Life history consequences of adaptation to larval nutritional stress in *Drosophila*

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Abstract

Many animal species face periods of chronic nutritional stress where the individuals must continue to develop, grow and/or reproduce despite low quantity or quality of food. Here we use experimental evolution to study adaptation to such chronic nutritional stress in six replicate *Drosophila melanogaster* populations selected for the ability to survive and develop within a limited time on a very poor larval food. In unselected control populations, this poor food resulted in 20 % lower egg-to-adult viability, 70 % longer egg-to-adult development and 50 % lower adult body weight (compared to the standard food on which the flies were normally maintained). The evolutionary changes associated with adaptation to the poor food were assayed by comparing the selected and control lines in a common environment for different traits after 29-64 generations of selection. The selected populations evolved improved egg-to-adult viability and faster development on poor food. Even though the adult dry weight of selected flies when raised on the poor food was lower than that of controls, their average larval growth rate was higher. No differences in proportional pupal lipid content were observed. When raised on the standard food, the selected flies showed the same egg-to-adult viability and the same resistance to larval heat and cold shock as the controls and a slightly shorter developmental time. However, despite only 4 % shorter development time, the adults of selected populations raised on the standard food were 13 % smaller and showed 20 % lower early-life fecundity than the controls, with no differences in lifespan. The selected flies also turned out less tolerant to adult malnutrition. Thus, fruit flies have the genetic potential to adapt to poor larval food, with no detectable loss of larval performance on the standard food. However, adaptation to larval nutritional stress is associated with trade-offs with adult fitness components, including adult tolerance to nutritional stress.

Introduction

Many animal species face periods of food shortage or have to get by with food of suboptimal quality. Therefore, the ability to cope with nutritional stress is likely to be under strong natural selection, presumably leading to adaptations impinging upon diverse aspects of the structure, function and life history of the organism (Hoffman and Parsons 1991; Iason and Van Wieren 1999; Blanckenhorn 2000; Dearing et al. 2005). Such adaptations have mostly been addressed by studying physiological (phenotypically plastic) responses thought to alleviate the consequences of nutritional stress for Darwinian fitness. Examples include maintenance of fat and carbohydrate reserves (Strasssmann and Dunbar 1999; Gluckman et al. 2005; Rion and Kawecki 2007) and plastic enlargement of the intestine surface in response to nutritionally poor diet (Koteja 1996). Much attention has also focused on life history responses to mild chronic nutritional stress, referred to as dietary or caloric restriction. In species ranging from yeast through *C. elegans* and *Drosophila* to mammals, dietary restriction leads to partially or completely suppressed reproduction, increased resistance to oxidative stress, and increased longevity (Kenyon 2001; Rogina et al. 2002; Mair et al. 2003; Tatar et al. 2003; Bross et al. 2005; Broughton et al. 2005; Pijpe et al. 2007). These changes can be interpreted as a shift of the physiology from the normal "reproduction mode" to a frugal "survival mode", which helps the organism to survive the hard times, when successful reproduction would be difficult (Tatar et al. 2003).

Studies of such plastic responses provide invaluable insights, but with regard to understanding how populations adapt to nutritional stress over evolutionary time they have two important limitations. First, it is often difficult to demonstrate which aspects of a phenotypically plastic response to nutritional stress are adaptive, and which are direct, maladaptive consequences of malnutrition (Stearns 1992; Schlichting and Pigliucci 1998). Second, some adaptations may evolve to be constitutively expressed (fixed) rather than induced as plastic responses (Stearns 1994). These limitations can be circumvented by the approach referred to as experimental evolution, whereby evolutionary changes induced in replicated experimental populations by controlled selection regimes are studied in real time. Experimental evolution studies can also directly address questions such as how readily a species can evolve a greater tolerance to nutritional stress, or what the associated costs or trade-offs would be.

Almost all experimental evolution studies on adaptation to nutritional stress have been carried out in *Drosophila melanogaster*; most of them have focused on adaptation to survive periods

of short-term acute starvation in adult flies (reviewed by Rion and Kawecki 2007). Selection regimes applied in those studies succeeded in increasing the survival time without food (but with access to water) several-fold (Chippindale et al. 1996; Harshman and Schmid 1998; Harshman et al. 1999a; Baldal et al. 2005; Harbison et al. 2005). They indicate that the evolution of increased starvation resistance in this species is largely mediated by increased storage of lipids and by a reduction of reproduction. Both absolute and relative lipid content of the starvation-resistant flies is already higher at the emergence from pupa, accounting for most, if not all, of the increase in emergence body weight observed in those flies relative to unselected controls (Chippindale et al. 1996; Borash and Ho 2001; Hoffmann et al. 2005). The need to accumulate those extra lipids likely explains why starvation-resistant flies typically have longer development (Chippindale et al. 1996; Harshman et al. 1999a; Bubliy and Loeschcke 2005; but see Hoffmann et al. 2005). Other correlated responses to selection for starvation resistance include reduced fecundity (Service et al. 1988; Leroi et al. 1994), longer lifespan (Rose et al. 1992; Bubliy and Loeschcke 2005) and greater resistance to desiccation (Harshman et al. 1999b; Hoffmann et al. 2005:); these evolved differences parallel plastic responses to dietary restriction. Perhaps surprisingly, reduction in the metabolic rate has not been consistently observed (Djawdan et al. 1997; Harshman et al. 1999a).

Another form of nutritional stress addressed with experimental evolution in *Drosophila* results from larval crowding. Populations maintained under high larval density typically evolve a faster feeding rate and a lower efficiency of converting food into biomass (Bierbaum et al. 1989; Mueller 1990; Joshi and Mueller 1996). The correlated responses of adult resistance to starvation have been inconsistent among the experiments (Borash and Ho 2001; Sanders et al. 2005). Rather than for tolerance to food of low nutritional quality, larval crowding seems to impose selection mostly on traits involved in scramble competition and tolerance to toxic waste products (Borash et al. 2000; Borash and Ho 2001)

In this paper we use experimental evolution to study adaptation to chronic nutritional stress, whereby animals have to cope with poor nutrition for prolonged periods. In contrast to short periods of acute starvation, under chronic nutritional stress waiting out until better or more food becomes available is not an option; the animals must not only survive, but also develop, grow and/or reproduce despite malnutrition. The mechanisms of adaptation to such chronic malnutrition are thus likely to differ from the mechanisms facilitating starvation resistance. They are also likely to differ form adaptations facilitating competition for a small amount of high-quality food, such as adaptation to larval crowding mentioned above.

We studied six populations of *Drosophila melanogaster* bred for several dozen generations on nutrient-poor larval food (the responses of different traits were assayed after 29-64 generations of selection). The poor food substantially slowed egg-to-adult development, which in nature would increase the risk of the food (i.e., a decomposing fruit) to either rot completely or dehisce, both of which would impose additional selection against very slow development. To emulate this selection, only flies that reached adulthood within 14 days from egg were allowed to breed under the selection regime. Thus, the selected flies had to grow and develop on the poor food within limited time, and the resulting adults had to be capable of reproduction despite the hardship endured in the larval stage. We report how stress resistance and life history traits evolved in these selected populations, using six control populations maintained on standard food as reference. We address several questions.

First, did the original base population have the genetic potential to adapt to the nutritional stress? Even though most traits do respond to experimental selection, some do not show any response or only respond to selection in one direction, which may be due to a lack of genetic variation or functional constraints (Blows and Hoffmann 2005). To address this question, we tested if the selected lines showed improved egg-to-adult viability, developmental time and growth rate when raised on the poor larval food.

Second, is adaptation to poor larval food associated with trade-offs in life history traits expressed if flies are raised on the standard food? Animals, including *Drosophila*, can detect their nutritional situation and use this information to induce adaptive plastic responses (Tatar et al. 2003; Partridge et al. 2005). It is therefore conceivable that selection for tolerance to poor food would act on the plasticity and only change the part of the reaction norm expressed on the poor food, without affecting the phenotype expressed on the standard food. If this were not the case, the correlated responses would give insights into potential trade-offs associated with adaptation to poor larval food. Such trade-offs might be manifested as lower survival, slower growth or longer larval development on the standard food, smaller adult body size, and/or as lower fecundity or shorter lifespan of the adult flies. To test for such trade-offs, we assayed the correlated responses in these larval and adult life history traits in flies raised on the standard food. We also assayed resistance of larvae to heat and cold stress. Adaptation to nutritional stress might involve "frugal" phenotypes that invest little in the maintenance of defense mechanisms against other stressors, resulting in a trade-off between tolerances to different stressors. On the other hand, larval crowding has been shown to induce elevated expression of heat shock proteins (Sorensen and Loeschcke 2001). If similar elevated

expression of heat shock proteins is induced by poor larval nutrition, adaptation to larval nutritional stress might be associated with increased constitutive tolerance to temperature extremes.

Third, do populations selected for tolerance to larval nutritional stress also become more tolerant to adult nutritional stress? If the adaptation to poor larval food involved improvements in the efficiency of nutrient extraction and use, they might also act in the adult, making it also more tolerant to nutritional stress. However, adaptation to poor food may also result in changes in adult body size and composition (e.g., the amount of lipid reserves) that would make the adult more susceptible to malnutrition. Because larvae and adults in *Drosophila* feed on the same substrates, such a trade-off between larval and adult tolerance to malnutrition would be an important constraint on adaptation to nutrient-poor environments. To address this issue, we measured the lipid content of the pupae and studied the tolerance of adult flies to both acute starvation and chronic malnutrition.

Material and Methods

Fly populations and selection regimes

The base population for this experiment was generated by mixing 200 adults from each of four populations maintained in our laboratory for about 100 generations at the size of 100-150 adults, and originally founded by several hundred flies caught in Basel (Switzerland) in 1999. (They were the control populations of Mery and Kawecki 2002.) These populations had been maintained in the lab on our standard cornmeal medium (15 g agar, 30 g sucrose, 60 g glucose, 12.5 g dry yeast, 50 g cornmeal, 0.5 g $MgSO_4$, 0.5 g $CaCl_2$, 30 ml ethanol, 6 ml propionic acid, and 1 g nipagin per 1 l water); we refer to this medium as the standard food. The base population was allowed to breed for seven generations before the start of the experiment to homogenize the gene pool.

Six replicate selection and six control lines were then derived from this base population and maintained on a 21-day generation cycle at 25 °C and 70 % humidity. The control populations were bred every generation on the standard food. Selection for tolerance to nutritional stress was imposed by raising larvae of the selected populations on nutritionally poor food, which only contained 1/4 of the amounts of sugars, yeast, and cornmeal of the standard medium. For

the first 18 generations these populations were part of a larger selection experiment (the "P" lines of Kolss and Kawecki 2008), and selection was only imposed every other generation; in the remaining generations the flies were bred on the standard food. At generation 18 the populations reported here were split from the "P" lines and subsequently bred on the poor food every generation. Flies of both selection and control lines were raised in vials containing 30 ml of the poor and standard food, respectively, and seeded with 200-250 eggs; multiple vials per selection line were used to ensure enough surviving adults. Only adults which had eclosed within 14 days of oviposition were used to breed the next generation. All adults eclosed within this time on the standard food, but only about 20-50% on the poor food; thus this procedure imposed additional selection for fast development on poor food. The adults were culled to 150 per line and transferred to standard food with live yeast for six days; the eggs for the next generation were collected on the following day.

Before all assays described below, the flies of all lines were bred for two or three generations on the standard food to reduce effects due to maternal or grand-maternal environment. For the assays, eggs were collected overnight in mass oviposition on grape or orange juice with agar and live yeast. Egg-to-adult viability, developmental time and adult body size were assayed after 29 generations of selection, pupal lipid content after 32 generations, larval tolerance to heat and cold shock after 48 generations, adult lifespan on the good and poor food after 30 generations, adult resistance to starvation after 63 generations and fecundity after 64 generations. (The numbers refer to the generations in which the selected populations were grown on the poor food; generations of relaxed selection are excluded.)

Viability, development and adult body size

To study the direct response to selection on poor larval food, we assayed egg-to-adult viability, developmental time, and adult dry body weight of flies raised on the poor food. Concomitantly, to see if these responses were specific to the poor food environment and to test for correlated responses in these traits between food levels, we assayed these traits in flies raised on the standard food. Four replicate 68-ml vials with 10 ml of food and 100 eggs were set up for each population and food treatment. Eclosing adults were collected on days 8 to 19 after oviposition in the standard food treatment. In the poor food treatment, adults were

collected on days 12 to 29 and once again on day 32; the very few flies emerging on days 30 to 32 were pooled and treated as eclosed on day 30.

All flies were deep-frozen within 24 hours upon emergence. To determine the dry body weight, flies from a given population and food treatment were pooled across vials and dried at 80 °C for 5 days. Twenty flies of each sex were picked randomly and weighed on a Mettler MT5 balance with a resolution of 1 µg. Note that the flies could feed for up to 24 h in the rearing vials, so the weight measured may be greater than the weight at the point of eclosion from the pupa.

Growth rate

Because we found that the selected lines evolved faster development and a smaller body size (see Results), we sought a way to combine these two traits in a measure of larval growth performance. One simple way to do this was to calculate an average exponential growth rate g over the entire developmental period:

$$g = \ln(w_A/w_0)/t \quad (1)$$

where t is the developmental time from egg to adult, w_A is the adult dry weight and w_0 is the egg dry weight, assumed to be 5 µg. This value is based on the fresh egg weight of 11.2 µg laid by females of both selected and control lines (unpublished data). We used the mean adult weight and mean developmental time to calculate a value of g for each line and sex. This measure is rather crude in that it ignores the complexities of larval growth and pupation (e.g., Santos et al. 1997), but we decided to use it here as a heuristic measure of the efficiency in which the time available for development is used to produce an adult of a particular size. Patterns of larval growth will be the subject of another study.

Larval resistance to heat and cold

Vials with eggs on 10 ml of standard food were set up after oviposition overnight. For the cold shock, at about 60 h from the oviposition the vials with the developing larvae were transferred to 0 °C for 20 h, and then transferred back to the 25 °C incubator and allowed to

complete development. For the heat shock, at about 60 h after oviposition the vials were placed for 3 h in an incubator heated to 39 °C, and subsequently were returned to 25 °C to complete development. There were four vials for each line and shock treatment; additional four vials were maintained throughout at 25 °C. The adults emerged from each vial were counted 20 days after the eggs were laid.

Pupal lipid content

Flies were raised both on standard and on poor medium as described above, and pupae (unsexed) were collected two to three days after pupation. The lipid extraction protocol was modified after Sanders et al. (2005). Pupae were placed singly in the pits of well-plates, pierced with a fine needle to facilitate lipid extraction in the subsequent step, and dried at 70 °C for ten days. They were weighed on the Mettler balance to get the total dry mass and soaked in petroleum ether for four days, with the ether replaced twice a day. Subsequently, the ether was allowed to evaporate for 24 hours, then the pupae were again dried at 70 °C for three days and weighed to obtain dry non-lipid mass. The absolute lipid content was calculated as the difference between the two weights, the proportional lipid content as the absolute lipid content divided by the total dry mass (before extraction). The larvae were raised in two replicate vials for each line and food type. Between 40 and 48 and between 27 and 40 pupae were assayed per line for the standard and poor food treatments, respectively.

Fecundity

Fecundity was assayed in young selected and control flies raised on standard food. Sixteen females eclosing on day 12 (counted from oviposition) from each line were transferred individually with two males into 60 ml vials containing 10 ml of standard food. Four days later the females were transferred to fresh vials with standard food and a small drop of yeast to stimulate oviposition. After 24 h and again after 48 h the females were transferred to fresh vials; after 72 h they were discarded. The eggs laid in each of these three 24 h periods (i.e., days 5-7 of adult life) were counted and used as estimates of early-life fecundity.

Lifespan and resistance to starvation

We assayed adult female lifespan on both the standard and on the poor food. Flies were raised on the standard medium under standard conditions. Three days after eclosion to allow for mating, female flies were separated using CO_2 and placed in 1 l demography cages in groups of 50. Three times a week dead flies were removed and counted, and food (of either standard or poor quality) was replaced. There were three replicate cages per line and food treatment.

A similar approach was used to assay starvation resistance. Flies were raised on the standard food. Flies of both sexes eclosing on day 12 from egg were collected and maintained (sexes mixed) on standard medium for two days. Females were then separated using CO_2 and put in the demography cages in groups of 50 (four cages per line), with constant access to 0.1% agarose. Dead flies were scored every six hours.

Crosses between replicate lines

The selected lines showed a reduction in adult body weight and survival on adult poor food. To exclude that these effects may be due to inbreeding depression, we assayed those traits in F_1 crosses between replicate selection lines and compared them to F_1 crosses between control lines. We used a circular crossing scheme: females of line 1 were crossed with males of line 2, females of line 2 with males of line 3, etc.; females of line 6 were crossed with males of line 1. There were thus six crosses between the selected lines and six between the control lines.

The F_1 offspring were raised on the standard medium, with three vials per cross. Flies emerging on days 13-14 were collected and frozen, six females per vial were randomly chosen and their dry weight was determined as described above.

For the lifespan of F_1 females under adult nutritional stress, flies were raised on the standard medium. Adults of both sexes eclosing on day 12 after egg laying were collected and transferred to fresh standard medium for two days to allow mating. Subsequently their lifespan on poor food was measured in the same way as the lifespan of original lines (see above). There were three replicate cages per cross, each with 50 flies.

Statistical analysis

We used SAS and JMP statistical software. For each trait at each food level we carried out an ANOVA. In general, the design involved two selection regimes (the main factor of interest), six replicate selection lines treated as a random factor nested within the selection regimes (biological replicates), and 2-4 replicate rearing vials or demography cages per selection line (technical replicates). The F-test for selection regime used line MS as the error term; for most traits the effect of replicate selection lines was tested over the term corresponding to variation among vials or cages. The exceptions are body weight, where flies emerging from different vials were pooled before a sample was selected for weighing, and fecundity, which was analyzed with a repeated measures ANOVA (see below). Where no significant effect of selection regime was detected, we provide the 95 % confidence intervals on the difference between selected and control lines as an indication of power to detect differences.

For traits measured in parallel on flies raised on the poor and standard food, we also carried out a single ANOVA to test for plastic responses to the food treatment (the main effect of food), and for differences in the effects of selection regime on the two food types (regime × food interaction). The significance of both effects was tested using line nested within regime × food interaction term as the denominator in the F-test (Snedecor and Cochran 1967).

Egg-to-adult viability (including that of flies in the heat and cold shock treatment) was expressed as the proportion of eggs that resulted in an eclosed adult, calculated separately for each replicate vial; this proportion was angularly transformed before the analysis. Individual values of egg-to-adult developmental time and body weight were log-transformed to normalize the distributions. Note that deviations from additivity of effects (i.e., interactions) estimated on log-transformed data correspond to deviations from multiplicative effects on the original (untransformed) scale. Proportional pupal lipid content was angularly transformed. Only one value of average growth rate was calculated for each line and food level, i.e., there is no replication within lines for this trait and the line effect could only be assessed in the joint analysis of growth on both food types.

Fecundity data were analyzed with a repeated-measures ANOVA (using the univariate approach), with females as subjects, selection regime and line (nested within regime) as between-subject factors and day as the within-subject factor. All factors except regime were treated as random. The number of eggs laid by a given females during each 24 h period was

the dependent variable. Four females from selected lines and one from a control line laid no eggs on any of the three days; they were excluded from the analysis reported in the Results, but including them only made a negligible quantitative difference to the statistics and did not affect any conclusions.

The time-to-death data (i.e., adult lifespan on standard and poor food, and starvation resistance) were analyzed using Cox regression (proportional hazard model), with lines nested within selection regimes and cages nested within lines. However, random effects are not implemented in Cox regression. Therefore, as a more conservative approach to test for the effects of selection regime taking lines as biological replicates, we also analyzed these data with an ANOVA, taking life expectancy and median lifespan calculated for each cage as the dependent variable. Even though the individual times to death have non-normal distribution, based on the central limit theorem the average lifespan of 50 individuals will be approximately normal. To correct for censoring, we used product-limit life expectancy estimates calculated for each cage with JMP statistical software. The ANOVA on median lifespan led to the same conclusions as the ANOVA on mean lifespan, so only the latter is reported.

Results

Potential to adapt. As expected, the direct effects of poor larval food included lower egg-to-adult viability, prolonged development, and a 50 % reduction in adult dry body weight, relative to flies reared on the standard food (Fig. 1A-D). The evolutionary adaptation to nutritional stress in our selected lines was opposite to the first two of those plastic responses: on the poor food the selected lines evolved 15 % higher viability (Fig. 1A) and 17 % faster development (Fig. 1B) compared to the unselected control lines (for results of statistical tests see Table 1). In turn the evolutionary change in body weight paralleled the plastic response: when raised on the poor food, the selected flies were smaller than the controls (body weight reduction 14 % for females, 11.5 % for males; Fig. 1C, D, Table 1). Nonetheless, our heuristic estimates of average egg-to-adult growth rates on the poor food were substantially higher for the selected than for the control populations (Fig. 1E, Table 1). Finally, the plastic response to poor larval food was characterized by an increase in the proportional pupal lipid fraction: pupae of both selected and control lines contained proportionally more lipids when raised on

poor than on good food (Fig. 1F, Table 1). In contrast, the evolutionary adaptation to poor food conditions did not lead to an increase in the proportional lipid content (95 % confidence interval for the difference selected – control: –5.1 %, 1.5 %); if anything, the lipid fraction of the selected lines tended to be lower on average than that of the controls (Fig. 1F, Table 1). Thus, even though both the plastic and the evolutionary response to poor food involved a reduction in body size, the contribution of lipid storage to these responses seems to have been different. To summarize, adaptation to our nutritional stress selection regime involved higher egg-to-adult viability and higher average growth rate of the selected lines on the poor food. Despite their faster growth the selected adults were smaller than the controls on poor food because of their substantially shorter development time.

Life history trade-offs

We hypothesized that the adaptation to develop on poor food might be associated with reduction of larval performance on the standard food. This was not the case. The selection regime had no effect on egg-to-adult viability on good food (Fig. 1A, Table 1; the 95 % confidence interval on the difference between the selected and control lines was (–1.9 %, 3.9 %)). Likewise, we detected no difference in the average growth rate on the standard food (Fig. 1E, Table 1). As on the poor food, the selected lines developed significantly faster than the control lines on the standard food, but the difference was much smaller than on the poor food (Fig. 1B, Table 1).

We also found no correlated responses in larval tolerance to heat (3 h at 39 °C) and cold (20 h at 0 °C). Both treatments killed over half of the larvae, but the percentage of survivors did not differ between the selection regimes (heat shock: selected 33.9 ± 2.0 %, control 32.1 ± 2.4 %, $F_{1,10} = 0.3$, $P = 0.58$; cold shock: selected 35.6 ± 3.3 %, control 39.2 ± 2.3 %; $F_{1,10} = 0.8$, $P = 0.39$). The 95 % confidence interval for the difference in percentage surviving the heat shock is (–5.3 %, 9.0 %); the analogous interval for the cold shock is (–12.8 %, 5.5 %). We did find a negative correlated response in an adult fitness component. When raised as larvae and maintained as adults on the standard food, females of the selected lines showed about 20 % lower fecundity over days 5-7 of adult life than the control lines (Fig. 2; $F_{1,10} = 58.5$, $P < 0.0001$). This effect was highly consistent among lines (line effect $F_{10,93.3} = 0.7$, $P = 0.70$) as well as among the three oviposition days (regime × day interaction $F_{2,20} = 0.1$, $P = 0.96$). It is

likely at least in part due to the smaller adult body size of the selected flies; the proportional reduction in body weight of the selected compared to the control lines on the standard food was as large as on the poor food (Fig. 1C,D, no regime × food interaction in Table 1). In spite of differences in body size and early fecundity, females of selected and control lines had on average essentially identical survival curves (Fig. 3A, Table 2). Thus, even though we detected no adverse effects of adaptation to the poor food for larval traits expressed on the standard food, the adults of the selected lines were smaller and had a lower fecundity than the controls.

Larval versus adult nutritional stress

To test if adaptation to larval nutritional stress affected tolerance to adult nutritional stress, we raised flies from the selected and control lines on the standard food and subsequently studied their adult survival on poor food. While mild nutritional stress (dietary restriction) in the adult stage is known to extend lifespan, the very low nutritional content of our poor food resulted in malnutrition, causing most flies to die within four weeks of adult life (Fig. 3B). The selected lines turned out to be more susceptible to this malnutrition (Fig. 3B, Table 2). Thus, the evolution of increased tolerance to larval nutritional stress was associated with reduced tolerance to chronic adult nutritional stress. The selected lines also tended to be somewhat less resistant to starvation (Fig. 3C). Even though Cox regression indicated a significant effect of selection regime on the survival curve under starvation, in the more conservative ANOVA on mean and median times to death the effect of regime was far from significant (Table 2), reflecting the large variation among replicate lines.

Tolerance to nutritional stress may be associated with lower lipid stores. As on the poor food, the selected lines did tend to have a lower proportional lipid content in pupae raised on the standard food (Fig. 1F), but the effect was not significant (95 % confidence interval for the difference selected – control is (–3,6 %, 0.5 %)). Nonetheless, because of their smaller body size the absolute amount of lipids per fly was substantially smaller in the selected than in the control lines (75.9 ± 3.1 versus 91.8 ± 4.3 μg; see Table 1 for statistical tests).

Crosses between replicate lines

Due to being subject to selection, the selected lines probably had a smaller effective population size, and so may have become more inbred than the control populations. Thus, rather than being a correlated response to selection, their reduced size and greater susceptibility to chronic adult nutritional stress might be signs of inbreeding depression. To address this alternative, we assessed these traits in F_1 crosses between replicate selected populations and between replicate control populations. Inbreeding depression is mostly due to random fixation of deleterious recessive alleles, and different alleles would be expected to become fixed in different populations. Crossing replicate populations would thus restore most of the heterozygosity lost to inbreeding. If the differences between the selection regimes were due to differential inbreeding, they should disappear, or at least be much smaller, in the F_1 crosses. This was not the case. F_1 crosses between different selected lines had similar body size (female dry weight) as the original selected lines, and were substantially smaller than crosses between control lines (Fig. 4A, $F_{1,10} = 25.9$, $P < 0.001$). Similarly, crosses between selected lines died on average 4.5 days earlier than crosses between replicate control lines when subject to adult nutritional stress (Fig. 4B,C; Table 2). Thus, the difference between the selected and control populations in body size and susceptibility to adult nutritional stress is not a result of differential inbreeding. (The lifespan of the crosses in this assay was higher than that of original lines reported above, but the two assays were carried out at different times and in different laboratories, so their results cannot be directly compared.)

Discussion

Potential to adapt

Our results indicate that our experimental populations harbored sufficient genetic variation to evolve, within several dozen generations, substantially increased tolerance to chronic larval nutritional stress. The selected populations showed a higher egg-to-adult viability and faster development when raised on the poor food. Even though they reached a smaller adult size, our heuristic measure of average juvenile growth rate was higher for the selected populations growing on the poor food (Fig. 1).

That these populations would adapt to our selection regime was not a foregone conclusion. The only experimental evolution study involving adaptation to poor larval nutrition in *Drosophila* (Bochdanovits and de Jong 2003) does not report if any adaptation to the poor

food in terms of improved viability or growth rate occurred at all. Even though numerous experiments demonstrated that many fitness-related traits do respond to selection (Partridge and Barton 2000; Brakefield 2003), some aspects of performance seem impossible to improve (reviewed in Blows and Hoffmann 2005). For example, apparently none of several natural populations of *Drosophila birchii* has the potential to evolve improved desiccation resistance in response to laboratory selection (Hoffmann et al. 2003; Kellermann et al. 2006).

The populations that gave rise to our base stock had been derived from a single field collection and kept in the laboratory on the standard food under moderate larval density for five years (about 100 generations) before the start of this study. They had thus presumably been already well adapted to the lab conditions and the standard food, we can be confident that these differences between the control and selected populations reflect the evolution of the latter in response to the nutritional stress. Our results also indicate that additive genetic variation for tolerance to nutritional stress can persist under standard laboratory conditions. This variation might have been neutral under those conditions and only persisted because there had not been enough time for drift to eliminate it. It is, however, tempting to speculate that some form of balancing selection has acted in our laboratory cultures to maintain polymorphism at loci relevant for the response to nutritional stress. There is some evidence that competition may result in balancing selection on loci involved in larval resource acquisition (Borash et al. 2000; Fitzpatrick et al. 2007), and similar mechanisms might have been at work in our lab cultures despite only moderate levels of competition. Irrespective of the forces that maintained the genetic variation in the laboratory, together with evolutionary experiments on starvation resistance (reviewed in Rion and Kawecki 2007) and larval crowding (Santos et al. 1997; Borash and Ho 2001), our study indicates that fruit flies have the genetic potential to adapt to various forms of nutritional stress.

The accelerated development of flies from the selected lines could be in part a consequence of physiological adaptation to poor food. However, this would not explain the fact that selection lines also developed slightly faster on the standard food (Fig. 1B). Therefore, the acceleration of development is presumably largely due to truncation selection on developmental time (only flies emerged within 14 days were allowed to reproduce). Selection for fast development on nutritionally poor food is also likely to act in nature. The control flies take much longer to develop to adulthood on the poor food than on the standard food, and, based on our preliminary data, it is clear that the main reason for this delay is a much longer time to pupation. Under natural conditions a decomposing fruit would be unlikely to remain suitable

for the 10-15 days it took the control larvae to reach the pupal stage. The ability to complete larval development relatively fast despite poor nutrition may thus be an important aspect of adaptation to larval malnutrition in nature. However, simultaneous improvement of viability and acceleration of development would have been difficult if there had been a trade-off between these traits; such a trade-off has been reported under good food conditions (e.g., Prasad et al. 2001). Thus, the fact that our flies could simultaneously evolve faster development and higher viability on poor food is an important facet of their potential to adapt to larval nutritional stress.

Life history trade-offs

We hypothesized that adaptation to development on poor food might involve changes in larval physiology that would adversely affect larval viability or growth on the standard food, or their ability to tolerate other forms of stress. We found no evidence for such trade-offs. On the standard food the larvae of the selected lines survived to adulthood as well as those of the control lines, showed the same average growth rate, and developed slightly faster than the control lines. They also showed the same tolerance to heat and cold shock applied to larvae developing on the standard food. These results are consistent with the hypothesis that some of the (at present unknown) physiological changes underlying adaptation to larval nutritional stress involve plastic responses induced by the nutritional stress, but not expressed on the standard food. Alternatively, the improved survival and the apparently faster average growth might involve little change of physiology of food consumption and processing, and be instead largely a by-product of faster development. In the latter case one would, however, expect a negative correlation among the selected lines between developmental time and egg-to-adult viability; if anything, this correlation tended to be positive ($r = 0.31$).

Rather, the costs of adaptation to poor food were manifested in adult fitness-related traits. Adult body size in *Drosophila* typically shows a strong positive genetic correlation with developmental time – faster developing larvae have less time for growth (e.g., Hillesheim and Stearns 1992; Partridge and Fowler 1993; Zwaan et al. 1995a; Nunney 1996; Prasad et al. 2001). It is therefore not surprising that the evolution of faster development in our selection lines was associated with a reduction in adult body size. The proportional reduction in body size of the selection lines was similar in flies raised under poor and standard food conditions

(13.8 versus 14.6 % for females, 11.3 versus 11.5 % for males). In contrast, the reduction in developmental time was much more pronounced on the poor food (17 %) than on the standard food (4 %). Hence, if the shorter developmental time were to be interpreted as a benefit and the smaller body size as a cost, the cost to benefit ratio would be greater on the standard food. The relationship between *Drosophila* body size and fitness under field conditions seems complex (Partridge et al. 1987; Hoffmann and Loeschcke 2006; Hoffmann et al. 2007), but body size is typically positively correlated with fecundity (e.g., Robertson 1957). Given the reduced size of the selected flies, it is thus not surprising that their early-life fecundity was reduced by 21 % relative to the controls. That their decline in fecundity is at least in part due to the smaller body size is corroborated by a marginally significant correlation between mean female body weight and mean fecundity across the six selected lines ($r = 0.80$, $P = 0.054$). Irrespective of the underlying mechanism, a 20 % reduction in fecundity represents a substantial fitness cost.

Early-life fecundity has often been found to show a negative genetic correlation with lifespan (Rose 1984; Partridge and Fowler 1992; Zwaan et al. 1995b), which is interpreted as an allocation trade-off between reproduction and somatic maintenance. However, we observed no such correlation; the selected and control lines did not differ in their lifespan on the standard food. Some studies also reported a positive genetic correlation between developmental time and longevity (Partridge and Fowler 1992; Chippindale et al. 1994). Yet, our selected flies did not have shorter lives despite having faster development. Our paper thus adds to the number of studies indicating that developmental time and adult longevity are evolutionarily independent (Hillesheim and Stearns 1992; Zwaan et al. 1995a, b; Partridge et al. 1999; Bubliy and Loeschcke 2005).

The assays reported here were carried out after varying number of generations of selection. Egg-to-adult viability, developmental time, body weight, lipid content and adult lifespan on standard and poor food were assayed early in the experiment, after 29-32 generations of selection. Larval resistance to heat and cold stress was assayed after 48 generations, while fecundity and adult resistance to starvation only after 63-64 generations. One might expect that the likelihood of detecting a correlated response would increase with the number of generations of selection, and so whether a correlated response in a particular trait was detected would reflect more the timing of the assay than the strength of the relationship of this trait with adaptation to poor food. However, there seems to be no relationship between detection of correlated responses and the number of generations of selection in our study. For example,

while viability on the good food shows no correlated response by generation 29, developmental time and body size measured in the same generation clearly do. Furthermore, no differences in egg-to-adult viability on standard food were observed in another assay carried out at generation 48 (data not shown). Adult lifespan on the standard and poor food were both assayed in generation 30, but only the latter showed a response (see below). Furthermore, assays of body size and developmental time carried out around generation 70 show that the correlated responses in those traits have been stable (unpublished data). Thus, while we cannot exclude future correlated responses in viability, larval thermal stress and lifespan on normal food to continuing selection, the current results suggest that the main life history cost of adaptation to poor food has been reduction in fecundity and possibly other deleterious consequences of smaller body size – and increased susceptibility to adult nutritional stress.

Larval versus adult nutritional stress

Even though the selected lines evolved improved tolerance to chronic larval malnutrition, they became less tolerant to chronic malnutrition at the adult stage, and also tended to be less resistant to acute starvation. This apparent trade-off between larval and adult tolerance to nutritional stress may at least in part be mediated by the differences in developmental time and/or body size. While the evolutionary relationship between these traits and tolerance to chronic adult malnutrition has, to our knowledge, not been addressed, several studies addressed the relationship between these traits and adult starvation resistance. Some of them found positive genetic correlations between starvation resistance and developmental time or body weight (Chippindale et al. 1996; Harshman et al. 1999a; Bubliy and Loeschcke 2005), but others did not (Zwaan et al. 1995b; Borash and Ho 2001; Hoffmann et al. 2005). Starvation resistance is most consistently genetically correlated with high proportional lipid content (Zwaan et al. 1995b; Chippindale et al. 1996; Djawdan et al. 1998; Hoffmann et al. 2005; Vermeulen et al. 2006), and where positive correlation of starvation resistance with body weight was found, it was largely explained by a higher amount of stored lipids rather than by greater structural size (Chippindale et al. 1996; Baldal et al. 2006).

If differences in developmental time, body size and/or lipid content were responsible for the differences in tolerance to adult nutritional stress between the selected and control lines, one

would expect them to be positively correlated with the tolerance across the six replicate selection lines. However, life expectancy on poor food (measured on females raised on standard food) was not correlated with mean female body weight across the selected lines (r = 0.05), and, if anything, tended to be negatively correlated with developmental time (r = –0.52, P = 0.29). The correlation with proportional lipid content on the standard food did tend to be positive (r = 0.42), but far from significant (P = 0.41); besides, the lipid content did not differ systematically between the selected and control lines. Thus, differences in body size and lipid content do not seem to be the principal reason for the lower tolerance of selected lines to chronic adult malnutrition.

Irrespective of the underlying mechanism, the trade-off between larval and adult tolerance to malnutrition would be an important constraint on adaptation to nutritional stress. Even though adult fruit flies tend to utilize a broader range of yeast species, they often feed on the same substrates (Shorrocks 1975; Vacek et al. 1985; Morais et al. 1994). The ecological circumstances leading to malnutrition of the larvae will thus likely impose nutritional stress on the adults as well.

Conclusions

Our study indicates that flies can readily become adapted to develop under chronic malnutrition, and that this adaptation is not associated with adverse effects on larval development in standard conditions. Rather, the ability to survive and develop on a nutritionally poor substrate trades-off with adult body size, fecundity, and adult tolerance to nutritional stress. Adult fruit flies largely feed on the same substrates as the larvae, and so both life stages will usually be affected by nutritional stress at the same time. Hence, a trade-off between larval and adult tolerance to malnutrition would impose an important constraint on adaptation to nutritional stress, especially if high quality food is not available within the adult flying distance.

While natural populations of *Drosophila* often show geographical and altitudinal gradients in starvation resistance, these gradients may be due to correlated responses to selection on other traits rather than differential selection on starvation resistance itself (for review and discussion see Rion and Kawecki 2007). Trade-offs between competitive ability and resistance to nutritional stress have also been proposed as a key factor facilitating coexistence of multiple

Drosophila species (Sevenster and van Alphen 1993a), but empirical support for this hypothesis is mixed (Sevenster and van Alphen 1993b; van der Linde and Sevenster 2006). Adult flies may also avoid laying eggs on poor food. It thus remains unclear how important nutritional stress is as a selective factor for *Drosophila* in nature. However, the mechanisms of response to altered nutritional conditions seem highly conserved over a broad range of animal taxa (Kenyon 2001; Tatar et al. 2003). Thus, even if nutritional stress turns out to be of minor importance in the natural life of fruit flies, they can still provide useful insights into how species that do regularly face nutritional stress adapt to it over evolutionary time.

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Table 1. Summary of analyses of variance (*F* statistics and their significance) on the developmental traits, analyzed jointly on both food types, and separately for each food. For the effect of line, the denominator degrees of freedom are *df* = 36 for viability and developmental time, *df* = 12 for pupal weight and lipid content, and *df* = 228 for body weight.

	Both food types			Poor food		Standard food	
Factor (*df*):	Regime (1)	Food (1)	Regime×Food (1)	Regime (1)	Line (10)	Regime (1)	Line (10)
Trait							
Egg-adult viability	18.4**	113.8***	28.7***	51.4***	1.8	0.5	1.4
Dev. time	178.4***	3781***	78.3***	156.4***	4.0***	38.1***	4.0***
Female weight	28.9***	1902***	0.2	12.3**	3.3***	47.5***	2.7**
Male weight	21.4***	1285***	0	8.8***	3.1***	30.3***	4.2***
Fem. growth rate	51.6***	6892***	28.9***	62.3***	–	0.6	—
Male growth rate	55.9***	5029***	20.4***	64.9***	—	1.9	—
Absolute lipids	11.9**	617.5***	0.6	12.4**	2.2†	9.2*	7.0**
Proportion lipids	2.4	286.9***	0	1.6	1.4	3	0.7

†$P < 0.1$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; all remaining $P > 0.1$.

Table 2. Analysis of adult survival of the selected and control lines on the standard food, the poor food, and under starvation; the last row refers to a comparison of lifespan on poor food of crosses between replicate selection lines with that of crosses between replicate control lines. The reported results come from a proportional hazard model (Cox regression) and F-tests in ANOVA on the mean time to death (life expectancy). ANOVA on median time to death led to the same qualitative conclusions and is not reported.

	Proportional hazards (χ^2)		ANOVA (F)	
Assay	Regime	Line	Regime	Line
Standard food	0.9	118.4***	0	16.4***
Poor food	59.9***	95.0***	5.7*	8.1***
Starvation	30.0***	214.2***	0.8	10.0***
Poor food crosses	62.6***	31.6***	16.1**	2.0†

*$P = 0.037$, **$P < 0.01$, ***$P < 0.001$, †$P = 0.09$; all other $P > 0.4$

Figure legends

Fig. 1. Developmental traits of the selected (gray bars) and control (white bars) populations on the poor and standard larval food (bars indicate selection regime means; dots mark means of individual replicate populations). (A) Viability from egg to adult. (B) Developmental time from egg to adult. (C) Female and (D) male dry body weight at eclosion. (E) Average growth rate from egg to adult, estimated for females (the pattern for males is almost identical). (F) Proportional lipid content in pupae (sexes mixed). Lipid content was measured after 32 generations of selection, the other traits after 29 generations. All populations were maintained for 2-3 generations on standard food before the assays. The *P*-values refer to the effect of selection regime within a given food type; for details of the statistics see Table 1.

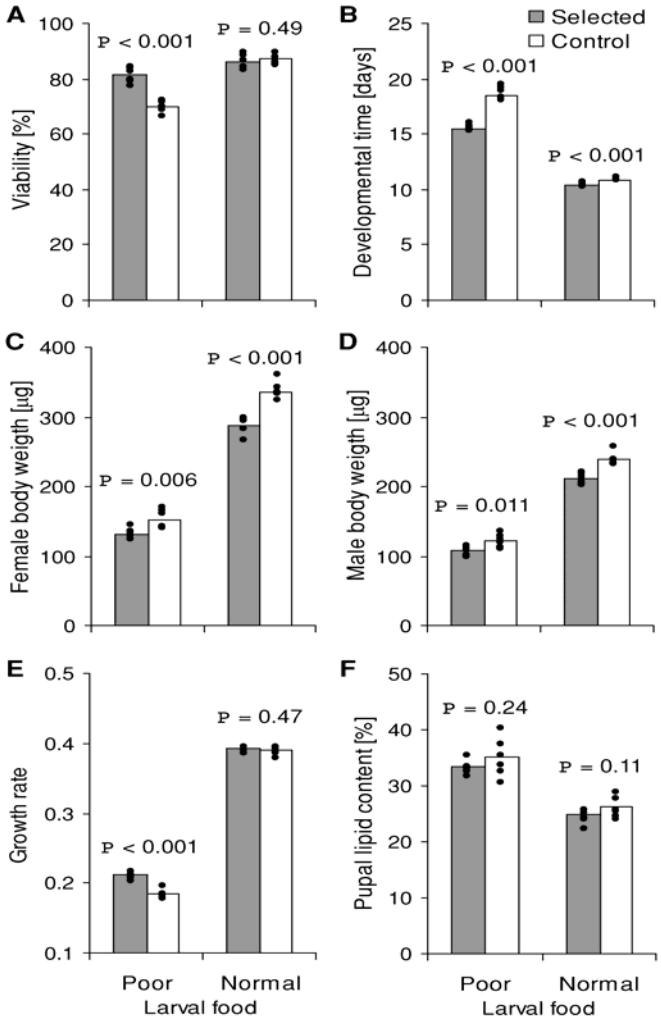

Fig. 2. Early-life fecundity of selected and control lines; "pooled" refers to the average over the three days of the assay. The assay was carried out after 64 generations of selection. Symbols as in Fig. 1.

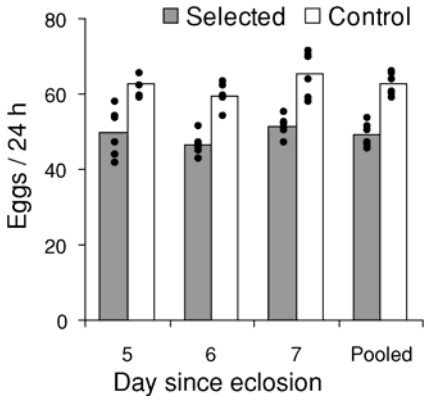

Fig. 3. Adult survival on standard food (A), on poor food (B), and with no food (C). All flies were raised on standard larval food. The top graphs show average survival curves of flies from the two selection regimes, error bars indicate standard errors based on variation among replicate lines. The bottom graphs show the mean time to death for the two regimes (bars) and means of individual replicate lines (dots). Data in panel (A) and (B) were obtained after 30 generations of selection, in panel (C) after 63 generations of selection.

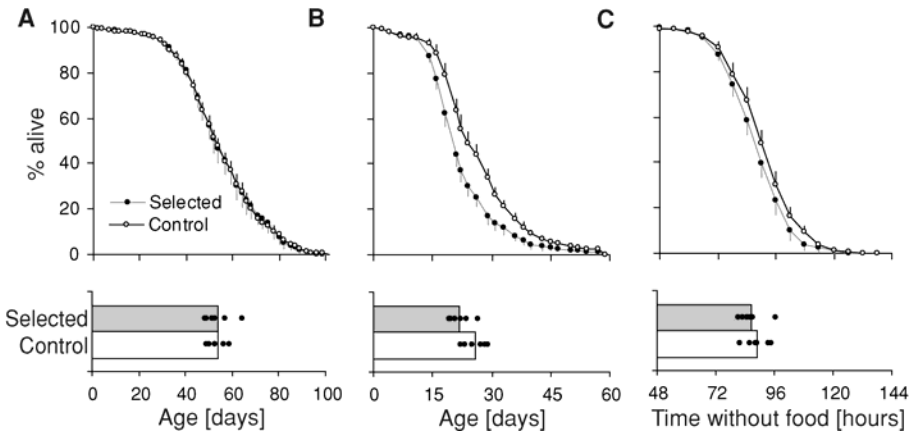

Fig. 4. Dry body weight (A), adult survival curve on poor food (B), and mean adult lifespan on poor food (C) of females of F_1 crosses between replicate selected lines and between replicate control lines. The flies were raised on the standard food. The dots in panels A and C indicate means of individual crosses, the bars overall selection regime means. The bars in panel B indicate standard errors.