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

Y. Henry\(^1,\)\(^2\), P. Tarapacki\(^1\), H. Colinet\(^1\)

\(^1\)ECOBIO - UMR 6553, Univ Rennes 1, CNRS, Rennes, France

\(^2\)Eawag - Swiss Federal Institute of Aquatic Science and Technology, Dübendorf, Switzerland

*Corresponding author: Youn Henry - Eawag, Überlandstrasse 133, 8600 Dübendorf, Switzerland

Email: youn.henry@eawag.ch

Running title: Larval microbiota remains stable during crowding
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 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 evidence 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 contrasted 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, but also their abiotic and biotic surroundings. Environmental bacterial community likely adapts 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). Consequences of crowding are multiple. High individual densities do not only generate a quantitative food shortage; 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). It results that larval density may represent an important environmental pressure, driving evolutionary process and adaptation (Horváth and Kalinka 2016; Sarangi et al. 2016).

Two main factors are supposed 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 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 submitted 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.
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.

The alteration of nutritional medium by the action of larval foraging is impacting flies, but is also likely to affect microorganisms 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). Contrary to 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 Leullier 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 environment (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 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 D. melanogaster (Douglas 2018), it is worth investigating whether 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 contrasted 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 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 microorganisms 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 under high...
larval density. Finally, we tested whether the supplementation of metabolic wastes in uncrowded conditions would result in similar phenotypic effects as under 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% RH (12L: 12D) on standard food comprising inactive brewer yeast (MP Bio 0290331205, 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 µg.L\(^{-1}\)) added in the food for three generations, followed by multiple untreated generations of recovery (>10). 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; d = 23 mm) were precisely filled with 2.0 mL of standard food, in order to achieve
all the desired larval density treatments (see below). Eggs 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). 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 contrasted 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 4h 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 in cubes of 5x5x5 mm, i.e including the whole
depth of the medium. Samples were then transferred in autoclaved tubes with sterile
tools, immediately snap-frozen in liquid nitrogen, and stored at -80°C until use.

1) 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-days-old adults) using a micro-balance (Mettler Toledo UMX2, Mettler Toledo, Greifensee, Switzerland; accurate to 1 µg). Dry mass was measured after individuals were dried for at least one week in oven at 60°C. Pupation height was measured in all vials from all densities using an electronic caliper. Because of very large number of pupae, measurements were only performed on half of the pupae for densities 200 and 300.

2) Metabolic wastes

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

Uric acid measurements were performed using uric acid assay kit (Sigma-Aldrich, MAK077) and following 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 µg. Samples were homogenized in
250 µL (larvae) or 500 µL (food) of PBS with two tungsten beads using a bead beating apparatus (20 Hz, 2 min). After dilutions when necessary, fluorometric measurements were realized with 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 uric acid standard. During all these experiments, samples were quickly processed and kept on ice to avoid degradation.

3) 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 vortex in 2.7% hypochlorite for two minutes, 70% ethanol for two minutes, and rinsed twice in autoclaved miliQ water. DNA extraction was performed in six independent replicates using FastDNA spin kit (MP Biomedicals), according to manufacturer’s instructions. We used PCRs to amplify V3/V4 16S RNA regions with universal bacterial primers: forward (5’-CTTTCCCTACACGACGCTCTTCCGATCTACGGRAGGCAGCAG-3’), and reverse (5’-GGAGTTGACACGTGTGCTCCCGATCTTCAGGGGTATCTAATCCTTCT-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 manufacturer’s instructions.
Metabolic wastes supplementation

To investigate phenotypic effects due to metabolic wastes per se in non-crowding situation (i.e. without nutrient depletion and intense inter-individual interactions), we designed a second experimental setup that is summarized in Fig. S2. 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, 105432) (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 condition (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 analyzed using mixed binomial generalized linear models (GLM) with logit link function and with replicates as 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 analyzed 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 analyzed 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) such as:

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

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, \(c\) is the range of the curve on the density axis.

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

Sequencing data were analyzed using a custom pipeline. Raw pair-end sequences files from Illumina were assembled using Flash software (Magoč and Salzberg 2011) using at least a 10bp-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 Silva132 16S database. When clearly incoherent affiliations were generated, a blast was performed in NCBI database and the identification was corrected if needed. A filter was applied to remove marginal diversity represented by low number of reads (< 0.05% of total number of reads per sample). Once these steps were performed, data were processed using ‘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 table was used to compute alpha and beta diversity tests (McMurdie and Holmes 2013). Differences in the community composition in function of the sample type (larvae or food) and of the density level (LD, MD, HD) were tested using PERMANOVA on 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 higher than 100 were about 12 h slower to reach 50% of emerged adults than levels below 100. Above density 100, 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) (χ²=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 to 20 showed the
maximal viabilities (80-90%), and each increasing density level diminished the viability until density 1000, where less than 3% of the deposited eggs turned into viable adults. Pupal and adult viabilities showed parallel decreasing patterns in 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 in higher densities, both in females and males and both in fresh and dry mass (Fig. 1C). Properties of the model allowed to highlight the lower limit of weight of flies, which appears to be around 400 eggs. mL⁻¹. The weight difference reached up to threefold change between extreme density levels (i.e. 1 vs 1000). Pupation height was also rapidly affected by density (Fig. 1D). Larvae from low densities (1 to 20) tended to pupate very close to the food substrate, whereas larvae from higher densities clearly pupated higher in the vials, 50 mm over 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 & F) (density * sample type effect: χ²=353, df=2, p<0.001; χ²=264, df=2, p<0.001; p<p<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 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; S3; S4). Bacterial communities were significantly different between larvae and their environment ($F_{1,30}=10.94$, $p=0.002$; $F_{1,30}=37.79$, $p<0.001$; for observed richness and Shannon diversity respectively) and were also significantly modified by larval density ($F_{2,30}=8.19$, $p=0.001$, $F_{2,30}=13.74$, $p<0.001$; 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, 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 significant effect on Bray-Curtis distances, explaining respectively 21, 12 and 19% of the total variance (Table S1). The figure 2D shows large and consistent differences in bacterial composition of the food according to larval density, while only subtle changes in bacterial composition were found in larval gut microbiota. Even if the global bacterial community in larvae did not drastically changed with density, we could note 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 to the control without supplementation (Dunn’s, $p<0.001$). Urea alone had smaller effect than ammonia, but still increased development time compared to control (Dunn’s, $p=0.019$). We found no significant effect of supplementation on viability or pupation height (Fig. 3B & 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 over 80% in all treatments, which is comparable with values found in the first experiment at similar larval density. Median pupation height was comprised 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: less than 3% of eggs on average should attain the adult stage when density is over 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 the density levels of 400 and above. This plateau suggests that a critical minimal viable mass was reached in our population at around one third of the normal mass. Although we observe a developmental delay at high densities, it did not exceed one day for the median time. However, above density level of 100, the distribution of emergence became much larger due to extreme individuals: from intervals of about two days between the first and the last emerging individual at low densities (1-20) to intervals sometimes over 7 days at the highest densities (200-1000). Higher variance at high densities is probably the consequence of contrasted responses: some individuals were rapid enough to profit from early non-degraded conditions while 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 damages 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 contrasted 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 with 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 had also 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
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404 to the excretion. Urea is supposed to occur naturally in *Drosophila* cultures (Joshi,
405 Shiotsugu and Mueller 1996) and previous studies actually found increasing levels of
406 urea in food in 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
410 Borash *et al.* (1998) reported that ammonia is the primary metabolic waste product of
412 *D. melanogaster* larvae but other studies rather posit that uric acid is the main waste
product of nitrogen metabolism (Botella *et al.* 1985; Winans *et al.* 2017). Finally,
414 products such as allantoin were also found in the food medium, but have not been
415 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,
417 though in variable amounts. Our results confirm the presence of ammonia in food, with
418 high levels detected in MD and HD conditions. On the other hand, we only found traces
419 of uric acid in the food but surprisingly higher amounts in larvae. This suggests that *D.
420 melanogaster* larvae produce and accumulate uric acid, probably in tissues such as
421 the fat body or in Malpighian tubules (Weihrauch, Donini and O’Donnell 2012), but this
422 compound seems to quickly degrade once in the environment. Consequently, the
423 presence of urea and ammonia in food may either result from an effective excretion of
424 these products by larvae, or from the degradation and transformation of uric acid in the
425 environment. In other words, uric acid could degrade either inside or outside the larvae
426 (fig. 4).
427 The classic pathway of uric acid degradation involves the urate oxidase enzyme, coded
428 by the *Uro* gene in *D. melanogaster* (Friedman 1973; Wallrath, Burnett and Friedman
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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 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 metabolism 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 flies cultures, but not in the medium of flies harboring bacteria with functional urate oxidase gene. We can expect that in crowded conditions, bacteria experienced even more 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 to *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). The analysis revealed no marked functional change in pathways related to nitrogen wastes regulation such as the purine and urea metabolism (fig. S6). However, this kind
of approach may not be well suited to evidence minute variation at the pathway level in samples collected from unusual environments, possibly explaining this 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 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 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 disentangling 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 to 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 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 was not strongly affecting 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 bacteria 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, while 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 to the association between larvae and bacteria, affecting the stability of the relationship. We evidenced 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 evidences accumulate 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.

Conflict of interest

Authors declare no conflict of interest

References


Staubach F, Baines JF, Künzel S et al. Host Species and Environmental Effects on Bacterial Communities Associated with Drosophila in the Laboratory and in the Natural Environment. *PLOS ONE* 2013;*8:e70749*.


1 **Figure legends**

2 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. Horizontal dashed lines: stabilization threshold of mass calculated from model properties. (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).

3 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: 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). (C) NMDS Bray-Curtis ordination of bacterial communities in LD, MD and HD conditions, split by sample type (larvae or...
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 (Dunnett 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.

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.
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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.

<table>
<thead>
<tr>
<th>Distance</th>
<th>Factors</th>
<th>Df</th>
<th>Sums of squares</th>
<th>Mean squares</th>
<th>F Model</th>
<th>R2</th>
<th>Pr(&gt;F)</th>
</tr>
</thead>
</table>
| 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        |         |       |        |
Fig S5: Relative abundance heatmap of the second-level functional KEGG pathways identified by PICRUSt2, in larvae and food samples, at each larval density level.

Fig S6: Relative abundance of purine metabolism and urea cycle KEGG modules (N=6 per sample type per density), 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. We found no significant differences between any treatments.

References
