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Abstract

Identifying which behavioural strategies maximize individual fitness is a key objective in ecology. Organisms are known to adapt their foraging behaviour to their environment in response to abiotic and biotic constraints, such as the distribution of resources or the presence of competitors. For instance, bees are known to avoid recently visited flowers and thus focus their foraging on more rewarding patches. Whether other flower-visiting insects adapt their foraging behaviour in response to exploitative competition for floral resources remains unknown. Here, we asked if a predatory hoverfly (*Episyrphus balteatus)* and a parasitoid (*Aphidius colemani)* 1) are physiologically impacted by flower resource limitation following exploitation of flowers by a competitor (either the bumblebee *Bombus terrestris* or *E. balteatu*s); 2) have the ability to discriminate flowers that were previously exploited by a competitor; and 3) modify their foraging behaviour accordingly. *Episyrphus balteatus* and *A. colemani* individuals foraging on previously exploited flowers were found to be less concentrated in sugar compounds, especially in fructose and glucose, suggesting that previously exploited flowers contained less available sugars. Nevertheless, individuals did not avoid previously exploited patches in the choice experiment. On the contrary, *E. balteatus* females preferentially landed on inflorescences that had previously been exploited by conspecifics (but not by *B. terrestris*), while *A. colemani* did not show preferences between

inflorescences. However, female hoverflies spent more time feeding on unexploited patches, suggesting that exploited patches were resource limited. To our knowledge, this study provides the first evidence of the use of social cues among *E. balteatus* individuals in food foraging strategies. It also shows that even insects with tiny nectar requirements, such as parasitoids, can suffer from heavy exploitative competition. Such results may have applied consequences for the understanding of natural enemy conservation, in particular in agroecosystems where competition with honeybees may be important.

Key words: Aphid parasitoid; Buckwheat; Bumblebee; Hoverfly; Natural enemy; Resource discrimination

1. Introduction

In natural environments, the spatio-temporal availability of trophic resources varies in terms of quality, quantity and distribution (Possingham, 1989). To maintain optimal fitness, organisms have developed a range of responses, including adjustments of their foraging strategy to provide the highest benefit (in terms of reproduction or energy intake) at the smallest cost (energy loss or acquisition risk) (the Optimal Foraging Theory (Charnov, 1976; Pyke *et al.*, 1977)). The plastic nature of this nutritional strategy has been demonstrated in a range of organisms and is adjusted in response to abiotic (e.g. temperature) or biotic (e.g. the presence of predators or competitors) constraints (e.g. Reader *et al.* (2006)).

Ecological interactions among organisms sharing common trophic resources may alter their subsequent fitness (Tilman, 1982). Often, the outcome of this interaction is deleterious to one or both competitors, as each can decrease the availability, distribution and quality of the resource to other foragers (Tilman, 1982). As such, this competition, known as exploitative competition, represents an important selection driver of the foraging strategies of insects, that triggers adaptations that reduce the level of inter-specific competition. For instance, reduced foraging time on depleted resources, spill-over on more rewarding resources, and diet breadth expansion, have been observed in honeybees (*Apis mellifera*) and bumblebees (*Bombus* spp.) (Balfour *et al.*, 2015; Fontaine *et al.*, 2008; Walther-Hellwig *et al.*, 2006). Importantly, these responses require the perception and integration of environmental cues

allowing the detection, and thus avoidance, of patches of resources that are supposedly less rewarding (Almohamad *et al.*, 2008; Stout *et al.*, 1998).

Competition for floral resources, in particular pollen and nectar, is likely to occur as they are exploited by a wide variety of organisms, mostly dominated by four insect orders: Hymenoptera, Lepidoptera, Diptera, and Coleoptera (Brown *et al.*, 1981). This is especially true in agroecosystems, where floral resources are less abundant and less diverse than in natural ecosystems (Fried *et al.*, 2009; Robinson and Sutherland, 2002). When already exploited, flowers can be, at least temporarily, less rewarding to other foragers due to diminished nectar and/or pollen resources. For instance, when visited by the insect community, buckwheat (*Fagopyrum esculentum*: Polygonaceae) flowers are empty after mid-day (Lee and Heimpel, 2003). These authors warn that this exploitative competition for buckwheat nectar resources may limit the ability of buckwheat strips to support flower-visiting natural enemies. In accordance with the Optimal Foraging Theory, some flower visitors have the ability to discriminate and avoid flowers that have been previously exploited by another organism (Goulson, 1999; Goulson *et al.*, 1998), thus increasing their foraging efficiency (Stout *et al.*, 1998). Such avoidance of the supposedly less rewarding resources seems to rely on the perception of cues indicating that flowers were recently depleted. These signals can be perceived visually (such as a modification of the flower colour (Willmer *et al.*, 2009)) or by olfaction, as for instance the perception of scent marks by bumblebees deposited by previous visitors (Stout *et al.*, 1998; Wilms and Eltz, 2008)).

Surprisingly, there has been little investigation on the existence of behavioural avoidance of formerly exploited flowers in flower visitor insects other than bees (Apoidea: Hymenoptera). Indeed, up to now, the honeybee *Apis mellifera* (Reader *et al.*, 2005; Stout and Goulson, 2001), several bumblebee species (Goulson *et al.*, 1998; Stout and Goulson, 2001), and a few solitary bee species (Yokoi and Fujisaki, 2009) are the only ones for which behavioural avoidance capacity has been explicitly tested. Most of those insects have the capacity to recognise cues emitted by both conspecific and heterospecifics (Stout and Goulson, 2001). Honeybees also possess the ability to detect and avoid flowers formerly visited by insects from other orders, e.g. Syrphidae (Diptera) (Reader *et al.*, 2005), thus suggesting that they can rely on general cues for detecting the most rewarding resources. Nevertheless, it is still unknown if this capacity is widespread, and thus exists in other flower-visiting insects (Reader *et al.*, 2005),

even though this information is crucial for understanding their population dynamics. Indeed, the amount and quality of pollen and nectar strongly drives reproduction and survival performance of many insect species (Wäckers and Van Rijn, 2012). This is particularly important for synovigenic insects for which the development of reproductive tissues mainly depends on the acquisition of resources by adults (Branquart and Hemptinne, 2000a; Godfray, 1994). For instance, adult hoverflies (Diptera: Syrphidae), which are common flower-visitors, allocate nitrogenous elements provided by pollen and nectar for the maturation of their reproductive system (Branquart and Hemptinne, 2000a) and the production of eggs (Gilbert, 1981). Most flower-visiting insects are also highly dependent on carbohydrates such as sucrose, glucose and fructose, which constitute the main metabolites of nectar (Nicolson and Thornburg, 2007), for survival and flight (Azzouz et al., 2004; Branquart and Hemptinne, 2000b; Gilbert, 1981; Laubertie et al., 2012; Wäckers and Van Rijn, 2012). For some insects, such as some parasitoids and hoverflies, carbohydrates are also involved in egg production and maturation (Araj and Wratten, 2015; Leroy *et al.*, 2010; Olson and Andow, 1998). In this context, GC-MS nutrimetabolomics, *i.e.* the description of the metabolic responses of organisms to dietary resources, represents a valuable tool for linking energetic allocations to nutritional foraging patterns of insects.

In this study, we aimed at understanding the physiological and behavioural consequences of exploitative competition for floral resources in two natural enemy species: a frequent flower visitor, the aphidophagous hoverfly *Episyrphus balteatus* (Diptera: Syrphidae) and a more occasional flower visitor, the aphid parasitoid *Aphidius colemani* (Hymenoptera: Braconidae) (Charles and Paine, 2016; Gilbert, 1981). To meet their energetic requirements, adults of these two species feed on flower resources, which has been shown to increase females' fecundity, longevity and dispersal capacities (Charles and Paine, 2016; Laubertie *et al.*, 2012; Pinheiro *et al.*, 2015; van Rijn and Wäckers, 2016). For both species, longevity without nectar intake is only one or two days (Charles and Paine, 2016; Pinheiro et al., 2015). Two competitors were used: the same hoverfly species *E. balteatus* and the bumblebee *Bombus terrestris* (Hymenoptera: Apidae). To address if the recognition capacity varies among species and with the previous visiting species, we compared the effects of competition between conspecifics (*E. balteatus – E. balteatus*) and between heterospecifics from the same taxonomic order (*A. colemani – B. terrestris*) and from a different order (*E. balteatus – B. terrestris* and *A. colemani*

– *E. balteatus*). Our specific expectations were that 1) individual insects fed on recently exploited flowers should be characterised by lower metabolite concentrations as compared with those fed on unexploited flowers; for instance, lower amounts of fructose and glucose sugars are expected due to the lower quantity of available nectar in exploited flowers. 2.a) If trophic resources are limiting when the flowers have been previously exploited by competitors, we expect secondary foragers to discriminate patch quality, and favour unexploited patches over exploited ones. 2.b) If the exploited patches are not completely avoided, we expect that foragers will spend less time foraging and feeding on patches that are less rewarding (*i.e.* the exploited ones). 3) The physiological consequences for the metabolic profiles and foraging patterns of the tested species might vary among the different species due to their different physiological needs and ecology, ability to recognise cues, and the previous competitor species.

2. Material and methods

2.1. Biological models

The foraging behaviour of two flower-visitor species, *Episyrphus balteatus* and *Aphidius colemani* (hereafter referred to as tested insects), on buckwheat flowers (*Fagopyrum esculentum*) was studied in response to previous exploitation of the flowers by two competitors, *E. balteatus* and *Bombus terrestris* (hereafter referred to as competitor insects).

Insect rearing:
<u>*Tested insects.*</u> Pupae of hoverflies and mummies of parasitoids were supplied by Katz Biotech® and Biobest France®, respectively. The insects were kept in a climatic chamber at 20±2 °C, 70±4% RH and a 16:8 h (Light: Dark) photoperiod until adult emergence. Pupae of hoverflies were individually stored on a layer of vermiculite in microcages (H=10.5cm, Ø=4.5cm) sealed with a net to allow a sufficient airflow. Mummies of parasitoids were individually stored in 1.5 mL safe-lock microtubes with caps pierced with two small holes. Only unmated female that were less than 24 hours-old were used in the experiments for both species. At this age, female hoverflies are sexually immature (Geusen-Pfister, 1987), and we thus assumed that they would not be attracted by male pheromones, as reported for many Diptera species (Katsoyannos, 1982; Lima *et al.*, 2001; Robacker *et al.*, 1990). As most of the

individuals emerged overnight, a cotton ball soaked with water was added at 8 AM in all microcages and tubes containing newly emerged individuals, providing them with a water supply prior to sucrose feeding.

Competitor insects. Adult hoverflies used as previous competitors were raised in a Plexiglas cage (L=50cm, l=50cm, H=100cm) kept at room temperature with natural light. They were provided with water, honey and plants of buckwheat (_Fagopyrum esculentum_: Polygonaceae, "Billy" cultivar), white mustard (_Sinapis alba_: Brassicaceae, "Rota" cultivar) and phacelia (_Phacelia tanacetifolia_: Hydrophyllaceae, "Stella" cultivar). Both males and females were used as competitors.

Adult bumblebees (_B. terrestris_) were provided by Biobest France® and kept in the greenhouse at 21±3 °C, 53±8% RH and a 16:8 h (Light: Dark) photoperiod. Bumblebees had access to the same three plant species as described above for hoverflies. The insects were supplied with water and supplementary pollen obtained from ground organic honeybee pollen loads.

Plants:

Following Leadbeater and Chittka (2011), natural flowers were used to maximise foraging behaviours as it has been previously reported that inexperienced insects require larger exploration periods before foraging when maintained on artificial flowers. All plants used for the experiments were 7-8 week old inflorescences of buckwheat, corresponding to the age at which flower and nectar production reaches its optimum (Cawoy _et al._, 2008). Buckwheat nectar is characterized by high sugar concentrations, which can represent up to 55% of the composition of nectar (Cawoy _et al._, 2008). This plant is commonly used in biological conservation programs (Lavandero _et al._, 2005; Lee and Heimpel, 2003; Quinn _et al._, 2017) and is visited by a large number of insects for nectar and pollen feeding in agroecosystems (Ambrosino _et al._, 2006; Hogg _et al._, 2011; Laubertie _et al._, 2012; Lee and Heimpel, 2003). Buckwheat has also been found to be one of the plant species that best increases _A. colemani_ and _E. balteatus_ females' longevity, parasitism rate of _A. colemani_ females, and duration of oviposition of _E. balteatus_ individuals (Jado _et al._, 2019; Laubertie _et al._, 2012). In a preliminary experiment aimed at describing buckwheat nectar production dynamics (Supplementary Information I.A and I.B), we found that nectar volume per flower is low (maximum of 0.16 μL), but that sucrose concentration is high (between 60 and 70% of sucrose in nectar mass). For our experiments, buckwheat plants were raised in a greenhouse at 21±3 °C, 53±8% RH and a

16h:8 Light: Dark photoperiod. They were placed under a net (L=200cm, l=100cm, H=180cm) to prevent any contact between plants and insects.

Preparation of exploited and unexploited flowers:

Inflorescences were freshly cut (less than 1 hour) before the experiments, and were checked to ensure that no aphids (which are prey for hoverfly larvae and hosts for parasitoids) were present on the plant. Parasitoid longevity was shown to be similar when individuals were fed with excised flowers as when fed with intact flowers (Wade and Wratten, 2007). Exploited buckwheat inflorescences were obtained by placing them into Plexiglas cages (L=35cm, l=35cm, H=50cm) with either 2 bumblebee workers (patch visited by bumblebees) or 10 hoverflies (patch visited by hoverflies). Once all the flowers had been visited at least once, the individuals were left to forage on the flowers for an additional 15 minutes. To make sure hoverfly competitors fed on the inflorescences, they were previously left fasting for 17-23h. Bumblebees did not need any fasting to quickly forage on the inflorescences. This design allowed us to obtain relevant experimental conditions, with flowers visited several times and possibly by different organisms, as observed *in natura* (e.g. Lee and Heimpel, 2003). Exploited inflorescences were presented to tested insects in the choice and no-choice experiments in the 15 minutes following their exploitation by competitors. Unexploited flowers were kept for the same duration and in the same conditions without competitors. A preliminary experiment showed that intact buckwheat flowers only moderately replenish after nectar removal, with a maximum of 34% of the initial nectar volume being restored two hours after nectar removal (Supplementary Information I.C).

2.2. No-choice experiment: metabolite contents of secondary foragers:

To assess if flower exploitation by competitors subsequently limited resources available to a secondary forager, metabolic profiles of individuals fed on exploited or unexploited flowers were compared. To that aim, newly emerged females (hoverflies and parasitoids) were exposed to one of the four following experimental treatments: (i) water (Water), (ii) water and unexploited flowers (Unexploited), (iii) water and flowers exploited by bumblebees (Exploited B), or (iv) water and flowers exploited by hoverflies (Exploited H). Water was provided by a soaked cotton ball in all microcages and tubes. The floral resources consisted of an inflorescence of five flowers for each hoverfly, and a single flower for each parasitoid. All tested individuals were left feeding for 15 minutes after the beginning of the feeding

behaviour was first observed. For each treatment, 16 to 22 hoverflies and 30 to 45 parasitoids were tested. Individuals were then immediately frozen in liquid nitrogen, and stored at -80 °C until processing. After having freeze-dried for over 48h, dry mass of the insects was measured with a Mettler Toledo XP2U precision balance with 0.1 μg resolution.

For GC-MS analyses, insect dry masses must be above 0.2 mg to ensure reliable detection and quantification of several primary metabolites. Hoverflies' dry mass ranged from 2.373 mg to 5.787 mg, and parasitoids' dry mass ranged from 0.0213 mg to 0.1258 mg. Therefore, hoverfly samples contained one individual, while parasitoids were pooled in groups of 2-5 individuals, resulting in 16-22 replicates for hoverflies and 10-13 replicates for pooled parasitoids per treatment. The samples were homogenized in 900μL [300μL] (respectively for hoverflies and [parasitoids]) of methanol-chloroform solution (2:1), and a volume of 600μL [220μL] of ultrapure water was added to each sample. After centrifugation, a 120μL [220μL] aliquot of the upper aqueous phase, which contains polar metabolites, was transferred to new glass vials. The GC-MS procedure used was first described by Khodayari *et al.* (2013), and modified by Thiébaut *et al.* (2020). Concentrations of each metabolite were divided by the dry mass of the individual tested (or the sum of the masses of pooled individuals), and are presented in nmol per mg of dry mass.

2.3. Choice experiment: Does previous flower exploitation impact patch choice, foraging and feeding behaviours of secondary foragers?

To determine the innate feeding preference of the secondary foragers for exploited and unexploited flower patches, a choice test was carried out with newly emerged insects. A preliminary sucrose feeding was carried out, as *E. balteatus* and *A. colemani* individuals are likely to die after 24 hours when they are not offered sugar intake (Charles and Paine, 2016; Pinheiro *et al.*, 2015). A cotton ball soaked with 0.5 mL of a 70% solution of sucrose was placed in each microcage or tube. Ten minutes after the individual had started feeding on the sucrose solution, the cotton ball was replaced by another one containing water only. Individuals that did not feed on the cotton ball with sugar were not used for the choice test. For both tested species (hoverfly and parasitoid), the feeding occurred at 5 PM on 1-23 h old individuals.

The choice test experiment was carried out in an air-conditioned room, 16-20 h after the preliminary sucrose feeding to stimulate the foraging behaviour of the tested insects (Fig. 1).

The two resource patches used during the choice test were an unexploited buckwheat inflorescence and an exploited one by either hoverflies or bumblebees. Each inflorescence contained five flowers (the other flowers were cut), and were selected to have a similar architecture. Both patches were placed under a Plexiglas cage (L=27cm, W=15cm, H=17cm) at a 10 cm distance from one another (Fig. 1). A 2 cm diameter hole was pierced at the centre of the cage, so that the tested individual (hoverfly or parasitoid) entered at an equal distance from both patches. The position of the two inflorescences was alternated from one test to the other to avoid an effect of the position of the patches on the insect's choice. The soil surface was covered with white paper changed between each test to prevent the presence of olfactory marks deposited by the individual previously tested. For the same reason, the Plexiglas cages used were washed with distilled water between each test and with ethanol at the end of each day. Individuals that did not forage within three minutes of their entrance into the Plexiglas cage were removed and tested again later. Each individual was given a maximum of three trials, after which they were discarded from the experiment.

The behaviour of each tested individual was observed for 10 minutes after it entered the cage (head out of tube) and was recorded with the *sequenceR* package (Hervé, 2013) of the R software. All observed behaviours are described in Table 2. At the end of the assay, individuals were frozen in liquid nitrogen and stored at -80 °C before being processed. After the samples were freeze-dried for 48 h, dry mass was measured with a Mettler-Toledo XP2U balance with 0.1µg resolution.

In total, 55 hoverfly females (N=29 in the hoverfly competitor trial and N=26 in the bumblebee competitor trial) and 40 parasitoid females (N=23 in the hoverfly competitor trial and N=17 in the bumblebee competitor trial) were tested.

*Table 1. Metabolites quantified in hoverflies (*E. balteatus*) and parasitoids (*A. colemani*), organized by metabolite families. Letters indicate the presence of the metabolite in hoverflies (H), parasitoids (P) or both (H+P).*

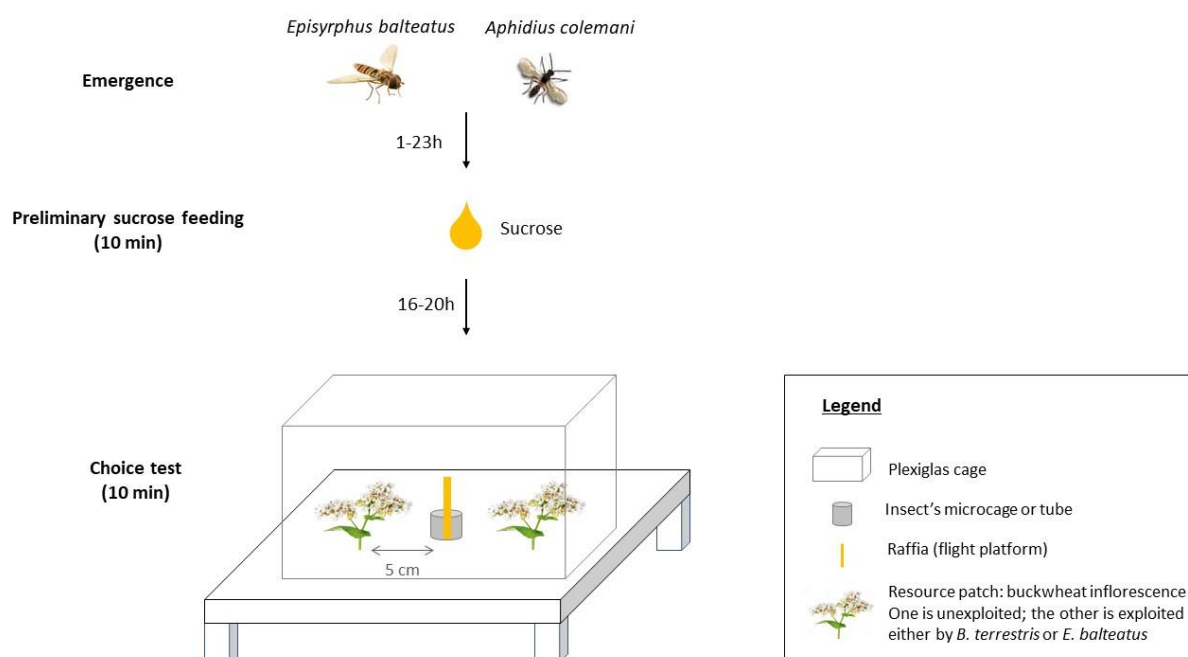| Sugars and sugar phosphates		Essential amino acids		Non-essential amino acids		Organic acids		Polyols		Amines		Other metabolites	
Fructose	H+P	Isoleucine	P	Alanine	P	Citric acid	H	Adonitol	P	Monoetha nolamine	P	Gamma aminobutyric acid (GABA)	H+P
Fructose-6-phosphate (F6P)	H	Leucine	H+P	Asparagine	P	Gluconic acid	P	Arabitol	P	Putrescine	P	Glycerol-3-phosphate (G3P)	H+P
Glucose	H+P	Methionine	H+P	Aspartic acid	H+P	Glyceric acid	H+P	Erythritol	H+P				
Glucose-6-phosphate (G6P)	H	Phenylalanine	H+P	Citrulline	H+P	Lactic acid	P	Glycerol	H+P				
Ribose	P	Threonine	P	Glutamic acid	P	Malic acid	H	Inositol	H+P				
Trehalose	H+P	Valine	H+P	Ornithine	P	Phosphoric acid	H+P	Sorbitol	H+P				
				Proline	H+P	Succinic acid	P	Xylitol	P				
				Serine	H+P								

Figure 1. Experimental design of the choice test. Female Episyrphus balteatus *and* Aphidius colemani *previously fed on sucrose were tested in a choice arena. The arena contained two resource patches: an unexploited inflorescence of buckwheat and an inflorescence previously exploited by bumblebees; or an unexploited inflorescence of buckwheat and an inflorescence previously exploited by hoverflies.*

Table 2: Description of recorded behaviours for hoverflies (E. balteatus*) and parasitoids (*A. colemani*) during the choice test.*

Observed behaviour (on a given patch)	Definition	Type
Feeding	Individual has either its tongue (hoverfly) or mandibles (parasitoid) in the corolla for more than 2 seconds	Duration
Foraging	Individual probes the patch with its tongue (hoverfly) or antenna (parasitoid)	
Immobility	Individual stays immobile, is grooming itself or is moving on the inflorescence without foraging	
First landing	Identity of the first patch the individual lands on	Binary
First feeding	Identity of the first patch the individual feeds on	
Entrance/Exit	Individual enters or exits a patch	Occurrence
Changing	Individual moves from a flower to another on the same patch	

2.4. Statistical analyses

All statistical analyses were conducted with the RStudio software (v.1.2.1335).

Metabolite contents of secondary foragers: To identify the metabolites that contributed to the separation of the four experimental treatments, Linear Discriminant Analyses (LDA) were performed on the log-transformed concentrations of all quantified metabolites for each tested species (hoverfly and parasitoid) (*lda* function from the "MASS" package) (Colinet and Renault, 2012). The effect of the treatment on metabolite concentrations was tested for each metabolite family (Table 1), as well as fructose, glucose and trehalose. When the concentrations of the metabolites/metabolite families were close to a Normal distribution (amino acids for both species, organic acids for hoverflies and polyols for parasitoids), a linear model was fitted to the data (*lm* function). For all other metabolites/metabolite families, the response variable was log-transformed. For hoverflies, the testing day was added as a random factor (*lmer* function from the "lme4" package), but not for parasitoids due to the pooling of individuals. When the effect of the experimental treatment was significant, a Tukey post-hoc test was carried out to distinguish which of the four treatments differed from the others with the "multcomp" package (*glht* and *cld* functions) (Hothorn *et al.*, 2008).

Patch choice of the secondary forager: The probability of first landing or feeding on an unexploited patch compared to an exploited patch was tested with an independent binomial test (function *binom.test*) for each tested species (hoverfly or parasitoid) and each previous competitor species (hoverflies or bumblebees). All observations were assumed to be independent and the null hypothesis was that individuals chose a patch at random (probability of 0.5 to choose the unexploited patch). As very few parasitoid individuals fed on the inflorescences (N=3 during the bumblebee competitor trial and N=7 during the hoverfly competitor trial), it was not possible to analyse feeding behaviours for parasitoids (neither first feeding patch choice nor feeding duration, see below). For hoverflies only, the number of visits per patch was analysed with GLMMs with a lognormal distribution (*glmer* function from the "lme4" package (Bates *et al.*, 2014)). The patch quality (*i.e.* exploited by a competitor species or unexploited) and the side of the visited patch (right or left) were considered as fixed factors, while the tested individual dry mass was added as a covariate. Each patch visited (the unexploited and the exploited) was considered an observation, so the same tested individual

could have been observed twice if both patches were visited during the choice test. Such a behaviour was recurrent for hoverflies, so individual identity was added as a random factor in the models. The number of visits per patch was not analysed for parasitoid individuals as among the 39 individuals observed, only 15, all treatments included, visited the same patch several times.

Foraging and feeding behaviours of the secondary forager: The durations of foraging and feeding behaviours per patch were analysed with Cox proportional hazard models, using the *coxph* function from the "survival" package. Explanatory variables were the same as for the models testing the number of visits per patch. Similar to above, hoverfly identity was added as a random factor as one individual often visited both patches. For parasitoids, the only individual who visited both patches during the choice test was removed from the data for this analysis, so each observation corresponded to one individual, and therefore the models did not contain any random factors. Feeding duration was not analysed for parasitoids as too few individuals actually fed on the inflorescences (see above). The mean number of flower changes per patch visit was analysed for hoverflies and parasitoids with mixed generalized linear models (GLMMs, *glmmPQL* function from the "MASS" package) and GLMs following quasipoisson distributions (log link function), respectively.

All models were simplified by removing variables (except "patch quality" as it was the main tested variable) for which significance was above the significance threshold (0.05). A Fisher test (for LMs) and a Wald Chi-square test (for LMMs and GLMMs) were used assuming a type II sum of squares (*Anova* function from the "car" package). Final model validity was checked to meet model assumptions according to Crawley (2005).

3. Results

3.1. Metabolite content of secondary foragers

Nineteen metabolites were quantified from *Episyrphus balteatus* and *Aphidius colemani* (Table 1). Four additional metabolites were found from hoverflies only and fifteen from parasitoids only (Table 1). When quantified in a given species, the metabolite was detected in all individuals from the four treatments. Both LDAs performed on the metabolite contents of hoverflies and parasitoids revealed a separation according to the feeding treatment experienced by individuals on the two first axes (representing 90.7% and 93.3% of the variance

for hoverflies and parasitoids respectively) (Fig. 2). In both species, sugars (in particular glucose, fructose and trehalose), many amino acids, and sorbitol highly contributed to separate tested individuals between treatments on LD1 (Fig. 2B, 2D). In particular, water-reared individuals were characterised by lower concentrations of metabolites in general, compared with flower-fed individuals (Fig. 2A, 2C). For parasitoids, there was a clear differentiation of the metabolic fingerprint of individuals fed with flowers exploited by bumblebees and those fed with flowers exploited by hoverflies (Fig. 2C).
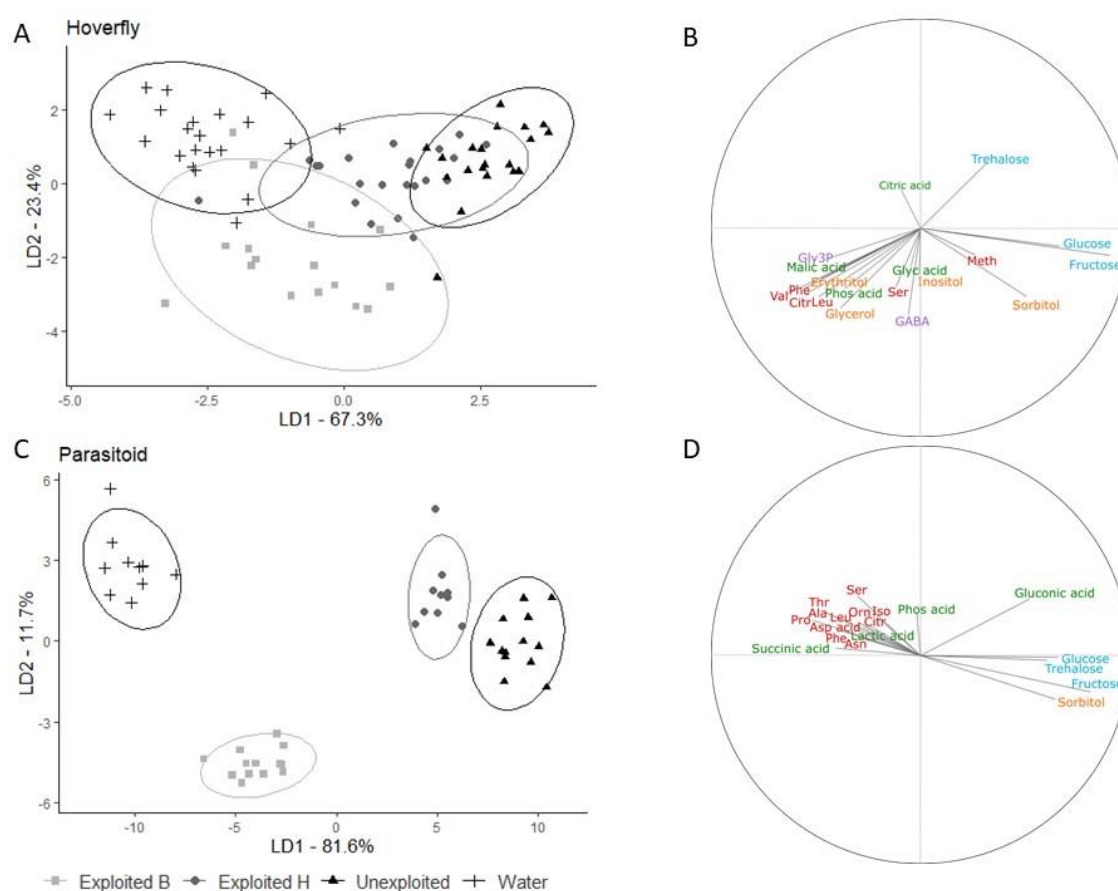

Figure 2. Sample projections of hoverflies (A) and parasitoids (C) onto the first discriminant plane of the multivariate analysis LDA (representing 90.7% and 93.3% of the variance for hoverflies and parasitoids, respectively) depending on their metabolic composition and treatment (Water (crosses), patch exploited by bumblebees (Exploited B, squares); patch exploited by hoverflies (Exploited H, round dots), or an unexploited patch (Unexploited, triangles)). Normal confidence ellipses are represented. The correlation circles (B, D) depict the normalized relation between each metabolite and LDA axes for hoverflies (B, 23 log-transformed variables) and parasitoids (D, 34 log-transformed variables). For readability, only metabolites contributing by more than 30% to both dimensions are presented.

Total sugar concentration was higher in insects fed on flowers compared with those reared on water, and in those fed with unexploited flowers compared with exploited ones (χ^2=83.1; df=3; p<0.001 for hoverflies and F=19.3; df1/df2=3/43; p<0.001 for parasitoids) (Fig. 3A, 3B). For

both tested species, there was no difference in total sugar concentration between individuals fed on flowers previously exploited by bumblebees or by hoverflies.
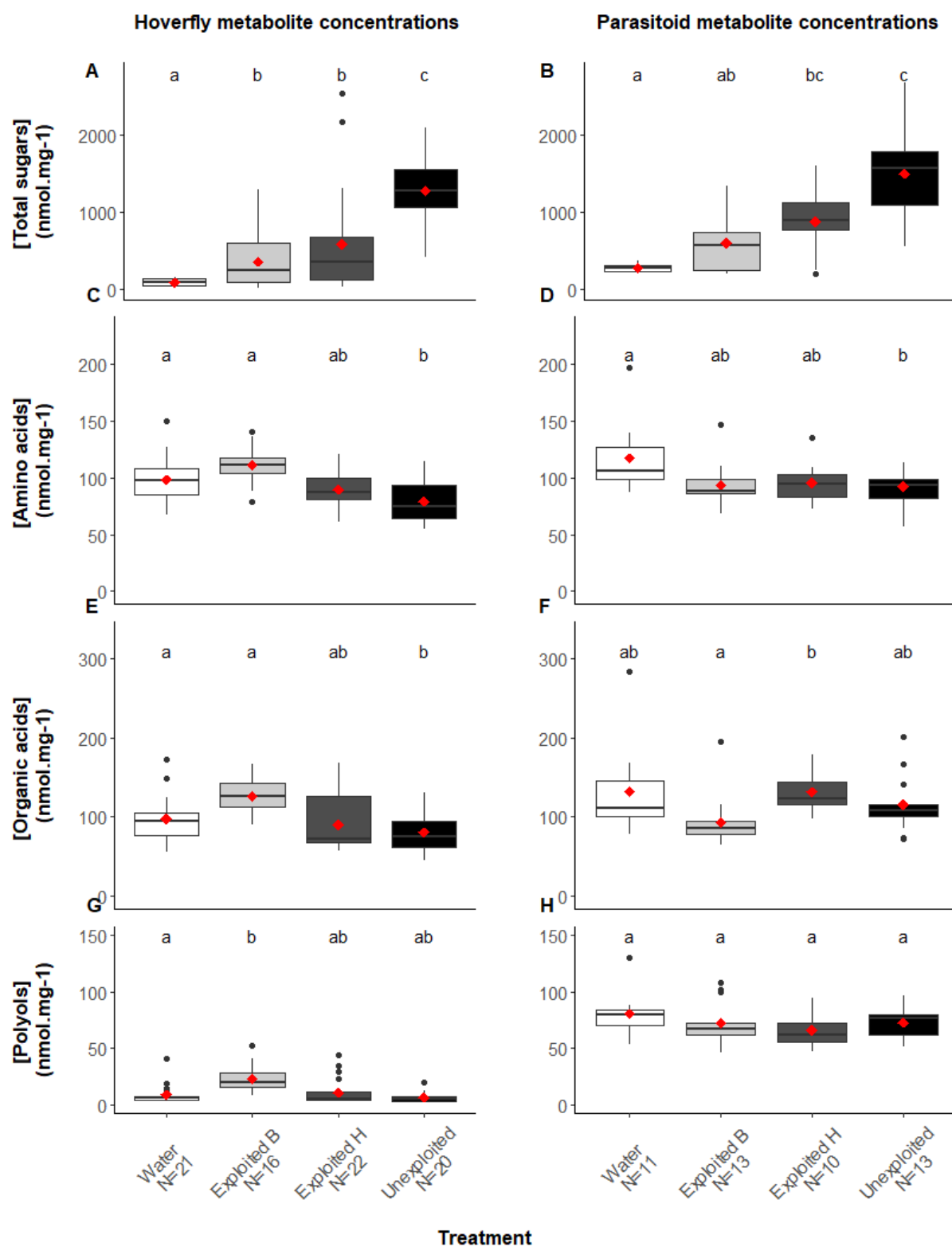

Figure 3. Effect of feeding treatment (Water (white), patch exploited by bumblebees (Exploited B, light grey); patch exploited by hoverflies (Exploited H, dark grey), or an unexploited patch (Unexploited, black)) on the concentrations (nmol.mg^{-1}) of total sugars (A, B), amino acids (C, D), organic acids (E, F) and polyols (G, H), of the hoverfly E. balteatus *(A, C, E, G)* and parasitoid A. colemani *(B, D, F, H)*. On

Hoverflies ingesting water or flower resources previously exploited by bumblebees consistently exhibited higher amino acid (χ^2=16.0; df=3; p=0.001) and organic acid (χ^2=10.1; df=3; p=0.017) concentrations than their relatives fed on unexploited flowers (Fig. 3C, 3E). Parasitoids reared on water had higher amino acid concentrations than individuals fed with unexploited flowers (F=3.38; df1/df2=3/43; p=0.027); no difference was found among individuals from the two other treatments (Fig. 3D). Parasitoids fed on flowers exploited by bumblebees showed lower concentrations of organic acids than those fed with flowers previously exploited by hoverflies, but no concentration difference was detected with water-reared parasitoids or parasitoids fed with unexploited flowers (F=3.65; df1/df2=3/43; p=0.020) (Fig. 3F). Water-reared hoverflies exhibited lower concentrations of polyols than hoverflies fed with flowers exploited by bumblebees (χ^2=9.57; df=3; p=0.023) (Fig. 3G). In parasitoids, polyol concentration was not affected by the feeding treatment (F=1.22; df1/df2=3/43; p=0.314) (Fig. 3H).

Fructose and glucose concentrations were higher for hoverflies fed on unexploited flowers than for those fed on exploited ones, themselves higher than for water-reared individuals (Fructose: χ^2= 139.0; df=3, p<0.001; Glucose: χ^2=57.0; df=3; p<0.001) (Fig. 4A, 4C). For parasitoids, fructose and glucose concentrations were higher for individuals fed on unexploited flowers than those fed on flowers exploited by bumblebees, but not on flowers exploited by hoverflies (Fructose: F=37.5; df1/df2=3/43; p<0.001; Glucose F=12.4; df1/df2=3/43; p<0.001) (Fig. 4B, 4D). No difference between treatments was detected for trehalose concentrations in hoverflies (χ^2=8.0; df=3; p=0.047) (Fig. 4E). The concentration of trehalose was higher in parasitoids fed on unexploited flowers than on the three other treatments (F=11.0; df1/df2=3/43; p<0.001) (Fig. 4F).

Hoverfly sugar metabolite concentrations

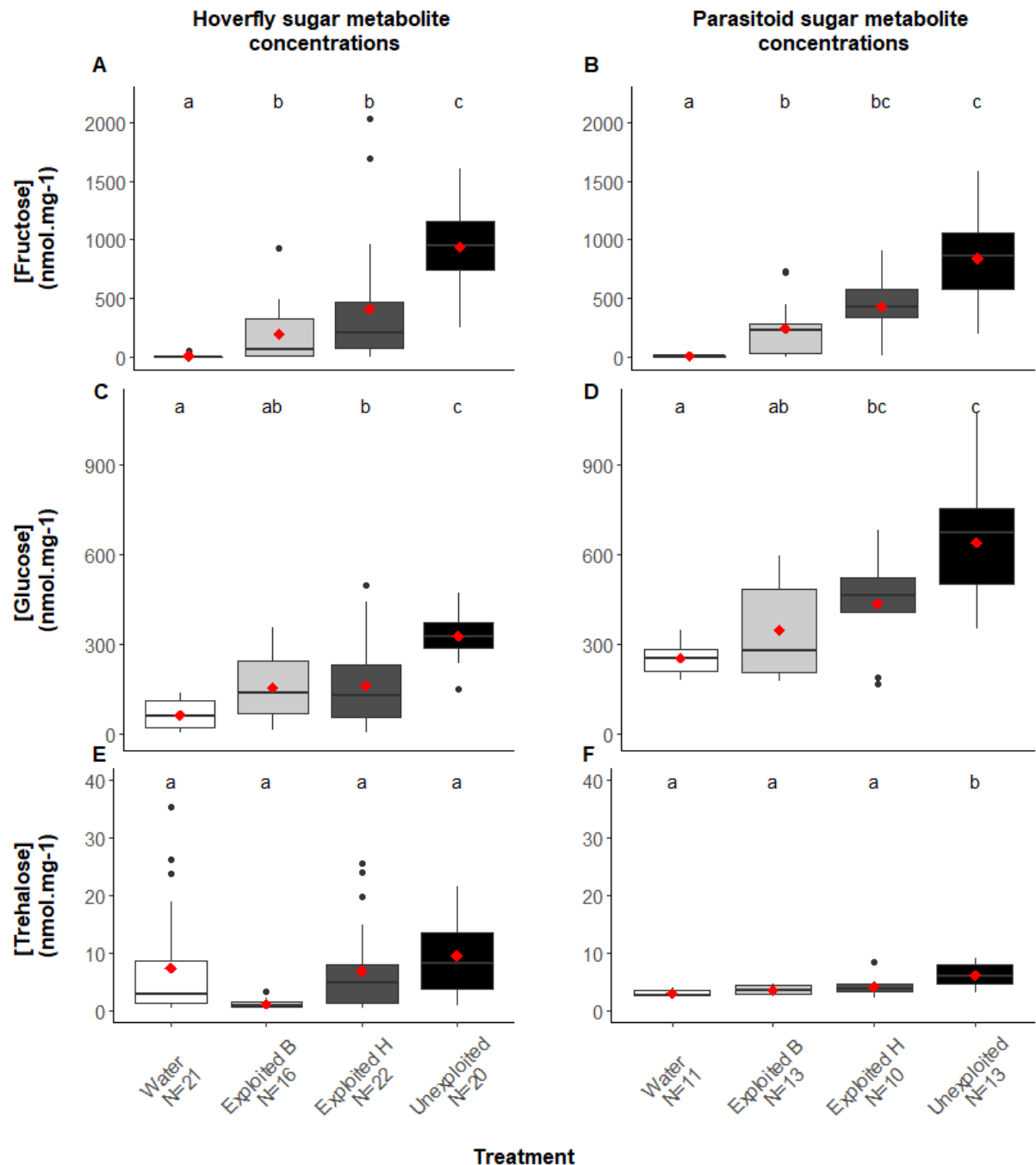**Parasitoid sugar metabolite concentrations**

Figure 4. Effect of feeding treatment (Water (white), patch exploited by bumblebees (Exploited B, light grey); patch exploited by hoverflies (Exploited H, dark grey), or an unexploited patch (Unexploited, black)) on the concentrations (nmol.mg^{-1}) of fructose (A, B), glucose (C, D), and trehalose (E, F) of the hoverfly E. balteatus *(A, C, E) and parasitoid* A. colemani *(B, D, F). On each boxplot, horizontal lines represent the median, first and third quartiles, the black points are extreme values, the red square is the mean and N the sample size. Different letters between treatments indicate significant differences (Tukey tests, p<0.05).*

3.2. Secondary forager patch choice

Hoverflies preferentially landed on the flower patch that had already been exploited by conspecifics (probability to first land on the patch exploited by hoverflies=0.83; p<0.001, Fig.

5A). This preference was not found when previous competitors were bumblebees (probability to first land on the patch exploited by bumblebees=0.38; p=0.327) (Fig. 5A). No preference was recorded for the first feeding patch exploited by hoverflies whatever the nature of the first competitor (probability to first feed on the patch exploited by hoverflies=0.61; p=0.405 and probability to first feed on the patch exploited by bumblebees=0.29; p=0.144). Furthermore, hoverflies visited the patch exploited by conspecifics significantly more times than the unexploited one (χ^2=20.69; df=1; p<0.001); this difference was not observed when the patches were previously exploited by bumblebees (χ^2=2.19; df=1; p=0.139).

Parasitoid individuals had no innate landing preference in our experimental design (probability to first land on a patch exploited by hoverflies=0.44; p=0.678; probability to first land on a patch exploited by bumblebees=0.35; p=0.332) (Fig. 5B).
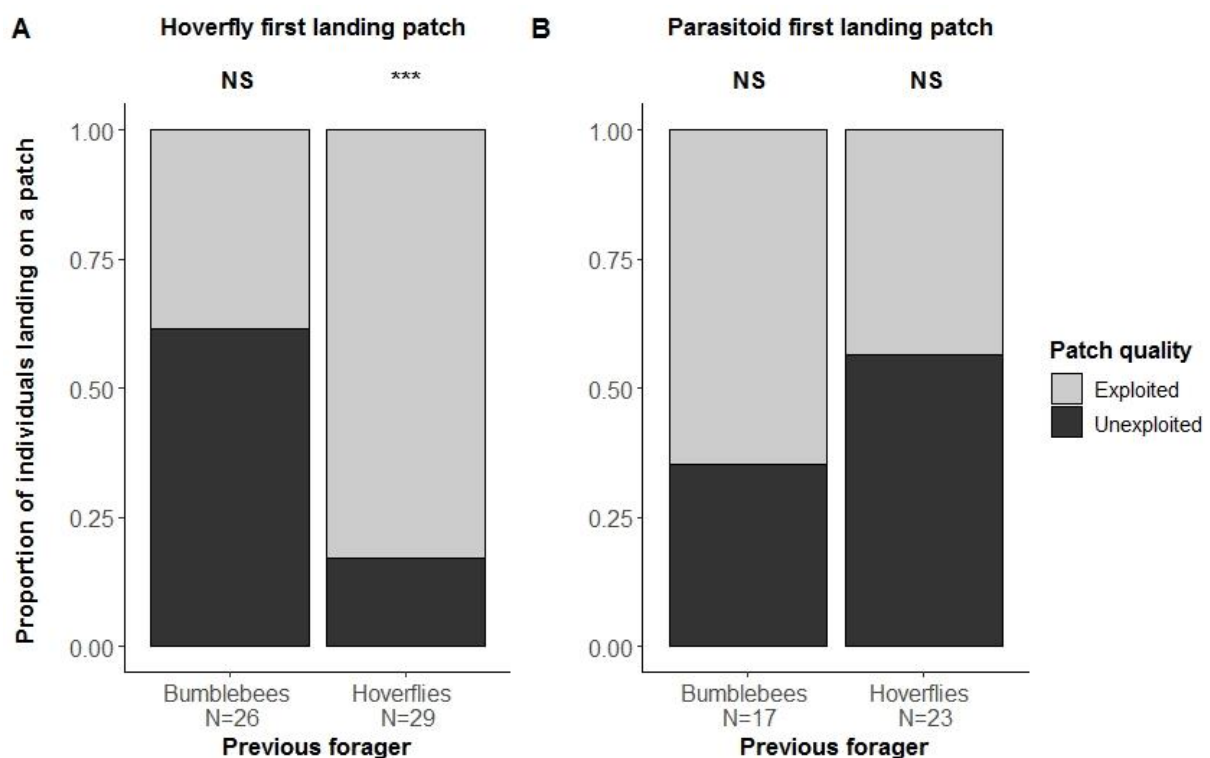

Figure 5. Proportion of hoverflies (A) and parasitoids (B) choosing first the unexploited patch or the patch exploited by either bumblebees (left) or hoverflies (right). N is the sample size. Significant differences between exploited and unexploited patches are represented above the plots: NS: not significant; *: p-value < 0.05; **: p-value < 0.01; ***: p-value < 0.001.

3.3. Foraging and feeding behaviours of secondary foragers

Foraging duration was similar in hoverflies visiting unexploited patches and patches previously exploited by other hoverflies (χ^2=2.27; df=1; p=0.132). However, hoverflies spent more time feeding on unexploited patches than on those previously exploited by their conspecifics (χ^2=16.8; df=1; p<0.001) (Fig. 6A, 6C). Additionally, hoverflies spent more time foraging and feeding on unexploited patches compared with patches previously exploited by bumblebees (foraging duration: χ^2=16.6; df=1; p<0.001; feeding duration: χ^2=4.10; df=1; p=0.043) (Fig. 6B, 6D).

For both competitor species, the foraging duration of parasitoids was not significantly different between exploited and unexploited patches (hoverfly: χ^2=3.52 df=1; p=0.060; bumblebee: χ^2=3.55; df=1; p=0.059) (Fig. 6E, 6F).

Hoverflies moved more from one flower to another within unexploited inflorescences than within inflorescences exploited by conspecifics (χ^2=7.77; df=1; p=0.005) or by bumblebees (χ^2=9.00; df=1; p=0.003). The number of flowers visited by parasitoids was not significantly different on unexploited and exploited patches (exploited by hoverflies: χ^2=0.25; df=1; p=0.618; exploited by bumblebees: χ^2=0.79; df=1; p=0.373).
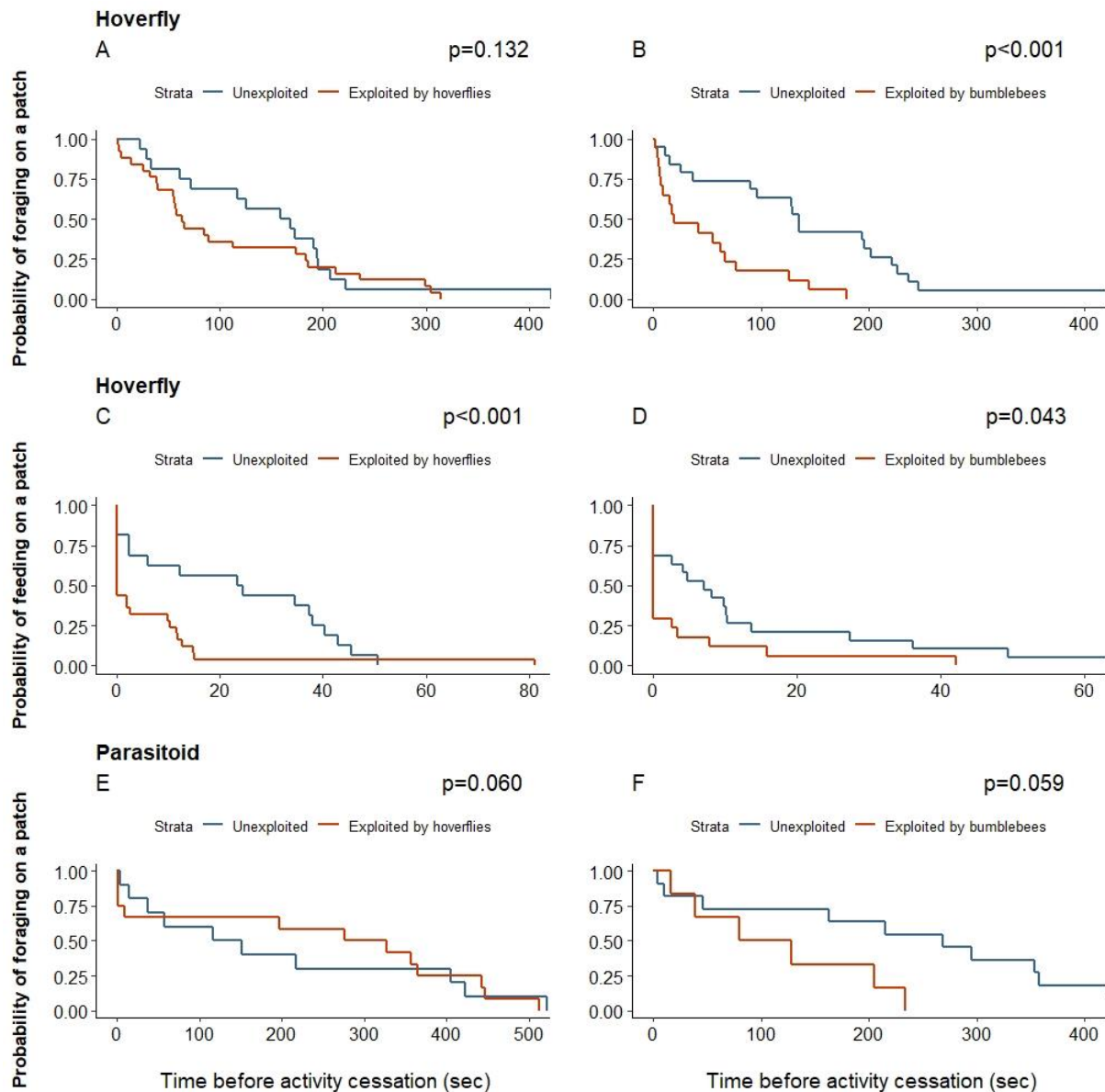
Figure 6. Probability of a hoverfly to still be foraging (A, B) or feeding (C, D) and of a parasitoid to still be foraging (E, F) after a given amount of time (in seconds) on unexploited patches (blue lines) or patches exploited by either hoverflies (A, C, E) or bumblebees (B, D, F) (red lines). P-values from Cox proportional hazard models are given.

4. Discussion

This study aimed to determine the effect of exploitative competition on the foraging behaviours of two common natural enemy species, the hoverfly *Episyrphus balteatus* and the aphid parasitoid *Aphidius colemani*, both flower visitors. As we considered two species with different physiological needs and ecology, yet both relying on the same floral resources for

their reproduction and survival, we were expecting that exploitative competition would trigger behavioural responses in favour of the most rewarding foraging strategy. The assumption that exploited flowers would limit food availability, and thus nutrient ingestion, was verified for sugars for both species. In particular, glucose and fructose levels were drastically reduced in insects fed on flowers previously exploited by conspecifics or heterospecifics. Nevertheless, and surprisingly, we did not find evidence of an innate avoidance of previously exploited inflorescences for either species. Our results even showed that hoverflies were more attracted to inflorescences previously exploited by conspecifics for their first landing, although they spent more time feeding on unexploited patches. Parasitoids did not seem to show any innate preference, nor adjustment in their foraging and feeding behaviours, according to patch quality. Differences in energy requirements of the two species may explain these different responses, as discussed below.

To assess if already exploited flowers limit the uptake of nutrients by secondary foragers, we performed nutrimetabolomics on hoverflies and parasitoids fed on previously exploited or unexploited flowers. The distinct metabolic fingerprints revealed that sugar concentration of the study insects was affected by the quality of flowers offered, whatever the species of the previous competitor (bumblebee or hoverfly). The exploitation of the flowers by a previous competitor is very likely to have reduced sugar amounts as nectar cannot be replenished by the plants within minutes after the insect's visit (Fig. SI.C). In this case, secondary visitors were certainly offered lower quality floral resources, with a lower possibility of sugar ingestion. Nevertheless, exploited flowers were not fully depleted of nectar and sugars, as individuals fed on exploited flowers consistently exhibited significantly higher sugar concentrations than water control individuals. This can be explained by the high sugar concentrations of buckwheat nectar (Cawoy *et al.*, 2008, Fig. SI.B). Buckwheat nectar is considered to be hexose-rich, according to the Percival's classification (1961), thus mainly encompassing fructose and glucose, at the detriment of sucrose. Accordingly, the decrease of sugar contents in hoverflies and parasitoids fed on exploited flowers resulted from an impoverishment of glucose and fructose compared to insects fed on unexploited flowers. On the other hand, trehalose, which is often found in high amounts in insects' haemolymph, is not found in buckwheat nectar (Wäckers *et al.*, 2006), yet this compound can be directly synthesized from glucose molecules in many insect species (Thompson, 2003). Thus, the higher trehalose concentrations in

parasitoids fed with unexploited flowers suggest that *A. colemani* can also synthesize this molecule. Trehalose concentrations were similar in hoverflies in all treatments, suggesting that *E. balteatus* individuals did not synthesized additional trehalose directly after feeding on floral resources in our experiment.

As buckwheat nectar and pollen are also sources of amino and organic acids for foragers (Pinheiro *et al.*, 2015), we expected hoverflies and parasitoids to be more concentrated in these two families of metabolites when fed on unexploited flowers. In particular, during the choice experiments, hoverflies were often observed feeding on pollen (but this was not the case for parasitoids). However, the opposite pattern was reported as individuals fed on unexploited flowers had identical or lower concentrations of organic acids and amino acids than the ones reared on water and exploited flowers. Such result could be explained by a direct metabolite use of those compounds for female egg maturation (Branquart and Hemptinne, 2000a).

Sugar content of the hoverflies and parasitoids was not influenced by either species of previous competitor. Nevertheless, the nutrimetabolomics approach and the subsequent discriminant analysis revealed a high differentiation of metabolic fingerprints between parasitoids fed on flowers exploited by bumblebees and flowers exploited by hoverflies. This suggests that the effect of competition on parasitoid physiology may vary depending on the previous competitor species.

Exploitative competition due to sugar resource limitation could lead to fitness costs for insects. Pinheiro *et al.* (2015) found that a higher glucose concentration of *E. balteatus* individuals increases their longevity, and the same result was reported for *Aphidius ervi*, a parasitoid species closely related to *A. colemani* (Azzouz *et al.*, 2004). Buckwheat nectar has also been shown to increase hoverfly and parasitoid females' longevity and fecundity (Jado *et al.*, 2019; Laubertie *et al.*, 2012; Vattala *et al.*, 2006). Therefore, in an environment where floral resource availability is limited, exploitative competition among flower visitors could potentially impact the fitness of *E. balteatus* and *A. colemani*. For instance, exploitative competition is likely to happen in summer, as already evidenced in agroecosystems for other flower visitors (Couvillon *et al.*, 2014). This is particularly true in agroecosystems with intensive beekeeping activities, as the honeybee *Apis mellifera* has been demonstrated to be a high

floral resources competitor (Henry and Rodet, 2018). Under these conditions, we could expect hoverfly and parasitoid foragers to show a preference for flowers which are more rewarding in terms of glucose content, *i.e.* that were not previously exploited, as shown for other flower visiting insects (Reader *et al.*, 2005; Stout and Goulson, 2001; Yokoi and Fujisaki, 2009). However, we did not observe such pattern. Parasitoid females did not show any preference between exploited or unexploited flowers, or did not have the capacity to discriminate between these two types of flowers. Conversely, hoverfly females showed a preference for flowers previously exploited by their conspecifics, in terms of first visited patch and also of numbers of visits per patch. The fitness cost incurred by hoverflies feeding on previously exploited resources might not be as strong as expected. Indeed, Charles & Paines (2016) found that the longevity and fecundity of *A. colemani* were not different when individuals were fed with honeydew or nectar, even if nectar was more concentrated in fructose and glucose. Therefore, the exploitation of flowers may lower the amount of nectar sugars available for *A. colemani* parasitoids, but not to a sufficient extent to incur fitness costs in the short term (as glucose and fructose contents were still higher than in the water treatment). Also, *A. colemani* are small insects (usually less than 0.1 mg dry mass), and their energetic needs, in particular for sugars, are lower than those of larger insects, as supported by the low amount of feeding behaviours in the trials. As sugar content in hoverflies showed exactly the same pattern, the same hypothesis could be proposed for this species. Nevertheless, it is less probable that hoverflies reached their energetic needs with exploited inflorescences, as it is known that hoverflies visit many flowers in sequence when foraging in nature (Gilbert, 1985). This observation is strengthened by our behavioural assays. Indeed, hoverflies spent more time foraging (in the trials where bumblebees were the previous competitor) and feeding (in both the trials where hoverflies and bumblebees were the previous competitors) on unexploited patches, showing that there were more resources available on those patches and that the amount of resources on exploited patches was not sufficient. Therefore, hoverflies might be more attracted to patches exploited by conspecifics at distance, but once on the patch, we suggest that they possess the ability to discriminate those flowers that are less rewarding.

Floral resources may not be sufficiently limiting in nature to impose selective pressure on hoverflies and parasitoids strong enough for the species to have adopted innate strategies to increase resource foraging efficiency. Among insects, the use of social information can either

be genetically determined or acquired by learning processes (Dukas, 2008). Leadbeater and Chittka (2011) suggested that innate rejection of previously visited flowers could be weakly selected, as the abundance and frequency of scent marks encountered *in natura* can allow quick and context-specific learning to occur. Accordingly, inexperienced bumblebees do not show any preference, nor rejection, of flowers previously visited by conspecifics, but such behaviour has been shown to develop over time by learning (Leadbeater and Chittka, 2011). Moreover, learning also depends on the competitive context: for instance, bumblebees learn to follow conspecific cues when competitive pressure is low, while they learn how to avoid conspecifics when competition pressure is high (Baude *et al.*, 2011; Leadbeater and Chittka, 2009). Our study species are non-social, contrary to bumblebees, which makes them difficult to compare. Nevertheless, concerning foraging for host/oviposition sites, some parasitoids and hoverflies can learn to detect and avoid competitors (Almohamad *et al.*, 2008; Le Lann *et al.*, 2008, 2011) by relying on olfactory cues for instance (Benelli et al 2004). Future work will have to be conducted to determine if the foraging strategies adopted by flower-visitors facing competition for floral resources are modified with experience resulting from learning processes.

Hoverfly preference for patches exploited by conspecifics was consistent between the first patch chosen for landing and the number of visits to the patch. Such attraction by inexperienced individuals suggests the use of cues, possibly scent marks, deposited (actively or not) by conspecifics. No such attraction was found for bumblebee-visited patches, which suggests that specialist cues are involved. Nevertheless, the experimental design does not allow us to identify which cues were used by female hoverflies for patch choice. We identify three different resource types that female hoverflies could be attracted to: males for mating, aphids for oviposition, and pollen/nectar foraging for feeding. Females used in this experiment were not sexually mature, as they were less than 24h old and unmated (Geusen-Pfister, 1987). In many Diptera species, immature females are not attracted to male pheromones (Katsoyannos, 1982; Lima *et al.*, 2001; Robacker *et al.*, 1990). Moreover, plants were protected by a net and checked before use to ensure they were not infested with aphids (Almohamad *et al.*, 2009). Therefore, it is very unlikely that one-day-old females were attracted by conspecific cues for mating or oviposition, but rather for feeding. It is known that *E. balteatus* individuals (and syrphids in general) preferentially use olfactory cues over visual cues to

localize floral resources (Majetic *et al.*, 2009; Primante and Dötterl, 2010), and that they are able to detect conspecific olfactory cues (eggs or larval) when foraging for oviposition sites (Almohamad *et al.*, 2009, 2010; Scholz and Poehling, 2000). Nevertheless, to our knowledge, this is the first evidence of conspecific use of information for floral resource foraging in *E. balteatus*. Such behaviour may be beneficial for inexperienced individuals to find rewarding patches of food (*i.e.* the presence of conspecifics on a patch may be a good indication that resources are present on the patch), and in that sense could be part of a learning process to forage more efficiently on flowers. It is interesting that this behaviour is innate in a non-social species, whereas social species such as bumblebees do not show this innate attraction to conspecifics (Leadbeater and Chittka, 2011).

Conclusion

By linking physiological and behavioural approaches, this study provides the first evidence that exploitative competition for floral resources can generate cascading effects on the metabolic intake and the foraging behaviour of two flower-visiting natural enemies. It demonstrates that even insects with very small nectar requirements, such as the parasitoid studied, can be physiologically affected by heavy exploitative competition for floral resources. Nevertheless, these flower-visiting insects do not innately discriminate the most rewarding resource, which could make natural enemies even more exposed to exploitative competition in nature. With high levels of competition for floral resources in agroecosystems linked to honeybee management and low flowering plant abundance and diversity, such experimental results suggest that we can expect flower-visiting natural enemies to suffer from exploitative competition in nature. Such understanding could help to improve the success of floral resource enhancement strategies to conserve the insects that provide essential ecosystem functions such as pollination and herbivore control.

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