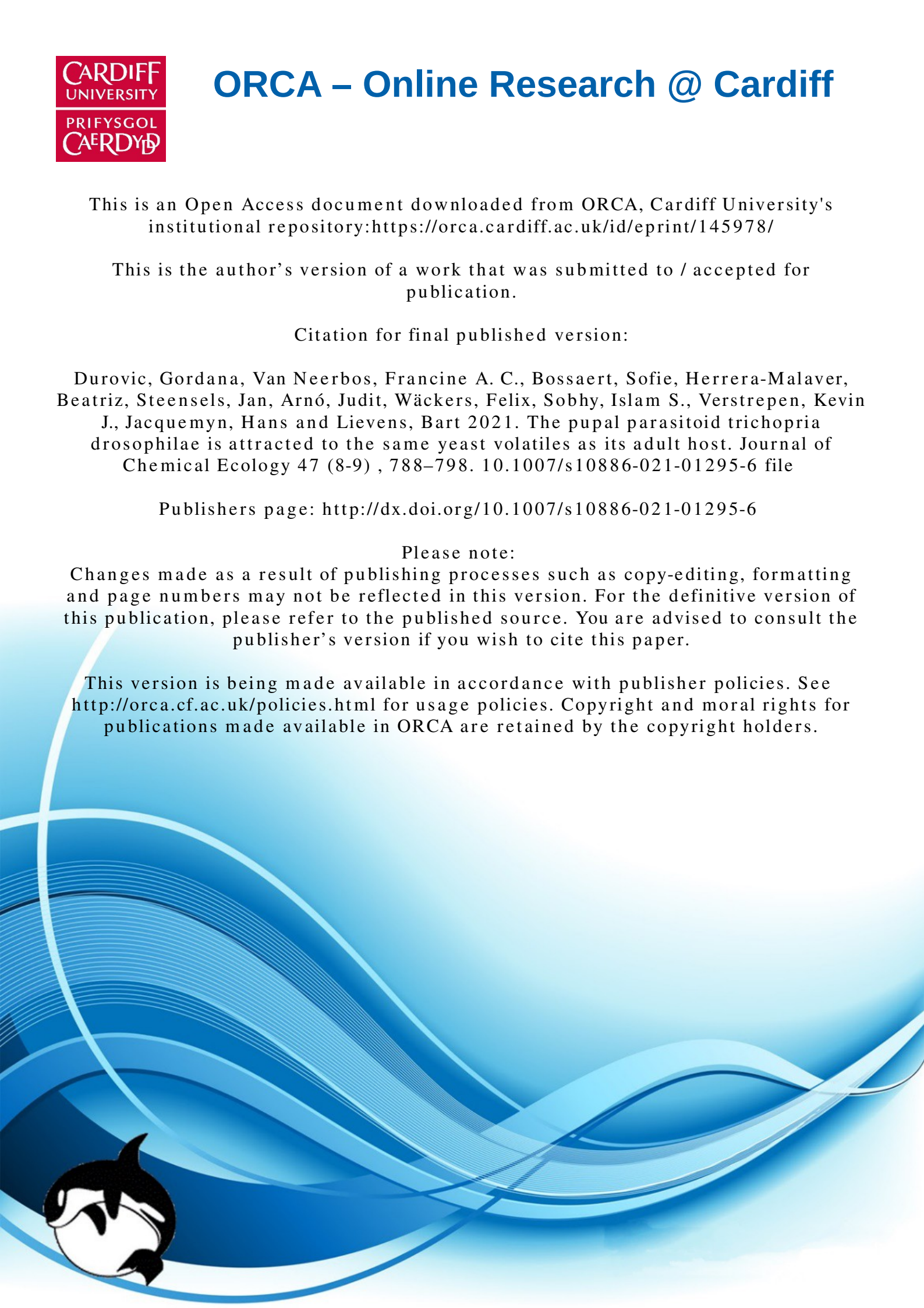

THE PUPAL PARASITOID *TRICHOPRIA DROSOPHILAE* IS ATTRACTED TO THE SAME YEAST VOLATILES AS ITS ADULT HOST

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ABSTRACT - There is increasing evidence that microorganisms, particularly fungi and bacteria, emit volatile compounds that mediate the foraging behaviour of insects and therefore have the potential to affect key ecological relationships. However, to what extent microbial volatiles affect the olfactory response of insects across different trophic levels remains unclear. Adult parasitoids use a variety of chemical stimuli to locate potential hosts, including those emitted by the host's habitat, the host itself and microorganisms associated with the host. Given the great capacity of parasitoids to utilize and learn odours to increase foraging success, parasitoids of eggs, larvae or pupae may respond to the same volatiles the adult stage of their hosts use when locating their resources, but compelling evidence is still scarce. In this study, using *Saccharomyces cerevisiae* we show that *Trichopria drosophilae*, a pupal parasitoid of *Drosophila* species, is attracted to the same yeast volatiles as their hosts in the adult stage, i.e. acetate esters. Parasitoids significantly preferred the odour of *S. cerevisiae* over the blank medium in a Y-tube olfactometer. Deletion of the yeast *ATF1* gene, encoding a key acetate ester synthase, decreased attraction of *T. drosophilae*, while addition of synthetic acetate esters to the fermentation medium restored parasitoid attraction. Bioassays with individual compounds revealed that the esters alone were not as attractive as the volatile blend of *S. cerevisiae*, suggesting that other volatile compounds also contribute to the attraction of *T. drosophilae*. Altogether, our results indicate that pupal parasitoids respond to the same volatiles as the adult stage of their hosts, which may aid them in locating oviposition sites.

Keywords - Acetate esters, behavioral response, *Drosophila*, parasitoid, *Saccharomyces cerevisiae*, *Trichopria drosophilae*, tritrophic interaction

Declarations

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Conflicts of interest/Competing interests

The authors declare that no conflicts of interest exist.

Availability of data and material

Data and materials are available upon request.

Code availability

Codes for statistical analysis were written in R and are available upon request.

Author contributions

BL, HJ and ISS conceived the ideas and designed methodology. GD, FACVN, SB and BM-H collected the data. JS and KJV contributed to equipment and reagents for the VOC analysis. KJV provided the yeast strains, JA and FW provided insects. FACVN, HJ and BL analyzed the data. HJ and BL led the writing of the manuscript. All authors contributed critically to the drafts of this manuscript and gave final approval for publication.

Ethical note

Experimental manipulation of parasitoids occurred according to the common and ethical requirements for animal welfare. All parasitoids were carefully handled during experiments and maintained in the laboratory under appropriate conditions.

Consent to participate

Not applicable

Consent for publication

Consent for publication was granted by all co-authors.

INTRODUCTION

Microorganisms release a wide variety of volatile organic compounds (VOCs), many of which play a crucial role in intra- and inter-kingdom interactions (Schulz-Bohm et al., 2017; Tilocca et al., 2020). Given their high vapor pressure and low molecular weight, microbial volatile organic compounds (mVOCs) can travel far from the point of production through the atmosphere, porous soils and liquids, making them ideal info-chemicals for mediating both short- and long-distance interspecific interactions (Bitas et al., 2013). Although knowledge about the biological and ecological roles of mVOCs is still limited, there is increasing evidence that mVOCs mediate the foraging behaviour of insects and therefore have the potential to affect key ecological relationships (Davis et al., 2013; Leroy et al., 2011).

Recent research has shown that the production of insect-attracting metabolites is a basic and general feature in yeasts (Becher et al., 2018). Yeast volatiles act as semiochemicals that attract insects by signalling the presence of suitable resources such as sugary food or oviposition sites (Davis et al., 2013; Dzialo et al., 2017). Furthermore, the yeasts themselves may provide an important source of dietary proteins to the insects (Begon, 1982; Skorupa et al., 2008). This chemical communication between yeasts and insects is believed to be the driving force of a strong mutualistic relationship, also referred to as the "dispersal–encounter hypothesis" (Madden et al., 2018). Early in their evolution, yeasts evolved biochemical pathways to obtain energy from sugars during which volatile compounds (e.g. ethanol and other alcohols, and fruity acetate esters) are produced as by-products (Dzialo et al., 2017). These volatiles thus signal the presence of sugar (or are energy sources themselves (Ogueta et al., 2010)) and attract sugar-feeding insects which can inadvertently transport the yeasts to another patch of sugar. The otherwise immotile yeasts benefit from getting transported to new habitats, the insects from a free and honest signal indicating an

127 available resource (Becher et al., 2012; Christiaens et al., 2014). Furthermore, the yeasts can use
128 the insects to survive unfavourable environmental conditions like a cold winter (Pozo et al., 2018;
129 Stefanini et al., 2012). The benefits that yeasts might reap from this interaction may go beyond
130 mere dispersal and survival. Low nutrient levels trigger the formation of sexual spores that can
131 survive passage through the insect gut and promote outbreeding and thus genetic variation (Freese
132 et al., 2007).

133 Although the benefits of this mutualistic interaction have become better understood in
134 recent years, very little is known about potential costs for both partners. Both the yeasts and the
135 insects may experience direct and indirect costs related to the interaction. For example, production
136 of certain volatiles may be costly, or some yeast propagules may be killed by feeding insects.
137 Likewise, sugar resources with high yeast densities may have decreased sugar quantities and
138 qualities, or have high alcohol concentrations which may be detrimental for the insects (Bouletreau
139 & David, 1981; Madden et al., 2018). Insects may experience indirect costs when natural enemies
140 are attracted to the same yeast volatiles helping them to find their hosts. Parasitoids (i.e. insects
141 whose larvae feed and develop within or on the bodies of other arthropods, eventually killing them)
142 use a variety of chemical cues to identify and locate potential hosts, including those emitted by the
143 host's habitat (Vet et al., 1984), host by-products (e.g. frass, Agelopoulos et al., 1995), the host
144 itself (Jumean et al., 2009), and organisms living in close association with the host (Sullivan et al.,
145 2000) or its habitat (Goelen et al., 2020). Given the extraordinary capacity of parasitoids to detect
146 and associate chemical information with a reward (Turlings et al., 1992; Vet et al., 1995; Vet, et
147 al., 2002), it is reasonable to assume that they may exploit the same volatiles as their hosts to
148 optimize foraging behaviour, but compelling evidence is still lacking. There are studies showing
149 that egg and larval parasitoids of Tephritid fruit flies are not only attracted to host-containing

infested, fermenting fruits (Carrasco et al., 2005), but also respond to odours from healthy, undamaged fruits which their adult hosts prefer for egg deposition (Altuzar et al., 2004; Eben et al., 2000; Leyva et al., 2012).

The objective of this study was to test the hypothesis that pupal parasitoids respond to the same yeast volatile compounds that the adult stage of their hosts use to locate suitable resources. Specifically, we evaluated the olfactory response of *Trichopria drosophilae* to acetate ester production in *Saccharomyces cerevisiae*. *Trichopria drosophilae* is a solitary, cosmopolitan pupal endoparasitoid that attacks many species of Drosophilidae (Carton et al., 1986; Yi et al., 2020), and is a very promising candidate for augmentative biocontrol of the invasive pest species *Drosophila suzukii*, for which it is already commercially available (Gabarra et al., 2015; Mazzetto et al., 2016). *Saccharomyces cerevisiae* was chosen as it is frequently used to study *Drosophila* – yeast interactions (Arguello et al., 2013; Christiaens et al., 2014; Ha et al., 2009; Murgier et al., 2019; Scheidler et al., 2015), and has been shown to produce acetate esters, particularly ethyl acetate and isoamyl acetate, that attract adult *Drosophila melanogaster* flies (Christiaens et al., 2014). To test the hypothesis that acetate esters also drive parasitoid attraction, Y-tube olfactometer bioassays were performed using cell-free fermentation media of a wild type *S. cerevisiae* strain and two mutants thereof in which the production of acetate esters was either reduced or enhanced. Studying mutant organisms that have acquired changes or deletions in their genome has the advantage of determining gene functions in a very efficient way, particularly in a community context to unravel ecological functions of genes without affecting other genome features (Christiaens et al., 2014). Our study not only provides new insights in the interactions that take place between different trophic levels, but may also lead to new tools that enhance the biocontrol efficacy of *T. drosophilae* against *D. suzukii* (Holighaus & Rohlfs, 2016).

MATERIALS AND METHODS

Study Organisms. Three strains of *S. cerevisiae* (Basidiomycota: Saccharomycetaceae) that differed in acetate ester production were used in this study. These included the wild type strain Y182 (WT-Y182) and two *ATF1* mutants of Y182, one in which the *ATF1* gene was deleted (DEL-KV3734) and one in which the *ATF1* gene is overexpressed (OE-KV3735) (Christiaens et al., 2014). The *ATF1* gene is one of two genes (*ATF1* and *ATF2*) encoding an alcohol acetyl transferase in *S. cerevisiae*, a key enzyme in the production of acetate esters from acetyl-coenzyme A and alcohol. Among these two genes, *ATF1* controls the bulk of the acetate ester formation in *S. cerevisiae* (Lilly et al., 2000; Verstrepen et al., 2003). Strain Y182 was originally isolated from a vineyard and has an average production of acetate esters (Christiaens et al., 2014). In the *ATF1* deletion mutant, both *ATF1* alleles were deleted using deletion cassettes based on pUG6, conferring resistance to either hygromycin B or G-418 disulfate. In the overexpression mutant, the native promoter is replaced by the strong, constitutive *TEF1* promoter, introduced using pYM-N18, which contains the KanMX antibiotic resistance marker for mutant selection. Markers were removed through the Cre/LoxP technique using pSH65. Deletions as well as marker removal were confirmed through (lack of) growth on selective media, as well as PCR (Christiaens et al., 2014). Strains were stored at −80°C in yeast extract peptone dextrose broth (YPDB; Difco, Le Pont-de-Claix, France) containing 37.5 % glycerol.

Trichopria drosophilae (Hymenoptera: Diapriidae) is a widespread solitary pupal endoparasitoid of *Drosophila* spp. (Carton et al., 1986; Yi et al., 2020). Adult females of *T. drosophilae* are commonly encountered in the habitat of *Drosophila* flies, seeking *Drosophila* pupae for oviposition. The vast majority of its *Drosophila* hosts lay eggs in damaged, overripe or

196 decaying fruit, where alcoholic fermentation abundantly occurs (Phaff & Starmer, 1987). Unlike
197 other species, female *D. suzukii* prefers to lay eggs in ripening fruit during the early stages of
198 fermentation (Walsh et al., 2011). When a suitable pupa is found, the parasitoid lays an egg in the
199 host hemocoel. The *T. drosophilae* larvae then feed on the tissues of the host, which are
200 subsequently killed (Carton et al., 1986).
201
202 ***Fermentations.*** Yeast fermentations were performed as outlined in Christiaens et al. (2014).
203 Briefly, fermentations were started by inoculating the yeasts from a YPD 2% plate into a test tube
204 with 5 mL YPDB 2%, and incubating the tubes at 30°C on a rotary shaker at 100 rpm. After one
205 overnight, 300 µL was inoculated into 50 mL YPDB 4% in a 250-mL Erlenmeyer flask, which
206 was then sealed with a water lock and incubated overnight at 30°C (100 rpm). Subsequently, the
207 OD_{600} was measured and the preculture was used to inoculate a 250-mL Erlenmeyer flask
208 containing 150 mL YPD 10% at a final OD_{600} of 0.5. Flasks were sealed with a water lock and
209 fermentations were allowed to continue for seven days at 30°C while shaking at 100 rpm.
210 Afterwards, fermentation media were spun down at 4,500 g for 5 min and subsequently filtered
211 (pore size 0.22 µm; Nalgene, Waltham, MA, USA) to obtain cell-free cultures. Obtained media
212 were then stored in small aliquots in sealed sterile dark glass vials (Fagron, Nazareth, Belgium) at
213 -20 °C until further use (VOC analysis and olfactometer bioassays). For each yeast strain, three
214 independent fermentations were performed, and a medium without yeast inoculation was included
215 as a control (sterility of the blank medium was confirmed after the incubation period).
216
217 ***VOC Analysis.*** To detect and determine the concentrations of various aroma compounds
218 associated with yeast fermentations, including higher alcohols and esters, a Headspace Gas

Chromatography system coupled with a Flame Ionization Detector (HS-GC-FID) was used. A headspace autosampler (PAL system, CTC analytics, Switzerland) was used and the GC contained a DB-WAXether column (length: 30 m; internal diameter: 0.32 mm; layer thickness: 0.50 µm) (Shimadzu, Kyoto, Japan). Nitrogen was used as the carrier gas. For each sample, 5 mL was put in a 20 mL glass vial containing 1.75 g of sodium chloride. The vials were immediately closed and kept at -20°C until their analysis in order to minimize evaporation and loss of volatile compounds. Prior VOC analysis, vials were thawed at room temperature for 30 min. During the chromatographic run, each vial with sample was heated at 70°C with continuous agitation (500 rpm) for 25 min, inside a heater unit of the autosampler. After this incubation period, 1 ml of the headspace sample was injected into the GC inlet using a 2.5-ml headspace syringe (Hamilton, Switzerland). The injector and FID were both kept 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^{-1}, followed by a second ramp of 4°C min^{-1} until 200°C. The temperature was then held for 3 min at 200°C and subsequently increased by 4°C min^{-1} until a temperature of 230°C was reached. Results were analyzed with the Shimadzu GCSolution software version 2.43.00. Stock solutions of authentic volatile standards (18 compounds) were prepared in ethanol. For all detected compounds, calibration curves were made prior to sample analysis. To this end, solutions of the target compounds were prepared in water by spiking the compounds while keeping 5% ethanol solution to obtain a 9-point calibration curve.

Olfactometer Bioassays. To investigate the olfactory response of *T. drosophilae* to the different fermentation media, naïve females (inexperienced to yeast smell and food; less than 24h old) were tested in a Y-tube olfactometer bioassay. Parasitoids were obtained in the form of parasitized *D.*

242 *suzukii* pupae from Bioplanet (Cesena, Italy). Upon receipt, parasitized pupae were placed in a
243 nylon insect cage (20×20×20 cm, BugDorm, MegaView Science Co., Ltd., Taichung, Taiwan) and
244 kept under controlled conditions (22°C, 70% relative humidity and a 16:8-h light:dark
245 photoperiod) until parasitoid emergence. One hour prior testing, the parasitoid cage was brought
246 from the rearing chamber to the olfactometer laboratory for acclimatization. The olfactometer, a
247 glass Y-tube (base: 20 cm; arms:12 cm with a 60° angle at the Y-junction; inner diameter: 1.5 cm)
248 connected to an air pump producing an unidirectional air flow of 400 mL min^{-1} from the arms to
249 the base, was put on a table that was homogeneously illuminated by four high frequency 24W T5
250 TL-fluorescent tubes with a 96% colour representation of true day light at a height of 0.45 m
251 (Goelen et al., 2020). To improve parasitoid responsiveness, the Y-tube was mounted at a 20°
252 incline stimulating insect movement towards the odour source. Additionally, to eliminate any
253 visual cues that could affect parasitoid response, the olfactometer was surrounded by white
254 curtains.

255 In a first set of experiments, parasitoid behaviour was evaluated by simultaneous
256 application of two odours in different conditions, including (i) odour of the blank medium vs water,
257 (ii) odour of the three yeast strains vs blank medium, (iii) odour of the *ATF1* deletion mutant
258 supplemented with acetate esters vs blank medium, and (iv) odour of the *ATF1* deletion mutant
259 supplemented with acetate esters vs the wild type strain or the *ATF1* overexpression mutant.
260 Experiments were performed with 1000× diluted cell-free fermentation medium as preliminary
261 experiments revealed suboptimal responses with higher concentrations (data not shown). The
262 supplemented samples of the *ATF1* deletion mutant contained either ethyl acetate (99.5%, Acros
263 Organics), isoamyl acetate (>95%, Sigma-Aldrich, Saint Louis, MO, USA) or phenylethyl acetate
264 (98%, Sigma-Aldrich) at concentrations that matched the ones present in the diluted media from

265 the wild type yeast (0.03 ppm, 0.0007 ppm and 0.0001 ppm, respectively) or the overexpression
266 mutant (0.1 ppm, 0.003 ppm and 0.0007 ppm, respectively), or combinations of these compounds
267 (Table 1). For each test, 150 µL medium was loaded on a filter paper (37 mm; Macherey-Nagel,
268 Düren, Germany) and subsequently put in one of the olfactometer odour chambers. In a second set
269 of experiments, parasitoid response was evaluated by subjecting the parasitoids to two
270 concentrations of ethyl acetate (0.1 ppm and 1 ppm), isoamyl acetate (0.001 ppm and 0.01 ppm)
271 or phenylethyl acetate (0.001 ppm and 0.01 ppm) dissolved in diethyl ether vs diethyl ether. Again,
272 150 µL was loaded on a filter paper, and 30 s later the filters were put in the odour chambers of
273 the olfactometer set-up.

274 All experiments were conducted with 60 female individuals, which were released in 12
275 cohorts of five individuals at the base of the olfactometer. Olfactory response was evaluated 10
276 min after their release. Wasps that had passed a set line in of one of the olfactometer arms (1 cm
277 from the Y-junction) at the time of evaluation were considered to have chosen the odour source
278 presented by that olfactometer arm (Goelen et al., 2020). All other parasitoids were considered as
279 non-responding individuals and were eliminated from statistical analysis. For every release, new
280 parasitoid females were used. To avoid positional bias, the arms of the Y-tube olfactometer were
281 flipped 180° every six releases. At the same time, the Y-tube was also renewed by a clean tube.
282 To maintain a high level of odour release, filter papers were replaced with fresh filter papers with
283 150 µL of the tested medium every two runs. At the end of the experiment, all olfactometer parts
284 were thoroughly cleaned with tap water, distilled water, acetone and finally pentane. After solvents
285 had evaporated, the glass parts were placed overnight in an oven at 150°C. All bioassays were
286 conducted at 23 ± 1°C and 65 ± 5% RH between 09h00 and 16h00. As the VOC composition of

287 the three biological replicates was highly similar, olfactory response was determined for one of the
288 three biological replicates.
291
292 ***Statistical Analysis.*** Differences in VOC profiles between the different yeast strains were
293 visualized by a principal component analysis (PCA) using the concentrations of the detected
294 volatiles as dependent variables. Additionally, a non-parametric multivariate analysis of variance
295 (*PERMANOVA*) was used to investigate whether the VOC profiles differed between the different
296 yeast strains and the blank medium. We performed 9999 permutations to assess the significance
297 of the observed *pseudo F*-statistic. All calculations were performed using the adonis function of
298 the vegan package (Oksanen et al., 2013) in R. Parasitoid olfactory response was analyzed using
299 a *Generalized Linear Mixed Model (GLMM)* based on a binomial distribution with a logit link
300 function (logistic regression) using the test treatment ((supplemented) fermentation medium or
301 compound) as fixed factor (performed in R with the 'glmer' function from the lme4 package).
302 Each release of one cohort of five parasitoid females served as a replicate (n = 12). To adjust for
303 overdispersion and to prevent pseudo-replication, the release of each cohort was included in the
304 model as a random factor. The number of parasitoids choosing the treatment side or the control in
305 each cohort was entered as response variable. To examine the preference of the parasitoids, we
306 tested the null hypothesis (H_0) that parasitoids showed no preference for any olfactometer arm (i.e.
307 50:50 response) by testing H_0: logit = 0, which equals a 50:50 distribution. In addition, an analysis
308 of variance *Type III Wald chi-square test* was performed on the *GLMM* to determine if there was
309 an overall difference between the olfactory responses for the different treatments. A significance
310 level of $\alpha = 0.05$ was used to determine significant attraction or repellence. The *GLMM* analysis
311 was performed in R (R Core Development Team, 2019).

RESULTS

VOC Profiles. Principal component analysis (PCA) showed a clear separation of the obtained VOC profiles between the three yeast strains and the blank medium. The first principal component (PC1) accounted for 60.6% of the total variation, the second component (PC2) for 21%. Yeast strains were separated from the blank medium along the first axis, and were separated from each other mainly along the second axis (Fig. 1). The overexpression mutant was characterized by high concentrations of acetate esters (e.g. ethyl acetate, isoamyl acetate, isobutyl acetate and phenylethyl acetate). The deletion mutant was characterized by relatively high concentrations of 1-hexanol (Fig. 1). *PERMANOVA* confirmed that the VOC profiles were significantly different (*pseudo-F* = 62.2, $P < 0.0001$). Univariate analyses of variance indicated that the levels of various acetate esters (ethyl acetate, isoamyl acetate, isobutyl acetate, phenylethyl acetate, and propyl acetate) were significantly reduced or even completely abolished in the medium of the *ATF1* deletion mutant, whereas they were abundantly produced by the overexpression mutant (Table 1). Likewise, the VOC blend of the deletion mutant was enriched in a number of alcohols which are acetate ester precursors (isoamyl alcohol, isobutanol, 1-hexanol and 1-propanol). No significant differences in the concentration of other volatile compounds were observed among the three yeast strains (Table 1).

Parasitoid Olfactory Response. Olfactory response of *T. drosophilae* varied significantly between the three yeast strains (*GLMM;* $\chi^2 = 13.5936$; df = 2; $P = 0.001$; Fig. 2). Parasitoids had a significant preference for the wild type strain ($P < 0.001$) and the *ATF1* overexpression mutant ($P < 0.001$), while a neutral response was obtained for the *ATF1* deletion mutant (Fig. 2). To verify whether

335 the observed behavioral differences were due to the lower acetate ester levels produced by the
336 *ATF1* deletion mutant, the *ATF1* deletion mutant medium was supplemented with three important
337 acetate esters affected by *ATF1* deletion (Table 1), i.e. ethyl acetate, isoamyl acetate or phenylethyl
338 acetate, and combinations of these compounds. Compounds were added in concentrations that
339 matched concentrations in the fermentation medium of the wild type strain or the overexpression
340 mutant. When performing the preference tests with the supplemented medium pitted against blank
341 medium, attraction of the parasitoids was restored, especially when the medium was supplemented
342 with ethyl acetate (Fig. 3). When the supplemented media were tested against the media of the
343 wild type strain (Fig. 4A) or the overexpression mutant (Fig. 4B), no significant difference in
344 preference for either medium was observed, supporting the central role of these esters in the altered
345 attraction phenotype. When testing the individual esters at two concentrations resembling those in
346 the original attractive media, no statistically significant effects were observed (Fig. 5).
348
349 DISCUSSION
350 In this study, we have shown that *T. drosophilae* is attracted to the same volatile compounds to
351 which its host in the adult stage is attracted, particularly acetate esters. Parasitoids significantly
352 preferred the odour of the wildtype *S. cerevisiae* strain or its *ATF1* overexpressing mutant over the
353 blank medium in a Y-tube olfactometer. By contrast, deletion of the *ATF1* gene decreased
354 attraction of *T. drosophilae*, while simple addition of synthetic acetate esters to the fermentation
355 medium restored parasitoid attraction. Previous studies have shown that fruit flies in the family
356 Drosophilidae strongly respond to acetate esters to orient themselves towards suitable food or
357 oviposition sites. Ethyl acetate and isoamyl acetate have been found to attract *D. melanogaster*
358 (Christiaens et al., 2014). Isoamyl acetate is also responsible for attraction of the closely related

359 species *D. simulans*, but attraction seems largely dependent on the background chemical matrix
360 (Günther et al., 2015). Likewise, *D. suzukii* is strongly attracted to isobutyl acetate and isoamyl
361 acetate (Revadi et al., 2015; Scheidler et al., 2015). Moreover, neurobiological research has shown
362 that *Drosophila* antennae possess specific receptors for acetate esters, indicating that they have
363 evolved specific mechanisms to detect and respond to the fruity yeast esters (Vosshall et al., 2000;
364 Hallem & Carlson, 2006; Hallem et al., 2004). Notably, some plants have taken advantage of the
365 drosophilids' ability to detect acetate esters. For example, the black calla lily (*Arum palaestinum*)
366 has evolved to mimic yeast fermentation volatiles specifically by producing 2,3-butanediol acetate
367 and acetoin acetate to lure drosophilids for pollination (Stökl et al., 2010).

368 In addition to *T. drosophilidae* other fruit fly parasitoids have been shown to respond
369 positively to acetate esters, including the *Drosophila* larval parasitoid *Leptopilina heterotoma*
370 (Dicke et al., 1983) and *Biosteres longicaudatus*, a parasitoid of the Caribbean fruit fly
371 (*Anastrepha suspensa*) (Greany et al., 1977). More generally, previous studies have shown that
372 various insect species are attracted to acetate esters (Davis et al., 2013), suggesting that responding
373 to acetate esters may be a general trait in insects. This is further supported by studies that have
374 shown that receptors for acetate esters are widespread in insects (Galizia et al., 1999; Zhao &
375 McBride, 2020). For example, the dusky sap beetle *Carpophilus lugubris* is attracted to ethyl
376 acetate, amongst some other tested volatiles (Lin & Phelan, 1991), while Alm et al. (1985)
377 employed butyl acetate to attract *Glischrochilus* beetles. Likewise, the aphid parasitoid *Aphidius*
378 *ervi* was found to be attracted by the wild type *S. cerevisiae* strain used in this study (Y182) (Sobhy
379 et al., 2018).

380 Insect foraging driven by yeast volatiles may be influenced by the yeast species and the
381 basal growth medium as the precise composition of the VOC blends largely depends on the yeast

382 species and the precursors available (Dzialo et al., 2017; Gonzalez et al., 2019). Several yeast
383 species have been isolated from the body of *Drosophila* flies and their food and oviposition sites,
384 including members of *Candida*, *Hanseniaspora* and *Pichia* (Buser et al., 2014; Christiaens et al.,
385 2014; Hamby et al., 2012; Phaff & Knapp, 1956; Quan & Eisen, 2018). However, it has been found
386 that the vast majority of these yeasts produce aroma-active esters like ethyl acetate and isoamyl
387 acetate (Christiaens et al., 2014; Scheidler et al., 2015), and that fruit flies did not respond
388 differentially to the yeast species (Quan & Eisen, 2018). It has been hypothesized that these yeast
389 species employ the same biosynthetic pathway as *S. cerevisiae* for their active dispersal by the
390 fruit flies (Christiaens et al., 2014). However, acetate esters are not exclusively produced by
391 *Drosophila*-associated yeasts, they generally occur in yeast fermentations (Dzialo et al., 2017).
392 Furthermore, these volatile esters are produced by many plant species contributing to the
393 characteristic aroma of several fruits and flowers (Macku & Jennings, 1987; Shalit et al., 2003;
394 Schwab et al., 2008). This would make acetate esters unreliable as a signal, leading parasitoids to
395 many habitats lacking hosts. However, chemical stimuli can achieve more specificity in several
396 ways, e.g. through a specific concentration or a specific combination of compounds (in specific
397 concentrations and ratios) (Bruce et al., 2005; Goelen et al., 2021; Mumm & Hilker, 2005; Olson
398 et al., 2012; Takemoto & Takabayashi, 2015). In our tests, none of the individual compounds tested
399 could compete with the VOC blend of *S. cerevisiae*, suggesting that other yeast volatiles also
400 contribute to attraction of *T. drosophilae*. Further research should therefore focus on testing other
401 (concentrations and combinations of) fermentation products, or other volatile compounds
402 associated with the host habitat, in combination with acetate esters to fully understand the
403 attractivity of yeast odours for *T. drosophilae* or its foraging behaviour in general.

Altogether, our results show that *T. drosophilae* is attracted to the same volatile cues that are exploited by the adult stage of its host. Whereas the mutualistic interaction between yeasts and fruit flies has been well documented over the last few years, our results indicate that the same yeast volatiles signaling appropriate resources for adult *Drosophila* flies may also give away the presence of its offspring to parasitoids, potentially disrupting this mutualistic relation.

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TABLES

Table 1. Microbial volatile profiles (ppm)[*] of the three yeast strains used in this study[†]

Compound[§]	N°[#]	Blank[$]	WT-Y182	DEL-KV3734	OE-KV3735	*P* value
Ethyl acetate	**1**	**1.59 ± 0.01^{a}**	**28.16 ± 0.68^{c}**	**15.36 ± 0.86^{b}**	**100.16 ± 2.30^{d}**	**<0.001**
Ethyl butyrate	2	ND^{a}	0.23 ± 0.01^{b}	0.23 ± 0.01^{b}	0.22 ± 0.01^{b}	<0.001
Ethyl isobutyrate	3	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.59
Ethyl-2-methylbutyrate	4	ND^{a}	0.23 ± 0.06^{b}	0.15 ± 0.05^{ab}	0.21 ± 0.06^{b}	0.04
Ethyl isovalerate	5	0.01 ± 0.01	0.07 ± 0.07	ND	0.49 ± 0.49	0.49
Ethyl hexanoate	6	ND^{a}	0.05 ± 0.00^{b}	0.05 ± 0.00^{b}	0.04 ± 0.00^{b}	<0.001
Ethyl octanoate	7	0.05 ± 0.00^{a}	0.10 ± 0.00^{b}	0.11 ± 0.00^{b}	0.11 ± 0.00^{b}	<0.001
Ethyl decanoate	8	0.02 ± 0.00^{a}	0.10 ± 0.01^{b}	0.12 ± 0.01^{b}	0.13 ± 0.01^{b}	<0.001
1-Propanol	9	0.66 ± 0.66^{a}	49.26 ± 1.09^{b}	53.76 ± 1.06^{c}	47.15 ± 0.78^{b}	<0.001
Propyl acetate	**10**	**ND^{a}**	**0.01 ± 0.00^{b}**	**ND^{a}**	**0.01 ± 0.00^{b}**	**0.006**
Isobutanol	11	ND^{a}	80.66 ± 1.98^{c}	81.03 ± 1.54^{c}	69.94 ± 1.10^{b}	<0.001
Isobutyl acetate	**12**	**0.01 ± 0.00^{a}**	**0.16 ± 0.01^{b}**	**0.03 ± 0.00^{a}**	**0.85 ± 0.05^{c}**	**<0.001**
Isoamyl alcohol	13	ND^{a}	128.24 ± 5.62^{b}	151.59 ± 2.76^{c}	117.93 ± 4.71^{b}	<0.001
Isoamyl acetate	**14**	**0.30 ± 0.00^{a}**	**0.74 ± 0.02^{b}**	**0.36 ± 0.01^{a}**	**3.19 ± 0.22^{c}**	**<0.001**
1-Hexanol	15	ND^{a}	ND^{a}	0.03 ± 0.00^{b}	ND^{a}	<0.001
Hexyl acetate	**16**	**ND^{a}**	**0.01 ± 0.00^{b}**	**0.01 ± 0.00^{b}**	**0.01 ± 0.00^{b}**	**<0.001**
Phenylethyl alcohol	17	1.59 ± 0.48^{a}	13.26 ± 2.85^{b}	11.70 ± 0.44^{b}	13.17 ± 3.11^{b}	0.01
Phenylethyl acetate	**18**	**ND^{a}**	**0.11 ± 0.00^{b}**	**0.00 ± 0.00^{a}**	**0.72 ± 0.08^{c}**	**<0.001**

[*]Average of three biological replicates ± SEM. Values with different superscript letters in a row are significantly different ($P < 0.05$). ND, not detected.

[†]Tested strains included the wild type *Saccharomyces cerevisiae* strain Y182 (WT-Y182), its *ATF1* deletion mutant (DEL-KV3734) and its *ATF1* overexpression mutant (OE-KV3735).

[§]Acetate esters are displayed in bold.

[#]Numbers refer to the vectors shown in Fig. 1.

[$]Non-inoculated cultivation medium.

FIGURE CAPTIONS

Figure 1. Principal component analysis (PCA) of the volatile profiles produced by the three *Saccharomyces cerevisiae* strains investigated, including the wild type strain Y182 (WT-Y182), the *ATF1* deletion mutant (DEL-KV3734) and the *ATF1* overexpression mutant (OE-KV3735), and the non-inoculated cultivation medium (blank). The closer the dots together, the more similar the VOC profiles. Vectors (red arrows) represent the loadings for each compound. The length of the arrows approximates the variance of the variables, whereas the angels between them approximate their correlations. Numbers refer to the different compounds measured: (1) ethyl acetate; (2) ethyl butyrate; (3) ethyl isobutyrate; (4) ethyl-2-methylbutyrate; (5) ethyl isovalerate; (6) ethyl hexanoate; (7) ethyl octanoate; (8) ethyl decanoate; (9) 1-propanol; (10) propyl acetate; (11) isobutanol; (12) isobutyl acetate; (13) isoamyl alcohol; (14) isoamyl acetate; (15) 1-hexanol; (16) hexyl acetate; (17) phenylethyl alcohol; and (18) phenylethyl acetate. All analyses were performed on cell-free fermentation media (three biological replicates).

Figure 2. Olfactory response of adult *Trichopria drosophilae* females (tested in 12 cohorts of five females) when given the choice between the odor of the non-inoculated cultivation medium (blank) and one of the three *Saccharomyces cerevisiae* strains investigated. Tested yeast strains included the wild type strain Y182 (WT-Y182), the *ATF1* deletion mutant (DEL-KV3734) and the *ATF1* overexpression mutant (OE-KV3735). The used parasitoids were naïve (*i.e.* inexperienced to smell and food). Non_responders were eliminated from statistical analysis. Error bars represent standard error of the mean. Mean parasitoid responsiveness was 72.8%.

685 **Figure 3.** Olfactory response of adult *Trichopria drosophilae* females (tested in 12 cohorts of five
686 females) when given the choice between the odor of the non-inoculated cultivation medium (blank)
687 and the *Saccharomyces cerevisiae ATF1* deletion mutant (DEL-KV3734) fermentation medium
688 supplemented with one or more acetate esters at concentrations that matched the levels in the
689 medium of the wild type strain (WT-Y182) (A) and the *ATF1* overexpression mutant (OE-
690 KV3735) (B). Tested esters included ethyl acetate (EA), isoamyl acetate (IA) and phenylethyl
691 acetate (PA). The used parasitoids were naïve (*i.e.* inexperienced to smell and food). Non-
692 responders were eliminated from statistical analysis. Error bars represent standard error of the
693 mean. Mean parasitoid responsiveness was 71.7%.
694
695 **Figure 4.** Olfactory response of adult *Trichopria drosophilae* females (tested in 12 cohorts of five
696 females) when given the choice between the odor of the wild type *Saccharomyces cerevisiae* strain
697 (WT-Y182) or the *ATF1* overexpression mutant (OE-KV3735) and the *ATF1* deletion mutant
698 (DEL-KV3734) fermentation medium supplemented with one or more acetate esters at
699 concentrations that matched the levels in the medium of the wild type strain (A) or the *ATF1*
700 overexpression mutant (B). Tested esters included ethyl acetate (EA), isoamyl acetate (IA) and
701 phenylethyl acetate (PA). The used parasitoids were naïve (*i.e.* inexperienced to smell and food).
702 Non-responders were eliminated from statistical analysis. Error bars represent standard error of
703 the mean. Mean parasitoid responsiveness was 69.2%.
704
705 **Figure 5.** Olfactory response of adult *Trichopria drosophilae* females (tested in 12 cohorts of five
706 females) when given the choice between two different concentrations of ethyl acetate (0.1 ppm
707 and 1 ppm), isoamyl acetate (0.001 ppm and 0.01 ppm) and phenylethyl acetate (0.001 ppm and

708 0.01 ppm) dissolved in diethyl ether and a diethyl ether blank in a Y-tube olfactometer bioassay.
709 The used parasitoids were naïve (*i.e.* inexperienced to smell and food). Non-responders were
710 eliminated from statistical analysis. Error bars represent standard error of the mean. Mean
711 parasitoid responsiveness was 67.4%.