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Felix Zajitschek

e-mail: felix@zajitschek.net

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Evolution under dietary restriction increases male reproductive performance without survival cost

Felix Zajitschek^{1,2}, Susanne R. K. Zajitschek^{3,4}, Cindy Canton², Grigorios Georgolopoulos², Urban Friberg^{3,5} and Alexei A. Maklakov²

¹School of Biological Sciences, Monash University, Clayton, Victoria 3800, Australia²Department of Animal Ecology, Evolutionary Biology Centre, and ³Department of Evolutionary Biology, Evolutionary Biology Centre, Uppsala University, Uppsala 75236, Sweden⁴Doñana Biological Station, Spanish Research Council CSIC, c/ Americo Vespucio, s/n, Isla de la Cartuja, 41092 Sevilla, Spain⁵IFM Biology, AVIAN Behavioural, Genomics and Physiology Group, Linköping University, Linköping 58183, Sweden

FZ, 0000-0001-6010-6112; SRKZ, 0000-0003-4676-9950; UF, 0000-0001-6112-9586

Dietary restriction (DR), a reduction in nutrient intake without malnutrition, is the most reproducible way to extend lifespan in a wide range of organisms across the tree of life, yet the evolutionary underpinnings of the DR effect on lifespan are still widely debated. The leading theory suggests that this effect is adaptive and results from reallocation of resources from reproduction to somatic maintenance, in order to survive periods of famine in nature. However, such response would cease to be adaptive when DR is chronic and animals are selected to allocate more resources to reproduction. Nevertheless, chronic DR can also increase the strength of selection resulting in the evolution of more robust genotypes. We evolved *Drosophila melanogaster* fruit flies on 'DR', 'standard' and 'high' adult diets in replicate populations with overlapping generations. After approximately 25 generations of experimental evolution, male 'DR' flies had higher fitness than males from 'standard' and 'high' populations. Strikingly, this increase in reproductive success did not come at a cost to survival. Our results suggest that sustained DR selects for more robust male genotypes that are overall better in converting resources into energy, which they allocate mostly to reproduction.

1. Introduction

Dietary restriction (DR) is to date one of the most robust interventions that prolong lifespan in a wide range of organisms [1], whereas excess-nutrient diets are associated with negative effects [2]. While the understanding of nutritional [3] and molecular [4] mechanisms of lifespan extension under DR has progressed immensely in recent years, the evolutionary basis of the DR effect remains ambiguous [5], and there have been few empirical studies that explicitly test existing theories. Most often, a *holding out for better times* argument is implicated as the adaptive cause for DR-mediated lifespan extension. More formally, this can be framed by applying the disposable soma theory of ageing [6]. According to this theory, the DR effect is an adaptive plastic response of organisms, which optimize their fitness by reallocating their resources from reproduction into somatic maintenance, and hence into survival, when resources are scarce [6].

Because the resource reallocation theory is based on the assumption that the DR effect is an adaptation, which allows surviving a temporary shortage of food until the resources become plentiful again, the corollary is that the DR effect will become maladaptive if food shortage is permanent. Under sustained DR, individuals that produce more offspring in such suboptimal conditions will be selected for rather than individuals that can survive for longer. Therefore, evolution under sustained DR is predicted to increase reproduction and reduce the DR effect, provided there is standing genetic variation for this plastic

response [7]. On the other hand, evolution under high resource availability should not select against plasticity under short-term DR (observed as the classic DR effect), unless simply carrying the gene(s) responsible for the DR effect is costly (i.e. if there is a cost of plasticity [8]).

The above scenario, however, ignores other potential sources of selection that a population may experience under DR. It is often suggested that environmental stress increases selection (e.g. [9], but see [10]). Two recent studies in different invertebrate taxa showed that evolution under temperature stress can result in the correlated evolution of increased fecundity accompanied by either increased or unaltered lifespan [11,12]. DR is a form of an environmental stressor, and might therefore result in stronger selection on genetic quality, compared with when nutrition is more plentiful.

Furthermore, Adler & Bonduriansky [5] recently proposed a controversial new theory, which maintains that the DR effect may be an unselected by-product of maximizing reproduction during famine. Specifically, this theory suggests that the DR effect is driven by a highly conserved nutrient recycling mechanism, autophagy, which maximizes the use of internal resources for reproduction under food shortage and as a by-product extends lifespan of organisms under protected laboratory conditions. Under this hypothesis, evolution under sustained DR will select for individuals that are better at converting low amounts of available energy into reproduction, hence leading to the evolution of increased reproduction under long-term DR accompanied by the evolution of increased lifespan.

Because the prolongevity effect of DR is a key target for applied research on lifespan extension in humans, it is important to understand its evolutionary foundation. To test how lifespan, reproduction and their relationship change during evolution under sustained DR, we set up experimental populations of fruit flies (Dahomey strain of *Drosophila melanogaster*), kept either on DR, standard (ancestral) or high diets ('evolution diets') as adults, with DR, standard and high referring to the manipulated dietary yeast content of 40 (classic DR diet for *Drosophila*), 100 and 270 g yeast per litre diet, respectively). Fly larvae from all populations were raised on the standard diet. Flies were kept in mixed-sex population cages (approximately 150 individuals per sex per population, four replicate populations per treatment) with overlapping generations (see electronic supplementary material for detailed experimental protocols). After 500 days of experimental evolution (approx. 25 generations), we measured male lifespan and reproductive performance of all 12 experimental populations from the three 'evolution diets' in all three corresponding 'assay diets'. We focused on male flies here, which are usually understudied in ageing research in invertebrate models, and estimate their reproductive performance in a well-established reproduction assay where both natural selection and sexual selection jointly determine focal male fitness in competition with standardized competitor males.

2. Methods

(a) Experimental design

(i) Set-up and maintenance of experimental evolution population cages

Flies used to set up the experimental evolution population cages were all derived from the wild-type, outbred and long-term

laboratory-adapted *D. melanogaster* population Dahomey. Founders of this population were originally collected in Dahomey (now Benin) in 1970. Since then, the fly population has been kept in population cages containing more than 3000 individuals with overlapping generations, on standard 1.0 sugar–yeast (SY) diet (with baker's yeast as protein source). Prior to populating cages, flies were raised on standard 1.0 SY diet (with brewer's yeast) with approximately 100 eggs per vial (plastic, 28.5 × 95 mm) for two generations. This was done to assure that there was no random phenotypic bias in founder flies. Emerging adult flies of the third generation were lightly anaesthetized with CO₂ and distributed into experimental population cages, made out of clear plastic (26.5 × 16.5 × 15.5 cm) with one opening, closed by fine nylon mesh. We populated each cage with 150 males and 150 females, and set up four replicate cages per diet. We used three diets that differed in their yeast concentration (40, 100 or 270 g per 1 litre of diet: from here on DR, standard and high protein diet, respectively; electronic supplementary material, table S1) and therefore in their protein-to-carbohydrate ratio. Diets were prepared by mixing required quantities of agar, sugar, yeast and water together. We then boiled the mixture in an autoclave at 121°C for 30 min. When the solution had cooled down to 65°C, we added propionic acid and nipagin solution to prevent fungal and bacterial growth, and dispensed it into plastic containers (10 × 10 × 4.5 cm; 120 ml in each container). Flies were maintained in a climate chamber at 25°C and 60% humidity, on a 12:12 h light:dark cycle. Food was exchanged twice every week (Monday and Thursday). On these occasions, dead flies were counted and removed from cages, without determining the sex of the dead flies. Cages were carefully cleaned on Mondays. On Thursdays, we transferred eggs laid on the surface of the food into two vials per cage, with approximately 100 eggs per vial. These vials contained ancestral diet on which the larvae developed. Offspring (1–2-day-old adults) from these vials were used each Monday to re-establish the original density of 300 individuals per cage, putting back the same number of flies that were counted dead over the last week, with a sex ratio of 1:1. This allowed the development of populations with overlapping generations (age-structured) over time. Owing to the higher mortality on the high evolution diet (electronic supplementary material, table S2), the number of generations for a given amount of days that the experimental evolution cages have been maintained might be slightly higher in high-diet cages, compared with ancestral and low-diet cages.

Experimental evolution has proven to be one of the most valuable experimental methods to study evolution in the laboratory [13]. Yet there are also potential problems associated with this experimental approach. Exposure to a novel environment, inbreeding and long-term selection can all contribute to obscure underlying trade-offs [14]. Although experimental evolution by definition always entails exposure to a novel environment, our experimental populations were exposed to a culturing protocol close to the one experienced by our base population, minimizing this problem. The size of our experimental populations should effectively prevent problems associated with inbreeding. Trade-offs between lifespan and fecundity should have been apparent in this relatively short experimental evolution study, even if such trade-offs may disappear over the long term. We will be able to assess the reported effects in long-term selected populations, as our lines are still maintained.

(ii) Assay design

After 500 days of experimental evolution in the cages described above (equivalent to approx. 25 generations, assuming a generation time of 20 days), we raised flies from each cage for two generations under standardized conditions (see set-up for population cages above), and used the third generation to set up 108 experimental vials (28.5 × 95 mm). Each vial was populated with 40 male flies. Flies were provided with one of the three

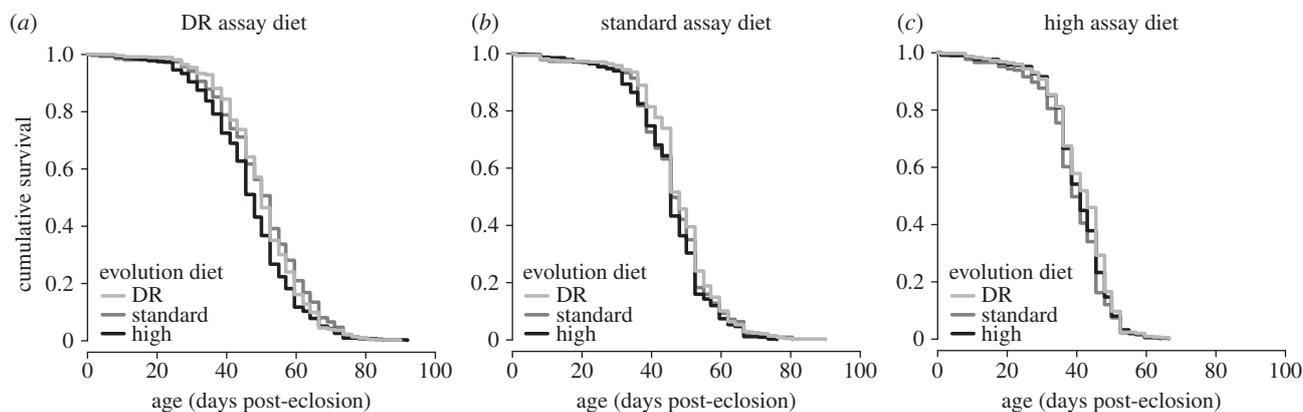


Figure 1. (a–c) No difference in male fruit fly survivorship between evolution diet populations. Each panel shows Kaplan–Meier survival curves for assay diet treatment groups. Separate curves depict survivorship of evolution diet populations, tested on different assay diets.

diets used in the experimental evolution cages, with three replicate vials per cage and diet combination (12 cages, three diets per cage, four replicates).

(iii) Mortality and reproductive performance assays

Mortality was checked each week on Monday, Wednesday and Friday, when flies were flipped into new food vials. We measured male reproductive performance at ages 7, 28 and 56 days post eclosion into adulthood. For this, we gave the males of each vial the opportunity to mate with 40 females. These females were homozygous for the recessive marker ebony (e), a mutation that darkens the body colour, which earlier had been introgressed into the Dahomey genetic background. Ebony females were allowed to mate with ebony males for the first 2 days after eclosion into adulthood, and were placed with the experimental males at age 4 days, for 4 h. Eggs laid by the ebony females over the next 24 h were trimmed back to 180 eggs per vial. Wild-type and ebony offspring were counted. Any wild-type offspring was sired by experimental wild-type males. We calculated the ratio of number of wild-type offspring to total offspring number as reproductive performance (reproductive fitness) measure.

(b) Statistical analysis

(i) Survival

We used mixed Cox proportional hazard models (function `coxme`, R package `coxme` [15]) to test for effects of evolution diet and assay diet on survival patterns, with a random effect that models the variation between vials of the same treatment, nested within population cage of origin. For model selection, we performed backward elimination of non-significant fixed effects (at a 0.05 significance level), using log-likelihood-ratio tests, with twice the difference in log-likelihoods of the models taken as chi-square distributed. To further analyse the significant interaction term between evolution diet and assay diet for male survival, we proceeded with separate analyses on subgroups of the data (with a significance level of 0.017, after a Bonferroni correction for multiple testing). For all reported results, diet was treated as a categorical variable. Lifespan summary statistics and sample sizes are given in electronic supplementary material, table S2.

(ii) Reproductive performance

We calculated total male reproductive performance per vial as the sum of the ratios of number of wild-type offspring to total number of offspring (the sum of the number of ebony and wild-type offspring), with the value 1 indicating the highest reproductive performance of experimental males and 0 the lowest, and, accordingly, the value 3 representing the highest value of total

reproductive performance (summed across the three samples, measured at ages 7, 28 and 56 days of adult life). This represents the most inclusive reproductive performance measure for a given vial population. Effects of evolution diet, assay diet and the interaction between evolution diet and assay diet on reproductive fitness were tested in linear-mixed models, with the variable ‘cage’ fitted as a random intercept (using function `lmer` in R package `lme4` [16]). We used the R package `lmerTest` to calculate *p*-values for `lmer` models, with degrees of freedom based on the Satterthwaite approximation [17]. Model selection was performed with the same rationale as for survival analyses. To further (*post hoc*) analyse the significant effect of evolution diet, we used the function `diffsmeans` in package `lmerTest` (see electronic supplementary material, table S3).

3. Results

Here, we report effects of experimental evolution under DR, standard and enriched adult diets, on male survival and reproduction. In the full model for male survival, the interaction between evolution diet and assay diet was significant (evolution diet \times assay diet, $\chi^2 = 14.72$, d.f. = 4, $p = 0.005$). Because the main point of interest was the effect of evolution diet on survival, we proceeded to test the evolution diet effects in separate analyses for assay diet. All initial models contained evolution diet as a main effect, and were tested against null models of no treatment effect. Evolution diet had no effect on male survival in any of the assay diet groups (figure 1; likelihood-ratio tests between mixed Cox proportional hazards models: DR assay diet: $\chi^2 = 4.39$, d.f. = 1, $p = 0.111$; standard assay diet: $\chi^2 = 4.03$, d.f. = 1, $p = 0.133$; high assay diet: $\chi^2 = 2.72$, d.f. = 1, $p = 0.257$). Assay diet, on the other hand, had the classic DR effect in all evolution diet groups, with a negative relationship between dietary yeast content and survival (tested separately in each evolution diet group: DR evolution diet: $\chi^2 = 81.64$, d.f. = 1, $p < 0.001$; standard evolution diet: $\chi^2 = 63.08$, d.f. = 1, $p < 0.001$; high evolution diet: $\chi^2 = 57.40$, d.f. = 1, $p < 0.001$; electronic supplementary material, figure S1). The significant interaction between evolution and assay diet for survival could be driven by flies from the high evolution diet living shorter than flies from DR and standard diet populations on DR assay diet (electronic supplementary material, figure S1). For male reproductive performance, evolution diet and assay diet had significant effects (figure 2; evolution diet: $F_{2,102.87} = 3.15$, $p = 0.047$; assay diet:

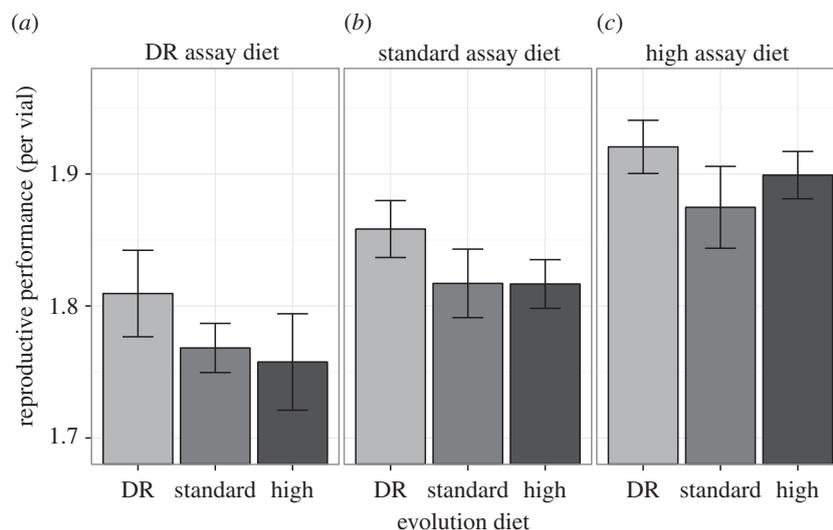


Figure 2. Male reproductive performance is highest in flies evolved on DR, compared with standard and high evolution diet populations. Bar graphs show means with 95% CIs for each evolution diet population, tested on different assay diets.

$F_{2,102.87} = 20.55$, $p < 0.001$). The effects of evolution diet and assay diet were independent of each other (evolution diet \times assay diet, tested in full model: $\chi^2 = 0.76$, d.f. = 4, $p = 0.951$). Males from DR populations had the highest reproductive performance on all tested assay diets, compared with males from standard and high-diet populations (figure 2; electronic supplementary material, table S3). Males evolved on ancestral diet showed the expected clear positive relationship between dietary yeast content and reproductive performance.

4. Discussion

How diet (and especially how DR) affects lifespan, reproduction and other fitness traits has been the focus of some theoretical [18,19] and of a large number of empirical studies (reviewed in [4,20]). A reallocation of resources from reproduction towards somatic maintenance and repair is the underlying mechanism commonly used to explain extended lifespan and reduced reproduction under DR [21]. If a DR-induced negative correlation between reproduction and survival is based on the reallocation of resources, and if it is adaptive as a way to overcome short periods of reduced resource availability, chronic DR should select for increased reproduction with a concomitant reduction in survival, compared with the levels observed under short-term DR. Our results do not support this scenario. While male reproductive performance was higher in the DR evolution populations, as predicted, survival rates were not affected significantly by evolution diet.

While this study is the first to indirectly test the reallocation hypothesis for DR effects using experimental evolution, empirical evidence against an obligatory tight negative correlation between reproduction and survival, based on reallocation of resources, has come from a number of studies in recent years. In female *D. melanogaster*, Mair *et al.* [22] showed that ovaries and hence the ability to produce eggs were not necessary to elicit lifespan extension under DR. Grandison *et al.* [23] showed that fecundity in female flies under DR could be increased to levels observed under standard full feeding diet conditions by adding one specific amino acid (methionine) to DR diet. While fecundity was increased on methionine supplemented DR diet, the typical

DR effect of lifespan extension was still observed. In the neriid fly *Telostylinus angusticollis* [24], adult DR diet in males did not reduce the fecundity or egg-to-adult viability of eggs laid by females mated to DR males. If the DR effects were based on an obligatory trade-off between male reproduction and survival through a reallocation of resources from reproduction to survival, males would be predicted to show decreased reproductive potential, which was not the case.

Two other good examples that do not directly support the reallocation hypothesis of DR effects come from a study on olfaction [25] and from a study on nutrient tracking [26], both in *D. melanogaster*. Libert *et al.* [25] showed that the smell of nutrient-rich food alone is sufficient to reverse the lifespan-extending effect of DR to a substantial degree, without having more dietary resources available for reproduction. This clearly shows that lifespan can be regulated independently of direct allocation of resources into reproduction under some circumstances. O'Brien *et al.* [26] marked nitrogen and carbon in dietary yeast with different stable isotopes and traced their allocation to either eggs or somatic tissue after ingestion. Absolute investment of marked nutrients into somatic tissue was smaller in DR females, compared with standard diet females. Therefore, longer lifespan of DR females seems not to be caused by greater absolute investment into somatic tissue. However, DR females invested relatively more resources into somatic tissue than into eggs, compared with standard diet females. The authors suggest that rather than absolute resource investment into somatic tissue and longer survival, it is this higher ratio between investment into the soma and into eggs that is causing extended lifespan under DR. In this interpretation, greater investment in soma and repair offsets damage through reproduction, leading to longer lifespan, and resource reallocation is still the underlying mechanism. Our selection regime could have resulted in the evolution of fitter males under DR by reducing mutation load in the population [27]. For example, DR males could have evolved to become more efficient at extracting and/or converting nutrients into energy. Because DR males have higher fitness, they could be investing relatively more in reproduction, as predicted by resource reallocation theory.

Do our results support the model for the evolutionary basis of DR brought forward by Adler & Bonduriansky [5]? These

authors assign autophagy and apoptosis a pivotal role in explaining the DR effect on longevity, with autophagy probably being the more important and prominent process [28] in largely post-mitotic tissues (but see [29]) of adult *D. melanogaster*. In fully fed animals, levels of this cellular degradation and recycling machinery are comparatively low, whereas nutrient-sensing pathways, for example IIS (insulin/Insulin-like growth factor 1 signalling) and target of rapamycin (TOR), are upregulated to promote cellular growth. In contrast, when resources are scarce, autophagy and apoptosis, which are controlled to a large degree by IIS/TOR signalling [30], are upregulated to more efficiently re-use internally available resources (organelles and long-lived proteins [31]), whereas nutrient-sensing pathways are less active. Our results of a generally higher reproductive fitness of DR evolution diet males could be explained by selection for more efficient autophagy, leading to optimal use of available resources. These resources, however, are allocated to reproduction while survival does not increase much, thus contradicting the hypothesis that life-span extension under DR is a by-product of scavenging for internal resources to increase reproduction [5].

If heightened levels of autophagy have positive fitness consequences, such as the observed beneficial effect on male reproductive performance, why would we not observe the evolution of higher or more efficient autophagy levels in nutrient-rich environments? This may be explained by trade-offs which are masked in a benign laboratory environment. When autophagy is induced directly by upregulation of Autophagy-specific gene 1 (*Atg1*), cell death is also induced [32]. Even though cell death and organismal death are not necessarily positively correlated, additional stressors such as high pathogen load, temperature fluctuations or higher activity levels, experienced under more natural conditions, might trigger a decrease in survival. Another explanation involves a cost for the other sex. While most prominent in the fat body of *D. melanogaster*, autophagy has recently been shown to also be upregulated in ovaries under starvation [33]. Even with normal nutrient intake, autophagy is a necessary process in follicle cells during oogenesis, but it is not required for germline development [33]. Therefore, altered levels of autophagy could have sex-specific effects, with negative effects that only manifest in

females potentially affecting egg production. The main apoptosis regulatory pathway p53 is also able to regulate autophagy [34] and has been shown to act in a sex-specific way on lifespan in *D. melanogaster*, with negative effects in females and positive effects in males [35], which gives tentative support to a possible sexually antagonistic effect of elevated autophagy levels and may explain increased male fitness that we observe in lines evolving under DR. Data on the evolution of female lifespan and reproductive fitness in our experimental evolution populations are forthcoming and will shed more light on this aspect.

Overall, evolution under sustained DR did not result in strong effects on male survival patterns, but if anything, males evolved on DR diet had higher, and not lower, survival compared with males evolving on higher diets. Importantly, this lack of response was observed despite ample additive genetic variation for lifespan segregating in the base population [36]. Competitive reproduction, in contrast, was highest in males from DR lines. The absence of an evolutionary decrease in lifespan in males from populations evolving under DR in the face of increased reproductive performance gives no direct support for the reallocation theory of DR. However, our results do suggest that sustained DR increases the strength of selection leading to the evolution of increased male fitness across a wide range of dietary conditions. Thus, it is possible that evolution under sustained DR selected for fitter males that allocate relatively more resources to reproduction, as predicted by resource reallocation theory. Whether this male fitness advantage in populations evolved under sustained DR is offset by disadvantages in females is under investigation in our laboratory.

Data accessibility. Life-history data: Dryad: <http://dx.doi.org/10.5061/dryad.2fp25>.

Authors' contributions. F.Z. designed the study, carried out the laboratory work, analysed the data and prepared the manuscript; S.R.K.Z. carried out laboratory work and prepared the manuscript; C.C. and G.G. carried out laboratory work; U.F. and A.A.M. designed the study and prepared the manuscript.

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