Associative learning and memory retention of nectar yeast volatiles in a generalist parasitoid

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Understanding how animals learn is crucial to interpreting animal behaviour. Flower-visiting insects, such as bees and parasitoids, are excellent animal models to study visual and olfactory learning, including memory phenomena. The diversity of resources flower-visiting insects exploit predisposes them to learn and remember the colours, shapes and odours associated with rewarding experiences (e.g. flowers), allowing them to focus on the most rewarding resources. Recent research has shown that nectar-living microbes release volatile organic compounds (VOCs) that contribute to overall flower scent. Nevertheless, little is known about the extent to which nectar microbiota mediate insect learning

of floral preferences. In this study, we investigated whether VOCs produced by nectar microbes serve as a learning cue to parasitoids and how long any developed preference is maintained. Experiments were performed using the generalist aphid parasitoid *Aphidius ervi* and three nectar yeasts, including the nectar specialist *Metschnikowia reukaufii* and the generalist species *Hanseniaspora uvarum* and *Sporobolomyces roseus.* Results showed that naïve parasitoids had an innate preference for nectar fermented by the nectar specialist *M. reukaufii*, but not by the other two yeasts which had either a neutral (*H. uvarum*) or deterrent (*S. roseus*) effect. When parasitoids were conditioned with yeast-fermented nectar, they were strongly attracted to their odours 2 and 24 h after conditioning, but not after 48 h. Furthermore, when parasitoids were conditioned to one yeast-fermented nectar, they also showed increased attraction to other yeast-fermented nectars. This generalization suggests that their learning ability may have broader ecological consequences. However, this generalized response to other yeast VOCs lasted for only 2 h. We conclude that parasitoids show conditioned responses to the scent of yeast-fermented nectar, and yeasts, therefore, may play an important but understudied role in shaping their foraging behaviour.

Keywords: *Aphidius*; associative learning; *Hanseniaspora*; memory retention; *Metschnikowia*; nectar yeast; olfactory; parasitoid foraging; *Sporobolomyces*; yeast volatiles

Animal learning is the ability to acquire neuronal representations of new spatial, sensory or olfactory information (Dukas, 2008), which allows animals to better exploit environmental resources across time and space (Smid & Vet, 2016). While learning seems to be a universal property of animals with a central nervous system, numerous insect species, despite possessing small nervous systems, also rely on learning and memorizing various types of sensory cues for their major life functions (Giurfa, 2013). Therefore, nectar-feeding insects, especially bees and parasitoids, are commonly used as animal models to study visual and olfactory learning, as well as memory phenomena (Chittka, 2017; Hoedjes et al., 2011; Turlings, Wackers, Vet, Lewis, & Tumlinson, 1993). The lifestyle of such insects predisposes them to learn and remember the colours, shapes and odours of different food-rewarding flowers so that they can keep returning to these flowers.

Parasitoids have been shown to quickly learn olfactory and visual cues that are associated with successful host location (Lewis & Tumlinson, 1988; Wäckers & Lewis, 1999) and to use this information for subsequent host-searching decisions (Bleeker et al., 2006). This associative learning (i.e. the ability to learn associations between a stimulus and a reward) allows parasitoids to find their hosts faster and therefore increase their reproductive success (Smid & Vet, 2016). Apart from searching for hosts, adult parasitoids also forage for carbohydrate-rich food to cover their energetic and nutritional needs (Lewis, Stapel, Cortesero, & Takasu, 1998). This food is typically associated with separate olfactory and visual stimuli (Wäckers & Lewis, 1994), and parasitoids can respond innately, or learn stimuli associated with food rewards, separately from host-associated stimuli (Takasu & Lewis, 1995; Wäckers, 1994). For feeding, parasitoids exploit a broad range of sugar resources, including floral and extrafloral nectar and, to a lesser extent, honeydew (Hogervorst, Wäckers, & Romeis, 2007; Lee & Heimpel, 2008; Vollhardt, Bianchi, Wäckers, Thies, & Tscharntke, 2010; Wäckers, 2000).

Floral nectar is a sweet aqueous solution that, in addition to sugars, usually contains a variety of other compounds, such as organic acids, lipids, proteins, antioxidants, inorganic ions, scents and other secondary compounds (Heil, 2011; Nepi et al., 2012; Raguso, 2004). Because of its high sugar content, floral nectar is an ideal habitat for diverse microbes, mostly yeasts, that can rapidly reach high densities after colonization (Brysch-Herzberg, 2004; Lievens et al., 2015; Pozo, Lievens, & Jacquemyn, 2015). In turn, nectar-inhabiting yeasts strongly affect nectar characteristics, for example by altering the concentration and composition of sugars and amino acids (Canto & Herrera, 2012; Herrera, García, & Perez, 2008), reducing the amount of secondary metabolites (Vannette & Fukami, 2016) and influencing acidity (Good, Gauthier, Vannette, & Fukami, 2014; Vannette, Gauthier, & Fukami, 2013). More recently, it has also been shown that, as a result of nectar fermentation, nectar yeasts produce volatile mixtures that are highly attractive to pollinators and parasitic wasps (Rering, Beck, Hall, McCartney, & Vannette, 2018; Sobhy, Baets,- et al., 2018). This suggests that volatile organic compounds (VOCs) produced by nectar yeasts could mediate insect foraging for food (Dzialo, Park, Steensels, Lievens, & Verstrepen, 2017). However, at present little is known about parasitoid learning of VOCs produced by nectar-inhabiting yeasts, and how this learning affects (long-term) parasitoid foraging behaviour.

In this study, we investigated the learning ability and memory retention of the solitary parasitoid *Aphidius ervi* (Hymenoptera: Braconidae) when exposed to VOCs emitted from synthetic nectars fermented by various nectar yeasts. *Aphidius ervi* is a generalist parasitoid of aphids that feeds preferentially on nectar as a main source of sugars (Vollhardt et al., 2010), and its efficiency in suppressing aphids strongly increases when individuals have been supplied with floral nectar (Araj, Wratten, Lister, Buckley, & Ghabeish, 2011). Using Y-tube olfactometer experiments, we first investigated the innate parasitoid response to the yeast-fermented nectars by using experimentally naïve wasps (inexperienced to smell and food). Next, we assessed whether the parasitoid response changed when wasps were trained to associate the presence of yeast odours with nectar as a food reward. First, wasps were tested 2 h following the conditioning to assess their learning ability. Then, we determined how long the memory persisted after the learning event. Finally, we tested whether conditioning to one yeast odour could also affect the parasitoid's response to another yeast odour.

<H1>METHODS

<*H2*>*Yeasts and Insects*

Three nectar yeasts (*Metschnikowia reukaufii* (ST12.14/017), *Hanseniaspora uvarum* (EHE_1_Y1) and *Sporobolomyces roseus* (ST12.14/075)) were used in this study. A previous study showed that parasitic wasps responded differently to VOC blends from nectar fermented by these yeast species (Sobhy, Baets, - et al., 2018). While the VOCs of *M. reukaufii* were attractive to *A. ervi* females, VOCs produced by *H. uvarum* were not attractive and those produced by *S. roseus* were repellent. Prior to the current experiments, yeast strains were stored at -80 °C in yeast extract peptone dextrose broth (YPDB; Difco, Le Pont-de-Claix, France) containing 37.5% glycerol.

In all experiments, adult female *A. ervi* were used; these were supplied as mummies by Biobest (Ervi-system, Westerlo, Belgium). Upon receipt, mummies were kept under controlled conditions (22 °C, 70% relative humidity, 16:8 h light:dark) in a nylon insect cage until adult emergence, as described in Sobhy et al. (2018). Once emerged, parasitoids received no food or hosts, so that they were naïve before the experiments.

<H2>Preparation of Yeast-fermented Nectars

Yeast-fermented nectars were prepared following the procedure outlined by Sobhy et al. (2018). Briefly, yeast strains were inoculated in test-tubes containing 5 ml YPDB and incubated at 25 °C on a rotary shaker at 150 rpm for one night. Afterwards, cells were washed and suspended in sterile NaCl solution (0.9%) until an optical density (OD 600 nm) of 1 was reached. Subsequently, 250 ml Erlenmeyer flasks containing 150 ml sterile synthetic nectar were inoculated with 1.5 ml of the suspension. Synthetic nectar was prepared by filter-sterilizing 15% w/v sucrose solution supplemented with 3.16 mM amino acids from digested casein (Lenaerts et al., 2017; Sobhy, Baets, - et al., 2018). Erlenmeyer flasks were sealed with fermentation water locks and incubated statically for 7 days at 25 °C. This was sufficiently long to obtain cell densities that are comparable with those commonly found in floral nectar (de Vega, Herrera, & Johnson, 2009). Three independent fermentations were performed for each yeast, and a control treatment (i.e. medium without yeast inoculation) was included in the experiment as well. Control treatments were checked for microbial growth after the fermentation period of 7 days and showed no signs of bacterial or fungal growth. To obtain cell-free cultures, yeast-fermented nectars were centrifuged at 5040 *g* for 3 min and subsequently filtered (pore size 0.22 µm; Nalgene, Waltham, MA, U.S.A.). Cell-free nectar media were then stored in small aliquots in sterile dark glass vials (Fagron, Nazareth, Belgium) at -20 °C until further use.

<H2>Chemical Analysis of Yeast-fermented Nectars

To compare the VOC profiles between the fermented nectars and the control, all media were analysed using a gas chromatograph (GC) coupled with flame ionization detector (FID) and flame photometric detector (FPD; Shimadzu, Kyoto, Japan). The GC was fitted with a polar column (DB-WAX 30 m length x 0.32 mm inner diameter x 0.5 µm film thickness, Agilent Technologies, Santa Clara, CA, U.S.A.) to the FID and a no-polar column (HP-5, Agilent, 30 m x 0.25 mm inner diameter, 0.25 µm thin layer) to the FPD. The GC was calibrated for 15 important yeast-specific volatiles, including higher alcohols, esters, acetaldehyde and sulphur compounds as described in Gallone et al. (2016). Nitrogen (N_2) was used as the carrier gas. For each sample, 5 ml cell-free nectar medium was added into a 20 ml

glass vial containing 1.75 g NaCl. Vials were immediately closed and stored at -20 °C until their analysis to minimize evaporation and loss of volatile compounds. To perform the analysis, 1 ml of each sample was automatically injected by means of a headspace autosampler (PAL system; CTC Analytics, Zwingen, Switzerland) in split mode at 250 °C. The GC oven temperature was first held at 50 °C for 5 min and then allowed to rise to 80 °C at a rate of 5 °C/min, followed by the second ramp of 4 °C/min until 200 °C. The temperature was then held for 3 min at 200 °C and subsequently increased by 4 °C/min until a temperature of 230°C was reached. Results were analysed with the GCSolution software version 2.4 (Shimadzu, Kyoto, Japan).

<H2>***Conditioning of Parasitoids***

Parasitoids were collected within 24 h of emergence. Soon after emergence, mating was frequently observed between males and females, reassuring us that the tested females were mated prior to the experiments. Parasitoids were subjected to a dark period of 8 h prior to being used in the experiments. For each conditioning treatment (see below), 12 groups of 7–10 newly emerged naïve females were conditioned in petri dishes (diameter = 9 cm) by allowing them to feed on a filter paper (diameter 37 mm, Macherey-Nagel, Düren, Germany) saturated with 550 µl cell-free synthetic nectar. Each group of parasitoids was given 2 min to feed on the nectar and associate its odour with the food reward. This procedure was repeated three times, at 1 min intervals, mimicking consecutive flower visits of the parasitoids in the field. In addition, this training scheme is known to stimulate the development of long-term memory in parasitic wasps (Smid et al., 2007). For all treatments, extensive nectar feeding was observed for all individuals used in the experiment. After conditioning, parasitoids were kept in cages for 2, 24 and 48 h and provided with 50% (w/v) sugar solution when tested after 24 and 48 h to provide them with necessary sugars to survive (Azzouz, Giordanengo, Wäckers, & Kaiser, 2004). Experienced parasitoids were starved 2 h before the olfactometer bioassay to increase their foraging activity (Scharf, 2016).

<H2>***Olfactometer Bioassays***

The behavioural response of parasitoids was assessed using the Y-tube olfactometer bioassay described by Sobhy et al. (2018). We used either naïve or experienced (i.e. conditioned to yeast-fermented or control nectar) adult females. The olfactometer was placed on a table that was evenly illuminated by four high-frequency 24W T5 TL-fluorescent tubes with a 96% colour representation of true daylight at a height of 0.45 m. Additionally, to improve parasitoid responsiveness, the Y-tube was positioned in an inclining position (angle 20° between Y-tube and horizontal plane) stimulating insect movement towards the light. To eliminate any visual cues that could affect the insect's response, the olfactometer was fully enclosed with white curtains. To determine the parasitoid's response to the different fermented nectars, 150 µl cell-free fermented nectar was loaded onto a filter paper (Macherey-Nagel, Düren, Germany), which was then placed into one of the olfactometer odour chambers, while in the second chamber another filter paper was placed on which 150 µl control nectar was added. For each treatment, on a given experimental day, the bioassay was carried out by releasing cohorts of 60 adult females in 12 groups of five individuals (N = 60) at the base of the olfactometer and evaluating their response 10 min after their release. Wasps that had entered and reached the end of an olfactometer arm and remained there at the time of evaluation (i.e. 10 min after release) were considered as responding females, while individuals that remained in the stem tube 10 min after release were considered as nonresponding individuals or individuals that had made 'no choice'. Nonresponding parasitoids were excluded from the statistical analysis. Most parasitoids walked back and forth between both olfactometer arms before making a final choice. To avoid parasitoids developing any experiences of the tested odours, we only tested them once. Further, to avoid positional bias, we rotated the odour chambers after six releases using a new set of Teflon tubes. At the same time, the Y-tube was also replaced by a cleaned tube to avoid choices based on odour residues or potential insect traces (Kang, Liu, Zhang, Tian, & Liu, 2018). Odour sources were also regularly (every second run) renewed to maintain a high level of odour release. At the end of each experiment, all olfactometer parts were thoroughly rinsed and then baked in an oven at 150 °C as described by Sobhy et al. (2018). All experiments were performed at 22 °C and 70% relative humidity between 0900 and 1600 hours.

In the first experiment, we investigated the innate response of naïve wasps to the yeast-fermented nectars using noninoculated nectar as a control. Next, we tested whether parasitoid

conditioning to yeast odours impacted their subsequent behavioural response to the same yeast odour 2 h after conditioning. We also assessed memory retention by assessing how long yeast odours (i.e. the conditioned stimulus) continued to elicit a response, testing experienced individuals 24 and 48 h after conditioning. As a control treatment, parasitoids were also conditioned to nonfermented nectar and the response of experienced parasitoids was tested against control nectar versus distilled water. Finally, we assessed whether conditioning to one yeast odour could also affect the parasitoid's response to another yeast odour, at both 2 and 24 h after conditioning.

<H2>Statistical Analysis

To get a general overview of the quantitative variation and correlations between compounds and the effect of yeast species on volatile profiles, we performed a principal component analysis (PCA) using each compound as a variable according to Rencher (2002). A biplot was created with the 'scores' matrix displaying the location of each sample along each principal component (PC). In addition, we used a matrix of 'loadings', which indicates the strength of correlation between individual compounds and each PC and the direction of the different compounds. Prior to analysis, data were cube-root transformed, using the online tool MetaboAnalyst 4.0 (Chong et al., 2018). To test whether the overall scent profile differed between the different yeast treatments and the control, a multivariate analysis of variance (MANOVA) was performed, with treatment as fixed factor and the concentrations of each compound as dependent variables. Subsequently, one-way analysis of variance (ANOVA) was used to test whether concentrations of individual compounds differed between treatments. Post hoc tests using the Student–Newman–Keuls method were used to see which individual compounds differed significantly between treatments. Data were first checked for normality and homogeneity using the Shapiro–Wilk test and Levene's test. If both assumptions were violated, the nonparametric Kruskal–Wallis test was used to investigate whether concentrations differed between treatments (SigmaPlot 12.3, SYSTAT Inc., Chicago, IL, U.S.A.). To test whether parasitoids were significantly attracted to a yeast odour, we used chi-square tests (IBM SPSS Statistics version 22.0, Armonk, NY, U.S.A.) under the null hypothesis that the parasitoids had no preference for either olfactometer arm (i.e. 50:50 response). Analyses were performed on the total number of parasitoids that chose either the control or the treatment

side of the Y-tube olfactometer as a dependent variable. A significance level of $\alpha = 0.05$ was used to determine significant attraction or repellence.

<H1>RESULTS

<*H2*>*Volatile Profiles of Nectar Yeasts*

All yeasts significantly changed the nectar volatile composition compared to the control nectar (MANOVA; Pillai's trace = 2.96, $F_{9,24} = 29.28$, $P < 0.001$). More particularly, of the nine detected volatile compounds, univariate ANOVA indicated that acetaldehyde, ethyl butyrate, amyl acetate, dimethyl sulphide, carbon disulphide and dimethyl disulphide were quantitatively different between the yeast-fermented nectars and the control nectar. More specifically, yeast-fermented nectars showed a significantly higher emission of ethyl butyrate ($F_{3,11} = 7.33$, $P = 0.011$) and dimethyl disulphide ($H_3 = 13.43$, $P = 0.004$) compared to the control nectar (Table 1). Especially, *H. uvarum* and *M. reukaufii* strongly altered nectar VOC profiles (Table 1), as can also be seen from the PCA in which the first two components (PC1 and PC2) explained 69.4% of the total variation in the volatiles data (Fig. 1). Further, the biplot showed that most VOCs were more associated with *H. uvarum*- and *M. reukaufii*-fermented nectars. The total amount of VOCs emitted by these fermented nectars was significantly higher than those of *S. roseus* and the control nectar ($F_{3,11} = 13.48$, $P = 0.002$), particularly due to the high emission of acetaldehyde ($F_{3,11} = 8.87$, $P = 0.006$), ethyl acetate ($F_{3,11} = 6.63$, $P = 0.003$) and propyl acetate ($F_{3,11} = 53.33$, $P < 0.001$). On the other hand, *S. roseus*-fermented nectar produced significantly higher amounts of amyl acetate ($F_{3,11} = 45.16$, $P < 0.001$).

<*H2*>*Parasitoid Response after Conditioning*

Naïve wasps showed a significant preference for volatiles from *M. reukaufii*-fermented nectar compared to control nectar ($X_1^2 = 4.12$, $P = 0.042$; Fig. 2a). In contrast, *S. roseus*-fermented nectar elicited a significant negative response by the parasitoid females leading them more towards the control ($X_1^2 = 4.57$, $P = 0.033$), while no attraction or repellence was recorded for parasitoid females towards *H. uvarum* ($X_1^2 = 0.21$, $P = 0.647$; Fig. 2a). When parasitoids had been exposed to a specific yeast odour

during feeding, they were subsequently strongly and equally attracted to this yeast odour at 2 h after conditioning (*H. uvarum*: X_1^2 = 4.26, P = 0.039; *M. reukaufii*: X_1^2 = 7.37, P = 0.007; *S. roseus*: X_1^2= 4.46, P = 0.035; Fig. 2b). Furthermore, as shown in Fig. 2c, observed effects lasted for at least 24 h after conditioning (*H. uvarum*: X_1^2 = 4.12, P = 0.042; *M. reukaufii*: X_1^2 = 6.15, P = 0.013; *S. roseus*: X_1^2 = 4.08, P = 0.043), showing that *A. ervi* is able to retain the learned response for at least 1 day. At 48 h after conditioning, the parasitoids were no longer significantly attracted to nectars fermented with *H. uvarum* (X_1^2 = 0.56, P = 0.456) and *S. roseus* (X_1^2 = 2.00, P = 0.157), but were still significantly attracted to *M. reukaufii*-fermented nectar (X_1^2 = 3.93, P = 0.047; Fig. 2d). Both naïve individuals and those exposed to control nectar showed no preference between water and control nectar (Fig. 2a, b, c, d).

<*H2*>*Effect of Different Yeast Stimuli*

When parasitoids were conditioned with one yeast and then tested with another yeast species 2 h later, in most cases they showed a strong preference for the yeast-fermented nectar (Fig. 3a). For instance, when the parasitoids were conditioned with nectar from *M. reukaufii* or *S. roseus*, they were subsequently also strongly attracted to *H. uvarum*-fermented nectar (*M. reukaufii*: X_1^2= 5.33, P = 0.021; *S. roseus*: X_1^2 = 4.33, P = 0.037). Similarly, *A. ervi* females were significantly attracted to *M. reukaufii* when conditioned with *H. uvarum*- or *S. roseus*-fermented nectar (*H. uvarum*: X_1^2 = 5.12, P = 0.024; *S. roseus*: X_1^2 = 4.12, P = 0.042). In contrast, the parasitoids showed neutral (X_1^2 = 1.28, P = 0.258) or negative responses (X_1^2 = 4.12, P = 0.042) to *S. roseus* after having been conditioned with *H. uvarum*- or *M. reukaufii*-fermented nectar, respectively (Fig. 3a). However, 24 h after conditioning parasitoids showed the same response to the tested yeast-fermented nectars as the naïve wasps (i.e. *M. reukaufii* was attractive*; H. uvarum* was neutral; *S. roseus* was repellent), irrespective of the nectar used for conditioning (Fig. 3b).

<H1>DISCUSSION

Parasitoids depend on sugar-rich food resources to survive and sustain their host-searching activity. The results of this study show that parasitoids can optimize nectar foraging through associative learning of odours from yeast-fermented nectars.

<H2>***Innate Parasitoid Responses to Nectar Yeast Odours***

In agreement with Sobhy et al. (2018), naïve females of *A. ervi* were highly attracted to *M. reukaufii*-fermented nectar, while they were not attracted to or even deterred by nectar fermented by *H. uvarum* and *S. roseus*, respectively. These findings could be explained by the ecology of the yeasts. While *M. reukaufii* is a nectar specialist which is strongly dependent on floral visitors for dispersal among flowers (Brysch-Herzberg, 2004), the other two yeast species occur in a wide variety of habitats and are less dependent on insect vectors for dispersal (Jolly, Varela, & Pretorius, 2014; Nakase, 2000). Nectar specialists such as *Metschnikowia* are therefore believed to be highly dependent on producing high levels of attractive VOC blends to attract suitable insect vectors that can transfer these otherwise immotile organisms to new flowers (Rering et al., 2018; Sobhy, Baets, et al., 2018). In turn, the insects may benefit from the yeast volatiles as a signal indicating the presence of a highly suitable food source such as nectar (Dzialo et al., 2017; Stefanini, 2018). This could explain why *A. ervi* exhibits a strong innate response to volatiles produced by a nectar specialist such as *M. reukaufii*, and not to more ubiquitous yeasts such as *Hanseniaspora* or *Sporobolomyces*.

<H2>***Associative Learning of Nectar Yeast-associated Olfactory Stimuli***

Although unconditioned insects were not attracted to *H. uvarum*-fermented nectars, and even repelled by *S. roseus*-fermented nectars, parasitoids allowed to feed on nectar fermented by these yeasts found them attractive. These results suggest that the insects were able to associate the yeast odour with a feeding experience. Learning was not observed when parasitoids were conditioned with control nectar, indicating that the observed response for the fermented nectars indeed involved volatiles associated with yeast fermentation. These findings are in agreement with previous studies demonstrating olfactory learning in parasitic wasps after they had associated an odour with a reward such as a suitable food source (Takasu & Lewis, 1996; Wäckers, Bonifay, & Lewis, 2002). *Aphidius ervi* did not show an

attraction to *H. uvarum*- and *S. roseus*-fermented nectars when fed with control nectar. This indicates that the observed learning response is a result of classical conditioning to yeast odours rather than sensitization, defined as a change in general responsiveness following exposure to the conditioned or neutral stimulus (nectar) only (McGuire, 1984).

Several studies have already shown that braconid parasitoids can learn to associate odours with a suitable food source (Olson et al., 2003; Takasu & Lewis, 1996; Wäckers et al., 2002). In these studies, parasitoid females that had experienced an odour while feeding on a sugar solution subsequently showed a strong preference for these odours. The generalist larval endoparasitoid *Microplitis croceipes* has been shown to be able to associate a very broad range of chemicals as foraging cues with sugar feeding, including chemicals not related to their natural history (Olson et al., 2003; Wäckers, Olson, Rains, Lundby, & Haugen, 2011). *Aphidius ervi* adults can also learn to associate odours that are not necessarily ecologically relevant, such as vanilla, with sugar feeding (Gutiérrez-Ibáñez, Villagra, & Niemeyer, 2007). In addition, the ability of *A. ervi* to learn odours also extends to males, which can learn to associate odours with rewards, including the presence of females (Villagra, Vásquez, & Niemeyer, 2005).

<H2>Memory Retention of the Learned Responses

It became clear from our results that *A. ervi* females responded to conditioned yeast odours for at least 24 h, but that this response faded 48 h after conditioning. In general, memory retention in insects can be highly variable and can range from a few hours to weeks (Bleeker et al., 2006; Kaiser, Pérez-Maluf, Sandoz, & Pham-Delègue, 2003; Müller, Collatz, Wieland, & Steidle, 2006). So far, however, it is unclear whether this variation is due to species-specific differences or to the training procedures used. Memory retention in insects tends to depend on the number of training sessions (Tully, Preat, Boynton, & Del Vecchio, 1994). A single training session generally induces short-term memory, while multiple training sessions give rise to long-term memory (Hoedjes et al., 2011). Therefore, it is important to consider the number of training sessions used when evaluating memory retention in parasitoids. For example, whereas a single experience induced a 1 day memory duration in *Leptopilina boulardi*, a parasitoid of *Drosophila* flies, memory retention doubled (2 days) when the parasitoids were given

multiple training sessions spread over 2 - 24 h (Kaiser et al., 2003). In our study, we also used repeated intermittent stimulus presentations mimicking consecutive flower visits of the parasitoids in the field. One possible interpretation of the reduced memory to yeast VOCs after 24 h, in spite of the repeated reinforcements during training, is that *A. ervi* is a generalist parasitoid having a wide range of aphid hosts that occur on many plants from which the parasitoids may also obtain nectar (Zemenick, Kula, Russo, & Tooker, 2018). Therefore, short-term memory retention may increase foraging efficiency in the short term, while providing flexibility to switch to other nectar plants (Sisterson & Averill, 2002).

<H2>Generalization of Learned Yeast Odours

When *A. ervi* adults were conditioned to yeast-fermented nectars, their behavioural response to other yeasts was almost always significantly changed compared to the nonconditioning treatment, with parasitoids showing increased attraction to yeasts other than the one to which they had been conditioned, the exception being odours of *S. roseus*-fermented nectar, which remained repellent. This suggests that conditioned responses in *A. ervi* can be generalized to odour blends that are different from the training odour. This generalization phenomenon was first described by Ghirlanda and Enquist (2003), who showed that once a particular behaviour has been established in response to a stimulus, novel stimuli resembling the first will elicit the same response. This learning generalization has previously been described for the parasitoid *M. croceipes* (Meiners, Wäckers, & Lewis, 2002, 2003). After these parasitoids were trained to an aliphatic alcohol, they generalized their response to other related chemicals, the response depending on the carbon chain length and the position of the functional group (Meiners et al., 2002). Similarly, when conditioned to a blend of volatiles, parasitoids subsequently also responded to odours representing part of the blend (Meiners et al., 2003). Nevertheless, in our study learning generalization to different VOCs lasted for only a few hours (effects were gone after 24 h, but note that this could be because, after conditioning, parasitoids were given sugar water without the conditioned odours when we tested them 24 or 48 h after conditioning; see also above). The fact that conditioning with yeast-fermented nectars caused a substantial increase in parasitoid attraction towards other nectar–yeast combinations suggests that the VOC profiles of different yeast-fermented nectars might show some similarities. Indeed, the volatile data show that *H. uvarum*- and *S. roseus*-fermented

nectars did not differ significantly in their emission of ethyl acetate, propyl acetate, ethyl butyrate and dimethyl disulphide. The same was also true for *H. uvarum*- and *M. reukaufii*-fermented nectars with regard to the emission of acetaldehyde, amyl acetate, ethyl butyrate and dimethyl disulphide. It has been shown that these compounds are key volatiles in attracting diverse insect taxa, including different species of fruit flies and parasitoids of solitary bees (Christiaens et al., 2014; Filella, Bosch, Llusià, Seco, & Peñuelas, 2011; Kleiber et al., 2014; Semmelhack & Wang, 2009).

Interestingly, no generalization was found when parasitoids were conditioned to *M. reukaufii* and *H. uvarum* and subsequently subjected to nectar fermented by *S. roseus*. This is even more remarkable, considering that the reverse situation (conditioning to *S. roseus*) resulted in a generalized response to *M. reukaufii* and *H. uvarum.* This may be explained by the differences in VOC profiles between this yeast and the other two species. For example, the total amount of VOCs emitted by *M. reukaufii*- and *H. uvarum*-fermented nectars was significantly higher than that collected from *S. roseus*-fermented nectar. This is also visualized by the PCA in which the loading vectors of most VOCs were more associated with *M. reukaufii*- and *H. uvarum*-fermented nectars, suggesting distinct VOC profiles compared to *S. roseus*-fermented nectar. Indeed, low VOCs released from *S. roseus*-fermented nectar may explain the poor olfactory responses of naïve and experienced parasitoids when tested against *S. roseus*-fermented nectar, suggesting limitations of the parasitoid perceptual learning of VOCs produced by *S. roseus*. Learning generalization in terms of odour concentration has been shown in *M. croceipes* (Olson, Wäckers, & Haugen, 2012). Alternatively, this yeast species may have produced specific VOCs which are deterrent to *A. ervi* or which could have masked the attracting effect of other compounds. Masking of parasitoid attractants has been reported for other parasitoids in response to high emission of certain plant volatiles (D'Alessandro, Brunner, von Mérey, & Turlings, 2009; Sobhy et al., 2012; Sobhy, Bruce, & Turlings, 2018). In this regard, dimethyl sulphide or amyl acetate, which *S. roseus* produced more than the other yeasts, could be such compounds, but further research is needed to confirm this.

<*H2*>*Conclusion*

In conclusion, our results clearly showed that attraction of the generalist aphid parasitoid *A. ervi* to nectar can be improved through associative learning of nectar-fermenting yeast odours, indicating that microbial cues can mediate both innate and learned components of parasitoid preference. Our results further showed that *A. ervi* parasitoids can rapidly learn to associate the volatiles produced by nectar microbes with the presence of a suitable food source, even if they were exposed to different nectar yeast odours after conditioning. This suggests that the frequent flower visits of parasitoids for nectar intake (Jervis, Kidd, Fitton, Huddleston, & Dawah, 1993; M. Russell, 2015; Zemenick et al., 2018) can be in part attributed to associative learning to yeast-scented nectar (Raguso, 2004). Cues derived from nectar microbes are likely to function in concert with plant-derived cues, such as flower patterns and colours, and could therefore be an important component of the complex floral display (Lawson, Chittka, Whitney, & Rands, 2018). Likewise, recent observations have suggested that microbes occurring on the petals of flowers affect the foraging behaviour of insects through associative learning (Russell & Ashman, 2019). Together, these results demonstrate that flower-inhabiting microbes provide important supplementary cues that may improve the foraging behaviour and feeding efficiency of flower-visiting insects. Further experiments and observational studies using real flowers and nectar are needed to confirm this scenario under field conditions.

Author contributions

I.S.S., H.J. and B.L. conceived the ideas and designed the experiments. I.S.S., T.G. and B.H.M. performed the experiments and collected the data. I.S.S., B.H.M., H.J. and B.L. analysed the data. F.W. and K.J.V. contributed to equipment, reagents and materials. B.H.M. contributed to nectar chemical analysis. I.S.S., H.J. and B.L. led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication. The authors have declared that no competing interests exist.

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637

638 **FIGURE CAPTIONS**

639

640 **Figure 1.** Principal component analysis (PCA) of the volatile profiles produced from the different
641 nectars: control = noninoculated, yeast-free nectar; H.u. = *Hanseniaspora uvarum*-fermented nectar;
642 M.r. = *Metschnikowia reukaufii*-fermented nectar; and S.r. = *Sporobolomyces roseus*-fermented nectar.
643 Score plots visualize the location of each collected sample on each PC with the percentage of explained
644 variation in parentheses, whereas vectors (red lines) visualize the loadings for each compound. Vector
645 numbers refer to the different volatile compounds measured: (1) acetaldehyde, (2) amyl acetate, (3)
646 ethyl acetate, (4) propyl acetate, (5) ethyl butyrate, (6) dimethyl sulphide, (7) carbon disulphide, (8)
647 diethyl sulphide and (9) dimethyl disulphide. All analyses were performed on cell-free nectar solutions
648 (three biological replicates).

649

650 **Figure 2.** Olfactory response of *Aphidius ervi* females when given the choice between two odours
651 (percentage, N = 60). (a) Parasitoids were naïve (i.e. had no experience of smell and food). (b)
652 Parasitoids were conditioned to the different yeast-fermented nectars and then tested against the same
653 nectars, 2 h after conditioning. (c) Parasitoids were conditioned to the different yeast-fermented nectars
654 and then tested against the same nectars, 24 h after conditioning. (d) Parasitoids were conditioned to the
655 different yeast-fermented nectars and then tested against the same nectars, 48 h after conditioning.
656 Control = noninoculated, yeast-free nectar; water = distilled water; H.u. = *Hanseniaspora uvarum*-
657 fermented nectar; M.r. = *Metschnikowia reukaufii*-fermented nectar; and S.r. = *Sporobolomyces roseus*-
658 fermented nectar. Experiments were performed with cell-free nectars. The bioassay was carried out by
659 releasing 60 adult females (in 12 groups of five individuals) at the base of a two-choice Y-olfactometer
660 and evaluating their response 10 min after their release. Numbers in parentheses inside each bar
661 represent the number of parasitoids that were in each olfactometer arm at the time of evaluation. Both
662 percentages and absolute numbers (in parentheses) of nonresponding parasitoids are presented on the
663 right-hand side ('no choice'). Asterisks indicate a preference that is significantly different (chi-square

664 test) from a 50:50 distribution within a choice test: *P < 0.05; **P < 0.01. Nonresponding parasitoids
665 were excluded from the statistical analysis.
666
667 **Figure 3.** Olfactory response of *Aphidius ervi* females when given the choice between two odours
668 (percentage, N = 60). (a) Parasitoids were conditioned to yeast-fermented nectar and then tested against
669 (i) the same or (ii) different yeast-fermented nectar, 2 h after conditioning. (b) Parasitoids were
670 conditioned to yeast-fermented nectar and then tested against (i) the same or (ii) different yeast-
671 fermented nectar, 24 h after conditioning. Control = noninoculated, yeast-free nectar; H.u. =
672 *Hanseniaspora uvarum*-fermented nectar; M.r. = *Metschnikowia reukaufii*-fermented nectar; and S.r. =
673 *Sporobolomyces roseus*-fermented nectar. Insect pictograms on the right indicate which yeast-
674 fermented nectar was used for the conditioning: blue = *H. uvarum*-fermented nectar; dark red = *M.*
675 *reukaufii*-fermented nectar; and orange = *S. roseus*-fermented nectar. Experiments were performed with
676 cell-free nectars. The bioassay was carried out by releasing 60 adult females (in 12 groups of five
677 individuals) at the base of a two-choice Y-olfactometer and evaluating their response 10 min after their
678 release. Numbers in parentheses inside each bar represent the number of parasitoids that were in each
679 olfactometer arm at the time of evaluation. Both percentages and absolute numbers (in parentheses) of
680 nonresponding parasitoids are presented on the right-hand side (‘no choice’). Asterisks indicate a
681 preference that is significantly different (chi-square test) from a 50:50 distribution within a choice test:
682 *P < 0.05; **P < 0.01. Nonresponding parasitoids were excluded from the statistical analysis.
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687 **Table 1.** Volatile organic compounds of the different yeast-fermented nectars investigated in this
688 study
689

Class	Compound	Unit	Yeast-fermented nectars				*P*
			Control	H.u.	M.r.	S.r.	
Aldehyde	Acetaldehyde	mg/litre	0.0153 [b]	0.1783 [a]	0.1475 [a]	0.0129 [b]	**0.006**
Ester	Amyl acetate	mg/litre	ND [c]	0.0032 [b]	0.0037 [b]	0.0051 [a]	**≤0.001**
	Ethyl acetate	mg/litre	0.007 [c]	0.0253 [b]	0.0771 [a]	0.0367 [b]	**0.003**
	Propyl acetate	mg/litre	ND [c]	0.0013 [b]	0.0108 [a]	0.0017 [b]	**≤0.001**
	Ethyl butyrate	mg/litre	ND [b]	0.0082 [a]	0.0081 [a]	0.0053 [a]	**0.011**
Containing sulphur	Dimethyl sulphide	µg/litre	ND	0.0003	0.0026	ND	0.530
	Carbon disulphide	µg/litre	ND	0.0138	0.0035	ND	0.082
	Diethyl sulphide	µg/litre	ND	0.0021	0.0011	ND	0.192
	Dimethyl disulphide	µg/litre	0.0083 [b]	0.0551 [a]	0.0552 [a]	0.0691 [a]	**0.004**

690 Volatile organic compounds were identified according to retention times on DB-
691 WAX column in comparison with synthetic standards. Presented values are means
692 of three biological replicates. ND = not detected; control = noninoculated, yeast-
693 free nectar; H.u. = nectar fermented with *Hanseniaspora uvarum*; M.r. = nectar
694 fermented with *Metschnikowia reukaufii*; S.r. = nectar fermented with
695 *Sporobolomyces roseus***.** Different letter superscripts within rows indicate
696 statistically significant differences ($P \leq 0.05$); when no letters are present there
697 were no significant differences between treatments. Significant *P* values are
698 shown in bold.
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