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Larval density affects phenotype and surrounding bacterial community without altering gut microbiota in *Drosophila melanogaster*

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4 1 **Larval density affects phenotype and surrounding bacterial**
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6 2 **community without altering gut microbiota in *Drosophila***
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8 3 ***melanogaster***
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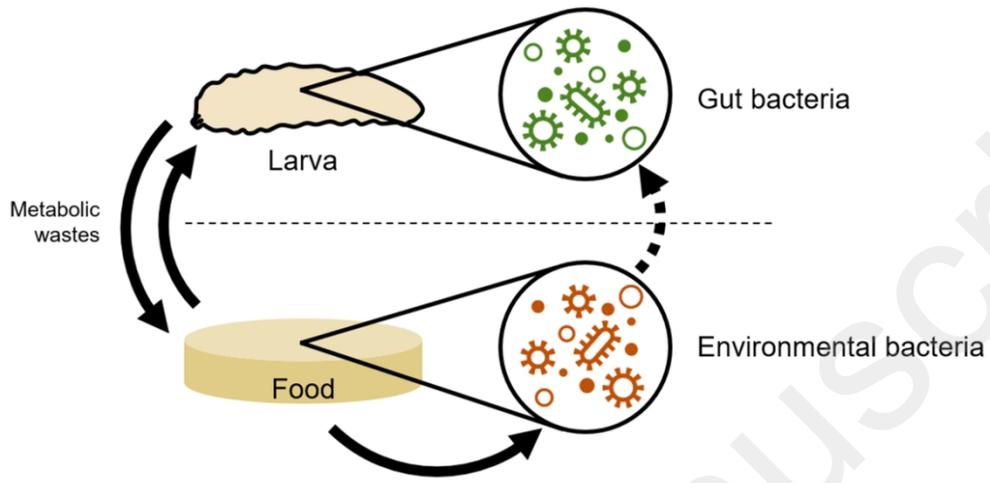
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20 12

21 13 Running title: Larval microbiota remains stable during crowding

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Accepted Manuscript

15 Abstract

16 Larval crowding represents a complex stressful situation arising from inter-individual
17 competition for time- and space-limited resources. The foraging of a large number of
18 individuals may alter chemical and bacterial composition of food, and in turn affect
19 individual's traits. Here we used *Drosophila melanogaster* to explore these
20 assumptions. First, we used a wide larval density gradient to evidence the impact of
21 crowding on phenotypical traits. We confirmed that high densities increased
22 development time and pupation height, and decreased viability and body mass. Next,
23 we measured concentrations of common metabolic wastes (ammonia, uric acid) and
24 characterized bacterial communities, both in food and in larvae, for three contrasted
25 larval densities (low, medium and high). Ammonia concentration increased in food from
26 medium and high larval densities, but remained low in larvae regardless of the larval
27 density. Uric acid did not accumulate in food but was detected in larvae. Surprisingly,
28 bacterial composition remained stable in guts of larvae whatever their rearing density,
29 although it drastically changed in the food. Overall, these results indicate that crowding
30 deeply affects individuals, but also their abiotic and biotic surroundings. Environmental
31 bacterial community likely adapts to altered nutritional situations resulting from
32 crowding, putatively acting as scavengers of larval metabolic wastes.

33 **Keywords:** larval density, crowding, microbiota, metabolic wastes, uric acid, ammonia

34

35 Introduction

36 Scramble competition may appear in insects feeding on discrete, spatially restricted
37 and ephemeral resources such as carrion, weeds or rotting fruits (Crawley and Gillman
38 1989; Nunney 1990; Ireland and Turner 2006). These limited resource patches are
39 colonized by opportunistic species as soon as they become available, and population
40 density may rapidly reach a crowded situation (Atkinson 1979). Consequences of
41 crowding are multiple. High individual densities do not only generate a quantitative food
42 shortage; foraging and excretion of conspecifics also degrade the nutritional quality of
43 the resource supply (Botella *et al.* 1985). In *Drosophila*, this typically results in marked
44 phenotypic effects such as reduced body mass and slower development (Lints and
45 Lints 1969; Scheiring *et al.* 1984; Borash *et al.* 2000; Kolss *et al.* 2009). Curiously,
46 crowding can also be beneficial to flies, for instance by promoting tolerance to stressors
47 such as starvation, toxic wastes, or thermal stress (Zwaan, Bijlsma and Hoekstra 1991;
48 Shiotsugu *et al.* 1997; Sørensen and Loeschcke 2001; Henry, Renault and Colinet
49 2018). It results that larval density may represent an important environmental pressure,
50 driving evolutionary process and adaptation (Horváth and Kalinka 2016; Sarangi *et al.*
51 2016).

52 Two main factors are supposed to underlie crowding effects in flies: food deprivation
53 and intoxication due to ingestion of metabolic wastes. In a recent study, Klepsatel *et*
54 *al.* (2018) showed that effects of increased larval density on life-history traits were likely
55 controlled by decreased yeast availability in food. They showed that yeast-poor diets
56 without crowding triggered equivalent changes in size, energy reserves and lifespan
57 as those observed in flies submitted to high larval density. Previous studies also
58 indicated that the accumulation of nitrogenous wastes, resulting from excretion (urea,
59 ammonia, uric acid), may also influence *Drosophila* development and life-history traits

1
2
3 60 (Botella *et al.* 1985; Joshi, Shiotsugu and Mueller 1996). However, it remains unclear
4
5 61 which nitrogenous wastes are actually excreted by larvae: uric acid, urea or ammonia
6
7 62 (Botella *et al.* 1985; Borash *et al.* 1998; Etienne, Fortunat and Pierce 2001; Henry,
8
9 63 Renault and Colinet 2018). Regardless of the exact compounds, multiple lines of
10
11 64 evidence suggest that exposure to nitrogenous wastes imposes a selection force
12
13 65 leading to the evolution of resistant genotypes (Borash *et al.* 2000). The combination
14
15 66 of both quantitative and qualitative food alterations thus remains a reasonable
16
17 67 hypothesis to explain phenotypic effects generated by crowding.
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22 68 The alteration of nutritional medium by the action of larval foraging is impacting flies,
23
24 69 but is also likely to affect microorganisms communities colonizing the food, such as
25
26 70 bacteria and yeasts (Chandler, Eisen and Kopp 2012; Wong *et al.* 2015; Erkosar *et al.*
27
28 71 2018). *Drosophila* individuals, whether at the larval or adult stage, establish mutualistic
29
30 72 relationships with these microorganisms (Erkosar *et al.* 2013). Contrary to obligate
31
32 73 symbiosis found in other insect species, host-bacteria interactions are facultative in
33
34 74 *Drosophila* (Erkosar *et al.* 2017). This explains the large observed variability in
35
36 75 microbiota composition, according to environmental parameters or artificial rearing
37
38 76 conditions in flies (Staubach *et al.* 2013; Erkosar and Leulier 2014; Bing *et al.* 2018;
39
40 77 Téfrit *et al.* 2018). The transient nature of this relationship implies a constant need for
41
42 78 replenishment via the feeding activity (Blum *et al.* 2013). However, this view was
43
44 79 recently challenged by the observation of stable and resilient associations between
45
46 80 fruit flies and bacteria, even in varying environment (Jehrke *et al.* 2018; Pais *et al.*
47
48 81 2018). Indeed, taxa diversity in the digestive tract of fruit flies rarely reflects the
49
50 82 microbial diversity of their close environment, suggesting an active control of
51
52 83 microbiota (Martinson, Douglas and Jaenike 2017). This is not surprising since best
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54 84 cooperators are likely to be favored over generations and co-adapt toward a
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3 85 maximization of the holobiont's fitness (Soen 2014). In this context, one can wonder to
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5 86 what extent crowding and its side-effects may alter the environmental microbial
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7 87 community as well as flies' gut microbiota. Life-history traits such as lifespan,
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9 88 morphology or development are under the influence of gut microbiota abundance and
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11 89 diversity (Brummel *et al.* 2004; Ryu *et al.* 2008; Shin *et al.* 2011; Wong, Dobson and
12
13 90 Douglas 2014). In addition, microbiota-related effects are generally dependent on
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15 91 nutrient concentration in the medium (Storelli *et al.* 2011; Yamada *et al.* 2015; Matos
16
17 92 *et al.* 2017; Bing *et al.* 2018), which may be severely reduced during crowding.
18
19 93 Considering the myriad of effects that gut microbiota has on phenotypical traits of *D.*
20
21 94 *melanogaster* (Douglas 2018), it is worth investigating whether effects of crowding may
22
23 95 be concurrently associated with microbial changes in both host and food. Larval
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25 96 crowding is an ecologically relevant and naturally occurring situation and represents a
26
27 97 great system to explore host-bacteria-environment interactions.

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33 98 In this study, we investigated the phenotypical consequences of crowding by using an
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35 99 artificial larval density gradient spanning from very low density to overcrowded
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37 100 conditions. Based on the data resulting from these phenotypic analyses, we selected
38
39 101 three contrasted densities (low, medium, and high) in which we sampled individuals
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41 102 (L3 larvae) and food substrate. In all these samples, we measured ammonia and uric
42
43 103 acid concentration, and we sequenced bacterial community based on V3/V4 16S
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45 104 regions. We assumed crowding-induced nutritional alteration would affect phenotypes
46
47 105 of individuals but also the composition of their surrounding microorganisms
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49 106 communities. We expected environmental bacterial communities in food to differ
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51 107 significantly according to larval density, and because the larvae feed on this substrate,
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53 108 we also expected their gut microbiota to show concomitant community changes. We
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55 109 supposed that ammonia and uric acid would accumulate in food especially under high
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3 110 larval density. Finally, we tested whether the supplementation of metabolic wastes in
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5 111 uncrowded conditions would result in similar phenotypic effects as under high larval
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7 112 density.
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10 11 113 **Material and methods**

12 13 14 114 **Fly stocks and rearing medium**

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16 115 We conducted the experiments on an outbred laboratory population of *D.*
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18 116 *melanogaster* derived from wild individuals collected in September 2015 in Brittany
19
20 117 (France). Fly stocks were maintained at 25°C and 70% RH (12L: 12D) on standard
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22 118 food comprising inactive brewer yeast (MP Bio 0290331205, MP Bio, 80 g.L⁻¹), sucrose
23
24 119 (50 g.L⁻¹), agar (Sigma-Aldrich A1296, 10 g.L⁻¹) and supplemented with Nipagin
25
26 120 (Sigma-Aldrich H5501; 10% 8 mL.L⁻¹). These conditions were also used for rearing of
27
28 121 flies in following experiments. *Wolbachia* symbionts were previously eliminated from
29
30 122 the population by submitting flies to a tetracycline treatment (Sigma-Aldrich T7660, 50
31
32 123 µg.L⁻¹) added in the food for three generations, followed by multiple untreated
33
34 124 generations of recovery (>10). Effectiveness of the procedure was previously
35
36 125 confirmed by PCR with *wsp* and *wspB* primers (Teixeira, Ferreira and Ashburner
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38 126 2008).
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46 128 **Larval density**

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48 129 Before all the experiments, we allowed adult flies from rearing stocks to lay eggs for
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50 130 <12 h on standard food supplemented with extra agar (15 g.L⁻¹) and food coloring.
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52 131 Using a stereomicroscope, eggs were delicately collected with a paint brush, counted
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54 132 on moistened fabric, and then transferred into new vials. Flat-bottom plastic vials (50
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56 133 mL; d = 23 mm) were precisely filled with 2.0 mL of standard food, in order to achieve
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3 134 all the desired larval density treatments (see below). Eggs manipulation was performed
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5 135 identically in all treatments, standardizing time spent under the stereomicroscope to
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7 136 15 min.
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11 138 **Effects of larval crowding**

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15 139 In a first experiment, we generated a broad range of larval densities: 1, 5, 20, 60, 100,
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17 140 200, 300, 500, and 1000 eggs per mL of food (see Fig. S1). To generate these nine
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19 141 density treatments, a total of 300, 300, 400, 480, 800, 1600, 2400, 4000, and 8000
20
21 142 eggs were counted and deposited in 150, 30, 10, 4, 4, 4, 4 and 4 replicated vials
22
23 143 respectively, each containing 2 mL of food. We did not adjust the number of deposited
24
25 144 eggs to account for embryo mortality.
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29 145 We characterized development, phenotypes, and behavior of individuals from all these
30
31 146 larval densities. For metabolic wastes measurements and microbiota characterization,
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33 147 we selected among the nine densities three densities showing contrasted phenotypes
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35 148 and referred as LD (low density; 5 eggs.mL⁻¹), MD (medium density; 60 eggs.mL⁻¹) and
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37 149 HD (high density; 300 eggs.mL⁻¹). For these three densities, we measured ammonia
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39 150 and uric acid in L3 larvae and in the food that sustained their development. We
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41 151 collected larvae and food samples over a time window of 4h and at the specific
42
43 152 occurrence peak of L3 instar for each larval density, in order to avoid sampling outlier
44
45 153 individuals. Larvae samples were L3 instar individuals picked on the surface of the
46
47 154 medium, and food samples consisted in cubes of 5x5x5 mm, i.e including the whole
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49 155 depth of the medium. Samples were then transferred in autoclaved tubes with sterile
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51 156 tools, immediately snap-frozen in liquid nitrogen, and stored at -80°C until use.
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59 158 1) Development, phenotypes and behaviors
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3 159 During development, pupation and adult emergence were checked twice a day to
4
5 160 estimate development durations (*i.e.* time to pupation and to emergence). Emerged
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7 161 adults were immediately removed from their tubes and transferred to new vials
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9 162 containing clean food. Viability was calculated based on the total number of emerged
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11 163 adults at the end of the experiment over the total number of deposited eggs. Adult fresh
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13 164 and dry masses were individually measured for both sexes from 30 randomly collected
14
15 165 individuals per density (3-days-old adults) using a micro-balance (Mettler Toledo
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17 166 UMX2, Mettler Toledo, Greifensee, Switzerland; accurate to 1 μg). Dry mass was
18
19 167 measured after individuals were dried for at least one week in oven at 60°C. Pupation
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21 168 height was measured in all vials from all densities using an electronic caliper. Because
22
23 169 of very large number of pupae, measurements were only performed on half of the
24
25 170 pupae for densities 200 and 300.

31 171 2) Metabolic wastes

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34 172 Ammonia measurements were performed using Ammonia Assay Kit (Sigma-Aldrich,
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36 173 AA100) and following manufacturer's instructions. Ten biological replicates of larvae
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38 174 (pools of ten individuals) and food were used for each of the three densities. Samples
39
40 175 were weighted using a microbalance. Food samples were adjusted to 50 μg . Samples
41
42 176 were homogenized in 250 μL (larvae) or 500 μL (food) of PBS with two tungsten beads
43
44 177 using a bead beating apparatus (20 Hz, 2 min). After dilutions when necessary,
45
46 178 colorimetric measurements were realized with microplate reader (VersaMax Molecular
47
48 179 Devices, San José, CA, USA) at 340 nm.

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52 180 Uric acid measurements were performed using uric acid assay kit (Sigma-Aldrich,
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54 181 MAK077) and following manufacturer's instructions. Eight biological replicates of 10
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56 182 larvae were used for each of the three densities. Samples were weighted using a
57
58 183 microbalance. Food samples were adjusted to 50 μg . Samples were homogenized in

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3 184 250 μ L (larvae) or 500 μ L (food) of PBS with two tungsten beads using a bead beating
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5 185 apparatus (20 Hz, 2 min). After dilutions when necessary, fluorometric measurements
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7 186 were realized with microplate reader (SAFAS Monaco Xenius XC, Monaco) set up at
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9 187 535 nm (emission) and 587 nm (excitation). Quantification was obtained by running
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11 188 serial dilution of uric acid standard. During all these experiments, samples were quickly
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13 189 processed and kept on ice to avoid degradation.
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19 191 3) Microbiota composition

22 192 For LD, MD and HD conditions, the bacterial composition of L3 larvae and of food that
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24 193 sustained their development was characterized using 16S Illumina MiSeq sequencing.
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26 194 To remove external bacteria in larvae, pools of 10 individuals were surface-sterilized
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28 195 with successive baths and quick vortex in 2.7% hypochlorite for two minutes, 70%
29
30 196 ethanol for two minutes, and rinsed twice in autoclaved milliQ water. DNA extraction
31
32 197 was performed in six independent replicates using FastDNA spin kit (MP Biomedicals),
33
34 198 according to manufacturer's instructions. We used PCRs to amplify V3/V4 16S RNA
35
36 199 regions with universal bacterial primers: forward (5'-
37
38 200 CTTCCCTACACGACGCTCTTCCGATCTACGGRAGGCAGCAG-3'), and reverse
39
40 201 (5'-GGAGTTCAGACGTGTGCTCTTCCGATCTTACCAGGGTATCTAATCCT-3') with
41
42 202 adapters for MiSeq (François *et al.* 2016). Thirty thermal cycles at 65°C annealing
43
44 203 temperature were performed. The PCR products were purified and loaded onto the
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46 204 Illumina MiSeq cartridge (Illumina, San-Diego, CA, USA) according to manufacturer's
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48 205 instructions.
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207 **Metabolic wastes supplementation**

208 To investigate phenotypic effects due to metabolic wastes *per se* in non-crowding
209 situation (*i.e.* without nutrient depletion and intense inter-individual interactions), we
210 designed a second experimental setup that is summarized in Fig. S2. In essence,
211 larvae were reared under low larval density but with high amounts of waste products.
212 Of the three putative nitrogenous wastes excreted by *Drosophila*, only ammonia (see
213 in the present study) and urea (see Henry, Renault and Colinet 2018) accumulated
214 substantially in food under crowding situation. Consequently, only these two molecules
215 (and not uric acid) were supplemented in food using nominal concentrations that were
216 experimentally found in HD food: 1.2 mg.mL⁻¹ for ammonia (Merck Millipore, 105432)
217 (see results section), and 5 mg. mL⁻¹ for urea (PanReac, PA6ACS) (see Henry et al.,
218 2018). The experimental design included four treatments: *Co* (control, no
219 supplementation), *Ur* (urea supplementation), *Am* (ammonia supplementation), *UrAm*
220 (urea and ammonia supplementation) (see Fig. S2). For all treatments, eggs were
221 deposited in LD condition (5 eggs.mL⁻¹, N=6 vials). Development duration, viability and
222 pupation height were measured as previously described.

224 **Data analysis**

225 In the density gradient experiment, development duration was analyzed using mixed
226 binomial generalized linear models (GLM) with logit link function and with replicates as
227 time-dependent random effect. Pairwise contrasts were checked using the 'emmeans'
228 package (Lenth *et al.* 2020). Viability (both in the density gradient and in wastes
229 supplementation experiments) was analyzed using binomial GLMs with logit link
230 function according to density, followed by Tukey post-hoc test to assess pairwise
231 differences using the 'Multcomp' package (Hothorn, Bretz and Westfall 2008). Mass

232 and pupation height were analyzed using non-linear models (NLS) as described in
 233 Henry, Renault and Colinet (2018). Briefly, we adapted the non-linear logistic equation
 234 proposed by Börger and Fryxell (2012) such as:

$$235 \quad \text{Mass} = d + \frac{a}{1 + \exp \left[\frac{\text{density} - b}{c} \right]}$$

236 where $(a+d)$ corresponds to the asymptotic mass at density=0, b is the inflection point
 237 expressed in density units, c is the range of the curve on the density axis, and d is the
 238 asymptotic mass at the highest density; and

$$239 \quad \text{Pupation height} = \frac{a}{1 + \exp \left[\frac{b - \text{density}}{c} \right]}$$

240 where a corresponds to the asymptotic mass at the highest density, b is the inflection
 241 point expressed in density units, c is the range of the curve on the density axis.

242 Ammonia and uric acid contents were analyzed using one-way ANOVA followed by
 243 post-hoc Tukey tests. In the waste-supplementation experiment, development duration
 244 was analyzed using Kruskal-Wallis test followed by post-hoc Dunn's test with
 245 Benjamini-Hochberg correction, and pupation height was analyzed using one-way
 246 ANOVA followed by post-hoc Tukey tests.

247 Sequencing data were analyzed using a custom pipeline. Raw pair-end sequences
 248 files from Illumina were assembled using Flash software (Magoč and Salzberg 2011)
 249 using at least a 10bp-overlap between the forward and reverse sequences, allowing
 250 5% of mismatch. Dereplicating, denoising, clustering and chimera removing steps were
 251 sequentially performed using Galaxy tool "FROGS" (Escudié *et al.* 2016). A
 252 comparison of normalized reads to the lower sample vs non-normalized reads showed
 253 it provided similar results in both cases. Thus, we kept non-normalized reads as
 254 normalization may induce statistical bias (McMurdie and Holmes 2014). Taxonomic

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3 255 affiliation was defined using Silva132 16S database. When clearly incoherent
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5 256 affiliations were generated, a blast was performed in NCBI database and the
6
7 257 identification was corrected if needed. A filter was applied to remove marginal diversity
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10 258 represented by low number of reads (< 0.05% of total number of reads per sample).
11
12 259 Once these steps were performed, data were processed using 'Phyloseq' package in
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14 260 R (McMurdie and Holmes 2013). Some sequences corresponding to *Wolbachia* were
15
16 261 found (the bacteria probably recovered from the elimination treatment thanks to rare
17
18 262 survivors). Yet, these sequences, represented only ~10% and ~1% in larvae and food,
19
20 263 respectively. They were discarded in the subsequent steps of the analysis. The
21
22
23 264 resulting OTU table was used to compute alpha and beta diversity tests (McMurdie
24
25 265 and Holmes 2013). Differences in the community composition in function of the sample
26
27
28 266 type (larvae or food) and of the density level (LD, MD, HD) were tested using
29
30 267 PERMANOVA on Bray-Curtis distance matrix (Anderson, Ellingsen and McArdle
31
32 268 2006). OTUs were clustered at the genus level for graphical representation.
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34
35 269

38 270 **Results**

41 271 **Effects of larval crowding on phenotype**

42 272 Development was significantly affected by larval density (Fig. 1A) ($F=162.43$, $df=8$,
43
44 273 $p<0.001$). Flies from density levels higher than 100 were about 12 h slower to reach
45
46 274 50% of emerged adults than levels below 100. Above density 100, the distribution and
47
48 275 variance of emergence events became much larger due to extreme individuals
49
50
51 276 emerging up to 8 days after the adults reared at low density. Viability was strongly
52
53 277 dependent on larval density (Fig. 1B) ($\chi^2=7907$, $df=1$, $p<0.001$). Within our tested
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55
56 278 range, we could capture the upper and the lower viability limit of our *D. melanogaster*
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59 279 population reared under crowded conditions. Density levels of 1 to 20 showed the

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3 280 maximal viabilities (80-90%), and each increasing density level diminished the viability
4
5 281 until density 1000, where less than 3% of the deposited eggs turned into viable adults.
6
7 282 Pupal and adult viabilities showed parallel decreasing patterns in function of density.
8
9 283 Adult viability was only slightly lower than pupal viability, indicating that larval density
10
11 284 mainly affects the egg to pupae part of development. Body mass was also strongly
12
13 285 reduced in higher densities, both in females and males and both in fresh and dry mass
14
15 286 (Fig. 1C). Proprieties of the model allowed to highlight the lower limit of weight of flies,
16
17 287 which appears to be around 400 eggs. mL⁻¹. The weight difference reached up to three-
18
19 288 fold change between extreme density levels (*i.e.* 1 vs 1000). Pupation height was also
20
21 289 rapidly affected by density (Fig. 1D). Larvae from low densities (1 to 20) tended to
22
23 290 pupate very close to the food substrate, whereas larvae from higher densities clearly
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25 291 pupated higher in the vials, 50 mm over the surface on average.
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33 293 **Effects of larval crowding on nitrogenous wastes**

34
35 294 Ammonia and uric acid concentrations were both dependent on larval density and on
36
37 295 sample type, with significant interaction between these factors (Fig. 1E & F) (density *
38
39 296 sample type effect: $\chi^2=353$, $df=2$, $p<0.001$; $\chi^2=264$, $df=2$, $p<0.001$; $p<p<$ for ammonia
40
41 297 and uric acid respectively). Ammonia was detected mostly in food, where
42
43 298 concentrations were significantly higher in MD and HD than in LD conditions (Tukey
44
45 299 HSD, $p<0.001$) (Fig. 1E). In contrast, ammonia was found at very low concentrations
46
47 300 in larvae and there was no change depending on density (Fig. 1E). Uric acid showed
48
49 301 a completely different pattern as it was barely detectable in food samples, but present
50
51 302 in larvae (Fig. 1F). Uric acid concentration in larvae was negatively correlated with
52
53 303 larval density, with highest value in LD, intermediate value in MD and lowest value in
54
55 304 HD individuals (all significantly different, Tukey HSD, $p<0.001$).
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306 Effects of larval crowding on bacterial communities

307 Microbiota diversity was affected by sample type and by larval density, whatever the
308 considered index (Fig. 2A-D; S3; S4). Bacterial communities were significantly different
309 between larvae and their environment ($F_{1,30}=10.94$, $p=0.002$; $F_{1,30}=37.79$, $p<0.001$; for
310 observed richness and Shannon diversity respectively) and were also significantly
311 modified by larval density ($F_{2,30}=8.19$, $p=0.001$, $F_{2,30}=13.74$, $p<0.001$; for observed
312 richness and Shannon diversity respectively). Pairwise comparison of larval density
313 treatments did not show differences in OTU richness for larvae or food samples (Fig.
314 2A, Tukey HSD, $p>0.05$ for all densities comparisons). Pairwise comparison of larval
315 density treatments did not show differences in Shannon diversity index for larvae
316 samples (Tukey HSD, $p>0.05$ for all densities comparisons), but significant differences
317 were detected among food samples, with higher diversity in HD than in LD and MD
318 foods (Tukey HSD, $p<0.001$). Beta diversity was largely impacted by larval densities in
319 food samples but not in larval gut samples (Fig. 2C, S4). Both larval density ($F_{2,30}=6.40$,
320 $p<0.001$), sample type ($F_{1,30}=7.58$, $p<0.001$), and their interaction ($F_{2,30}=5.97$, $p<0.001$)
321 had significant effect on Bray-Curtis distances, explaining respectively 21, 12 and 19%
322 of the total variance (Table S1). The figure 2D shows large and consistent differences
323 in bacterial composition of the food according to larval density, while only subtle
324 changes in bacterial composition were found in larval gut microbiota. Even if the global
325 bacterial community in larvae did not drastically changed with density, we could note
326 the apparition of *Lactobacillus* OTUs in MD larvae (and to a lesser extent in HD); these
327 *Lactobacillus* were almost absent in LD.

328

329 **Effects of metabolic wastes supplementation**

330 Supplementation of metabolic wastes had a subtle but significant effect on
331 development (Fig. 3A) ($\chi^2=54.02$, $df=3$, $p<0.001$). Supplementation of ammonia (in Am
332 or UrAm) consistently increased the development time by about half a day compared
333 to the control without supplementation (Dunn's, $p<0.001$). Urea alone had smaller
334 effect than ammonia, but still increased development time compared to control
335 (Dunn's, $p=0.019$). We found no significant effect of supplementation on viability or
336 pupation height (Fig. 3B & C) ($\chi^2=3.90$, $df=3$, $p=0.272$; $\chi^2=3.34$, $df=3$, $p=0.342$; for
337 viability and pupation height respectively). Viability was over 80% in all treatments,
338 which is comparable with values found in the first experiment at similar larval density.
339 Median pupation height was comprised between 10 and 15 mm in all conditions, which
340 is also comparable to heights found in the first experiment for the same density.

341 **Discussion**

342 Crowding is usually regarded as a source of nutritional stress. When a fixed amount of
343 food has to be shared by an increasing number of individuals, the quantity available
344 per individual unavoidably shrinks. Klepsatel et al. (2018) investigated this
345 phenomenon in flies, showing that marked phenotypic changes generated by a gradual
346 nutrient depletion during crowding could be rescued by yeast supplementation, or
347 conversely induced without crowding by yeast deprivation. For traits such as
348 development, lifespan, or metabolic state, it implies that crowding as a stressor could
349 almost be considered equivalent to a simple dietary restriction. In this study, we first
350 characterized the strong phenotypical consequences of different larval densities, and
351 then, we explored some least characterized aspects of crowding, such as the role of
352 toxic metabolic wastes and the impact of larval density on bacterial communities in
353 food and larval guts.

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3 354 Larval crowding generated large variations in all measured phenotypic traits
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5 355 (development, survival and morphology). The present data are therefore in agreement
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7 356 with our previous observations (Henry, Renault and Colinet 2018) but also provide
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10 357 additional descriptions of crowding effects on behavioral traits (*i.e.* pupation site
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12 358 selection). We can now rather precisely predict the extreme limit of viability under
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14 359 crowding: less than 3% of eggs on average should attain the adult stage when density
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16 360 is over 1000 eggs.mL⁻¹. Strikingly, about 20% viability is still attainable with 500
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18 361 eggs.mL⁻¹, highlighting the great competitive ability of fruit flies. Decreased viability
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20 362 with larval density likely reflects, at least in part, that an increasing number of
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22 363 individuals were unable to reach critical mass for pupation (Mirth, Truman and
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24 364 Riddiford 2005). In line with this observation, body mass showed a clear decline with
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26 365 increasing larval density (Scheiring *et al.* 1984; Shenoj, Ali and Prasad 2016), followed
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28 366 by a plateau at the density levels of 400 and above. This plateau suggests that a critical
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30 367 minimal viable mass was reached in our population at around one third of the normal
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32 368 mass. Although we observe a developmental delay at high densities, it did not exceed
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34 369 one day for the median time. However, above density level of 100, the distribution of
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36 370 emergence became much larger due to extreme individuals: from intervals of about
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38 371 two days between the first and the last emerging individual at low densities (1-20) to
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40 372 intervals sometimes over 7 days at the highest densities (200-1000). Higher variance
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42 373 at high densities is probably the consequence of contrasted responses: some
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44 374 individuals were rapid enough to profit from early non-degraded conditions while the
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46 375 others were exposed to increasingly degraded food which generated a developmental
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48 376 delay.
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56 377 Nitrogenous wastes are generally toxic. Ammonia is known for its cytotoxic activity,
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58 378 impeding respiratory metabolism and membrane ion transporters (Weihrauch, Donini
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3 379 and O'Donnell 2012; Henry *et al.* 2017) and urea, although being less toxic, can impair
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5 380 development and survival of species through protein denaturation (David *et al.* 1999).
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7 381 Uric acid can show positive effects by preventing oxidative damages or water loss
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9 382 (Hilliker *et al.* 1992; Andersen *et al.* 2010), but may also trigger detrimental
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11 383 inflammatory reactions (Sautin and Johnson 2008). Consequently, we expected that
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13 384 crowding, by increasing wastes concentration, would be stressful to larvae. Notably,
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15 385 we observed changes in the larval behavior, with a significant increase in the pupation
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17 386 height at high densities. This behavior is likely the outcome of contrasted larval foraging
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19 387 strategies that have been similarly observed in density-selected lineages (Sokolowski,
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21 388 Pereira and Hughes 1997) and we speculated that increased pupation height at higher
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23 389 densities could result from a need to avoid proximity with toxic products (Belloni *et al.*
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25 390 2018). Yet, we found that pupation height was unchanged in larvae reared at low
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27 391 density, when toxic metabolic wastes were artificially supplemented in the food to
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29 392 mimic larval crowding conditions. Therefore, the increase in pupation height could be
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31 393 a consequence of nutritional restriction or inter-individual pressures such as
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33 394 cannibalism (Vijendravarma, Narasimha and Kawecki 2012), rather than an avoidance
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35 395 of toxic wastes. Wastes supplementation had also limited impact on life-history traits,
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37 396 inducing only a minor developmental delay and no additional mortality. Our results thus
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39 397 seem to corroborate the conclusions of Klepsatel *et al.* (2018) that, for these traits,
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41 398 nutritional restriction as a stressor outshines other stress resulting from larval
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43 399 crowding.

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45 400 In insects, the main waste products of nitrogenous metabolism are uric acid, urea and
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47 401 ammonia (Bursell 1967; O'Donnell and Donini 2017). Even in model species like *D.*
48
49 402 *melanogaster*, it remains unclear whether uric acid is the major waste product, as in
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51 403 many Dipterans (Bursell 1967; Dow and Davies 2003) or if other compounds contribute

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3 404 to the excretion. Urea is supposed to occur naturally in *Drosophila* cultures (Joshi,
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5 405 Shiotsugu and Mueller 1996) and previous studies actually found increasing levels of
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7 406 urea in food in case of overfeeding (Botella *et al.* 1985) or with increasing larval
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9 407 densities (Henry, Renault and Colinet 2018). On the other hand, Etienne *et al.* (2001)
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11 408 suggested that *D. melanogaster* is unable to produce this compound. The presence of
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13 409 urea in food may thus result from conversion of products such as uric acid by the action
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15 410 of aerobic *bacteria* (Bachrach 1957; Potrikus and Breznak 1981; Winans *et al.* 2017).
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17 411 Borash *et al.* (1998) reported that ammonia is the primary metabolic waste product of
18
19 412 *D. melanogaster* larvae but other studies rather posit that uric acid is the main waste
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21 413 product of nitrogen metabolism (Botella *et al.* 1985; Winans *et al.* 2017). Finally,
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23 414 products such as allantoin were also found in the food medium, but have not been
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25 415 measured in flies (Borash *et al.* 1998; O'Donnell and Donini 2017). According to this
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27 416 literature, we can speculate that all these compounds could be present in the medium,
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29 417 though in variable amounts. Our results confirm the presence of ammonia in food, with
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31 418 high levels detected in MD and HD conditions. On the other hand, we only found traces
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33 419 of uric acid in the food but surprisingly higher amounts in larvae. This suggests that *D.*
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35 420 *melanogaster* larvae produce and accumulate uric acid, probably in tissues such as
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37 421 the fat body or in Malpighian tubules (Weihrauch, Donini and O'Donnell 2012), but this
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39 422 compound seems to quickly degrade once in the environment. Consequently, the
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41 423 presence of urea and ammonia in food may either result from an effective excretion of
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43 424 these products by larvae, or from the degradation and transformation of uric acid in the
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45 425 environment. In other words, uric acid could degrade either inside or outside the larvae
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47 426 (fig. 4).

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49 427 The classic pathway of uric acid degradation involves the urate oxidase enzyme, coded
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51 428 by the *Uro* gene in *D. melanogaster* (Friedman 1973; Wallrath, Burnett and Friedman
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3 429 1990). *Uro* already showed increased expression with increased larval densities
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5 430 (Henry, Renault and Colinet 2018), and this could have played a role in the reduction
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7 431 of uric acid content observed in MD and HD, in comparison with LD (fig. 4A). In
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9 432 addition, the progressive nutrient depletion in crowded conditions limited the amount
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11 433 of digestible material (particularly purines) and therefore may have reduced uric acid
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13 434 production. Alternatively, uric acid could be excreted by larvae and used as a nitrogen
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15 435 resource by surrounding microorganisms (fig. 4B). This last hypothesis is supported
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17 436 by previous observations in termites (Potrikus and Breznak 1981) and by recent
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19 437 findings showing a functional selection for uric acid metabolization in some bacterial
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21 438 taxa associated with laboratory cultures of *Drosophila* flies (Winans *et al.* 2017).
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23 439 Indeed, Winans *et al.* (2017) reported that uric acid accumulated substantially in the
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25 440 medium of axenic flies cultures, but not in the medium of flies harboring bacteria with
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27 441 functional urate oxidase gene. We can expect that in crowded conditions, bacteria
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29 442 experienced even more this selection pressure with unusual high wastes levels. The
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31 443 changes between bacterial communities identified in the environment of LD, MD and
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33 444 HD are in line with this assumption. From a community largely dominated by
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35 445 *Acetobacterales* (*Acetobacter sp* and *Gluconobacter sp*) in LD, environment changes
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37 446 modified the species composition with the apparition of *Lactobacillus sp* in MD. In HD,
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39 447 changes totally reshaped the community, reducing the abundance of *Acetobacterales*
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41 448 but allowing to *Enterococcus* and *Pseudomonas* species to thrive. In a first attempt to
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43 449 explore the functional diversity of bacterial communities from the different larval density
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45 450 conditions, we extrapolated the expression level of metabolic pathways using our
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47 451 taxonomic diversity data and the PICRUSt2 pipeline (Douglas *et al.* 2019) (fig. S5).
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49 452 The analysis revealed no marked functional change in pathways related to nitrogen
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51 453 wastes regulation such as the purine and urea metabolism (fig. S6). However, this kind
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3 454 of approach may not be well suited to evidence minute variation at the pathway level
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5 455 in samples collected from unusual environments, possibly explaining this apparent lack
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7 456 of functional changes.
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11 457 The presence of *Lactobacillus* almost exclusively in MD food is of particular interest:
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13 458 this genus is known to be a good cooperator with flies (Storelli *et al.* 2011; Newell and
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15 459 Douglas 2014) and shows a high dependency upon host's diet as a driving evolutionary
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17 460 force (Martino *et al.* 2018). Hence, previously reported beneficial effects of
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19 461 intermediate larval densities on flies (Sørensen and Loeschcke 2001; Moghadam *et*
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21 462 *al.* 2015; Sheno, Ali and Prasad 2016; Henry, Renault and Colinet 2018) could be the
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23 463 result of an Allee effect favoring development of specific bacteria that happen to be
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25 464 advantageous (Wertheim *et al.* 2002). Conversely, *Pseudomonas* genus is known for
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27 465 its pathogenicity towards *Drosophila*, and could have intensified detrimental effects of
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29 466 high larval densities (Vodovar *et al.* 2005; Apidianakis and Rahme 2009). Whether the
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31 467 species changes are related to wastes concentration changes or to other factors has
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33 468 to be clarified in future studies, for instance by sequencing food and larvae samples
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35 469 coming from similar set-ups as we used in our supplementation experiment.
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37 470 Additionally, performing new larval crowding experiments in axenic conditions could
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39 471 help disentangling the importance of these adverse bacteria in the detrimental effects
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41 472 of high larval densities.
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45 473 Larval density mainly affected the environmental bacterial composition, with few
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47 474 impacts on gut microbiota. The only modification we concomitantly observed in both
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49 475 food and larvae was the apparition of *Lactobacillus sp* starting from intermediate
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51 476 densities. Aside from this minor alteration, the gut community was surprisingly
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53 477 independent to external changes. Therefore, in our laboratory population, *D.*
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55 478 *melanogaster* larvae may have established stable mutualistic relationships with
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3 479 bacteria able to cope with occasional dietary alterations and mitigate opportunistic
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5 480 colonization by new species. Stable mutualistic relationships between flies and gut
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7 481 microbiota have previously been observed in wild *Drosophila* species feeding on
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9 482 mushrooms, and harboring bacteria that are almost absent in the environment
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11 483 (Martinson, Douglas and Jaenike 2017). Our observation of stable relationships
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13 484 between flies and gut microbiota in a laboratory context shows that flies may gain
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15 485 advantages controlling their microbiota, even in favorable environment assumed to
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17 486 apply limited selection force and consequently lead to transient bacterial communities
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19 487 (Blum *et al.* 2013). Soen (2014) proposed that rapid environmental changes would
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21 488 change microbiota; this would affect the host in return, snowballing into a dysbiosis
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23 489 state. Here, we found that the constant ingestion of food was not strongly affecting gut
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25 490 microbiota composition. Mechanisms underlying the control of microbiota by larvae
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27 491 remain elusive (Erkosar and Leulier 2014). Co-adaptation over many generations in
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29 492 laboratory conditions is probably involved, especially the selection for functional traits
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31 493 improving bacteria fitness such as the loss of motility and uric acid degradation ability
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33 494 (Winans *et al.* 2017).

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35 495 Larval crowding is a complex nutritional situation that has strong ecological relevance.
36
37 496 Here, while we measured large metabolic wastes variability depending on larval
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39 497 density, we could not establish a link between wastes and the observed phenotypical
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41 498 and behavioral changes. Nonetheless, wastes may still apply a strong pressure to the
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43 499 association between larvae and bacteria, affecting the stability of the relationship. We
44
45 500 evidenced microbial composition modifications in food associated with density-
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47 501 dependent changes in metabolic wastes. This represents one of the few examples
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49 502 where macroorganisms action can actually shape the biotic micro-environment through
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51 503 abiotic alterations (Stamps *et al.* 2012; Wong *et al.* 2015). Future studies will need to
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3 504 solve the apparent paradox of larval microbiota stability in changing environments,
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5 505 when evidences accumulate in favor of diet-centered rather than host-centered
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7 506 adaptation (Winans *et al.* 2017; Martino *et al.* 2018). The key here is to understand
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10 507 why some bacteria are transient whereas some others can persist in the gut, and why
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12 508 this dichotomy parallels laboratory vs wild bacteria (Pais *et al.* 2018). The use of germ-
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14 509 free eggs and gnotobiotic individuals at high larval densities could help to unravel the
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17 510 dynamics of the relationship in these complex situations.
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20 511 **Data accessibility**

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23 512 Sequencing data has been deposited in the NCBI Sequence Read Archive (SRA)
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25 513 database under project number PRJNA611582. Other datasets are available on the
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28 514 Figshare repository under the DOI doi.org/[10.6084/m9.figshare.11956095](https://doi.org/10.6084/m9.figshare.11956095).
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31 515 **Conflict of interest**

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34 516 Authors declare no conflict of interest
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37 517 **References**

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1 **Figure legends**

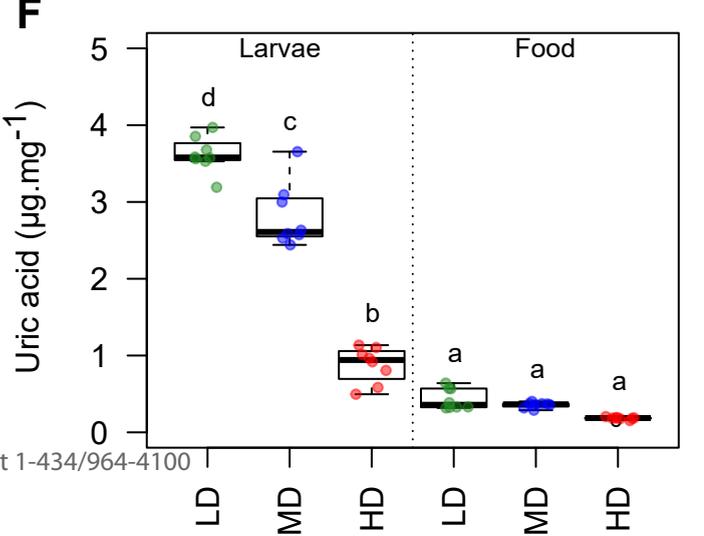
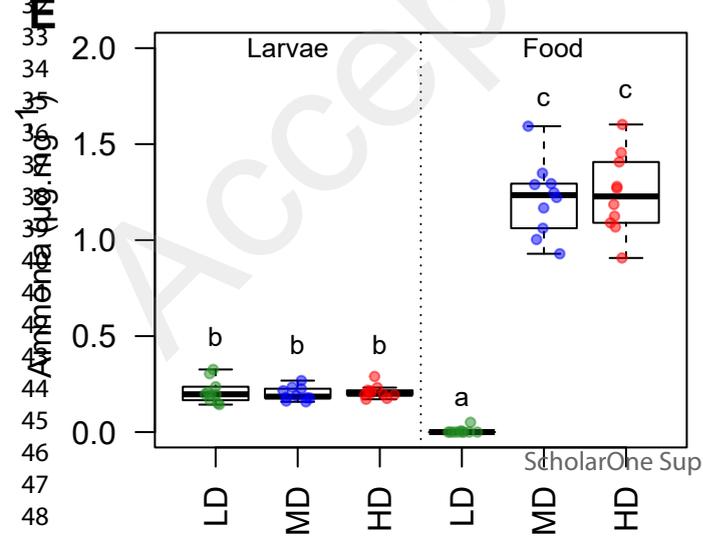
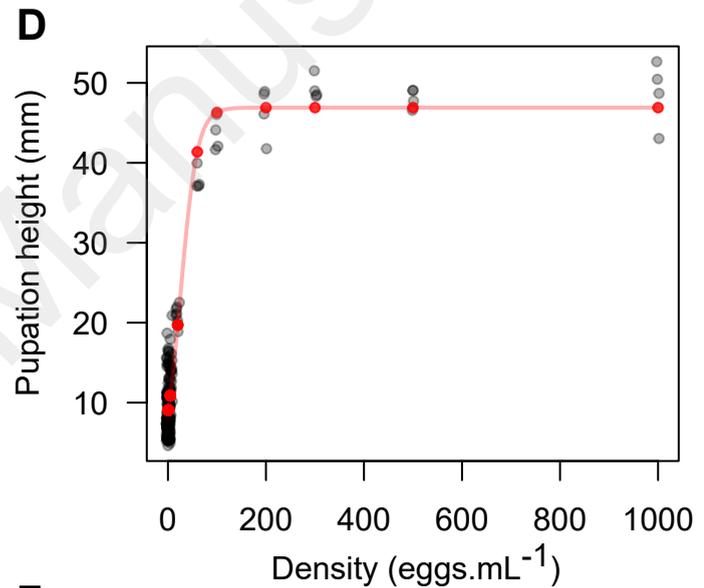
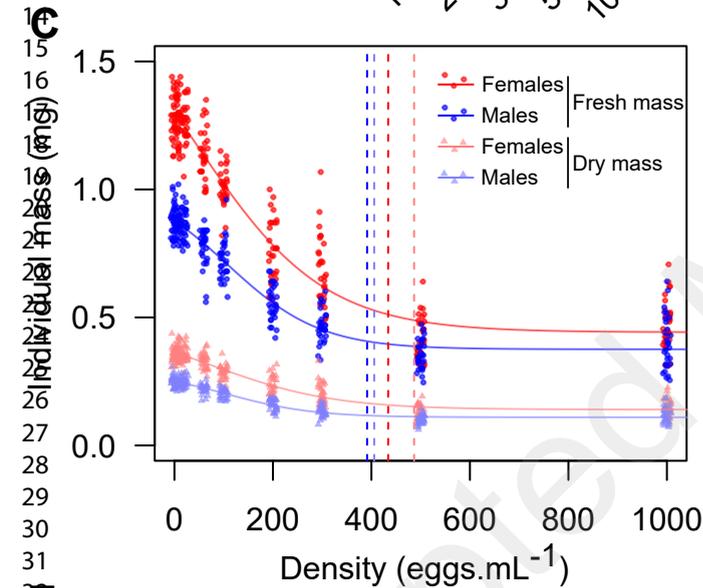
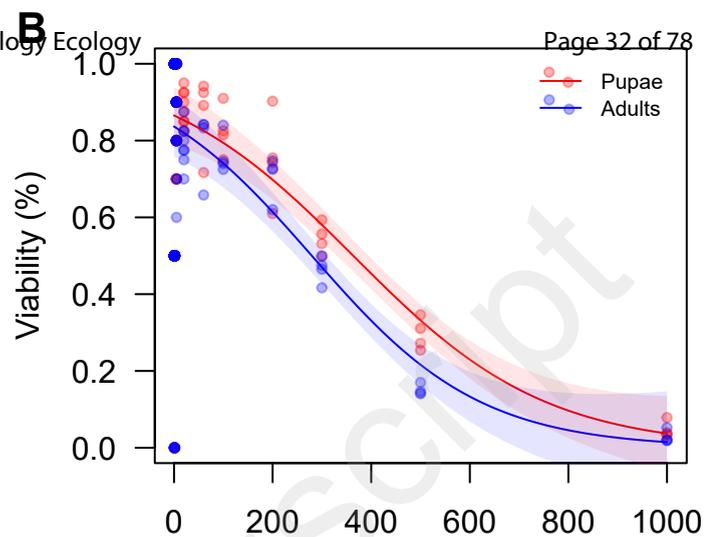
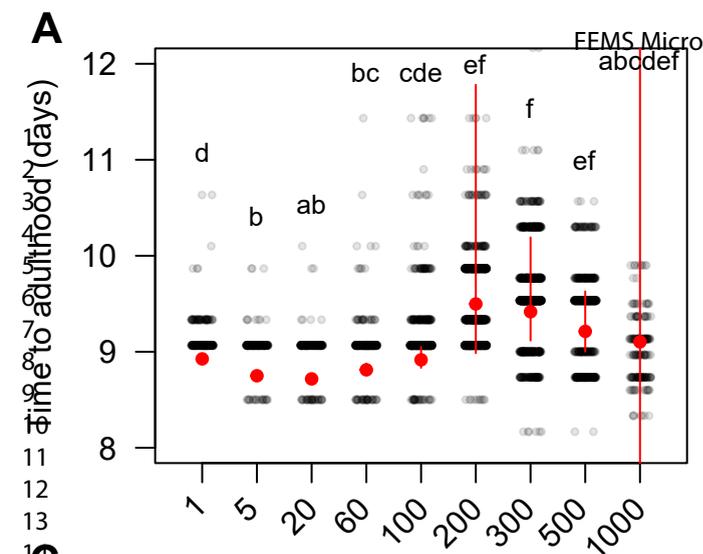
2 Figure 1: Direct consequences of larval crowding in *D. melanogaster*. (A) Development
3 time to adulthood as a function of larval density. Black dots: individual adult emergence
4 events. Red dots: mean development duration predicted using NLS model. Red error
5 bars: 95% confidence intervals around the prediction. Different letters indicate non-
6 overlapping confidence intervals. (B) Viability from egg to pupae (red) and from egg to
7 adult (blue) as a function of larval density. Dots: mean viability per culture vial. Lines:
8 predictions from binomial GLM. Shaded areas: 95% confidence intervals around
9 predictions. (C) Fresh and dry masses of female and male adult individuals as a
10 function of larval density (N=30 per sex per density). Dots: individual mass
11 measurements. Lines: predictions from NLS model. Horizontal dashed lines:
12 stabilization threshold of mass calculated from model proprieties. (D) Pupation height
13 as a function of larval density. Dots: mean pupation height per vial. Red line and dots:
14 prediction from NLS model. (E) and (F) Boxplots of ammonia (N=10 per sample type
15 per density) and uric acid (N=8 per sample type per density) concentrations, in larvae
16 and food samples from LD, MD and HD conditions. Boxes: first and third quartiles of
17 the distribution. Black horizontal line: median of the distribution. Different letters
18 indicate significant differences (Tukey test, $p < 0.01$).

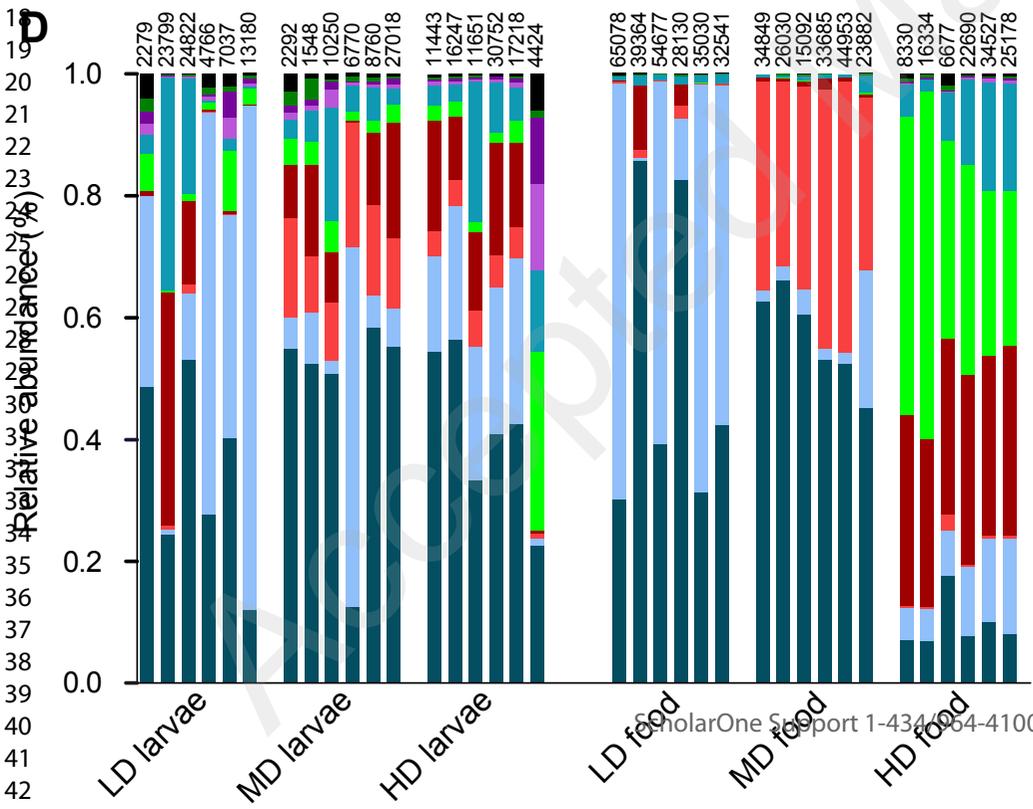
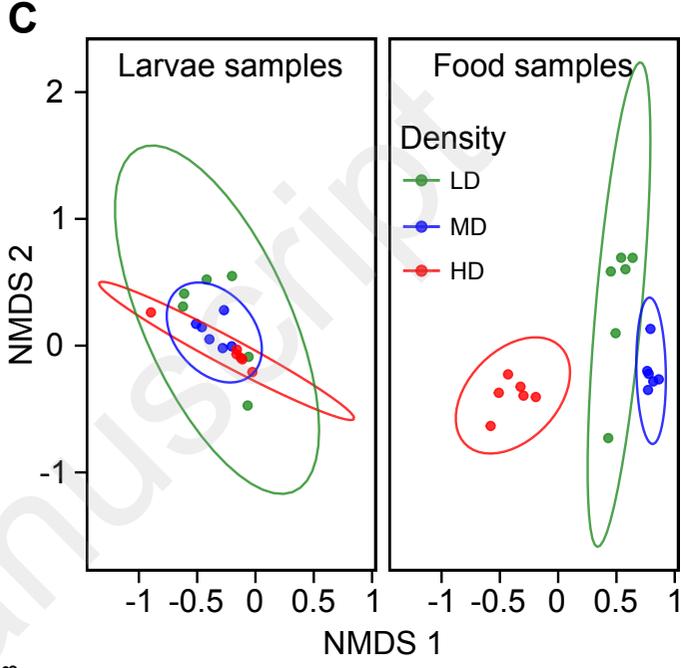
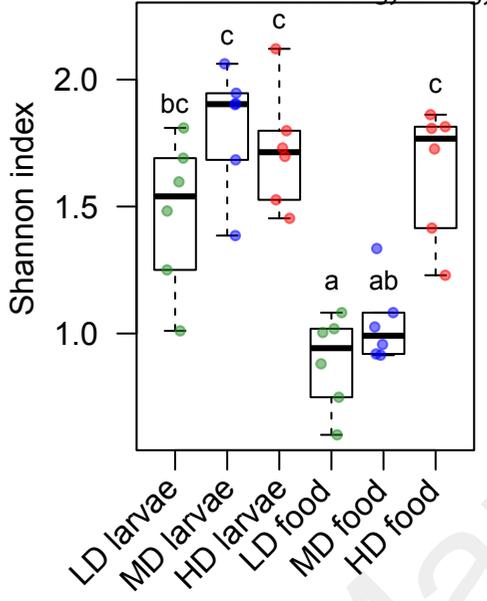
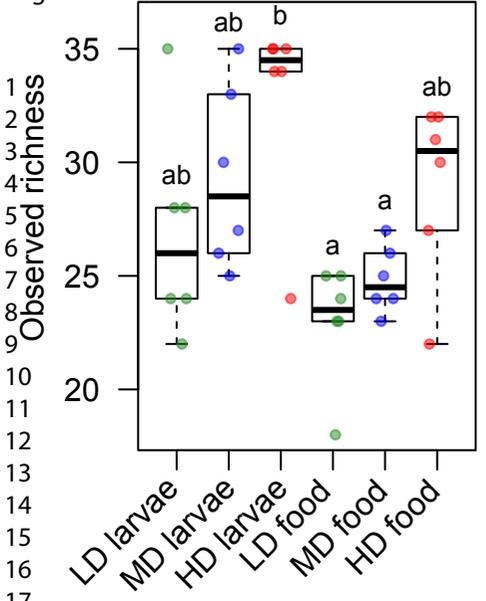
19 Figure 2: Bacterial community variations in *D. melanogaster* larvae and in its
20 environment, at increasing population densities. (A) and (B) Boxplots of observed
21 richness and of Shannon diversity respectively in LD, MD and HD conditions. Dots:
22 sequencing replicates diversity values. Boxes: first and third quartiles of the
23 distribution. Black horizontal line: median of the distribution. Different letters indicate
24 significant differences (Tukey test, $p < 0.01$). (C) NMDS Bray-Curtis ordination of
25 bacterial communities in LD, MD and HD conditions, split by sample type (larvae or

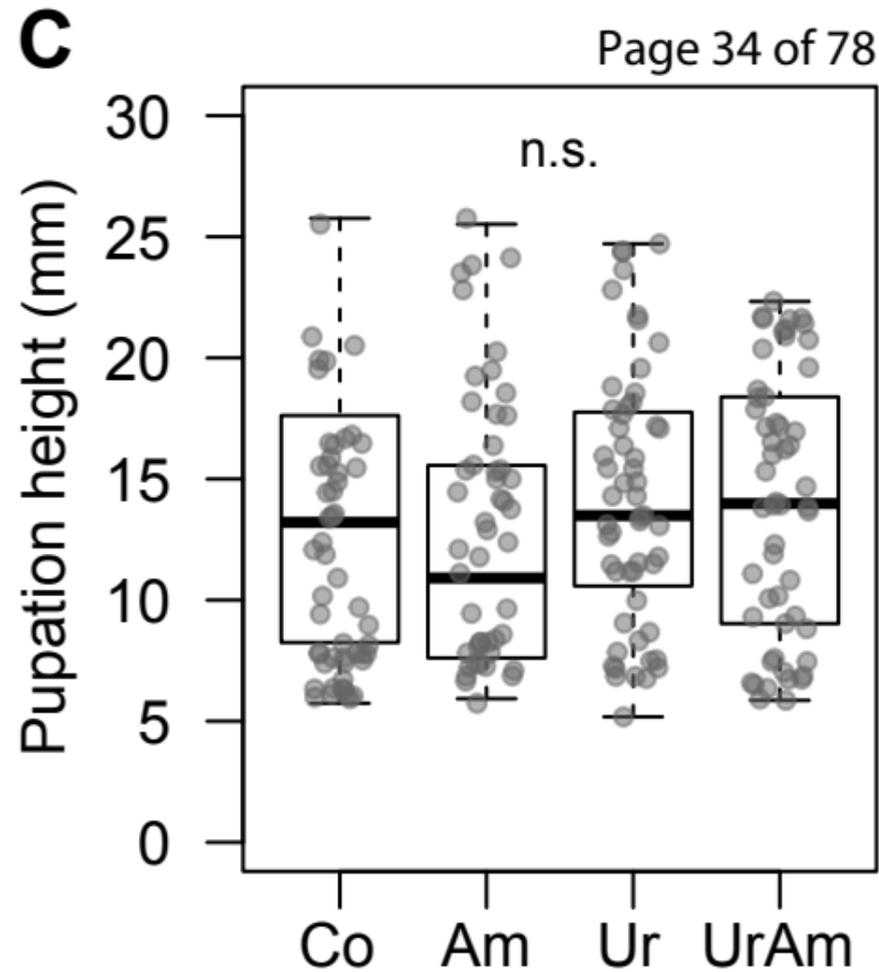
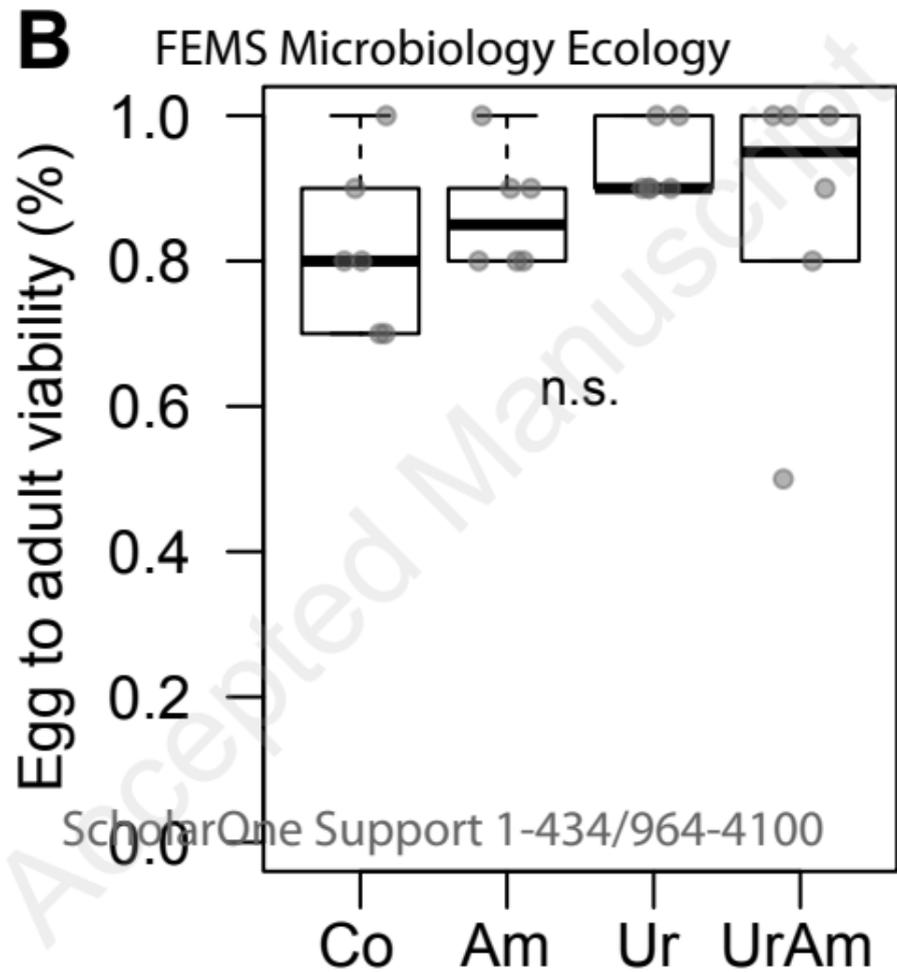
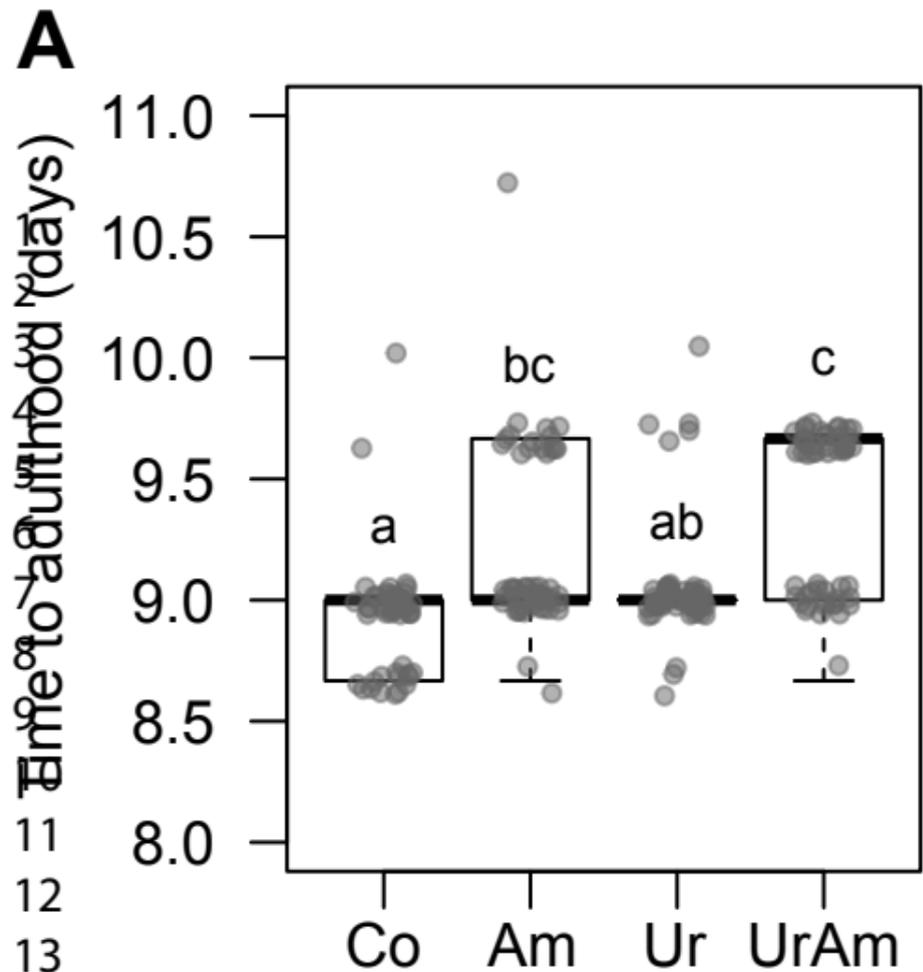
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3 26 food). Dots: sequencing replicates, colored by rearing density. Ellipses represent 95%
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5 27 confidence zones. (D) Stacked barplot of sequenced OTU, grouped at the genus level.
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8 28 Number of reads effectively used per replicate is displayed on top of bars.
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11 29 Figure 3: Effects of artificial supplementation of metabolic wastes on development and
12
13 30 pupating behavior in *D. melanogaster*. (A) Development time to adulthood in control
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15 31 flies and in the three supplementation treatments. Dots: individual adult emergence
16
17 32 events. Different letters indicate significant differences (Dunnett test, $p < 0.01$). (B)
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19 33 Percentage of viable adults emerged from deposited eggs. Dots: mean viability per vial
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21 34 (N=6). (C) Pupation height. Dots: individual pupation heights. For all plots, boxes: first
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23 35 and third quartiles of the distribution. Black horizontal line: median of the distribution.
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25 36 “n.s.” indicates no statistical differences.
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30 37 Figure 4: Conceptual visualization of hypothetical nitrogen cycle in the lab *D.*
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32 38 *melanogaster* system. (A) Larva is metabolizing nutrients, producing uric acid in the
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34 39 process. Larva then degrades uric acid into intermediate products and finally to
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36 40 ammonia or urea through the uricolytic pathway before defecating in the food. (B) Larva
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38 41 is metabolizing nutrients, producing uric acid in the process. This uric acid is directly
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40 42 excreted in the hindgut or defecated in the food, before microorganisms scavenge it
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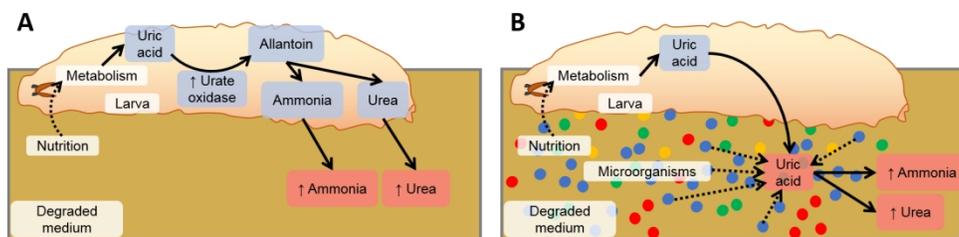


Figure 4: Conceptual visualization of hypothetical nitrogen cycle in the lab *D. melanogaster* system. (A) Larva is metabolizing nutrients, producing uric acid in the process. Larva then degrades uric acid into intermediate products and finally to ammonia or urea through the uricolytic pathway before defecating in the food. (B) Larva is metabolizing nutrients, producing uric acid in the process. This uric acid is directly excreted in the hindgut or defecated in the food, before microorganisms scavenge it into ammonia and urea.

301x80mm (150 x 150 DPI)

Supplementary material

Functional profiling of the bacterial metacommunity

We extrapolated functional characteristics of bacterial communities from their taxonomic composition using the PICRUST2 program ver 2.2.0 (Douglas *et al.* 2019). We used the same OTU tables than those used for taxonomic diversity analyses as inputs (build with SILVA 132 16S database), and then run the PICRUST2 script with default settings (picrust2_pipeline.py). We extracted KEGG Orthology (KO) abundances and normalized them relative to the total expression level in each individual sample. We grouped KO in KEGG pathways and KEGG modules (Kanehisa and Goto 2000) and calculated functional differences between treatments using Kruskal-Wallis test followed by post-hoc Dunn's test with Benjamini-Hochberg correction.

Supplementary figures

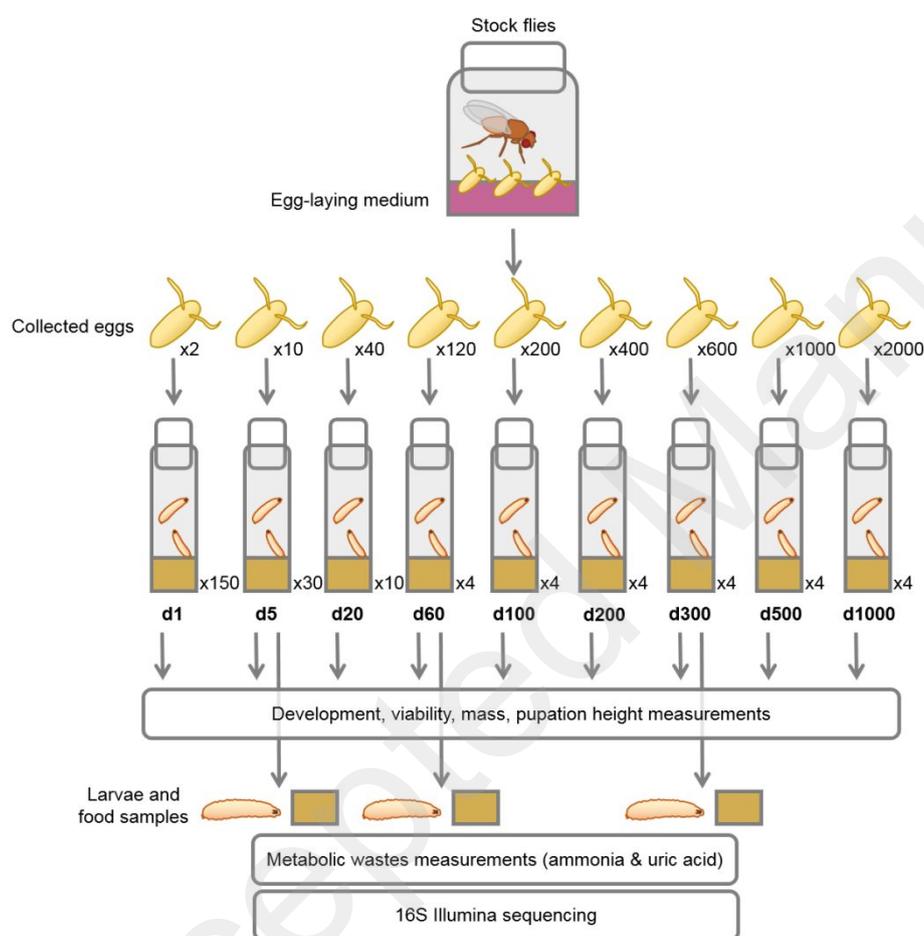


Figure S1: Experimental procedure for testing life-history traits, development, behavior, toxic wastes concentration, and bacterial community variations under increasing larval densities.

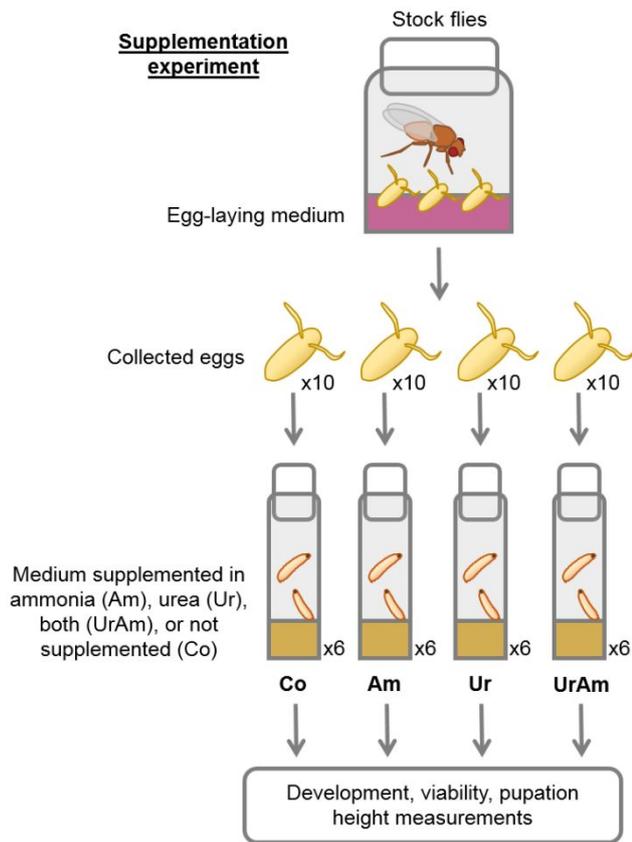


Figure S2: Experimental procedure for testing toxic effects of nitrogenous wastes independently from larval density.

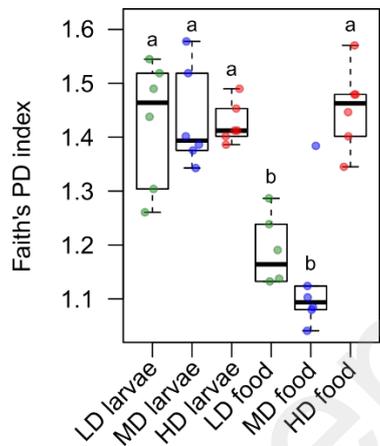


Figure S3: Boxplots of Faith's phylogenetic alpha diversity index in LD, MD and HD conditions, from either larvae or food samples. Dots: sequencing replicates diversity values. Boxes: first and third quartiles of the distribution. Black horizontal line: median of the distribution. Different letters indicate significant differences (Tukey test, $p < 0.01$).

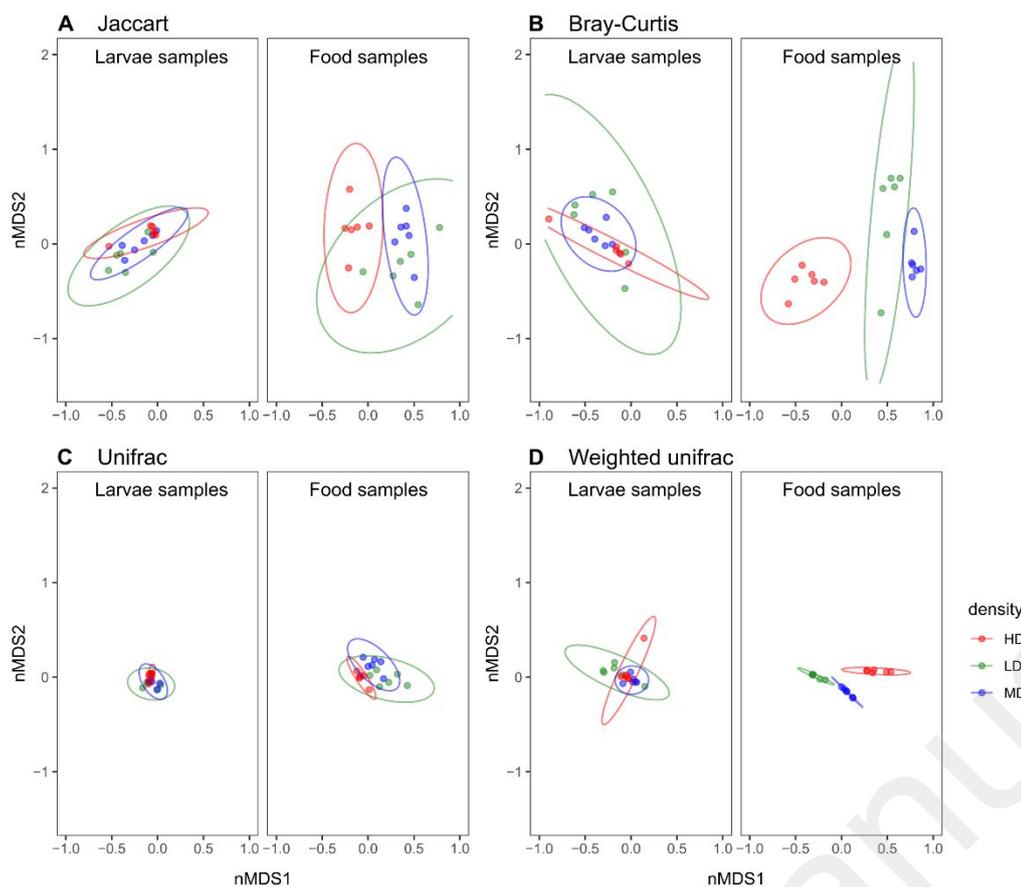
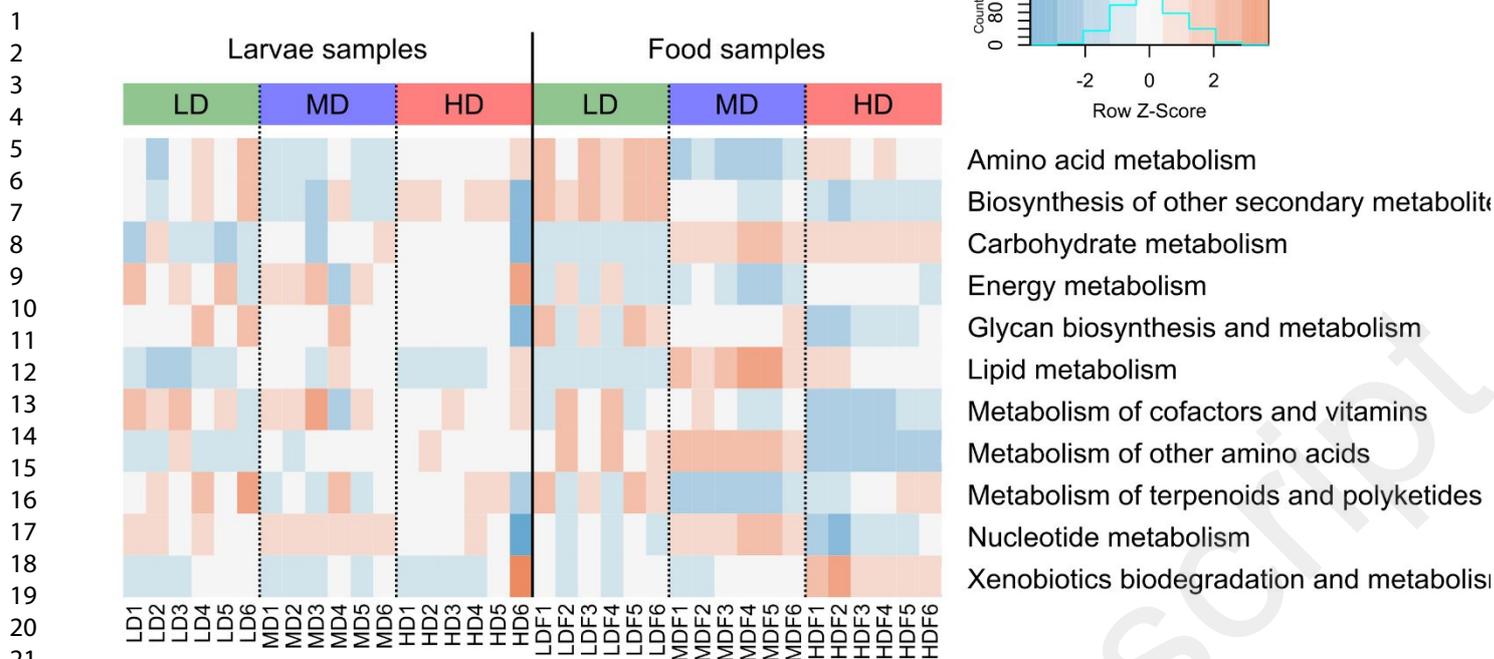


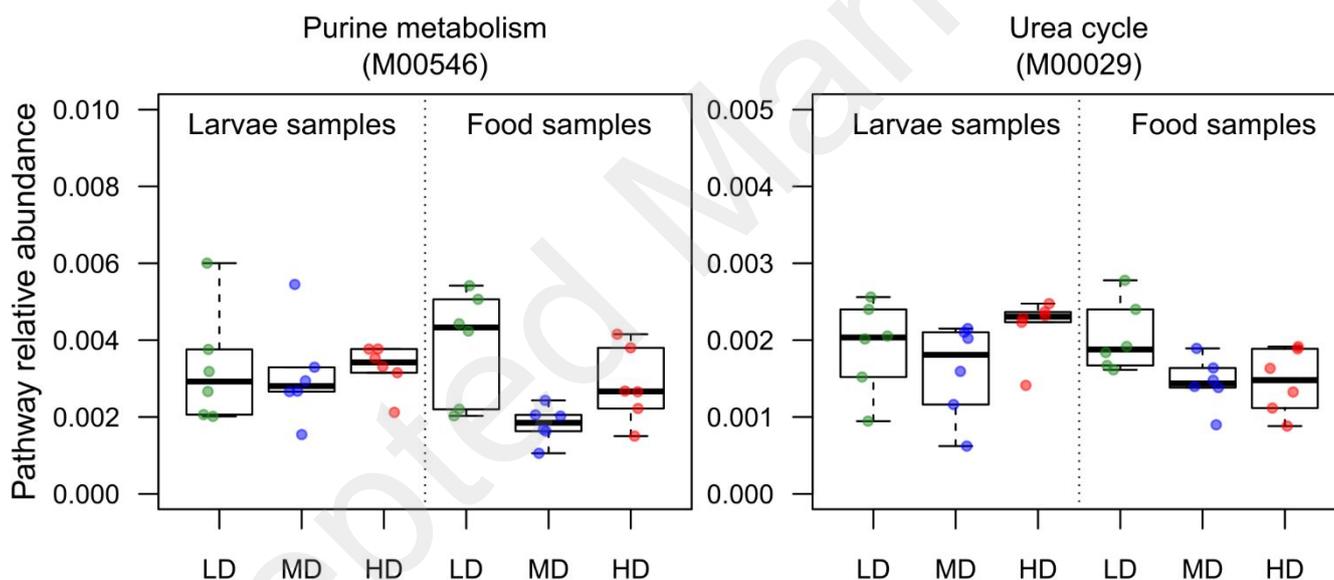
Figure S4: nMDS ordinated distances of bacterial communities in LD, MD and HD conditions, split by sample type (larvae or food). Plots A-D respectively show Jaccard, Bray-Curtis, unifrac, and weighted unifrac distances. Dots: sequencing replicates, colored by rearing density. Ellipses: 95% confidence zones. Plot B is identical to the Fig. 2C in article.

Table S1: PERMANOVA table showing the effect of larval density and sample type, whether alone or in interaction, on different distance metrics describing the beta diversity between bacterial communities.

Distance	Factors	Df	Sums of squares	Mean squares	F Model	R2	Pr(>F)	
Jaccard	density	2	0,307	0,154	4,542	0,163	2e-04	***
	sample_type	1	0,307	0,307	9,086	0,163	1e-04	***
	density:sample_type	2	0,254	0,127	3,755	0,135	1e-03	***
	Residuals	30	1,015	0,034	0,539			
	Total	35	1,884	1,000				
Bray-Curtis	density	2	1,727	0,863	6,402	0,205	1e-04	***
	sample_type	1	1,023	1,023	7,581	0,122	1e-04	***
	density:sample_type	2	1,612	0,806	5,974	0,192	1e-04	***
	Residuals	30	4,046	0,135	0,481			
	Total	35	8,407	1,000				
Unifrac	density	2	0,191	0,096	3,977	0,154	0.0016	**
	sample_type	1	0,199	0,199	8,268	0,160	0.0001	***
	density:sample_type	2	0,132	0,066	2,746	0,106	0.0173	*
	Residuals	30	0,722	0,024	0,580			
	Total	35	1,244	1,000				
Weighted unifrac	density	2	1,297	0,649	29,267	0,493	0.0001	***
	sample_type	1	0,188	0,188	8,460	0,071	0.0011	**
	density:sample_type	2	0,484	0,242	10,928	0,184	0.0001	***
	Residuals	30	0,665	0,022	0,252			
	Total	35	2,634	1,000				



22 Fig S5: Relative abundance heatmap of the second-level functional KEGG pathways identified by
23 PICRUSt2, in larvae and food samples, at each larval density level.



45 Fig S6: Relative abundance of purine metabolism and urea cycle KEGG modules (N=6 per sample type
46 per density), in larvae and food samples from LD, MD and HD conditions. Boxes: first and third quartiles of
47 the distribution. Black horizontal line: median of the distribution. We found no significant differences
48 between any treatments.

50 References

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