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OPINION

Neglecting larval rearing conditions in *Drosophila melanogaster* can negatively impact research on ageing

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Abstract The developmental conditions of *Drosophila melanogaster* flies can modify the phenotypic traits of adults. However, the control of these conditions is neglected by some authors in their articles and the readers are unaware, for instance, whether flies developed in crowded cultures or fed on a new or used medium. Controlling developmental conditions allows to know precisely the viability of flies, their duration of development and sex-ratio, which can be warning signals of bad rearing conditions. As developmental conditions can modify the results of experiments on the effects of ageing it is necessary to strictly control them.

Keywords *Drosophila melanogaster* · Early life · Rearing conditions · Larval density · Viability · Duration of development

Introduction

It has been shown for decades that early-life events can have effects in adult *Drosophila melanogaster* flies, a well-known animal model used to study a plethora of phenotypes. As these events can modulate many phenotypes, one would expect that experimenters tightly control conditions of development of flies but it is not always not the case. The purpose of this article is to show that this can have negative consequences for the results of experiments, particularly those linked to the ageing process, and to convince colleagues to systematically adopt controlled developmental conditions.

Adult flies observed in any experiment have first to develop as egg, larva and pupa before the emergence of the imago. This part of the experimental method is neglected by some authors to the point they do not give details on their rearing procedures. In such conditions, the reader does not know whether flies developed in crowded cultures or not, fed on a new or used medium, or even what was the composition of the medium. Thus, it seems that some authors consider that preadult development has no effect on the trait they measure in adult flies and that their results will not be modified by these uncontrolled conditions of development. This is maybe a highly optimistic view.

The pioneering studies of Lints and Lints (1969ab) showed that, in females, increasing preimaginal density decreased mean daily fecundity, weight, thoracic size and increased duration of development and lifespan. Preimaginal density also decreased larval viability (Moya and Botella 1985; Zwaan et al. 1991) or fecundity (Lushchak et al. 2019). These results have been replicated in both sexes and may probably be

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explained at least partly by a lack of yeast, as supplementing crowded cultures with yeast decreased some effects of crowding and, conversely, removing yeast mimicked the effects of crowding in non-crowded cultures (Klepsatel et al. 2018). Urea content, a toxic waste produced by larvae, increases in crowded cultures (Henry et al. 2018), and adding urea to the feeding medium of larvae decreases viability and increases development duration like crowding does (Botella et al. 1985). Obviously, flies emerging late from crowded cultures are feeding on an old medium replete with wastes produced by the flies that emerged sooner.

These studies thus strongly indicate that preimaginal density can have major effects in adult flies and argue for strictly controlling it. Indeed, recording lifespan, adult weight or its components (Klepsatel et al. 2018), or fecundity, can lead to biased results if preimaginal environment is badly controlled. However, preimaginal density is not the only issue when preimaginal living conditions are not controlled. The parental age can also be involved because offspring viability decreases when the maternal age increases (Kern et al. 2001), and the protein-carbohydrate ratio in the feeding medium of parents can slightly modify lifespan of female offspring and the metabolic pool of offspring (Strilbytska et al. 2020).

An example of a study with poorly controlled conditions is that of Everman et al. (2018). These authors allowed 5 pairs of flies to lay eggs for 3 days in 25 by 95 mm vials, before transferring the pairs to new vials for 3 days. Experimental non-virgin flies were harvested from both sets of vials. Thus, experimental flies were coming from vials where maybe ca. 1500 eggs had been laid, a young female being able to lay more than 100 eggs a day (e.g. Lints and Lints 1969b). The first larvae had a fresh medium and the last ones a used medium with wastes of the first larvae. It cannot be excluded that these developmental differences increased the variability of adult traits.

Early-life events and adult life: a cautionary tale

One study clearly showed that neglecting developmental conditions can even strongly modify its conclusions. In a study of the DIF transcription factor (a NF-kB-like factor in the Toll innate immunity pathway), the *Dif*^{1} mutant was compared to control *cn bw* flies (Le Bourg 2011). The developmental conditions were strictly controlled in one set of experiments: 3–5 day-old parents grown in the absence of larval crowding on a fresh medium, experimental flies grown at a larval density of 25 eggs/vial, experimental flies harvested at emergence and thus virgin and of a known age. In another set, "experimental flies are coming from bottles in which flies had laid for a few days. Larval crowding is thus not controlled and high, the age of parents is unknown, and experimental flies are collected lately after the beginning of emergence, which implies that they are not virgin and their age is not precisely known" (Le Bourg 2011), similar conditions being used in the previous articles studying the *Dif*^{1} mutant (e.g. Rutschmann et al. 2000). Flies were infected with the fungus *Beauveria bassiana* and the lifespan was observed: if developed in uncontrolled conditions, infected *Dif* flies survived for 2–4 days and *cn bw* ones for 6–7 days, as reported by the authors of previous experiments (e.g. Rutschmann et al. 2000), and the lifespan of non-infected *Dif* flies was much lower than that of *cn bw* ones (ca. one vs. three weeks). However, when flies were developing under controlled conditions, the non-infected *Dif* flies lived much longer and for the same time or slightly longer than *cn bw* ones (e.g. 35 vs. 25 days) and the survival time after infection done at various ages was slightly lower in *Dif* flies than in *cn bw* ones (e.g. 11 vs. 13 days) or not different. These results show that lifespan of both infected and non-infected flies of the two strains increased when compared to uncontrolled conditions, and to a larger extent in *Dif* flies than in *cn bw* ones. Therefore, *Dif* flies were severely impaired if developing in uncontrolled conditions as these conditions decreased the lifespan of infected or non-infected adults. Thus, the effect of the DIF transcription factor is weaker than previously thought and is depending on preimaginal rearing conditions, to the extent that the non-infected mutant may outlive its control if flies developed in controlled conditions.

Controlling the rearing conditions is also a tool to check whether the developmental conditions are optimal. An unpublished study of the author compared two rearing media at 25 °C. This experiment used 5 day-old parents of the Canton-S wild-type strain and a density of 25 eggs/vial (10 vials, 250 eggs), all flies being stored in the same incubator (25 °C, Light-Dark 12:12). The viability was 88.0 % on one medium and 78.4 % on the second one, a significant but minor

difference (Chi^2 = 8.24, 1 d.f., p = 0.0041), the sex-ratio was similar (respectively, 49.5 vs. 43.9 % of females, Chi^2 = 1.34, 1 d.f., n.s.), and development was strongly delayed on the second medium (respectively, 218 flies emerging with a 9 or 10 days duration of development, and only 2 ones later on the first medium, vs. 85 and 111 flies on the second medium). Thus, using controlled conditions made possible to know that the second medium strongly delays emergence when compared to the usual 9 days at 25 °C and thus is not the best medium. This result is not so surprising, as Ormerod et al. (2017) have shown, by comparing two commercial food media, that larvae (e.g. development duration) and adult traits (e.g. body size, lifespan) were highly dependent on the larval diet. Similarly, high concentrations of glucose or fructose in the rearing medium of larvae increase the median time of pupation and decrease viability: these results would have been unknown if the authors had not strictly controlled developmental conditions (Rovenko et al. 2015).

Consequences for future research with flies

The previous studies show that, in addition to the temperature and light conditions that are kept constant, it is necessary to control the age of parents and the larval density, for instance by using a constant number of eggs in a vial. These previous studies are a warning signal: uncontrolled developmental conditions or some rearing media can be an issue for any study. For instance, if one compares the effect of treatments on lifespan, uncontrolled rearing conditions can make individual lifespan to be highly variable in each experimental group, which could be an issue to detect a significant effect of the treatment under study. A controlled egg density allows routinely measuring the duration of development, viability, and sex-ratio to check that conditions of development are optimal. An abrupt decrease of viability is a signal that something got wrong and that a careful examination of the rearing conditions is needed, the same being true if the duration of development increases. During the

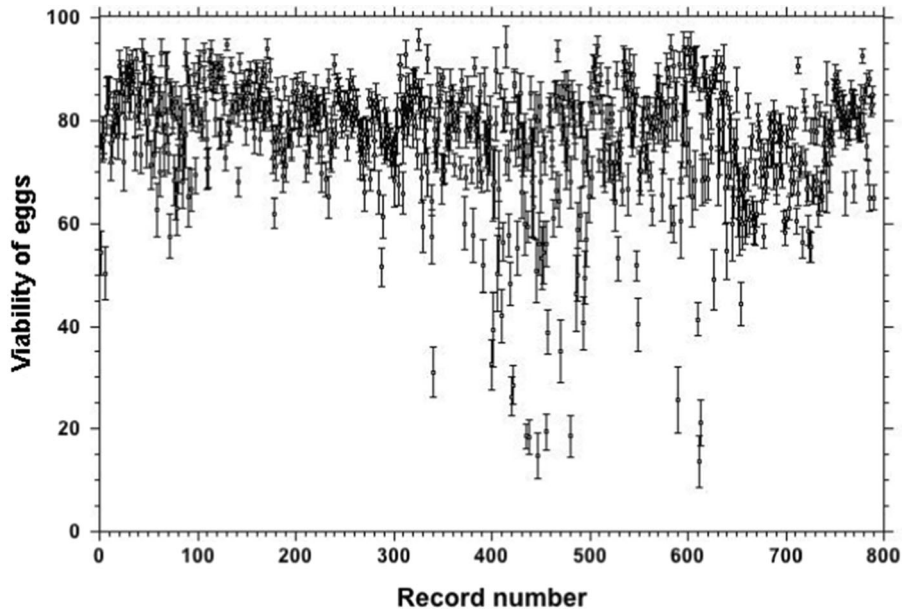

Fig. 1 Viability from 1988 to 2016 (± confidence intervals at the p = 0.05 level) of the wild-type strain Meyzieu, caught at the end of the 1970s near the city of Lyon (France). In order to obtain the parents of the experimental flies, flies were allowed to lay eggs for one night in a bottle. Flies emerging 9–10 days after egg-laying were transferred to fresh bottles: they are the parents of the experimental flies. Experimental flies were obtained as follows: eggs laid by ca. 5 day-old parents during one night on a Petri dish containing the medium coloured with charcoal and a drop of live yeast were transferred by batches of 25 into 80 ml glass vials. New flies were obtained during hundreds of weeks (record number on the figure) and the number of harvested eggs each week was in the 75–2975 range. The viability of the 550,026 eggs was 76.97 ± 0.11 % (range of weekly records: 13.71–95.71 %), and the sex-ratio of the 423,358 flies was 50.71 ± 0.15 % of females (range of weekly records : 35.95–63.30 %). The figure shows that, in some cases, viability was very low. These flies were not used in experiments, as it could be concluded that the developmental conditions, for unknown reasons, were not optimal these very weeks

experiments of the author, it happened that viability of wild-type strains was very low rather than the usual ca. 75–95 %, which could be linked for instance to a degraded live yeast and required not using these very flies (Fig. 1). The unpublished experiment using two media described above also shows that the duration of development could be strongly delayed, which is a signal for a poorly nutritious medium, even if viability and sex-ratio were barely modified.

Authors are well aware that temperature during development or adult life must be kept constant: observing, e.g., lifespan in flies whose rearing temperature would not be controlled would have no sense. However, it happens that some articles do not report the other rearing conditions of flies, or only poorly control them: these authors implicitly consider that early-life events have no importance and that the life of a fly begins at emergence. The good news is that it is very easy to control the conditions of development of flies and each experimenter can afford that, which implies that the published results are less open to criticism, and this is particularly true when studying ageing and longevity that can be modulated by time-remote factors.

In fact, it is rather strange that some authors do not consider as useful to strictly control the developmental conditions of flies at a time when many results show that, in humans, these conditions can have long-lasting effects (a review in Vaiserman et al. 2018). Osmond et al. (1993) showed that low birth weight is linked to death from cardiovascular disease in both sexes. It is thus not surprising that women suffering from the 1944 Dutch winter famine during their mid-to late gestation gave birth to low-weight babies, and, in addition, that famine during early gestation, even if birth weight was normal, was also associated with cardio-vascular disease at middle age (review in Preston et al. 2018) and death rate in women, but not in men (van Abeelen et al. 2012). On the whole, early-life events, and particularly foetal nutrition, can have long-lasting adverse consequences in humans and results in animal models are not at variance (Tarry-Adkins and Ozanne 2017). This is particularly the case in *D. melanogaster* and it is thus needed that biogerontologists pay attention to developmental conditions, whatever the animal model they use.

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