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Honeydew composition and its effect on life-history parameters of hyperparasitoids

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Abstract. 1. Diets that maximise life span often differ from diets that maximise reproduction. Animals have therefore evolved advanced foraging strategies to acquire optimal nutrition and maximise their fitness. The free-living adult females of parasitoid wasps (Hymenoptera) need to balance their search for hosts to reproduce and for carbohydrate resources to feed.

2. Honeydew, excreted by phloem-feeding insects, presents a widely available carbohydrate source in nature that can benefit natural enemies of honeydew-producing insects. However, the effects of variation in honeydew on organisms in the fourth trophic level, such as hyperparasitoids, are not yet understood.

3. This study examined how five different honeydew types influence longevity and fecundity of four hyperparasitoid taxa. *Asaphes* spp. (Pteromalidae) and *Dendrocerus* spp. (Megaspilidae) are secondary parasitoids of aphid parasitoids and are thus associated with honeydew-producing insects. *Gelis agilis* and *Acrolyta nens* (both Ichneumonidae) are secondary parasitoids of species that do not use honeydew-producing hosts.

4. Most honeydew types had a positive or neutral effect on life span and fecundity of hyperparasitoids compared with controls without honeydew, although negative effects were also found for both aphid hyperparasitoids. Honeydew produced by aphids feeding on sweet pepper plants was most beneficial for all hyperparasitoid taxa, which can partially be explained by the high amount of honeydew, but also by the composition of dietary sugars in these honeydew types.

5. The findings of this study underline the value of aphid honeydew as a carbohydrate resource for fourth-trophic-level organisms, not only those associated with honeydew-producing insects but also 'interlopers' without such a natural association.

Key words. Carbohydrates, foraging behaviour, Hymenoptera, life-history trade-off, nutritional ecology.

Introduction

All animal species need food in order to survive and to reproduce. In animals, diets that maximise life span often differ considerably from diets that maximise reproduction. Studies on the impacts of human diet have traditionally focused on ageing and maximising life span (e.g. Solon-Biet *et al.*, 2015), often motivated by issues such as human health and disease. However, in nature, animals have evolved to maximise individual fitness, and reproduction is crucial. This requires advanced foraging

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strategies to acquire optimal nutrition, and foraging decisions must balance life span against opportunities for reproduction (Simpson & Raubenheimer, 2012; Piper *et al.*, 2017; Raubenheimer & Simpson, 2018). In this regard, insects have proved to be model organisms in studies of nutritional ecology (Raubenheimer *et al.*, 2009). For example, in fruit fly (*Drosophila melanogaster*) adult females, a low ratio of protein to carbohydrates maximises life span, while a high ratio favours egg laying (Lee *et al.*, 2008; Jensen *et al.*, 2015). Relative investment of these macronutrients in somatic maintenance and reproduction is one of the physiological mechanisms that underlies the classical trade-off between these two important life-history traits (Partridge & Farquhar, 1981; Tatar & Carey, 1995; Zera & Harshman, 2001; Solon-Biet *et al.*, 2015).

Among insects, parasitoid wasps (Hymenoptera) are important study organisms in optimal foraging research. Adult females lay their eggs in or on the body of a host (insect) and the offspring develop at the expense of the host (Godfray, 1994). All nutritional resources for development of parasitoid larvae are thus contained within a single individual host (Godfray, 1994), making host selection by the adult female parasitoid an important component of fitness (Harvey, 2005). In contrast with immature stages, adult parasitoids are free-living and need to forage for food to support somatic maintenance and reproduction (Jervis & Kidd, 1986; Jervis *et al.*, 2008). To maximise their fitness, parasitoid females therefore need to balance foraging for hosts and food. Adult parasitoids typically feed on food sources that are rich in sugars, such as floral or extrafloral nectar, or on honeydew excreted by phloem-feeding insects, including aphids, whiteflies, mealybugs, psyllids, and soft scales among others (e.g. Jervis *et al.*, 1993; Wackers *et al.*, 2008). Whereas sugar provides energy and can increase life expectancy of parasitoids, its effects on reproduction are less straightforward (Jervis *et al.*, 2008). Egg production, or ovigenesis, requires protein and lipids (Chapman, 1998), which are mainly acquired during larval development (Visser *et al.*, 2010). However, adult parasitoid females may supplement these nutritional elements by feeding on hosts, a behaviour that has evolved in several hymenopteran parasitoid families (Jervis & Kidd, 1986; Heimpel & Collier, 1996).

The relative importance of different sources of adult nutrition for parasitoids depends on multiple context- and trait-dependent factors. Although the sugar composition of honeydew may be less favourable for parasitoids than that of nectar, honeydew may be particularly important for parasitoids of honeydew-producing insects, certainly in environments where flowers are scarce (Wackers *et al.*, 2008; Lundgren, 2009; Vollhardt *et al.*, 2010; Tena *et al.*, 2016). When honeydew is present near their hosts, parasitoids have to invest less time in searching for sugar sources, and they can minimise energy and risks associated with searching for nectar, which may be located further away (Wackers *et al.*, 2008). Indeed, longevity of aphid parasitoids is generally supported by honeydew, but not all honeydew types are equal in this respect (Hogervorst *et al.*, 2007; Tena *et al.*, 2018), probably because some honeydew carbohydrates have a larger impact on longevity than do others (Lenaerts *et al.*, 2016). Indeed, many factors contribute to variation in the composition of dietary sugars in honeydew, including host plant species (e.g. Fischer & Shingleton, 2001; Pringle *et al.*, 2014) and aphid species (e.g. Woodring *et al.*, 2004; Hogervorst *et al.*, 2007). Aphid honeydew is also used as a food source by natural enemies of insects that do not produce it (Wackers *et al.*, 2008; Lundgren, 2009; Tena *et al.*, 2013), and it can indeed enhance the longevity and fecundity of non-aphid parasitoids (e.g. Faria *et al.*, 2008). However, it is not yet understood if and how variation in honeydew composition affects this group of parasitoids.

In this study, our objective was to determine the effect of different honeydew types (i.e. combinations of different aphid and plant species) on longevity and fecundity of four hyperparasitoid taxa. Hyperparasitoids lay their egg(s) in or on the developing stages of another parasitoid species and are therefore in the fourth trophic level or even higher (Godfray, 1994; Sullivan & Völkl, 1999). Most hyperparasitoids are in the Hymenoptera and their life histories and behaviour are in many ways similar to those of their hymenopteran hosts. *Gelis agilis* (Ichneumonidae) and *Acrolyta nens* (Ichneumonidae) are hyperparasitoids of *Cotesia glomerata* (Harvey & Witjes, 2005; Harvey *et al.*, 2009), which itself is a gregarious endoparasitoid of caterpillars of the large cabbage white butterfly, *Pieris brassicae*. *Dendrocerus* spp. (Megaspilidae) and *Asaphes* spp. (Pteromalidae) are aphid hyperparasitoids that use mummies of many combinations of aphid and primary parasitoid species (Buitenhuis *et al.*, 2017; de Boer *et al.*, 2019). We are thus using two hyperparasitoid taxa that coevolved with honeydew-producing insects (*Dendrocerus* spp. and *Asaphes* spp.) and two species that are associated with other insects and are 'interlopers' when feeding on honeydew (*G. agilis* and *A. nens*). Furthermore, the four hyperparasitoid taxa include two that feed on their host (*Asaphes* spp. and *G. agilis*), and two that do not (*Dendrocerus* spp. and *A. nens*) (Harvey & Witjes, 2005; Harvey *et al.*, 2009; Buitenhuis *et al.*, 2017).

We predicted that: (i) honeydew supports longevity and fecundity of the four hyperparasitoids because it is known to contain carbohydrates that benefit a wide range of arthropods; (ii) hyperparasitoids associated with aphids benefit more from honeydew compared with non-aphid hyperparasitoids because they are coevolved with aphids; (iii) non-host-feeding hyperparasitoids benefit more from honeydew than do host-feeding hyperparasitoids because they may be more efficient in processing nutrition from honeydew (conversely, host feeders can also obtain nutrition from hosts and may be less efficient in processing honeydew); and (iv) honeydew type affects longevity and fecundity of hyperparasitoids through the composition of dietary sugars.

Materials and methods

Honeydew and hyperparasitoids

Honeydew was obtained from five combinations of plant and aphid species. Sweet pepper (*Capsicum annuum* var. Maranello), radish (*Rhaphanus raphanistrum sativus* var. Cherry Belle) and winter wheat (*Triticum aestivum* var. Premio) were grown from seeds in a greenhouse (22 ± 2 °C during the day, 16 ± 1 °C at night, 50–70% RH, LD 16:8 h) for *c.* 2 months (pepper) or 3 weeks (wheat and radish). Tobacco plants *c.* 5 weeks old were provided by Koppert Biological

Systems (Berkel en Rodenrijs, The Netherlands). Plants were infested with aphids in large mesh cages holding aphid colonies. The tobacco aphid *Myzus persicae nicotianae* was used to infest pepper, radish and tobacco (we further refer to honeydew from these combinations as MP, MR and MT, respectively). The foxglove aphid *Aulacorthum solani* was used to infest pepper (AP), and the bird cherry oat aphid *Rhopalosiphum padi* was used to infest winter wheat (RW). MP and RW were kept in climate-controlled chambers (22 ± 1 °C, 60% RH, LD 16:8 h), while MR, MT, and AP were kept in the laboratory at room temperature and under ambient light conditions. To obtain honeydew for experiments, we visually inspected plants that had been placed in our aphid colonies for several days and selected a leaf with honeydew. The leaf was cut to a size of *c*. 16 cm^2 and aphids and exuviae were brushed off. Except for wheat, the main vain was excluded to minimise exposure of hyperparasitoids to fluids leaking from the leaves. Leaves were presented with the adaxial surface up, because honeydew mostly accumulated on this side. We used this method to provide honeydew to hyperparasitoids in a comparable way to how they encounter it in the field, i.e on the leaf on which it was deposited by the aphids. A disadvantage of this method is that quantity and quality of honeydew are less well controlled, and therefore may vary between replicates.

Laboratory colonies of aphid hyperparasitoids were obtained from a commercial sweet pepper greenhouse in 2015 and 2016. Although identified as *Dendrocerus aphidum* and *Asaphes vulgaris* (Graham, 1969; Fergusson, 1980), it became apparent that colonies consisted of several closely related sister species after completion of the experiments. *Dendrocerus laticeps* and *Dendrocerus carpenteri* were additionally present in the *Dendrocerus* colony, and *Asaphes suspensus* in the *Asaphes* colony, in unknown ratios (tentative identifications by Frank van Veen, University of Exeter) (de Boer *et al.*, 2019). We assume that this will not affect our conclusions because any differences between the two genera, and between the aphid hyperparasitoids and 'interlopers' are likely to be substantially larger than those within each genus of aphid hyperparasitoids. We further refer to the aphid hyperparasitoids as *Dendrocerus* spp. and *Asaphes* spp. They were reared on mummies of *M. persicae* parasitised by *Aphidius colemani* following methods of de Boer *et al.* (2019). *Gelis agilis* and *A. nens* were reared on cocoons of *C. glomerata* developed on larvae of *P. brassicae* on Brussels sprouts plants (for detailed methods see Harvey, 2008; Harvey *et al.*, 2009). All hyperparasitoids were used within 24 h of emergence and kept without food or water until experiments began.

Experimental procedures

Longevity. Newly emerged hyperparasitoid females were individually placed in Petri dishes with a circular mesh membrane in the lid (diameter 10 cm, height 4 cm; SPL Lifesciences Co. Ltd, Gyeonggi-do, Pocheong-si, Korea). A moist filter paper (Whatman, grade 1) and a small ball of wet cotton were placed on the bottom. To test the effects of honeydew on longevity, we presented a leaf with honeydew from one of the five combinations as described above (MP, MR, MT, AP or RW), and replaced it three times per week. A control without honeydew and plant material was also included (NO). Cotton balls were remoistened three times per week, and filter papers and cotton balls replaced once per week. Survival was monitored daily. Ten replicates were done per treatment, except for *Asaphes* spp. on MP honeydew ($N = 21$), *Dendrocerus* spp. on MP ($N = 20$), MT ($N = 23$) and NO ($N = 24$) (these treatments had a higher sample size because they partly overlapped with a recently published study from our group: de Boer *et al.*, 2019), and *A. nens* on NO ($N = 12$).

Fecundity. The fecundity assay was set up as the longevity assay with individual females in Petri dishes as described earlier. During the first 24 h, each *Dendrocerus* and *Asaphes* female was kept with three males and 20 aphid mummies (*M. persicae* parasitised by *A. colemani*). *Acrolyta nens* females were each kept with one male and 25 *C. glomerata* cocoons. Ten *C. glomerata* cocoons and no males were included with each *G. agilis* female because it reproduces asexually. Males were removed after 24 h. *Dendrocerus* spp. and *Asaphes* spp. were then provided with 30 fresh aphid mummies every Monday, Wednesday and Friday, and with 20 mummies on Sunday. *Acrolyta nens* and *G. agilis* received, respectively, 25 and 10 fresh *C. glomerata* cocoons every Monday, Wednesday, Friday, and Sunday. These numbers of hosts were based on expected rates of reproduction in these hyperparasitoid taxa (Harvey, 2008; Harvey *et al.*, 2009; de Boer *et al.*, 2019). Hosts were offered on moist filter paper and a moist cotton ball was added to every dish. Five combinations of honeydew and a control were used. Leaves with honeydew were replaced three times per week until death of the female, and cotton balls were remoistened at the same time. Filter papers and cotton balls were replaced once per week. When hosts were replaced, the 'used' set of hosts was transferred to a clean Petri dish sealed with Parafilm. Hosts were kept at 22 °C for a minimum of 4 weeks after which we counted the number of emerged primary parasitoids and hyperparasitoids. Female longevity was also recorded. Ten replicates were done per treatment, except for *A. nens* on MP ($N = 11$), and *Asaphes* spp. on MP, MR and NO ($N = 11$).

Carbohydrate composition. To assess the availability of carbohydrates in honeydew, honeydew was collected onto aluminium discs (diameter 3 cm). The discs were first washed with 70% ethanol and air-dried, and then randomly placed around the aphid-infested plants. Honeydew was deposited onto the discs naturally by aphids feeding on the plants, and amounts cm^{-2} were comparable to the amounts of honeydew collected on leaves for the life-history assays. After 48 h, we removed the discs and brushed off any aphids and exuviae. Control discs without honeydew were used as well. Ten replicates were collected per treatment. Discs with honeydew were weighed on a Mettler Toledo MT5 microbalance (Columbus, Ohio) (± 1 µg), and stored at −20 °C until use. Honeydew was removed by ripping each disc into small pieces and placing it in a 15-ml tube with 5 ml of 98% methanol, after which the tubes were spun overnight at 15 rpm. Pieces of aluminium foil were

then removed, air-dried, and weighed again to determine the mass of honeydew that was washed off. Methanol extracts were vacuum-dried (Rapidvap; Labconco, Kansas City, Missouri) for 16 h at 16 kPa and 31 °C. Samples were then resuspended in 1 ml milliQ water (MilliQ Gradient A10; Millipore, Molsheim, France), filtered (0.2 µm polytetrafluoroethylene filter; VWR, Radnor, Philadelphia), and kept at −20°C until analysis. Carbohydrate analysis was performed on a high-performance liquid chromatograph with an electrochemical detector [LC: Bio-Inert 1260 Infinity (Agilent, Santa Clara, California); ECD: Decade elite detector (Antec Scientific, Zoeterwoude, The Netherlands)], with a CarboPac guard column (Thermo Scientific, Breda, The Netherlands) (PA1,2, 2 × 50 mm) and main column (PA1, 2 × 50 mm), and a mobile phase of 100% 0.1 M NaOH. The flow rate was 0.25 ml min^{-1} and total run time was 35 min. Five microlitres of each sample were injected, and dilutions (10-fold and 100-fold) were made in milliQ water when concentrations were too high. Carbohydrates were identified by comparing the detected retention times with those of a reference mixture of carbohydrates run on the same column (sorbitol, mannitol, trehalose, glucose, fructose, melibiose, sucrose, melezitose, raffinose and maltose; all from Sigma-Aldrich, Saint Louis, Missouri). This mixture was run at four concentrations (2.5, 5, 7.5, and 10 ppm) to determine the limit of detection and the amount of carbohydrates measured in honeydew samples. The concentration of each carbohydrate (µg mg^{-1}) was normalised per mg of honeydew collected by multiplying the measured value (ppm) by the appropriate dilution factor and dividing by the mass of honeydew collected per sample.

Data analysis

Hyperparasitoid survival was checked daily from the day of eclosion. Separate generalised linear models (GLMs; Poisson distribution, log link function) were constructed per hyperparasitoid taxa to test the effect of honeydew type on longevity. When the main effect of honeydew type was significant, a Fisher's protected least significant difference test (LSD) was performed for pairwise comparisons of longevity on different honeydew types using the R package AGRICOLAE (de Mendiburu, 2017). Longevity in the presence of hosts was analysed similarly, with realised fecundity as a covariate. To further investigate how the proportional hazard for mortality changes over time for each honeydew type in the absence or presence of hosts, Cox proportional hazard regression models were performed per hyperparasitoid taxa, followed by multiple comparisons of the survival curves using R packages SURVMINER (Kassambara & Kosinski, 2018) and SURVIVAL (Therneau, 2018).

The costs of reproduction in terms of longevity were investigated by testing the effect of honeydew type, host presence and their interaction with GLMs as described earlier. Multiple comparisons (LSD) were done when the main effect of honeydew type was significant, focusing on direct comparisons of longevity in the presence or absence of hosts per honeydew type. We also tested whether fecundity was correlated with longevity by calculating Pearson's product moment correlation coefficient per honeydew type per hyperparasitoid taxa.

Total realised fecundity of each hyperparasitoid female was calculated by summing the offspring produced over her lifetime. The data included zeros because some hyperparasitoids did not produce any offspring, and we assumed linearity. We used linear models (LMs) per hyperparasitoid taxa to test the effect of honeydew type on fecundity, with longevity as a covariate. When the main effect of honeydew type was significant, Tukey honestly significant difference (HSD) *post hoc* tests were performed for pairwise comparisons (R package AGRICOLAE) between honeydew types, using the most parsimonious model.

Linear models were used to test the effect of honeydew type on the total amount of honeydew, on the total concentration of carbohydrates and on the concentration of each carbohydrate separately. When the main effect of honeydew type was significant, pairwise comparisons between honeydew types were made (Tukey HSD, R package AGRICOLAE). No carbohydrates were detected in controls, with a few exceptions (two and four carbohydrates in two samples), and these were excluded from the analyses. To test whether mean collected amount of honeydew and carbohydrate concentrations per honeydew type were correlated with longevity (with or without hosts) and fecundity, we calculated Pearson's product moment correlation coefficients. This was done using means of each life-history parameter per hyperparasitoid taxa per honeydew type, excluding controls without honeydew. All statistics were performed in R v.3.4.0. (R Development Core Team, 2014).

Data accessibility

We intend to archive all data belonging to this manuscript in Dryad.

Results

Longevity

In the absence of hosts, longevity of hyperparasitoids was significantly affected by honeydew type (GLM per taxa, all $P < 0.001$; Fig. 1; Tables S1 and S2). The life span of all hyperparasitoid taxa was significantly enhanced by providing them with honeydew of both aphid species feeding on pepper as compared with controls (NO) without honeydew (MP and AP; Fisher's LSD, all pairwise $P < 0.001$). In *Asaphes* spp., *Dendrocerus* spp. and *G. agilis*, longevity on *M. persicae* honeydew (MP) was significantly higher than longevity on *A. solani* honeydew (AP) (all pairwise $P < 0.001$). The opposite effect was found for *A. nens*, which had the highest longevity on AP compared with any other treatment (all pairwise $P < 0.001$; Fig. 1d). Tobacco honeydew (MT) also significantly enhanced longevity of *Dendrocerus* spp., *G. agilis* and *A. nens* hyperparasitoids ($P_{MT\text{-}NO} < 0.039$), albeit by only 1 or 2 days. Conversely, honeydew on radish (MR) and wheat (RW) did not enhance longevity of any hyperparasitoid taxa (all $P > 0.05$). These effects of honeydew on longevity were supported by Cox proportional hazard regression models for each hyperparasitoid taxa [Likelihood ratio test (LRT), all $P < 0.001$; Table S3].

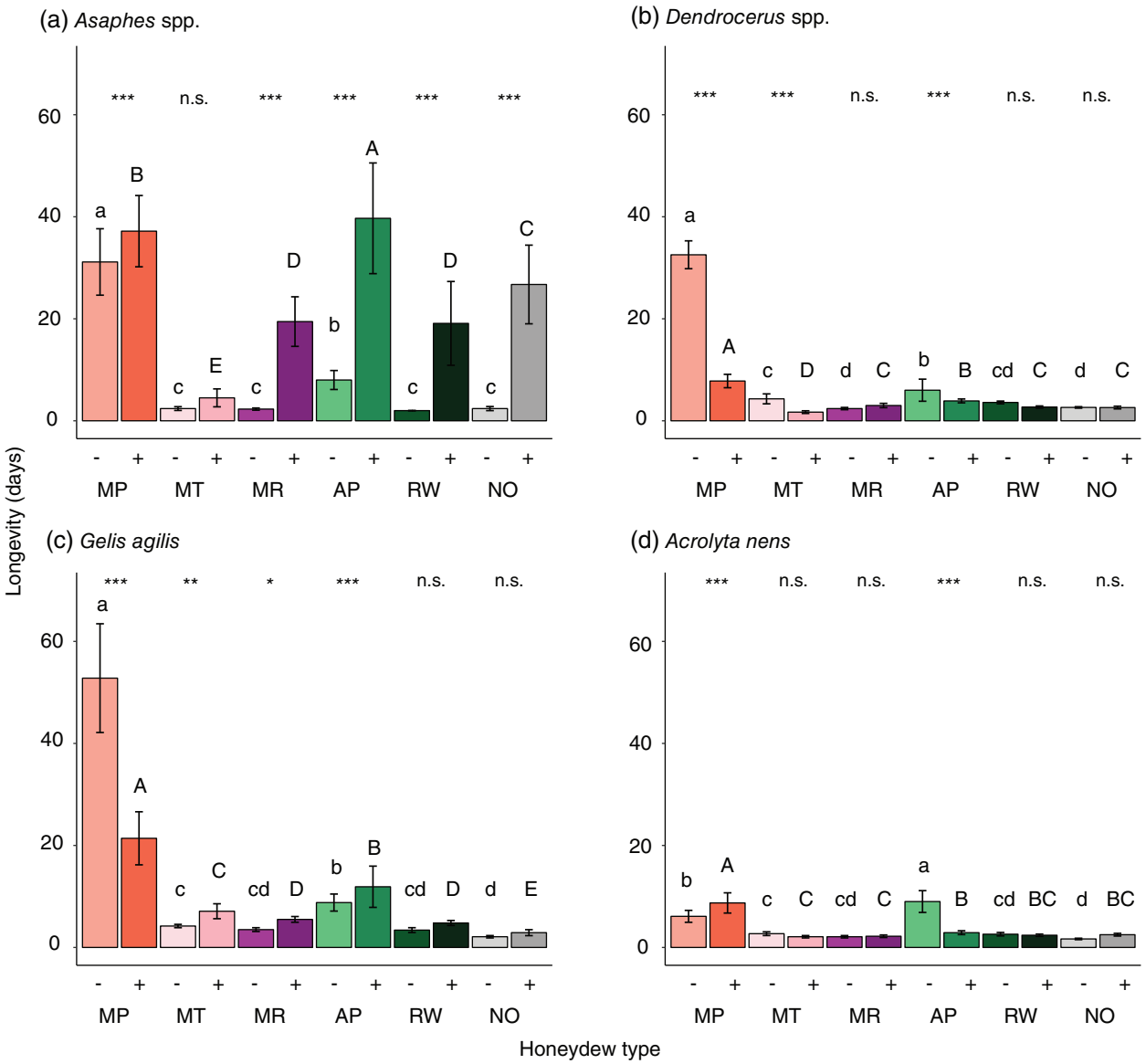

Fig. 1. Effect of honeydew types on longevity of hyperparasitoids: (a) *Asaphes* spp.; (b) *Dendrocerus* spp.; (c) *Gelis agilis*; (d) *Acrolyta nens*. Honeydew types included *Myzus persicae* on pepper (MP), tobacco (MT), and radish (MR), *Aulacorthum solani* on pepper (AP), and *Rhopalosiphum padi* on wheat (RW), and there was a control without honeydew (NO). Bars represent mean longevity with '−' and '+' below bars reflecting the absence and presence of hosts, respectively (error bars represent standard errors of the mean). Pairwise significant differences [Fisher's least significant difference (LSD), $P \leq 0.05$] are indicated with different lower-case letters above bars for longevity in the absence of hosts and with different capital letters for the longevity in the presence of hosts (Supporting Information Tables S2 and S4). The pairwise effect of host presence in terms of longevity are shown per honeydew type as follows: ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; or n.s., $P > 0.05$ (Fisher's LSD; see Table S6 for full matrix of P-values). All treatments were repeated 10 times ($N = 10$), except as follows: in the absence of hosts, *Asaphes* spp. on MP ($N = 21$), *Dendrocerus* spp. on MP ($N = 20$), MT ($N = 23$), and NO ($N = 24$) [these treatments partially overlapped with another study, published in de Boer *et al.* (2019), and therefore had a higher sample size], and *A. nens* on NO ($N = 12$); and in the presence of hosts, *Asaphes* spp. on MP, MR and NO ($N = 11$) and *A. nens* on MP ($N = 11$).

In the presence of hosts, honeydew type also significantly affected longevity of hyperparasitoids (GLM per taxa, all $P < 0.001$; Fig. 1; Tables S1 and S4). Life span was significantly enhanced by MP honeydew for *Dendrocerus* spp. and *A. nens* (LSD, $P_{\text{MP-NO}} < 0.001$ for both taxa), i.e. the two hyperparasitoids that do not host-feed. In *Dendrocerus* spp. but not in *A. nens*, AP honeydew also significantly enhanced longevity ($P_{\text{AP-NO}} < 0.001$ and $P_{\text{AP-NO}} = 0.151$, respectively). Instead, MT honeydew significantly decreased longevity in *Dendrocerus* spp. ($P_{\text{MT-NO}} = 0.004$), but not in *A. nens* ($P_{\text{MT-NO}} = 0.151$). Results were less straightforward for the host-feeding taxa. In *Asaphes* spp., longevity was high in the presence of hosts in most treatments, including the control. Remarkably, life span was significantly shorter on MT honeydew than on all other

treatments (all pairwise $P < 0.001$). Life span was also significantly shorter on RW and MR honeydew ($P_{\text{MR-NO}}$ and $P_{\text{RW-NO}} < 0.001$). Honeydew from both aphid species on pepper, on the other hand, resulted in significantly higher longevity of *Asaphes* spp. compared with all other treatments, with AP resulting in the highest longevity (all pairwise $P < 0.001$, except $P_{\text{MP-AP}} = 0.004$). In *G. agilis*, all honeydew types significantly enhanced longevity (all pairwise $P \leq 0.001$). The highest longevity of *G. agilis* was found on MP and AP honeydew, with longevity on MP significantly higher than longevity on AP ($P_{\text{MP-AP}} < 0.001$). For each hyperparasitoid taxa, significant effects of honeydew type were supported by Cox proportional hazard regression analyses (LRT, *Asaphes* spp., $P = 0.002$; other taxa, $P < 0.001$; Table S5).

Costs of reproduction in terms of longevity depended on honeydew type, as suggested by the significant interaction between honeydew type and the presence of hosts in each hyperparasitoid taxa (GLM per taxa, all $P_{\text{honeydew} \times \text{hosts}} < 0.001$; Fig. 1; Table S6). For all taxa, except *A. nens* (GLM, $P_{\text{hosts}} = 0.144$), life span was significantly affected by host presence (GLM, $P_{\text{hosts}} \leq 0.001$). Host presence reduced longevity in *Dendrocerus* spp. on MP, AP, and MT honeydew (LSD, all pairwise $P < 0.001$). Overall, host presence also reduced life span of *G. agilis*, but this effect was significant only on MP honeydew ($P < 0.001$), whereas host presence increased longevity on AP, MR, and MT honeydew ($P \leq 0.028$). By contrast, host presence increased longevity of *A. nens* on MP honeydew ($P < 0.001$) but decreased longevity on AP honeydew ($P < 0.001$). Finally, host presence significantly increased life span of *Asaphes* spp. on four honeydew types (MP, AP, MR, and RW) and on the control (all $P < 0.001$), but not on MT honeydew ($P = 0.203$).

We also examined the relationship between fecundity and longevity for each hyperparasitoid taxa per honeydew type. For *Asaphes* spp., there was a significant positive correlation on all honeydew types and the control (Pearson's correlation, all $P < 0.001$; Table S7), suggesting that, independent of additional sugar sources, the longer these hyperparasitoids live, the more offspring they produce. In *Dendrocerus* spp., *G. agilis* and *A. nens*, the relationship between fecundity and longevity clearly depended on honeydew type, with positive correlations found on MP honeydew for all three hyperparasitoid taxa ($P < 0.001$). Positive correlations were also found for *G. agilis* on MT and AP honeydew ($P \leq 0.001$), and non-significantly on RW honeydew ($P = 0.057$), and for *A. nens* on AP honeydew ($P = 0.001$).

Fecundity

Honeydew type significantly affected hyperparasitoid fecundity (ANOVA per taxa, all $P_{\text{honeydew}} < 0.001$; Fig. 2; Tables S1 and S8). The realised fecundity of hyperparasitoids was significantly higher on MP honeydew than on the control (Tukey's HSD, all pairwise $P \leq 0.002$). For *A. nens* and *Dendrocerus* spp., i.e. the non-host-feeding hyperparasitoids, this was the only honeydew type that significantly increased fecundity. In *Dendrocerus* spp., fecundity on AP honeydew was significantly similar to fecundity on MP honeydew but also to the control ($P_{\text{AP-MP}} = 0.810$, $P_{\text{AP-NO}} = 0.070$). In *G. agilis*, fecundity was significantly increased by MP and AP honeydew ($P_{\text{AP-NO}} = 0.020$). Finally, in *Asaphes* spp., fecundity was also significantly increased by MP and AP honeydew ($P < 0.001$), whereas MT and MR honeydew significantly reduced fecundity compared with the control ($P_{\text{MT-NO}}$ and $P_{\text{MR-NO}} \leq 0.001$).

Carbohydrate composition

Honeydew type significantly affected the amount of honeydew collected (LM, $P < 0.001$; Table 1), with the highest amount produced by *M. persicae* feeding on pepper, and the lowest by the same aphids on tobacco. Honeydew of the different plant–aphid combinations also differed significantly in carbohydrate concentrations and ratios (LM, all $P \leq 0.01$; Table 1; Fig. 3). Melibiose and raffinose were not detected in any honeydew type. The other eight carbohydrates were present in most samples of all honeydew types. The concentrations of mannitol and maltose were significantly higher in MP honeydew than in the other honeydew types. Sucrose and trehalose were present in significantly higher concentrations in MP honeydew than in MR, AP and RW honeydew. Fructose was also present in significantly higher concentration in MP honeydew than in MR and AP honeydew, while concentrations in MT and RW honeydew were intermediate. MT honeydew had a significantly higher sorbitol concentration compared with other honeydew types. RW honeydew had the highest concentrations of glucose and melezitose compared with the other honeydew types, although glucose was significantly higher only when compared with AP and MT honeydew, and melezitose only when compared with MT honeydew.

Correlations between life-history traits and honeydew quantity and quality

To investigate whether honeydew quantity and dietary composition can explain differential effects on hyperparasitoids, we examined correlations between life-history parameters and these honeydew factors. Longevity in the absence of hosts was significantly positively correlated with the total amount of honeydew collected (Pearson's correlation, $P \leq 0.006$, $R^2 \geq 0.971$; Fig. S1), and the concentration of mannitol ($P \leq 0.003$, $R^2 \geq 0.982$) for all taxa, except *A. nens*. For *Dendrocerus* spp., longevity was also significantly correlated with maltose ($P = 0.041$, $R^2 = 0.894$). No significant correlations were found for *A. nens*. In the presence of hosts, longevity of *G. agilis* and *A. nens* was significantly correlated with the amount of honeydew ($P \leq 0.017$, $R^2 \geq 0.941$; Fig. S2) and the concentration of mannitol ($P \leq 0.025$, $R^2 \geq 0.923$), while for *A. nens* there was also a significant correlation with maltose ($P = 0.050$, $R^2 = 0.878$). For *Dendrocerus* spp., longevity in the presence of hosts was significantly correlated with the amount of honeydew ($P < 0.001$, $R^2 = 0.992$) and mannitol ($P = 0.004$, $R^2 = 0.977$). No significant correlations were found for longevity of *Asaphes* spp. in the presence of hosts. Fecundity was significantly correlated with the amount of honeydew ($P \leq 0.006$, $R^2 \geq 0.971$; Fig. S3) and the concentration of mannitol ($P \leq 0.007$, $R^2 \geq 0.966$) for *G. agilis* and *A. nens*. No significant correlations were found for fecundity of *Asaphes* spp. and *Dendrocerus* spp.

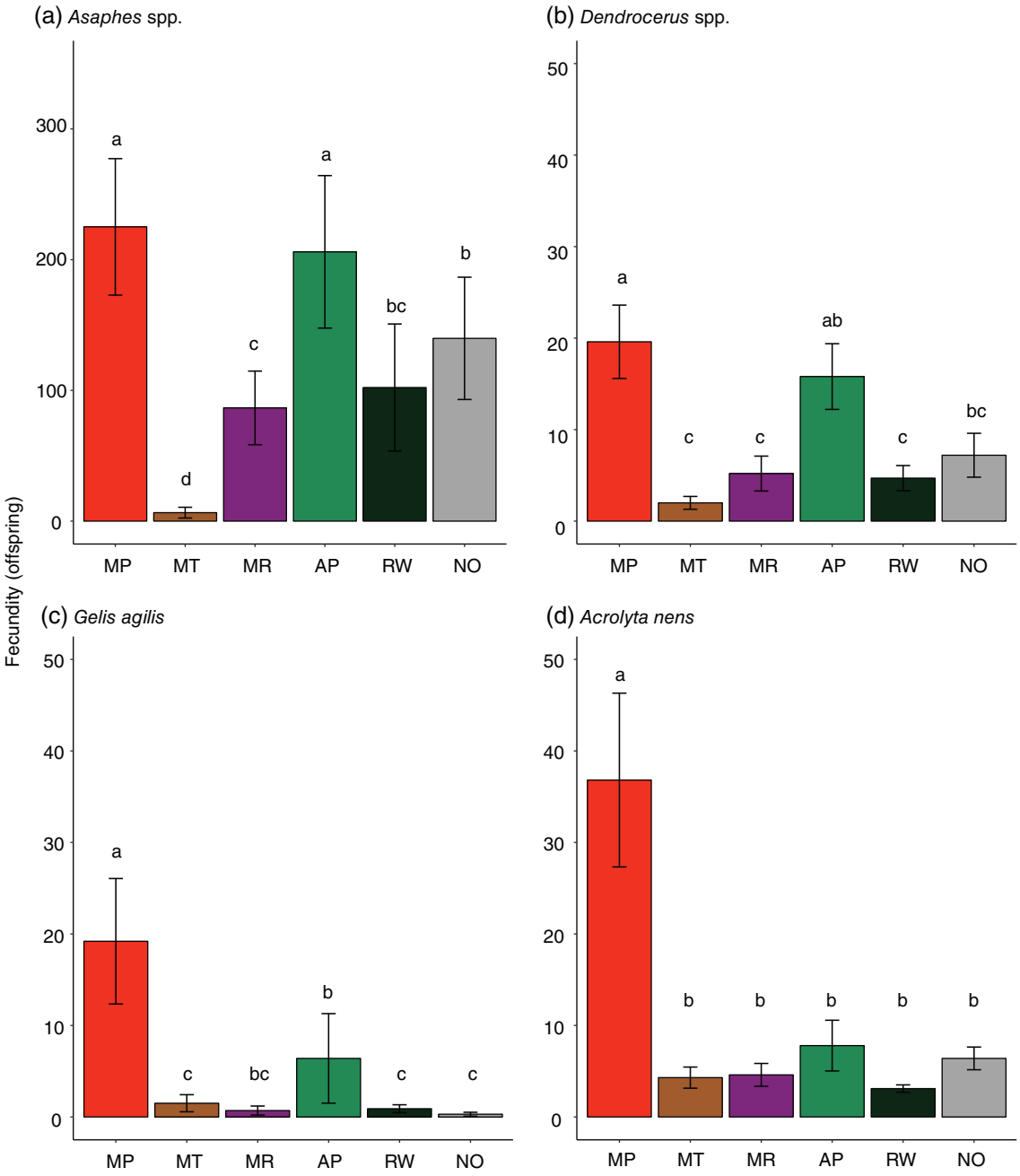

Fig. 2. Effect of honeydew type on hyperparasitoid fecundity: (a) *Asaphes* spp.; (b) *Dendrocerus* spp.; (c) *Gelis agilis*; (d) *Acrolyta nens*. Honeydew types included *Myzus persicae* on pepper (MP), tobacco (MT), and radish (MR), *Aulacorthum solani* on pepper (AP), and *Rhopalosiphum padi* on wheat (RW), and there was a control without honeydew (NO). Bars show mean realised fecundity with error bars representing standard errors of the mean. Lower-case letters indicate pairwise significant differences between honeydew types (Tukey's honestly significant difference, $P \leq 0.05$). All treatments were repeated 10 times ($N = 10$), except for *A. nens* on MP ($N = 11$), and *Asaphes* spp. on MP, MR and NO ($N = 11$).

Discussion

Honeydew is known to be an important food source for insect natural enemies in nature (Wackers *et al.*, 2008), including predators and parasitoids of honeydew-producing insects as well as those not intimately associated with such insects (Lundgren, 2009). However, it is not well understood how honeydew impacts the latter group and hyperparasitoids in the fourth trophic level. Here, we used honeydew of five different combinations of plant and aphid species and showed that honeydew influences longevity and fecundity of four hyperparasitoid taxa. The extent, and even direction, of this effect depended on the specific life-history parameter that we measured, on hyperparasitoid taxa, and on honeydew type. Honeydew quantity

Table 1. Effect of honeydew type on the amount of honeydew collected, the concentrations of total and individual carbohydrates ($N = 10$, except for MR, where $N = 9$).

		Honeydew type				
	F, P-value*	MP†	MT	MR	AP	RW
Amount collected (mg)	$F_{4,45} = 21.57$, $P < 0.001$	4.59 ± 0.80^{a}	0.02 ± 0.01^{b}	0.90 ± 0.24^{b}	1.17 ± 0.32^{b}	0.42 ± 0.21^{b}
Total CH (mg mg^{-1})	$F_{4,40} = 8.45$, $P < 0.001$	0.23 ± 0.01^{a}	$0.13 \pm 0.04^{ab,‡}$	0.07 ± 0.02^{b}	0.05 ± 0.02^{b}	0.14 ± 0.04^{ab}
Sorbitol (μg mg^{-1})§	$F_{4,40} = 43.23$, $P < 0.001$	0.25 ± 0.05^{a}	5.87 ± 0.83^{b}	0.17 ± 0.04^{a}	0.90 ± 0.39^{a}	0.45 ± 0.12^{a}
Mannitol (μg mg^{-1})	$F_{4,40} = 23.89$, $P < 0.001$	11.01 ± 1.65^{a}	0.15 ± 0.10^{b}	1.03 ± 0.20^{b}	2.29 ± 0.45^{b}	2.14 ± 0.62^{b}
Trehalose (μg mg^{-1})	$F_{4,40} = 5.57$, $P = 0.001$	12.30 ± 2.55^{a}	7.37 ± 2.21^{ab}	2.45 ± 0.85^{b}	2.23 ± 0.79^{b}	4.30 ± 2.09^{b}
Glucose (μg mg^{-1})	$F_{4,40} = 4.08$, $P = 0.007$	23.37 ± 4.17^{ab}	6.61 ± 2.80^{a}	18.93 ± 5.14^{ab}	9.34 ± 3.54^{a}	48.68 ± 14.99^{b}
Fructose (μg mg^{-1})	$F_{4,40} = 6.44$, $P < 0.001$	78.50 ± 3.30^{a}	50.42 ± 13.31^{abc}	26.97 ± 7.65^{bc}	20.65 ± 7.11^{b}	58.79 ± 14.52^{ac}
Sucrose (μg mg^{-1})	$F_{4,40} = 15.38$, $P < 0.001$	86.33 ± 6.98^{a}	55.67 ± 17.92^{ab}	17.11 ± 7.38^{b}	17.29 ± 6.95^{b}	18.66 ± 4.59^{b}
Melezitose (μg mg^{-1})	$F_{4,40} = 3.82$, $P = 0.010$	0.14 ± 0.06^{ab}	0.00 ± 0.00^{ab}	0.08 ± 0.04^{ab}	0.02 ± 0.02^{b}	0.66 ± 0.28^{a}
Maltose (μg mg^{-1})	$F_{4,40} = 9.26$, $P < 0.001$	20.17 ± 4.24^{a}	7.02 ± 3.82^{b}	0.40 ± 0.29^{b}	2.04 ± 0.38^{b}	8.77 ± 2.31^{b}

∗The effect of honeydew type on each parameter was analysed with linear models (ANOVA, F-distribution), followed by pairwise comparisons (Tukey's honestly significant difference). Different lower-case letters per row indicate significant pairwise differences ($P < 0.05$).
†Honeydew types included *Myzus persicae* aphids feeding on pepper (MP), tobacco (MT) or radish (MR), *Aulacorthum solani* aphids on pepper (AP), and *Rhopalosiphum padi* aphids on wheat (RW).
‡For MT honeydew, mean concentrations of total and individual carbohydrates were based on six replicates because no measurable amount of honeydew was collected in the other four samples.
§Per carbohydrate type, concentrations were normalised per mg of honeydew collected.

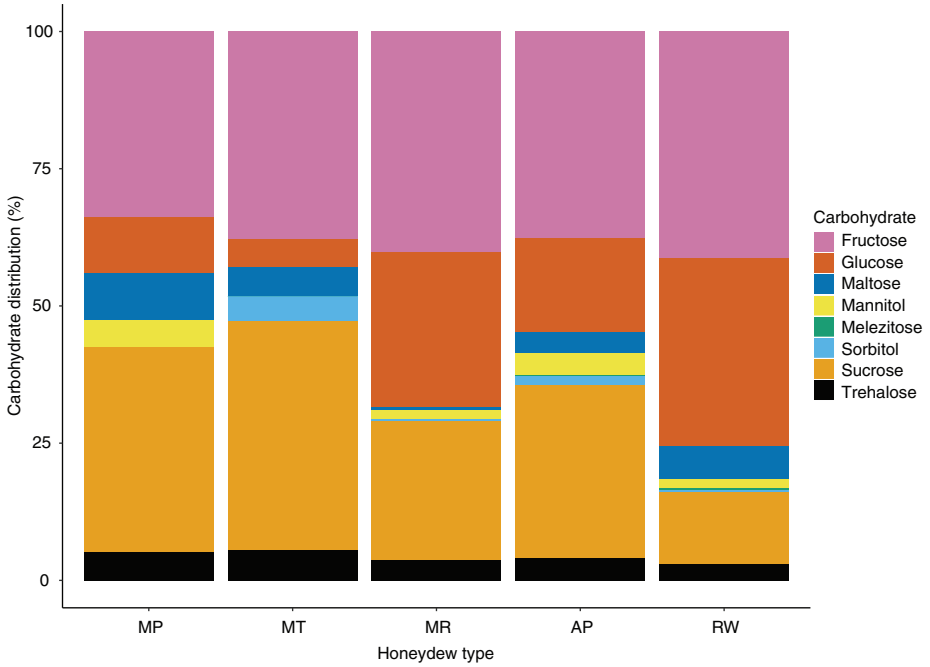

Fig. 3. Overview of the proportional distribution of carbohydrate concentrations per honeydew type. Percentages of each carbohydrate were calculated by dividing the mean carbohydrate concentrations (μg mg^{-1}; Table 1) by the total amount of carbohydrates collected for each honeydew type. Honeydew types included *Myzus persicae* on pepper (MP), tobacco (MT), and radish (MR), *Aulacorthum solani* on pepper (AP), and *Rhopalosiphum padi* on wheat (RW).

and carbohydrate composition both contributed to differential effects of honeydew on hyperparasitoids.

Our data clearly demonstrate that feeding on honeydew can extend the life span and increase fecundity of hyperparasitoids, supporting our first prediction. We next hypothesised that the relative importance of honeydew depends on hyperparasitoid taxa and their life-history traits and is primarily related to coevolution with honeydew-producing insects and host-feeding behaviour. However, the different types of honeydew had surprisingly similar effects on longevity of the four hyperparasitoid taxa. Life span of all hyperparasitoids was extended significantly by honeydew from sweet pepper plants infested with two different aphid species (*M. persicae* and *A. solani*) and by honeydew from tobacco in three hyperparasitoid taxa

(Fig. 1). Honeydew of *M. persicae* feeding on sweet pepper was particularly beneficial, with life spans of *Asaphes* spp., *Dendrocerus* spp. and *G. agilis* in the range of other studies where hyperparasitoids were provided with unlimited honey (Harvey, 2008; Buitenhuis *et al.*, 2017; Harvey *et al.*, 2017; de Boer *et al.*, 2019). *Acrolyta nens* was the exception, however, as this species lives much longer when provided with honey than on any of the honeydews provided here (Harvey *et al.*, 2009). Our findings with three of four hyperparasitoids studied are remarkable because honeydew was generally thought to be inferior compared with non-honeydew carbohydrate sources (Wackers *et al.*, 2008; Tena *et al.*, 2016). Nevertheless, direct comparisons, using standardised amounts and more controlled quality (e.g. in terms of age) of honeydew and a standardised positive control (one or more pure carbohydrates), are necessary to support this conclusion.

Interestingly, adverse effects of honeydew were observed for both hyperparasitoid taxa but not for the 'interlopers'. The life spans of *Asaphes* spp. and *Dendrocerus* spp. were reduced on tobacco honeydew, and that of *Asaphes* spp. was also reduced by radish and wheat honeydew. Compared with the control, fecundity of *Asaphes* spp. was reduced 20-fold by tobacco honeydew and 1.6-fold by radish honeydew, whereas they were increased *c*. 1.5-fold on both types of pepper honeydew. Tobacco and radish plants contain secondary metabolites that, when ingested by aphids, can be excreted in honeydew. Indeed, glucosinolates are present in honeydew of *M. persicae* feeding on brassicaceous plants (Francis *et al.*, 2001), and cardenolides can be present in honeydew of *Aphis nerii* feeding on milkweed (Pringle *et al.*, 2014; Zuest & Agrawal, 2016). Alternatively, tobacco and radish leaves, on which honeydew was presented, may have directly affected the aphid hyperparasitoids because secondary plant metabolites may be present on the leaf surfaces (Roda *et al.*, 2003). We do not know why aphid hyperparasitoids would be more sensitive to such compounds than the 'interlopers'. In fact, it has been shown that dietary nicotine has a negative effect on adult body weight of the hyperparasitoid *Lysibia nana* developed in *C. congregata* cocoons emerged from tobacco hornworm larvae (Harvey *et al.*, 2007). Nevertheless, these findings suggest that selection of carbohydrate food sources can be an important component of fitness, particularly for aphid hyperparasitoids, and hence influence foraging strategies.

There were no clear differences in the relative importance of honeydew between the host-feeding taxa *Asaphes* spp. and *G. agilis*, and the non-host-feeding taxa *Dendrocerus* spp. and *A. nens*. As expected, both host-feeding species benefited from access to hosts in terms of longevity. *Asaphes* spp. lived (much) longer in the presence of hosts, even without access to honeydew, while the 'interloper' *G. agilis* also lived longer with hosts on three types of honeydew but not when fed on honeydew of *M. persicae* on pepper on which it had the highest fecundity. In agreement with previous research, the life span of *G. agilis* was not extended by the presence of hosts in the absence of carbohydrates (Harvey, 2008). The non-host-feeding hyperparasitoids *Dendrocerus* spp. and *A. nens* lived for a shorter time in the presence than in the absence of hosts, with the exception of *A. nens* when fed honeydew of *M. persicae* on pepper, confirming that reproduction is costly in terms of longevity for these two taxa (Harvey *et al.*, 2009; de Boer *et al.*, 2019).

Although diets that maximise life span often differ from diets that maximise fecundity (Lee *et al.*, 2008; Jensen *et al.*, 2015), this does not seem to be the case for the honeydew types that we tested, as we found that honeydew from both aphid species on pepper generally best supported both life-history parameters (Figs 1 and 2), a result that was also obtained with the parasitoid *Aphytis melinus* (Tena *et al.*, 2013). We predicted that differential effects between honeydew types could be explained by the composition of dietary sugars. Our data partially support this hypothesis but the amounts of honeydew also varied between treatments. *Myzus persicae* aphids fed on sweet pepper plants clearly produced the highest amount of honeydew, roughly four times more than *A. solani* on sweet pepper, which yielded the next highest amount. Life-history parameters were generally positively correlated with the amount of honeydew for all hyperparasitoids, with a few exceptions, suggesting that honeydew quantity is the main driver of its effect on hyperparasitoids, although experiments with a standardised range of amounts are necessary to support this conclusion. However, in contrast to honeydew of *A. solani* on pepper, honeydew collected from radish, wheat and tobacco did not substantially benefit hyperparasitoids, even though statistically similar amounts of honeydew were produced. This suggests that honeydew quality also contributes to its effect on hyperparasitoid life history. Indeed, we found concentrations of mannitol and maltose, and to a lesser extent trehalose and sucrose, to be positively correlated with life-history parameters. Sucrose and its components glucose and fructose are common carbohydrates in honeydew and nectar and are well known for their nutritional value to hymenopteran insects (e.g. Lundgren, 2009; Tompkins *et al.*, 2010; Goelen *et al.*, 2018). Not much is known about the nutritional roles of the other carbohydrates on fitness of hymenopteran insects, although insect-synthesised carbohydrates, such as trehalose, may be relatively unsuitable for parasitoid survival (Wackers, 2001). Goelen *et al.* (2018) recently reported that sucrose enhances longevity of the aphid hyperparasitoid *D. aphidum* to a significantly greater extent than does trehalose, although maltose and mannitol were not included in their study. Another interesting finding is that the concentration of sorbitol was negatively correlated with longevity and fecundity, albeit non-significantly. Adverse effects of high concentrations of this carbohydrate were recently also reported in an anthocorid bug that uses psyllid honeydew as an alternative food source to herbivorous prey (Ge *et al.*, 2019). Controlled experiments with pure carbohydrates or artificial honeydews of known composition are needed to further investigate the relationship between honeydew quality and hyperparasitoid life history. It is also possible that honeydew characteristics that we did not measure here, such as amino acid concentrations, viscosity (Faria *et al.*, 2008) or plant secondary metabolites (see earlier), contributed to the suitability of the different honeydew types as a food source for hyperparasitoids.

In nature, sources of carbohydrates important for maintenance (e.g. floral nectar) may be located a considerable distance from suitable hosts (e.g. Tenhumberg *et al.*, 2006; Vollhardt *et al.*, 2010; Jamont *et al.*, 2014). For aphid primary parasitoids and hyperparasitoids, the benefits of honeydew feeding are obvious,

as it reduces the need to leave host patches to search for floral nectar. Although sugars obtained from nectar may be of higher quality than honeydew, this may be offset by the metabolic costs incurred by moving between host patches and flowers. For 'interlopers' like *A. nens* and *G. agilis*, the benefits of honeydew over floral nectar are less clear, because neither species is intimately associated with aphids or their parasitoids and it is not currently known whether they feed on honeydew in nature. Winged species like *A. nens* are strong fliers and can probably cover a significant area when searching for food and hosts. They are therefore expected to feed on the most profitable carbohydrate resource. However, the relative benefits of carbohydrates from honeydew or nectar are not yet known for this species. By contrast, *G. agilis* is wingless and can cover a smaller area per unit of time compared with *A. nens*. Wingless *Gelis* species appear to prefer searching for hosts on or close to the ground instead of in the canopy of forbs (Harvey *et al.*, 2014; Heinen & Harvey, 2019). Honeydew excreted by aphids not only accumulates on leaf surfaces of the food plant, but also falls to the ground (Moller & Tilley, 1989). In this situation, it may serve as an important food source for *G. agilis* and other wingless (hyper)parasitoids that reduces the need to climb plants to search for floral nectar.

In conclusion, our experiments demonstrate that aphid honeydew can be a valuable food source for hyperparasitoids in the fourth trophic level. This is true for hyperparasitoids associated with aphids as well as those that use insects that do not produce honeydew. By supporting life span and fecundity of hyperparasitoids, honeydew can influence species interactions that cascade through multiple trophic levels and structure food webs (Bukovinszky *et al.*, 2008). Nevertheless, not all honeydew types were equal with respect to their nutritional value to hyperparasitoids. These fourth-trophic-level organisms may therefore be under selection in their natural environment to forage for honeydew that best supports their longevity and fecundity, especially when other food sources are scarce.

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Author Contributions

FACvN, MK, LEMV, and JAH conceived the ideas and designed the methodology. FACvN, JGdB, LS, and WT performed the experiments and collected the data. FACvN and JGdB analysed data and interpreted data together with JAH. JGdB and FACvN led the writing of the manuscript. All authors contributed critically to the drafts and approved the final manuscript.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Effect of five honeydew types and control without honeydew (NO) on three life-history parameters of hyperparasitoids.

Table S2. Matrices of pairwise *P*-values for longevity in the absence of hosts of hyperparasitoids provided with five types of honeydew and a control.

Table S3. Hazard ratio of hyperparasitoids per honeydew source compared with control for longevity in the absence of hosts.

Table S4. Matrices of pairwise *P*-values for longevity in the presence of hosts of hyperparasitoids provided with five types of honeydew and a control (NO) as measured in the fecundity experiment.

Table S5. Hazard ratio per taxa per honeydew source when given hosts, compared with no honeydew control treatment.

Table S6. Matrices of pairwise *P*-values for the effect of the presence of hosts on longevity of four hyperparasitoid taxa when provided with five different types of honeydew or a control.

Table S7. Correlations between longevity and fecundity were calculated with Pearson's product moment correlation coefficient (R^2) per honeydew type for each hyperparasitoid taxa.

Table S8. Matrices of pairwise *P*-values for realised fecundity of hyperparasitoids provided with five types of honeydew and a control.

Fig. S1. Correlation matrix between concentrations of individual carbohydrates or total concentration of honeydew and amount of honeydew collected and hyperparasitoid longevity in the absence of hosts.

Fig. S2. Correlation matrix between concentrations of individual carbohydrates or total concentration of honeydew and amount of honeydew collected and hyperparasitoid longevity in the presence of hosts.

Fig. S3. Correlation matrix between concentrations of individual carbohydrates or total concentration of honeydew and amount of honeydew collected and hyperparasitoid fecundity.

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