

RESEARCH ARTICLE

Hormesis-like effect of mild larval crowding on thermotolerance in *Drosophila* flies

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ABSTRACT

Crowding is a complex stress that can affect organisms' physiology, especially through decreased food quality and accessibility. Here, we evaluated the effect of larval density on several biological traits of *Drosophila melanogaster*. An increasing gradient, from 1 to 1000 eggs per milliliter of food, was used to characterize life-history traits variations. Crowded conditions resulted in striking decreases of fresh mass (up to 6-fold) and viability, as well as delayed development. Next, we assessed heat and cold tolerance in L3 larvae reared at three selected larval densities: low (LD, 5 eggs ml⁻¹), medium (MD, 60 eggs ml⁻¹) and high (HD, 300 eggs ml⁻¹). LT₅₀ values of MD and, to a lesser extent, HD larvae were repeatedly higher than those from LD larvae, under both heat and cold stress. We investigated potential physiological correlates associated with this density-dependent thermotolerance shift. No marked pattern could be drawn from the expression of stress-related genes. However, a metabolomic analysis differentiated the metabolotypes of the three density levels, with potential candidates associated with this clustering (e.g. glucose 6-phosphate, GABA, sugars and polyols). Under HD, signs of oxidative stress were noted but not confirmed at the transcriptional level. Finally, urea, a common metabolic waste, was found to accumulate substantially in food from MD and HD larvae. When supplemented in food, urea stimulated cold tolerance but reduced heat tolerance in LD larvae. This study highlights that larval crowding is an important environmental parameter that induces drastic consequences on flies' physiology and can affect thermotolerance in a density-specific way.

KEY WORDS: Cold stress, Heat stress, Larval density, Metabolic response, Stress response, Urea

INTRODUCTION

The density of individuals in a population is likely to fluctuate with food accessibility. A sudden increase of trophic resources can result in a burst of reproduction, leading later to a crowding situation (Atkinson, 1979; Nunney, 1990). This is especially true for species with a short life cycle and high reproductive capacity such as many insects. When the population density overruns a given threshold (i.e. crowding), severe detrimental effects may arise (Kovács and Csermely, 2007). In *Drosophila* species, for instance, delayed development, lower fecundity, cannibalism and decreased emergence success are common consequences of crowding (Lints and Lints, 1969; Scheiring et al., 1984; Borash et al., 2000; Kolss

et al., 2009; Vijendravarma et al., 2012). These defects result from both a quantitative (e.g. food restriction) and a qualitative (e.g. overconsumption of toxic wastes) deterioration of food supply (Scheiring et al., 1984; Botella et al., 1985; Borash et al., 1998) as well as inter-individual scramble competition. Crowding can thus be considered as a complex multifactorial stressor.

While crowding can be deleterious to insects, it can also have positive effects, sometimes viewed as Allee effects (Wertheim et al., 2002). Several studies have reported increased lifespan (e.g. Miller and Thomas, 1958; Lints and Lints, 1969; Zwaan et al., 1991; Dudas and Arking, 1995; Shenoi et al., 2016), enhanced starvation tolerance (Mueller et al., 1993), increased additive genetic variance (Imasheva and Bublik, 2003) or elimination of fungal growth (Wertheim et al., 2002) under crowded conditions. However, some of these effects remain controversial as a result of inconsistent observations or limited experimental support (Baldal et al., 2005; Moghadam et al., 2015).

In the wild, crowding occurs in ephemeral and isolated habitats; for example, rotting fruits, where fruit fly larvae are forced to cope with multiple stressors, including thermal stress (see Feder et al., 1997; Warren et al., 2006). Several authors have reported that heat stress tolerance could be promoted by crowding in *Drosophila* adults (Quintana and Prevosti, 1990; Bublik et al., 1998; Sørensen and Loeschcke, 2001; Arias et al., 2012), while other studies reported no effect (Oudman et al., 1988) or even decreased stress tolerance (Loeschcke et al., 1994). These discrepancies may result from different methodological approaches among studies, and from the focus on adult stage despite the fact that crowding occurs at the larval stage and thus primarily affects larvae.

Hypotheses to explain the promoting effect of crowding on heat tolerance are diverse. For example, Quintana and Prevosti (1990) proposed the existence of a close pleiotropism between lifespan traits and thermal stress traits, Bublik et al. (1998) suggested that metabolic alterations could be responsible for enhanced heat knockdown resistance at high densities, and Loeschcke et al. (1994) argued that size/surface ratio variations may affect thermotolerance. Sørensen and Loeschcke (2001) first tested the existence of mechanistic links between crowding and thermal stress tolerance. These authors observed small but significant larval heat-shock protein 70 (HSP70) up-regulation correlated with enhanced heat stress tolerance of adults raised under high larval density. This induction of stress-related proteins could be triggered by increased amount of toxic waste, like urea, produced by larvae during intense crowding (Botella et al., 1985), urea being known as a protein denaturant (Yancey and Somero, 1980; Somero and Yancey, 1997). Larval crowding can also induce activation of the antioxidant defense system (Dudas and Arking, 1995), involving ubiquitous genes from the cellular stress response able to limit the detrimental effects of many stressors (Kültz, 2005). More recently, a study found that larval crowding affected lipid profiles (Moghadam et al., 2015); the study provides a putative, yet

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unexplored, mechanism explaining this effect, as changes in membrane lipid composition have been associated with thermotolerance shifts in insects (Overgaard et al., 2006; Shreve et al., 2007).

In the available literature, experimental designs of crowding-related studies were often based on comparisons between two larval densities, referred as low and high density (see Table S1 for a brief review). The choice of these larval densities was often heterogeneous, and not clearly justified. As the relationship between crowding and biological traits is not necessarily linear (Prout and McChesney, 1985), insightful interpretations strongly depend upon the initial selected densities.

In the present study, we aimed to decipher the effects of larval crowding on several biological traits of *D. melanogaster*. The first step was to find a 'viability threshold', i.e. a larval density over which viability becomes insignificant (<10%). To do so, we used a broad larval density gradient, and characterized the consequences of larval density on life-history traits (e.g. viability, development duration and body mass). Based on these life-history data, we selected three contrasted larval densities (considered as non-, mildly and highly stressful), under which we tested larval thermotolerance (using cold and heat stress). Because of the non-linearity of effects of larval density on biological traits (Prout and McChesney, 1985), we assumed that intermediate density may trigger a hormetic-like response improving thermotolerance, while intense crowding would become deleterious. As metabolic by-products accumulate in food under crowding (Botella et al., 1985), we measured urea concentration, assuming that it would accumulate in both food and larvae at high rearing densities. Next, we measured thermotolerance of *D. melanogaster* larvae reared on urea-supplemented food to test whether urea could mediate the observed effects on thermotolerance.

We also searched for physiological correlates of crowding. Antioxidant enzymes and HSPs have been linked to crowding stress (Dudas and Arking, 1995; Sørensen and Loeschcke, 2001). The activity of some enzymes associated with oxidative stress has been shown to vary with larval density (superoxide dismutase but not catalase or glutathione *S*-transferase), but in a genotype- and stage-specific manner (Dudas and Arking, 1995). In addition, despite an increase in HSP70 level in larvae reared under crowded conditions (Sørensen and Loeschcke, 2001), the regulation of other members of the HSP family has not been investigated. Here, we tested whether crowding caused the accumulation of oxidant molecules in larvae (H_2O_2), and whether it could impact the expression of various genes coding for antioxidant enzymes and members of the HSP family. Finally, considering that dietary modifications can strongly affect the fly metabolome (Colinet et al., 2013; Colinet and Renault, 2014), we tested whether different levels of crowding, associated with quantitative and qualitative alteration of nutritional supply, would also result in different metabolotypes.

MATERIALS AND METHODS

Stock population

We conducted the experiments on a laboratory population of *D. melanogaster* Meigen derived from wild individuals collected in September 2015 in Brittany (France). Fly stocks were maintained on standard fly medium comprising inactive brewer's yeast (MP Bio 0290331205, MP Bio, Santa Ana, CA, USA; 80 g l⁻¹), sucrose (50 g l⁻¹), agar (Sigma-Aldrich A1296, Sigma-Aldrich, St Louis, MO, USA; 10 g l⁻¹), supplemented with Nipagin (Sigma-Aldrich H5501; 8 ml l⁻¹) in 100 ml bottles, at 25°C (12 h light:12 h dark). Unless stated otherwise, these conditions were also used for rearing of flies in the following experiments.

Larval density treatments

Prior to the experiment, we allowed adult flies from rearing stock to lay eggs for 6 h on Petri dishes filled with standard food. Using a binocular microscope, eggs were then counted, delicately collected with a paint brush, and transferred into new 50 ml vials (23 mm diameter) containing 2 ml of food, in order to achieve the desired larval density (Fig. S1).

Effects of larval crowding on life-history traits

In the first experiment, we tested a broad range of larval densities: 1, 5, 20, 60, 100, 200, 300, 500 and 1000 eggs ml⁻¹ food (see pictures of the different treatments in Fig. S1). To generate these nine density treatments, a total of 180, 180, 200, 240, 200, 400, 600, 1000 and 2000 eggs were counted and deposited in 90, 18, 5, 2, 1, 1, 1 and 1 vials respectively, each with 2 ml of food. Viability was calculated based on the number of emerged adults over the total number of deposited eggs. The development duration (from egg to adult) was recorded from emerging individuals by checking their emergence twice a day. Adult fresh and dry masses were 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 µg). Dry mass was measured after individuals were dried out for at least 1 week at 60°C.

Larval density effects on thermotolerance

In the second part of the study, we reared flies under three larval density conditions: low (LD; 5 eggs ml⁻¹), medium (MD; 60 eggs ml⁻¹) and high density (HD; 300 eggs ml⁻¹). These three densities were selected based on life-history measurements (see above) and represent non-, mildly and strongly stressful larval crowding conditions. When individuals reached the wandering third instar (L3), they were delicately collected with a paint brush (larvae from different rearing vials were randomly pooled by density), and transferred by groups of 10 individuals into fresh food vials (the sex was not determined for larvae). For each density condition, 18 of these vials were then directly immersed into thermoregulated bath, filled with 70% ethylene glycol mixed with 30% water, and set at 38±0.1°C for assessment of heat stress, or at -3±0.1°C for cold stress (*N* per density per assay: 18 vials×10 individuals=180). In both tests, the acute stress exposure lasted for a maximum of 120 min. Over this period, two vials per condition were removed from the bath every 15 min, thus resulting in nine exposure durations. After the stress, vials were transferred to standard conditions for larval development (25°C, 12 h light:12 h dark), and survival was scored as the number of emerged adults. Within a given generation of experimental flies, we performed pairwise comparisons of either LD versus MD or LD versus HD (hereafter, the use of 'LDa' refers to LD flies used for the comparison between LD and MD, and 'LDb' refers to LD flies used for the comparison between LD and HD). LDa versus MD comparison was replicated three times, and LDb versus HD comparison was replicated twice, all replicates being from distinct generations. Third instar wandering larvae from the three density treatments were also collected, snap-frozen in liquid nitrogen, and stored at -80°C.

Urea effects on thermotolerance

We conducted additional thermotolerance assays with larvae reared on media supplemented with increasing amounts of urea (Sigma-Aldrich U5378) to obtain the following urea concentrations: 0 (control), 2, 5 and 10 mg of urea added per milliliter of food. These larvae were all reared under the LD condition (i.e. 5 eggs ml⁻¹), and acute cold and heat tolerance were tested as described above.

Triglyceride assay

Triglycerides (TAGs) and glycerol measurements were performed using the colorimetric method with triglyceride reagent (Sigma-Aldrich T2449) as described in Tennesen et al. (2014). Briefly, five frozen third instar larvae were homogenized in liquid nitrogen using a pestle to obtain a fine powder which was then diluted in 300 μ l of PBS-Tween 0.05%. Enzymes were heat inactivated (10 min at 70°C) and optical density was measured at 540 nm using a 96-well plate reader (Molecular Devices VersaMax, Molecular Devices, Sunnyvale, CA, USA) after the addition of free glycerol reagent (Sigma-Aldrich F6428). Quantification was done by a calibration curve using a glycerol standard (0–1 mg ml⁻¹ range). Ten biological replicates were performed for each larval density.

Urea assay

Urea concentration was determined using a Urea Assay Kit (Abnova KA1652, Abnova, Taipei, Taiwan) according to the manufacturer's instructions. Briefly, larvae (10 individuals) and food (50 mg) from each experimental condition were homogenized in, respectively, 250 and 500 μ l of cold PBS, using a tungsten-bead beating apparatus (Retsch MM301, Retsch GmbH, Haan, Germany; 20 Hz, 3 min). All samples were kept on ice and processed shortly afterwards (<15 min) to avoid melanization of homogenates. Optical density was measured at 530 nm using a 96-well plate reader (Molecular Devices VersaMax). Quantification was done based on a calibration curve using a urea standard (0–5 mg ml⁻¹ range). Ten biological replicates were performed for the larvae samples, and 7–10 replicates for the food samples.

Hydrogen peroxide assay

The hydrogen peroxide (H₂O₂) concentration in larvae samples was measured using Amplex Red (Invitrogen A 12222, Invitrogen, Carlsbad, CA, USA), following the protocol described in Chakrabarti et al. (2014). Briefly, for each replicate, 10 larvae were homogenized in 300 μ l of cold Ringer solution, using a tungsten-bead beating apparatus (Retsch MM301; 20 Hz, 3 min). All samples were kept on ice and processed shortly afterwards (<15 min) to avoid melanization of homogenates. Fluorescence was measured at 550 nm (excitation) and 590 nm (emission) using a spectrofluorometer (SAFAS Monaco Xenius XC, Monaco). Quantification was done by a calibration curve using a H₂O₂ standard (0–10 μ mol l⁻¹ range). Preliminary tests showed that a 30- to 100-fold dilution was necessary to stay within the linear range of the standard curve. Ten biological replicates were performed per density condition.

Gene expression assays

RNA extraction was performed using a Nucleospin Kit (Macherey-Nagel, Düren, Germany) following the protocol described in Colinet et al. (2010). For the three larval densities (LD, MD, HD), RNA was extracted from four replicates, each consisting of 10 larvae. RNA was then diluted in RNase-free water in order to standardize concentrations at 500 ng of total RNA, and then reverse-transcribed to cDNA using the Superscript III First-Strand Synthesis System (Invitrogen) following the manufacturer's instructions. We quantified the transcript abundance of 15 genes involved in protein chaperoning, oxidative stress defense or the urea cycle as well as three housekeeping genes as reference through qRT-PCR (for primer sequences, see Table S2). Only *RpS20* was kept as a reference housekeeping gene as it showed high stability in all experimental conditions. Reactions were performed in a LightCycler® 480 system (Roche, Basel, Switzerland) with

SybrGreen I mix (Roche) according to Colinet et al. (2010). Relative expression ratios were computed using the $\Delta\Delta$ Ct method (Pfaffl, 2001).

Metabolic profiling

Metabolic profiles of larvae from LD, MD and HD treatments were compared. Two different LD controls were used, LDa and LDb, corresponding to control larvae from the same generations as larvae from MD and HD treatments, respectively. Fresh mass of each sample was measured (Mettler Toledo UMX2) before metabolite extractions. Sample preparation and derivatization were performed as previously described in Colinet et al. (2016) with minor modifications. Briefly, after homogenization in 750 μ l of ice-cold methanol–chloroform solution (2:1, v:v) and phase separation with 500 μ l of ultrapure water, a 100 μ l aliquot of the upper phase was vacuum-dried. The dry residue was resuspended in 30 μ l of 20 mg ml⁻¹ methoxyamine hydrochloride in pyridine before incubation under automatic orbital shaking at 40°C for 60 min. Then, a volume of 30 μ l of BSTFA was added and the derivatization was conducted at 40°C for 60 min under agitation. A CTC CombiPal autosampler (PAL System, CTC Analytics AG, Zwingen, Switzerland) was used, ensuring standardized sample preparation and timing. Metabolites were separated, identified and quantified using a GC/MS platform consisting of a Trace GC Ultra chromatograph and a Trace DSQII quadrupole mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The temperature was increased from 70 to 170°C at 5°C min⁻¹, from 170 to 280°C at 7°C min⁻¹, and from 280 to 320°C at 15°C min⁻¹, and then the temperature was held at 320°C for 4 min. We completely randomized the injection order of the samples. All samples were run under the SIM mode rather than the full-scan mode. We therefore only screened for the 63 pure reference compounds included in our custom spectral database. Calibration curves for 63 pure reference compounds at 1, 2, 5, 10, 20, 50, 100, 200, 500, 750, 1000 and 1500 μ mol l⁻¹ concentrations were run concurrently. Chromatograms were deconvoluted using XCalibur 2.0.7, and metabolite levels were quantified using the quadratic calibration curve for each reference compound and concentration. Quality controls at concentrations of 200 μ mol l⁻¹ were run every 15 samples. A total of 44 metabolites was detected and quantified from our samples.

Data analysis

Data analysis was mainly performed using R software v3.3.1 (R Development Core Team 2016). Binomial generalized linear models (GLM) were fitted to survival data from thermotolerance assays using a logit link function, followed by a deviance analysis and Tukey tests computed using the 'multcomp' package (Hothorn et al., 2008) in order to test for pairwise differences. Masses (fresh and dry), as well as viability and development duration, were analyzed using non-linear models, with custom formulations of the logistic equation proposed by Börger and Fryxell (2012). For mass, we used the formulation:

$$\text{Mass} = d + \frac{a}{1 + \exp[(\text{larval density} - b)/c]}, \quad (1)$$

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. We used properties of the equation to define 'decreasing mass threshold' ($=b-3\times c$) and 'stabilization threshold' ($=b+3\times c$).

For viability and development duration, we used the formulation:

$$\% \text{Emerging adults} = \frac{a}{1 + \exp[(b - \text{time})/c]}, \quad (2)$$

where a corresponds to the endpoint viability, b is the inflection threshold expressed in time units, and c is the range of the curve on the time axis. This equation was convenient because at $\text{time} = b + 3c$, we knew that measurements reached 95% of a , and we used this time value as an estimation of development duration for 95% of total emerging adults. Relative gene expression differences between treatments were tested using t -tests. Metabolomics data were analyzed using between-principal components analysis (PCA) (ade4 package) followed by Monte-Carlo test to look for significant differences between treatments. ANOVA were computed on TAG, urea and H_2O_2 measurements using Prism software v5.01 (GraphPad, La Jolla, CA, USA).

RESULTS

Effect of larval density on life-history traits

At the highest density (1000 eggs ml^{-1} of food), we observed very low adult emergence (1.25% viability); only extreme individuals (outliers) that developed fast enough to limit the crowding stress were able to emerge. Because of the limited number of adults (6 females and 19 males out of 2000 individuals), data from this condition were not included in development, viability and body mass analyses. Emergence rate was strongly affected by the interaction of time and larval density (deviance analysis, $\chi^2 = 289.10$, d.f.=8, $P < 0.001$). The flies that were reared under extreme crowding conditions took about one more day to emerge compared with other crowding conditions (Fig. 1A). Moreover, viability dropped from

ca. 90% at the lowest larval density (1 egg ml^{-1} of food) to ca. 40% at high larval density (500 eggs ml^{-1} of food) (Fig. 1B). Both fresh and dry masses drastically decreased in both sexes with increasing larval density (Fig. 1C,D). While the dry mass remained rather stable at densities of 1, 5, 20 and 60 eggs ml^{-1} of food, it showed a clear drop-off from the 60 eggs ml^{-1} of food condition onwards [decreasing threshold ($b - 3c$) = 71 females, 51 males], before stabilizing at densities above 300 eggs ml^{-1} of food [stabilization threshold ($b + 3c$) = 339 females, 398 males] (Fig. 1D). Fresh mass decreased by more than 6-fold between extreme densities (e.g. 0.895 ± 0.062 versus 0.135 ± 0.013 mg for males at 1 and 500 eggs ml^{-1} , respectively) (Fig. 1C). Despite excluding the density of 1000 eggs ml^{-1} from analyses, mass measurements were consistent with those from a density 500 eggs ml^{-1} of food.

For all subsequent experiments, we focused on three contrasting densities: (1) low density (LD) at 5 eggs ml^{-1} of food, considered as not stressful (i.e. 80% viability, unaffected development duration and mass), (2) medium density (MD) at 60 eggs ml^{-1} of food, considered as mildly stressful (i.e. 55% viability but little to no effect on mass and development duration) and (3) high density (HD) at 300 eggs ml^{-1} of food, considered as stressful because of the dramatic phenotypic changes it caused (i.e. 55% viability, 60% mass decrease and >12 h delay of development duration).

Thermotolerance of larvae reared under crowded conditions

For all thermotolerance assays, survival decreased with duration of exposure ($P < 0.05$, Fig. 2). Cold tolerance was consistently higher in MD treatments compared with LD controls (Fig. 2) (deviance analysis, $\chi^2 = 44.09$, d.f.=1, $P < 0.001$; $\chi^2 = 31.09$, d.f.=1, $P < 0.001$; $\chi^2 = 44.92$, d.f.=1, $P < 0.001$; for replicates 1, 2, 3, respectively). Cold

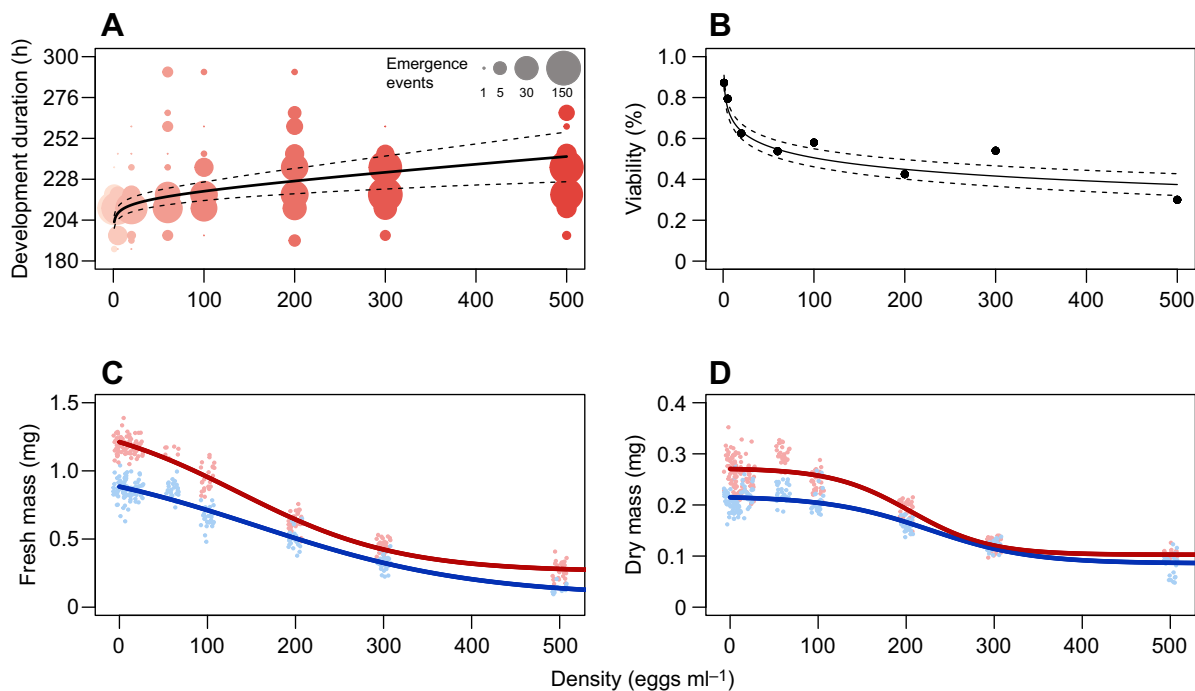


Fig. 1. Life-history traits of flies reared at increasing larval density. (A) Development duration from egg to adult. Circles: pooled observed emergence events at a given density of larvae and at a given scoring time. The color gradient indicates increasing larval density. Solid line: time needed for 95% of individuals to emerge (predicted using a non-linear model). Dotted lines: 95% confidence intervals. $N = 180, 180, 200, 240, 200, 400, 600, 1000$ at densities from 1 to 500 eggs ml^{-1} . (B) Egg to adult viability ratio. Circles: raw experimental observed egg to adult viability ratio. Solid line: viability ratio predicted using a non-linear model. Dashed lines: 95% confidence interval. $N = 147, 153, 125, 129, 116, 170, 324, 300$ at densities from 1 to 500 eggs ml^{-1} . (C) Wet mass and (D) dry mass of adult flies reared at increasing larval density (expressed as initial number of eggs ml^{-1}). Circles indicate raw data. Lines: non-linear model based on these data. Females are represented in red and males in blue. For mass, $N = 30$ in all treatments.

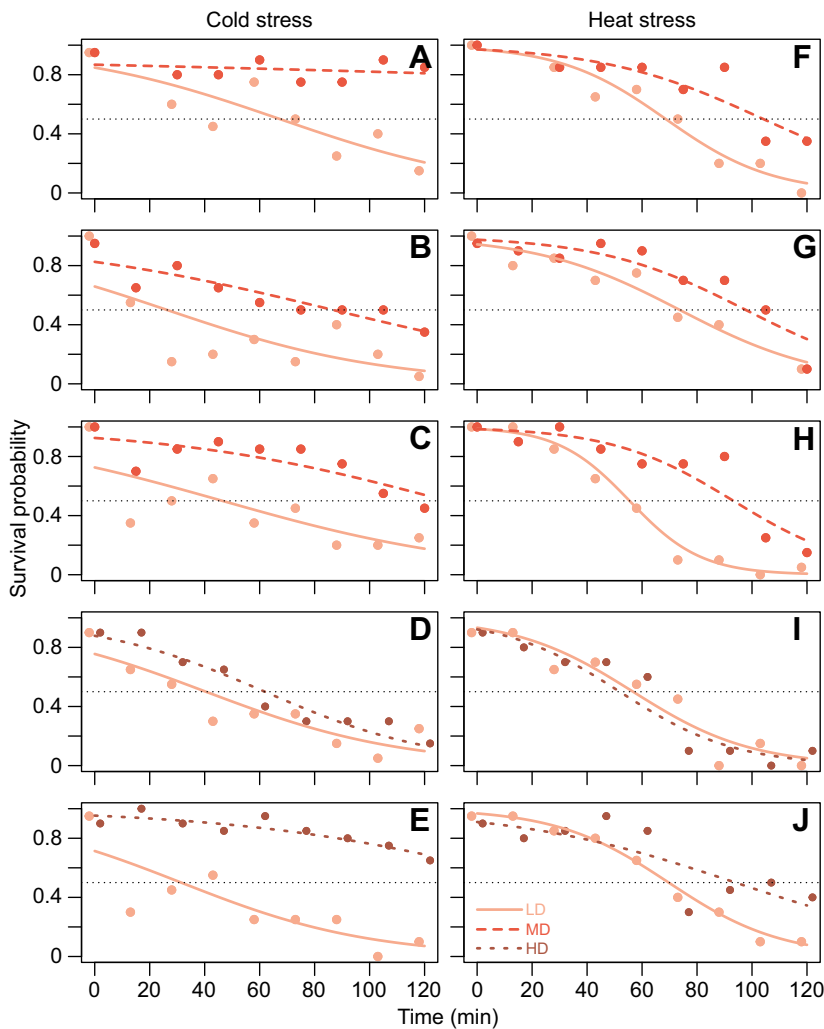


Fig. 2. Survival of third instar larvae as a function of exposure duration to acute cold ($-3\pm 0.1^\circ\text{C}$) or heat ($38\pm 0.1^\circ\text{C}$) for three experimental larval density conditions. Mortality was scored on adults 5–6 days after thermal stress. Data for increasing larval density (low, LD, 5 eggs ml^{-1} ; medium, MD, 60 eggs ml^{-1} ; and high, HD, 300 eggs ml^{-1}) are presented (see key). Left column: acute cold tests. Right column: acute heat tests. A–C for cold and F–H for heat are three replicates of the pairwise comparison of LD versus MD; D and E for cold and I and J for heat are two replicates of the pairwise comparison LD versus HD. Each replicate was performed on separate generations. Lines: predictions from GLM models. Circles: raw data of survival proportion for a given exposure duration, based on 20 larvae. Nine exposure durations were tested (i.e. $N=180$ per treatment, per replicate).

tolerance was also higher in HD compared with LD larvae in both replicates ($\chi^2=6.48$, d.f.=1, $P<0.05$; $\chi^2=115.46$, d.f.=1, $P<0.001$), although large inter-generational differences were observed (Fig. 2D,E). LT_{50} (lethal time) values of LD larvae were consistently inferior to those of the other treatments (based on lack of overlap in 95% confidence intervals; Fig. 3).

Heat tolerance was higher for larvae reared under MD than under LD control, and this pattern was repeatedly observed for all replicated assays ($\chi^2=22.30$, d.f.=1, $P<0.001$; $\chi^2=6.76$, d.f.=1, $P<0.01$; $\chi^2=43.69$, d.f.=1, $P<0.001$; for replicates 1, 2, 3,

respectively) (Fig. 2F–H). The effect of crowding appeared less robust for HD larvae, with no significant difference between HD and LD larvae in one of the two replicates ($\chi^2=0.70$, d.f.=1, $P=0.41$; $\chi^2=5.31$, d.f.=1, $P<0.05$; for replicate 1, 2; Fig. 2I,J).

Urea production during crowding and effects on thermotolerance

First, we monitored urea concentration in food from the nine rearing conditions of the larval density gradient (Fig. 4A). We found that urea accumulated in food with increasing larval density

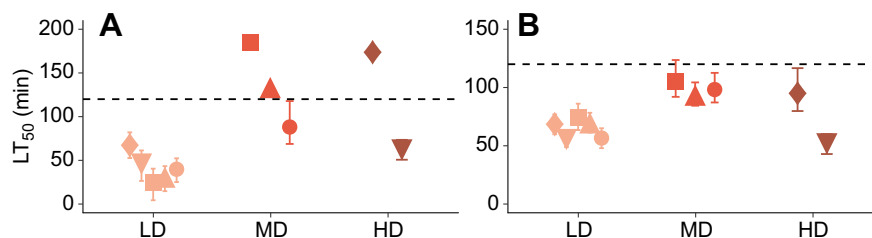


Fig. 3. Mean LT_{50} for third instar larvae exposed to increasing durations of acute cold ($-3\pm 0.1^\circ\text{C}$) or heat ($38\pm 0.1^\circ\text{C}$) stress. (A) Cold stress; (B) heat stress. Mortality was scored on adults 5–6 days after thermal stress. Data for increasing larval density (LD, MD and HD) are presented. Each symbol corresponds to one generation of flies. Error bars show 95% confidence intervals. Mean LT_{50} values and their 95% confidence intervals were calculated by simulating 1000 times the GLM model fitted on survival data. The dashed horizontal line shows the maximum stress exposure duration tested in the experiments. LT_{50} values above the dashed line are estimations based on conditions where 50% mortality was actually not reached by the end of the experiment. These points are thus shown without error intervals and need to be considered as extrapolations from models. For each LT_{50} , $N=180$.

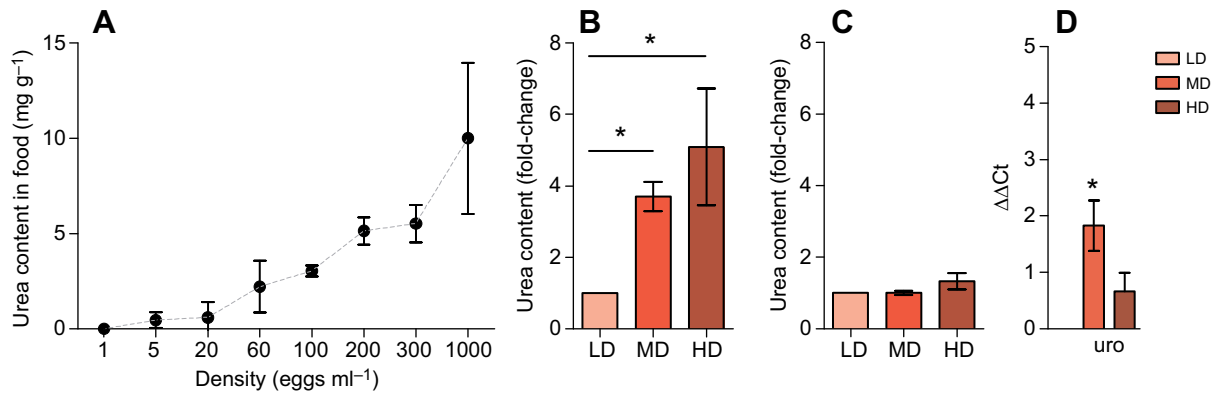


Fig. 4. Urea content in food and in larvae reared under crowded or uncrowded conditions. (A) Urea content in food, after the development of larvae, under conditions of increased crowding. Error bars show 95% confidence intervals. $N=7-10$ depending on the density treatment. (B,C) Urea content in (B) food and (C) L3 larvae reared at three larval densities (LD, MD and HD). Urea content is presented as a fold-change relative to the LD condition. Error bars show 95% confidence intervals. *Significant difference (Tukey HSD, $P<0.001$). In B, $N=7$ per density treatment; in C, $N=10$ per density treatment. (D) $\Delta\Delta\text{Ct}$ of qPCR Ct values, i.e. the \log_2 fold-change relative to the LD condition ($=0$) of the expression of the urate oxidase gene, *uro*. The values are expressed relative to expression of a reference housekeeping gene (*RpS20*). Each Ct was measured on four biological replicates. Error bars show s.e.m. *Significant difference (*t*-test, $P<0.05$).

($F_{7,15}=746.9$, $P<0.001$), reaching up to 10 mg of urea per gram of food at the highest larval density. Next, we measured urea concentration in both food and wandering larvae from LD, MD and HD conditions (Fig. 4B,C). We observed no significant increase of urea concentration in larvae ($F_{2,27}=1.92$; $P=0.16$; Fig. 4C), despite significant accumulation of this compound in food ($F_{2,23}=29.48$, $P<0.001$; Fig. 4B). Consistent with the previous assay, the urea level increased 5 times between the LD and HD conditions (Fig. 4B).

Artificial urea supplementation in food had a significant effect on cold survival of larvae ($\chi^2=22.98$, d.f.=3, $P<0.001$) (Fig. 5A). Survival was enhanced at intermediate concentrations (2 and 5 mg urea ml^{-1} of food) compared with the control with no added urea (Tukey HSD, $P<0.05$) (Fig. 5A). No difference was found between the highest concentration (10 mg urea ml^{-1} of food) and control (Tukey HSD, $P=0.21$). A global detrimental effect of urea supplementation was detected on heat tolerance ($\chi^2=303.08$, d.f.=3, $P<0.001$) (Fig. 5B).

Molecular changes induced by larval crowding

Expression of *uro* gene, coding for urate oxidase, showed significant up-regulation (*t*-test, $P<0.05$) in response to crowding, but only in MD larvae ($\Delta\Delta\text{Ct}=1.83$ corresponding to a 3.56-fold change compared with LD larvae, Fig. 4D). No significant change in expression was detected for genes implicated in the degradation

of reactive oxygen species [superoxide dismutase (*sod*) and glutathione synthase (*gs*)], except for the catalase gene (*cat*), which showed a significant upregulation in MD versus LD larvae (*t*-test; $P<0.05$) (Fig. 6A). For the other generic stress-related genes (i.e. molecular chaperones or co-chaperones), several significant differences were observed (Fig. 6C): (1) in MD versus LD larvae, upregulation of starvin gene (*stv*), heat-shock cognate 70 gene (*hsc70*) and *hsp40*, and downregulation of *hsp60*, *hsp27*, *hsp23*, *hsp26*, and *hsp68* (*t*-test; $P<0.05$), and (2) in HD versus LD larvae, upregulation of *stv* and downregulation of *hsp68* (*t*-test; $P<0.05$). However, all detected transcriptional changes aside from *uro* remained of relatively small magnitude, ranging from 2.12-fold (*stv* in MD) to 0.27-fold (*hsp68* in HD). Finally, fluorometric H_2O_2 measurements showed higher concentrations (2 times) in flies from HD conditions than in their relatives from LD or MD conditions (Tukey HSD, $P<0.001$) (Fig. 6B).

Forty-four metabolites were detected and quantified in our larval fly samples. A list of detected metabolites with their abbreviations is provided in Table S3. Trehalose and glucose were the most abundant detected metabolites. Changes in the level of each metabolite in relation to larval density treatments are shown in Fig. S2. The first and second principal components (PC1 and PC2) of the PCA represented 65.4% and 24.1% of total inertia, respectively. Monte-Carlo randomizations confirmed the significance of the differences among classes (observed $P<0.001$). PC1 mostly

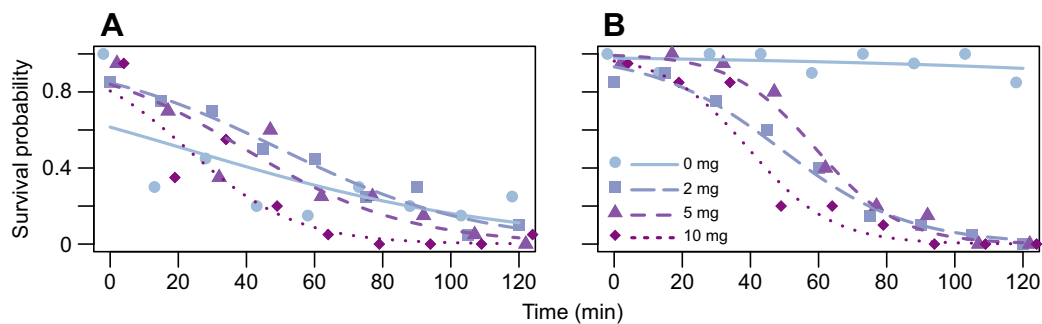


Fig. 5. Survival of larvae reared at LD with increasing urea supplementation following acute cold or heat exposure. Urea was present at 2, 5 and 10 mg ml^{-1} of food (control had no urea supplementation). (A) Cold stress; (B) heat stress. Symbols: raw data of survival proportion based on 20 larvae. Lines: predictions from GLM models. Nine exposure durations were tested (i.e. $N=180$ individuals per treatment, per stress). Survival was scored on emerging adult flies.

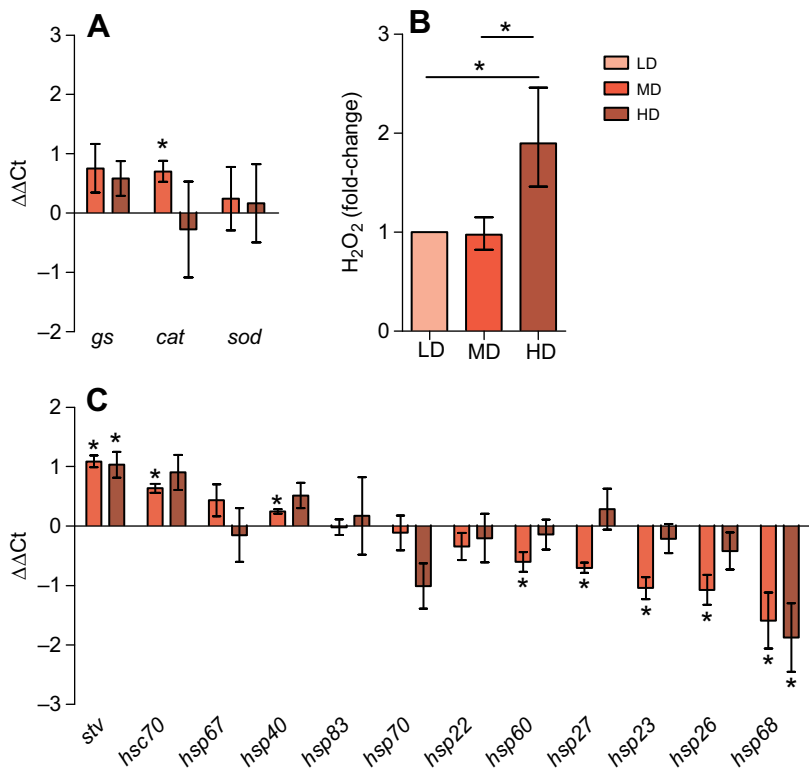


Fig. 6. Gene expression and oxidative stress levels during crowding. (A) $\Delta\Delta C_t$ of qPCR Ct values, i.e. \log_2 fold-change relative to the LD condition ($\Delta\Delta C_t$ LD=0), of the expression of genes involved in oxidative stress regulation. Error bars show s.e.m. (B) Relative internal H_2O_2 concentration in third instar larvae raised in LD, MD or HD conditions, and expressed as fold-change relative to the LD condition ($N=10$). Error bars show 95% confidence intervals. Density significantly impacts H_2O_2 concentration ($F_{2,27}=19.31$, $P<0.001$). *Significant difference (Tukey HSD, $P<0.001$). (C) $\Delta\Delta C_t$ of qPCR Ct values of the expression of genes involved in the general stress response. The values are expressed relative to expression of a reference housekeeping gene (*RpS20*). Each Ct was measured from four biological replicates. Error bars show s.e.m. *Significant difference from control LD condition (t -tests, $P<0.05$). gs, glutathione synthase; cat, catalase; sod, superoxide dismutase; stv, starvin; hsc, heat-shock cognate; hsp, heat-shock protein.

represented the increasing larval density, with a clear opposition between LD and HD larvae. Sugars (e.g. ranked by contribution to axis: maltose, trehalose, mannose, glucose), polyols (e.g. arabinol, galactitol, erythritol, mannitol) and acids (pipecolic acid, fumaric acid and malic acid) were positively correlated to PC1 (i.e. more abundant in MD and HD than in LD larvae), whereas amino acids were in general negatively correlated to PC1 (Fig. 7B). PC2 differentiated MD from LD and HD larvae, and some polyols were positively correlated to this axis (xylitol, sorbitol and glycerol 3-phosphate) as well as fructose (Fig. 7C). We also completed the metabolic analysis by measuring lipid content using colorimetric assays. No effect of density treatment was detected on TAG content ($F_{3,36}=1.79$, $P=0.16$) of larvae, but a significant effect of density treatment was detected on glycerol content ($F_{3,36}=14.29$, $P<0.001$), with larvae from the MD treatment showing higher concentrations than all other treatments (Tukey HSD, $P<0.001$) (Fig. S3).

DISCUSSION

In the present study, we assessed the effects of larval crowding on several biological traits of *D. melanogaster*. We found that both viability and development duration were impaired, even at moderate larval densities, and that viability was close to 0% at the highest tested density. Interestingly, body mass was rather stable for larval densities ranging from 1 to 60 eggs ml^{-1} of food, before dramatically decreasing to reach stable minimum values at larval densities over 300 eggs ml^{-1} of food.

In previous studies, simple pairwise comparison of control versus crowded conditions was often used, with larval densities generally ranging from 1 to 50 individuals ml^{-1} of food (Table S1). On this crowding range, our life-history traits measurements were consistent with the literature (e.g. Botella et al., 1985; Bierbaum et al., 1989). However, our data highlight that larvae of *D. melanogaster* can cope with much higher levels of larval crowding than those formerly considered as highly crowded. Hence, it is likely that the larval

densities used in many studies were only mildly stressful, and far from the tolerable limit for *Drosophila* flies. Accordingly, we showed that the highest tolerable larval crowding threshold (<90% mortality) falls in the range 500–1000 individuals ml^{-1} of food for *D. melanogaster*. This corresponds to the densities that were excluded from analyses as a result of excessive mortality in the work of Sarangi et al. (2016). Based on these observations, we selected three larval densities that we considered as non-, mildly and highly stressful, and subsequently investigated the thermotolerance of larvae.

Consistent with previous studies (Quintana and Prevosti, 1990; Bubli et al., 1998; Sørensen and Loeschcke, 2001; Arias et al., 2012), heat tolerance was superior in MD larvae compared with that in LD larvae, whereas this increase was less obvious in HD larvae. In parallel, cold tolerance was enhanced in MD and HD larvae compared with that of LD larvae, but the inter-replicate variance was high in individuals from HD conditions. As mortality increased with larval density, it is plausible that we selected extreme individuals, which could explain some of the phenotypic variation observed here. Limited and inconsistent effects of HD conditions on thermotolerance of larvae suggested that the priming effect was more effective at moderate (MD) than at very high (HD) larval densities (Figs 2 and 3). At very high larval density, the hardening-like effect generated by crowding could have been overwhelmed by the nutritional stress (i.e. food restriction and higher ingestion of toxic metabolic waste). In other words, our findings support the hypothesis that larval density may trigger a dose-dependent benefit on thermotolerance, which can be assimilated to a cross-tolerance hormesis response (Costantini et al., 2010).

The concept of hormesis is based on the genericity of stress response pathways (Calabrese and Baldwin, 2002; Sulmon et al., 2015), in which HSPs and proteins related to oxidative stress defense are prominent actors (Kültz, 2005). For instance, both the chaperoning activity of HSPs and antioxidant enzymes help cells to counterbalance cellular homeostasis disruption provoked by various

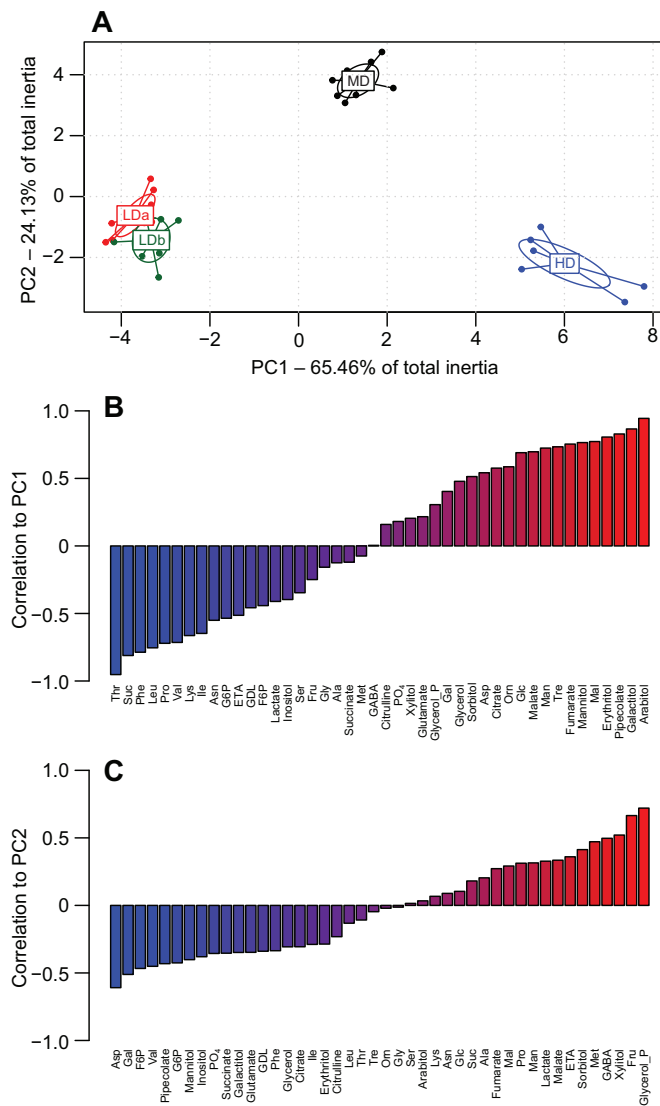


Fig. 7. Principal component analysis (PCA). (A) Between-PCA of metabolic profiles of *D. melanogaster* larvae reared at LD, MD or HD ($N=7-8$ per treatment). The first two principal components (PC1 and PC2) accumulated 89% of total variance (65% and 24%, respectively), and only these two first axes were kept in the analysis. The two LD treatments, LDa and LDd, correspond, respectively, to control flies from the same generations as MD and HD treatments. (B) Ranking of molecules according to their correlation to PC1. (C) Ranking of molecules according to their correlation to PC2.

stresses (Korsloot et al., 2004; Li and Srivastava, 2004). Despite the link between crowding and oxidative stress resistance (Buck et al., 1993; Dudas and Arking, 1995), we found no evidence of clear-cut increased regulation of antioxidant genes, or of *hsp* genes, in larvae reared under crowded conditions (MD and HD). Unlike Sørensen and Loeschcke (2001), Dudas and Arking (1995) also failed to observe increased regulation of HSPs in adults of *D. melanogaster* from their HD conditions. It is possible that regulation of stress genes occurred at different time scales (especially post-transcriptionally; see Bahrndorff et al., 2009; Košťál and Tollarová-Borovanská, 2009), or in other larval stages (i.e. L1 or L2, as we monitored gene expression in L3).

Crowding changes the chemical composition of the nutritional environment, mainly via metabolic waste excreted by the large number of larvae (Botella et al., 1985; Borash et al., 1998). While

this waste is deleterious at high doses, it could be stimulating at low doses, i.e. inducing hormetic effects (Hayes, 2007; Mattson, 2008; Calabrese and Mattson, 2009; Costantini, 2014). We found increasing concentrations of urea in food with increasing larval density. Yet, despite being present in food, urea was not detected in wandering larvae, most probably because this is a non-feeding stage, and urea might have been either metabolized or excreted earlier. Interestingly, we found upregulation of *uro* gene, but only under the MD condition. There are three known urea metabolizing enzymes: arginase in the ornithine–urea cycle, and allantoinase and urate oxidase in the uricolytic pathway. Urate oxidase is coded by the *uro* gene. Even though this gene is known to be present in *D. melanogaster* (Friedman and Baker, 1982), fruit flies are not capable of metabolizing urea (Etienne et al., 2001). Therefore, the upregulation of *uro* under MD conditions might not be directly linked to dietary urea. *uro* is involved in many stress-related pathways, such as exposure to ethanol (Logan-Garbisch et al., 2014) or oxidative stress (Terhzaz et al., 2010). Other studies have also reported an upregulation of *uro* in cold-exposed *Drosophila* adults (Zhang et al., 2011; Boardman et al., 2017); therefore, this gene probably has multiple functions related to stress-tolerance mechanisms.

To evaluate the potential role of urea in enhancing thermotolerance of *D. melanogaster*, we used larvae reared under low density, and fed with urea-supplemented medium. Small doses of urea supplementation, corresponding to the amounts we quantified in the MD treatment, enhanced cold tolerance of the larvae, while high doses of urea were deleterious. These causal changes indicate that dietary accumulation of metabolic waste may directly affect (positively or negatively) stress tolerance. Both heat stress and urea exposure induce protein unfolding (David et al., 1999). The combination of these two protein-denaturing treatments was probably overly cytotoxic, therefore explaining the poor survival of urea-supplemented larvae exposed to high temperature. Conversely, beneficial effects of urea supplementation might have been linked to increased hemolymph osmolarity (Pierce et al., 1999). In particular, amino acids such as proline and alanine, which have been directly linked to *Drosophila* cold tolerance (Olsson et al., 2016; Košťál et al., 2012), showed increased concentrations in flies fed on urea-supplemented media (Pierce et al., 1999). Overall, we consider that the positive effect of urea supplementation is insufficient to explain the large differences in cold tolerance between LD and MD larvae, but it is possible that it partly contributed to the observed effects.

Finally, crowding can directly affect the physiology of the larvae by altering diet quality and causing a scarcity of essential nutrients, leading to mild or more severe nutritional/caloric restriction (Botella et al., 1985). It has been shown that nutritional balance disruption such as yeast deprivation or sugar deprivation can generate large variations in life-history traits (Chippindale et al., 1993; Imasheva et al., 1999; Mair et al., 2005) together with metabolic shifts (Skorupa et al., 2008; Matzkin et al., 2011; Colinet and Renault, 2014). Thus, we performed a quantitative metabolic profiling that compared (i) control LDa versus MD larvae in one generation and (ii) control LDd versus HD larvae in a subsequent generation. The metabolic phenotypes of the two uncrowded controls (LDa and LDd) overlapped, which suggests a consistency of metabolic patterns between generations. We observed, however, a clear discrimination of the three experimental treatments (LD, MD and HD larvae), which indicates that larval crowding generated physiological phenotypes that were dissimilar. This discrimination was mainly characterized by high concentrations of sugars and low amounts of amino acids at higher densities. Interestingly, the second

axis of the PCA, still accounting for 24% of the inertia, only discriminated the MD condition from the two others, with a high content of polyols (xylitol, sorbitol, glycerol phosphate) and fructose in MD larvae. All these compounds could potentially be linked to cold tolerance of larvae (Overgaard et al., 2007; Teets et al., 2012; Colinet et al., 2013; Williams et al., 2014; Košťál et al., 2016). Therefore, our metabolomic results represent further support of the hormesis effect hypothesis, as some variations were only present at intermediate larval density, hence depicting a non-gradual effect of increased crowding on metabolism. Although we can only report correlations between a metabolic profile shift and enhanced thermotolerance, our findings are similar to results from artificial diet manipulation experiments. When fruit flies were fed with reduced amounts of yeasts, an increase in carbohydrate content was observed (Simmons and Bradley, 1997; Skorupa et al., 2008; Matzkin et al., 2011; Galenza et al., 2016). Higher cold tolerance was also detected in these flies (Burger et al., 2007; Andersen et al., 2010; Sisodia and Singh, 2012), as well as in mildly raised ones (Le Bourg, 2013, 2015). Consequently, it is possible that larvae from crowded conditions had to feed on a yeast-limited diet because of competition, leading to overconsumption of carbohydrates, and resulting in stress-resistant phenotypes. At very high larval density, however, the hardening-like effect generated by crowding could be contained by the excessive nutritional stress.

Conclusions

Larval crowding is an important component of the environment in natural populations of *Drosophila* (Bubli et al., 1998). At high larval density, the amount of food available is much reduced and of poor nutritional quality. This condition hampers the ability of larvae to attain the critical body mass needed for successful pupation, thus increasing larval mortality (Bakker, 1962). Our study conclusively showed that the thermotolerance of larvae can strongly vary with larval density. To our knowledge, we have provided here the first record of increased tolerance to cold stress triggered by larval density.

Crowding is a complex stress that results from many constraints such as competition for food resources and intoxication by waste generated by larval metabolism. We found multiple phenotypical and physiological correlates pointing to non-gradual effects of crowding on larvae. We did not succeed in replicating the observation of *hsp70* upregulation in crowding, but we did find interesting patterns of expression of antioxidant genes and *uro*. Metabolic profiles were dependent on crowding intensity, and showed similar patterns to the ones described in fasting or diet alteration experiments, which could explain their convergence regarding cold tolerance. Thermotolerance effects could have also been triggered by exposure to toxic waste and, more specifically, intermediate urea supplementation without crowding improved cold tolerance of *D. melanogaster* larvae. Collectively, these results may indicate a cross-tolerance hormetic effect of mild crowding, although they might also be linked to selection of extreme larvae. Many parameters potentially linked to population density like inter-individual chemical communication (Vijendravarma et al., 2012), food consumption rate (Ireland and Turner, 2006) or even microbial community (Leonardo and Mondor, 2006), remain almost untested in this context, and could be of crucial importance in shaping the effects of crowding.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: H.Y., H.C.; Methodology: H.Y., H.C.; Formal analysis: H.Y.; Investigation: H.Y., H.C.; Writing - original draft: H.Y.; Writing - review & editing: H.Y., D.R., H.C.; Supervision: D.R., H.C.; Funding acquisition: H.C.

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Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.169342.supplemental>

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