Targeting environmental and genetic aspects affecting life history traits

Baldal, E.A.

Chapter 2

A test of the thrifty phenotype hypothesis in *Drosophila melanogaster*

Submitted to Mechanisms of Ageing and Development

A test of the thrifty phenotype hypothesis in *Drosophila melanogaster*

E.A. Baldal[1, 2, 4], P.M. Brakefield[1,4], R.G.J. Westendorp[3,4]and B.J. Zwaan[1,4]

[1] Section of Evolutionary Biology, Institute of Biology, Leiden University, P.O. Box 9516, 2300 RA Leiden, The Netherlands

[2] Section of Animal Ecology, Institute of Biology, Leiden University, P.O. Box 9516, 2300 RA Leiden, The Netherlands

[3] Department of Gerontology and Geriatrics, Leiden University Medical Center CO-P, P.O. Box 9600, 2300 RC Leiden, The Netherlands

[4] On behalf of the "Lang Leven" consortium. The "Lang Leven" consortium consists of A. Ayrinhac, E.A. Baldal, M. Beekman, G.J. Blauw, D.I. Boomsma, P.M. Brakefield, B.W. Brandt, R. Bijlsma, D. van Heemst, B.T. Heijmans, J. van Houwelingen, D.L. Knook, I. Meulenbelt, P.H.E.M. de Meijer, S.P. Mooijaart, J. Pijpe, P.E. Slagboom, R.G.J. Westendorp, L.P.W.G.M. van de Zande, B.J. Zwaan

Abstract

Adverse pre-natal conditions in humans have been found to affect adult metabolism in a way that the individuals show an increased incidence of metabolic syndrome late in life. Consequently, the average life span of these individuals should be lower than that of the average population. This observation was termed the thrifty phenotype hypothesis by Hales and Barker, and is known as the Barker hypothesis. Here, we examine whether a similar process could be detected in *Drosophila melanogaster*. By rearing flies under adverse conditions, we tried to mimic adverse conditions in the human uterus. Medium with half the sugar and yeast concentrations (“half”) of standard medium was used as an adverse pre-adult condition. The standard medium (“standard”) was the normal rearing condition and the double medium (“double”) contained twice the amounts of sugar and yeast, making it affluent. We were careful not to mix up the thrifty phenotype with the thrifty genotype hypothesis, which works in the same direction but has an ultimate rather than a proximate cause. We showed that animals reared on half medium weighed less, developed slower and had reduced egg-to-adult survival. The half medium was thus truly adverse. The animals reared at adverse medium displayed significantly shortened adult longevity, caused especially by increased mortality late in life. These data indicate that similar mechanisms underpin the Barker hypothesis and the phenomena found in our flies. Further research into this system should reveal whether the adults suffer from metabolic disease or not.

Keywords

Barker hypothesis, thrifty phenotype hypothesis, thrifty genotype hypothesis, proximate, ultimate, *Drosophila melanogaster*, scar, adaptive plasticity, longevity, metabolic syndrome

Introduction

In 1962, Neel proposed the thrifty genotype hypothesis that during human evolution, when food conditions were relatively poor, selection favoured individuals whom could endure long periods of starvation. Subsequently, in periods of food abundance, these humans would acquire as much resources as possible as a reserve against more adverse times. The adaptation to these conditions is hypothesized to be regulated by insulin metabolism which directs several key processes in response to food conditions, such as metabolism, storage and growth. Neel proposed that the increased number of diabetics observed already during his time could be attributed to the moulding of the genetic regulation of insulin metabolism in evolutionary history. As a result of this adaptation, the abundant presence of food in the Western world leads to an increased incidence of diabetes mellitus and other metabolic diseases, in turn reducing fitness and longevity (Diamond 2003).

The thrifty phenotype hypothesis (Hales and Barker 1992, 2001), also known as the Barker hypothesis, is based on the observation that pre-birth food conditions modify insulin metabolism in the human foetus and subsequently in the resulting adult. Specifically, this hypothesis was put forward to explain the observation that poor nutrition in the mother's womb would increase the likelihood of metabolic disease later in life, when food conditions are relatively better, and therewith reduce fitness and longevity. The poor nutrition can arise from adverse environmental conditions where the mother does not have access to food. Another mechanism would be the situation where the mother does not allocate sufficient food to the foetus in her womb via the placenta. Two potential mechanisms could explain the increased incidence of metabolic disease in these individuals (Brakefield et al. 2005). One mechanism is 'scar', i.e. a disruption of homeostasis. The alternative is based on the hypothesis that as the result of natural selection, developmental plasticity of insulin regulation is adaptive using the pre-natal environment as a predictor of future food conditions (Zwaan 2003). This study does not aim to distinguish between these, but mentions them for the context.

The thrifty genotype and thrifty phenotype hypotheses work in the same direction. They both predict that poor food conditions experienced early in the life history will lead to malfunctioning metabolic regulation when food conditions have become better later in the life history. Yet, the thrifty phenotype hypothesis takes place on a physiological (proximate) level, whereas the thrifty genotype hypothesis takes place on a genetic (ultimate) level. Nevertheless, the thrifty phenotype hypothesis and thrifty genotype hypothesis are easily mixed up and thus Zwaan (2003) proposed a clear separate testing of these theories.

Baldal et al. (2005) found that larval crowding in three *Drosophila* species negatively affects adult life histories. This finding could be explained by the thrifty phenotype hypothesis, as crowding involves a relative food shortage, but there were other factors involved (e.g. nitrogenous waste intoxication, competition) that prevented a firm conclusion. We sought a method that specifically tested the thrifty phenotype hypothesis without any potential interaction with the thrifty genotype hypothesis. Furthermore, the method had to exclude any other physiological responses than those involving food adversity in the pre-adult stage. To this end, here we examine whether different pre-adult food conditions significantly affect several life history and

physiological traits in the fruit fly *Drosophila melanogaster*, and whether our findings are in line with the observations in humans.

Materials and methods

Flies

One hundred and eleven females of *Drosophila melanogaster* were caught and used to initiate isofemale lines. Of these, 17 were collected in France by B. Pannebakker, 22 in Panama by K. van der Linde and C. Krijger (all from Leiden University, The Netherlands), 4 from Groningen (The Netherlands; kindly provided by R. Bijlsma and A. Boerema from the University of Groningen), and 68 were collected in the Leiden area (The Netherlands). The iso-female lines were mixed to form a Stock-population, which was maintained for over 30 generations before the onset of this experiment. Flies were allowed to lay eggs for three hours on agar medium with ample wet yeast, to induce reproduction. All experiments took place at 25ºC, 50% relative humidity and a 12/12 hour day/light regime.

Larval food conditions and crowding

In previous work, we found that high larval density reduced longevity and starvation resistance in three species of *Drosophila* (Baldal et al. 2005). In the larval environment, crowding is a stress factor that involves multiple factors such as nitrogenous waste, food competition and food depletion (Graves and Mueller 1993; Joshi et al. 1996; Borash et al. 1998). The reduced longevity and starvation resistance as an effect of high larval density in Baldal et al.'s study could thus be caused by such factors. The thrifty phenotype hypothesis revolves around malnutrition and does not take such factors into account. To test the thrifty phenotype hypothesis, we thus focused on food conditions and tested life histories of adults raised on adverse, standard and affluent conditions as larvae (figures 1 and 2).

The rationale of the experimental design

Paralleling the human womb and insect models
Extrapolating life history directly from holo-metabolous insects to man is tenuous. It would be highly contentious to simply define the larval instar stages as childhood, pupation as puberty and imago as adulthood. We regard early human development *in utero* as a phase of resource acquisition and early development that forms the infant that is supposed to eventually become a reproductive adult. We deduce that the larval stage of the fly is a developmental phase in which the organism acquires resources. After that, it metamorphoses into a stage from where it develops further, comparable to human childhood and adolescence. This reasoning is based on the traits of interest in this work: food intake, development and adult life history. Thus, we considered the larval stages as comparable to the intra-uterine environment in terms of development. Therefore, we manipulated the larval food environment to test the

thrifty phenotype hypothesis (figures 1 and 2).

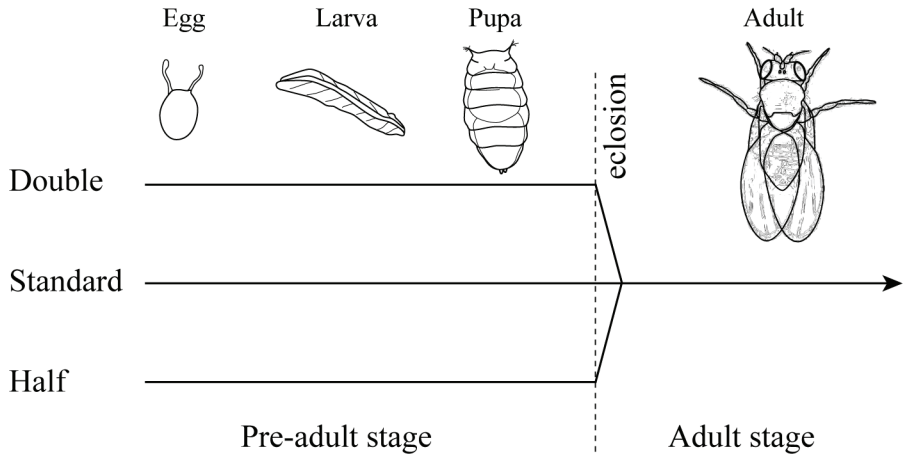

Figure 1. Overview of the experimental set-up. Eggs were put in vials containing one of three media -half, standard and double- hatched as larvae, became pupae and were from eclosion transferred to standard medium as adults. *Drosophila* stages redrawn from http://www.neosci.com/demos/10201_AP%20Lab%207/Presentation_2.html

The adult diet

The thrifty phenotype hypothesis revolves around the physiological response to adverse pre-adult conditions, independent of their evolutionary history. The effects of this process are easily confounded with the effects of a thrifty genotype, which also involve metabolic syndrome. For a clear testing of the thrifty phenotype hypothesis (i.e. examining physiological, proximate responses) we thus need to match the adult environment with the environment in the evolutionary history of the population (figure 2), being the standard medium.

Food conditions

Eggs were placed in vials containing either 6 ml of half, standard or double medium. For each of the three food conditions, 20 vials were set up containing 100 eggs each. We interpret these conditions for the larvae that hatched as adverse, standard and abundant food conditions, respectively. Pupation and subsequent eclosion took place in the same vials. At eclosion, the flies were transferred to fresh standard medium vials (figure 1). Standard medium consisted of 20 g agar, 9 g kalmus (10 parts acidum tartaricum, 4 parts ammonium sulphate, 1 part magnesium sulphate and 3 parts potassium phosphate), 10 ml nipagin (100 g 4-methyl hydroxy benzoate per liter ethanol), 50 g saccharose and 35 g granulated yeast per litre water. In half and double medium the amounts of yeast and sugar are respectively half and double of standard medium, whereas the concentrations of the other ingredients were

maintained as in standard medium. This was done in order to introduce as little variation as possible into the experiment.

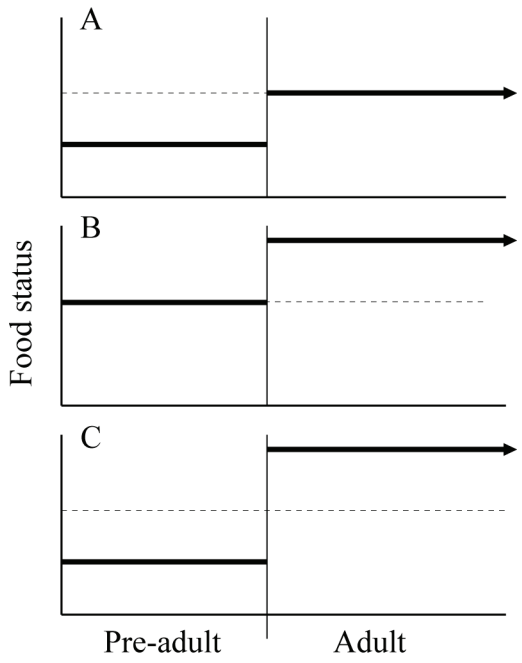

Figure 2. The difference between testing the thrifty genotype and thrifty phenotype hypotheses lies mainly in the evolutionary history of the population. When testing the thrifty phenotype hypothesis, it is important to examine the pre-adult effect on the adult phase under the food conditions to which the population has adapted by natural selection (dotted line), as is depicted in A, because then the only variable is the pre-adult environment. If an adult population is put in a situation to which it is not genetically adapted (B), we are testing the effect of the thrifty genotype hypothesis because the only variable is the condition of adult feeding. When one tries to test the Barker hypothesis by offering adverse pre-adult and affluent adult conditions (C), a confounding of proximate and ultimate mechanisms is inevitable. Both mechanisms work in the same direction, only the causality of the effect is different, resulting in similar phenotypes. Distinguishing between these causes is the aim of the present study.

Traits measured

Development time

Development time was determined by examining the number of flies that eclosed on a specific day. All flies were allowed to eclose and then counted at the same time each day. Development time was measured by taking the number of days between

egg-laying and adult eclosion. Subsequently, we calculated egg to adult survival for each of the media by dividing the number of eclosed adults by the number of eggs.

Wet weight
We measured wet weight of 5 groups of 5 females of each larval food condition directly from the peak of eclosion on a Sartorius® ultra microbalance.

Longevity
Adult longevity was measured in virgin flies as the number of days from eclosion to death. The sexes were maintained separated throughout the entire experiment. Flies were initially maintained at a density of 5 flies per vial, because this yields longevity data without the negative effects of adult density (Baldal, unpublished results).

Statistics

All data were analyzed in JMP 5.0.1 (SAS Institute). For longevity testing, the animals that died within 10 days from eclosion were censored, as they usually died by other than intrinsic causes. We used Cox Proportional hazards analysis to analyze the longevity data and used ANOVA with full factorial designs for all other data. Relative order and significant differences among the groups tested were determined by *post hoc* Tukey testing. Replicate vials were always nested in the factor sex and treated as a random factor. The age specific survival was determined by calculating the log(-log(Survival)). Age specific mortality was tested using ANCOVA. The mortality curve (fig. 4) showed two distinct phases with a linear relationship between mortality and age within each phase. Therefore, the data were analysed for 0-30 days and from 30 days onwards, separately, allowing us to obtain insight into early and late mortality patterns.

Results

The effects of larval media on development

Development time and number of eclosed adults
Females showed a significant effect of the larval media on their development time ($F_{2,2145}$=1658, P<0.0001), as did males ($F_{2,2085}$=1580, P<0.0001). Double medium yielded the fastest developing flies, followed by standard medium and then half medium. The latter medium also showed a strong increase in the variance (table 1). The differences in the number of eclosed adults from the media were striking; out of 2000 eggs put on half medium we obtained 1126 adults (56.3%), in the case of standard medium this is 1388 (69.4%) adults, and in the case of double medium 1722 (86.1%).

Wet weight
Wet weight of females was significantly lower when flies were raised on half medium ($F_{2,12}$=48, P<0.0001) compared to standard and double medium, which is also reflected in *post hoc* Tukey testing (see table 1).

Table 1. Proportion of the total number of adults of each sex that eclosed each day from egg-laying onwards on different larval diets. Peak days of eclosion are highlighted in bold. Average wet weight after eclosion in mg of 5 female flies with standard error for each of the larval media is shown in the right-hand column.

Medium	Sex	Hatching (proportion)								Wet weight (SE)
		Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15	Day 16	
Half	male	0	0.01	0.12	**0.30**	0.22	0.20	0.12	0.04	-
Half	female	0	0.03	**0.25**	0.21	0.19	0.17	0.13	0.02	3.85 (0.17)
Standard	male	0.02	**0.53**	0.33	0.07	0.03	0.01	0.01	0	-
Standard	female	0.06	**0.63**	0.23	0.05	0.01	0.01	0.01	0	6.13 (0.25)
Double	male	0.13	**0.81**	0.05	0.01	0.01	0	0	0	-
Double	female	0.48	**0.49**	0.02	0	0	0	0	0	5.87 (0.09)

Longevity

The longevity of adults was significantly affected by the larval medium (Chi^2_2=17.0, P<0.001). Both females and males showed an effect of the different larval media (Chi^2_2=7.65, P<0.05; Chi^2_2=8.9, P<0.05, respectively, see also figure 3). We found that in both sexes there was no difference between the standard and the double media (female; Chi^2_1=0.28, n.s., male; Chi^2_1=0.005, n.s.), however, flies reared on the half medium were significantly shorter-lived than those reared on standard medium (female; Chi^2_1=4.9, P<0.05, male; Chi^2_1=8.7, P<0.01) or double medium (female; Chi^2_1=7.2, P<0.01, male; Chi^2_1=4.6, P<0.05).

Analysis of the age specific mortality rate with age as a covariate reveals a pattern similar to the results of the Cox proportional hazards analysis on survivorship (see figure 4).The analysis of early and late life mortality (see materials and methods) revealed the following. Female early mortality did not differ significantly among the groups ($F_{2,20}$=0.26, P=0.77), but *post hoc* Tukey testing revealed a tendency of females reared at half medium to have slightly increased mortality. Female late mortality showed a significantly higher mortality for females reared on the half medium ($F_{2,43}$=52, P<0.0001). Male early and late mortality both showed a negative effect of the half medium ($F_{2,19}$=24, P<0.0001, $F_{2,43}$=46, P<0.0001, respectively). This indicates that there is a negative effect on longevity when larvae are reared at half medium. This effect is present throughout life, but is most significant at a later age. This confirms that life span in *Drosophila* can be affected by the pre-adult environment in a similar way as was reported in some human studies (Hales and Barker 1992, 2001) and one on butterflies (Boggs and Freeman 2005). It is noteworthy, that also in the human situation it is the late life mortality that is increased.

Discussion

General

These experiments showed that our half medium represented adverse larval conditions relative to the standard conditions. Flies from the double medium behaved in a similar manner to those from the standard medium. Adult longevity was significantly decreased by adverse larval medium. Mortality analysis showed elevated mortality, especially later in life.

The adversity of half medium and its effects on adult body size

Two physiological traits (development time and female wet weight) and the number of eclosed adults (cf. Borash et al. 1998; 2000) were negatively affected by the half medium in the larval stages. Preliminary data suggest that mass specific metabolic rates do not differ among the groups. Insects require a minimum amount of resources to initiate pupation (Ashburner et al. 1989; Davidowitz et al. 2003). Increased development time can be explained by the requirement of resources to successfully complete metamorphosis. Food intake from the critical weight onwards determines the final adult size. This explains the great reduction in adult weight of females reared on half medium; there are less resources to contribute to adult size. It may also explain the decreased egg-to-adult survival we observed in flies reared at the half medium; if it becomes more difficult to gather resources, fewer animals reach critical weight and as a result, fewer adults will eclose. Therefore, we think it appropriate to speak of adverse conditions in the case of the lowest food concentrations.

An important comparison

In the study of Tu and Tatar (2003) a significant increase in life expectancy is present in the animals that underwent juvenile dietary restriction. This holds when life expectancy is considered from eclosion onwards, not when considered from day 15 of adult life onwards. In our experiment we do not find an increase in longevity in animals that had poorer larval conditions (see figures 3 and 4). The difference between the studies implies that the condition that Tu and Tatar created, yeast deprivation in the third larval instar, is not similar to our adverse larval medium. In the third larval instar, yeast deprivation most probably commences after the critical weight (0.3 mg) has been reached for *Drosophila melanogaster*. As argued above, the critical weight is an important point in insect development. When an individual has bridged this critical point in development, disturbance results in a situation where the individual is still capable of becoming a fully functional adult. In this stage, the individual displays high plasticity and can make physiological “decisions” depending on the conditions it meets. Because in the present experiment caloric restriction commenced from hatching onwards, we judge these larval conditions as more severe than those of Tu and Tatar (2003), and also likely to affect only partly the same biological processes.

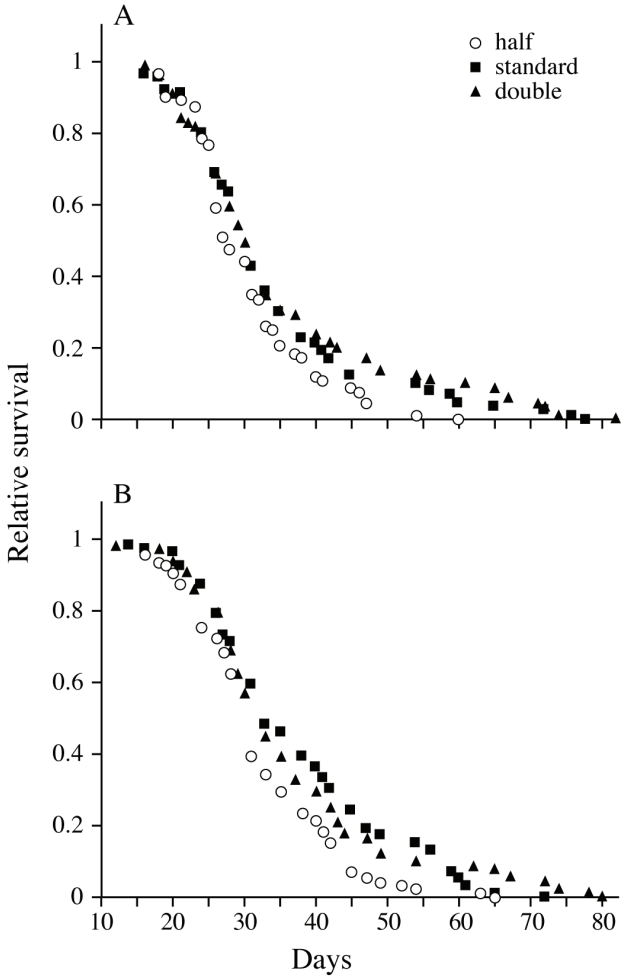

Figure 3. Survival curves of A) females and B) males on the different media. Mortality before day 10 was regarded as an effect of 'developmental hangover' and was discarded from the analyses.

Pre-adult condition dependent adult longevity

Brakefield et al. (2005) argued that environmental variation during early development determines adult features either by the mechanism of 'imprinting', which implies adaptive developmental plasticity (cf. Brakefield et al. 1998), or by the mechanism of 'scar', where homeostasis is disturbed (as proposed by Hales and Barker, 1992, 2001).

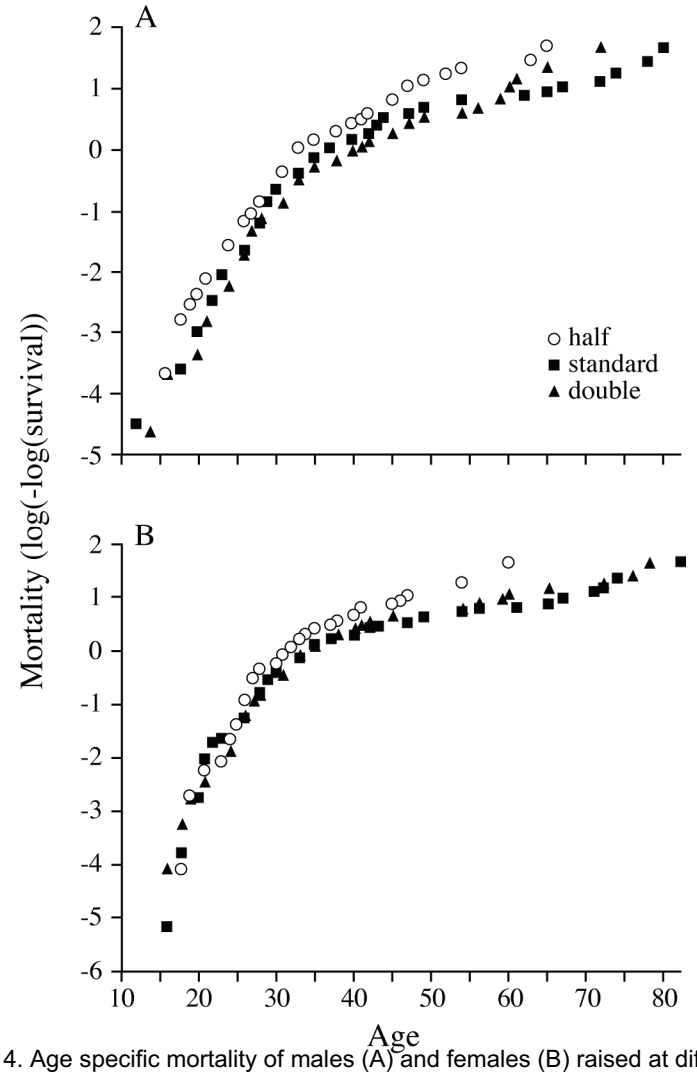

Figure 4. Age specific mortality of males (A) and females (B) raised at different larval media.

We tested the thrifty phenotype hypothesis by examining the proximate response of *Drosophila* to adverse developmental conditions. We hypothesized a response involving metabolic disease that would in turn lead to reduced longevity, the trait monitored. Mortality was elevated in animals that were reared under adverse conditions, especially late in life. This is consistent with the thrifty phenotype hypothesis, where adult metabolic disease typically appears late in life. In this experiment on *D. melanogaster*, we provide some additional credibility to the observation in humans, but the mechanism underlying this effect remains unknown. If

the reduction in longevity can be attributed to metabolic disease, then the Barker hypothesis is proven in flies.

On the basis of the literature, the thrifty phenotype is hypothesised to be a developmental scar, because it leads to a mal-adapted phenotype, relative to well-fed animals, under affluent adult conditions. Further research should focus on distinguishing between the mechanisms of ‘scar’ and ‘imprint’ by measuring lifespan, life history traits in a variety of adult conditions.

Conclusion

In summary, we tested whether the response to larval pre-adult adversity as predicted by the proximate thrifty phenotype hypothesis exists in *D. melanogaster*. Analysis of key life history traits indicates that the half medium represents an adverse larval environment. Longevity of the flies was significantly reduced when reared under adverse larval rearing conditions. This matches the observation in humans that formed the basis of the thrifty phenotype hypothesis. The next step in this line of research should be to establish whether larval adversity leads to metabolic syndrome in flies. Only then, can *D. melanogaster* become a model to investigate the mechanisms behind the thrifty phenotype hypothesis, including distinguishing between the mechanisms of ‘scar’ and ‘imprint’.