Dietary nutrient balance shapes phenotypic traits of Drosophila melanogaster in interaction with gut microbiota

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1 # Dietary nutrient balance shapes phenotypic traits of *Drosophila*
2 # *melanogaster* in interaction with gut microbiota

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9 ## Abstract

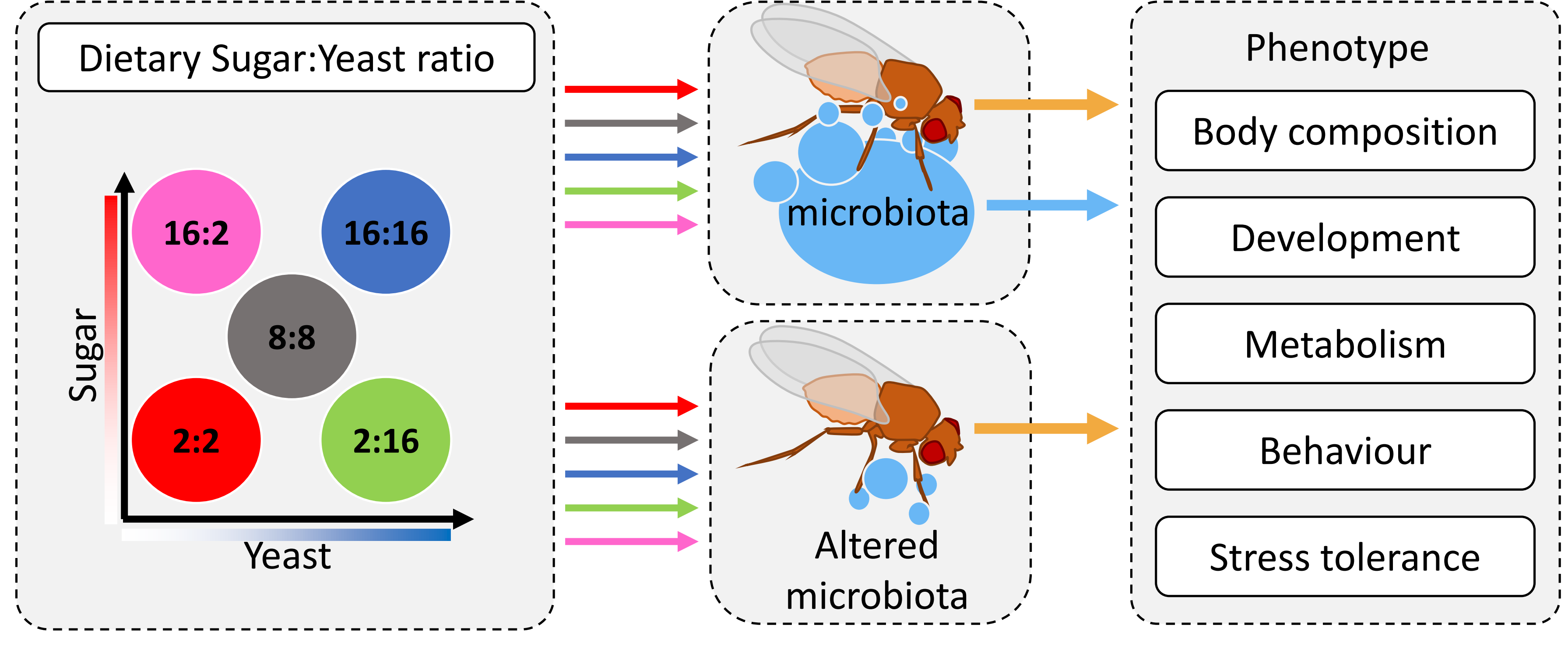
10 The dietary nutrient composition can affect insects' phenotypes by modulating their physiology.
11 Furthermore, diet can affect gut microbiota composition and abundance, with indirect consequences for
12 the host. In this study, we reared *Drosophila melanogaster* on five different diets; three with balanced
13 sugar:yeast ratio, but with increasing caloric content (2:2, 8:8, 16:16, in weight %), and two with
14 imbalanced sugar:yeast ratio, either with low sugar and high yeast content (2:16) or vice-versa (16:2).
15 In each of these diets, we compared flies with conventional vs. artificially altered gut microbiota with
16 antibiotics that reduced the bacterial load. The antibiotic treatment also had the surprising effect of
17 increasing the amount of live yeast associated with the flies. We characterized flies from these ten
18 treatments (5 diets x 2 microbiota) in terms of development, body mass, food preference, body reserves,
19 metabolic rate and a range of stress tolerance traits (heat, cold, starvation and desiccation tolerance).
20 Diets, and to a lesser extent antibiotic treatment, affected development rate, weight, and cold tolerance
21 of adult flies. Other traits such as energy reserves, metabolic rate, food preference, or starvation
22 tolerance were affected by diet alone. When detected, the effect of antibiotic treatment was stronger in
23 yeast-poor diets, suggesting that gut bacterial community might help to counterbalance nutritional
24 deficiencies. These results show that changes in dietary factors lead to a global re-organization of fly's
25 physiology and development while the manipulation of gut microorganisms had minor effects that were
26 mainly seen in case of protein restriction.

27 Keywords: nutrition; microbiota; developmental plasticity; stress tolerance; metabolic rate

28 Short title: Diet and microbiota shape phenotypes

Highlights

- We expected interactions between diet balance and microbiota on phenotypes.
- The physiological impact of microbiota was limited in comparison with the one of diets.
- Yeast content in food drove most nutritional effects on development and metabolism.
- Sugar-rich diets improved cold and starvation tolerance.


Dietary Sugar:Yeast ratio
Sugar
Yeast
16:2
16:16
8:8
2:2
2:16
microbiota
Altered
microbiota
Phenotype
Body composition
Development
Metabolism
Behaviour
Stress tolerance

29 # Introduction

30 Most animals have a preferred dietary target in terms of macronutrient composition and amount
31 (Simpson and Raubenheimer, 2012). In insects, the inability to meet this nutritional target represents a
32 great challenge as nutrition affects the physiology and performance of individuals, including
33 reproduction, development, and lifespan (Lee et al., 2008; Matzkin et al., 2011). Numerous studies
34 addressed nutritional questions focusing on caloric restriction (Bartke et al., 2001; Masoro, 2003; Rogina
35 et al., 2002). Food shortage may result in energy trade-off that manifests as reduced or delayed
36 investment in development and reproduction (Edgar, 2006; Koyama et al., 2013). The shift from
37 reproduction to survival mode is assumed to be a way to cope with harsh environment by increasing
38 lifespan and promoting stress resistance, at the cost of a slow development (Burger et al., 2007; Carvalho
39 et al., 2012; Kolss et al., 2009; Rion and Kawecki, 2007). Any change in nutritional supply can have
40 considerable consequences for ecologically relevant traits of species (Raubenheimer et al., 2009), and
41 these changes may also be driven by nutritional balance (sugar:protein ratio), independent of the caloric
42 content (Fanson et al., 2009; Mair et al., 2005; Solon-Biet et al., 2014). Experiments using artificial food
43 with controlled nutrient compositions have helped to disentangle the contributions of specific
44 macronutrients on insects' phenotype. For example, the "geometric framework" represents a robust
45 method to analyze the consequences of protein/carbohydrate ratios (Lee et al., 2008; Simpson and
46 Raubenheimer, 2012) and studies have demonstrated how low protein:carbohydrate ratios reduce
47 development rate but increase lifespan across diverse insect species (Fanson et al., 2009; Lee et al.,
48 2008; Simpson and Raubenheimer, 2012; Skorupa et al., 2008). Insects display various strategies to
49 counterbalance nutritional deficiencies. Behavioral shifts represent a first response to dietary pressures,
50 whether they manifested in food preference changes, or increased foraging (*e.g.* Corrales-Carvajal et al.,
51 2016; Simpson et al., 2006). Insects may also deal with nutritional imbalance through internal
52 physiological adjustments that can help to maintain nutritional homeostasis. For instance, nutrient
53 sensing in the gut is regulating molecular signaling pathways such as IIS/TOR (for insulin-like growth
54 factor signaling/target of rapamycin) that are responsible for growth (Kapahi et al., 2004; Koyama et al.,
55 2013; Layalle et al., 2008).

56 Mutualistic relationships with microorganisms may provide advantageous nutritional functions to
57 insects, including the degradation and detoxification of indigestible food and synthesis of essential
58 nutrients (Douglas, 2009). Understanding the complex tripartite interaction among diet, microbiota and
59 host traits represents an exciting and novel challenge in the field of nutritional ecology (Jehrke et al.,
60 2018). In *Drosophila* flies, some bacterial taxa like *Lactobacillus plantarum* or *Acetobacter sp* can
61 promote growth through the activation of insulin pathways and partially compensate for detrimental
62 effects of protein-poor nutrition (Matos et al., 2017; Shin et al., 2011; Storelli et al., 2011). Commensal
63 bacteria have also been found to influence behavioral decisions by limiting yeast appetite and buffer for

64 specific amino-acid depletion (Leitão-Gonçalves et al., 2017). Furthermore, the gut microbiota can
65 directly contribute to the nutrient supply of *D. melanogaster*, and thereby affect both lipid and
66 carbohydrate metabolism (Ridley et al., 2012; Wong et al., 2014). Other roles of gut microbiota include
67 improved peptidase activity (Erkosar et al., 2015) and provision of secondary metabolites, vitamins, and
68 amino acids (Sannino et al., 2018; Yamada et al., 2015).

69 The aforementioned studies suggest that the presence of a functional microbiota could promote
70 the host's nutritional balance and fitness. However, benefits are likely to vary according to diet, bacterial
71 taxa, as well as host genotype (Dobson et al., 2015; Newell and Douglas, 2014; Wong et al., 2014). For
72 instance, in *D. suzukii,* the presence of a functional microbiota was found to be mandatory for survival
73 on specific poor diets, but deleterious for lifespan on balanced diets (Bing et al., 2018). Likewise,
74 multiple studies have also reported links between stress tolerance and microbiota in insects (Ferguson
75 et al., 2018; Henry and Colinet, 2018; Moghadam et al., 2018; Montllor et al., 2002; Russell and Moran,
76 2006). Since nutritional composition and microbiota may both affect insect phenotypes, including stress
77 tolerance, it is of interest to examine how these factors compare and if one factor is more dominant than
78 the other. As an example, it is known that carbohydrate and lipid reserves vary according to nutrition
79 (Lee and Jang, 2014; Wong et al., 2014) but also according to gut microbiota (Huang and Douglas,
80 2015; Ridley et al., 2012; Wong et al., 2014), and that these reserves are important for tolerance to
81 thermal, desiccation, and starvation stress (Arrese and Soulages, 2010; Ballard et al., 2008; Colinet et
82 al., 2013; Colinet and Renault, 2014; Klepsatel et al., 2016). Similarly, nutritional scarcity or altered gut
83 microbiota can both increase development duration leading to hardened phenotypes that are better able
84 to tolerate stress (Kolss et al., 2009; Storelli et al., 2011). To our knowledge there are only a handful of
85 studies that have investigated the impact of nutritional variables in insects harboring contrasted gut
86 microbiota compositions or abundances (Chaston et al., 2016; Huang and Douglas, 2015; Ridley et al.,
87 2012; Wong et al., 2014).

88 Here, we tested i) the effect of dietary restriction using balanced diet and ii) the effect of isocaloric
89 but imbalanced diets with skewed sugar:yeast ratios (S:Y), on *D. melanogaster*. These dietary
90 manipulations were combined (or not) with an antibiotic treatment altering the gut microbiota. This
91 experiment aimed to disentangle the role microorganisms in the host-nutrition interaction. We first
92 hypothesized that moderate nutrient scarcity of particular proteins (here added as yeast in diets) would
93 decrease development rate, body weight, and metabolic rate, but provide larger energy reserves and *in*
94 *fine* higher stress tolerance. In addition, we expected that flies could be able to show preferences for
95 specific nutrient in two-choices experiments, with a more marked preference for the limiting nutrient in
96 imbalanced diets. Knowing that gut microorganisms may provide nutrients for their host, we also
97 predicted that microbial depletion would amplify phenotypical and behavioral responses in poor
98 nutritional situations, including low-calorie diets but also imbalanced diets. As a result, we expected the

99 importance of microbiota depletion to vary according to diet: on rich diets, microbiota alteration could
100 be neutral or beneficial, whereas it could strengthen the nutritional stress on poor diets.

101

102 Methods

103 **Fly stocks and culture medium**

104 We conducted the experiments on an outbred laboratory population of *Drosophila melanogaster*
105 derived from wild individuals collected in September 2015 in Brittany (France). Fly stocks were
106 maintained at 25°C under a 12:12 L:D photoperiod, on standard fly medium comprising 80 g. L^{-1} of
107 inactive brewer yeast (MP Biochemicals 0290331205), 50 g. L^{-1} of sucrose, 10 g. L^{-1} of agar (Sigma-
108 Aldrich A1296), supplemented with 8 mL. L^{-1} of 10% methyl 4-hydroxybenzoate (Sigma-Aldrich
109 H5501). *Wolbachia* symbiont was absent from the population.

110 **Dietary and microbiota manipulations**

111 Five diets comprising different amounts of sugar and yeast were tested. The experimental design
112 was adapted from Zhu et al. (2014) (see fig. S1). The following S:Y ratios were used: 2:2, 8:8, 16:16,
113 2:16 and 16:2 (amounts expressed in weight %). 2:2, 8:8, and 16:16 represented balanced diets with
114 gradual increase in the caloric content, whereas 2:16 and 16:2 represented isocaloric diets (Min et al.,
115 2007) but with imbalanced compositions (poor in sugar and rich in yeast, and vice-versa). Except for
116 sugar and yeast concentrations, diets were prepared similarly to standard fly medium, and 40 mL of food
117 was poured per 230 mL plastic bottle.

118 For each tested S:Y ratio, we compared flies with intact microbiota to flies with altered
119 microbiota, resulting in a total of 10 treatments (see fig. S1). The five diet treatments with microbiota
120 alteration are indicated with single quotes (S:Y ratios: 2:2', 8:8', 16:16', 2:16' and 16:2'). Microbiota
121 alteration was obtained by egg dechorionation using successive two minutes wash in 2.7% hypochlorite
122 and two minutes in 70% ethanol, followed by three rinses in sterile miliQ water. In addition,
123 dechorionated eggs were placed on food supplemented with an antibiotic cocktail: 50 µg ampicillin
124 (Sigma-Aldrich A9518), 50 µg kanamycin (Sigma-Aldrich K4000), 50 µg tetracyclin (Sigma-Aldrich
125 T7660) and 15 µg erythromycin (Sigma-Aldrich E5389) per liter of fly food (Téfit et al., 2018). Eggs
126 for the untreated condition were not washed at all, as a simple water wash may affect bacterial abundance
127 on the chorion. For simplicity, we use the term "antibiotic treatment" in the manuscript to refer to this
128 microbiota alteration procedure including dechorionation and antibiotics. We roughly counted eggs to
129 get approx. 500 eggs per bottle, hence avoiding crowding (Henry et al., 2018). After emergence, we
130 changed bottles for new ones every second day.

131 **Bacterial and yeast abundance measurement**

132 We counted colony forming units (CFU) on MRS media to estimate microorganisms load in
133 flies' gut and control the efficiency of the antibiotic treatment with a standard method adapted from
134 Koyle et al. (2016). See ESM for detailed methods.

135 **Mass, water content, and total lipid content measurement**

136 Mated females (5-d old) from all treatments were snap frozen in liquid nitrogen. Five to seven
137 pools of *ca* 20 flies per treatment were randomly picked and then weighed using a microbalance.
138 Samples were dried for 48 h at 60 °C and re-weighed to determine their dry mass. Finally, samples were
139 washed repeatedly in boiling petroleum ether for 72 h in order to remove all lipids, and re-dried to
140 determine lean mass.

141 **Glucose and glycogen assays**

142 Glucose and glycogen measurements were performed using a colorimetric method adapted from
143 Tenessen et al. (Tennessen et al., 2014). Six replicates, each consisting of pools of five 5-d old mated
144 females were used. Flies were snap frozen in liquid nitrogen, dried at 60 °C for 48 h, and weighed using
145 a microbalance. Flies were crushed in 500 µL of PBS using a bead biting apparatus (TissueLyzer LT,
146 Qiagen, Hilden, Germany). Enzymes in samples were heat-inactivated by a 10 min incubation at 70 °C.
147 Thirty microliters of these samples were dispensed in duplicates in a 96-well plate. In the first well of
148 each duplicate, 100 µL of GO reagent (Sigma Aldrich GAGO-20) were added, and in the second well,
149 100 µL of GO reagent plus 0.1% amyloglucosidase (Sigma Aldrich 1602) were added. After 60 min
150 incubation at 37 °C, 100 µL of 12 N sulfuric acid was dispensed in all wells to stop the reaction.
151 Absorbance was measured at 540 nm. Glycogen concentration for each sample was determined by
152 subtracting samples without aminoglucosidase (corresponding to glucose concentration) from samples
153 with aminoglucosidase (corresponding to glucose plus glycogen concentration). Quantification was
154 based on glucose and glycogen standard curves. See ESM for detailed methods.

155 **Development time**

156 Adult flies were allowed to lay eggs on standard medium for 8 h before being discarded. Eggs
157 were then collected using a paint brush, treated with the dechorionation treatment or left untouched.
158 Eggs were then placed on the surface of the food, in controlled density of 25 eggs per 40 mL vial filled
159 with 5 mL of medium (N = 4 vials per treatment). The end of the oviposition period was considered as
160 the t = 0 for the development of flies. Pupation and emergence events were checked twice a day.

161 **Metabolic rate assay**

162 Metabolic rate of the flies was indirectly estimated by the rate of CO_2 production ($\dot{V}CO_2$) and
163 O_2 consumption ($\dot{V}O_2$) using repeated measurements of stop-flow respirometry (Lighton and Halsey,
164 2011). Here we used an experimental setup similar to that described by Jensen et al. (Jensen et al., 2014).
165 Briefly, 4-d old mated females from the different treatments were randomly assigned to one of 16

166 cylindrical glass chambers (dimensions: D: 20 mm L: 70 mm, 11-21 individuals in each chamber).
167 Within each chamber, flies had access to a piece of filter paper (15 mm × 15 mm) with a droplet of the
168 appropriate food to avoid starvation and desiccation. Chambers without flies, with or without filter paper
169 and the droplet of food were also tested to correct measurements from background fluctuations. Two
170 parallel 8-channels-multiplexers (RM Gas Flow Multiplexer, Sable Systems, Las Vegas, NV, USA)
171 controlled the sequentially flushing and closing of the chambers such that the stop-flow respirometry
172 system enabled us to obtain repeated measures of $\dot{V}CO_2$ and $\dot{V}O_2$ over 24 h. Measurements were
173 performed independently at two experimental temperatures by placing the metabolic chambers in a
174 climate chamber maintained at 15 °C or 25 °C (12:12 L:D). The low temperature ensured measurements
175 at which locomotor activity is much reduced, allowing to infer the basal levels of gas exchange.

176 At the end of the experiment, flies were collected, killed by placing them briefly at -20°C after
177 which they were weighed using a microbalance (Sartorius Laboratory Balance type 1712, Göttingen,
178 Germany). Analysis of respiratory data and estimation of basal metabolic rate were performed as in
179 Jensen et al. (2014). See ESM for detailed methods.

180 **CAFE assay**

181 Mated female flies from all treatment combinations were used to measure feeding rates and
182 preferences using capillary feeding method (CAFE) adapted from (Ja et al., 2007). Flies were left in
183 empty vials for one hour before starting the experiments. Next, seven individuals were placed in 40 mL
184 vials with access to food via 2*2 10 µL glass capillaries, containing either 8% sucrose or 8% autoclaved
185 yeast solutions (N = 5-8 vials per treatment). These foods were randomly supplemented with blue and
186 red commercial food coloring (Vahiné, France) as this improved precision of food intake measurements
187 (Diegelmann et al., 2017). Vials were placed in a hermetic tank with humidified towels to limit
188 evaporation (25°C, 12:12 L:D). Eight vials with capillaries but without flies were used to control for
189 evaporation. Food consumption was measured using an electronic caliper from the reduction in “liquid
190 height” in the capillaries after 10 h.

191 **Thermal stress assays**

192 Adult flies from all the diets*microbiota combinations were sexed under light CO_2 anesthesia
193 (< 2 min), allowed to recover at least 48 h, and 5-7-d mated females were used to assess thermal stress
194 tolerance.

195 For acute stress survival, 180 flies per treatment were placed into 50 mL vials and immersed in
196 a thermoregulated bath filled with a glycol-water mix set at -3.5°C or 38.5°C (± 0.1°C) for cold and heat
197 stress respectively. From t = 0 to t = 120 min, 20 individuals per treatment were removed from the bath
198 each 15 min and transferred into fresh vials with standard food medium to recover at 25°C. Survival
199 was scored 40 h after the stress. Individuals were considered alive when able to stand on their legs.

200 For critical minimal and maximal temperatures (CT_{min} and CT_{max}), 25 mated females per treatment were
201 individually placed in 5-mL glass vials immersed in a thermoregulated glycol-water bath set at 20 °C.
202 Next, flies were exposed to a slow ramping down or up at the rate of $\pm$ 0.1 °C min^{-1} and the temperature
203 at which all movement ceased was recorded as the upper or lower thermal limit for a given individual.

204 **Starvation and desiccation assays**

205 Adult flies from all the diets*microbiota combinations were sexed under light CO_2 anesthesia,
206 allowed to recover at least 48 h, and 5-7 d mated females were used to measure starvation tolerance.
207 Twenty-five flies were individually placed in 40 mL vials filled with 5 mL of 1,5% agar-water medium
208 to avoid desiccation (25°C, 12:12 L:D). Survival was checked each two hours for the first 48 h, and each
209 four hours afterwards, until 100% mortality was reached. The time at which all movement ceased was
210 recorded as survival time.

211 For desiccation, 25 mated females per treatment were individually placed in 5-mL glass vials
212 closed with fine mesh to allow air circulation. These vials were then placed in a hermetic glass tank with
213 a four-centimeter layer of silica-gel that ensured a low air humidity (< 5% RH, 25°C, 12:12 L:D).
214 Survival of flies was checked each hour for 24 h. The time at which all movement ceased was recorded
215 as survival time.

216 **Statistical analyses**

217 All statistical analyses were performed with R (R Core Team, 2017). CFU counting was
218 analyzed using negative binomial regression accounting for replicates random effect. Survival was
219 analyzed using cox models for tolerance to starvation and desiccation, that were both measured over
220 time with repeated observations. Pairwise differences were assessed using log-rank Mantel-Cox test
221 with Benjamini-Hochberg correction for multiple comparisons. Acute stress tolerance (cold and heat)
222 were analyzed using binomial GLM with logit link function (binomial
223 survival~diet*microbiota*exposure duration) followed by Tukey HSD post-hoc test. ANOVA were
224 used for all other metrics, (metric~diet*microbiota), followed by Tukey HSD post-hoc test. The part of
225 the variance attributed to diet, microbiota, and their interaction was extracted using 'relaimpo' package
226 (Groemping, 2006). For all tests, significance threshold was fixed at $p<0.01$.

227 Results

228 **Bacterial and yeast load**

229 The antibiotic treatment used in the present study almost completely killed the microbial
230 community in the treated flies (fig. 1A; fig. S2). The bacterial load was already rather low in
231 conventional flies from several diets, especially those with low yeast supply (2:2 and 16:2). Yeast
232 colonization was also much dependent on diet and microbiota alteration treatment (fig. 1B; table 1).
233 While the antibiotics markedly reduced bacterial load, yeasts were more abundant in bacteria-depleted

234 flies. Some yeasts colonies were observed in conventional flies, but mostly in yeast-rich diets (16:16
235 and 2:16) (fig. 1B; fig. S2).

236 **Body composition**

237 Fresh mass was affected by dietary treatments such that yeast-poor diets (2:2 and 16:2) were
238 significantly smaller (by about 40%) in comparison with yeast-rich diets (fig. 2A; table 1). Balanced
239 diets 8:8 and 16:16 did not show significant mass differences linked to caloric content (table S1). Even
240 if the main effect of microbiota and the diet*microbiota interaction were significant, they only accounted
241 for a small part of the mass total variance (table 1), and pairwise differences could not detect clear
242 pattern linked to these two variables (table S1). Water content relative to mass was increased in sugar-
243 poor diets (2:2 and 2:16) in comparison with others diets, but no significant effect of microbiota was
244 observed (fig. 2B; table 1). Similarly, total lipid content was linked to the dietary sugar content and not
245 to microbiota status (fig. 2C; table 1). Thus, flies from sugar-poor diets (2:2 and 2:16) had half the lipid
246 content of flies from the others diets (fig. 2C; table S1). Carbohydrate reserves (glucose and glycogen)
247 were highly influenced by diet (fig. 2D & E; table 1). Flies that developed on diets with low yeast content
248 (2:2 and 2:16) were characterized by high levels of glucose and glycogen compared to other treatments
249 (table S1). Sugar-rich diets did not result in carbohydrate rich flies when the S:Y ratio was balanced (8:8
250 and 16:16). Diet*microbiota interactions were significant for both glucose and glycogen contents,
251 accounting for about 10% of the total variance. This manifested in higher levels of carbohydrates in 2:2'
252 compared to 2:2, but lower glucose level in 16:2' compared to 16:2 (fig. 2D & E; table S1). Overall,
253 yeast content of the food drove most of the body composition, including weight and carbohydrates
254 reserves. Sugar content affected lipid reserves, and we did not find evidence to suggest that the
255 antibiotics treatment affected this relationship.

256 **Development, metabolism and nutritional behavior**

257 Development time (fig. 3A) was significantly impaired by diet and diet*microbiota interaction
258 (table 1). Diets with low yeast content (2:2 and especially 16:2) increased development time compared
259 to the other treatments (pairwise comparisons in table S1). Antibiotic treatment also tended to delay
260 development (fig. 3A) but here the effect was diet-specific with considerable delay of development in
261 the sugar rich 16:2' diet but only minor delay in the others (fig. 3A; table S1). Metabolic rate, measured
262 as oxygen consumption, was high in yeast-rich diets (8:8; 16:16; 2:16), and low in yeast-poor ones (fig.
263 3B; table S1). Caloric content of the diets seemed to have limited effect on oxygen consumption since
264 the highly caloric but yeast-poor diet (16:2) showed a rather low oxygen consumption, not different
265 from the 2:2 diet (table S1). There were no significant effects of microbiota on metabolic rate either
266 (table 1). As expected, balanced diets (2:2; 8:8; 16:16) were characterized by an intermediate RQ (0.9-
267 0.94), sugar-rich diets (16:2) had a RQ very close to 1 and yeast-based diets (2:16) had a RQ of 0.8-0.85
268 (fig. 3C; table S1). We could not evidence pairwise differences between the RQ of flies treated or not
269 with antibiotics (table 1). Food preference varied between diets in a pattern that indicated compensation

270 for lacking nutrient related to the rearing diet (fig. 3D; table 1). Sugar consumption was lower than yeast
271 consumption in all diets except in the flies reared on yeast-rich diet (2:16). Conversely, flies from yeast-
272 poor diets (2:2 and 16:2) were those most attracted by yeast (fig. 3D; table S1). Total food consumption
273 also showed compensatory feeding as flies reared on reduced caloric content (2:2) fed more than all
274 other dietary groups (fig. 3E). Food preference and total food consumption variations mostly resulted
275 from diet composition, with no significant effect of microbiota (table 1). In conclusion, all
276 developmental, metabolic, and behavioral effects were mainly linked to yeast amount in the food. The
277 antibiotic treatment increased the developmental delay only in yeast-poor diets that already showed slow
278 development.

279 **Stress tolerance**

280 Cold survival was significantly and mainly affected by diets (fig. 4A; table 1). Flies from
281 balanced diets with medium or large caloric content (8:8 and 16:16) were sensitive to cold while flies
282 from yeast-poor ones (2:2 and 16:2) were especially tolerant (fig. 4A; table S1). Significant effect of
283 microbiota was detected on cold survival, as flies from antibiotics diets tended to survive longer
284 exposures than conventional flies, but its impact was weak considering it only represented 3 % of
285 explained variance (table 1). Interestingly, we did not observe that differences in cold survival reflected
286 in CT_{min} differences (fig. 4B). Although there was a significant effect of diet, it only accounted for a
287 limited part of the explained variance (fig. 4B; table 1). CT_{min} was mainly affected by the microbiota
288 alteration and the diet*microbiota interaction (table 1). Except for the 2:16 diet, flies treated with
289 antibiotics entered chill coma at lower temperatures than flies with intact microbial communities (fig.
290 4B; table S1). Heat tolerance (acute & knockdown) was almost unaffected by diet and microbiota (fig.
291 4C & D; table 1). Diet, microbiota, and diet*microbiota interaction all affected starvation tolerance (fig.
292 4E; table 1). Survival to starvation was greatly enhanced in 16:2 flies, reduced in 2:16, and intermediate
293 in all balanced diets (2:2, 8:8, 16:16) (fig. 4E; table S1). The antibiotic treatment globally reduced the
294 starvation tolerance (*e.g.* 16:16 vs 16:16'), although no pairwise differences were found significant
295 (table 1; table S1). For desiccation tolerance, we observed a small but significant effect of diet (fig. 4F;
296 table 1). The different dietary groups varied by less than five hours in survival but this was sufficient to
297 separate the most (16:16 and 8:8) from the least resistant flies (2:16) (fig. 4F; table S1). No specific
298 combination of diet and antibiotic treatment lead to globally more tolerant individuals against all
299 stressors. Antibiotics treatment either improved tolerance to cold or reduced starvation tolerance. Flies
300 from yeast-poor diets tolerated cold and starvation better, but heat and desiccation tolerance remained
301 almost unaffected.

302 Discussion

303 Although dietary effects have been broadly explored in *Drosophila* (Burger et al., 2007;
304 Kristensen et al., 2016; Matzkin et al., 2011), it is not yet clear whether phenotypical changes result

305 exclusively from dietary factors *per se* or whether they also entail some indirect influences from gut
306 microbiota. In this study we investigated how both the caloric content of diets (sugar and yeast
307 concentration) and the composition (sugar:yeast ratio) affected phenotypes of flies harboring
308 conventional or altered microbiota.

309 **Dietary effects on nutritional phenotype**

310 Differences in diets were clearly responsible for the largest part of observed phenotypes,
311 representing 80-99% of the explained variance in almost all of the measured traits (table 1). A key result
312 is that dietary deprivation or supplementation of one specific nutrient (carbohydrates or proteins) often
313 lead to similar responses as global caloric deprivation or supplementation of both nutrients. This is
314 consistent with the idea introduced by Mair et al. (2005), and followed by many studies using the
315 geometric framework (Lee et al., 2008; Simpson and Raubenheimer, 2012), that nutritional balance is
316 the main limiting nutritional factor. Yeast content generally drove organisms to the extreme phenotypes,
317 irrespective of the sugar content. Yeast-poor diets generated leaner individuals, displaying only half the
318 size of most other developmental groups, and with relative high glucose and glycogen content (fig. 2A,
319 D, E). This result is consistent with previous studies in which protein deprivation switched the
320 metabolism to carbohydrates-based reserves (Simmons and Bradley, 1997; Wong et al., 2014). We
321 noticed flies being offered a choice between two nutrients would try to compensate for the yeast scarcity
322 via increased preference for yeast over sugar, and global increased hunger (fig. 3D, E). Such
323 compensatory feeding behavior probably indicates that flies reared on imbalanced diets suffered from
324 malnutrition, and were unable to meet their target nutrient supply in a food where everything is mixed
325 (Fanson et al., 2012; Leitão-Gonçalves et al., 2017). We observed slow development at the larval stage
326 and a low metabolic rate in flies reared on yeast-poor diets (fig. 3A, B). These results contrast with the
327 metabolic rate stability previously observed in individuals subjected to dietary restriction at the adult
328 stage (Hulbert et al., 2004). However, in our experimental setup, we performed dietary manipulations
329 for the entire larval development. This demonstrates that nutrition may deeply affect physiology of
330 individuals during their early development, and carry over to the adult stage, as it has also been found
331 for many other phenotypic traits (De Block and Stoks, 2008; Gandolfi et al., 2003; Hahn, 2005; Hoover
332 et al., 2005). Sugar content in food also influenced flies' phenotypes, though less obviously than yeast.
333 High amounts of sugar increased total lipid content and decreased water content in flies, as previously
334 observed (fig. 2B, C) (Jehrke et al., 2018; Matzkin et al., 2011; Musselman et al., 2011; Wong et al.,
335 2014). In addition, yeast-poor and sugar-rich diet (16:2) prolonged the developmental time, as compared
336 to the low caloric but balanced diet (2:2). This is in agreement with the results of Musselman et al.
337 (2011) who reported that high-sucrose feeding resulted in a significant developmental delay. Indeed,
338 excessive dietary sugar tends to suppress protein storage in *Drosophila* (Skorupa et al., 2008). It is
339 possible that the high sugar content generated energetic satiety before the protein supply target was met.

340 Such a response would indirectly reduce the uptake of proteins (as dietary yeast) and slow development
341 on a diet that is rich in carbohydrate but limited in protein (Lee et al., 2008).

342 **Microbiota effects on nutritional phenotype**

343 Combining eggs dechorionation and antibiotics successfully reduced gut bacterial load, but lead
344 to an unexpected accumulation of gut yeasts (fig. 1A, B). Therefore, instead of comparing conventional
345 vs. axenic flies, we rather compared flies with intact microbiota (high gut bacteria load, low gut yeast
346 load) to flies with manipulated microbiota (low gut bacteria load, high gut yeast load). As yeast are a
347 known source of dietary protein and micronutrients (Keebaugh et al., 2018), it is possible that the
348 phenotypes resulted either from the lack of gut bacteria or from the increased abundance of gut yeasts.
349 This prevent us from drawing unequivocal conclusions about microbiota effect as a whole, and we
350 therefore limit our discussion to the effect of antibiotic treatment in general.

351 Bacteria and yeast abundances in the gut were positively correlated with yeast concentration in
352 the food, as was also found by Galenza et al. (2016) and Keebaugh et al. (2018). We only observed
353 contrasted microbiota load and compositions in the yeast-rich diets (8:8, 16:16, 2:16), resulting in strong
354 diet-dependency of most phenotypic traits affected by the antibiotic treatment. Our procedure did not
355 provide the desired control over the microbiota, but we still observed phenotypic effects that were
356 consistent with the literature. In line with findings from Wong et al. (2014b) we observed a slight
357 increase of mass in antibiotic treated flies from low calorie diets (2:2') and slight decrease when they
358 were reared on energy rich diets (16:16') (fig: 2A). Unlike in vertebrate models, axenic flies generally
359 show hyperlipidemia when reared on poor diets (Wong et al., 2014). This is possibly because axenic
360 individuals miss bacteria that usually consume dietary sugars in place of their host (Bäckhed et al.,
361 2004). In the present study, we did not observe such response. Here, the antibiotic treatment did not
362 change the lipid content under the poorest nutritional conditions (2:2) (fig. 2C-E), which is consistent
363 with the findings of Ridley et al. (2012). Interestingly, antibiotics increased the carbohydrate content
364 (glucose and glycogen) of flies reared on poor diets (2:2) and conversely reduced glucose content of
365 flies reared under sugar-rich conditions (16:2). We did not detect any significant impact of the antibiotic
366 treatment on oxygen consumption or RQ, whatever the diet (fig. 3C). This is surprising because one
367 may expect changes in carbohydrates reserves to affect the available energy fuel and consequently to
368 trigger metabolic rate changes as observed by Ridley et al. (2012). One possible explanation is that the
369 effect of diet differences outweighed a putative minor effect of antibiotics, especially given that flies
370 had access to their specific food during measurements of metabolic rate.

371 Alteration of energy reserves by microbiota could result from many kinds of host-microbiota
372 relationships, including changes in host's behavior and dietary preference (Douglas, 2018). However,
373 we failed to identify any "buffering" effect of our antibiotic treatment on yeast appetite as it is described
374 by Leitão-Gonçalves et al. (2017), even when the nutrient supply was low. It is possible that high live

375 yeasts abundances in antibiotic-treated conditions represented a protein source and prevented us from
376 observing such behaviors. The most striking effect of the antibiotic treatment was on development (fig.
377 3A). Developmental time increased for flies treated with antibiotic, especially in flies reared on food
378 overloaded with sugar (from +1d in balanced diets 8:8 and 16:16 to +4d in 16:2), but without affecting
379 the poorest diet (2:2). Whether the microbiota affected the supply of available sugars in the food by
380 consuming them (Chaston et al., 2016; Huang and Douglas, 2015; Ridley et al., 2012), or changed flies'
381 use of energy reserves acting on hormonal pathways (Ridley et al., 2012; Shin et al., 2011; Storelli et
382 al., 2011) needs future investigations.

383 **Consequences of diet-microbiota interactions on stress tolerance**

384 Considering the role of microbiota in nutrition (Douglas, 2009) and the role of nutrition in stress
385 tolerance (Andersen et al., 2010; Sisodia and Singh, 2012), we expected to observe marked responses
386 in stress tolerance traits related to both diet and microbiota. We did not detect any effect of diet or
387 antibiotic treatment on heat stress tolerance (fig. 4C, D). These results contrast with those of Andersen
388 et al. (2010) and Sisodia and Singh (2012) who found that protein-rich diets improved heat stress
389 tolerance, although diets and metrics used to assess tolerance are not directly comparable (Jørgensen et
390 al., 2019). In contrast, resistance to cold stress was much more responsive to dietary conditions and
391 manipulation of microbiota. Cold knockdown temperature showed rather small effect of diets (2:16 flies
392 fell into coma at slightly lower temperatures than other diets), and a larger and consistent beneficial
393 effect of the antibiotics treatment (fig. 4B). Conversely, acute cold stress survival was only weakly
394 dependent on antibiotics treatment, but strongly dependent on nutrition, with low caloric content being
395 beneficial for cold survival in comparison with rich diets (fig. 4A). The divergence between the two
396 cold tolerance metrics is of interest because they rely on different physiological mechanisms: cold
397 knockdown is linked to the loss of neurological function (mainly in the brain), and the survival after
398 harsh cold exposure depends upon the ability to maintain ion balance (in the whole body) (Armstrong
399 et al., 2012; Overgaard and MacMillan, 2017). There are energetic costs associated with ion balance
400 regulation and one could speculate that dietary-induced changes in the metabolic rate and nutritional
401 status affected the energy allocation and prevented the maintenance of ion homeostasis after harsh stress.
402 We observed a positive effect of dietary restriction on cold survival that diverges from previous findings
403 (Burger et al., 2007). Our restriction was more severe than that of Burger et al (2007) who used a
404 restrictive diet comparable to our intermediate diet (8:8), which could explain this result. The lower
405 knockdown temperature in yeast-rich and sugar-poor diet 2:16 diet is consistent with previous
406 observations that excess in dietary sugar is not necessarily beneficial for cold tolerance (Colinet et al.,
407 2013) and that yeasts, especially live ones, favor cold tolerance (Colinet and Renault, 2014; Le Bourg
408 et al., 2015). Although increased cold tolerance of microbiota-altered flies is surprising (Henry and
409 Colinet, 2018), it could be linked to the unexpected increased yeast gut content that resulted from our
410 antibiotic treatment. The focus is often on bacteria as the main gut symbionts but the role of yeasts

411 should not be neglected (Hoang et al., 2015). Live yeasts can affect *Drosophila* physiology and increase
412 survival to infections and starvation, in addition to cold stress (Le Bourg et al., 2015; Le Rohellec and
413 Le Bourg, 2009). The difference between the effects of dead and live yeasts suggests these
414 microorganisms provide an active protection that goes beyond being a simple food source.

415 Starvation tolerance was improved in sugar rich diet particularly when dietary yeast was absent.
416 This is not surprising since the sugar fed flies have a higher lipid content which has been shown to
417 provide better tolerance to starvation in other *Drosophila* species (*e.g*. Ballard et al., 2008). Interestingly
418 we did not find any effect of caloric restriction on starvation tolerance. Here, the lower metabolic rates
419 likely offset the lower body mass of calorie-restricted individuals, leading to a neutral outcome. In
420 addition, Burger et al. (2007) found that caloric restriction effects were time-dependent, and only turned
421 detrimental after 20 days post-emergence. It is possible that we tested our individuals at an intermediate
422 time, without marked trend for beneficial or detrimental effects. Antibiotic treatment had only little
423 influence on starvation tolerance, which is coherent with its limited effect on body reserves.

424 Desiccation tolerance responded less than expected to dietary manipulation and antibiotics
425 treatment. Both diet and microbiota have previously been shown to play a role in synthesis of cuticular
426 lipids (Fedina et al., 2012; Heys et al., 2018), a family of molecules affecting water retention of insects
427 (Gibbs et al., 1997). In addition, changes in metabolic rate between diets should also affect tolerance of
428 flies since increased gas exchanges tend to increase dehydration (Gibbs et al., 2003). We observed little
429 support for these effects in our results. At most, flies from diets with higher content in sugar were slightly
430 better at dealing with dehydration stress than those with reduced amount of sugar in their diet. Total
431 lipid content was higher in these flies, and this type of reserves is well suited for tolerance to hydric
432 stress since lipids oxidation represent a decent source of water (Arrese and Soulages, 2010).

433 **Conclusions**

434 Overall, we showed that for most of the tested traits, diet was more determining than microbiota.
435 The antibiotic treatment interacted with diets to alter several traits, but, except for CT_{min}, the strength of
436 these effects was generally minor compared to the one of diets alone, encompassing over 80% of the
437 explained variance (table 1). We failed to replicate the observation of hyperlipidemia, hyperglycemia
438 and reduced metabolic rate previously detected in axenic flies (Wong et al., 2014). The absence of
439 marked microbial effects could be related to our experimental procedure. Though antibiotics effectively
440 eliminated bacteria, they also allowed yeasts to thrive even more than in conventional flies. Hence, it is
441 unclear whether the relatively modest changes in phenotype associated with antibiotic treatments were
442 a consequence of bacteria elimination or of yeast growth. Additionally, we cannot rule-out the
443 importance of genetic background in our observations, possibly hampering our ability to replicate
444 previous findings (Chaston et al., 2016). Regarding diet, we showed that its composition was often more
445 important than its caloric content. Flies exposed to imbalanced diets with similar intermediate caloric

contents exhibited divergent developmental responses, energy metabolism and stress tolerance. The combined restriction of sugar and yeasts often led to similar responses as yeast-deprived but sugar-rich diets, suggesting protein content (as dietary yeasts) was the main driver of observed changes in phenotype. Flies reared on yeast-poor diets, irrespective of the sugar content, were characterized by slow development, low body mass, large relative carbohydrates reserves, high metabolic rate and increased stress tolerance. Further investigations should aim to disentangle the role of live yeasts from the one of bacteria in various dietary conditions. This could give a finer understanding of these complex nutritional interactions and particularly improve our understanding of yeasts, both as members of the microbiota and as an important protein resource.

Data accessibility

Datasets are available in online repository https://doi.org/10.6084/m9.figshare.7594343

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711

712

713 # Figure captions

714 Figure 1: Bacterial and yeast load of adult female flies. Colors show the different diets. Dots represent
715 replicated CFU countings (N=6*5 flies per condition). Filled dots are for individuals with
716 unmanipulated microbiota, and open dots for individuals with antibiotic treatment. Horizontal black
717 lines are medians.

718 Figure 2: Weight and body composition of adult female flies. Colors represent the different diets. Filled
719 dots represent measurements for individuals with unmanipulated microbiota, and open dots
720 measurements for individuals with antibiotic treatment. In A, B and C, dots represent means for 14-21
721 individuals (N=7 per condition); in D and E, dots represent pools of 5 individuals (N=6 per condition).
722 Horizontal black lines are means, and error bars are 95% confidence intervals around the mean.

723 Figure 3: Developmental, metabolic and behavioral characteristics of adult female flies. Colors represent
724 the different diets. Filled dots represent measurements for individuals with unmanipulated microbiota,
725 and open dots measurements for individuals with antibiotic treatment. In A, dots are individual adult
726 emergence events; in B and C, dots represent the mean for 14-21 individuals (N=7 per condition); in D
727 and E, dots are the mean for 7 individuals (N=8 per condition). Food preference index is the subtraction
728 of sugar consumption to yeast consumption in a choice experiment. Total consumption is the addition
729 of sugar and yeast consumption in the same choice experiment. Horizontal black lines are means, and
730 error bars are 95% confidence intervals around the mean.

731 Figure 4: Stress tolerance characteristics of adult female flies. Colors represent the different diets. Filled
732 dots/plain lines represent measurements for individuals with unmanipulated microbiota, and open
733 dots/dashed lines measurements for individuals with antibiotic treatment. A and B are for cold stress, C
734 and D are for heat stress, E is for starvation, F is for desiccation. In A and C, lines represent survival
735 predictions of non-linear binomial model; in B and D, dots represent individual coma events (N=25), in
736 E and F, lines and dots represent survival proportion timepoints of 25 individualized flies. Horizontal
737 black lines in B and D represent the mean. Shades in A and error bars in B and D represent 95%
738 confidence intervals around the mean. No confidence intervals shades are presented in C, as they all
739 overlapped.

740

741 # Table

742 Table 1: General table of effects of diet, microbiota, and diet*microbiota, for all measured metrics.

743

	Diet			Microbiota			Diet*microbiota			
	Chi statistic (df=4)	p value	Variance explained	Chi statistic (df=1)	p value	Variance explained	Chi statistic (df=4)	p value	Variance explained	Variance explained by the model (%)
Bacterial CFU	50,34	p<0.001	0,25	117,41	p<0.001	0,59	30,01	p<0.001	0,15	-
Yeast CFU	60,73	p<0.001	0,46	57,07	p<0.001	0,43	14,07	p=0.007	0,11	-
Fresh mass	330,17	p<0.001	0,95	0,13	p=0.716	<0.01	17,36	p=0.002	0,05	86,34
Water content	291,27	p<0.001	0,97	2,72	p=0.099	0,01	4,57	p=0.333	0,02	84,52
Total lipid	908,72	p<0.001	0,99	0,21	p=0.649	<0.01	4,23	p=0.374	<0.01	94,32
Glucose content	861,86	p<0.001	0,92	0,01	p=0.913	<0.01	70,57	p<0.001	0,07	94,91
Glycogen content	146,99	p<0.001	0,86	1,57	p=0.210	<0.01	21,4	p<0.001	0,12	77,27
Development rate	3901,09	p<0.001	0,82	378,67	p<0.001	0,08	446,12	p<0.001	0,09	86,61
Dioxygen consumption	572,49	p<0.001	0,98	0,32	p=0.571	<0.01	11,63	p=0.020	0,02	91,4
RQ	90,36	p<0.001	0,95	0,73	p=0.391	<0.01	3,89	p=0.421	0,04	63,24
Food preference	177,93	p<0.001	0,94	3,18	p=0.074	0,01	7,97	p=0.092	0,04	74,03
Food consumption	58,73	p<0.001	0,88	0,17	p=0.679	<0.01	7,56	p=0.109	0,11	50,17
Cold stress survival	555,21	p<0.001	0,96	17,11	p<0.001	0,03	6,53	p=0.163	0,01	82,46
Cold knockdown t°C	28,45	p<0.001	0,11	149,54	p<0.001	0,59	73,18	p<0.001	0,29	51,31
Heat stress survival	28,13	p=0.032	0,73	0,02	p=0.898	<0.01	10,27	p=0.036	0,27	96,85
Heat knockdown t°C	10,85	p=0.028	0,3	16,85	p=0.049	0,47	7,93	p=0.094	0,22	12,93
Starvation tolerance	262,22	p<0.001	0,86	19,36	p<0.001	0,06	21,81	p<0.001	0,07	-
Dessication tolerance	29,69	p<0.001	0,78	1,56	p=0.210	0,04	6,8	p=0.146	0,18	-

744

745

746

747

748

Figure

Cold

Heat

A

Survival probability

1.0
0.8
0.6
0.4
0.2
0.0

0 20 40 60 80 100 120

Exposure duration (min)

B

Knockdown temperature (C°)

10
8
6
4
2

2:2 2:2' 8:8 8:8' 16:16 16:16' 2:16 2:16' 16:2 16:2'

C

Survival probability

1.0
0.8
0.6
0.4
0.2
0.0

0 20 40 60 80 100 120

Exposure duration (min)

D

Knockdown temperature (C°)

44
43
42
41
40
39
38
37

2:2 2:2' 8:8 8:8' 16:16 16:16' 2:16 2:16' 16:2 16:2'

E

Percent survival to starvation

100
80
60
40
20
0

0 50 100 150

Time (h)

F

Percent survival to desiccation

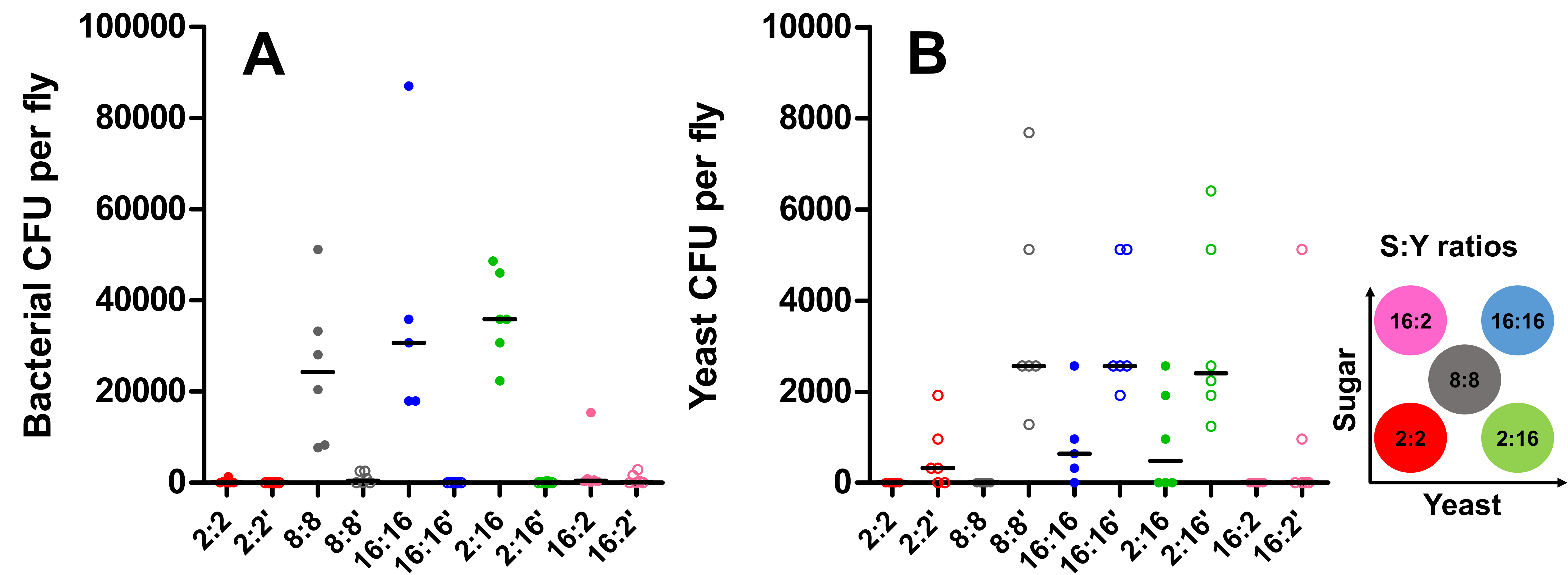
100
80
60
40
20
0

0 5 10 15 20 25

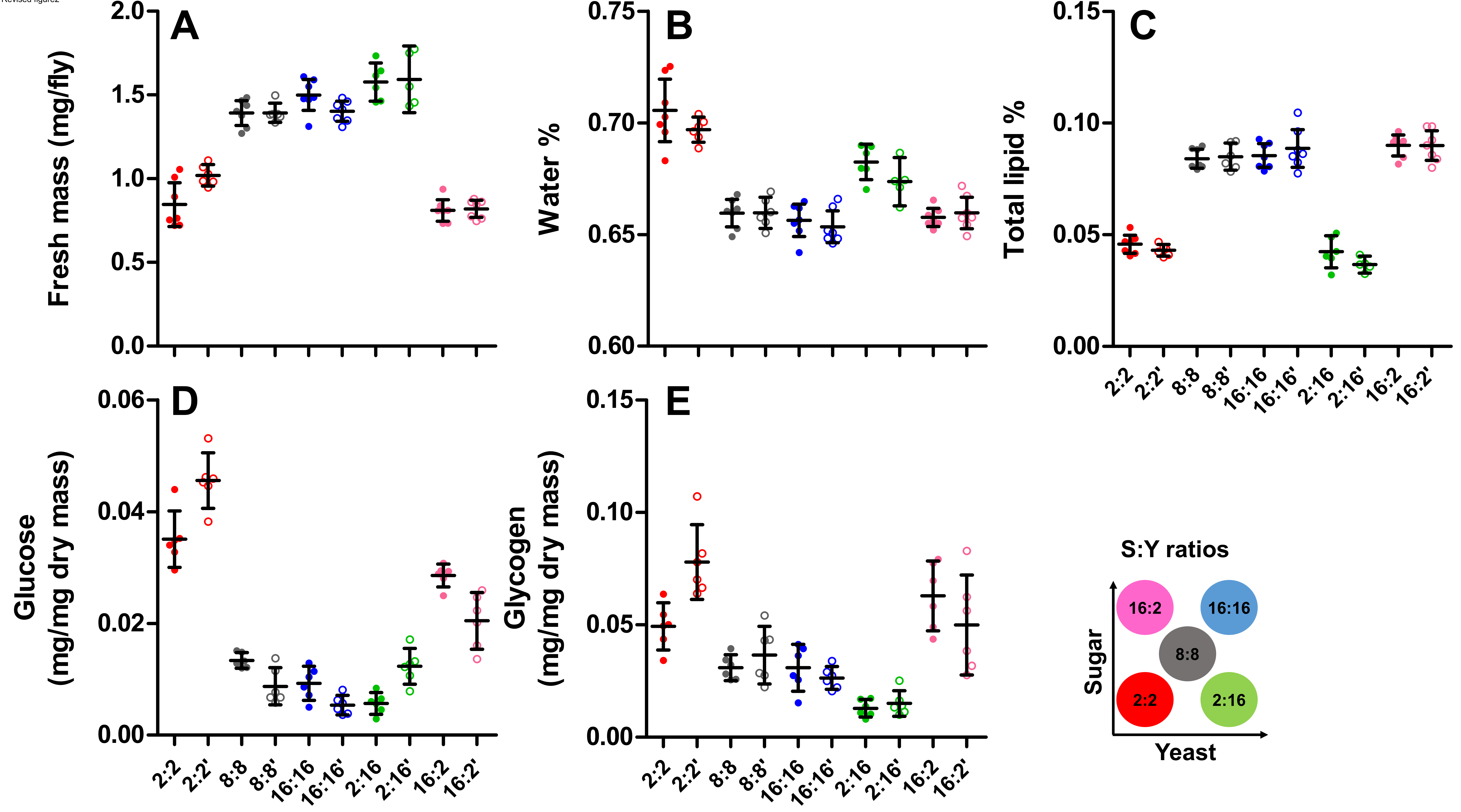
Time (h)

2:2 2:2'
8:8 8:8'
16:16 16:16'
2:16 2:16'
16:2 16:2'

Revised figure1



Revised figure2



Revised figure3

A

Developement duration (hours)

480
456
432
408
384
360
336
312
288
264
240
216

B

Basal O_2 consumption (µL/h/mg of fresh mass)

6
4
2
0

C

Respiratory quotient

1.1
1.0
0.9
0.8
0.7

100% carbohydrates metabolism

100% fatty acids metabolism

2:2 2:2' 8:8 8:8' 16:16 16:16' 2:16 2:16' 16:2 16:2'

D

Food preference (µL/fly/h)

2
1
0
-1
-2

Yeast

Sugar

2:2 2:2' 8:8 8:8' 16:16 16:16' 2:16 2:16' 16:2 16:2'

E

Total food consumption (µL/fly/h)

3
2
1
0

2:2 2:2' 8:8 8:8' 16:16 16:16' 2:16 2:16' 16:2 16:2'

S:Y ratios

16:2
16:16
8:8
2:2
2:16

Sugar

Yeast

ESM

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