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# Parental age influences developmental stability of the progeny in *Drosophila*

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The stochastic nature of biochemical processes is a source of variability that influences developmental stability. Developmental instability (DI) is often estimated through fluctuating asymmetry (FA), a parameter that deals with within-individual variation in bilateral structures. A relevant goal is to shed light on how environment, physiology and genotype relate to DI, thus providing a more comprehensive view of organismal development. Using *Drosophila melanogaster* isogenic lines, we investigated the effect of parental age, parental diet and offspring heterozygosity on DI. In this work, we have uncovered a clear relationship between parental age and offspring asymmetry. We show that asymmetry of the progeny increases concomitantly with parental age. Moreover, we demonstrate that enriching the diet of parents mitigates the effect of age on offspring symmetry. We show as well that increasing the heterozygosity of the progeny eliminates the effect of parental age on offspring symmetry. Taken together, our results suggest that diet, genotype and age of the parents interact to determine offspring DI in wild populations. These findings provide us with an avenue to understand the mechanisms underlying DI.

## 1. Introduction

Organisms are exposed to a changing environment during development. It is known that environmental fluctuations affect biochemical reactions [1] and morphogenetic processes [2], a fact that could, in theory, generate a considerable amount of phenotypic variation or facilitate the appearance of aberrant phenotypes. However, phenotypic variation within populations is limited, and, in general, individuals do not deviate much from a norm. Bearing this in mind, it has been hypothesized that species have buffering mechanisms that canalize [3] the phenotype in the face of environmental variation. These mechanisms should be the active players that provide developmental robustness. In effect, molecular mechanisms that canalize development exist, and have been described recently [4–6]. These mechanisms can also function in genetic canalization, protecting developmental processes from the impact of genetic mutations or negative epistatic interactions.

Under laboratory conditions, it is feasible to measure phenotypic variation among genotypically identical individuals, grown under the same environmental conditions. Even in these controlled conditions (same environment and same genotype), it is possible to find substantial phenotypic variation both between and within individuals (differences in replicated bilateral structures). In this case, the causes of variation cannot be attributed to fluctuations in the external environment and/or differences in genetic constitution. The source of this phenotypic variation is thought to be the stochastic nature of developmental processes, a phenomenon known as ‘developmental noise’ [7,8]. It is also common to use the term ‘developmental instability’ (DI) when referring to noise in developmental processes. Most often, DI is estimated through fluctuating asymmetry (FA), a parameter that deals with within-individual variation in bilateral organisms [9]. FA is calculated by measuring differences in morphology between sides (left versus right) in a group of individuals. The rationale behind FA is simple: left and right structures of a bilateral individual are generated by the same genetic

system that interacts with the same environment and, therefore, the remaining sources of variation have to be related to the stochasticity associated with developmental processes.

Shedding light on the processes that influence DI would allow us to have a more comprehensive view of organismal development. If we are to understand the mechanistic underpinnings of DI we first need to determine which (and to what extent) environmental, physiological and genetic factors affect DI. Inevitably, we have to think about the internal environment of the organism when discussing DI. For example, differences in the genetic make-up of populations could affect the levels of FA: diverse allelic combinations might make development more or less stable. In addition, we have to consider that variables of the external environment might modify the internal environment of the organism, thereby affecting the stability of development. For example, genotypically identical organisms grown under different temperatures might exhibit different levels of FA [10].

Although a vast number of studies have examined the effect of various factors on developmental stability [11–13], it would be relevant to identify additional variables that may impact on DI. Moreover, it would be important to elucidate the role of factors with equivocal effects. For instance, it is still debated whether there is a connection between heterozygosity and DI [14,15]. Nevertheless, there is evidence that links parasitic infection [16] and environmental pollution [17] with DI.

To better understand what variables influence DI, here we investigate the effect of parental age on the developmental stability of the descendants. In this work, we have uncovered a clear relationship between parental age and offspring FA in three isogenic lines of *Drosophila melanogaster*. We show as well that parental diet and offspring heterozygosity modulate the effect of parental age on offspring FA. The identification of factors that influence FA stimulates further experiments that might bring us closer to the mechanisms underlying DI.

## 2. Material and methods

### (a) Fly strains

We used lines 335 and 852 from the *D. melanogaster* Genetic Reference Panel [18] and a third line named L25 (kindly provided by J. J. Fanara). The first two lines derive from a Raleigh, North Carolina (USA) population, and the latter stems from a population of Lavelle, Mendoza (Argentina). These lines were constructed by collecting mated females from the population of origin, followed by 20 generations of full-sibling crosses, thus achieving approximately 99% homozygosity [19].

### (b) Collection and ageing of the parental generation

Fly lines were expanded in flasks with cornmeal–molasses–yeast medium. Three hundred virgin females and two hundred virgin males (2 day old) were collected and transferred to 11 plastic egg-lay cages that accommodate a standard Petri dish (Ø90 mm). The Petri dish contained grape agar (the egg-laying substrate) and an excess of yeast paste (the food source). Every day, we changed the dish for each cage. At the chosen time-points (see below), eggs were collected from the dishes. In one of the experiments, we enriched the diet of ageing adults by adding cornmeal–molasses–yeast medium in excess to the dishes. In order to investigate the contribution of each sex to the age-effect, females and males were aged separately in 11 cages and after 28 days, 40 virgin females or 20 virgin males were mated with young flies

of the opposite sex (40 virgin females or 20 virgin males). We also crossed old males with old females and young males with young females for this experiment. Cages were kept in a dark incubator at  $25 \pm 0.5^\circ\text{C}$ .

### (c) Embryo collection and development of the progeny

Embryos were collected at different parental ages. For each treatment, we collected 60–100 embryos that were transferred to vials containing cornmeal–molasses–yeast medium. In order to avoid crowding conditions during growth, we placed 15 embryos per vial. Vials were immediately transferred to a dark incubator at  $25 \pm 0.5^\circ\text{C}$ . After eclosion, adults were maintained in 70% ethanol and stored until use.

### (d) Morphological measurements

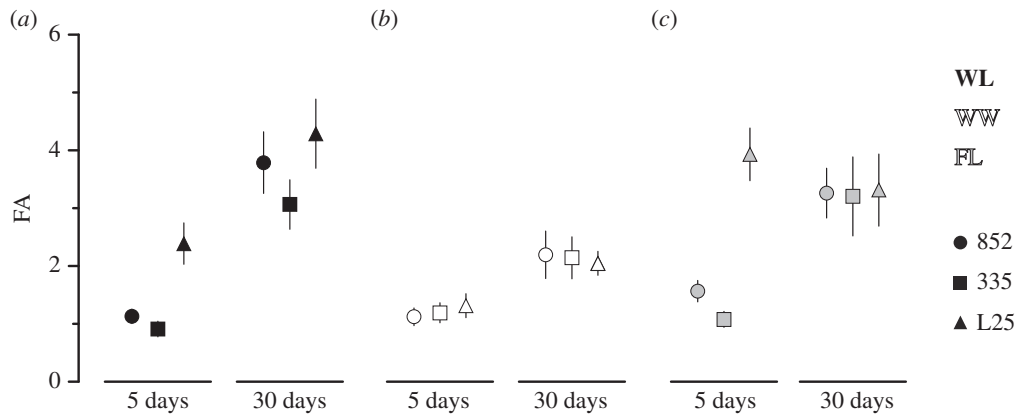
Both wings and T2 legs were dissected and mounted on a microscope slide using Hoyer's medium and covered with a coverslip. Images were captured with a Retiga-2000R camera (QImaging) attached to an Eclipse E200 microscope (Nikon). Measurements were made in pixels using tpsDIG1 (<http://life.bio.sunysb.edu/morph/>). We estimated wing length (WL), wing width (WW) and femur length (FL) in the right and left wing/leg of each individual as described [20,21]. WL was calculated as the distance from the intersection of veins L2 and L3 to the intersection of vein L3 and the wing margin (see the electronic supplementary material, figure S1). WW was calculated as the distance from the intersection of vein L2 and the wing margin to the intersection of vein L5 and the wing margin (see the electronic supplementary material, figure S1). FL was estimated as the distance between the anterior ventral campaniform sensilla and the distal tip of the femur (see the electronic supplementary material, figure S1). We made two measurements per side for WL, WW and FL in samples of our first experiment and calculated measurement error (ME1, [22]). We concluded that ME was less than 5% of the respective FA mean (data not shown). We also determined that between-sides variation is significantly larger than ME (the interaction term was significant in a two-way ANOVA with side and individual as factors; data not shown). Hence, we decided to use one measurement per side to calculate FA. All measurements were made by the same person. Twenty individuals (10 males and 10 females) were used to calculate all FA means and errors.

### (e) Fluctuating asymmetry

We did not observe evidence for either directional asymmetry or antisymmetry (data not shown). The presence of directional asymmetry was investigated by means of a Student's *t*-test for each data point ( $H_0 = \text{mean}(R - L) = 0$ ). The existence of antisymmetry was evaluated using Shapiro–Wilk's test of normality. We did not detect a correlation between body size and asymmetry (data not shown). Given all mentioned above, we chose the FA1 index to estimate FA [22]. FA1 is defined as the mean value of  $|R - L|$ , *R* and *L* being the right and left measurements of the same trait in the same individual. Unsigned asymmetry values were used for all statistical tests.

## 3. Results

In order to examine the effect of parental age on offspring FA, we maintained a large number of flies in egg-laying cages, changing the agar–yeast plate every day. At different time-points (parental ages), we collected the progeny, which developed in rich medium under controlled conditions from embryo to adult. We used estimators of wing and leg size with the aim of studying the symmetry of the progeny. First, we measured FA in the offspring of young (5 days) and old (30 days) parents

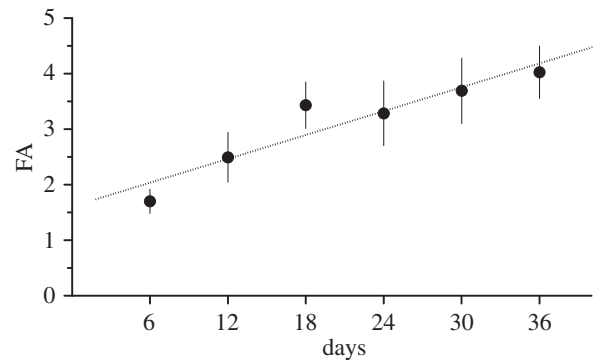


**Figure 1.** Parental age influences the symmetry of the progeny. Levels of FA in (a) wing length (WL), (b) wing width (WW) and (c) femur length (FL) were measured in the progeny of 5 and 30 day old parents. The data correspond to lines 852 (circles), 335 (triangles) and L25 (squares). Symbols show FA means and lines represent  $\pm 1$  s.e.

in three isogenic lines of *D. melanogaster* (figure 1). We observed a large increase in WL-FA and WW-FA at 30 days in all lines (figure 1*a,b*). On average, offspring WL-FA of old parents is 2.8 times higher than that of young parents, whereas WW-FA is 1.7 times greater. For two of the assayed lines, we also detected an increase in FL-FA in the progeny of 30 day old parents (figure 1*c*). On average (lines 852 and 335), FL-FA is 2.7 times greater in the progeny of old parents. Accordingly, statistical analyses showed that age is a significant factor affecting FA in the three characters (two-way ANOVA, with age and line as fixed factors; see the electronic supplementary material, table S1). Moreover, pairwise comparisons within lines (see the electronic supplementary material, table S2) showed that age has a significant effect for WL-FA in all lines. However, pairwise comparisons for WW-FA and FL-FA were not significant (except for FL-FA in line 335), which is probably caused by the stringency of Tukey's test (see the electronic supplementary material, table S2). Altogether, our results indicate that parental ageing has a profound impact in the symmetry of body structures of the progeny.

Bearing in mind that FA was observed both in leg and wing, we set out to investigate whether individuals with high WL asymmetry also had high FL asymmetry. To this end, we performed a correlation analysis that did not yield significant results (see the electronic supplementary material, table S3). We also examined the effect of age on trait averages and interindividual variation. Comparisons of mean trait sizes and coefficients of variation for each line and trait did not reveal differences between the progeny of old and young parents (see the electronic supplementary material, tables S4 and S5). Given the consistency of our results across lines, we decided to use only one line for our following set of experiments.

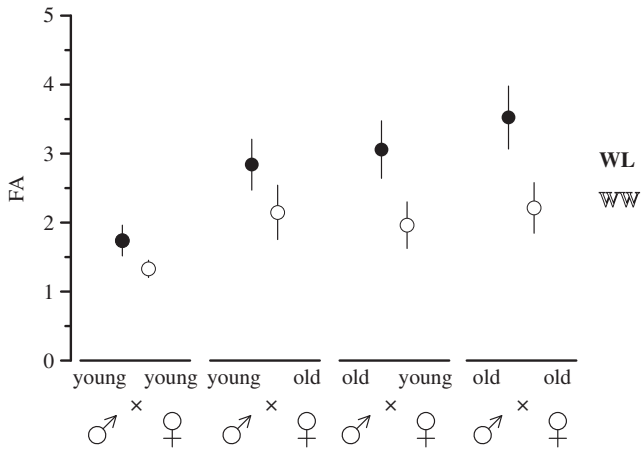
Next, we sought to investigate the temporal dynamics of the age effect, as our previous experiment dealt with only two time-points (5 and 30 days). To this end, we measured WL-FA at six parental ages using line 852. We observed that offspring FA increased as a function of parental age (figure 2). The data were fitted to a linear regression model that was highly significant ( $p = 0.0002$ ,  $r^2 = 0.11$ , slope = 0.072; figure 2). Pairwise comparisons between ages indicated that FA values were significantly different only at the extremes (6 days versus 30 or 36 days, see the electronic supplementary material, table S6). Because FA seemed to plateau at 18 days (figure 2), we also tried fitting polynomial or exponential



**Figure 2.** FA of the progeny increases gradually with parental age. Levels of WL-FA in the progeny of parents aged 6–36 days (line 852). The dotted line shows the fit of a linear mean-squares regression ( $p = 0.0002$ ,  $r^2 = 0.11$ , slope = 0.072). Black circles depict FA means and lines represent  $\pm 1$  s.e.

regressions. Nonetheless, these analyses did not significantly improve  $r^2$  (data not shown). Hence, it can be hypothesized that FA of the progeny increases gradually with parental age.

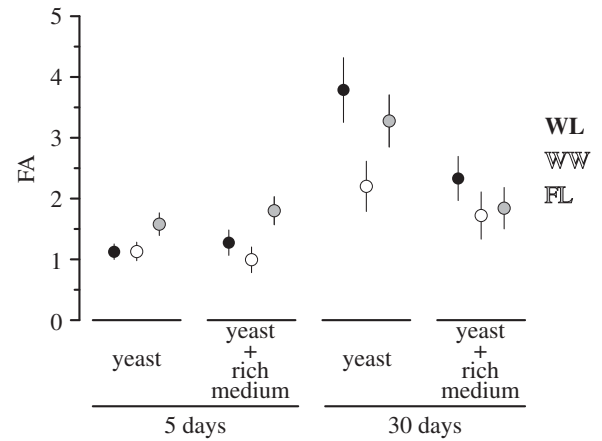
In view of this interesting pattern, we wondered whether the effect would be caused by maternal and/or paternal ageing. Thus, in order to investigate this issue, we modified the experimental design. Males and females of line 852 were aged in separate cages and later brought together with members of the opposite sex according to the following crossing scheme: young ♀  $\times$  young ♂, young ♀  $\times$  old ♂, old ♀  $\times$  young ♂ and old ♀  $\times$  old ♂. The parental age at the time of embryo collection remained the same; young males or females were 5 days old and old males and females were 30 days old. As a consequence of the new experimental design, at 30 days of embryo collection, the parents had been mating for just 2 days, whereas in the experiments described above, they had been mating for 25 days. Despite these differences, in this experiment, we found the same general pattern (figure 3). For both WL and WW, we saw increased offspring FA when only one or both parents were old, when compared with the cross of young males and females. In fact, offspring WL-FA of the old ♀  $\times$  old ♂ cross is two times higher than that of the young ♀  $\times$  young ♂ cross. In addition, offspring WL-FA of the two crosses with only one old parent is 1.7 times higher than that of the young ♀  $\times$  young ♂ cross (see the electronic supplementary material, table S7). A two-way ANOVA (with age of male and age of female as fixed factors)



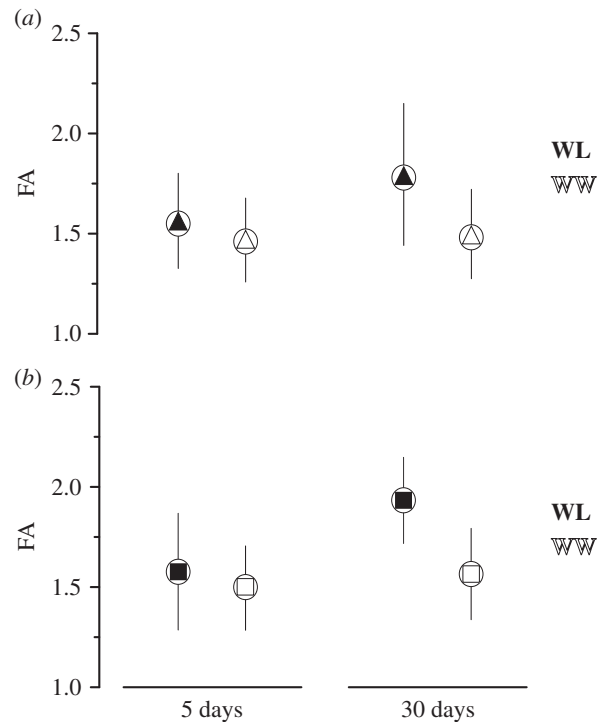
**Figure 3.** Both maternal and paternal age affect FA of the progeny. Levels of WL-FA (black circles) and WW-FA (white circles) in young ♀ × young ♂, young ♀ × old ♂, old ♀ × young ♂ and old ♀ × old ♂ crosses. Young flies were 5 days old and old flies were 30 days old at the time of embryo collection. Circles (line 852) depict FA means and lines represent  $\pm 1$  s.e.

confirmed that WL-FA is significantly increased when only one of the parents is old, whether male or female (see the electronic supplementary material, table S8). Even though we observed the same trend for WW, we did not detect significant differences between FA means (see the electronic supplementary material, tables S7 and S8).

We next explored whether the effect of ageing could be reversed by changing the conditions in which parents age or the genetic constitution of the progeny. First, we modified the diet of the parents (line 852) by supplementing yeast paste, a food source with a low carbohydrate to protein ratio (C:P ratio), with a medium that is rich in carbohydrates. It has been shown that a diet with a high C:P ratio is preferred by adult *D. melanogaster* flies [23]. Moreover, it has been reported that raising the C:P ratio with sucrose increases adult lifespan in *D. melanogaster* [24]. In our experiment, the two diets produced similar levels of FA in the progeny of young parents (figure 4). As expected, the levels of WL-FA, WW-FA and FL-FA augmented in the progeny of old parents that were fed yeast only (figure 4). In effect, offspring WL-FA of old parents (fed yeast only) is 3.3 times higher than that of young parents (fed yeast only), whereas WW-FA and FL-FA are 1.9 and 2 times higher, respectively (see the electronic supplementary material, table S9). Interestingly, there was a marked decrease in WL-FA, WW-FA and FL-FA in the offspring of old parents that were fed yeast + rich medium during ageing. Offspring WL-FA of old parents that were fed yeast + rich medium is two times higher than that of young parents (fed yeast only), whereas WW-FA and FL-FA is 1.5 and 1.2 times higher, respectively (see the electronic supplementary material, table S9). Statistical analyses demonstrated that diet significantly mitigates the effect of ageing on WL-FA and FL-FA. Namely, the age × diet interaction was significant in a two-way ANOVA (with age and diet as fixed factors; see the electronic supplementary material, table S10). Furthermore, for both WL-FA and FL-FA, pairwise Tukey's tests indicated that the offspring of old parents fed with yeast only were significantly more asymmetrical than all other progenies (see the electronic supplementary material, tables S11–S13). By contrast, Tukey's tests also showed that the progeny of old parents fed with yeast + rich medium



**Figure 4.** Enrichment of parental diet increases the symmetry of the progeny. Levels of WL-FA (black circles), WW-FA (white circles) and FL-FA (grey circles) in the progeny of 5 and 30 day old parents that were fed yeast only or yeast + cornmeal–molasses–yeast medium. Circles (line 852) depict FA means and lines represent  $\pm 1$  s.e.



**Figure 5.** Heterozygosity eliminates the effect of parental age on FA. Levels of WL-FA (black symbols) and WW-FA (white symbols) in the hybrid progeny obtained from crossing (a) line 852 ♀ × line L25 ♂ and (b) line 852 ♀ × line 335 ♂. Parents were 5 and 30 days old at the time of embryo collection. Triangles within circles and squares within circles show FA means and emphasize the hybrid nature of the progeny. Lines represent  $\pm 1$  s.e.

was no different from the progeny of young parents (see the electronic supplementary material, tables S11–S13).

Finally, we crossed males and females of the isogenic lines used before with the aim of testing the effect of heterozygosity. Specifically, we crossed females of line 852 with males of either line 335 or line L25. These two crosses yielded intrapopulation (852 × 335) and interpopulation hybrids (852 × L25), giving us the chance to analyse possible consequences of genetic distance and genomic compatibility. Strikingly, the effect of parental age on FA disappeared in both crosses (figure 5 and electronic

supplementary material, table S14). The differences in WL-FA and WW-FA between the progeny of young and old parents were no longer significant (one-way ANOVA with age as fixed factor; see the electronic supplementary material, table S15). Remarkably, both a change in parental diet and the hybrid genetic constitution of the progeny decreased the levels of asymmetry.

#### 4. Discussion

In this work, we have demonstrated that parental age impinges on developmental stability of the progeny in *D. melanogaster*. We observed that the progeny of 30 day old parents is significantly more asymmetric than the progeny of 5 day old parents. Subsequently, we confirmed the effect of age on FA by measuring the asymmetry of the progeny at six different parental ages. It is important to note that the increase in asymmetry was revealed in both wings and legs (two structures derived from different imaginal discs), suggesting that parental age is likely to affect the symmetry of many bilateral body structures.

Then, we tried to determine whether the age-effect was caused by both parents or, alternatively, was sex-specific. Thus, we analysed FA in the progeny derived from crosses in which only one, none or both of the parents were old. In principle, we can conclude that, in terms of asymmetry, having an old mother and a young father is the same as having an old father and a young mother. In other words, the age of both sexes influenced independently the symmetry of the progeny.

As humans, we would like to know whether we can reduce the problems associated with ageing by changing life habits. It is known that diet can have a significant impact on many aspects of ageing in different organisms [25]. Thinking in these terms, we wondered if it would be possible to diminish the asymmetry of the progeny by changing the rearing conditions of the parents. We hypothesized that the poor diet we had used so far (yeast paste) might be stressful for reproductive ageing. Thus, we supplemented the diet of parents with a carbohydrate-rich medium. Interestingly, we observed that the progeny of old flies fed with a rich diet exhibited reduced FA compared with the offspring of old flies fed with yeast only. This suggests that diet affects the quality of gametes (in an unknown manner) and, eventually, the quality of gametes decides the symmetry of the individual. Likewise, infection/disease is another facet of parental wellness that influences offspring symmetry in both humans and flies. *Drosophila nigrospiracula* female flies that are infected with mites have sons that are more asymmetric than those born to mothers that are free of parasites [26]. In the same vein, women that suffer from certain diseases (e.g. diabetes) during pregnancy give birth to asymmetric daughters [27].

We thought that another factor which may be altering the stability of development is the genetic constitution of the progeny. It would not be unreasonable to think that the high homozygosity of isogenic lines may be a feature that escalates the noise of a developmental system [14]. Besides, the benefits of heterosis (hybrid vigour) have been acknowledged for ages [28]. Would it be possible to increase the symmetry by outbreeding the lines? To answer this question, we analysed the symmetry of F<sub>1</sub> hybrids obtained in two different crosses that had the same mother (line 852) but different father. In the first cross, the male line was derived from the

maternal population (line 335) and in the second cross, the male line was derived from a distant population (line L25). In agreement with our hypothesis, hybridization completely purged the effect of ageing on FA, irrespective of the male line used. This implies that inbreeding is a variable which is somehow connected to the effect of age on developmental stability. Moreover, it is clear that whether or not the parental lines are from the same population does not change the result of the experiment. Hence, a plausible scenario is that intra- or interpopulation hybridization simply alleviates the effects of deleterious mutations present in isogenic lines. However, because we did not measure FA of controls (isogenic lines) together with the FA of hybrids, it cannot be ruled out that uncontrolled experimental conditions, and not heterozygosity, affected the levels of FA.

Taken together, our results suggest that diet, genotype and age of the parents interact to determine offspring DI in wild populations. In our laboratory experiments, offspring DI is detectable in very specific conditions: old and highly homozygous parents that aged with a poor diet. It is conceivable that the combination of multiple stressors (irrespective of their nature) is necessary to reach a threshold level that destabilizes development. This might be the reason why previous attempts to link parental age and DI in *D. melanogaster* were inconclusive [29,30]. Parsons [29] detected a very subtle effect of maternal age on symmetry, whereas Wakefield *et al.* [30] did not find such a correlation between FA and parental age. In both these studies, though, parents were fed with rich medium. Moreover, although the fly stocks used in both studies had been in the laboratory for many generations, they were not isogenized by full-sibling mating (our lines are approx. 99% homozygous).

In this paper, we have not only described an interesting phenomenon, but we have also established a robust experimental design to further investigate the mechanisms behind the phenomenon. In this context, it will be relevant to determine the physiological and/or genetic causes underlying the effect of parental age on offspring symmetry. We can hypothesize that the increase in FA is owing to the reduction in egg quality during ageing [31,32], a decline caused by damaged RNAs and proteins. Furthermore, instability could also result from the accumulation of DNA damage in gametes, a process that occurs both in humans [33–35] and flies [36]. Interestingly, it was recently shown that eggs from *D. melanogaster* females fed with a poor diet were deficient in correcting damage in sperm DNA [37]. A major challenge for the future is to link patterns and mechanisms, shedding light on how environment, physiology and genotype relate to developmental noise, thus, providing a more comprehensive view of organismal biology.

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**Author contributions.** V.C., E.H. and N.F. designed the experiments. B.C. and N.C.R. performed the experimental work. N.F. wrote the manuscript. V.C. and E.H. commented on the manuscript at all stages.

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**Competing interests.** We have no competing interests.

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