The analysis of several factors influencing *Drosophila melanogaster* embryo laying rates

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Abstract. *Drosophila melanogaster* is an essential model organism due to features such as a short life cycle, high reproductive rate, low maintenance costs and well-characterized genetics. We evaluated the oviposition and the number of laid embryos in three experimental setups, finding significant differences based on the genetic background, culture medium composition and female ages. In the first experiment, we compared two standard strains (Oregon R and Canton S) with two mutant lines ($As^{m1.5\text{-}R}$ and w1118). The results showed that the standard strains have statistically significant superior prolificacy compared to the mutant lines, most probably because of several locomotion impairments experienced by the latter category. The second experimental setup assessed oviposition on three different media formulations with grape (standard), orange or blueberry juice addition. We observed significantly more embryos laid on orange juice medium, a feature that can be related to evolutionary preferences of *D. melanogaster*. In addition, we showed that very young females laid significantly more embryos compared to older females, indicating a rapidly evolving age-related decline in egg laying rates during midday. This study provides insights into the interactions between genetics, specific phenotypic traits, nutritional preferences and the age of *D. melanogaster*, reinforcing the importance of this model for understanding its ecological adaptability and reproductive success.
Key Words: age, *Drosophila melanogaster*, egg laying, environment, fruit fly.

Introduction. For more than a century, *D. melanogaster* has been a fundamental experimental platform for life sciences due to its unique characteristics, including short life cycle, small genome size, well-characterized genetics, and ease of manipulation in the laboratory (Fernández-Moreno et al 2007). The use of *D. melanogaster* model covers fields such as genetics, developmental biology and biomedicine, providing valuable insights into fundamental biological processes (Eickelberg et al 2022).

Over 77% of the human genes associated with human pathologies have orthologs in *D. melanogaster* (Reiter et al 2001). Among them, several conserved genes and metabolic signaling pathways are relevant to the study of human disease (Pandey & Nichols 2011). In addition, this experimental model is very effective for testing personalized nutritional strategies prior to their application in human clinical trials (Ruden et al 2009).

Another field of interest is represented by oviposition behavior, a direct indicator of the reproductive success and ecological adaptability of *D. melanogaster*. The females are recognized for their remarkable selectivity of oviposition sites, an adaptive behavior that optimizes offspring survival and development. In the absence of suitable substrate, they may withhold eggs until conditions become favorable (Yang et al 2008). Studies have shown that although there is a preference for substrates of the genus *Citrus*, the females are actually able to use a diverse range of fruit hosts for oviposition, demonstrating significant ecological flexibility (Dweck et al 2013). In nature, oviposition sites often include fermenting fruit, where factors such as the presence of acetic acid act as strong olfactory signals, suggesting the presence of an environment conducive to larval development (Higa & Fuyama 1993; Joseph et al 2009).

Oviposition in insects involves a series of complex behavioral and sensory steps in which females evaluate the quality of the available substrate. The relevant signals include chemical and physical feedback, which contribute to the final decision of females to lay eggs (Verschut et al 2017). In the case of *D. melanogaster*, oviposition provides a simple, but highly informative method to assess environmental preferences, as each laid egg marks a specific behavioral choice. Previous studies have used this behavior to identify conditions favorable to offspring development, as well as to distinguish the ecological preferences of different species or populations of *Drosophila* (Richmond & Gerking 1979; Chess & Ringo 1985; Amlou et al 1998).

In addition to substrate preferences, the genetic background differences between *D. melanogaster* populations or strains significantly influence reproductive behavior and prolificacy. This feature provides an essential framework for investigating how interactions between genetics and environment affect reproductive success. Despite the abundance of studies using oviposition site selection as a behavioral measure, the analysis of the sensory circuits involved in this process and how the oviposition schedule varies among genetic lines has been systematically investigated only relatively recently (Yang et al 2008).

The present study aims to analyze the influence of environment and genetic background over oviposition and number of laid embryos through rigorous experimental design and comparative analysis methodologies. This knowledge can contribute to a deeper understanding of the mechanisms that regulate oviposition substrate selection, a prerequisite for evaluating the factors that determine reproductive success under various ecological conditions.

Material and Method

D. melanogaster strains. To test the *D. melanogaster* prolificacy, two standard strains (Oregon R and Canton S) and two mutant lines ($As^{m1.5\text{-}R}$ and w1118) were used. These lines were provided by the *Drosophila* Laboratory, Faculty of Biology, University of Bucharest, which arguably possesses the only continuously perpetuated collection of *D. melanogaster* strains from Romania. Before being subjected to experimental conditions, the flies were maintained on standard banana-yeast-sugar-propionic acid culture medium (Ratiu et al 2025).

Experimental design. In order to assess the efficiency of embryo laying, we performed a series of experiments, each designed to focus on specific factors that could influence this process. Each experiment took place during the day after the adaptation period described below, between 12:00 and 14:00 PM and the embryos laid on the surface of the experimental plates were rigorously counted.

For the first experiment, we collected groups of 200 virgin individuals from each of Oregon R, Canton S, $As^{m1.5\text{-}R}$ and w1118 lines, a single group comprising 120 females and 80 males. Of note, the $As^{m1.5\text{-}R}$ females included both heterozygous and homozygous individuals harboring one or two, respectively, $As^{m1.5\text{-}R}$ alleles. The selection and sexing of the flies were achieved using an Olympus SZ51 stereo microscope and, consecutively, we placed the gathered groups on standard rearing medium. After the collection and before running the actual experiment, we implemented an adaptation period of 72 h at 25°C, 65% humidity and a photoperiod of 12 light (L):12 dark (D). After this initial adaptation stage, the flies were transferred to specially designed egg-collection cages for *Drosophila* (Genesee Scientific, cat. no. 59-101), under CO_2 anesthesia. Here, the flies were subjected for 24 h to a second period of adaptation to the new environment and the Grape-Agar collection mixture (Scientific Labs, cat. no. FLY1082). For stimulating egg laying, we added yeast paste to the center of the medium. In order to evaluate the egg laying proficiency, we placed the approximatively four-day old flies on new Petri dishes (with Grape-Agar mixture and freshly prepared yeast paste) every 40 min until three Petri dishes were available for each experimental group.

For the second experiment we used three batches of Oregon R flies, each consisting of 120 females and 80 males. The individuals were sexed using CO_2 anesthesia, a method that does not affect fly viability. By using a light microscope, the specific phenotypic traits of males and females can be easily scored and thus facilitating their proper selection.

The three batches were individually placed on distinct types of embryo collection media: the Grape-Agar collection medium (control group), and two experimental formulations containing either orange or blueberry juice. The experimental media were prepared according to an original recipe, as follows: 100 mL fresh orange/blueberry juice, 6 g agar and 0.6 g sucrose were mixed and heated to 80°C with continuous stirring following cooling to 60°C and then reheating to 80°C, before being poured into sterilized Petri dishes.

Similarly to the first experiment, before the actual evaluations, the virgin flies were subjected to the previously established adaptation periods. Yeast paste was used to stimulate collection on all embryo collection media. During the experimental assessing of laying proficiency of the four-day old females, the Petri dishes with different media were replaced after each 40 minutes intervals, until a total of three plates was available.

To establish whether there are differences between flies of different ages concerning the number of laid embryos, two batches of 120 females and 80 males each were analyzed. The Oregon R virgin individuals forming the first batch of so-called young flies were kept for the first adaptation period a total of two days on standard medium, then placed for another 24 h in the egg-laying cages on Grape-Agar collection medium. The virgin flies making the second batch were subjected to a six days first adaptation period, then placed for a second 24 h long accommodation in cages and on collection medium, forming the adult flies group. Thus, the embryo laying evaluations were performed for three and, respectively, seven days old females and consisted of three series of embryo deposition every 40 minutes.

Statistical analyses. Statistical analyses and graphical visualizations were performed with GraphPad Prism 8.4.2 (GraphPad Prism version 8.4.2 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com). The normality of data was evaluated with Shapiro-Wilk test. Differences between data groups were assessed by using either one sample or unpaired t tests. The actual mean values are presented as mean ± SD (standard deviation), whilst for the graphical representations we showed the mean ± SEM (standard error of the mean). The level of significance was set at $\alpha=0.05$.

Results and Discussion. The present study examines egg/embryo laying efficiency and preferences of *D. melanogaster* females pertaining to four laboratory strains, each having a specific genotype. Oregon R and Canton S lines are frequently used as reference or control groups in a variety of studies that involve both behavioral and molecular biology experiments. Oregon R is a wild-type strain believed to have a longer life span than wild-type Canton S (Ganetzky & Flanagan 1978). The Canton S strain was originally established by Calvin Bridges in the 1920s using wild flies collected from the Canton, Ohio region (Stern & Schaeffer 1943). Later, this strain was widely adopted as a control strain in biological studies (Benzer 1967). The $As^{m1.5\text{-}R}$ line was obtained consecutive to P{EP} transposon-mediated mutagenesis and the specific mutant allele, symbolized $As^{m1.5\text{-}R}$, is characterized by retaining an approximately 200 nucleotide-long transposon remnant within the 5'UTR region of the *Ube3a* gene (Ratiu et al 2015). Consequentially, the majority of mutant homozygous males fail to reach adulthood developmental stages and the few imago escapers have significant mobility impairments. On the other hand, the females harboring two mutant alleles are fully viable, but do exhibit minor mobility decline (Ratiu et al 2015). The w1118 line is routinely used as a white eye marker parental stock and occasionally used as in the control group in certain experiments. The w1118 line originates from the Oregon R wild strain and has a partial deletion in the *w* gene, which causes white eyes (Hazelrigg et al 1984). The *w* gene, located on the X chromosome of *D. melanogaster*, encodes a member of the ATP-binding cassette transporter superfamily (Qiu et al 2017). Relatively recently, a growing number of evidences showed that the w1118 mutants undergo problematic walking and climbing behavior, low stress resistance, shortened life span, and retinal degeneration (Ferreiro et al 2018; Qiu & Xiao 2018).

Thus, we consider of practical interest to investigate the impact of a specific genotype over the egg laying rate, a feature that could have an effect on the viability of distinctive *Drosophila* strains, as well as on a great variety of experimental studies. In addition, we tested both the egg laying stimulus of three different fruit juices supplemented to collection media, and the egg laying efficiency according to female age. Our results can be summed as a practical guide for efficient practice in the context of experimental designs involving the eggs/embryos collection from *D. melanogaster* specific females.

The power of a statistical test is the probability of detecting a given effect using a given test in a given context. The selection of the appropriate statistics for analyzing given data sets can be challenging, especially when the respective sets contain few actual values. Since experimental endeavors cannot gather exhaustive information regarding the population of values specific to a certain variable, at least two main challenges have to be addressed. Firstly, are there sufficient data to establish if the variable has a normal distribution? And secondly, have the standard deviations (SDs) of the considered populations comparable values?

In our study, the sought after variable was the number of embryos laid by groups of 120 females. From a biological perspective, we consider that for a particular genotype this variable should have a normal distribution. Nonetheless, we tested this by using the Shapiro-Wilk statistics and the obtained p values indicated that the four data sets pass the normality test. At the same time, there are no sufficient background data to establish if the SD statistics have similar or identical values between two distinct female genotypes. Therefore, because there are no SD prerequisites we opted for running one sample t tests in order to assess the embryo laying performances of Canton S, $As^{m1.5\text{-}R}$ and w1118 females compared to the mean performance scored for Oregon R. Since the other two experiments involved exclusively the use of Oregon R female groups that are expected to have identical or highly similar SD values, the differences between them were evaluated by using unpaired t tests.

To evaluate the egg laying efficiency of the four considered *D. melanogaster* lines, three grape-based collection plates or replicates were gathered from each female group. In accordance, a total number of 482 embryos were collected and grouped according to their parental lineage (Figure 1).

A noticeably higher number of embryos was obtained from Oregon R and Canton S females, with means of 82±38.31 and 59±38.20, respectively. In contrast, the $As^{m1.5\text{-}R}$ and w1118 females exhibited lower prolificacy, with means of 8±1.73 and 11.67±11.24, respectively. In these categories, the maximum number of 35 embryos was obtained for the w1118 line, but, curiously, the same line also provided the minimum number of two embryos. To establish the statistical significance of these results, we performed a series of one sample t tests, considering both the mean values of Oregon R and Canton S as hypothetical values. The results are summarized in Table 1. There are significant differences between the results obtained for Oregon R or Canton S and the two mutant lines, $As^{m1.5\text{-}R}$ and w1118. The embryo laying performances do not significantly differ between Oregon R and Canton S, as well as between $As^{m1.5\text{-}R}$ and w1118.

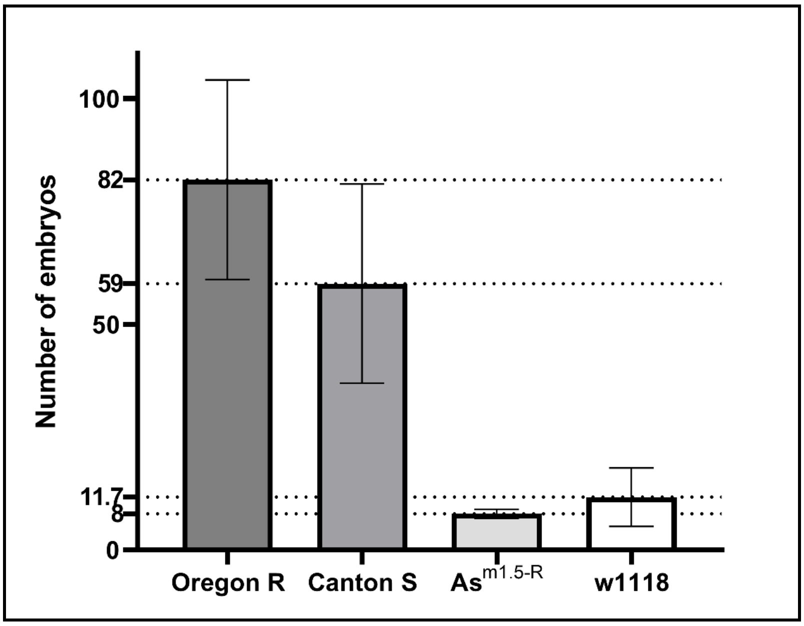

Figure 1. The number of *Drosophila melanogaster* embryos collected on three grape-agar plates from each of the Oregon R (82), Canton S (59), As$^{m1.5-R}$ (8) and w1118 (11.7) female groups; the bar plots show the mean embryo number, while the error bars stand for the SEM.

Table 1

P values generated with one sample t tests when comparing Oregon R, Canton S and Asm1.5-R means against the replicate values of the remaining three lines, the mutant lines and w1118

Genotypes	*Oregon R*	*Canton S*	*As$^{m1.5-R}$*	*W1118*
Oregon R	>0.9999			
Canton S	0.4065	>0.9999		
As$^{m1.5-R}$	0.0002	0.0004	>0.9999	
W1118	0.0084	0.0183	0.6290	>0.9999

The results of this experiment are consistent with previous reports of Oregon R increased prolificacy. For example, the study by Godoy-Herrera (1986) revealed that this strain has a superior reproductive capacity. Similarly, Markow (1987) pointed out that wild-type lines, such as Oregon R, tend to produce a higher number of eggs compared to other lines. Similarly, Canton S line is frequently used as a standard line in multiple experimental setups. The egg laying proficiency of the Canton S females was previously evaluated in fully fertile females (8-10 days) at around 40 eggs/female/24 hours (Lylyk et al 2018), which is somehow lower than other results concerning Oregon R, where the similar age fertility was estimated at a mean of 60 eggs/female/24 hours (Boulétreau-Merle 1974). We noticed a similar trend, where the egg laying performances of Canton S are lower than that of Oregon R, although the differences are not statistically significant. It is worth mentioning that behavioral traits can vary between sub-strains of *D. melanogaster* lines. A collection of five sub-strains derived from the same laboratory population of Canton S exhibited markedly different locomotor behavior when the individuals were tested in the so-called Buridan’s paradigm (Colomb & Brembs 2014). This test consists in a multiparameter evaluation of the trajectory of the flies within a specially designed platform, therefore is amenable to principal components analysis. The relevance to our study is that although the results presented here are representative for the specific tested genotypes, care should be taken if aiming to generally extrapolate them to any of the considered line, especially the standard ones, Oregon R, Canton S and w1118, which are maintained in multiple copies in laboratories around the world.

Furthermore, our data support the observations of Krstic et al (2013) showing that, in the presence of natural light and receptive virgin females, w1118 males show reduced sexual arousal compared to Oregon R males. In addition, the *w* gene mutation particular to w1118 line may influence male courtship (Zhang & Odenwald 1995). These aspects suggest that the Oregon R strain not only has a higher prolificacy, but also a more sexually active behavior, which may contribute to its reproductive success (Krstic et al 2013). Studies focusing on Canton S and w1118 found that there are noticeable behavioral and physiological differences between them, especially when it comes to locomotor performances, lifespan and copulation success (Qiu et al 2017; Xiao et al 2017). The latter trait is negatively influenced by mutant *w* alleles and could impact the egg laying processes. Thus, regardless of the apparent absence of other mutations, various phenotypic traits are impaired in *w* mutant backgrounds and this could lead to affected courtship behavior and drops in egg laying. In the respective females and males, the $As^{m1.5\text{-}R}$ mutation that affects locomotion and dendritic branching development (Wu et al 2008; Lu et al 2009; Ratiu et al 2015) is coupled with a *w* null allele, the individuals having white eyes. Therefore, regardless if a female is heterozygous or homozygous for $As^{m1.5\text{-}R}$ allele, it is surely homozygous for the *w* mutant allele. This condition may well lead to more severe behavioral outcomes than those produced by either of the mutations alone. In our experiment, the $As^{m1.5\text{-}R}$ females produced the lowest number of embryos, but the respective mean value did not significantly differ from that particular to w1118.

Overall, we confirmed the higher prolificity of Oregon R and Canton S in comparison with the standard w1118 line, but also with another mutant line harboring mutant alleles that affect locomotor behavior. Although not specifically tested, this first experiment adds support to the hypothesis that mutant phenotypes affecting locomotion may also impact courtship and copulation, prerequisites for efficient egg laying. Also, since the experiments were conducted during day-time and not at dusk, when the egg depositions are maximal (Ashburner et al 2005), the obtained values might not be representative for the whole embryo laying potential of the tested lines.

The Oregon R females provided the best embryos collection. Thus, we decided to use this category only for the following experiments involving media organoleptic properties preferences and age-related prolificity.

We appraised the efficiency of egg laying on a standard collection medium formula based on grape extract versus two different collection media with added orange and, respectively, blueberry juice. The results showed that the mean embryo deposits of 234.7±34.65 on orange supplemented medium exceeds those counted for grape and blueberry complemented media, which provided values of 129±28.58 and 142.3±38.59, respectively (Figure 2). The results obtained for the orange juice-based medium are significantly superior to the performances attained when using either the grape-based standard ($p=0.0152$) or blueberry-based ($p=0.0368$) media. There are no significant differences when grape vs blueberry supplemented media are compared ($p=0.658$).

The highest number of embryos collected was obtained using orange supplementation, being consistent with the results obtained by Dweck et al (2013), which showed that *D. melanogaster* chose oranges as an oviposition substrate in favor of 15 other tested fruits. The total number of embryos recovered from orange juice medium was approximately 82% and, respectively, 65% higher than the number of embryos collected on the standard grape or blueberry formula. This preference suggests an evolutionary imprinting, due to the fact that *D. melanogaster* likely had a close association with specific fruits having similar characteristics to citrus (Mansourian et al 2018). Actually, *D. melanogaster* was previously noted to naturally prefer citrus-like fruits as a substrate for embryo laying, as these fruits have a thick epicarp that provides protection against parasites, thus ensuring their safe reproduction (Fleury et al 2004). Although the number of gathered embryos on the grape-based medium had the lowest value, it still remains a reliable solution for embryo collection endeavors, as confirmed by its use in numerous studies (Yang et al 2008; Becher et al 2012; Linford et al 2013; Hailstock et al 2023). If a compromise between egg laying efficiency and ease of visualization is desired, then using the blueberry medium variant could be a solid alternative.

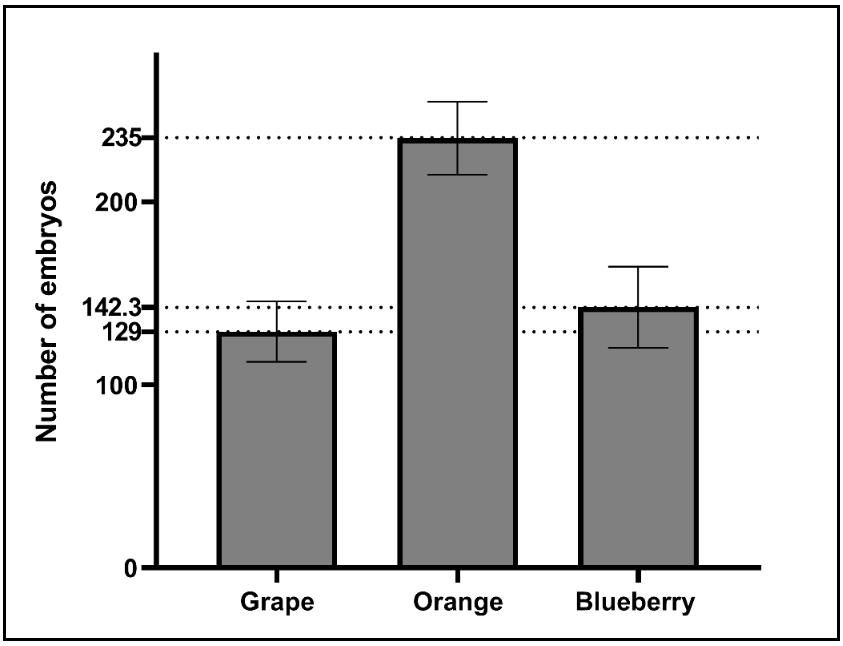

Figure 2. The number of *Drosophila melanogaster* embryos counted from the three grape-agar plates of each of the grape (129), orange (≈235), and blueberry (142.3) supplemented media; the bar plots show the mean embryo number, while the error bars stand for the SEM.

Considering that virgin females are commonly used in experiments involving *D. melanogaster*, we also wanted to test the egg laying proficiency of very young vs relatively young (old) individuals on grape-agar standard medium. The results are presented in Figure 3 and, to our surprise, they show that the three-day old females laid a mean number of 115±15.72 embryos that is statistically significantly higher ($p=0.0102$) than the mean of 62.33±12.22 embryos obtained for the seven-day old ones.

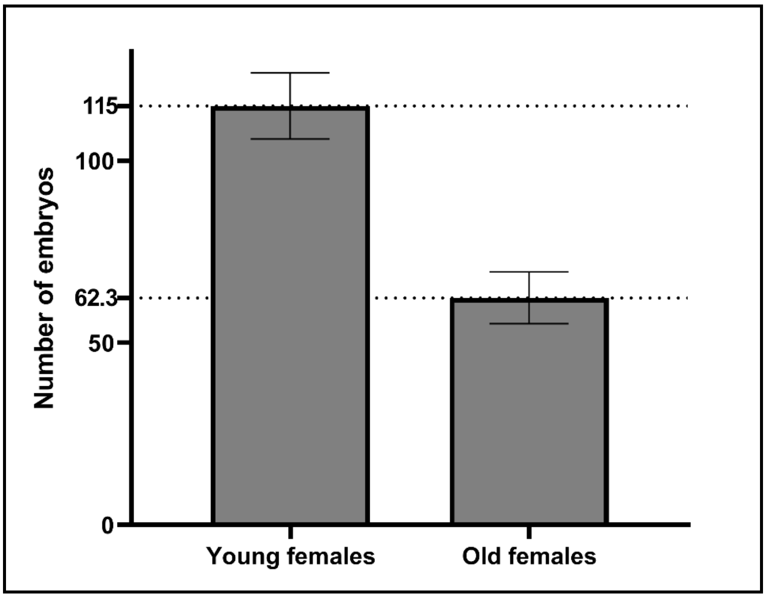

Figure 3. The number of *Drosophila melanogaster* embryos laid by the three-day old (young) and the seven-day old (old) Oregon S females on grape-agar plates; the bar plots show the mean embryo numbers (115 and, respectively, 62.3), while the error bars stand for the SEM.

The laying efficiency of *D. melanogaster* females during their first 10 days of life should not dramatically differ, although there are reports of an approximatively 15% decline of total eggs laid/day when comparing three to four days-old females with eight days-old ones (Ashburner et al 2005). In our study, it is possible that the almost two-fold difference in favor of young females is a consequence of gathering data during the middle of the day, but supplementary information is needed in order to test such a hypothesis. Then again, the females that mate once with a male experience a severe decline in fertility and, consequently, egg laying performance about eight days post-fertilization if no sperm supplementation is allowed (Kaufman & Demerec 1942). Thus, in spite of the presence of males within the collection cages, the females could exhibit strong repulsive behavior towards subsequent male courtship (Kubli 2003; Yang et al 2009), a trait that, coupled with fertility decrease, could at least partially explain the embryo laying differences between "young" and "old" females. In addition, it is known that usually the older males have a reduced mating capacity and therefore induce weaker reproductive responses in females, which in turn may negatively affect fertilization and egg laying (Ruhmann et al 2018). *D. melanogaster* females experience a progressive decrease in fecundity as they age, a phenomenon also highlighted in other studies that reported a reduction in the number of eggs laid in older individuals (Xie & Spradling 2000; Zhao et al 2008).

General observations in iteroparous insects show that the reproductive potential declines with age, leading to a significant reduction in the number of offspring (David et al 1975). In the case of *D. melanogaster*, Mossman et al (2019) demonstrated that older individuals produce fewer offspring, which can be attributed to both a general physiological decline and a reduced efficiency of the mating and oviposition process.

Conclusions. Genetic differences between *D. melanogaster* lineages played a critical role in determining embryonic prolificacy. The Oregon R line was distinguished by superior reproductive behavior, indicating a genetic advantage for reproduction and thus being the better choice for tackling embryo development studies. Substrate composition significantly influenced oviposition, with the orange juice medium proving to be the most effective, whilst blueberry juice supplemented and the grape-agar standard deposition medium were less attractive, but still provided useful number of embryos. The preference of *D. melanogaster* for natural and fermented substrates highlights the ecological adaptability of the species. These findings suggest that an orange juice-based medium is a suitable choice in order to obtain a high number of embryos in a short period. Regarding age, younger flies showed higher embryonic prolificacy, most probable due to a combination of post-mating behavior and age-related fertility decline in both females and males. The results highlight the importance of genetic, age and nutritional preferences interactions in optimizing the reproductive success of *D. melanogaster*, which may have applications in biomedical and ecological research.

Conflict of Interest. The authors declare that there is no conflict of interest.

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