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RESEARCH ARTICLE

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

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**One sentence summary:** Larval microbiota remains stable during crowding.

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## ABSTRACT

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

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

## INTRODUCTION

Scramble competition may appear in insects feeding on discrete, spatially restricted and ephemeral resources such as carrion, weeds or rotting fruits (Crawley and Gillman 1989; Nunney 1990; Ireland and Turner 2006). These limited resource patches are colonized by opportunistic species as soon as they become available, and population density may rapidly reach a crowded situation (Atkinson 1979). The consequences of crowding are multiple. High individual densities not only generate a quantitative food shortage, but foraging and excretion of conspecifics also degrade the nutritional quality of the resource supply (Botella *et al.* 1985). In *Drosophila*, this typically results in marked phenotypic effects such as reduced body mass and slower development (Lints and Lints 1969; Scheiring *et al.* 1984; Borash *et al.* 2000; Kolss *et al.* 2009). Curiously, crowding can also be beneficial to flies, for instance by promoting tolerance to stressors such as starvation, toxic wastes or thermal stress (Zwaan, Bijlsma and Hoekstra 1991; Shiotsugu *et al.* 1997; Sørensen and Loeschcke 2001; Henry, Renault and Colinet 2018). As a result, larval density may represent an important

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environmental pressure, driving the evolutionary process and adaptation (Horváth and Kalinka 2016; Sarangi *et al.* 2016).

Two main factors are thought to underlie crowding effects in flies: food deprivation and intoxication due to ingestion of metabolic wastes. In a recent study, Klepsatel *et al.* (2018) showed that the effects of increased larval density on life-history traits were likely controlled by decreased yeast availability in food. They showed that yeast-poor diets without crowding triggered equivalent changes in size, energy reserves and lifespan as those observed in flies exposed to high larval density. Previous studies also indicated that the accumulation of nitrogenous wastes, resulting from excretion (urea, ammonia, uric acid), may also influence *Drosophila* development and life-history traits (Botella *et al.* 1985; Joshi, Shiotsugu and Mueller 1996). However, it remains unclear which nitrogenous wastes are actually excreted by larvae: uric acid, urea or ammonia (Botella *et al.* 1985; Borash *et al.* 1998; Etienne, Fortunat and Pierce 2001; Henry, Renault and Colinet 2018). Regardless of the exact compounds, multiple lines of evidence suggest that exposure to nitrogenous wastes imposes a selection force leading to the evolution of resistant genotypes (Borash *et al.* 2000). The combination of both quantitative and qualitative food alterations thus remains a reasonable hypothesis to explain phenotypic effects generated by crowding.

Alteration of the nutritional medium by the action of larval foraging is impacting flies, but is also likely to affect microorganism communities colonizing the food, such as bacteria and yeasts (Chandler, Eisen and Kopp 2012; Wong *et al.* 2015; Erkosar *et al.* 2018). *Drosophila* individuals, whether at the larval or adult stage, establish mutualistic relationships with these microorganisms (Erkosar *et al.* 2013). Unlike obligate symbiosis found in other insect species, host–bacteria interactions are facultative in *Drosophila* (Erkosar *et al.* 2017). This explains the large observed variability in microbiota composition, according to environmental parameters or artificial rearing conditions, in flies (Staubach *et al.* 2013; Erkosar and Leulier 2014; Bing *et al.* 2018; Téfit *et al.* 2018). The transient nature of this relationship implies a constant need for replenishment via the feeding activity (Blum *et al.* 2013). However, this view was recently challenged by the observation of stable and resilient associations between fruit flies and bacteria, even in varying environments (Jehrke *et al.* 2018; Pais *et al.* 2018). Indeed, taxa diversity in the digestive tract of fruit flies rarely reflects the microbial diversity of their close environment, suggesting an active control of microbiota (Martinson, Douglas and Jaenike 2017). This is not surprising since the best cooperators are likely to be favored over generations and co-adapt toward a maximization of the holobiont's fitness (Soen 2014). In this context, one can wonder to what extent crowding and its side-effects may alter the environmental microbial community as well as flies' gut microbiota. Life-history traits such as lifespan, morphology or development are under the influence of gut microbiota abundance and diversity (Brummel *et al.* 2004; Ryu *et al.* 2008; Shin *et al.* 2011; Wong, Dobson and Douglas 2014). In addition, microbiota-related effects are generally dependent on nutrient concentration in the medium (Storelli *et al.* 2011; Yamada *et al.* 2015; Matos *et al.* 2017; Bing *et al.* 2018), which may be severely reduced during crowding. Considering the myriad of effects that gut microbiota has on phenotypical traits of *Drosophila melanogaster* (Douglas 2018), it is worth investigating whether the effects of crowding may be concurrently associated with microbial changes in both host and food. Larval crowding is an ecologically relevant and naturally occurring situation and represents a great system to explore host–bacteria–environment interactions.

In this study, we investigated the phenotypical consequences of crowding by using an artificial larval density gradient spanning from very low density to overcrowded conditions. Based on the data resulting from these phenotypic analyses, we selected three contrasting densities (low, medium and high) in which we sampled individuals (L3 larvae) and food substrate. In all these samples, we measured ammonia and uric acid concentration, and we sequenced the bacterial community based on V3/V4 16S regions. We assumed crowding-induced nutritional alteration would affect phenotypes of individuals but also the composition of their surrounding microorganism communities. We expected environmental bacterial communities in food to differ significantly according to larval density, and because the larvae feed on this substrate, we also expected their gut microbiota to show concomitant community changes. We supposed that ammonia and uric acid would accumulate in food especially at high larval density. Finally, we tested whether the supplementation of metabolic wastes in uncrowded conditions would result in similar phenotypic effects as at high larval density.

## MATERIAL AND METHODS

### Fly stocks and rearing medium

We conducted the experiments on an outbred laboratory population of *D. melanogaster* derived from wild individuals collected in September 2015 in Brittany (France). Fly stocks were maintained at 25°C and 70% relative humidity (12h light: 12h darkness) on standard food comprising inactive brewer's yeast (MP Bio 029 033 1205, MP Bio, 80 $g.L^{-1}$), sucrose (50 $g.L^{-1}$), agar (Sigma-Aldrich A1296, 10 $g.L^{-1}$) and supplemented with Nipagin (Sigma-Aldrich H5501; 10% 8 $mL.L^{-1}$). These conditions were also used for rearing of flies in following experiments. *Wolbachia* symbionts were previously eliminated from the population by submitting flies to a tetracycline treatment (Sigma-Aldrich T7660, 50 $\mu g.L^{-1}$) added in the food for three generations, followed by multiple untreated generations of recovery (>10). The effectiveness of the procedure was previously confirmed by PCR with *wsp* and *wspB* primers (Teixeira, Ferreira and Ashburner 2008).

### Larval density

Before all the experiments, we allowed adult flies from rearing stocks to lay eggs for <12 h on standard food supplemented with extra agar (15 $g.L^{-1}$) and food coloring. Using a stereomicroscope, eggs were delicately collected with a paint brush, counted on moistened fabric and then transferred into new vials. Flat-bottom plastic vials (50 mL; diameter = 23 mm) were precisely filled with 2.0 mL of standard food, in order to achieve all the desired larval density treatments (see below). Egg manipulation was performed identically in all treatments, standardizing time spent under the stereomicroscope to 15 min.

### Effects of larval crowding

In a first experiment, we generated a broad range of larval densities: 1, 5, 20, 60, 100, 200, 300, 500 and 1000 eggs per mL of food (see Fig. S1, see online supplementary material). To generate these nine density treatments, a total of 300, 300, 400, 480, 800, 1600, 2400, 4000 and 8000 eggs were counted and deposited in 150, 30, 10, 4, 4, 4, 4 and 4 replicated vials respectively, each containing 2 mL of food. We did not adjust the number of deposited eggs to account for embryo mortality.

We characterized development, phenotypes and behavior of individuals from all these larval densities. For metabolic wastes measurements and microbiota characterization, we selected among the nine densities three densities showing contrasting phenotypes and referred as LD (low density; 5 eggs.mL$^{-1}$), MD (medium density; 60 eggs.mL$^{-1}$) and HD (high density; 300 eggs.mL$^{-1}$). For these three densities, we measured ammonia and uric acid in L3 larvae and in the food that sustained their development. We collected larvae and food samples over a time window of 4 h and at the specific occurrence peak of L3 instar for each larval density, in order to avoid sampling outlier individuals. Larvae samples were L3 instar individuals picked on the surface of the medium, and food samples consisted of cubes of 5 × 5 × 5 mm, i.e. including the whole depth of the medium. Samples were then transferred to autoclaved tubes with sterile tools, immediately snap-frozen in liquid nitrogen and stored at −80°C until use.

#### *Development, phenotypes and behaviors*

During development, pupation and adult emergence were checked twice a day to estimate development durations (i.e. time to pupation and to emergence). Emerged adults were immediately removed from their tubes and transferred to new vials containing clean food. Viability was calculated based on the total number of emerged adults at the end of the experiment over the total number of deposited eggs. Adult fresh and dry masses were individually measured for both sexes from 30 randomly collected individuals per density (3-day-old adults) using a micro-balance (Mettler Toledo UMX2, Mettler Toledo, Greifensee, Switzerland; accurate to 1 $\mu$g). Dry mass was measured after individuals were dried for at least 1 week in an oven at 60°C. Pupation height was measured in all vials from all densities using an electronic caliper. Because of the very large number of pupae, measurements were only performed on half of the pupae for densities of 200 and 300 eggs per mL.

#### *Metabolic wastes*

Ammonia measurements were performed using Ammonia Assay Kit (Sigma-Aldrich, AA100) and following the manufacturer's instructions. Ten biological replicates of larvae (pools of 10 individuals) and food were used for each of the three densities. Samples were weighted using a microbalance. Food samples were adjusted to 50 $\mu$g. Samples were homogenized in 250 (larvae) or 500 (food) $\mu$L of PBS (Phosphate Buffered Saline) with two tungsten beads using a bead beating apparatus (20 Hz, 2 min). After dilutions when necessary, colorimetric measurements were carried out with a microplate reader (VersaMax Molecular Devices, San José, CA, USA) at 340 nm.

Uric acid measurements were performed using a uric acid assay kit (Sigma-Aldrich, MAK077) and following the manufacturer's instructions. Eight biological replicates of 10 larvae were used for each of the three densities. Samples were weighted using a microbalance. Food samples were adjusted to 50 $\mu$g. Samples were homogenized in 250 (larvae) or 500 (food) $\mu$L of PBS with two tungsten beads using a bead beating apparatus (20 Hz, 2 min). After dilutions when necessary, fluorometric measurements were carried out with a microplate reader (SAFAS Monaco Xenius XC, Monaco) set up at 535 nm (emission) and 587 nm (excitation). Quantification was obtained by running serial dilution of a uric acid standard. During all these experiments, samples were quickly processed and kept on ice to avoid degradation.

#### *Microbiota composition*

For LD, MD and HD conditions, the bacterial composition of L3 larvae and of food that sustained their development was characterized using 16S Illumina MiSeq sequencing. To remove external bacteria in larvae, pools of 10 individuals were surface-sterilized with successive baths and quick vortexed in 2.7% hypochlorite for 2 min, 70% ethanol for 2 min, and rinsed twice in autoclaved miliQ water. DNA extraction was performed in six independent replicates using a FastDNA spin kit (MP Biomedicals), according to the manufacturer's instructions. We used PCRs to amplify V3/V4 16S RNA regions with universal bacterial primers: forward (5′-CTTTCCCTACACGACGCTCTTCCGATC TACGGRAGGCAGCAG-3′) and reverse (5′-GGAGTTCAGACGTGT GCTCTTCCGATCTTACCAGGGTATCTAATCCT-3′) with adapters for MiSeq (François *et al.* 2016). Thirty thermal cycles at 65°C annealing temperature were performed. The PCR products were purified and loaded onto the Illumina MiSeq cartridge (Illumina, San-Diego, CA, USA) according to the manufacturer's instructions.

### Metabolic wastes supplementation

To investigate phenotypic effects due to metabolic wastes *per se* in a non-crowding situation (i.e. without nutrient depletion and intense inter-individual interactions), we designed a second experimental set-up that is summarized in Fig. S2, see online supplementary material. In essence, larvae were reared under low larval density but with high amounts of waste products. Of the three putative nitrogenous wastes excreted by *Drosophila*, only ammonia (see in the present study) and urea (see Henry, Renault and Colinet 2018) accumulated substantially in food under crowding situation. Consequently, only these two molecules (and not uric acid) were supplemented in food using nominal concentrations that were experimentally found in HD food: 1.2 mg.mL$^{-1}$ for ammonia (Merck Millipore, 105 432) (see Results section) and 5 mg. mL$^{-1}$ for urea (PanReac, PA6ACS) (see Henry *et al.* 2018). The experimental design included four treatments: Co (control, no supplementation), Ur (urea supplementation), Am (ammonia supplementation), UrAm (urea and ammonia supplementation) (see Fig. S2). For all treatments, eggs were deposited in LD conditions (5 eggs.mL$^{-1}$, $n = 6$ vials). Development duration, viability and pupation height were measured as previously described.

### Data analysis

In the density gradient experiment, development duration was analysed using mixed binomial generalized linear models (GLM) with logit link function and with replicates as a time-dependent random effect. Pairwise contrasts were checked using the 'emmeans' package (Lenth *et al.* 2020). Viability (both in the density gradient and in wastes supplementation experiments) was analysed using binomial GLMs with logit link function according to density, followed by Tukey post-hoc test to assess pairwise differences using the 'Multcomp' package (Hothorn, Bretz and Westfall 2008). Mass and pupation height were analysed using non-linear models (NLS) as described in Henry, Renault and Colinet (2018). Briefly, we adapted the non-linear logistic equation proposed by Börger and Fryxell (2012):

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

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

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

where $a$ corresponds to the asymptotic mass at the highest density, $b$ is the inflection point expressed in density units and $c$ is the range of the curve on the density axis. Ammonia and uric acid contents were analysed using one-way ANOVA followed by post-hoc Tukey tests. In the waste-supplementation experiment, development duration was analysed using Kruskal–Wallis test followed by post hoc Dunn's test with Benjamini–Hochberg correction, and pupation height was analysed using one-way ANOVA followed by post hoc Tukey tests.

Sequencing data were analysed using a custom pipeline. Raw pair-end sequence files from Illumina were assembled using Flash software (Magoč and Salzberg 2011) using at least a 10 bp overlap between the forward and reverse sequences, allowing 5% of mismatch. Dereplicating, denoising, clustering and chimera removing steps were sequentially performed using Galaxy tool 'FROGS' (Escudié *et al.* 2016). A comparison of normalized reads to the lower sample vs non-normalized reads showed it provided similar results in both cases. Thus, we kept non-normalized reads as normalization may induce statistical bias (McMurdie and Holmes 2014). Taxonomic affiliation was defined using the Silva132 16S database. When clearly incoherent affiliations were generated, a blast was performed in the NCBI database and the identification was corrected if needed. A filter was applied to remove marginal diversity represented by a low number of reads (<0.05% of the total number of reads per sample). Once these steps were performed, data were processed using the 'Phyloseq' package in R (McMurdie and Holmes 2013). Some sequences corresponding to *Wolbachia* were found (the bacteria probably recovered from the elimination treatment thanks to rare survivors). Yet, these sequences, represented only ~10 and ~1% in larvae and food, respectively. They were discarded in the subsequent steps of the analysis. The resulting OTU (Operational Taxonomic Unit) table was used to compute alpha and beta diversity tests (McMurdie and Holmes 2013). Differences in the community composition as a function of the sample type (larvae or food) and of the density level (LD, MD, HD) were tested using PERMANOVA on a Bray–Curtis distance matrix (Anderson, Ellingsen and McArdle 2006). OTUs were clustered at the genus level for graphical representation.

## RESULTS

### Effects of larval crowding on phenotype

Development was significantly affected by larval density (Fig. 1A) ($F = 162.43$, df = 8, $P < 0.001$). Flies from density levels >100 eggs per mL were about 12 h slower to reach 50% of emerged adults than levels <100. Above density 100 eggs per mL, the distribution and variance of emergence events became much larger due to extreme individuals emerging up to 8 days after the adults reared at low density. Viability was strongly dependent on larval density (Fig. 1B) ($\chi^2 = 7907$, df = 1, $P < 0.001$). Within our tested range, we could capture the upper and the lower viability limit of our *D. melanogaster* population reared under crowded conditions. Density levels of 1–20 eggs per mL showed the maximal viabilities (80–90%), and each increasing density level diminished the viability until density 1000 eggs per mL, where <3% of the deposited eggs turned into viable adults. Pupal and adult viabilities showed parallel decreasing patterns as a function of density. Adult viability was only slightly lower than pupal viability, indicating that larval density mainly affects the egg to pupae part of development. Body mass was also strongly reduced at higher densities, both in females and males and both in fresh and dry mass (Fig. 1C). Model proprieties allowed to identify 400 eggs per mL as the density theshold above which no further mass decrease was observed. The weight difference reached up to a three-fold change between extreme density levels (i.e. 1 vs 1000 eggs per mL). Pupation height was also rapidly affected by density (Fig. 1D). Larvae from low densities (1–20 eggs per mL) tended to pupate very close to the food substrate, whereas larvae from higher densities clearly pupated higher in the vials, 50 mm above the surface on average.

### Effects of larval crowding on nitrogenous wastes

Ammonia and uric acid concentrations were both dependent on larval density and on sample type, with significant interaction between these factors (Fig. 1E and F) (density * sample type effect: $\chi^2 = 353$, df = 2, $P < 0.001$; $\chi^2 = 264$, df = 2, $P < 0.001$; for ammonia and uric acid respectively). Ammonia was detected mostly in food, where concentrations were significantly higher in MD and HD than in LD conditions (Tukey HSD, $P < 0.001$) (Fig. 1E). In contrast, ammonia was found at very low concentrations in larvae and there was no change depending on density (Fig. 1E). Uric acid showed a completely different pattern as it was barely detectable in food samples but present in larvae (Fig. 1F). Uric acid concentration in larvae was negatively correlated with larval density, with the highest value in LD, intermediate value in MD and lowest value in HD individuals (all significantly different, Tukey HSD, $P < 0.001$).

### Effects of larval crowding on bacterial communities

Microbiota diversity was affected by sample type and by larval density, whatever the considered index (Fig. 2A–D; Figs S3 and S4, see online supplementary material). Gut bacterial community inside larvae was significantly different from the bacterial community of the food ( $F_{1,30} = 10.94$, $P = 0.002$; $F_{1,30} = 37.79$, $P < 0.001$; for observed richness and Shannon diversity respectively). Larval density also had a significant effect on bacterial communities ( $F_{2,30} = 8.19$, $P = 0.001$, $F_{2,30} = 13.74$, $P < 0.001$; for observed richness and Shannon diversity respectively). Pairwise comparison of larval density treatments did not show differences in OTU richness for larvae or food samples (Fig. 2A, Tukey HSD, $P > 0.05$ for all densities comparisons). Pairwise comparison of larval density treatments did not show differences in Shannon diversity index for larvae samples (Tukey HSD, $P > 0.05$ for all densities comparisons), but significant differences were detected among food samples, with higher diversity in HD than in LD and MD foods (Tukey HSD, $P < 0.001$). Beta diversity was largely impacted by larval densities in food samples but not in larval gut samples (Fig. 2C, Fig. S4). Both larval density ($F_{2,30} = 6.40$, $P < 0.001$), sample type ($F_{1,30} = 7.58$, $P < 0.001$) and their interaction ($F_{2,30} = 5.97$, $P < 0.001$) had a significant effect on Bray–Curtis distances, explaining respectively 21, 12 and 19% of the total variance (Table S1, see online supplementary material). Fig. 2D shows large and consistent differences in bacterial composition of the food according to larval density, whereas only subtle changes in bacterial composition were found in larval gut microbiota. Even if the global bacterial community in larvae did

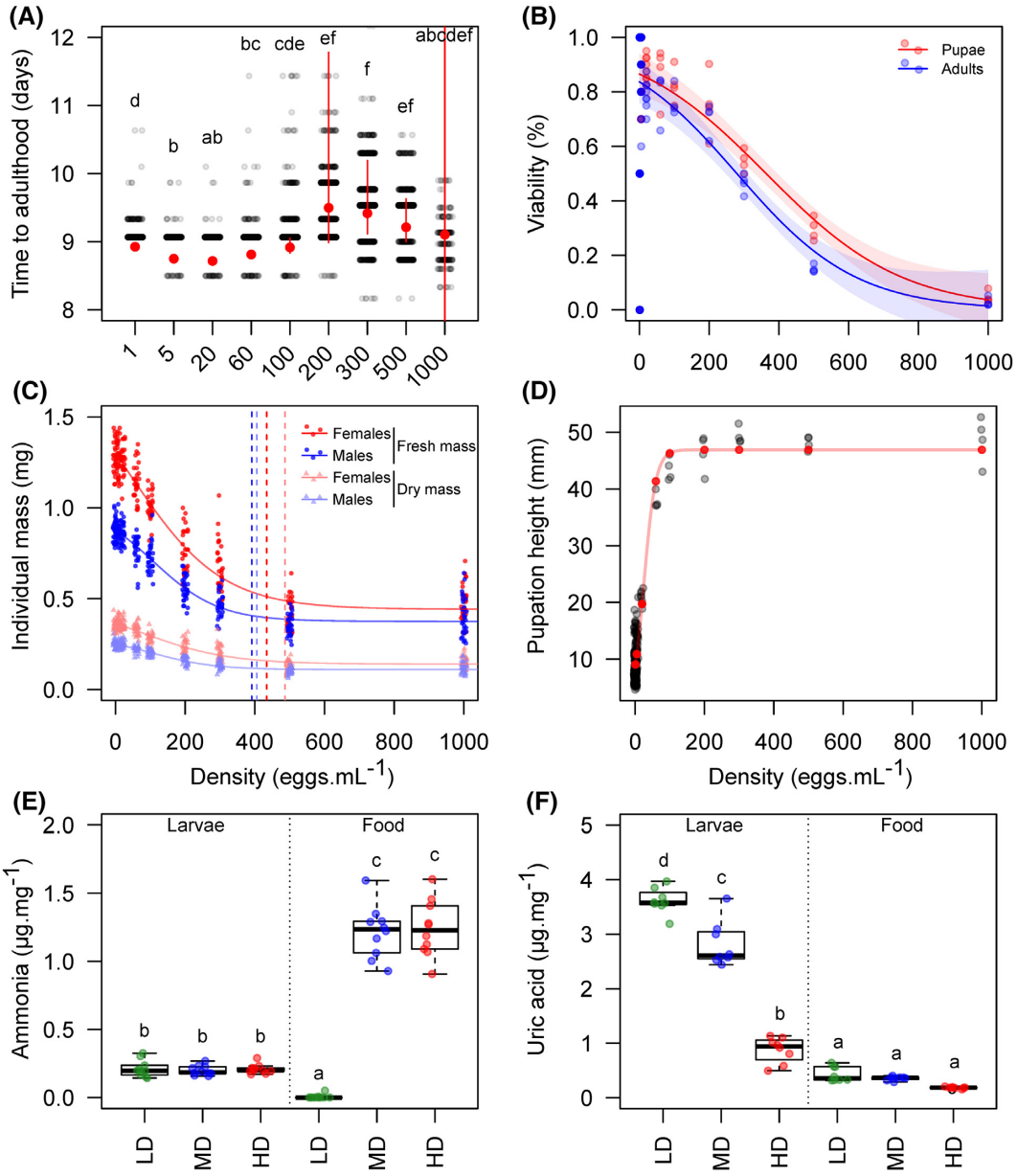


**Figure 1.** Direct consequences of larval crowding in *D. melanogaster*. (**A**) Development time to adulthood as a function of larval density. Black dots: individual adult emergence events. Red dots: mean development duration predicted using NLS model. Red error bars: 95% confidence intervals around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

not drastically change with density, we noted the apparition of *Lactobacillus* OTUs in MD larvae (and to a lesser extent in HD); these *Lactobacillus* were almost absent in LD.

## Effects of metabolic wastes supplementation

Supplementation of metabolic wastes had a subtle but significant effect on development (Fig. 3A) ($\chi^2 = 54.02$, df = 3, $P < 0.001$). Supplementation of ammonia (in Am or UrAm) consistently increased the development time by about half a day compared with the control without supplementation (Dunn's test, $P < 0.001$). Urea alone had smaller effect than ammonia, but still increased development time compared with control (Dunn's test, $P = 0.019$). We found no significant effect of supplementation on viability or pupation height (Fig. 3B and C) ($\chi^2 = 3.90$, df = 3, $P = 0.272$; $\chi^2 = 3.34$, df = 3, $P = 0.342$; for viability and pupation height respectively). Viability was >80% in all treatments, which is comparable with values found in the first experiment at sim-

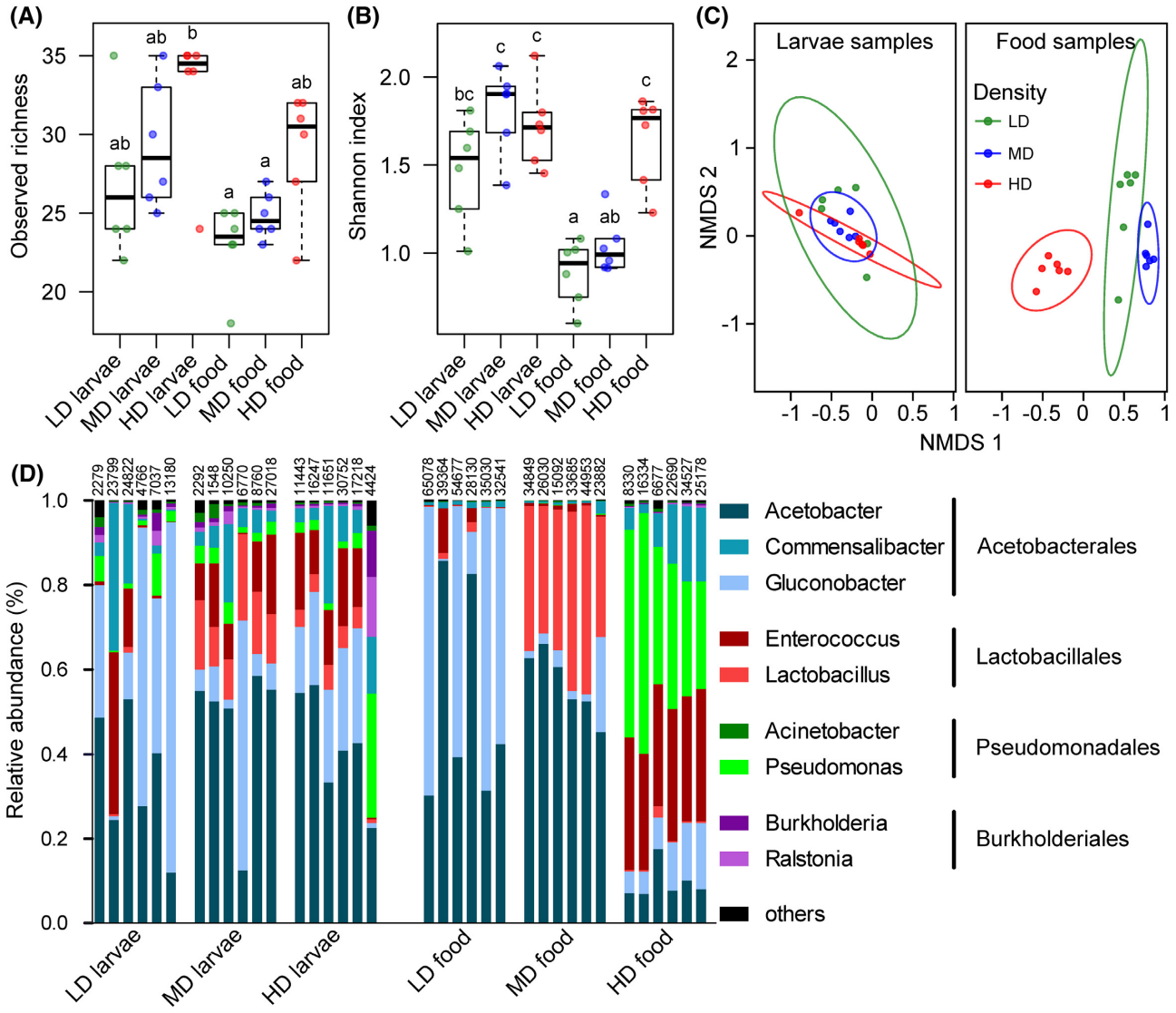


**Figure 2.** Bacterial community variations in *D. melanogaster* larvae and in its environment, at increasing population densities. (**A**) and (**B**) Boxplots of observed richness and of Shannon diversity respectively in LD, MD and HD conditions. Dots: diversity values of sequencing replicates. Boxes: first and third quartiles of the distribution. Black horizontal line: median of the distribution. Different letters indicate significant differences (Tukey test, $P < 0.01$). (**C**) NMDS Bray–Curtis ordination of bacterial communities in LD, MD and HD conditions, split by sample type (larvae or food). Dots: sequencing replicates, colored by rearing density. Ellipses represent 95% confidence zones. (**D**) Stacked barplot of sequenced OTUs, grouped at the genus level. Number of reads effectively used per replicate is displayed on top of the bars.

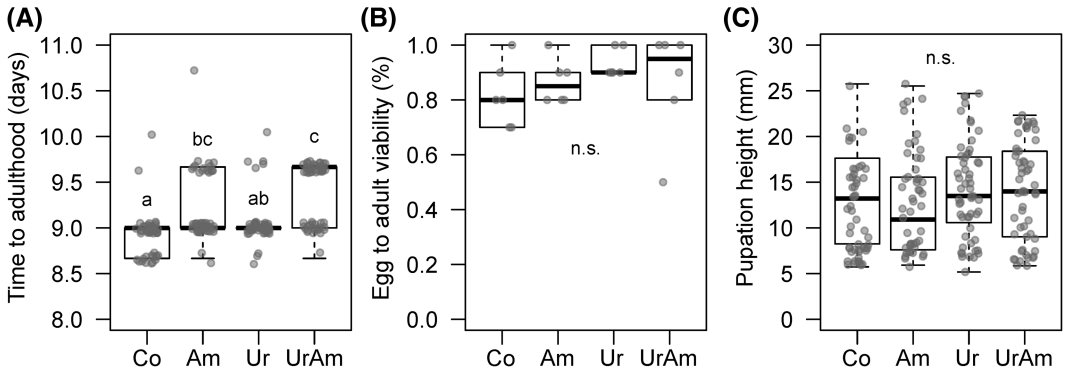


**Figure 3.** Effects of artificial supplementation of metabolic wastes on development and pupating behavior in *D. melanogaster*. (**A**) Development time to adulthood in control flies and in the three supplementation treatments. Dots: individual adult emergence events. Different letters indicate significant differences (Dunn's test, $P < 0.01$). (**B**) Percentage of viable adults emerged from deposited eggs. Dots: mean viability per vial ($n = 6$). (**C**) Pupation height. Dots: individual pupation heights. For all plots, boxes: first and third quartiles of the distribution. Black horizontal line: median of the distribution. 'n.s.' indicates no statistical differences.

ilar larval density. Median pupation height was between 10 and 15 mm in all conditions, which is also comparable to heights found in the first experiment for the same density.

## DISCUSSION

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

Larval crowding generated large variations in all measured phenotypic traits (development, survival and morphology). The present data are therefore in agreement with our previous observations (Henry, Renault and Colinet 2018) but also provide additional descriptions of crowding effects on behavioral traits (i.e. pupation site selection). We can now rather precisely predict the extreme limit of viability under crowding: <3% of eggs on average should attain the adult stage when density is >1000 eggs.mL$^{-1}$. Strikingly, about 20% viability is still attainable with 500 eggs.mL$^{-1}$, highlighting the great competitive ability of fruit flies. Decreased viability with larval density likely reflects, at least in part, that an increasing number of individuals were unable to reach critical mass for pupation (Mirth, Truman and Riddiford 2005). In line with this observation, body mass showed a clear decline with increasing larval density (Scheiring *et al.* 1984; Shenoi, Ali and Prasad 2016), followed by a plateau at density levels of ≥400 eggs per mL. This plateau suggests that a critical minimal viable mass was reached in our population at around one-third of the normal mass. Although we observed a developmental delay at high densities, it did not exceed 1 day for the median time. However, above density level of 100 eggs per mL, the distribution of emergence became much larger due to extreme individuals: from intervals of about 2 days between the first and the last emerging individual at low densities (1–20 eggs per mL) to intervals sometimes >7 days at the highest densities (200–1000 eggs per mL). Higher variance at high densities is probably the consequence of contrasting responses: some individuals were rapid enough to profit from early non-degraded conditions whereas the others were exposed to increasingly degraded food which generated a developmental delay.

Nitrogenous wastes are generally toxic. Ammonia is known for its cytotoxic activity, impeding respiratory metabolism and membrane ion transporters (Weihrauch, Donini and O'Donnell 2012; Henry *et al.* 2017), and urea, although being less toxic, can impair development and survival of species through protein denaturation (David *et al.* 1999). Uric acid can show positive effects by preventing oxidative damage or water loss (Hilliker *et al.* 1992; Andersen *et al.* 2010), but may also trigger detrimental inflammatory reactions (Sautin and Johnson 2008). Consequently, we expected that crowding, by increasing wastes concentration, would be stressful to larvae. Notably, we observed changes in the larval behavior, with a significant increase in the pupation height at high densities. This behavior is likely the outcome of contrasting larval foraging strategies that have been similarly observed in density-selected lineages (Sokolowski, Pereira and Hughes 1997), and we speculated that increased pupation height at higher densities could result from a need to avoid proximity to toxic products (Belloni *et al.* 2018). Yet, we found that pupation height was unchanged in larvae reared at low density, when toxic metabolic wastes were artificially supplemented in the food to mimic larval crowding conditions. Therefore, the increase in pupation height could be a consequence of nutritional restriction or inter-individual pressures such as cannibalism (Vijendravarma, Narasimha and Kawecki 2012), rather than an avoidance of toxic wastes. Wastes supplementation also had limited impact on life-history traits, inducing only a minor developmental delay and no additional mortality. Our results thus seem to corroborate the conclusions of Klepsatel *et al.* (2018) that, for these traits, nutritional restriction as a stressor outshines other stress resulting from larval crowding.

In insects, the main waste products of nitrogenous metabolism are uric acid, urea and ammonia (Bursell 1967; O'Donnell and Donini 2017). Even in model species like *D. melanogaster*, it remains unclear whether uric acid is the major waste product, as in many Dipterans (Bursell 1967; Dow and Davies 2003), or if other compounds contribute to the excretion. Urea is thought to occur naturally in *Drosophila* cultures (Joshi, Shiotsugu and Mueller 1996) and previous studies actually found increasing levels of urea in food in the case of overfeeding (Botella *et al.* 1985) or with increasing larval densities (Henry, Renault and Colinet 2018). On the other hand, Etienne *et al.* (2001) suggested that *D. melanogaster* is unable to produce this compound. The presence of urea in food may thus result from conversion of products such as uric acid by the action of aerobic bacteria (Bachrach 1957; Potrikus and Breznak 1981; Winans *et al.* 2017). Borash *et al.* (1998) reported that ammonia is the primary metabolic waste product of *D. melanogaster* larvae but other studies posit that uric acid is the main waste product of nitrogen metabolism (Botella *et al.* 1985; Winans *et al.* 2017). Finally, products such as allantoin were also found in the food medium, but have not been measured in flies (Borash *et al.* 1998; O'Donnell and Donini 2017). According to this literature, we can speculate that all these compounds could be present in the medium, though in variable amounts. Our results confirm the presence of ammonia in food, with high levels detected in MD and HD conditions. On the other hand, we only found traces of uric acid in the food but surprisingly higher amounts in larvae. This suggests that *D. melanogaster* larvae produce and accumulate uric acid, probably in tissues such as the fat body or in Malpighian tubules (Weihrauch, Donini and O'Donnell 2012), but this compound seems to quickly degrade once in the environment. Consequently, the presence of urea and ammonia in food may either result from an effective excretion of these products by larvae, or from the degradation and transformation of uric acid in the environment. In other words, uric acid could degrade either inside or outside the larvae (Fig. 4).

The classic pathway of uric acid degradation involves the urate oxidase enzyme, coded by the *Uro* gene in *D. melanogaster* (Friedman 1973; Wallrath, Burnett and Friedman 1990). *Uro* already showed increased expression with increased larval densities (Henry, Renault and Colinet 2018), and this could have played a role in the reduction of uric acid content observed in MD and HD, in comparison with LD (Fig. 4A). In addition, the progressive nutrient depletion in crowded conditions limited the amount of digestible material (particularly purines) and therefore may have reduced uric acid production. Alternatively, uric

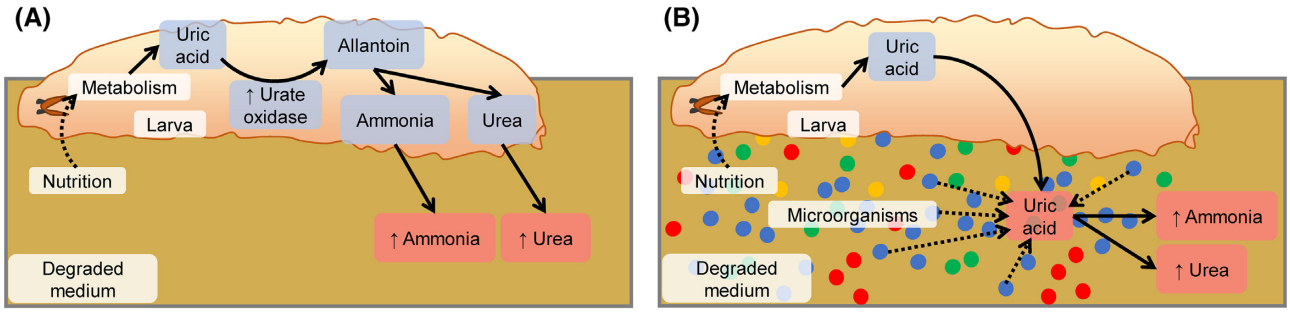


**Figure 4.** Conceptual visualization of a hypothetical nitrogen cycle in the lab *D. melanogaster* system. (**A**) Larva is metabolizing nutrients, producing uric acid in the process. Larva then degrades uric acid into intermediate products and finally to ammonia or urea through the uricolytic pathway 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.

acid could be excreted by larvae and used as a nitrogen resource by surrounding microorganisms (Fig. 4B). This last hypothesis is supported by previous observations in termites (Potrikus and Breznak 1981) and by recent findings showing a functional selection for uric acid metabolization in some bacterial taxa associated with laboratory cultures of *Drosophila* flies (Winans *et al.* 2017). Indeed, Winans *et al.* (2017) reported that uric acid accumulated substantially in the medium of axenic fly cultures, but not in the medium of flies harboring bacteria with a functional urate oxidase gene. We can expect that in crowded conditions, bacteria experienced even more of this selection pressure with unusual high wastes levels. The changes between bacterial communities identified in the environment of LD, MD and HD are in line with this assumption. From a community largely dominated by *Acetobacterales* (*Acetobacter sp* and *Gluconobacter sp*) in LD, environment changes modified the species composition with the apparition of *Lactobacillus sp* in MD. In HD, changes totally reshaped the community, reducing the abundance of *Acetobacterales* but allowing *Enterococcus* and *Psedomonas* species to thrive. In a first attempt to explore the functional diversity of bacterial communities from the different larval density conditions, we extrapolated the expression level of metabolic pathways using our taxonomic diversity data and the PICRUSt2 pipeline (Douglas *et al.* 2019) (Fig. S5, see online supplementary material). The analysis revealed no marked functional change in pathways related to nitrogen wastes regulation such as purine and urea metabolism (Fig. S6, see online supplementary material). However, this kind of approach may not be well suited to provide evidence of minute variation at the pathway level in samples collected from unusual environments, possibly explaining the apparent lack of functional changes.

The presence of *Lactobacillus* almost exclusively in MD food is of particular interest: this genus is known to be a good cooperator with flies (Storelli *et al.* 2011; Newell and Douglas 2014) and shows a high dependency upon the host's diet as a driving evolutionary force (Martino *et al.* 2018). Hence, previously reported beneficial effects of intermediate larval densities on flies (Sørensen and Loeschcke 2001; Moghadam *et al.* 2015; Shenoi, Ali and Prasad 2016; Henry, Renault and Colinet 2018) could be the result of an Allee effect favoring development of specific bacteria that happen to be advantageous (Wertheim *et al.* 2002). Conversely, *Pseudomonas* genus is known for its pathogenicity towards *Drosophila*, and could have intensified the detrimental effects of high larval densities (Vodovar *et al.* 2005; Apidianakis and Rahme 2009). Whether the species changes are related to wastes concentration changes or to other factors has to be clarified in future studies, for instance by sequencing food and larvae samples coming from similar set-ups as we used in our supplementation experiment. Additionally, performing new larval crowding experiments in axenic conditions could help disentangle the importance of these adverse bacteria in the detrimental effects of high larval densities.

Larval density mainly affected the environmental bacterial composition, with few impacts on gut microbiota. The only modification we concomitantly observed in both food and larvae was the apparition of *Lactobacillus sp* starting from intermediate densities. Aside from this minor alteration, the gut community was surprisingly independent of external changes. Therefore, in our laboratory population, *D. melanogaster* larvae may have established stable mutualistic relationships with bacteria able to cope with occasional dietary alterations and mitigate opportunistic colonization by new species. Stable mutualistic relationships between flies and gut microbiota have previously been observed in wild *Drosophila* species feeding on mushrooms and harboring bacteria that are almost absent in the environment (Martinson, Douglas and Jaenike 2017). Our observation of stable relationships between flies and gut microbiota in a laboratory context shows that flies may gain advantages controlling their microbiota, even in a favorable environment assumed to apply limited selection force, and consequently lead to transient bacterial communities (Blum *et al.* 2013). Soen (2014) proposed that rapid environmental changes would change microbiota; this would affect the host in return, snowballing into a dysbiosis state. Here, we found that the constant ingestion of food did not strongly affect gut microbiota composition. Mechanisms underlying the control of microbiota by larvae remain elusive (Erkosar and Leulier 2014). Co-adaptation over many generations in laboratory conditions is probably involved, especially the selection for functional traits improving bacterial fitness, such as the loss of motility and uric acid degradation ability (Winans *et al.* 2017).

Larval crowding is a complex nutritional situation that has strong ecological relevance. Here, although we measured large metabolic wastes variability depending on larval density, we could not establish a link between wastes and the observed phenotypical and behavioral changes. Nonetheless, wastes may still apply a strong pressure on the association between larvae and bacteria, affecting the stability of the relationship. We observed microbial composition modifications in food associated with density-dependent changes in metabolic wastes. This represents one of the few examples where macroorganisms action can actually shape the biotic micro-environment through abiotic alterations (Stamps *et al.* 2012; Wong *et al.* 2015). Future studies will need to solve the apparent paradox of larval microbiota

stability in changing environments, when evidence accumulates in favor of diet-centered rather than host-centered adaptation (Winans *et al*. 2017; Martino *et al*. 2018). The key here is to understand why some bacteria are transient whereas some others can persist in the gut, and why this dichotomy parallels laboratory vs wild bacteria (Pais *et al*. 2018). The use of germ-free eggs and gnotobiotic individuals at high larval densities could help to unravel the dynamics of the relationship in these complex situations.

## DATA ACCESSIBILITY

Sequencing data has been deposited in the NCBI Sequence Read Archive (SRA) database under project number PRJNA611582. Other datasets are available on the Figshare repository under the DOI doi.org/10.6084/m9.figshare.11956095.

## SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

***Conflict of Interest***. None declared.

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