THE CONTEXT OF CHEMICAL COMMUNICATION DRIVING A MUTUALISM

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Abstract- Recent work suggests that *Drosophila* and *Saccharomyces* yeasts may establish a mutualistic association, and that this is driven by chemical communication. While individual volatiles have been implicated in the attraction of *D. melanogaster*, the semiochemicals affecting the behavior of the sibling species *D. simulans* are less well characterised. Here, we comprehensively scrutinize a broad range of volatiles produced by attractive and repulsive yeasts to experimentally evaluate the chemical nature of communication between these species. When grown in liquid or on agar-solidified grape juice, attraction to *S. cerevisiae* was primarily driven by 3-methylbutyl acetate (isoamyl acetate) and repulsion by acetic acid, a known attractant to *D. melanogaster* (also known as vinegar fly). Using T-maze choice tests and synthetic compounds we show that these responses were strongly influenced by compound concentration. Moreover, the behavioral response is further impacted by the chemical context of the environment. Thus, chemical communication between yeasts and flies is complex, and is not simply driven by the presence of single volatiles, but modulated by compound interactions. The ecological context of chemical communication needs to be taken into consideration when testing for ecologically realistic responses.

Key Words- Chemical communication, *Drosophila*, Fermentation, Mutualism, *Saccharomyces.*

INTRODUCTION

Chemical communication is the most ancient and widespread form of information transfer among organisms (Haldane 1955). As with other forms of two-way communication, such as sight and sound, chemical communication can influence behavior if the sender and receiver inherently and/or through learning 'agree' upon a signal-response relationship (Bergström 2008). True signals are directed and intentional and are thought to have evolved from unintentional precursors (cues), such as metabolic waste products (Steiger et al. 2011, Weiss et al. 2013).

It has long been known that *Drosophila* is attracted to fermenting yeasts (Dobzhansky et al. 1956), which produce a range of volatile metabolites, especially during fermentation. These volatiles have been most well studied for *Saccharomyces cerevisiae*, as this species is both a research model and a key microbe in the production of wine and beer where aroma-active fermentation volatiles are major contributors to flavour (Styger et al. 2011; Cordente et al. 2012). However, the biological role of yeast volatile production remains elusive (Saerens et al. 2008). Recent work demonstrates that yeast volatiles might act as semiochemicals mediating the attraction of insect vectors (Becher et al. 2012; Buser et al 2014; Christiaens et al. 2014; Palanca et al. 2013; Witzgall et al. 2012). It is not only *Drosophila* that derives fitness benefits from accessing yeast-infested fruits (Anagnostou et al. 2010; Becher et al. 2012); insect attraction has also been shown to be selectively advantageous for yeasts in terms of increased dispersal (Buser et al. 2014; Christiaens et al. 2014). Experimental work suggests that: 1) the production of acetates by yeast can mediate attraction of *Drosophila* (Christiaens et al. 2014); 2) there is variance in attraction among different yeast species and genotypes of *S. cerevisiae* (Buser et al. 2014; Palanca et al. 2013); 3) attraction correlates with yeast dispersal both in the laboratory (Buser et al. 2014; Christiaens et al. 2014) and in

the field (Buser et al. 2014); 4) increased attraction by yeasts is associated with increased *Drosophila* fecundity in fruits, demonstrating that volatile emission by attractive *Saccharomyces* initiates a mutualism with *Drosophila* (Buser et al. 2014).

Recent research in this area has primarily focussed on the receptors involved in insect olfaction and the volatiles that activate them using *D. melanogaster* as a model species. Systematic characterisation of these receptors demonstrates that *D. melanogaster* is capable of sensing at least 100 volatiles (Hallem and Carlson 2006). A number of common yeast fermentation products, such as ethanol, acetic acid, ethyl acetate, 2-phenylethanol, 3-hydroxy-2-butanone (acetoin), 3-methylbutanol (isoamyl alcohol) and 3-methylbutyl acetate (isoamyl acetate) have been implicated in the attraction of *D. melanogaster* (Becher et al. 2012, Hutner et al. 1937, Joseph et al. 2009), which has much of its sensory apparatus tuned to volatiles produced by yeasts, especially esters (Hallem and Carlson 2004; Hallem and Carlson 2006; Vosshall and Stocker 2007). Indeed, when yeasts' ability to synthesise acetates is compromised, *D. melanogaster* attraction is significantly affected (Christiaens et al. 2014). While this research has been a significant step forward, such a gross change in volatile production capability might not reflect the complex ecological subtleties of the drivers of this interaction in nature.

Drosophila simulans belongs to the same subgroup as *D. melanogaster* (Drosophila 12 Consortium 2007), and is known to form hybrids and live in sympatry with its evolutionary sibling (Capy and Gibert 2004). One study (Stökl et al. 2010) describes the chemical drivers of deceptive pollination attraction of *D. melanogaster* and *D. simulans* to Solomon's Lily (*Arum palaestinum),* and suggests attraction is mediated by a more complex bouquet of at least six compounds (2,3-butanediol acetate, acetoin acetate, hexyl acetate, ethyl hexanoate, 2-phenylethyl acetate, 2-phenylethanol). The most parsimonious hypothesis would be that the chemical language shaping yeast-fly mutualism is "simple" and mediated by either a single

semiochemical or compound class, such as acetates. An alternate hypothesis might be that attraction is “complex” or multifactorial and context specific, perhaps comprising blends of behaviorally active volatiles (Becher et al. 2012; Stökl et al. 2010). Consequently, the inherent information of a chemical message would not only be contingent upon the chemical nature of the volatiles, but also on their relative abundance, and interaction with other semiochemicals and the background chemical matrix.

Buser et al. (2014) assayed the behavioral response of *D. simulans* to 100 genetically and ecologically diverse strains of *S. cerevisiae* and demonstrated a mutualistic association with the *S. cerevisiae* isolate ‘fly_KR_78.3’, which is attractive to this species, but not with the ‘DBVPG6044’ isolate (Liti et al. 2009), which is repulsive. Here we analyze the volatile profiles of these attractive and repulsive yeast isolates and experimentally evaluate whether the mutualistic association between *S. cerevisiae* and *D. simulans* is driven by simple or more complex forms of chemical communication.

METHODS AND MATERIALS

Study organisms

The *D. simulans* employed here originated from a natural vineyard population near Auckland, New Zealand, and is the same isofemale *D. simulans* line used by Buser et al. (2014). We follow Buser et al (2014) and assay the interaction between yeast and flies when grown in liquid and solidified (2:1 with 20% agar) Sauvignon Blanc grape juice (derived from Marlborough, New Zealand), sterilized with 400 µL dimethyl dicarbonate (Sigma-Aldrich; dissolved in 800 µL ethanol) per litre.

111 Flies were kept in polypropylene *Drosophila* vials (www.flystuff.com) on plain Formula 4-
112 24® instant *Drosophila* medium (Carolina, www.Carolina.com) and propagated at 25˚C and
113 12:12 light:dark cycle. The attractive *Saccharomyces cerevisiae* strain ('fly_KR_78.3') was
114 isolated from a single *D. simulans* fruit fly (Buser et al. 2014) sampled at a different vineyard
115 near Auckland, New Zealand, from which the isofemale *D. simulans* line was sourced. The
116 repulsive *S. cerevisiae* isolate ('DBVPG6044') was kindly provided by Prof Edward Louis
117 (University of Leicester, UK) and originated from a West African wine ferment (Liti et al.
118 2009). Yeasts were grown for 48h (28˚C) in standard liquid YPD-medium (1% yeast extract,
119 2% peptone, 2% glucose; BD-Difco). Sterilised liquid and agar-solidified grape juice was
120 inoculated with 10^5 cells per mL and incubated for 48h (28˚C; 200 rpm for liquid cultures).

121

122 *Volatile analysis*

123 Total headspace volatile profiles from yeast ferments were analyzed to screen for compounds
124 that may mediate *D. simulans* attraction and repulsion. Attractive and repulsive *S. cerevisiae*
125 isolates were inoculated into liquid and solidified grape juice in triplicate to constitute
126 biological replicates. In addition, three un-inoculated controls and one empty tube (blank)
127 were included and sampled in parallel. All samples were analyzed using gas chromatography
128 coupled with mass spectrometry (GC/MS).

129 After sample preparation 1.25 µL of the internal standard (0.2 mg mL^{-1} [D8]-methyl benzoate
130 in 70% ethanol; Sigma-Aldrich) was added to each cell-free liquid ferment (2.5 mL
131 supernatant in 100 mL glass tube), on the surface of the solidified 2.5 mL juice-agar plates
132 (35 x 10mm; in 500 mL preserving jar) and respective sterile controls. A dynamic (purge and
133 trap) headspace sampling approach was employed (23-25ºC), using purified air (BOC; 25 ±
134 0.2 mL min^{-1}) to concentrate volatiles in adsorbent-filled (Tenax®-TA resin; 100 mg) direct

thermal desorption vials (ATAS GL International). The sampling time was set for 2 h, and the Tenax® traps were immediately submitted for automated (Focus auto sampler, ATAS GL; PAL cycle composer software 1.5.4) GC-MS injection. Trapped volatiles were thermally desorbed (175ºC; ramp rate of 50ºC min^{-1}; Optic 3 thermal desorption system, ATAS GL) and then cryo-focused at -120ºC using liquid nitrogen. The sample was injected in split mode (1:15split for 3 min, then 1:25 split) to allow rapid homogenisation with the carrier gas (Helium). Volatiles were transferred onto a 30 m x 0.25 mm x 0.25 µm film thickness DB-Wax (J&W Scientific, Folsom, CA, USA) capillary column in a HP6890 GC (Agilent Technologies). A linear GC-program of 3ºC min^{-1} from 35ºC hold for 2 min to 220ºC hold for 5 min was applied with a column flow of 1 ml min^{-1}.

Time-of-flight mass spectrometry (TOF-MS, Leco Pegasus III, St. Joseph, MI, USA) was used for structure elucidation. The transfer line temperature was set to 220ºC, and a detector voltage of 1700 V was applied. The ion source temperature was kept at 200ºC, and an ionization energy of 70 eV was used for electron impact ionization. Spectra were collected from 26 to 250 amu with a data acquisition rate of 20 Hz s^{-1}. Spectra of target compounds were matched to the National Institute of Standards and Technology (NIST) library. The identity of a compound present in different runs was based on comparison of its mass spectrum and retention time. Single peaks were selected manually for integration (LECO chromaTOF software) and analyzed in equivalence to the internal standard. In total, the relative concentrations of 143 volatiles were semi-quantitatively evaluated in this way. 2-methylbutyl acetate (2-MBA), 3-methylbutyl acetate (3-MBA, isoamyl acetate) and acetic acid were verified using authentic standards (Sigma-Aldrich) and directly quantified using a dilution series in grape juice following headspace sampling and GC-MS analysis as described above.

159 The separation of 2-MBA and 3-MBA was poor using a polar DB-wax column and 2-MBA
160 was found to contribute to a minor portion of the 3-MBA peak.
161
162 Volatile profiles of semi-quantified compounds were visualized with heat maps using the
163 heatmap.2 function in R 2.15.0 (R Development Core Team, 2008). The variance of
164 individual compound levels (corrected against internal standard) from attractive and repulsive
165 ferments was evaluated with Principal Component Analysis (PCA) using PAST 3.x
166 (http://folk.uio.no/ohammer/past/). Differences in these semi-quantitative data were further
167 analysed using *t*-tests applying Benjamini-Hochberg multiple testing correction with $\alpha = 0.2$,
168 (P_{BH}).
169
170 *Behavioral study*
171 To further study the context dependency of semiochemicals on *D. simulans* behavior, two-
172 way (T-maze) choice tests (replicated six to eight times) were performed (30 min in the dark,
173 80 females, 3-6 days old; 25 h starved). An attraction index (AI) was calculated following
174 Buser et al. (2014), which calculates the proportion of flies found in either arm of the T-
175 maze. Controls which assayed fly choice between sterile grape juice were included in every
176 suite of assays to evaluate whether the experimental apparatus introduced a bias. The
177 binominal distribution was used to test whether the dispersal of flies between both arms of
178 the T-maze apparatus was significantly different from random expectations.
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183 RESULTS

184 *Chemical communication is modulated by compound concentrations*

185 The original experiment demonstrating the mutualistic interaction between attractive yeast
186 isolates and *Drosophila simulans* utilized a liquid environment for yeast growth in the T-
187 maze choice assays, and a solid environment for dispersal assays (Buser et al. 2014). We
188 repeated these assays, and the AIs for both liquid and solid media are almost perfectly
189 associated when tested against un-inoculated grape juice control. The attractive *S. cerevisiae*
190 (fly_KR_78.3) had an AI of 0.29 in liquid and 0.30 on solidified grape juice, and the
191 repulsive *S. cerevisiae* (DBVPG6044) had an AI of -0.22 in liquid and -0.20 on solidified
192 grape juice. We analysed the volatile profiles of these attractive and repulsive *S. cerevisiae*
193 isolates to screen for semiochemicals putatively involved in attraction and repulsion. GC/MS-
194 analysis showed the liquid ferments were 92%, and solid ferments 100% identical, in terms of
195 the presence/absence of 143 volatiles (measured across both systems; Fig 1). However, the
196 similarity of quantitative compositions of volatiles in liquid compared to solid grape juice
197 medium was below 50% for both yeast isolates with varying amounts of individual
198 compounds. Previously reported semiochemicals for yeast-mediated *Drosophila* attraction
199 include ethanol, acetic acid, ethyl acetate, 2-phenylethanol, 3-hydroxy-2-butanone (acetoin),
200 3-methylbutanol (isoamyl alcohol) and 3-methylbutyl acetate (isoamyl acetate) (Becher et al.
201 2012, Cha et al. 2012; Christaens et al. 2014). All of these compounds were identified in
202 both, attractive and repulsive *S. cerevisiae* isolates when grown on liquid and agar-solidified
203 grape juice. This first observation suggests that it might not just be the presence or absence of
204 one or several compounds that drives chemical attraction and repulsion, but perhaps the
205 relative abundance of compounds or some function of more complex compound interactions
206 (“pattern recognition”, additive, antagonistic, synergistic masking effects).

We went on to analyze both concentrations including single volatiles and volatile profile compositions to evaluate if any of these might be associated with insect attraction and repulsion. Analysis of the variance in concentrations of each of the compounds from both liquid and solid ferments with a simple *t*-test with a false discovery rate of 80%, revealed that just 22 and 12 of the 120 (volatiles measured from liquid cultures) and 93 volatiles (quantified from juice-agar) differed significantly between the profiles of attractive and repulsive yeasts at $P_{BH}<0.05$ when grown on liquid and solid grape juice, respectively. Of those volatiles that significantly differed between attractive and repulsive yeasts, just three were common to both liquid and solid ferments. Concentrations of 2-phenylethyl acetate were different between attractive and repulsive yeasts but inconsistent between growth environments: in liquid environments 2-phenylethyl acetate levels from attractive yeasts were 2.5-fold higher but in solid environments they were 2.7-fold lower. However, acetic acid (AA) was consistently associated with repulsive yeast in both liquid and solid ferments and levels were on average 3.2-fold (liquid) and 15-fold (solid) higher in the repulsive yeast's profile ($P_{BH}<0.03$). In comparison to the repulsive strain, the attractive yeast consistently produced higher amounts of the predominant isomer 3-methylbutyl acetate (isoamyl acetate; 3-MBA) and of the minor component 2-methylbutyl acetate (2-MBA) which were combined 2.6-fold and 3.5-fold higher ($P_{BH}<0.04$) in liquid and solid ferments, respectively.

We next employed Principle Component Analysis (PCA) to simultaneously analyze all data to dissect the impact of subtle shifts in volatile composition on the chemical message impacting fruit fly behavior. The first component explains 94.3% and 92.6% of the variance in volatile profiles in liquid and solid ferments, respectively. The results of this multivariate approach are consistent with the univariate analyses in that the subset of fermentation volatiles correlating with differential *Drosophila* behavior differs depending on whether the ferments are conducted in a liquid or solid environment. According to these analyses, a subset

of volatiles are associated with attraction across both systems: P15 (1,1 diethoxyethane), P37 (2-methylpropanol), P40 (3-MBA/2-MBA), P57 (3-methylbutanol) and P122 (2-phenylethanol). In comparison, P64 (3-hydroxy-2-butanone) and P80 (AA) were associated with repulsion. The same two compounds implicated in the analyses of single volatiles are also highlighted in the PCA analyses: 3-MBA/2-MBA and AA. Further, the polarity of these – greater concentrations of AA in the repulsive ferments and 3-MBA/2-MBA in the attractive ferments – are also in line with the previous analyses. Thus, the two different analytical approaches consistently reveal that , 3-MBA/2-MBA and AA, are associated with attractive and repulsive behavior of *D. simulans* to different genotypes of *S. cerevisiae*.

The behavioral read-out of single volatiles is modulated by its chemical environment

The analyses so far implicate AA in repulsion and 3-MBA as the primary compound mediating attraction of *D. simulans*, respectively. We suggest that selection for yeast volatile production instigating a mutualism will have operated more strongly on yeast traits that attract insect vectors, not those that repel them, as these are positively correlated with reproductive success for both species. Thus, we focussed on disentangling the ecological scenarios under which 3-MBA attracts flies. There were two main questions we went on to evaluate: 1) what concentrations elicit a response; and 2) are the behavioral stimuli affected by the background chemical context?

We directly quantified the concentrations of 3-MBA from liquid ferments in the attractive and repulsive yeast's profile as 0.5 mg L^{-1} and 0.2 mg L^{-1}, respectively. We first removed any effect of a background matrix and tested the behavioral response of flies to a range of 3-MBA concentrations (1 µg L^{-1} to 1 mg L^{-1}; Fig. 2A) diluted in water against water. We observed no

significant response of flies to any of these concentrations (all $P > 0.06$; see Fig 2A). However, when the background matrix was increased in complexity by testing the response of flies to a range of concentrations of 3-MBA diluted in the same but unfermented grape juice against unfermented grape juice, significantly different behavioral responses were apparent (Fig 2B). Flies were repelled by low concentrations of 1 µg L^{-1} ($P = 0.048$) and 10 µg L^{-1} ($P < 0.001$) 3-MBA, attracted to 25 µg L^{-1} 3-MBA ($P = 0.028$; Fig 2B grey circle) and indifferent to 1 mg L^{-1} ($P = 0.33$). Thus, the lack of a behavioral response to 3-MBA in water, but a significant, although complex response in grape juice, indicates that both the background matrix and concentration play a role in attraction.

Next we evaluated whether it is the absolute concentration of 3-MBA or the relative difference in concentration that stimulates fly attraction. Since the T-maze system is an enclosed environment with limited airflow, the compound diffusion from both samples is likely to form a spatial gradient across both arms. We, therefore, tested a 3-MBA dilution series (1, 5, 10, 100, 1000 µg L^{-1}) against a 'high' (1 mg L^{-1}) 3-MBA background matrix (Fig. 2C). Here, the behavioral response changed significantly from repulsion at 5 µg L^{-1} (AI: -0.2; $P = 0.007$) to attraction (AI: 0.34; $P < 0.001$) at 10 µg L^{-1} 3-MBA in grape juice. This later concentration was highly repulsive (AI: -0.36; $P < 0.001$; Fig 2B) when tested against grape juice, indicating a shift in response to lower 3-MBA concentrations (Fig 2C). This observation, together with the finding that up to 20-fold lower levels of 3-MBA than those measured from natural ferments were behaviorally active when tested in a system with reduced volatile complexity, is consistent with the hypothesis that it is differential 3-MBA concentrations that are ecologically important, not absolute concentrations.

Finally, we evaluated the role of AA in this system. This compound was found at levels of 0.4 g L^{-1} and 0.1 g L^{-1} in liquid ferments of the repulsive and attractive yeast, respectively. The fly choice between a range of AA concentrations (from 0.25 µg L^{-1} - 500 mg L^{-1}) against

grape juice was tested and significant repulsion observed at AA concentrations of 2.5, 5 and 25 µg L^{-1} (all $P < 0.03$). We then evaluated the behavior of flies when exposed to varying concentrations of 3-MBA (5, 10, 25, 100, 500, 1000 µg L^{-1}) against a repulsive AA matrix (25 µg L^{-1}). Here flies were either indifferent (5 µg L^{-1}; 25 µg L^{-1}; 0.1 mg L^{-1}) or attracted to 3-MBA at concentrations of 10 µg L^{-1} ($P = 0.009$), 0.5 mg L^{-1} ($P = 0.01$) and 1 mg L^{-1} ($P = 0.003$; Fig 2D). This indicates an interference effect of a repulsive background matrix (AA) to 3-MBA attraction. Moreover, no repulsion of 3-MBA was observed at any concentration against AA, suggesting that AA might be a more universal signal for repulsion in *D. simulans*.

DISCUSSION

This study examines the ecological context of chemical communication between microbes and insects with particular focus on a mutualistic association. Here we use the established and demonstrated interaction between *Saccharomyces* yeasts and *Drosophila* flies to evaluate whether the mode of chemical communication between them is 'simple' or 'complex' by scrutinizing single chemical components of their signals. We build on a recent study showing differential attraction between a range of *S. cerevisiae* genotypes and *D. simulans* (Buser et al. 2014). This behavior is beneficial for both parties as flies have a demonstrable fitness increase when accessing yeast-infested fruits (Anagnostou et al. 2010, Becher et al. 2012), and are more fecund when associated with more attractive yeast isolates (Buser et al. 2014). More attractive yeasts are in turn more frequently dispersed by flies (Buser et al. 2014, Christiaens et al. 2014). For this study we presupposed that attraction is a prerequisite for mutualism, and those volatiles eliciting attraction shape the chemical recognition of mutualistic partners, whether coevolved or by chance.

In contrast to most other studies, here we analyze attraction and volatile compositions of yeasts when grown on natural, fruit-derived and non-artificial media. First we attempted to narrow down the list of components that are associated with attraction by making use of the observation that attraction and repulsion of two *S. cerevisiae* isolates are similar when grown in liquid and solid fruit environments, despite considerable difference in volatile composition. Whether the environment is homogeneous or structured might affect the types of volatiles that yeast releases for at least two reasons. First, while the grape juice was identical, the physical nature of the matrix (fluid or solid) the yeast are growing in could reasonably affect the diffusion equilibrium of metabolic precursors to the cells, as well as the release of volatiles from the matrix and, thus, their concentrations in the headspace. Second, theory and some data suggest that the metabolic strategies employed by yeasts differ according to whether the environment is homogeneous or structured (Pfeiffer et al. 2001; MacLean and Gudelj 2006).

Drosophila simulans was able to discern between two *S. cerevisiae* isolates that produce an odour-space of qualitatively almost identical composition when grown in either liquid or solidified grape juice. Of the concentrations of 143 volatiles evaluated across liquid and solid ferments for both attractive and repulsive yeasts, just acetic acid and the two isomers 3-MBA (major component) and 2-MBA (minor component) were universally consistent in terms of their relative concentrations between attractive and repulsive yeasts: 3-MBA/2-MBA were associated with attraction and acetic acid with repulsion of *D. simulans*. At first glance it might, therefore, appear that the nature of chemical communication between these organisms is relatively simple.

Single compounds and blends thereof have been classified as attractive or repulsive for *D. melanogaster* in previous studies and suggest a core set of proposed semiochemicals that can

influence *D. melanogaster* behavior (Becher et al. 2012; Christiaens et al. 2014; Hutner et al. 1937; Knaden et al. 2012). It is of note that acetic acid has been consistently linked to *D. melanogaster* attraction in these experiments; not surprising given the common name of this species - vinegar fly. While 2-MBA is rarely discussed in literature, there are differences among *D. melanogaster* studies describing the response to 3-MBA, also known as banana oil or isoamyl acetate. Knaden et al. (2012) reported that 3-MBA was behaviorally neutral, whereas Christaens et al. (2014) implicated this compound in *D. melanogaster* attraction. Ruebenbauer et al. (2008) studied variance in attraction of different *D. melanogaster* genotypes to various food sources and single compounds and found a low response to synthetic 3-MBA, but high attraction of all strains was observed for banana and rotten banana, suggesting that single synthetic compounds confer only part of the odour information transmitted by complex, natural sources.

Using the less-well studied sibling species *D. simulans*, we find that 3-MBA is a likely semiochemical driving the yeast : fly mutualism, but only if presented in the context of a natural fruit source. Dilutions of the synthetic compound in water did not elicit any behavioral response in contrast to dilutions in grape juice. Further, the relative concentration showed a stronger effect on *Drosophila* behavior than the presence or absence of the compound itself. In natural ferments attractive yeast consistently produced 3-fold increased levels of 3-MBA compared to repulsive yeast; *Drosophila* attraction, repulsion and neutral behavior towards synthetic 3-MBA in grape juice was observed, and this was concentration-dependent. Lastly, the response to 3-MBA was altered further still when a repulsive compound was added to the system as part of the background odour, demonstrating context-dependent specificity of 3-MBA attraction to the chemical environment. Consequently, *D. simulans* requires 3-MBA to be part of a chemical blend to elicit attraction, and a behavioral response cannot be predicted by the presence or ultimate quantities of the compound *per se*. It

354 is of note that levels of the minor isomer 2-MBA were correlated to 3-MBA production and
355 the ratio of the two might impact attraction more strongly than the predominant ester alone.
356 This implies that studies evaluating the allelochemical effect of single compounds might not
357 achieve ecologically realistic responses.

358 In this study, 3-MBA concentrations from actual yeast ferments were 10-20- fold higher than
359 those eliciting attraction of the synthetic compound in grape juice, suggesting antagonistic
360 effects from other fermentation volatiles that were not present in spiked grape juice. Our
361 experiments show that acetic acid is repulsive to *D. simulans* and can interfere with 3-MBA
362 attraction when present as a background odour, shifting 3-MBA attraction towards
363 concentrations that more closely resemble levels measured from natural ferments. Thus, the
364 nature of chemical communication between these microorganisms and insects appears to be
365 complex involving a subtle interplay between semiochemicals, their relative concentrations
366 and context in terms of a suite of the background chemical matrix. These data are consistent
367 with reports that insect behavior can be modulated by background odour (Schröder and
368 Hilker 2008).

369 That *D. simulans* is repelled by acetic acid contrasts with consistent reports of this compound
370 being attractive to *D. melanogaster*. One possible explanation for the opposite behavioral
371 response in these sympatric species is that this difference may have evolved as a mechanism
372 to mitigate competition. Because ethanol tolerance is correlated to acetic acid tolerance in *D.*
373 *melanogaster* (Chakir et al. 1993), acetic acid can be hypothesized to effect selection of the
374 ethanol-sensitive *D. simulans* in nature. In addition, acetic acid might be an indicator that
375 fruits have been overrun by possibly less preferential microorganisms. The conversion of
376 ethanol to vinegar by *Acetobacter* spp. is a natural end point of fermenting fruits, and so it
377 seems plausible that yeast preferring flies might avoid this compound or show temporal

separation of resource utilization (Joseph et al. 2009). The fruit substrate plays a crucial role in this interaction between yeasts and flies since it provides the precursors of volatiles as well as an energy source, but we did not investigate this third aspect. It will be of interest to evaluate how the semiochemicals involved in behavioral responses here translate to other types of fruit.

Pollinators discriminate between floral phenotypes according to changes in odour intensity (same compounds, same ratio, different concentration), relative abundance (same compounds, same concentration, different ratio) and changes of composition (Cunningham et al. 2004; Sachse and Galizia 2006; Wright et al. 2005). Similar to floral scent, microbial volatile production can be viewed as a dynamic composite, changing its phenotype over time and in response to environmental factors such as temperature and nutrient availability (Smid and Kleerebezem 2014; Styger et al. 2011). The biological function of this mosaic of semiochemicals is likely to change accordingly.

From this study and experimental data we conclude that 1) single compounds (acetic acid and 3-MBA) can elicit different responses in the same or closely related species 2) single volatiles and blends thereof can act on members of different insect families. For example, *Drosophilidae* (Becher et al. 2012) and *Nitidulidae* (Phelan and Lin 1991) were shown to be attracted to an almost identical blend of typical *S. cerevisiae* produced fermentation volatiles. Our study further demonstrates that *D. simulans* can be attracted and repelled by natural yeast ferments containing volatile blends of similar composition; thus illustrating the difficulty in attempting to understand chemical communication by analysing its constituents in isolation. Organisms navigate through a complex odour space that is influenced by background odours from the environment in addition to the olfactory targets. Therefore, it seems plausible that communication will have evolved to take place in this more complex ecological scenario. In

summary this study provides a first step towards understanding the ecological context, and subtleties of chemical communication systems driving mutualistic interactions of microorganisms and insects.

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FIGURES

Figure 1

Volatile profiles of liquid (**Juice**) and solid (**Agar**) grape juice medium inoculated with *Saccharomyces cerevisiae* isolates attractive (**Sc**$^{+}$) and repulsive (**Sc**$^{-}$) to *Drosophila simulans* and sterile controls (**C**). Colour-intensity indicates the mean relative volatile concentration (n=3) as measured from the headspace of the ferment and uninoculated controls. Peak numbers are assigned according to GC-retention time.

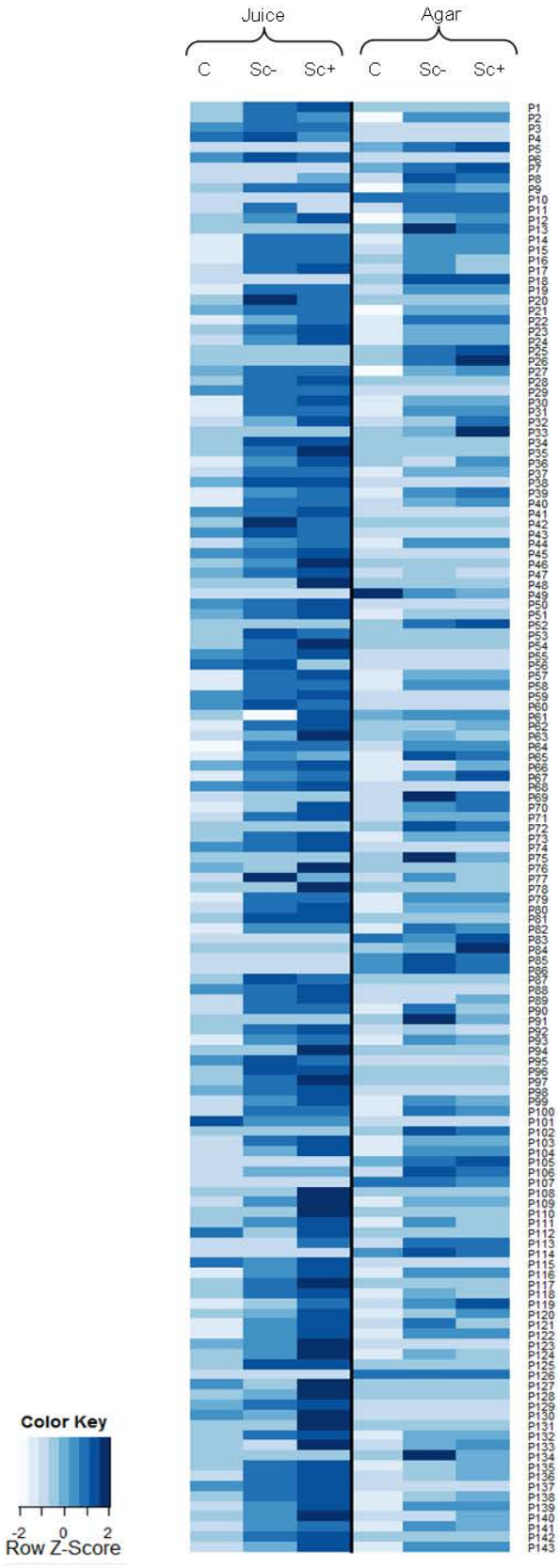

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Figure 2

Choice test response of *Drosophila simulans* to different concentrations (log-scale) of 3-methylbutyl acetate (3-MBA) in the context to the chemical environment (n = 6). Significantly different binominal distributions of flies are indicated by an asterisk α = 0.05). (A) Synthetic 3-MBA diluted in water and tested against water; (B) 3-MBA diluted in grape juice tested against grape juice; (C) 3-MBA diluted in grape juice and tested against 3-MBA (1 mg L^{-1}) in grape juice; (D) 3-MBA diluted in grape juice and tested against acetic acid (25 µg L^{-1}) in grape juice. 10-fold dilutions of 1 mg L^{-1} 3-MBA are indicated by black symbols and others by grey symbols.