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6   # THE CONTEXT OF CHEMICAL COMMUNICATION DRIVING A

7   # 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.

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

64 the field (Buser et al. 2014);   4) increased attraction by yeasts is associated with increased

65 *Drosophila* fecundity in fruits, demonstrating that volatile emission by attractive

66 *Saccharomyces* initiates a mutualism with *Drosophila* (Buser et al. 2014).

67 Recent research in this area has primarily focussed on the receptors involved in insect

68 olfaction and the volatiles that activate them using *D. melanogaster* as a model species.

69 Systematic characterisation of these receptors demonstrates that *D. melanogaster* is capable

70 of sensing at least 100 volatiles (Hallem and Carlson 2006). A number of common yeast

71 fermentation products, such as ethanol, acetic acid, ethyl acetate, 2-phenylethanol, 3-

72 hydroxy-2-butanone (acetoin), 3-methylbutanol (isoamyl alcohol) and 3-methylbutyl acetate

73 (isoamyl acetate) have been implicated in the attraction of *D. melanogaster* (Becher et al.

74 2012, Hutner et al. 1937, Joseph et al. 2009), which has much of its sensory apparatus tuned

75 to volatiles produced by yeasts, especially esters (Hallem and Carlson 2004; Hallem and

76 Carlson 2006; Vosshall and Stocker 2007). Indeed, when yeasts' ability to synthesise acetates

77 is compromised, *D. melanogaster* attraction is significantly affected (Christiaens et al. 2014).

78 While this research has been a significant step forward, such a gross change in volatile

79 production capability might not reflect the complex ecological subtleties of the drivers of this

80 interaction in nature.

81 *Drosophila simulans* belongs to the same subgroup as *D. melanogaster* (Drosophila 12

82 Consortium 2007), and is known to form hybrids and live in sympatry with its evolutionary

83 sibling (Capy and Gibert 2004). One study (Stökl et al. 2010) describes the chemical drivers

84 of deceptive pollination attraction of *D. melanogaster* and *D. simulans* to Solomon's Lily

85 (*Arum palaestinum),* and suggests attraction is mediated by a more complex bouquet of at

86 least six compounds (2,3-butanediol acetate, acetoin acetate, hexyl acetate, ethyl hexanoate,

87 2-phenylethyl acetate, 2-phenylethanol). The most parsimonious hypothesis would be that the

88 chemical language shaping yeast-fly mutualism is "simple" and mediated by either a single

89 semiochemical or compound class, such as acetates. An alternate hypothesis might be that

90 attraction is "complex" or multifactorial and context specific, perhaps comprising blends of

91 behaviorally active volatiles (Becher et al. 2012; Stökl et al. 2010). Consequently, the

92 inherent information of a chemical message would not only be contingent upon the chemical

93 nature of the volatiles, but also on their relative abundance, and interaction with other

94 semiochemicals and the background chemical matrix.

95 Buser et al. (2014) assayed the behavioral response of *D. simulans* to 100 genetically and

96 ecologically diverse strains of *S. cerevisiae* and demonstrated a mutualistic association with

97 the *S. cerevisiae* isolate 'fly_KR_78.3', which is attractive to this species, but not with the

98 'DBVPG6044' isolate (Liti et al. 2009), which is repulsive. Here we analyze the volatile

99 profiles of these attractive and repulsive yeast isolates and experimentally evaluate whether

100 the mutualistic association between *S. cerevisiae* and *D. simulans* is driven by simple or more

101 complex forms of chemical communication.

102

103 METHODS AND MATERIALS

104 *Study organisms*

105 The *D. simulans* employed here originated from a natural vineyard population near Auckland,

106 New Zealand, and is the same isofemale *D. simulans* line used by Buser et al. (2014). We

107 follow Buser et al (2014) and assay the interaction between yeast and flies when grown in

108 liquid and solidified (2:1 with 20% agar) Sauvignon Blanc grape juice (derived from

109 Marlborough, New Zealand), sterilized with 400 µL dimethyl dicarbonate (Sigma-Aldrich;

110 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

135 thermal desorption vials (ATAS GL International). The sampling time was set for 2 h, and

136 the Tenax$^®$ traps were immediately submitted for automated (Focus auto sampler, ATAS GL;

137 PAL cycle composer software 1.5.4) GC-MS injection. Trapped volatiles were thermally

138 desorbed (175ºC; ramp rate of 50ºC min^{-1}; Optic 3 thermal desorption system, ATAS GL)

139 and then cryo-focused at -120ºC using liquid nitrogen. The sample was injected in split mode

140 (1:15split for 3 min, then 1:25 split) to allow rapid homogenisation with the carrier gas

141 (Helium). Volatiles were transferred onto a 30 m x 0.25 mm x 0.25 µm film thickness DB-

142 Wax (J&W Scientific, Folsom, CA, USA) capillary column in a HP6890 GC (Agilent

143 Technologies). A linear GC-program of 3ºC min^{-1} from 35ºC hold for 2 min to 220ºC hold

144 for 5 min was applied with a column flow of 1 ml min^{-1}.

145 Time-of-flight mass spectrometry (TOF-MS, Leco Pegasus III, St. Joseph, MI, USA) was

146 used for structure elucidation. The transfer line temperature was set to 220ºC, and a detector

147 voltage of 1700 V was applied. The ion source temperature was kept at 200ºC, and an

148 ionization energy of 70 eV was used for electron impact ionization. Spectra were collected

149 from 26 to 250 amu with a data acquisition rate of 20 Hz s^{-1}. Spectra of target compounds

150 were matched to the National Institute of Standards and Technology (NIST) library. The

151 identity of a compound present in different runs was based on comparison of its mass

152 spectrum and retention time. Single peaks were selected manually for integration (LECO

153 chromaTOF software) and analyzed in equivalence to the internal standard. In total, the

154 relative concentrations of 143 volatiles were semi-quantitatively evaluated in this way. 2-

155 methylbutyl acetate (2-MBA), 3-methylbutyl acetate (3-MBA, isoamyl acetate) and acetic

156 acid were verified using authentic standards (Sigma-Aldrich) and directly quantified using a

157 dilution series in grape juice following headspace sampling and GC-MS analysis as described

158 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).

9

207   We went on to analyze both concentrations including single volatiles and volatile profile

208   compositions to evaluate if any of these might be associated with insect attraction and

209   repulsion. Analysis of the variance in concentrations of each of the compounds from both

210   liquid and solid ferments with a simple *t*-test with a false discovery rate of 80%, revealed that

211   just 22 and 12 of the 120 (volatiles measured from liquid cultures) and 93 volatiles

212   (quantified from juice-agar) differed significantly between the profiles of attractive and

213   repulsive yeasts at $P_{BH}<0.05$ when grown on liquid and solid grape juice, respectively. Of

214   those volatiles that significantly differed between attractive and repulsive yeasts, just three

215   were common to both liquid and solid ferments. Concentrations of 2-phenylethyl acetate

216   were different between attractive and repulsive yeasts but inconsistent between growth

217   environments: in liquid environments 2-phenylethyl acetate levels from attractive yeasts were

218   2.5-fold higher but in solid environments they were 2.7-fold lower. However, acetic acid

219   (AA) was consistently associated with repulsive yeast in both liquid and solid ferments and

220   levels were on average 3.2-fold (liquid) and 15-fold (solid) higher in the repulsive yeast's

221   profile ($P_{BH}<0.03$). In comparison to the repulsive strain, the attractive yeast consistently

222   produced higher amounts of the predominant isomer 3-methylbutyl acetate (isoamyl acetate;

223   3-MBA) and of the minor component 2-methylbutyl acetate (2-MBA) which were combined

224   2.6-fold and 3.5-fold higher ($P_{BH}<0.04$) in liquid and solid ferments, respectively.

225   We next employed Principle Component Analysis (PCA) to simultaneously analyze all data

226   to dissect the impact of subtle shifts in volatile composition on the chemical message

227   impacting fruit fly behavior. The first component explains 94.3% and 92.6% of the variance

228   in volatile profiles in liquid and solid ferments, respectively. The results of this multivariate

229   approach are consistent with the univariate analyses in that the subset of fermentation

230   volatiles correlating with differential *Drosophila* behavior differs depending on whether the

231   ferments are conducted in a liquid or solid environment. According to these analyses, a subset

232 of volatiles are associated with attraction across both systems: P15 (1,1 diethoxyethane), P37

233 (2-methylpropanol), P40 (3-MBA/2-MBA), P57 (3-methylbutanol) and P122 (2-

234 phenylethanol). In comparison, P64 (3-hydroxy-2-butanone) and P80 (AA) were associated

235 with repulsion. The same two compounds implicated in the analyses of single volatiles are

236 also highlighted in the PCA analyses: 3-MBA/2-MBA and AA. Further, the polarity of these

237 – greater concentrations of AA in the repulsive ferments and 3-MBA/2-MBA in the attractive

238 ferments – are also in line with the previous analyses. Thus, the two different analytical

239 approaches consistently reveal that , 3-MBA/2-MBA and AA, are associated with attractive

240 and repulsive behavior of *D. simulans* to different genotypes of *S. cerevisiae*.

241

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

243 The analyses so far implicate AA in repulsion and 3-MBA as the primary compound

244 mediating attraction of *D. simulans*, respectively. We suggest that selection for yeast volatile

245 production instigating a mutualism will have operated more strongly on yeast traits that

246 attract insect vectors, not those that repel them, as these are positively correlated with

247 reproductive success for both species. Thus, we focussed on disentangling the ecological

248 scenarios under which 3-MBA attracts flies. There were two main questions we went on to

249 evaluate: 1) what concentrations elicit a response; and 2) are the behavioral stimuli affected

250 by the background chemical context?

251

252 We directly quantified the concentrations of 3-MBA from liquid ferments in the attractive

253 and repulsive yeast's profile as 0.5 mg L^{-1} and 0.2 mg L^{-1}, respectively. We first removed any

254 effect of a background matrix and tested the behavioral response of flies to a range of 3-MBA

255 concentrations (1 µg L^{-1} to 1 mg L^{-1}; Fig. 2A) diluted in water against water. We observed no

256   significant response of flies to any of these concentrations (all $P > 0.06$; see Fig 2A).

257   However, when the background matrix was increased in complexity by testing the response

258   of flies to a range of concentrations of 3-MBA diluted in the same but unfermented grape

259   juice against unfermented grape juice, significantly different behavioral responses were

260   apparent (Fig 2B). Flies were repelled by low concentrations of 1 µg L^{-1} ($P = 0.048$) and 10

261   µg L^{-1} ($P < 0.001$) 3-MBA, attracted to 25 µg L^{-1} 3-MBA ($P = 0.028$; Fig 2B grey circle) and

262   indifferent to 1 mg L^{-1} ($P = 0.33$). Thus, the lack of a behavioral response to 3-MBA in water,

263   but a significant, although complex response in grape juice, indicates that both the

264   background matrix and concentration play a role in attraction.

265   Next we evaluated whether it is the absolute concentration of 3-MBA or the relative

266   difference in concentration that stimulates fly attraction. Since the T-maze system is an

267   enclosed environment with limited airflow, the compound diffusion from both samples is

268   likely to form a spatial gradient across both arms. We, therefore, tested a 3-MBA dilution

269   series (1, 5, 10, 100, 1000 µg L^{-1}) against a 'high' (1 mg L^{-1}) 3-MBA background matrix

270   (Fig. 2C). Here, the behavioral response changed significantly from repulsion at 5 µg L^{-1} (AI:

271   -0.2; $P = 0.007$) to attraction (AI: 0.34; $P < 0.001$) at 10 µg L^{-1} 3-MBA in grape juice. This

272   later concentration was highly repulsive (AI: -0.36; $P < 0.001$; Fig 2B) when tested against

273   grape juice, indicating a shift in response to lower 3-MBA concentrations (Fig 2C). This

274   observation, together with the finding that up to 20-fold lower levels of 3-MBA than those

275   measured from natural ferments were behaviorally active when tested in a system with

276   reduced volatile complexity, is consistent with the hypothesis that it is differential 3-MBA

277   concentrations that are ecologically important, not absolute concentrations.

278   Finally, we evaluated the role of AA in this system. This compound was found at levels of

279   0.4 g L^{-1} and 0.1 g L^{-1} in liquid ferments of the repulsive and attractive yeast, respectively.

280   The fly choice between a range of AA concentrations (from 0.25 µg L^{-1} - 500 mg L^{-1}) against

281   grape juice was tested and significant repulsion observed at AA concentrations of 2.5, 5 and

282   25 µg L^{-1} (all $P < 0.03$). We then evaluated the behavior of flies when exposed to varying

283   concentrations of 3-MBA (5, 10, 25, 100, 500, 1000 µg L^{-1}) against a repulsive AA matrix

284   (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

285   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} ($P =$

286   0.003; Fig 2D). This indicates an interference effect of a repulsive background matrix (AA)

287   to 3-MBA attraction. Moreover, no repulsion of 3-MBA was observed at any concentration

288   against AA, suggesting that AA might be a more universal signal for repulsion in *D.*

289   *simulans*.

290

291   DISCUSSION

292   This study examines the ecological context of chemical communication between microbes

293   and insects with particular focus on a mutualistic association. Here we use the established and

294   demonstrated interaction between *Saccharomyces* yeasts and *Drosophila* flies to evaluate

295   whether the mode of chemical communication between them is 'simple' or 'complex' by

296   scrutinizing single chemical components of their signals. We build on a recent study showing

297   differential attraction between a range of *S. cerevisiae* genotypes and *D. simulans* (Buser et

298   al. 2014). This behavior is beneficial for both parties as flies have a demonstrable fitness

299   increase when accessing yeast-infested fruits (Anagnostou et al. 2010, Becher et al. 2012),

300   and are more fecund when associated with more attractive yeast isolates (Buser et al. 2014).

301   More attractive yeasts are in turn more frequently dispersed by flies (Buser et al. 2014,

302   Christiaens et al. 2014). For this study we presupposed that attraction is a prerequisite for

303   mutualism, and those volatiles eliciting attraction shape the chemical recognition of

304   mutualistic partners, whether coevolved or by chance.

305   In contrast to most other studies, here we analyze attraction and volatile compositions of

306   yeasts when grown on natural, fruit-derived and non-artificial media. First we attempted to

307   narrow down the list of components that are associated with attraction by making use of the

308   observation that attraction and repulsion of two *S. cerevisiae* isolates are similar when grown

309   in liquid and solid fruit environments, despite considerable difference in volatile composition.

310   Whether the environment is homogeneous or structured might affect the types of volatiles

311   that yeast releases for at least two reasons. First, while the grape juice was identical, the

312   physical nature of the matrix (fluid or solid) the yeast are growing in could reasonably affect

313   the diffusion equilibrium of metabolic precursors to the cells, as well as the release of

314   volatiles from the matrix and, thus, their concentrations in the headspace. Second, theory and

315   some data suggest that the metabolic strategies employed by yeasts differ according to

316   whether the environment is homogeneous or structured (Pfeiffer et al. 2001; MacLean and

317   Gudelj 2006).

318   *Drosophila simulans* was able to discern between two *S. cerevisiae* isolates that produce an

319   odour-space of qualitatively almost identical composition when grown in either liquid or

320   solidified grape juice. Of the concentrations of 143 volatiles evaluated across liquid and solid

321   ferments for both attractive and repulsive yeasts, just acetic acid and the two isomers 3-MBA

322   (major component) and 2-MBA (minor component) were universally consistent in terms of

323   their relative concentrations between attractive and repulsive yeasts: 3-MBA/2-MBA were

324   associated with attraction and acetic acid with repulsion of *D. simulans*. At first glance it

325   might, therefore, appear that the nature of chemical communication between these organisms

326   is relatively simple.

327   Single compounds and blends thereof have been classified as attractive or repulsive for *D.*

328   *melanogaster* in previous studies and suggest a core set of proposed semiochemicals that can

329   influence *D. melanogaster* behavior (Becher et al. 2012; Christiaens et al. 2014; Hutner et al.

330   1937; Knaden et al. 2012). It is of note that acetic acid has been consistently linked to *D.*

331   *melanogaster* attraction in these experiments; not surprising given the common name of this

332   species - vinegar fly. While 2-MBA is rarely discussed in literature, there are differences

333   among *D. melanogaster* studies describing the response to 3-MBA, also known as banana oil

334   or isoamyl acetate. Knaden et al. (2012) reported that 3-MBA was behaviorally neutral,

335   whereas Christaens et al. (2014) implicated this compound in *D. melanogaster* attraction.

336   Ruebenbauer et al. (2008) studied variance in attraction of different *D. melanogaster*

337   genotypes to various food sources and single compounds and found a low response to

338   synthetic 3-MBA, but high attraction of all strains was observed for banana and rotten

339   banana, suggesting that single synthetic compounds confer only part of the odour information

340   transmitted by complex, natural sources.

341   Using the less-well studied sibling species *D. simulans*, we find that 3-MBA is a likely

342   semiochemical driving the yeast : fly mutualism, but only if presented in the context of a

343   natural fruit source. Dilutions of the synthetic compound in water did not elicit any

344   behavioral response in contrast to dilutions in grape juice. Further, the relative concentration

345   showed a stronger effect on *Drosophila* behavior than the presence or absence of the

346   compound itself. In natural ferments attractive yeast consistently produced 3-fold increased

347   levels of 3-MBA compared to repulsive yeast; *Drosophila* attraction, repulsion and neutral

348   behavior towards synthetic 3-MBA in grape juice was observed, and this was concentration-

349   dependent. Lastly, the response to 3-MBA was altered further still when a repulsive

350   compound was added to the system as part of the background odour, demonstrating context-

351   dependent specificity of 3-MBA attraction to the chemical environment. Consequently, *D.*

352   *simulans* requires 3-MBA to be part of a chemical blend to elicit attraction, and a behavioral

353   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

378   separation of resource utilization (Joseph et al. 2009). The fruit substrate plays a crucial role

379   in this interaction between yeasts and flies since it provides the precursors of volatiles as well

380   as an energy source, but we did not investigate this third aspect. It will be of interest to

381   evaluate how the semiochemicals involved in behavioral responses here translate to other

382   types of fruit.

383   Pollinators discriminate between floral phenotypes according to changes in odour intensity

384   (same compounds, same ratio, different concentration), relative abundance (same

385   compounds, same concentration, different ratio) and changes of composition (Cunningham et

386   al. 2004; Sachse and Galizia 2006; Wright et al. 2005). Similar to floral scent, microbial

387   volatile production can be viewed as a dynamic composite, changing its phenotype over time

388   and in response to environmental factors such as temperature and nutrient availability (Smid

389   and Kleerebezem 2014; Styger et al. 2011). The biological function of this mosaic of

390   semiochemicals is likely to change accordingly.

391   From this study and experimental data we conclude that 1) single compounds (acetic acid and

392   3-MBA) can elicit different responses in the same or closely related species 2) single volatiles

393   and blends thereof can act on members of different insect families. For example,

394   *Drosophilidae* (Becher et al. 2012) and *Nitidulidae* (Phelan and Lin 1991) were shown to be

395   attracted to an almost identical blend of typical *S. cerevisiae* produced fermentation volatiles.

396   Our study further demonstrates that *D. simulans* can be attracted and repelled by natural yeast

397   ferments containing volatile blends of similar composition; thus illustrating the difficulty in

398   attempting to understand chemical communication by analysing its constituents in isolation.

399   Organisms navigate through a complex odour space that is influenced by background odours

400   from the environment in addition to the olfactory targets. Therefore, it seems plausible that

401   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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508

509   FIGURES

510

511   **Figure 1**

512   Volatile profiles of liquid (**Juice**) and solid (**Agar**) grape juice medium inoculated with

513   *Saccharomyces cerevisiae* isolates attractive (Sc^+) and repulsive (Sc^-) to *Drosophila simulans*

514   and sterile controls (**C**). Colour-intensity indicates the mean relative volatile concentration

515   (n=3) as measured from the headspace of the ferment and uninoculated controls. Peak

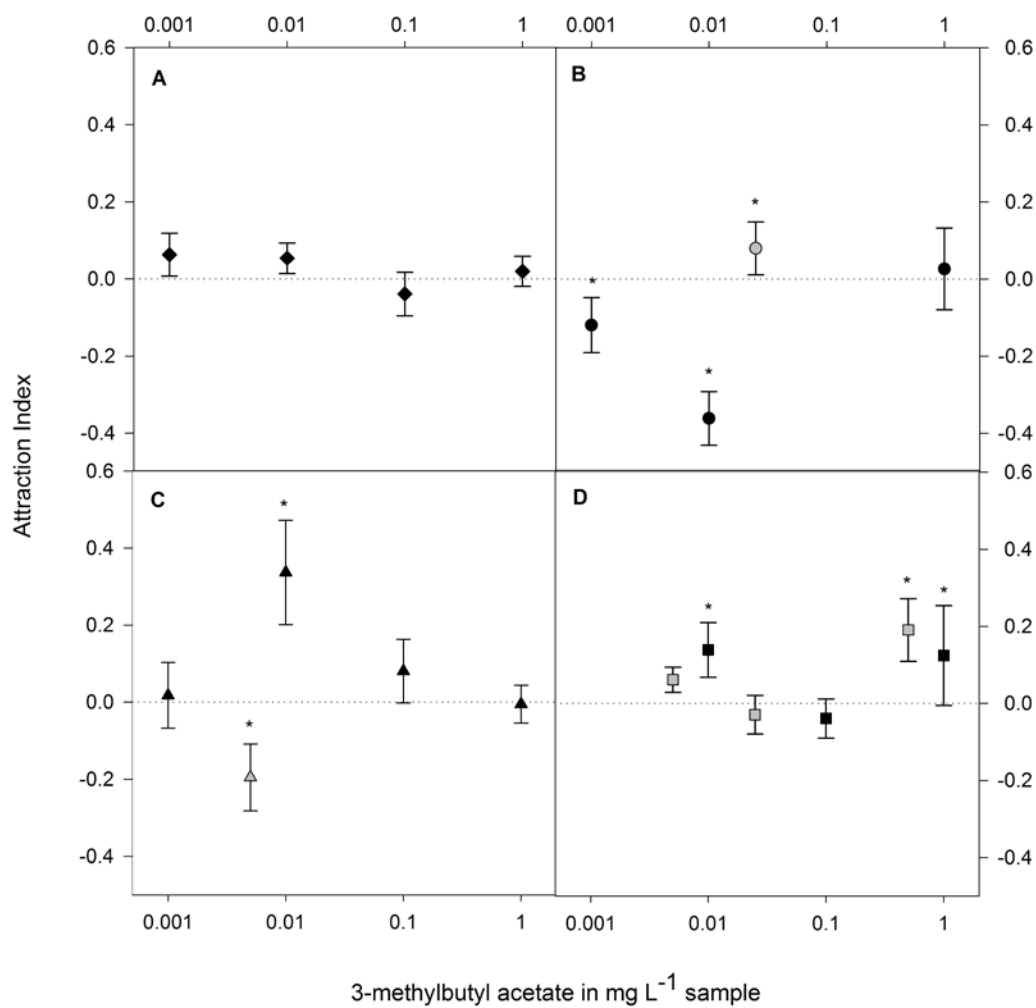516   numbers are assigned according to GC-retention time.

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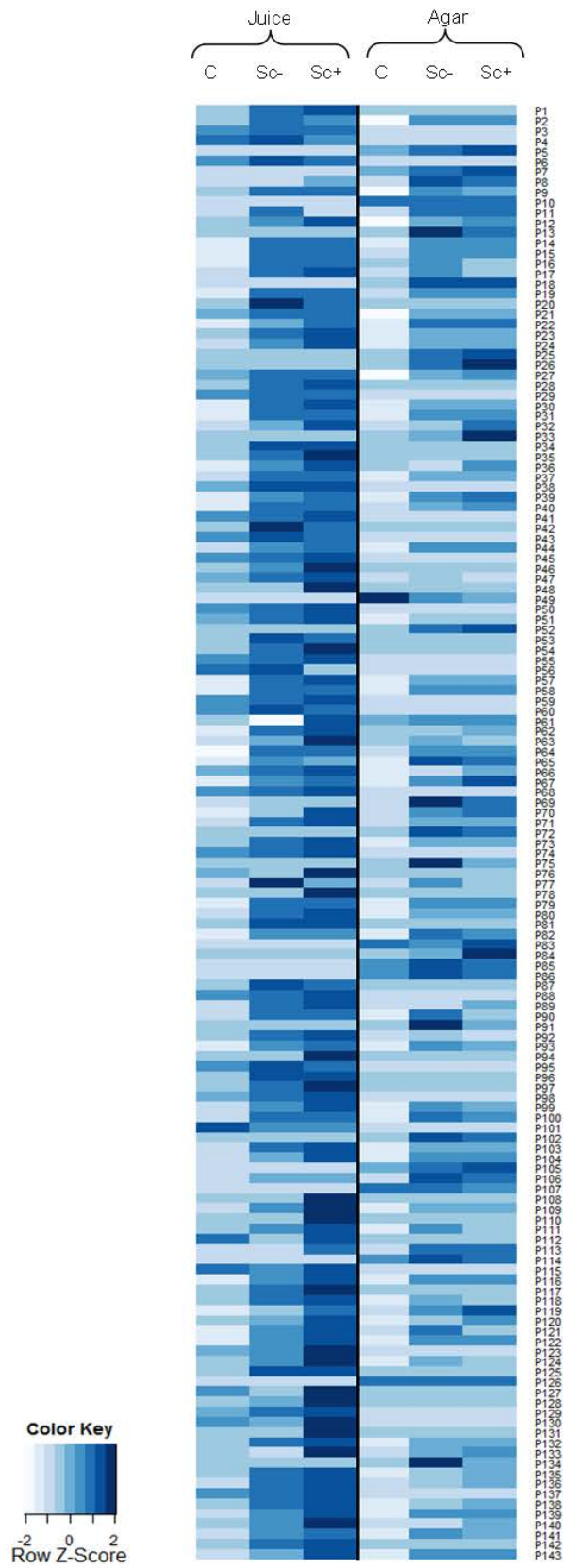
Figure 2

520 Choice test response of *Drosophila simulans* to different concentrations (log-scale) of 3-

521 methylbutyl acetate (3-MBA) in the context to the chemical environment (n = 6).

522 Significantly different binominal distributions of flies are indicated by an asterisk α = 0.05).

523 (A) Synthetic 3-MBA diluted in water and tested against water; (B) 3-MBA diluted in grape

524 juice tested against grape juice; (C) 3-MBA diluted in grape juice and tested against 3-MBA

525 (1 mg L^{-1}) in grape juice; (D) 3-MBA diluted in grape juice and tested against acetic acid (25

526 µg L^{-1}) in grape juice. 10-fold dilutions of 1 mg L^{-1} 3-MBA are indicated by black symbols

527 and   others   by   grey   symbols.

528

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