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The influence of aphid-produced honeydew on parasitoid fitness and nutritional state: A comparative study

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Abstract

Honeydew is a sugar-rich resource excreted by many hemipteran species and is a key food source for other insect species such as ants and parasitoid wasps. Here, we evaluated the nutritional value of 14 honeydews excreted by 13 aphid species for the generalist aphid parasitoid *Lysiphlebus testaceipes* to test a series of hypotheses concerning variation in the nutritional value of honeydew. There was a positive correlation between the body sugar content of honeydew-fed parasitoids and their longevity. This information is valuable for biological control researchers because it demonstrates that the nutritional state of honeydew-fed parasitoids in the wild can indicate their fitness, independently of the honeydew source they have fed on.

Although the carbohydrate content and longevity of *L. testaceipes* differed greatly among the different honeydews, we did not find a significant effect of aphid or host plant phylogeny on these traits. This result suggests that honeydew is evolutionarily labile and may be particularly subject to ecological selection pressures. This becomes apparent when considering host aphid suitability: *Schizaphis graminum,* one of the most suitable and commonly used hosts of *L. testaceipes,* produced honeydew of the poorest quality for the parasitoid whereas *Uroleucon sonchi*, one of the few aphids tested that cannot be parasitized by *L. testaceipes*, excreted the honeydew with the highest nutritional value. These data are consistent with the hypothesis that hemipterans are subject to selection pressure to minimize honeydew quality for the parasitoids that attack them.

Keywords: Hymenoptera; Aphididae; Nutritional ecology; Biological control; Carbohydrates; *Lysiphlebus testaceipes*

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Introduction

Honeydew is a sugar-rich resource excreted by many hemipteran species, attracting insects belonging at least to 49 families, including bees and natural enemies (Zoebelein, 1956a, 1956b; Lundgren, 2009). Many adults of dipteran and hymenopteran parasitoids depend on honeydew as a food source, often the most common source of available sugar in agroecosytems (Wäckers, van Rijn, & Heimpel, 2008; Lundgren, 2009; Tena, Wäckers, Heimpel, Urbaneja, & Pekas, 2016). A great number of ant species, which tend and protect hemipterans, also feed on and would not survive without honeydew (Way, 1963; Styrsky & Eubanks, 2007; Pekas, Tena, Aguilar, & Garcia-Marí, 2011). However, the quality of honeydew as a diet for insects is highly variable (Avidov, Balshin, & Gerson, 1970; Völkl, Woodring, Fischer, Lorenz, & Hoffmann, 1999; Wäckers et al., 2008; Tena, Llácer, & Urbaneja, 2013; Tena et al., 2016).

Debate on the quality of honeydew as a diet for insects has been ongoing since before the publications of Zoebelein (1956a, 1956b). Honeydew is considered to be a mixture of plant-derived compounds that vary with several biotic and abiotic factors (Maltais & Auclair, 1962; Fischer & Shingleton, 2001; Wäckers et al., 2008). However, the composition of honeydew has a genetic component with high heritability (Völkl et al. 1999; Wäckers, 2000). Consequently, it is expected that evolution has also shaped the quality of honeydew as carbohydrate source for insects. On the one hand, high-quality honeydew may be favored to attract and retain mutualistic ant species that tend and protect hemipteran colonies, but on the other hand, it may attract and retain natural enemies and increase their fitness with potential negative consequences on hemipteran populations if ants are absent (Evans & England 1996; Wäckers et al., 2008; Tena et al., 2016). In these cases, low-quality honeydew may be favored to limit honeydew feeding by natural enemies (Wäckers, 2000; Wäckers et al., 2008). Here, we determine whether aphid honeydew varies in quality for the generalist aphid parasitoid *Lysiphlebus testaceipes* and also whether the quality exhibits a phylogenetic signal. To do this, we categorized the quality of honeydews produced by 13 aphid species as food sources for *L. testaceipes* by measuring: (i) the content of fructose and total sugars of the honeydews and (ii) their effect on the longevity and nutritional state of *L. testaceipes* females fed on these honeydews.

L. testaceipes is a solitary koinobiont and pro-ovigenic (or with a very high ovigenic index) endoparasitoid of aphids (van Steenis, 1994). It does host feed (Desneux, Barta, Delebeque, & Heimpel 2009) and parasitizes more than 100 aphid species in various genera, tribes and subfamilies (Pike et al., 2000). It is native to North America where it is an effective natural enemy of the greenbug, *Schizaphis graminum* (Royer, Pendleton, Elliott, & Giles, 2015). The parasitoid was introduced to Europe (Starý, Lyon, & Leclant, 1988) where it has achieved some success as a biological control agent (Costa & Starý, 1988; Starý et al., 1988) and also has been found to attack numerous aphid species in various ecosystems (Starý et al., 1988; Starý, Lumbierres, & Pons, 2004; Mitrović et al., 2013; Zikic et al., 2015). Therefore, this parasitoid likely feeds on honeydews from various aphid species that differ greatly in their quality as a sugar source. Moreover, some of these aphids feed on different host plants (Holman, 2009) and therefore may produce different honeydew quality for insects depending on the plant. *L. testaceipes* is thus a highly suitable model for a comparison of the quality of aphid honeydews on parasitoid fitness.

To determine whether parasitoids feed on honeydew in the field, as well as to evaluate their nutritional state, researchers have used high-performance liquid chromatography (HPLC), or anthrone tests over the last decade (Casas et al., 2003; Steppuhn & Wäckers, 2004; Heimpel et al., 2004; Lee, Andow, & Heimpel, 2006; Hogervorst, Wäckers, & Romeis, 2007a; Winkler, Wäckers, & Pinto, 2009; Tena, Pekas, Wäckers, & Urbaneja, 2013; Dieckhoff, Theobald, Wäckers, & Heimpel, 2014; Tena, Pekas, Cano, Wäckers, & Urbaneja, 2015; Calabuig et al., 2015). This information is valuable for biological control researchers, especially when parasitoid carbohydrate contents are low; as this indicates that parasitoids are sugar-limited and that providing a sugar source may increase their fitness and biological control potential (Heimpel & Jervis, 2005; Tena et al., 2016). However, an observation of high energy reserves may be misleading if low-quality carbohydrates for insects have been ingested. This is especially important when multiple hemipteran species with different honeydew qualities are present at a given field site (Pekas et al., 2011; Tena, Hoddle et al., 2013; Tena, Llácer et al., 2013; Tena, Pekas et al., 2013).

Here, we used anthrone tests to quantify the carbohydrate levels (fructose, other sugars, glycogen and total carbohydrates) of *L. testaceipes* females fed on different honeydews, and we determined (i) whether the sugar content of different honeydews is correlated with the nutritional state and longevity of parasitoids fed on these honeydews; and (ii) whether the nutritional state of parasitoids fed on different honeydews is correlated with their longevity. Positive correlations would indicate that carbohydrate level of honeydews and/or parasitoids reflect also the fitness of honeydew-fed parasitoids.

Materials and methods

Insect colonies

Our culture of *L. testaceipes* was initiated with individuals collected from parasitized soybean aphids, *Aphis glycines* in Minnesota, USA in the year preceding these studies and had thus been in culture for approximately 25 generations on soybean aphid. Just prior to these studies, the parasitoid population was transferred to *Schizaphis graminum*

on barley at 25 °C, 65% relative humidity (RH) and 16:8 h light:dark (L:D). This *S. graminum* colony had been held in the laboratory for 10 years and was collected from a rye field in St. Paul, Minnesota, USA. The other aphid colonies were similarly collected from surrounding field settings and kept for between 1 and 10 years. All were kept as asexually reproducing populations. Parasitized aphids that had died and contained *L. testaceipes* pupae ('mummies') were removed from barley leaves and kept in plastic tubes (8 cm height × 2.5 cm diameter) until the emergence of adult parasitoids. Between 40 and 60 parasitoid mummies were introduced per tube, while no food or water was supplied. Newly emerged parasitoids were collected at 9:00 and 17:00, but only the former were used in the experiments. Therefore, females used for all experiments were less than 16 h old, had been in contact with males, and had never been fed or been in contact with living aphids. These females were placed individually into glass vials (3.0 cm height and 0.8 cm diameter) sealed with a moistened cotton plug and randomly assigned to the different treatments (see below).

Thirteen aphid species were tested, all of which were reared at the Entomology Department of the University of Minnesota on their respective host plants at 25 °C, 65% relative humidity (RH) and 16:8 h light:dark (L:D). Table 1 shows the aphid species, host plants and sample sizes for each assay. *Aphis gossypii* was fed on two plants: cotton (*Gossypium hirsutum*) and common milkweed (*Asclepias syriaca*). Thus, we obtained fourteen different honeydews from 13 aphid species. These aphid species were chosen because they cover a broad phylogenetic range of aphids within Aphididae. More information on the aphids used in this study can be found in Blackman and Eastop (2000, 2008).

Honeydew collection

To collect honeydew, we followed a protocol similar to the one described by Tena, Llácer et al. (2013), Tena, Hoddle et al. (2013) and Tena, Pekas et al. (2013). Pieces of Parafilm© were placed below aphid colonies on the different infested plants such that honeydew excreted by the aphids accumulated on the Parafilm©. The next day, the Parafilm© containing droplets of honeydew was collected, placed in a Petri dish (2.5 cm height and 9 cm diameter) with a piece of moistened paper and frozen until further use (Hogervorst et al., 2007a; Tena, Llácer et al., 2013; Tena, Hoddle et al., 2013; Tena, Pekas et al., 2013).

Adult survival

To assess the influence of continuous access to honeydew on *L. testaceipes* longevity, a piece of Parafilm© with droplets of honeydew prepared as described above was provided. Vials were checked at 8:00 and 18:00 daily to determine parasitoid survivorship. Treatments with only water and with honey streaked lightly onto the wall of the vial plus water were used as negative and positive control diets, respectively. Sugar sources were replaced every two days to prevent crystallization and desiccation but honeydew excreted by *R. padi* was renewed daily because sooty mold was observed after 24 h in preliminary assays. Cotton was moistened with water twice daily after checking the survival. Parasitoids that were lost or succumbed to accidental deaths were excluded from the analyses.

Honeydew analyses

Cold and hot anthrone tests were used to determine the content of fructose and total sugars present in the honeydews, respectively. For this, Parafilm© with accumulated honeydew was dried in an oven for 24 to 48 h

Table 1. Aphid species, phylogenetic relationships, host plants (from which aphids were collected and on which they were cultured), and number of replicates in the assays of longevity and carbohydrates.

Phylogeny	Aphid species	Host plant species	Replicates	
			Longevity	Carbohydrates
	Aphis oestlundi Gillete	*Oenothera biennis*	10	-
	Aphis monardae Oestlund	*Monarda fistulosa*	19	8
	Aphis gossypii Glover	*Gossypium hirsutum*	23	18
	Aphis gossypii Glover	*Asclepias syriaca*	27	18
	Aphis glycines Matsumara	*Glycine max*	20	18
	Aphis asclepiadis F.	*Asclepias incarnata*	19	-
	Aphis nerii Boyer de Fonscolombe	*Asclepias incarnata*	16	9
	Aphis fabae Scopoli	*Vicia fabae*	27	17
	Rhopalosiphum maidis F.	*Hordeum vulgare*	16	18
	Rhopalosiphum padi L.	*Hordeum vulgare*	26	15
	Schizaphis graminum Rondani	*Hordeum vulgare*	25	18
	Myzus persicae Sulzer	*Brassica oleracea*	23	18
	Uroleucon sonchi (L.)	*Sonchus spp.*	20	9
	Myzocallis asclepiadis (Monell)	*Asclepias incarnata*	26	15
	Water control		40	7
	Honey control		41	18

at 40 °C and dried honeydew was collected into 0.6 mL microcentrifuge tubes and dissolved into 25% ethanol at a concentration of 1 mg/mL. The cold anthrone test was done on 50 μL of each dissolved honeydew sample placed into separate 1.5 mL centrifuge tubes into which 950 μL anthrone solution was added (anthrone solution was prepared as described below). Anthrone was also added to the sucrose standard tubes so that each contained a final volume of 1000 μL. All sample and standard tubes were vortexed and then left to incubate at room temperature for 1.5 h. The same procedure was followed for the hot anthrone test for sugars but samples and standards were incubated in a dry bath at 90 °C for 12 min after adding the anthrone solution. Samples and standards were then cooled on ice. Incubated samples and standards from both tests were vortexed and placed in a 96-well plate. Three 200 μL replicates of each sample were placed in separate wells. Absorbance of samples and standards were recorded at 620 nm. The absorbance was compared to absorbance values of known fructose or sucrose standards with two replicates per read.

In addition to the contents of fructose and total sugars obtained with the anthrone tests, we also used the fructose:total sugars ratio to estimate the relative fructose content of the honeydews.

Parasitoid nutritional analyses

Living parasitoids were frozen (−80 °C) at 9:00 am two days after they were exposed to the nutritional treatment. The hind tibia length was measured as a proxy for parasitoid size before the carbohydrate content was analyzed since parasitoid size was likely to affect nutrient storage capacity (Briegel, 1990; Olson, Fadamiro, Lundgren, & Heimpel, 2000). Parasitoids were rinsed by vortexing individually for 10 s in 1 mL warm (60 °C) autoclaved deionized water in a 1.5 mL centrifuge tube. Parasitoids were then carefully transferred to a new 1.5 mL centrifuge tube and kept on ice.

The carbohydrate content of parasitoids, in particular fructose, total sugars and glycogen were analyzed using a quantitative anthrone assay (modified after Olson et al., 2000). After adding 5 μL sodium sulfate 2% (w/v) to the centrifuge tube, the parasitoids were crushed using autoclaved glass pestles. Pestles were rinsed with 45 μL of methanol:chloroform (2:1), which was added to the solution. After centrifuging at 13,000 rpm for 2 min, the supernatant, containing all soluble sugars, was transferred to 100 mm glass test tubes. The white precipitate containing insoluble high molecular sugars (e.g. glycogen) was kept on ice until used for the glycogen assay.

A cold anthrone test was used to analyze the fructose content. The supernatant was boiled at 90 °C for approximately 1.5 min in a dry bath incubator until all of the liquid was gone. After cooling on ice for 15 min, 100 μL of anthrone reagent as prepared in Olson et al. (2000) (375 mg anthrone dissolved in 70% concentrated sulfuric acid) was added and vortexed for 10 s. After incubating for 1.5 h at room temperature, 75 μL were transferred to wells in a 96-well plate and the absorbance at 620 nm was measured. Then, the same samples were heated at 65 °C for 2 h (hot anthrone test) to analyze the total sugar content of the parasitoid and the absorbance was again read at 620 nm.

The white precipitate containing insoluble high molecular weight sugars (glycogen) was analyzed by adding 200 μL of the anthrone reagent. The resulting solution was vortexed and 100 μL was transferred to a new 1.5 mL centrifuge tube while avoiding floating body parts. The solution was heated at 90 °C for 3 min in a dry bath incubator, after which the tubes were placed on crushed ice for 15 min, and 75 μL were transferred to a 96-well plate. The absorbance was again read at 620 nm. The absorbance of all three assays was compared to absorbance values of known fructose, sucrose standards dissolved in 25% ethanol or glycogen standards dissolved in dH_2O with two replicates per read.

The cold anthrone test is based upon the property of the anthrone reagent, which reacts with only fructose within 1 h at room temperature (Heimpel et al. 2004). Since fructose is not present at detectable levels in the hemolymph or most or all insects, exposing insects to the anthrone reagent at room temperature for limited periods of time can be used to indicate the quantity of gut sugars present in a sample (Heimpel et al., 2004). The hot anthrone test, on the other hand detects all sugars and can therefore be interpreted to measure the sum of sugars present in the gut and hemolymph. The glycogen test uses a hot anthrone approach to measure the glycogen content. Glycogen represents a long-term energy storage for insect parasitoids. We report here these three measurements as well as a sum of all three that we refer to as 'total carbohydrates'.

The aphid phylogeny and phylogenetic signal

We determined whether the sugar content in the honeydews and the sugar content in the parasitoids that were offered the honeydews clustered on the aphid phylogeny. This was done using the analysis of traits mode in the software package Phylocom to test for a phylogenetic signal for the various measures of sugar composition on the aphid phylogeny (Webb, Ackerly, & Kembel, 2007;). The phylogeny came from Desneux, Blahnik, Delebecque, and Heimpel (2012) with *Aphis fabae* inserted according to Coeur d'Acier, Jousselin, Martin, and Rasplus (2007). For simplicity, this analysis was done without branch lengths, utilizing only the topography of the tree. Phylogenetic signal is detected by Phylocom using randomization of trait values across the tips of the phylogeny to compare observed and randomized patterns for signs of clustering. We conducted 10,000 randomizations for each trait and considered clustering to be significant (indicating phylogenetic signal) if >9500 randomizations showed lower levels of variance for the observed

than the randomized values. Because the sugar composition of the honeydews may be affected by the host plant of the aphid (Fischer & Shingleton, 2001; Pringle, Novo, Ableson, Barbehenn, & Vannette, 2014) we also tested for phylogenetic signal of the host plants (as in Desneux et al., 2012) using the same procedures in Phylocom.

Statistical analysis

Carbohydrate levels were compared using ANOVA and Tukey's HSD for multiple comparisons. The normality assumption was assessed using Shapiro's test, and homoscedasticity assumption was assessed with the Levene test. The fructose:total sugars ratio was arcsine square root transformed in order to fulfill normality and homoscedasticity assumptions. In addition, simple linear regressions were used to relate (i) carbohydrate levels of honeydew and parasitoids, (ii) carbohydrate levels of honeydew and parasitoid longevity and (iii) carbohydrate levels of parasitoids and their longevity. Statistical analyses were run on absolute amounts of nutrients instead of on absorbance values. In the case of the parasitoids, the absolute amounts were divided by the tibia length of each parasitoid.

The longevity was analyzed using the survival functions of the "OIsurv" package (Diez, 2013) with standard specification in R, version 3.2.2 (R Core Team, 2015). We used the "survfit" function to calculate estimates of the different survival curves using the Kaplan–Meier method. A non-parametric cox proportional hazards model ("coxph" function) was used to analyse the effect of the food source treatment on parasitoid survival. The assumption of proportional hazards was tested by visual inspections using the "cox.zph" function. The Kaplan–Meier survival curves of individual treatments were compared with the log-rank test "survdiff". The latter uses the G-rho family of tests of Harrington and Fleming (1982) and weights $S(t)^{\hat{\rho}}$ho on each death (S is the Kaplan-Meier estimate of survival). Rho was set to zero for the log-rank test.

Results

Carbohydrate content of 14 honeydews

M. asclepiadis, U. sonchi and *A. nerii* produced honeydews with the highest content of fructose (measured with the cold anthrone test), followed by that of *R. maidis* and a group of six species ($F_{13, 41} = 243.7$; $P < 0.0001$) (Fig. 1A). *M. persicae*, *A. monardae*, *A. gossypii* (fed on cotton) and *R. padi* produced the honeydew with the lowest content of fructose. This analysis showed that the fructose content in honeydew produced by *A. gossypii* was affected by host plant. However, we found no evidence that fructose content is clustered either on the aphid phylogeny (phylogenetic signal analyses in Phylocom: $P = 0.64$; see Supplementary Appendix A: Fig. 1) nor on the plant phylogeny ($P = 0.13$).

For total sugar content measured by the hot anthrone test, honeydews can be divided into three groups ($F_{13, 41} = 65.6$; $P < 0.0001$) (Fig. 1B). The honeydew produced by *A. gossypii* fed on common milkweed, *M. asclepiadis* and *U. sonchi* contained the highest concentration of sugars, whereas *A. monardae*, *A. glycines*, *S. graminum* and *A. asclepiadis* produced the honeydew with the lowest sugar concentration. We found no evidence that the mean total sugar content is clustered either on the aphid phylogeny (phylogenetic signal analyses in Phylocom: $P = 0.38$; see Supplementary Appendix A: Fig. 1) or on the plant phylogeny ($P = 0.21$).

The fructose:total sugars ratio also varied significantly among honeydews from 0.55 ± 0.01 in *M. asclepiadis* and *A. nerii* to 0.05 ± 0.002 in *M. persicae* ($F_{13, 41} = 142.3$; $P < 0.0001$) (Fig. 1C). The graph represents a staircase from *M. asclepiadis* to *A. gossypii* fed on cotton (0.23 ± 0.01). The fructose:total sugars ratio was not significantly correlated with the total sugar content of the honeydews ($R^2 = 0.28$; $F_{1, 13} = 0.03$; $P = 0.86$).

Effect of honeydew produced by 13 aphid species on *L. testaceipes* longevity

We found significant differences among parasitoid longevities depending on the offered food source (Cox proportional hazards: likelihood ratio = 165.1; df = 15; $P < 0.0001$). Parasitoids fed on honeydew or honey lived significantly longer than parasitoids fed on water only (Table 2; Fig. 2; and see Supplementary Appendix A: Fig. 2). However, only the survival of parasitoids fed on honeydew of *A. oestlundi* [4.7 ± 0.3 days (mean $\pm$ SE)] and *U. sonchi* (4.6 ± 0.4 days) was not significantly different from the survival of parasitoids fed on honey (4.8 ± 0.3 days). There were marginally significant differences between the survivorship of *L. testaceipes* fed on honeydew of the same aphid species, *A. gossypii*, reared on two plant species (cotton and common milkweed). The lifespan of parasitoids fed honeydew from *A. gossypii* reared on cotton or on common milkweed was of 3.3 ± 0.2 days and 3.9 ± 0.2 days, respectively (Fig. 2, species; Table 2). When comparing the effect of honeydews from different aphid species (*A. nerii*, *A. asclepiadis* and *M. asclepiadis*) reared on the same host plant (swamp milkweed), *A. asclepiadis* (2.6 ± 0.2 days) produced honeydew of poorer quality (here and throughout the results section: we use "honeydew quality" as a measurement of the increment of parasitoid longevity) than *A. nerii* (3.5 ± 0.2 days) (Fig. 2, host plant; Table 2). *M. asclepiadis* produced honeydew of intermediate quality (2.9 ± 0.2 days). Similarly, when comparing the effect of honeydews from different aphid species (*R. maidis*, *R. padi* and *S. graminum*) reared on barley, *R. padi* (2.6 ± 0.2 days) and *S. graminum* (2.7 ± 0.3 days) produced honeydew of poorer quality than *R. maidis* (3.4 ± 0.2 days). When comparing

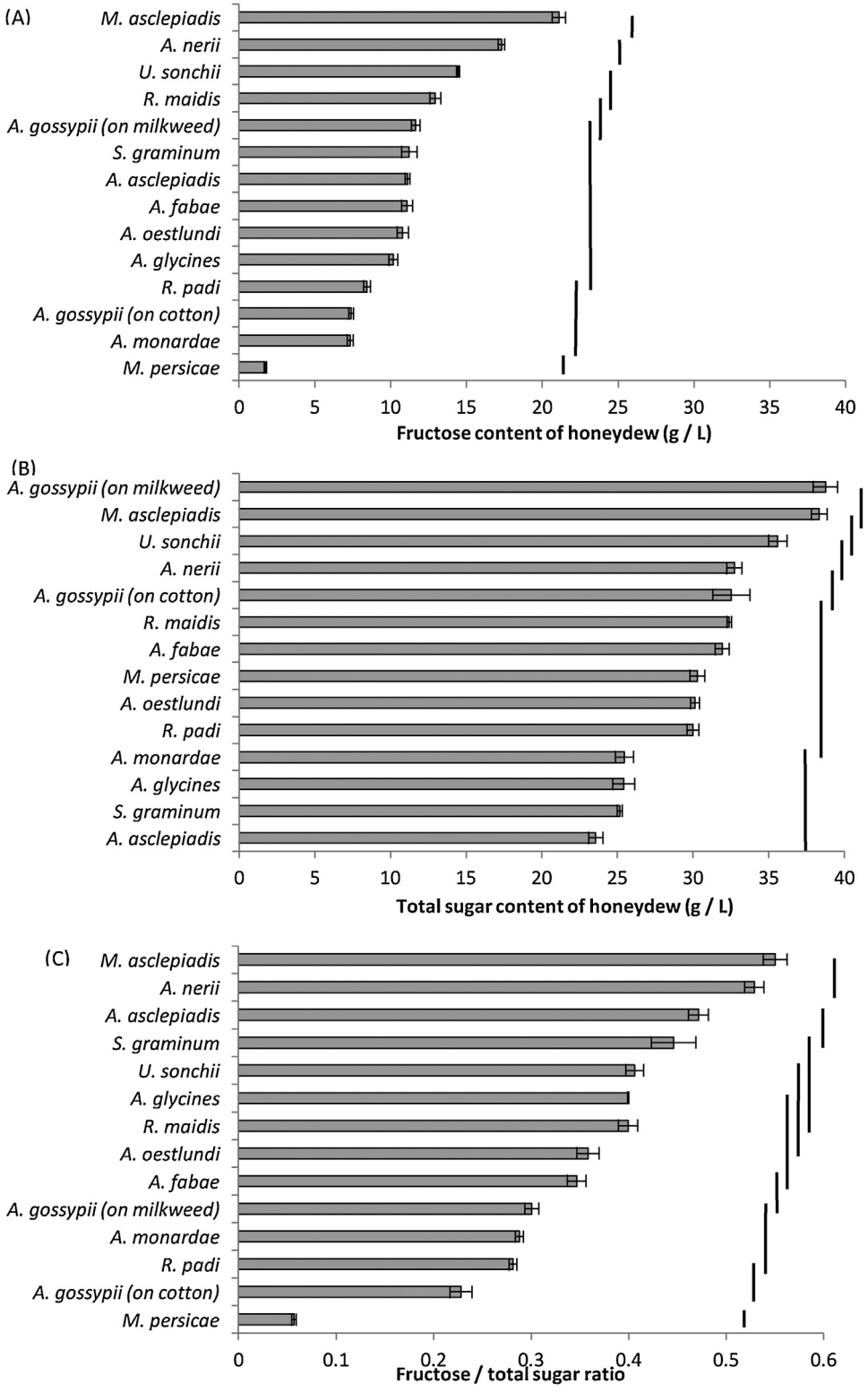

Fig. 1. Sugar content (mean ± SE) of 14 honeydews produced by 13 aphid species. (A) Fructose content (measured using the cold anthrone test). (B) Total sugar content (measured using the hot anthrone test). (C) Fructose:total sugar ratio. Sugar contents and ratio for species subtended by lines do not differ ($P > 0.05$, Tukey test).

Table 2. Pairwise differences between survival curves of *Lysiphlebus testaceipes* females subjected to 16 diet treatments: water, honey and 14 honeydews. Survival curves were estimated by using the Kaplan-Meier method and pairwise compared using a log-rank test using the "survdiff" function in R. Values are significance levels; bold indicates significance $P < 0.05$.

Treatment	Water	*A. fabae*	*A. glycines*	*A. monardae*	*A. neri*	*A. oestlundi*	*A.gossypii* (cotton)	*A.gossypii* (milkweed)	*A. asclepiadis*	*M. asclepiadis*	*M. persicae*	*R. maidis*	*R. padi*	*S. graminum*	*U. sonchi*	Honey
Water	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
A. fabae	**<0.001**	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
A. glycines	**<0.001**	0.1117	–	–	–	–	–	–	–	–	–	–	–	–	–	–
A. monardae	**<0.001**	0.2401	0.8128	–	–	–	–	–	–	–	–	–	–	–	–	–
A. nerii	**<0.001**	0.7319	0.1832	0.2908	–	–	–	–	–	–	–	–	–	–	–	–
A. oestlundi	**<0.001**	**<0.05**	0.1838	0.3524	**<0.05**	–	–	–	–	–	–	–	–	–	–	–
A. gossypii (cotton)	**<0.001**	0.9051	0.0653	0.1528	0.6646	**<0.01**	–	–	–	–	–	–	–	–	–	–
A. gossypii (milkweed)	**<0.001**	0.1469	0.8735	0.8908	0.1439	0.194	0.0616	–	–	–	–	–	–	–	–	–
A. asclepiadis	**<0.01**	0.054	**<0.001**	**<0.01**	**<0.05**	**<0.001**	**<0.05**	**<0.001**	–	–	–	–	–	–	–	–
M. asclepiadis	**<0.001**	0.3951	**<0.05**	**<0.05**	0.228	**<0.01**	0.4072	**<0.01**	0.2584	–	–	–	–	–	–	–
M. persicae	**<0.001**	0.7938	0.1589	0.3479	0.7681	**<0.05**	0.8215	0.1791	**<0.05**	0.3577	–	–	–	–	–	–
R. maidis	**<0.001**	0.7513	<0.05	0.1416	0.9082	**<0.01**	0.8418	0.0704	**<0.01**	0.2763	0.7334	–	–	–	–	–
R. padi	**<0.001**	**<0.05**	**<0.001**	**<0.001**	**<0.05**	**<0.001**	**<0.05**	**<0.001**	0.9711	0.2084	**<0.05**	**<0.01**	–	–	–	–
S. graminum	**<0.001**	0.0953	**<0.001**	**<0.01**	**<0.05**	**<0.001**	0.1083	**<0.001**	0.6289	0.4667	0.0711	**<0.05**	0.5684	–	–	–
U. sonchi	**<0.001**	**<0.05**	0.2479	0.1993	**<0.05**	0.8749	**<0.01**	0.1503	**<0.001**	**<0.001**	**<0.05**	**<0.01**	**<0.001**	**<0.001**	–	–
Honey	**<0.001**	**<0.001**	**<0.01**	**<0.05**	**<0.01**	0.2094	**<0.001**	**<0.01**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.5758	–

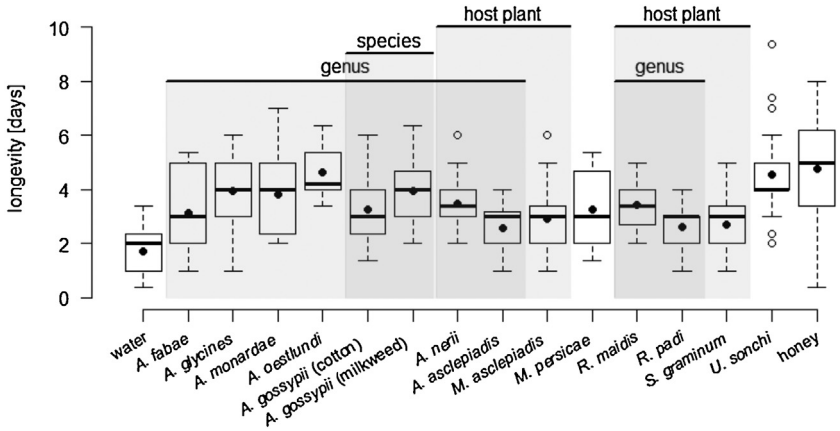

Fig. 2. Survivorship of *Lysiphlebus testaceipes* females subjected to 16 diet treatments: water, honey and 14 honeydews. Within the boxes, black dots represent the average and horizontal lines the median. Horizontal lines are used to group aphids of the same species (species), genus (genus), or reared on the same host plants (host plant). Statistical differences between treatments are presented in Table 2.

the effect of honeydews from different aphid species within the same genus, there were significant differences between the honeydew of *R. maidis* [3.4 ± 0.2 days (mean ± SE)] and *R. padi* (2.6 ± 0.2 days) ($\chi^2_1 = 7.96$, $P < 0.01$) (Fig. 2, genus; Table 2). There were also significant differences among the honeydews excreted by the aphid species of genus *Aphis* (Fig. 2, genus; Table 2). *A. oestlundi* produced the honeydew of highest quality and *A. asclepiadis* the poorest.

Finally, we found no evidence that mean survival of *L. testaceipes* fed on honeydew of different aphid species is clustered either on the aphid phylogeny (phylogenetic signal analyses in Phylocom: $P = 0.23$) or on the plant phylogeny ($P = 0.3$) (Supplementary Appendix A: Fig. 3).

Effect of honeydew produced by 12 aphid species on *L. testaceipes* nutritional state

The source of honeydew had a significant effect on the amount of fructose (cold anthrone test; $F_{13, 188} = 8.13$; $P < 0.0001$), total sugars (hot anthrone test; $F_{13, 188} = 4.06$; $P < 0.0001$), glycogen ($F_{13, 188} = 4.41$; $P < 0.0001$) and total carbohydrates ($F_{13, 188} = 6.51$; $P < 0.0001$) in *L. testaceipes* females (Fig. 3). Fructose was not detected in unfed parasitoids and these parasitoids contained the lowest levels of total carbohydrates.

Parasitoids fed on honeydew produced by *U. sonchi* had the highest content of fructose (Fig. 3A); followed by those fed on honeydew from *A. fabae, A. glycines, A. monardae,* and *A. nerii*; a third group was composed by *R. maidis, M. asclepiadis,* and *A. gossypii* (on common milkweed and cotton); and finally honeydew from *M. persicae, R. padi* and *S. graminum* were the poorest according to the fructose content of *L. testaceipes.* The content of fructose measured with the cold anthrone test was ten times lower than the total sugar content of the insect.

The pattern was slightly different for the total sugar content (Fig. 3B). Parasitoids fed on honeydew produced by *U. sonchi* again had the highest content of total sugars; followed by those fed on honeydew excreted by *A. glycines, A. fabae, A. monardae, M. asclepiadis, M. persicae* and *A. gossypii* (on cotton); the third group was composed of *A. nerii, R. maidis, R. padi* and *A. gossypii* (on common milkweed); and finally honeydew produced by *S. graminum* resulted in the lowest levels of total sugars within *L. testaceipes.*

Although the pattern was less clear for the content of glycogen, the consumption of honeydew produced by *S. graminum* also resulted in the lowest levels of glycogen within *L. testaceipes* (Fig. 3C). The amount of glycogen was generally lower than that of sugars.

According to the amount of total carbohydrates (total sugars + glycogen) that parasitoids contained, honeydews could be divided in four main groups (Fig. 3D). Parasitoids fed on honeydew produced by *U. sonchi, A. fabae, A. glycines* and *A. monardae* had the highest levels of carbohydrates, followed by those fed on honeydew excreted by *A. gossypii* (on cotton), *M. persicae*, *M. asclepiadis*, *A. nerii* and *R. maidis*. The third group was composed of *A. gossypii* (on common milkweed) and *R. padi*, and finally honeydew produced by *S. graminum* was the lowest according to the carbohydrate content of *L. testaceipes*.

Finally, we found no evidence for phylogenetic clustering of fructose (phylogenetic signal analyses in Phylocom: $P = 0.65$), total sugars ($P = 0.21$), total carbohydrates ($P = 0.45$) or glycogen ($P = 0.081$) in *L. testaceipes* females fed on honeydew of different aphid species (Supplementary Appendix A: Fig. 4).

Relationship between the sugar content of honeydew produced by 12 aphid species and the nutritional state and longevity of *L. testaceipes* when fed on them

The fructose content of honeydew was not correlated with fructose content of parasitoids fed on honeydew ($P = 0.41$).

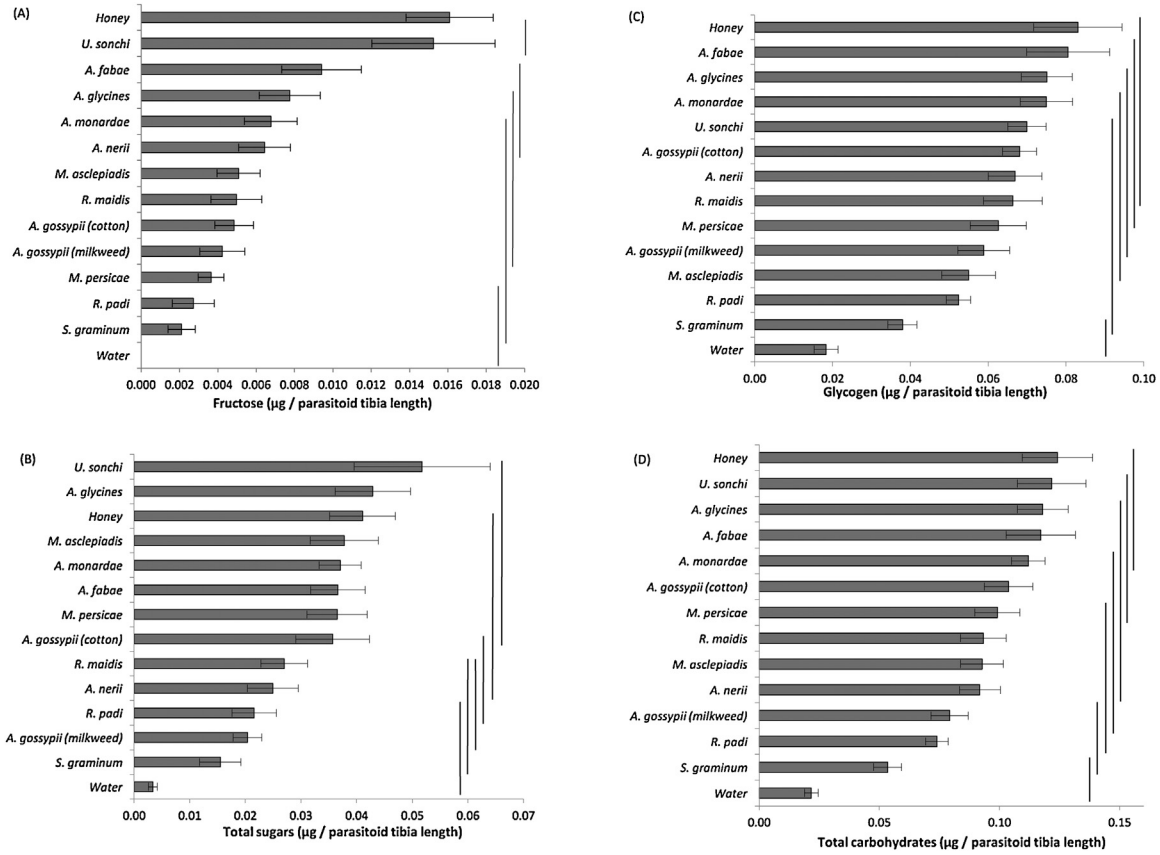

Fig. 3. Mean content (±SE) of (A) fructose, (B) total sugars, (C) glycogen and (D) total carbohydrates in *Lysiphlebus testaceipes* females that were two days old and had access to 14 diet treatments: water, honey and 12 honeydews. Carbohydrate contents for species subtended by lines do not differ ($P > 0.05$, Tukey test).

Similarly, the total content of sugars in the honeydew was correlated with neither the total content of sugars ($P = 0.71$) nor total content of total carbohydrates (sugars + glycogen) ($P = 0.84$) of the parasitoids fed on honeydew.

Neither the content of fructose ($P = 0.88$) nor the content of total sugars ($P = 0.59$) were correlated with the longevity of honeydew fed parasitoids.

Relationship between the nutritional state of *L. testaceipes* and its longevity when fed on honeydew excreted by 12 aphid species

We detected a significant positive relation between the mean longevity and the mean content of the three carbohydrates in the parasitoids (fructose: $P < 0.0001$; total sugars: $P = 0.0042$; glycogen: $P = 0.001$), as well as with total content of carbohydrates ($P < 0.0001$) (Fig. 4). A stronger relationship was observed for fructose ($R^2 = 75.5\%$), followed by the total content of carbohydrates ($R^2 = 71.3\%$), glycogen ($R^2 = 61.1\%$) and total sugars ($R^2 = 50.9\%$). Since the correlations could be greatly amplified by the presence of negative and positive controls (water and honey), we re-ran them without the controls to evaluate the variance among honeydews only. The correlation remained positive and significant for fructose ($P = 0.006$; $R^2 = 54.1\%$), and total content of carbohydrates ($P = 0.0036$; $R^2 = 58.8\%$), and positive and marginally significant for total sugars ($P = 0.0498$; $R^2 = 33.2\%$), and glycogen ($P = 0.052$; $R^2 = 32.6\%$).

Discussion

We analyzed the sugar content of 14 honeydews produced by 13 species of aphids and evaluated their nutritional value for the generalist aphid parasitoid *L. testaceipes*. Although there was a high variation among the different honeydews, none of the honeydews appeared to be toxic (i.e. causing higher mortality of parasitoids than a water control) and none were of a higher quality for parasitoid performance than honey. The lack of phylogenetic signal for the nutritional value of honeydew suggests that this characteristic is evolutionarily labile and may be particularly subject to ecological selection pressures. Finally, for the first time, we provide evidence that the longevity of honeydew-fed parasitoids is positively correlated with their carbohydrate contents, especially their content of fructose. The latter result may be due to direct positive effects of fructose, or indirectly due to higher oligosaccharide levels in honeydews with lower fructose levels.

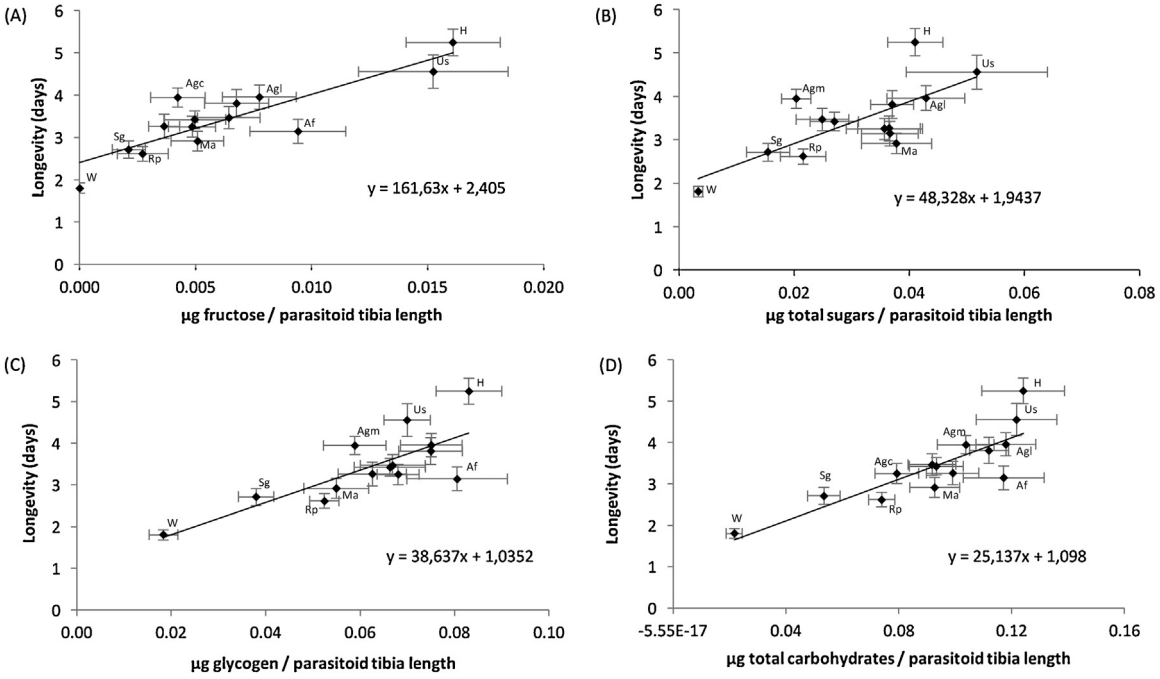

Fig. 4. Correlation between the longevity of *Lysiphlebus testaceipes* females and the content of carbohydrates [(A) fructose, (B) other sugars, (C) glycogen and (D) total carbohydrates] when they fed on 14 diet treatments: water, honey and 12 honeydews. Each point represents the mean ± SE longevity (Y-axis) and mean carbohydrate content (X-axis) of females fed with one of the 14 treatments: water = W; honey = H; *A. fabae* = *Af*; *A. glycines* = *Agl*; *A. gossypii* on cotton = *Agc*; *A. gossypii* on common milkweed = *Agm*; *A. monardae* = *Am*; *A. nerii* = *An*; *M. asclepiadis* = *Ma*; *M. persicae* = *Mp*; *R. maidis* = *Rm*; *R. padi* = *Rp*; *S. graminum* = *Sg*; *U. sonchi* = *Us*.

Honeydew composition

Other studies have shown that honeydew composition within the same aphid species can vary with host plant (as demonstrated herein and by Fischer & Shingleton, 2001; Pringle et al., 2014), phenological and physiological status of the plant (Maltais & Auclair, 1962), presence of ants (Fischer & Shingleton, 2001; Yao & Akimoto, 2002) and aphid age (Fischer,Völkl, Schopf, & Hoffmann, 2002). Although honeydew composition can vary with the host plant and honeydew reflects the host plant amino acid composition (Leroy et al., 2011), we did not observe a significant effect of plant phylogeny on the sugar and fructose content of the honeydews produced by 13 aphid species.

Apart from host-plant-mediated factors, the presence of microorganisms in aphids and honeydew is another factor that may affect the quality of honeydew as carbohydrate source for insects and explain the lack of phylogenetic signals. For example, in a proteomic study, almost 30% of the 96 proteins identified in the honeydew of the pea aphid *Acyrthosiphon pisum* (Harris) were homologous to bacterial sequences (Sabri et al., 2013). This result highlights the importance of other organisms (i.e. the host aphid and its microbiota, including endosymbiotic bacteria and gut flora) on the composition of honeydew. Recently, it has been demonstrated that the facultative endosymbionts *Hamiltonella defensa* and *Regiella insecticola* modify the content of *A. fabae* honeydew, in this case, reducing the concentrations of amino acids (Schillewaert, Vantaux, Parmentier, Vorburger, & Wenseleers, 2017). Moreover, honeydew can be a nutrient-rich resource for insect pathogens, reducing the quality of honeydew as a diet for insects (Lundgren, 2009; Lievens et al., 2015).

Finally, it is worth mentioning that we used the content of fructose and total sugars to measure honeydew quality for *L. testaceipes*. Previous studies have used the sucrose:hexose ratio to measure the quality of several nectars for parasitoid fitness without finding a clear relation (Tompkins, Wratten, & Wäckers, 2010). HLPC (high-performance liquid chromatography) could be used in future studies because this technique allows determination of the presence of characteristic hemipteran-synthesized sugars, such as melezitose or erlose (Tena, Llácer et al., 2013; Tena, Hoddle et al., 2013; Tena, Pekas et al., 2013). Although it has been recently demonstrated that some parasitoids have specifically adapted to the consumption of specific sugars present in honeydew, these sugars tend to reduce the nutritional value of honeydew (Wäckers, 2001; Lenaerts et al., 2016; Goelen et al., 2018).

Effect of honeydew on *L. testaceipes* nutritional state and longevity

The fourteen aphid honeydews showed high variation in their effect on nutritional state and longevity of *L. testaceipes*. As expected, sugar and glycogen levels of honeydew-fed parasitoids were higher compared to unfed ones, and the

latter did not contain fructose, consistent with previous field and laboratory studies (Olson et al., 2000; Lee et al., 2004; Heimpel et al., 2004; Lee et al., 2006; Wäckers, Lee, Heimpel, Winkler, & Wagenaar, 2006; Hogervorst et al., 2007a; Hogervorst, Wäckers, & Romeis, 2007b; Wyckhuys, Strange-George, Kulhanek, Wäckers, & Heimpel, 2008; Tena, Llácer et al., 2013; Tena, Hoddle et al., 2013; Tena, Pekas et al., 2013; Tena et al., 2015; Calabuig et al., 2015). *L. testaceipes* contained the highest sugar levels (both fructose and total) when females fed on honeydew produced by *U. sonchi.* This honeydew raised *L. testaceipes* fructose and total sugar content by a factor greater than 5 and 3, respectively, compared to the poorest honeydew. The high level of fructose, a sugar that is mostly present in the digestive system of insects (Heimpel et al. 2004), suggests that *L. testaceipes* fed on *U. sonchi* honeydew at a high rate. This hypothesis is also supported by the fact that this honeydew did not contain a high fructose-total sugar ratio. *L. testaceipes* also reached the greatest longevity when fed *U. sonchi* honeydew, which reinforces the high nutritional value of this honeydew for this parasitoid. It is interesting to highlight that *U. sonchi,* together with *M. asclepiadis,* are not parasitized by *L. testaceipes* (N. Desneux & G.E. Heimpel, unpublished data).

On the other hand, our data show that *S. graminum,* which is a highly suitable host for *L. testaceipes* (N. Desneux & G.E. Heimpel, unpublished data) and a commonly attacked in the native range of the parasitoid (Royer et al., 2015), produced the poorest honeydew for this parasitoid. These data support the hypothesis that hemipterans, and especially aphids, are subjected to strong selection pressure to minimize the quality of honeydew for insects, as any nutritional benefit to natural enemies has the potential to negatively impact fitness of the aphid species producing it (Wäckers, 2000, 2001; Wäckers et al., 2008). In the case of *S. graminum* honeydew, the effect of host plant cannot be disregarded because this aphid and *R. padi* were both reared on barley (*H. vulgare*) and both produced honeydew with relatively low nutritional value for *L. testaceipes.* The honeydew of these aphids might contain some of the secondary metabolites produced by *H. vulgare* and/or its endophytes (Schulz & Boyle, 2005), as it is known that aphids sequester secondary metabolites when feeding on toxic plants and also excrete them in the honeydew (Malcolm, 1990; Zust & Agrawal, 2015). These compounds could be detrimental to aphid natural enemies. Despite this caveat, there was no overall effect of host–plant phylogeny on honeydew composition or quality for insects as noted above.

To better understand the role of toxic plants on honeydew quality, we compared honeydew of three aphid species reared on swamp milkweed *A. incarnata,* which likely contains high levels of cardenolide toxins (Agrawal & Malcolm, 2002; Agrawal, 2005; Agrawal, Petschenka, Bingham, Weber, & Rasmann, 2012). In terms of parasitoid longevity, the specialist aphid *A. asclepiadis* excreted honeydew of poorer nutritional value for *L. testaceipes* compared to that excreted by the generalist aphid *A. nerii.* This result is in concordance with a recent study, which showed that *M. asclepiadis* – despite producing the lowest amounts of cardenolides in its honeydew – produces higher concentrations of an apolar cardenolide compared to generalist aphids reared on the common milkweed *A. syriaca* (Zöst & Agrawal, 2015). Apolar cardenolides are considered more toxic because contrary to polar ones they can pass through cell membranes via passive diffusion (Frick & Wink, 1995; Agrawal et al., 2012; Züst & Agrawal, 2015).

Relation between the nutritional state of *L. testaceipes* and its longevity when fed on honeydew

Our results show that the nutritional state of honeydew-fed parasitoids when they were two days old was positively correlated with their longevity; i.e. there was a positive correlation between carbohydrate contents and parasitoid fitness. This information is valuable for biological control researchers because it demonstrates that the nutritional state of honeydew-fed parasitoids in the wild also provides information on their fitness. So far, HPLC or anthrone tests have been used to determine the nutritional state of parasitoids that have fed on honeydew and nectar in the field (Casas et al. 2003; Heimpel et al. 2004; Lavandero, Wratten, Shishehbor, & Worner, 2005; Lee et al., 2006; Hogervorst et al. 2007a; Lee & Heimpel 2008; Winkler et al. 2009; Desouhant, Lucchetta, Giron, & Bernstein, 2010; Tena, Llácer et al., 2013; Tena, Hoddle et al., 2013; Tena, Pekas et al., 2013; Tena et al. 2015; Dieckhoff et al. 2014; Calabuig et al., 2015). This information is useful when carbohydrate contents of parasitoids are low; as this indicates that parasitoids are sugar-limited and it is necessary to provide a sugar source to increase their fitness and biocontrol potential (Tena et al., 2016). However, if energy reserves (sugar content) in field parasitoids are high, this might only show part of the picture, lacking information about the quality of the sugar source and eventually the impact on parasitoid fitness (Tena, Llácer et al., 2013; Tena, Hoddle et al., 2013; Tena, Pekas et al., 2013). In our data set, for example, parasitoids fed on *A. fabae* honeydew contained high sugar contents but their longevity was lower than expected given the overall relationship. A potential explanation is that *A. fabae* honeydew might be a phagostimulant for *L. testaceipes* as occurs in ants, even if its nutritional value is poor for the parasitoid (Völkl et al. 1999).

Interestingly, Wyckhuys et al. (2008) also observed that carbohydrate content was positively related with the longevity of the aphid parasitoid *Binodoxys communis* when it had access to three carbohydrate sources: honey, sucrose and honeydew excreted by the soybean aphid, *A. glycines*. Parasitoids fed on honey had the highest sugar content when they were 2-4-days old and lived the longest, whereas those fed on honeydew had the lowest sugar content and lived the shortest. Parasitoids fed on sucrose had an intermediate sugar content and lifespan. Lee et al. (2004) found similar results when comparing buckwheat nectar and soybean

aphid honeydew in the parasitoid *Diadegma insulare* Cresson (Hymenoptera: Ichneumonidae). Therefore, the positive correlation between carbohydrate content and longevity found here may not be exclusive to honeydew-fed parasitoids, but also include those fed on other sugar sources. A potential explanation for this correlation is that the toxic metabolites of honeydew, such as cardenolides (Malcolm, 1990), also deter feeding as has been demonstrated in herbivores that feed on plants with high contents of these secondary metabolites. In fact, a wide range of animals show preingestive (gustatory) sensitivity to cardenolides (Agrawal et al., 2012).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.baae.2018.04.003.

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