetry in forewing shape (Lima *et al.*, 2016). Here, despite that mortality did not differ among treatments, most of them occurred after handling in the infected microcolonies (Pers. Obs.). Next research should consider handling as a confounding effect and therefore handle all the treatment blocks in the same manner to alleviate this undesirable factor.

4.4.6. A NEGLECTED METACYCLOGENESIS

Despite the numerous research that have been conducted on *Crithidia bombi*, the fundamental knowledges around this trypanosomatid parasite are rather limited (Mark JF Brown, Pers. Comm.). Particularly, the understanding of the metacyclogenesis of this species is blurry (Gorbunov, 1996; Kaufer *et al.*, 2017), despite that its morphological stages seem well-recognised (i.e., promastigote, choanomastigote and amastigote; Hoare & Wallace, 1966; Wheeler *et al.*, 2013). Among more than a hundred *Crithidia*-related studies cited in this

manuscript, only a handful of them considered the different morphological forms of this parasite (Brown *et al.*, 2003a; Logan *et al.*, 2005; Richardson *et al.*, 2015; Koch *et al.*, 2019; Giacomini *et al.*, 2021). It must be noted that distinguishing the three stages could be tricky for non-specialist observers and could substantially increase under-microscope counting time (Pers. Obs.). However, a significant enhancement would be done by discriminating flagellate *vs.* aflagellate *C. bombi* cells (e.g., Giacomini *et al.*, 2021) as it would enable to discriminate promastigote / choanomastigote *vs.* amastigote forms, respectively. Next observers would thus like to put into practice such a differentiation, since discriminating *Crithidia* cells with or without flagellum is quite easy and time efficient.

5. CONCLUSION AND PERSPECTIVES

In our study, we wanted to assess (i) whether *Helianthus annuus* may distinctly allocate phenolamide (HCAA) compounds among its tissues, (ii) whether *Helianthus annuus* pollen or its HCAA extracts may lead to poor microcolony development and may induce stress responses in *Bombus terrestris*, and (iii) whether *Crithidia bombi*-infected *Bombus terrestris* may benefit from *Helianthus annuus* pollen or its HCAA extracts.

Phenolamide profiles differed among *H. annuus* tissues. No phenolamide compound was found in the petals or the leaves, while phenolamides differed qualitatively and quantitatively between pollen and nectar, despite some shared compounds. Therefore, any HCAA leakage across all tissues appears unlikely, but whether HCAA profiles in floral resources are due to pleiotropic effects – as well as their roles – has still to be investigated. We can only conclude that there is a tissue-specific allocation of phenolamides in *H. annuus*.

H. annuus pollen led to poor microcolony development and induced significant stress responses in *B. terrestris* at both microcolonial and individual levels, namely reduced offspring production and pollen efficacy, greater larval ejection and pollen dilution, lower fat body content, as well as smaller body size and altered wing shape in newly emerged males. HCAA extracts also induced stress responses in *B. terrestris*, but to a lesser extent, namely a greater pollen dilution, a lower fat body content, as well as slightly smaller newly emerged males that showed minor wing shape modifications. *H. annuus* pollen is thus a poor-quality diet for *B. terrestris*, but it is only partially explained by its HCAA profile.

H. annuus pollen did not reduce parasite load in *C. bombi*-infected bumblebees while unexpectedly, HCAA extracts increased it. The reason lying behind this increase, whether it is related to the host physiology, to the gut environment, to the microbiome or directly related to the parasite cell, is still to be investigated.

C. bombi infection did not alter microcolony development but induced stress responses in *B. terrestris*, namely a reduced pollen efficacy in microcolonies provided with pure *Salix* pollen, a lower fat body content in workers and newly emerged males, as well as wing shape modifications in newly emerged males. By contrast, *C. bombi* infection increased newly emerged male body size, which may hide a parasite manipulation of the host phenotype.

Most importantly, while *H. annuus* pollen did not alleviate pollen efficacy-related *Crithidia*-induced stress responses, HCAA extracts did, despite that they increased parasite load in bumblebee faeces. Some studies claim that detrimental effects against the parasite should be

compulsory in a medicative context, and clarifications are therefore needed with regards to this condition to determine if HCAAs had proper medicative effects on *Crithidia*-infected bumblebees.

We emphasise that in infected bumblebees, HCAAs led to lower fat body content and great phenotypic variation in newly emerged males, but alleviated parasite-induced costs by increasing pollen efficacy. In other words, HCAA consumption by infected bumblebees was beneficial at the microcolonial level but detrimental at the individual level. In uninfected individuals, HCAA consumption was detrimental both at the individual and at the microcolonial level. Therefore, at the individual level, HCAAs cannot be considered as a medicative resource but by contrast, at the microcolonial level, HCAAs may be seen as a potential medicative resource for *Crithidia*-infected bumblebees. Nevertheless, as outlined by Spivak *et al.* (2019), clarity is needed concerning the relative costs and benefits of medication from individual and colonial levels in social species. For instance, what parameters should be assessed to describe these costs and benefits at both levels? Our results further suggest that there is an urgent need to properly define the boundaries between self- and social-medication. Moreover, to assess if self-medicative behaviours towards HCAA resources may indeed occur, further behavioural experiments are also required to demonstrate that bumblebees deliberately increase their HCAA collection when they are infected.

The fact that HCAAs are beneficial for both the parasite and the microcolony sounds counterintuitive and as already described, the effect that the medicative resource must have towards the parasite in a self-medicative context is still debated in the scientific community. This double-edged interplay opens doors on many ecological questions when considering plant-pollinator-parasite interactions, such as:

- Could parasites modify their host's behaviour to increase the consumption of infection-boosting phytochemicals?
- Could the host detect and adjust its consumption of infection-boosting phytochemicals depending on the parasite load?
- Could some phytochemicals both increase and decrease parasite load depending on environmental factors (e.g., gut pH, gut microbial composition)?
- Could the production of such phytochemical confer evolutionary advantages or disadvantages to the host plant depending on the environmental context (e.g., prevalence of the parasite within pollinators' populations)?

In the current context of global change and pollinator decline (Goulson *et al.*, 2008; Nieto *et al.*, 2014; Mathiasson & Rehan, 2019; Eggleton, 2020), there is an crucial need to understand how specialised metabolites found in host plant pollen and nectar impact pollinators and their parasites since it could be important for the selection of plant species for mitigating strategies (e.g., composition of floral strips; Vaudo *et al.*, 2015; Duez, Gerbaux & Michez, 2018; Adler *et al.*, 2019), potentially including exotic invasive plant species (Vanderplanck *et al.*, 2019a). Moreover, poor nutrition and parasite infection have cumulative negative effects on bee selective value, which is a crucial interaction in the current monofloral culture spreading (Siviter *et al.*, 2021). Because parasite infections – and parasite spreading by human activities – have been blamed as a driver of global bee decline (Potts *et al.*, 2010; Vanbergen *et al.*, 2013; Goulson *et al.*, 2015; Graystock *et al.*, 2016a; Martin *et al.*, 2021), spotting plant species that could alleviate parasite burden – or at least that do not have cumulative negative effects with it – would be of great interest for bee conservation strategies. Further works in this research field should attempt to figure out the molecular and physiological mechanisms of pollen and nectar specialised metabolites on bee individuals and their parasite cells. A focus on the cytology and biochemistry of bee parasite cells is also required for a complete picture, as it still remains quite unexplored.

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7. APPENDICES

7.1. Appendix A – Pilot experiment

7.1.1. SUMMARY

During our pilot bioassays, we tested different pollen diets (i.e., four commercial pollen substitutes and two natural pollen sources) to find the most suitable phytochemical-free diet for our main experiments. We assessed diet quality through microcolony development and individual fat body content. Although none of the substitute diets gave results as satisfying as the natural diets, we decided to further mix Megabee®, the slightly best of the four artificial diets, with a natural diet in three different proportions. Nevertheless, no mix diet was adequate for microcolony development. Pure artificial substitutes as well as mixed diets led to poor microcolony development (i.e., reduced offspring production) and great stress responses (i.e., high larval ejection and low pollen efficacy). By contrast, only one pure artificial diet led to a lowered fat body content in comparison with the natural diets. Strikingly, the Megabee:*Cistus* 75:25 ratio led to a greater fat body content than the pure *Cistus* pollen, yet it did not exceed the fat body content of workers provided with *Salix* pollen. Due to the poor suitability of the tested phytochemical-free diets, we decided to use the natural *Salix* pollen source as control for our main experiment since it has been described as a highly suitable diet for bumblebees, even though it contains specialised metabolites. We thus took great care of this limitation when interpreting our results.

7.1.2. BACKGROUND

Since the 1980s, buff-tailed bumblebee *Bombus terrestris* colonies have been extensively used in commercial pollination systems (e.g., tomato crops in greenhouses; Velthuis & Doorn, 2006) and in laboratory experiments, notably as study model in ecotoxicological assessments (Banks *et al.*, 2020). In the wild, bumblebee nutrition is partitioned between nectar and pollen of flowering plants: the former represents the major energy source (i.e., sugar) while the latter is the prime nutrient resources (i.e., lipids, proteins, amino acids, vitamins and other micronutrients; Roulston & Cane, 2000). These resources are crucial for colony success since they are essential for larval and adult development (Genissel *et al.*, 2002; Vanderplanck *et al.*, 2014a). Bumblebees seem even able to regulate their protein, amino acid as well as lipid intakes when foraging for pollen, and such distinct nutrient profiles

shape colony development (Moerman *et al.*, 2015; Roger *et al.*, 2017; Vaudo *et al.*, 2017; Ruedenauer *et al.*, 2020; Vanderplanck *et al.*, 2020b). Hence, in laboratory rearing, experimenters aim to provide their colonies with adequate nutrient resources (Velthuis & Doorn, 2006). While nectar is usually substituted by a basic sugar syrup to provide carbohydrates, pollen substitution is more challenging because of its chemically complex composition (Roulston & Cane, 2000). Subsequently, bumblebee commercial colonies are generally fed with honeybee-collected pollen pellets to mimic natural nutrient intakes while foraging (Ptácek, 2001). However, pollen provisioning in bumblebee mass rearing is greatly problematic because honeybee-collected pollen (i) is expensive, (ii) varies in quantity and quality, (iii) contains unwanted pollutants (e.g., pesticides) and (iv) carries honeybee pathogens (Tasei & Aupinel, 2008a; Graystock *et al.*, 2013; Tosi *et al.*, 2018). Here, more importantly, we had to consider that honeybee-collected pollen displays a range of phytochemicals (i.e., specialised metabolites; Gardana *et al.*, 2018) that could interfere with the plant extracts we would like to study. For these reasons, we assessed four commercially available artificial diets to determine the most adequate phytochemical-free pollen substitute for our microcolony rearing.

7.1.3. MATERIALS & METHODS

7.1.3.1. POLLEN DIETS

In the first assay, we selected four different artificial paste-typed pollen diets, namely Megabee (Tucson, Arizona), Feedbee, Nutri-bombus medium and Nutri-bombus high (Nutrifeed Canada Inc.; Ajax, Canada) as well as two natural pathogen- and pesticide-free honeybee-collected pollen, namely a *Salix* dominant blend ('Ruchers de Lorraine'; France) and a *Cistus* dominant blend ('Pollenergie France'; France). Diets with a dominance of *Salix* sp. and *Cistus* sp. are known to be highly suitable and suitable diets for bumblebees, respectively (Tasei & Aupinel, 2008a; Vanderplanck *et al.*, 2018), and thus served as control diets. In the second assay, we tested three other diets based on the artificial diet that had given the best result in the first assay (i.e., Megabee) mixed with *Cistus* pollen in different ratios. We decided to use *Cistus* pollen in the mixture because it contains less phytochemicals (i.e., flavonoids and HCAAs) than *Salix* pollen (Irène Semay, Pers. Comm.). The diets were prepared to be provided to the microcolonies as candies (Table S1).

Table S1: Pollen candy preparation for the nine different diets (w/w/w/w). *Salix* sp. and *Cistus* sp. pollen were used as control diets. **Syrup**. Sugar syrup (water:sugar 65:35 w/w). **Raw**. Candies were made directly from the purchased diets without any water or sugar mixing. **Falcon cap**. These diets were provided into 50 mL Falcon caps because of their viscous texture.

DIET (1ST ASSAY)	PREPARATION (POLLEN/WATER/SYRUP)	NOTE
Salix sp.	100:40:1	Highly suitable control
Cistus sp.	100:25:1	Suitable control
Megabee	Raw	/
Feedbee	20:1:0	/
Nutri-bombus medium	Raw	Falcon cap
Nutri-bombus high	Raw	Falcon cap
DIET (2ND ASSAY)	**PREPARATION (MEGABEE/*CISTUS*/WATER/SYRUP)**	
Megabee:*Cistus* 50:50	125:125:25:1	
Megabee:*Cistus* 65:35	162.5:87.5:17.5:1	
Megabee:*Cistus* 75:25	375:125:25:1	

7.1.3.2. COLONY REARING AND MICROCOLONY MANAGEMENT

We purchased five *Bombus terrestris* colonies from our commercial supplier (Biobest *bvba*; Westerlo, Belgium) and divided them into 60 microcolonies (i.e., 12 microcolonies per colony) of five workers placed in different plastic boxes (10 × 16 × 16 cm) for the first assay and into 30 microcolonies (i.e., 6 microcolonies per colony) for the second assay. We allocated these microcolonies to the nine different diets (see section 7.1.3.1) and reared them in a dark room (26-28°C; 60 ± 10% humidity) during a 27-day period. The remainder of the microcolony management was the same as for the main experiment.

7.1.3.3. MICROCOLONY DEVELOPMENT AND WORKER FAT BODY

Estimation of the microcolony development as well as worker fat body content analyses were conducted in the same way as for the main experiment, except that fat body from three workers per microcolony (n = 30 per pollen diet) were examined.

7.1.3.4. DATA ANALYSES

Since the two pilot assays were not run simultaneously, we decided to run in parallel two distinct statistical analyses on microcolony development and fat body content. In the first assay, the artificial diets were compared to the natural diets. In the second assay, the artificial diet tested in different proportions was compared to the natural diets from the first assay.

Resource collection, number of individuals per developmental stage, larval ejection, drone mass, total mass of hatched offspring and fat body content were analysed with models, parameters and conditions identical to the ones in the main experiment, except that parasite infection was not an explanatory variable in this pilot experiment.

Here, pollen efficacy was analysed via a Gauss-Hermite quadrature (25 iterations) generalised linear mixed-effect model (GLMM) with gamma distribution and log link function ('glmer' command, R-package mlmRev; Bates *et al.*, 2020) with Diet as a fixed effect and Colony as a random effect.

Post-hoc pairwise comparisons and plots were performed in the same way as for the main experiments in R version 4.0.3 (R Core Team, 2020). Hereafter, all the statistical outputs are reported in the text.

7.1.4. RESULTS AND DISCUSSION

7.1.4.1. FAT BODY CONTENT

First assay *(Salix, Cistus, Megabee, Feedbee, Nutri-bombus medium, Nutri-bombus high)*

A significant difference in fat body content was detected between Diets (GLMM, $\chi 2 = 23.147$, df = 5, $p < 0.001$). Multiple pairwise comparisons showed that fat body content was higher in all diets, excepted Feedbee, in comparison with the Nutri-bombus high diet. No other significant difference was found (Figure S1). Megabee and Nutri-bombus medium diets were the artificial diets that led to the greatest fat body contents in comparison with the natural control diets, even though they did not exceed the fat body content of the latter.

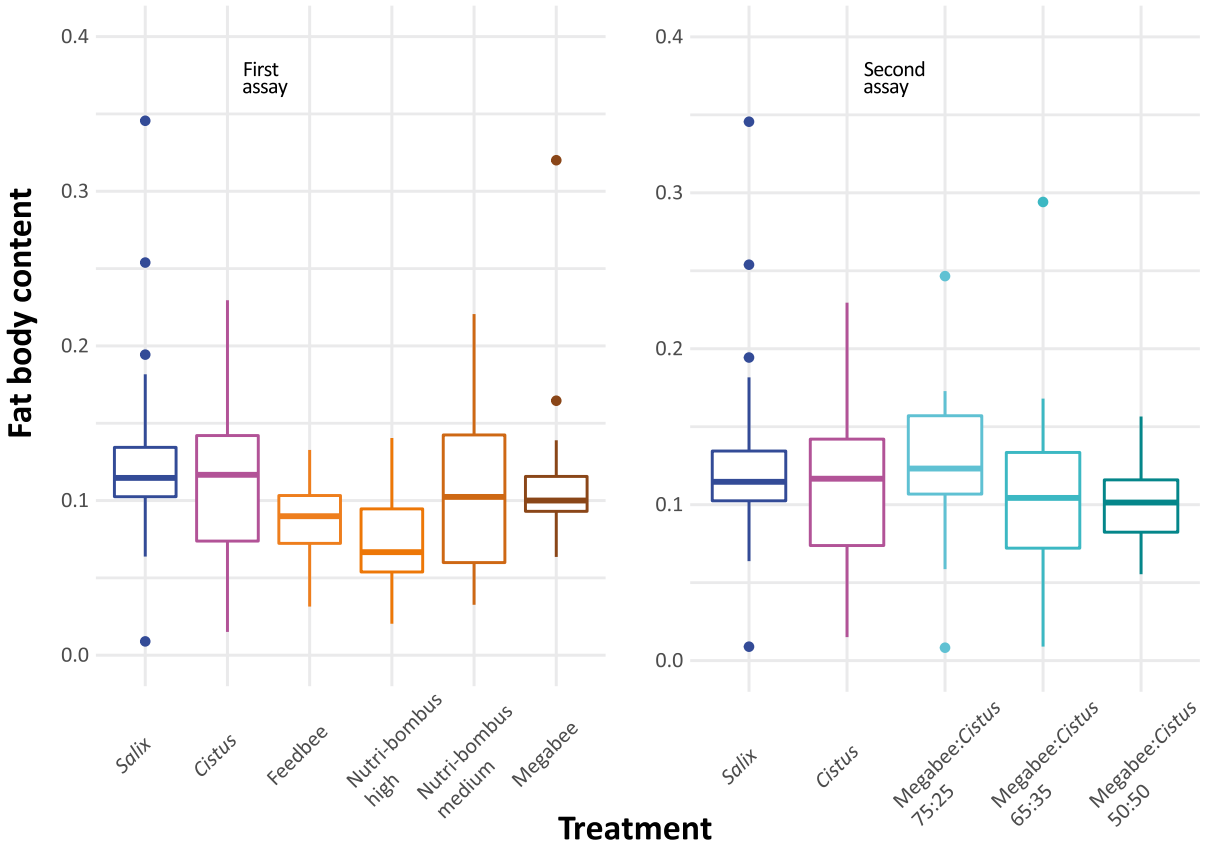


Figure S1: Worker fat body content. Fat body content is calculated as the fat body mass divided by the dry abdomen mass. The boxplots show medians (solid line, 50th percentile) and inter-quartile ranges (colored box, 25th and 75th percentile). The whiskers give the range except for "outliers" (circles) that are more than ±1.5 times the inter-quartile range larger or smaller than the median. **First assay**. Treatments consisted of two natural control diets (i.e., *Salix* and *Cistus*) and four artificial diets (i.e., Megabee, Feedbee, Nutri-bombus high and Nutri-bombus medium). **Second assay**. Treatments consisted of three Megabee:*Cistus* diets mixed in different proportions and were compared with the two natural control of the first assay.

Second assay *(Salix, Cistus, Megabee:Cistus 50:50, Megabee:Cistus 65:35, Megabee:Cistus 75:25)*

As for the first assay, we found a significant difference in fat body content between Diets (GLMM, $\chi 2 = 96.633$, df = 4, $p < 0.001$). Multiple pairwise comparisons showed a significant difference in fat body content between the natural control *Cistus* diet and the mixed diets Megabee:*Cistus* 50:50 and 75:25, but not Megabee:*Cistus* 65:35. Workers fed with the Megabee:*Cistus* 75:25 diet had a higher fat body content than workers fed with the natural control *Cistus* diet, while workers fed with the Megabee:*Cistus* 50:50 diet displayed the opposite trend. In addition, workers fed with the Megabee:*Cistus* 75:25 diet had a higher fat body content than workers fed with any of the two other mixed diets (Figure S1). Thus, interestingly, Megabee increased the fat body content when added in large proportions to *Cistus* pollen (i.e., 75:25) while it reduced fat body content when added in smaller proportion (i.e.,

50:50), in comparison with pure *Cistus* pollen. However, as seen in the first assay, pure Megabee diet did not increase the fat body content. Hence, one may test ratios around Megabee:*Cistus* 75:25 if one wants to increase fat body content when feeding bumblebee workers with Megabee and *Cistus* pollen.

7.1.4.2. MICROCOLONY DEVELOPMENT

First assay *(Salix, Cistus, Megabee, Feedbee, Nutri-bombus medium, Nutri-bombus high)*

We found a significant effect of Day (GLMM, $\chi2 = 459.070$, df = 12, $p < 0.001$) and Diet by Day (GLMM, $\chi2 = 325.620$, df = 60, $p < 0.001$) on syrup collection by *B. terrestris* microcolonies. Post-hoc analyses indicated that syrup collection increased significantly over time for the *Salix* and *Cistus* diets while it increased slightly but non-significantly for the Megabee, Feedbee, Nutri-bombus medium and Nutri-bombus high diets. Overall, no significant difference in syrup collection was found between Diets (GLMM, $\chi2 = 10.020$, df = 5, $p = 0.075$).

We found a significant effect of Diet (GLMM, $\chi2 = 212.160$, df = 5, $p < 0.001$), Day (GLMM, $\chi2 = 406.120$, df = 12, $p < 0.001$) and Diet by Day (GLMM, $\chi2 = 605.810$, df = 60, $p < 0.001$) on pollen collection by *B. terrestris* microcolonies (Figure S2). Post-hoc analyses indicated that pollen collection increased over time for the *Salix* and *Cistus* treatments while it remained stable for the other diets. Post-hoc analyses also showed that pollen collection in the Nutri-bombus medium and Nutri-bombus high diets was significantly lower than pollen collection in the control *Salix* and *Cistus* diets over the whole course of the experiment. Pollen collection in the Feedbee and Megabee diets was significantly lower than pollen collection in the control *Salix* and *Cistus* diets after Day 9 and 19, respectively.

We found a significant effect of Diet on drone mass (GLMM, $\chi2 = 192.260$, df = 5, $p < 0.001$), total offspring mass (GLMM, $\chi2 = 524.330$, df = 5, $p < 0.001$) and pollen efficacy (GLMM, $\chi2 = 303.560$, df = 5, $p < 0.001$). Post-hoc tests clearly revealed a greater total offspring mass and pollen efficacy in microcolonies fed the *Salix* and *Cistus* diets (i.e., natural controls) than in microcolonies fed with artificial diets. Besides, for the artificial diets, these analyses showed a greater total offspring mass and pollen efficacy in the Megabee, Feedbee and Nutri-bombus medium diets than in the Nutri-bombus high diet. Drones only emerged in the *Salix* diet.

A significant lower number of eggs (GLMM, $\chi2 = 23.700$, df = 4, $p < 0.001$), non-isolated larvae (GLMM, $\chi2 = 60.389$, df = 5, $p < 0.001$), pre-defecating larvae (GLMM, $\chi2 = 228.280$, df = 5, $p < 0.001$), post-defecating larvae (GLMM, $\chi2 = 60.389$, df = 5, $p < 0.001$) and

pupae (GLMM, $\chi2 = 100.290$, df = 4, $p < 0.001$) were found in the artificial diets in comparison with the natural diets, but these differences were more pronounced considering the *Salix* diet. By contrast, the number of post-defecating larvae (GLMM, $\chi2 = 2.331$, df = 5, $p = 0.8017$) did not significantly differ among diets. A few non-emerged males were only found in microcolonies fed with *Salix* pollen (Figure S3).

Larval ejection was significantly different among diets (GLMM, $\chi2 = 356.69$, df = 5, $p < 0.001$) with post-hoc analyses showing that microcolonies fed with artificial diets displayed higher larval ejection than microcolonies fed with natural diets (Figure S3).

Hence, the first assay showed that no artificial diet was as appropriate as the natural diets to breed *B. terrestris* microcolonies. However, it seemed like Megabee was slightly more suitable than the other artificial diets and that is why we decided to run a second assay in which we mixed Megabee with *Cistus* pollen in different proportions.

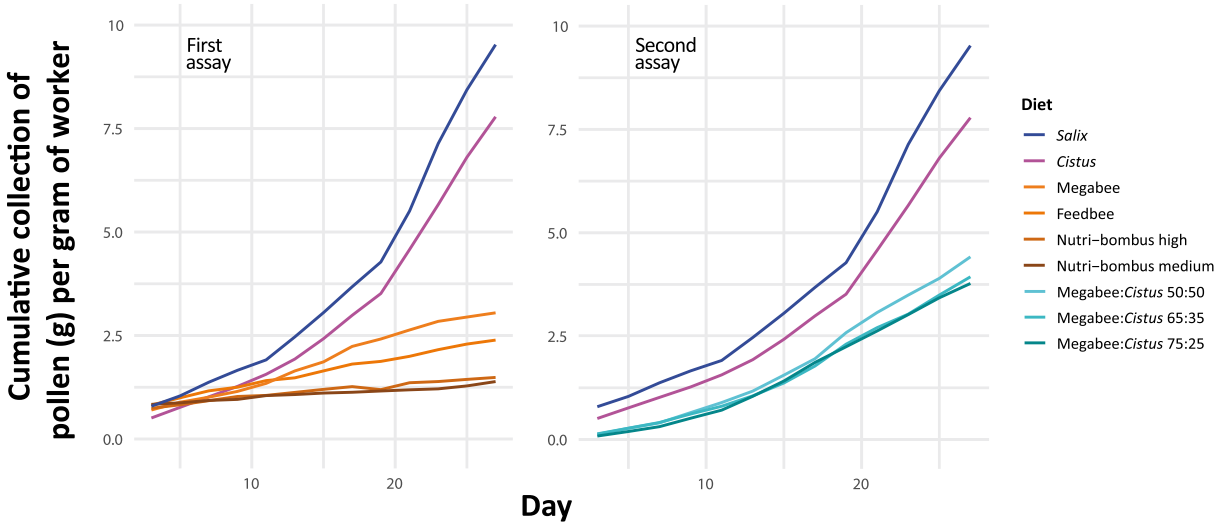


Figure S2: Worker mass-standardised cumulative collection of pollen (g) in microcolonies of *B. terrestris* over time. **First assay**. Treatments consisted of two natural control diets (i.e., *Salix* and *Cistus*) and four artificial diets (i.e., Megabee, Feedbee, Nutri-bombus high and Nutri-bombus medium). **Second assay**. Treatments consisted of three Megabee:*Cistus* diets mixed in different proportions and were compared with the two natural control of the first assay.

Second assay *(Salix, Cistus, Megabee:Cistus 50:50, Megabee:Cistus 65:35, Megabee:Cistus 75:25)*

In the same manner as for the first assay, we found a significant effect of Day (GLMM, $\chi2 = 261.720$, df = 12, $p < 0.001$) and Diet by Day (GLMM, $\chi2 = 325.477$, df = 48, $p < 0.001$) on syrup collection by *B. terrestris* microcolonies. Post-hoc analyses indicated that syrup collection increased slightly but non-significantly for all the Megabee:*Cistus* diets. Syrup collection was not significantly different between Diets (GLMM, $\chi2 = 2.136$, df = 4, $p = 0.711$).

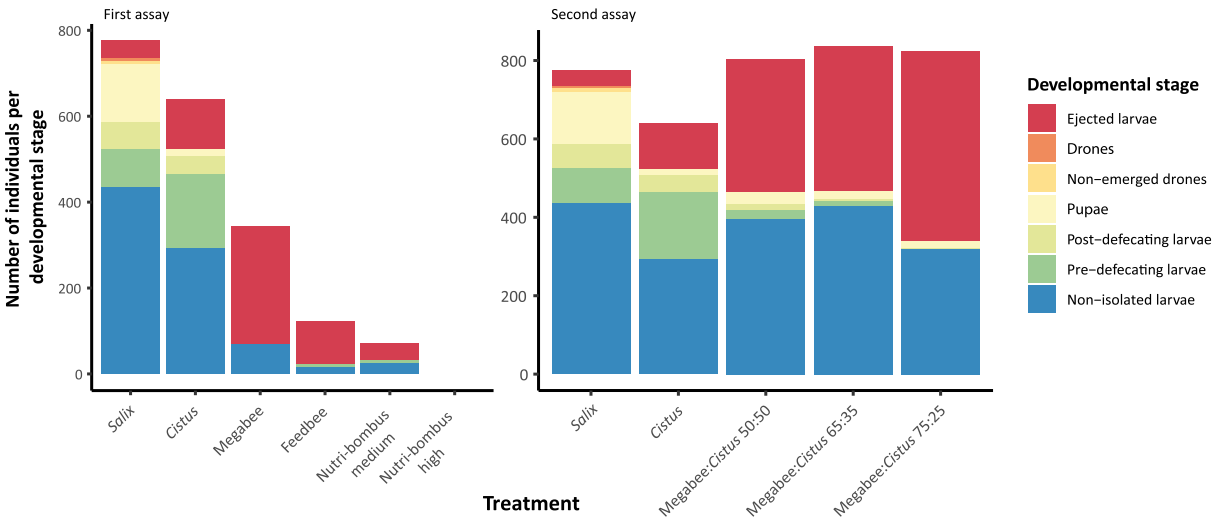


Figure S3: Number of individuals per developmental stage among treatments. Individuals in microcolonies were pooled by treatment. **First assay**. Treatments consisted of two natural control diets (i.e., *Salix* and *Cistus*) and four artificial diets (i.e., Megabee, Feedbee, Nutri-bombus high and Nutri-bombus medium). **Second assay**. Treatments consisted of three Megabee:*Cistus* diets mixed in different proportions that were compared to the two natural control of the first assay.

We found a significant effect of Diet (GLMM, $\chi2 = 120.540$, df = 4, $p < 0.001$), Day (GLMM, $\chi2 = 1145.930$, df = 12, $p < 0.001$) and Diet by Day (GLMM, $\chi2 = 257.050$, df = 48, $p < 0.001$) on pollen collection by *B. terrestris* microcolonies (Figure S2). As in the first assay, post-hoc tests indicated that pollen collection increased over time for the *Salix* and *Cistus* treatments while its remained stable for the Megabee:*Cistus* diets. Such post-hoc analyses also revealed that pollen collection was significantly higher in the *Salix* and *Cistus* diets than in the Megabee:*Cistus* diets, except in the middle of the experiment (from Day 9 to Day 19) where it was non-significant. Different proportions of Megabee:*Cistus* did not lead to significant differences in pollen collection over the whole course of the experiment.

We found a significant effect of Diet on drone mass (GLMM, $\chi2 = 184.570$, df = 4, $p < 0.001$) and total offspring mass (GLMM, $\chi2 = 38.564$, df = 4, $p < 0.001$), and a slightly significant effect of Diet on pollen efficacy (GLMM, $\chi2 = 13.561$, df = 4, $p = 0.0088$). Post-hoc tests indicated a greater total offspring mass in microcolonies fed with *Salix* and *Cistus* diets (i.e., natural controls) than in microcolonies fed with Megabee:*Cistus* 65:35 and 75:25. This difference was not significant for the Megabee:*Cistus* 50:50 diet. Besides, pollen efficacy was greater in the *Salix* diet than in the Megabee:*Cistus* 75:25 diet. No drone emerged in the *Cistus* nor in the three Megabee:*Cistus* diets.

A significant lower number of pre- (GLMM, $\chi2 = 118.780$, df = 4, $p < 0.001$) and post-defecating larvae (GLMM, $\chi2 = 47.911$, df = 4, $p < 0.001$) were found in the Megabee:*Cistus* diets in comparison with the *Salix* and *Cistus* diets. The number of eggs was lower in the

Megabee:*Cistus* diets in comparison with the *Salix* diet (GLMM, $\chi2 = 18.152$, df = 4, $p = 0.001$), but this difference was not significant in comparison with the *Cistus* diet. Besides, the number of pupae was greater in the *Salix* diet than in any other treatment (GLMM, $\chi2 = 101.630$, df = 4, $p < 0.001$). By contrast, the number of non-isolated larvae (GLMM, $\chi2 = 7.820$, df = 4, $p = 0.098$) and non-emerged drones (GLMM, $\chi2 = 5.307$, df = 4, $p = 0.257$) was not significantly different between the diets (Figure S3).

Larval ejection was significantly different among diets (GLMM, $\chi2 = 181.810$, df = 4, $p < 0.001$) with post-hoc analyses showing that microcolonies fed with Megabee:*Cistus* diets displayed higher larval ejection than microcolonies fed with natural diets.

Hence, the second assay yielded results in accordance with the first assay in that the greater was the Megabee part in the Megabee:*Cistus* ratio, the lower was the microcolony development. We thus did not achieve to find any pure or mixed-with-natural-pollen artificial diets as suitable as natural *Salix* pollen for *B. terrestris* rearing.

7.2. Appendix B – HCAA solution and treatment composition

Both control *Salix* pollen and *Helianthus* pollen that were provided to the microcolonies were analysed in triplicates via HPLC-MS/MS. The total phenolamide content was measured as triferuloyl spermidine equivalent (TSE). We found that *Salix* pollen contained 23.21 ± 3.22 mg TSE / pollen g while *Helianthus* pollen contained 54.8 ± 3.74 mg TSE / pollen g (mean ± SD).

The *Helianthus* pollen HCAA extract was obtained via a solid-liquid extraction into methanol solvent using a Soxhlet extractor and was quantified through HPLC-MS/MS. Thus, from 602.58 g of *Helianthus* pollen, 341.75 g crude methanol extract was collected with a concentration of 161.22 ± 2.24 mg TSE / pollen g. The chemical extract was resuspended in 75mL of aqueous ethanol (1:1 v/v) and 43mL of distilled water. The details of the final HCAA solution were the following:

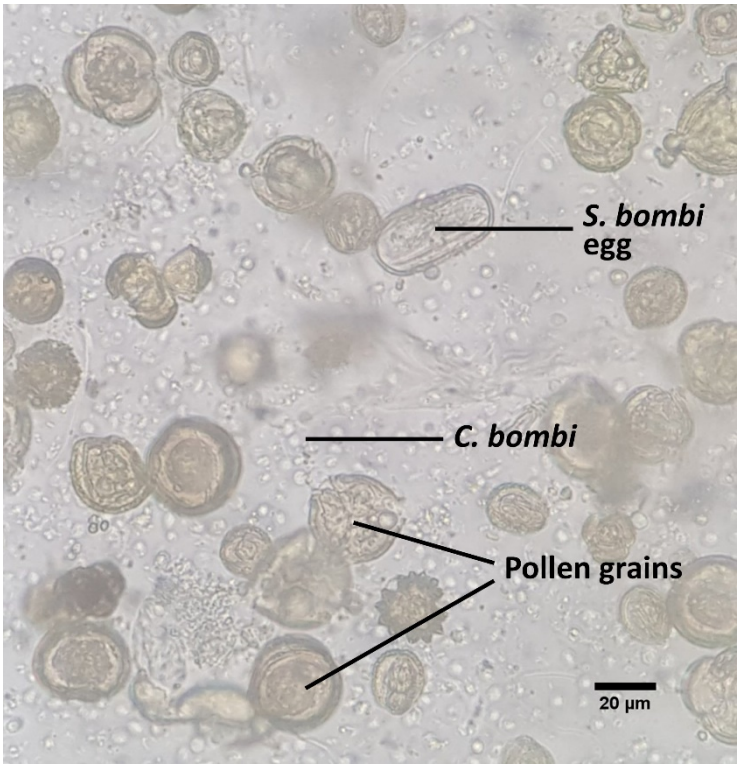
- Solution volume: 395.82 mL
- Solution mass: 451.789 g
- Solution density: 1.14 g/mL
- HCAA mass: 55096.935 mg
- HCAA concentration: 139.197 mg/mL

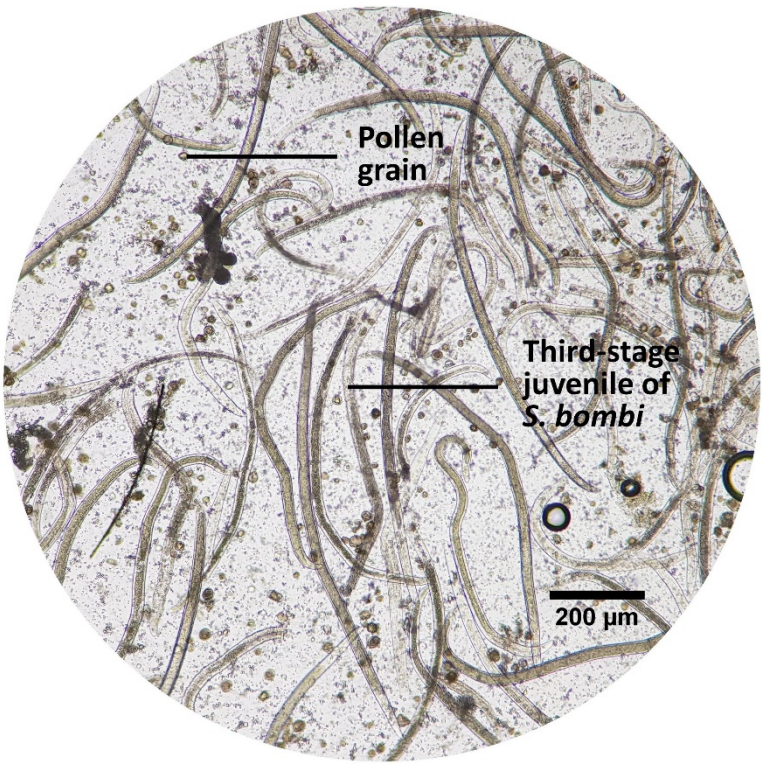
The HCAA solution was ready to be mixed with *Salix* pollen to feed microcolony in the '*Salix* + HCAA' treatment. The treatment composition (Table S2) was made for the HCAA in the '*Salix* + HCAA' treatment to mimic the HCAA concentrations in natural *Helianthus* candies. Hence, microcolonies in the '*Salix* + HCAA' treatment were provided with candies containing 42.371 HCAA mg / candy g which is nearly identical to the HCAA concentration in natural *Helianthus* candies (i.e., 42.615 HCAA mg / candy g).

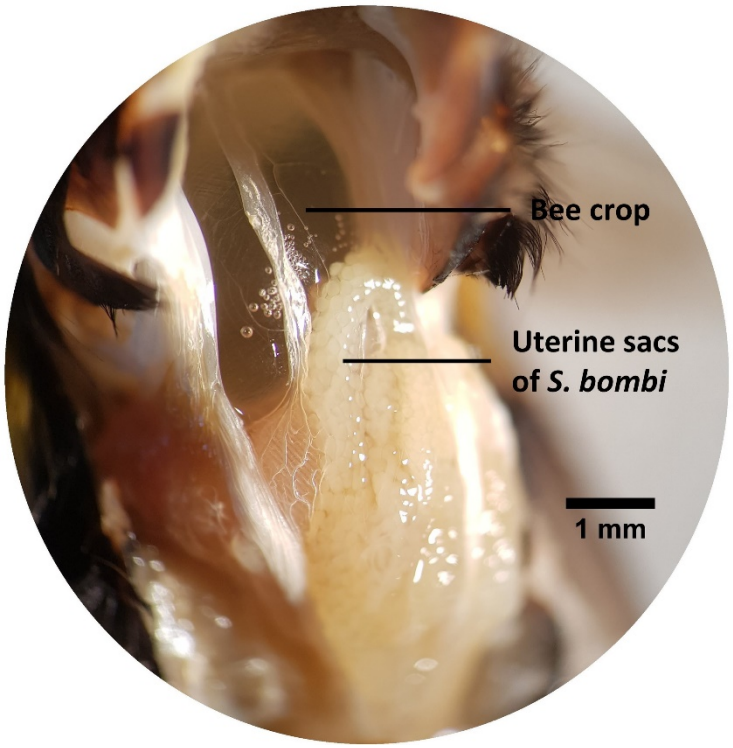
Table S2: Treatment composition. Three treatments were prepared on an every-two-day basis to be provided to the microcolonies, namely '*Salix'*, '*Helianthus'* and '*Salix* + HCAA'. The by-treatment quantities showed here enabled to feed 15 microcolonies at the onset of the experiment (i.e., when each microcolony was provided with 1 g of pollen every other day). These quantities and ratios were modified along the 35-day follow-up period according to the development of the microcolonies. All the treatments contained roughly the same amount of extract solvent (i.e., 26 – 34 ethanol µL / candy g) to consider its potential effects on parasite load and microcolony development.

	TREATMENT		
	Salix	***Helianthus***	***Salix* + HCAA**
Pollen (g)	15	15	15
65% sugar solution (drop)	8	8	/
Aqueous ethanol 1:1 (mL)	1.5	1	/
Distilled water (mL)	5.5	3	/
HCAA solution (mL)	/	/	7
Candy mass (g)	22.23	19.29	23
Ethanol µL / candy g	34	26	29
Pollen g / candy g	0.67	0.78	0.65
HCAA mg / candy g	/	42.615	42.371

7.3. Appendix C – *Sphaerularia bombi* (Nematoda: Tylenchoidea: Allantonematidae)







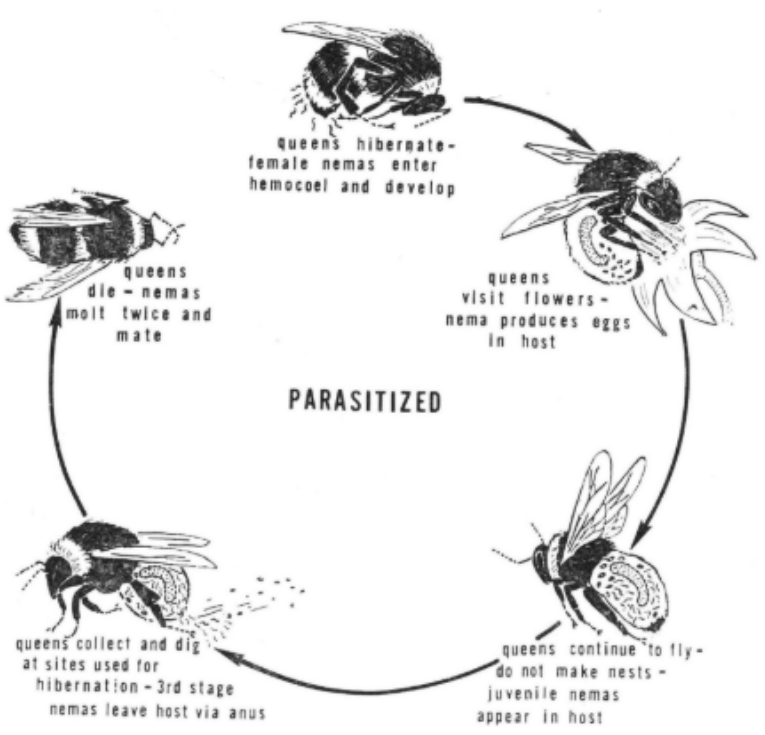


Figure S4: *Sphaerularia bombi*. Upper left. *S. bombi* egg in a *Crithidia*- and *Sphaerularia*-infected queen's faeces. **Upper right**. *S. bombi* third-stage juvenile in a *Sphaerularia*-infected queen's faeces. **Lower left**. Uterine sacs of a *S. bombi* adult female in a *Sphaerularia*-infected queen's abdomen. **Lower right**. *S. bombi* life history in a bumblebee queen (Poinar & Van der Laan, 1972). Pictures taken by A. Gekière.

7.4. Appendix D – *Crithidia bombi* purification protocol

Prior to microcolony inoculation, the faeces of the *Crithidia*-infected stock bees were collected and purified following the method used by Baron *et al.* (2014) and Martin *et al.* (2018) adapted from a 'triangulation' protocol developed by Cole (1970). Basically, this method is based on a purification used by organic chemists and the principles of countercurrent distribution chromatography. Eventually, eight tubes were made and the tubes 3, 4, 5 and 6 contained most of the *Crithidia* cells while the tubes 1, 2, 7 and 8 contained unwanted debris (e.g., pollen grains and bacteria). The procedure can be described as follows:

1. Pool the faeces and dilute them with NaCl 0.9% solution to make 1 ml of total solution (tube 1);
2. Centrifuge tube 1 at 800g for 2min;
3. Remove the supernatant and place it into another centrifuge tube (tube 2);
4. Dilute the remaining pellet (tube 1) and re-suspend it with another 1 ml of NaCl 0.9% solution;
5. Centrifuge tubes 1 and 2 at 800g for 2min;
6. Transfer the supernatant from tube 2 to tube 3 and the supernatant from tube 1 to tube 2;
7. Dilute the remaining pellet (tube 1) and re-suspend it with another 1 ml of NaCl 0.9% solution;
8. Centrifuge tubes 1, 2 and 3 at 800g for 2min;
9. Transfer the supernatant from tube 3 to tube 4, the supernatant from tube 2 to tube 3 and the supernatant from tube 1 to tube 2;
10. Repeat this process until 8 tubes are prepared (no need to spin tube 8);
11. Centrifuge tubes 4, 5, and 6 at 8000g for 1min and remove the supernatants;
12. Re-suspend tube 4 in 100µL of NaCl 0.9% solution;
13. Transfer solution from tube 4 to tube 5 and mix;
14. Transfer solution from tube 5 to tube 6 and mix;
15. Place the resulting solution from tube 6 in a Neubauer chamber, allowing for the *Crithidia* cells to be counted;
16. Adjust the solution (tube 6) to 2,500 cells μL^{-1} with 40% sugar solution.

7.5. Appendix E – Discarded data and photography setup

Table S3: Discarded males for wing morphometry analyses. Five males were discarded when observing the pictures of their wings, due to different causes. If one of the two wings (left or right) was not usable, both wings of the individual were discarded from the dataset.

MICROCOLONY	COLONY	TREATMENT	CAUSE
C9	C	*Helianthus* pollen (no parasite)	Unusual vein
E6	B	*Salix* pollen + HCAA (no parasite)	Damaged wing when placed on a glass slide
E8	C	*Salix* pollen + HCAA (no parasite)	Missing vein
E9	C	*Salix* pollen + HCAA (no parasite)	Damaged wing when placed on a glass slide
E11	D	*Salix* pollen + HCAA (no parasite)	Damaged wing when placed on a glass slide

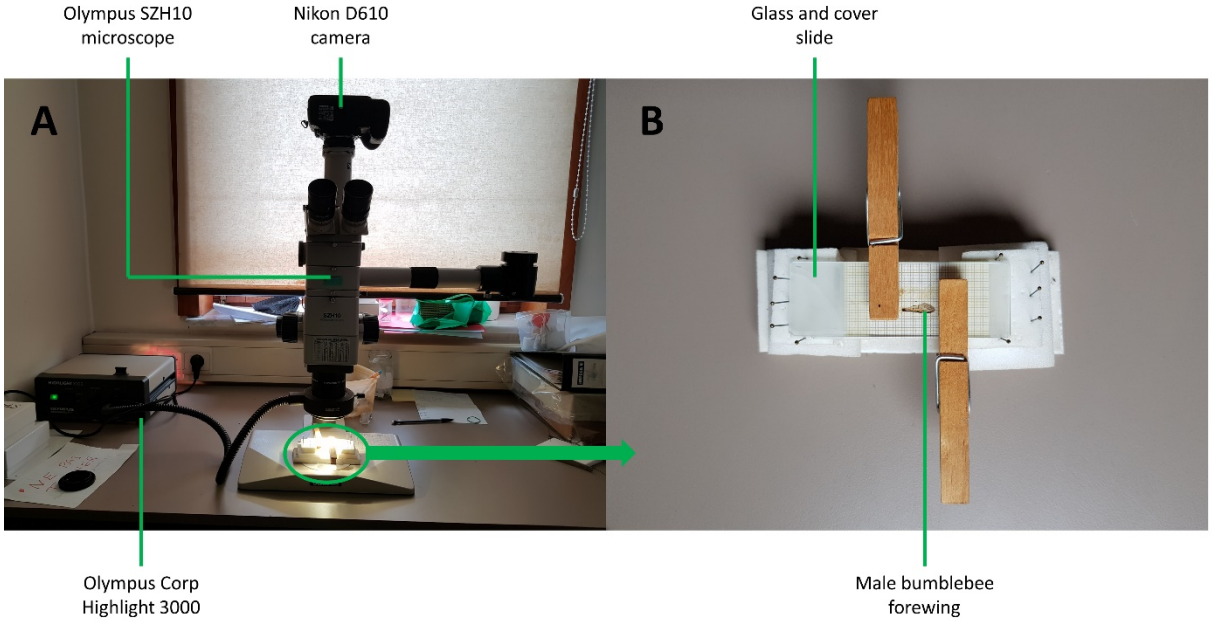


Figure S5: Wing photography setup. A. Olympus light source and microscope as well as Nikon camera used to take pictures of male bumblebee forewings. **B.** Wing flattened between a glass and a cover slide squeezed with clothespins.

6.6. Appendix F – Statistical outputs

Table S4: Outputs from the statistical analyses of this study. Significant *p*-value (<0.05) are in bold. **HCAA**. Hydroxycinnamic acid amide. **GLMM**. Generalised linear mixed-effect model. **GLMMzi**. Zero-inflated generalised linear mixed-effect model. **LMM_{λ}**. Linear mixed-effect model with Box-Cox transformation. **Cox model**. Cox proportional-hazard model. **LM_{RRPP}**. Linear model evaluation with a randomised residual permutation procedure. **$\chi 2$.** Chi-square statistic. **DF.** Degree of freedom.

RESPONSE VARIABLE (MODEL– DISTRIBUTION)	EXPLANATORY VARIABLE	$\chi 2$	DF	*P*-VALUE
Total phenolamide content (Kruskal-Wallis)	Tissue	16.573	3	**<0.001**
HCAA profile (perMANOVA)	Tissue	33.158	3	**<0.001**
Syrup collection (GLMM – Gamma)	Diet	36.999	2	**<0.001**
	Parasite	0.643	1	0.423
	Day	1902.663	16	**<0.001**
	Diet * Parasite	52.851	2	0.878
	Diet * Day	916.385	32	**<0.001**
	Parasite * Day	37.923	16	**0.002**
	Diet * Parasite * Day	52.851	32	**0.012**
Pollen collection (GLMM – Gamma)	Diet	47.983	2	**<0.001**
	Parasite	1.609	1	0.205
	Day	600.089	16	**<0.001**
	Diet * Parasite	1.358	2	0.507
	Diet * Day	198.151	32	**<0.001**
	Parasite * Day	78.914	16	**<0.001**

	Diet * Parasite * Day	71.643	2	**<0.001**
Number of eggs (GLMMzi – Poisson)	Diet	1.336	2	0.513
	Parasite	5.960	2	**0.015**
	Diet * Parasite	0.465	2	0.793
Mass of non-isolated larvae (GLMM – Gamma)	Diet	2.015	2	0.365
	Parasite	0.001	1	0.976
	Diet * Parasite	1.222	2	0.543
Number of non-isolated larvae (GLMM – Poisson)	Diet	10.429	2	**0.005**
	Parasite	0.724	1	0.395
	Diet * Parasite	0.474	2	0.789
Mass of pre-defecating larvae (GLMM – Gamma)	Diet	74.947	2	**<0.001**
	Parasite	0.955	1	0.329
	Diet * Parasite	9.309	2	**0.010**
Number of pre-defecating larvae (GLMM – Poisson)	Diet	44.190	2	**<0.001**
	Parasite	0.256	1	0.613
	Diet * Parasite	8.186	2	**0.017**
Mass of post-defecating larvae (GLMM – Gamma)	Diet	105.081	2	**<0.001**
	Parasite	0.011	1	0.916
	Diet * Parasite	0.040	2	0.980
Number of post-defecating larvae (GLMM – Poisson)	Diet	36.510	2	**<0.001**
	Parasite	0.507	1	0.477

	Diet * Parasite	1.168	2	0.558
Mass of pupae (GLMM – Gamma)	Diet	105.081	2	**<0.001**
	Parasite	1.031	1	0.310
	Diet * Parasite	3.910	2	0.142
Number of pupae (GLMM – Poisson)	Diet	63.567	2	**<0.001**
	Parasite	0.180	1	0.672
	Diet * Parasite	7.904	2	**0.019**
Mass of non-emerged males (GLMM – Gamma)	Diet	5.416	2	0.067
	Parasite	0.005	1	0.945
	Diet * Parasite	4.252	2	0.119
Number of non-emerged males (GLMM – Poisson)	Diet	4.280	2	0.118
	Parasite	0.001	1	0.970
	Diet * Parasite	2.590	2	0.274
Mass of emerged males (GLMM – Gamma)	Diet	64.862	2	**<0.001**
	Parasite	0.059	1	0.809
	Diet * Parasite	0.049	2	0.976
Number of emerged males (GLMMzi – Poisson)	Diet	70.500	2	**<0.001**
	Parasite	1.168	1	0.280
	Diet * Parasite	0.509	2	0.775
Mass of hatched offspring (GLMM – Gamma)	Diet	202.6878	2	**<0.001**
	Parasite	1.011	1	0.315

	Diet * Parasite	4.860	2	0.088
Larval ejection (GLMM – Poisson)	Diet	71.996	2	**<0.001**
	Parasite	4.250	1	**0.039**
	Diet * Parasite	5.451	2	0.065
Pollen efficacy (LMM_{λ}– Gaussian)	Diet	908.305	2	**<0.001**
	Parasite	0.480	1	0.489
	Diet * Parasite	8.219	2	**0.016**
Pollen dilution (LMM_{λ}– Gaussian)	Diet	78.997	2	**<0.001**
	Parasite	3.001	1	0.083
	Diet * Parasite	1.679	2	0.432
Mortality rate (Cox model)	Diet	4.330	2	0.115
	Parasite	1.995	1	0.158
	Diet * Parasite	3.282	2	0.194
Fat body content (GLMM – Gamma)	Diet	28.016	2	**<0.001**
	Parasite	25.3363	1	**<0.001**
	Caste	29.3460	1	**<0.001**
	Diet * Parasite	0.866	2	0.649
	Diet * Caste	15.219	2	**0.001**
	Parasite * Caste	2.058	1	0.151
	Diet * Parasite * Caste	0.650	2	0.723
Centroid size (GLMM – Gamma)	Diet	1030.071	2	**<0.001**

	Parasite	15.093	1	**<0.001**
	Diet * Parasite	4.469	2	0.107
Wing shape (LM_{RRPP})	Diet	22.252	2	**<0.001**
	Parasite	1.906	1	**0.039**
	Diet * Parasite	0.4331	2	0.996
Parasite load between treatments (GLMM – Gamma)	Diet	19.908	2	**<0.001**
	Day	429.275	10	**<0.001**
	Diet * Day	23.025	20	0.288
Parasite load within *Salix* treatment (GLMM – Gamma)	Colony	2.648	4	0.618
	Colony:Microcolony	8.427	10	0.587
Parasite load within *Helianthus* treatment (GLMM – Gamma)	Colony	22.131	4	**<0.001**
	Colony:Microcolony	12.258	10	0.268
Parasite load within HCAA treatment (GLMM – Gamma)	Colony	4.290	4	0.368
	Colony:Microcolony	22.164	10	**0.014**
Parasite load within microcolonies (Fligner-Killeen)	Microcolony	6.851	9	0.553
Parasite load between microcolonies and diets (LM – Gaussian)	Diet	7.370	2	**0.002**
	Diet:Microcolony	2.058	6	0.085