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

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

5  
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11       **Abstract**

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

19

20       **Key-words**

21       *Drosophila melanogaster* — early life — rearing conditions — larval density — viability —  
22 duration of development

23

24       **Introduction**

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

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

39       The pioneering studies of Lints and Lints (1969ab) showed that, in females, increasing  
40 preimaginal density decreased mean daily fecundity, weight, thoracic size and increased duration of  
41 development and lifespan. Preimaginal density also decreased larval viability (Moya and Botella  
42 1985; Zwaan et al. 1991) or fecundity (Lushchak et al. 2019). These results have been replicated in  
43 both sexes and may probably be explained at least partly by a lack of yeast, as supplementing  
44 crowded cultures with yeast decreased some effects of crowding and, conversely, removing yeast

45 mimicked the effects of crowding in non-crowded cultures (Klepsatel et al. 2018). Urea content, a  
46 toxic waste produced by larvae, increases in crowded cultures (Henry et al. 2018), and adding urea  
47 to the feeding medium of larvae decreases viability and increases development duration like  
48 crowding does (Botella et al. 1985). Obviously, flies emerging late from crowded cultures are  
49 feeding on an old medium replete with wastes produced by the flies that emerged sooner.

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

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

65

### 66 **Early-life events and adult life: a cautionary tale**

67 One study clearly showed that neglecting developmental conditions can even strongly modify  
68 its conclusions. In a study of the DIF transcription factor (a NF- $\kappa$ B-like factor in the Toll innate  
69 immunity pathway), the *Dif<sup>f</sup>* mutant was compared to control *cn bw* flies (Le Bourg 2011). The  
70 developmental conditions were strictly controlled in one set of experiments: 3-5 day-old parents  
71 grown in the absence of larval crowding on a fresh medium, experimental flies grown at a larval  
72 density of 25 eggs/vial, experimental flies harvested at emergence and thus virgin and of a known  
73 age. In another set, “experimental flies are coming from bottles in which flies had laid for a few  
74 days. Larval crowding is thus not controlled and high, the age of parents is unknown, and  
75 experimental flies are collected lately after the beginning of emergence, which implies that they are  
76 not virgin and their age is not precisely known” (Le Bourg 2011), similar conditions being used in  
77 the previous articles studying the *Dif<sup>f</sup>* mutant (e.g. Rutschmann et al. 2000). Flies were infected with  
78 the fungus *Beauveria bassiana* and the lifespan was observed: if developed in uncontrolled

79 conditions, infected *Dif* flies survived for 2-4 days and *cn bw* ones for 6-7 days, as reported by the  
80 authors of previous experiments (e.g. Rutschmann et al. 2000), and the lifespan of non-infected *Dif*  
81 flies was much lower than that of *cn bw* ones (ca one vs three weeks). However, when flies were  
82 developing under controlled conditions, the non-infected *Dif* flies lived much longer and for the  
83 same time or slightly longer than *cn bw* ones (e.g. 35 vs 25 days) and the survival time after  
84 infection done at various ages was slightly lower in *Dif* flies than in *cn bw* ones (e.g. 11 vs 13 days)  
85 or not different. These results show that lifespan of both infected and non-infected flies of the two  
86 strains increased when compared to uncontrolled conditions, and to a larger extent in *Dif* flies than  
87 in *cn bw* ones. Therefore, *Dif* flies were severely impaired if developing in uncontrolled conditions  
88 as these conditions decreased the lifespan of infected or non-infected adults. Thus, the effect of the  
89 DIF transcription factor is weaker than previously thought and is depending on preimaginal rearing  
90 conditions, to the extent that the non-infected mutant may outlive its control if flies developed in  
91 controlled conditions.

92 Controlling the rearing conditions is also a tool to check whether the developmental  
93 conditions are optimal. An unpublished study of the author compared two rearing media at 25°C.  
94 This experiment used 5 day-old parents of the Canton-S wild-type strain and a density of 25  
95 eggs/vial (10 vials, 250 eggs), all flies being stored in the same incubator (25°C, Light-Dark 12:12).  
96 The viability was 88.0% on one medium and 78.4% on the second one, a significant but minor  
97 difference ( $\text{Chi}^2 = 8.24$ , 1 d.f.,  $p = 0.0041$ ), the sex-ratio was similar (respectively, 49.5 vs 43.9% of  
98 females,  $\text{Chi}^2 = 1.34$ , 1 d.f., n.s.), and development was strongly delayed on the second medium  
99 (respectively, 218 flies emerging with a 9 or 10 days duration of development, and only 2 ones later  
100 on the first medium, vs 85 and 111 flies on the second medium). Thus, using controlled conditions  
101 made possible to know that the second medium strongly delays emergence when compared to the  
102 usual 9 days at 25°C and thus is not the best medium. This result is not so surprising, as Ormerod et  
103 al. (2017) have shown, by comparing two commercial food media, that larvae (e.g. development  
104 duration) and adult traits (e.g. body size, lifespan) were highly dependent on the larval diet.  
105 Similarly, high concentrations of glucose or fructose in the rearing medium of larvae increase the  
106 median time of pupation and decrease viability: these results would have been unknown if the  
107 authors had not strictly controlled developmental conditions (Rovenko et al. 2015).

108

### 109 **Consequences for future research with flies**

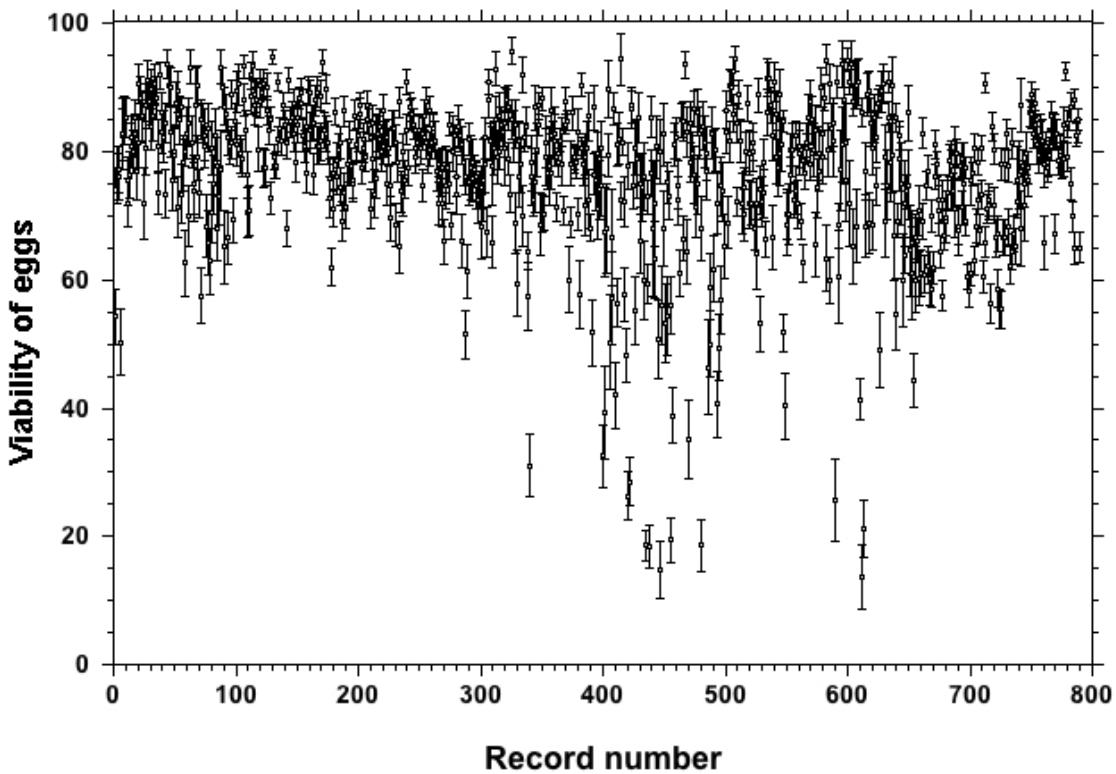
110 The previous studies show that, in addition to the temperature and light conditions that are  
111 kept constant, it is necessary to control the age of parents and the larval density, for instance by  
112 using a constant number of eggs in a vial. These previous studies are a warning signal: uncontrolled

113 developmental conditions or some rearing media can be an issue for any study. For instance, if one  
114 compares the effect of treatments on lifespan, uncontrolled rearing conditions can make individual  
115 lifespan to be highly variable in each experimental group, which could be an issue to detect a  
116 significant effect of the treatment under study. A controlled egg density allows routinely measuring  
117 the duration of development, viability, and sex-ratio to check that conditions of development are  
118 optimal. An abrupt decrease of viability is a signal that something got wrong and that a careful  
119 examination of the rearing conditions is needed, the same being true if the duration of development  
120 increases. During the experiments of the author, it happened that viability of wild-type strains was  
121 very low rather than the usual ca 75-95%, which could be linked for instance to a degraded live  
122 yeast and required not using these very flies (Figure 1). The unpublished experiment using two  
123 media described above also shows that the duration of development could be strongly delayed,  
124 which is a signal for a poorly nutritious medium, even if viability and sex-ratio were barely  
125 modified.

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

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

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



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