Adaptation to developmental diet influences the response to selection on age at reproduction in the fruit fly

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Abstract

Experimental evolution (EE) is a powerful tool for addressing how environmental factors influence life-history evolution. While in nature different selection pressures experienced across the lifespan shape life histories, EE studies typically apply selection pressures one at a time. Here we assess the consequences of adaptation to three different developmental diets in combination with classical selection for early or late reproduction in the fruit fly *Drosophila melanogaster*. We find that the response to each selection pressure is similar to that observed when they are applied independently, but the overall magnitude of the response depends on the selection regime experienced in the other life stage. For example, adaptation to increased age at reproduction increased lifespan across all diets, however, the extent of the increase was dependent on the dietary selection regime. Similarly, adaptation to a lower calorie developmental diet led to faster development and decreased adult weight, but the magnitude of the response was dependent on the age-at-reproduction selection regime. Given that multiple selection pressures are prevalent in nature, our findings suggest that trade-offs should be considered not only among traits within an organism, but also among adaptive responses to different – sometimes conflicting – selection pressures, including across life stages.

Introduction

One of the central tenets of life-history evolution is that individuals cannot simultaneously optimize all fitness-related traits due to constraints (Roff, 1992, Roff, 2001, Stearns, 1992). These constraints can emerge because individuals have limited resources at their disposal and must make allocation decisions between competing functions (physiological constraints; Van Noordwijk & de Jong, 1986, de Jong & van Noordwijk, 1992) or because traits have a shared genetic basis (genetic constraints). Such constraints can lead to trade-offs between traits, such that an increase in one trait comes at the expense of another (Stearns, 1992).

A powerful approach for understanding how life histories and trade-offs evolve in response to specific environments is through the use of experimental evolution (EE). EE allows the

43 experimenter to impose carefully controlled selective conditions in the laboratory and then
44 observe evolutionary responses in real time (Kawecki et al., 2012). Two areas in which EE
45 studies have been applied to great effect are in understanding how available nutrition influences
46 life history evolution (Kolss et al., 2009, Kristensen et al., 2010, Leftwich et al., 2016,
47 Chippindale et al., 1996, Bubliy & Loeschcke, 2005, Baldal et al., 2006, Zajitschek et al., 2016)
48 and in testing the classical theories of the evolution of ageing (Luckinbill et al., 1984, Rose,
49 1984, Partridge & Fowler, 1992).
50
51 EE studies manipulating available nutrition have identified several correlated changes in life
52 history traits, with the magnitude and direction of the response depending on whether the dietary
53 manipulation is applied during development or in adulthood. Adaptation to low resource
54 availability during development typically results in decreased adult weight (Kolss et al., 2009,
55 Kristensen et al., 2010), faster development (Kolss et al., 2009, Leftwich et al., 2016), and lower
56 fecundity (Kolss et al., 2009), while effects on lifespan are small or absent (Kolss et al., 2009). In
57 contrast, adaptation to low resource availability or starvation resistance during adulthood leads to
58 slower development, increased lipid accumulation, larger adult size, increased lifespan, and
59 increased male fitness (Chippindale et al., 1996, Bubliy & Loeschcke, 2005, Baldal et al., 2006,
60 Zajitschek et al., 2016, but see Hoffmann et al., 2005).
61
62 EE studies testing the classical theories of ageing have applied selection for later ages at
63 reproduction and show that increased lifespan can reliably evolve. (Luckinbill et al., 1984, Rose,
64 1984, Partridge & Fowler, 1992). In most cases, decreased early or life-long fecundity is observed
65 as a correlate of lifespan extension, suggesting a trade-off between lifespan extension and
66 fecundity, as predicted by the disposable soma theory (Zwaan, 1999, Kirkwood & Holliday, 1979,
67 Kirkwood & Rose, 1991).
68
69 Notably, the experiments described above each address the life history consequences of
70 adaptation within a single life-stage and to a single selection pressure (variation in diet or

71 selection on increased age at reproduction). However, in nature individuals will need to cope
72 with multiple, potentially conflicting selection pressures (e.g. Lankau, 2007, Tarwater &
73 Beissinger, 2013) experienced at different stages across the lifespan. Thus, they must balance the
74 relative costs and benefits of adaptation and resource allocation made at one life stage with those
75 at other stages (reviewed in Schluter *et al.,* 1991). Indeed, EE studies applying more than one
76 selection pressure within a single life stage reveal that the responses to multiple selection
77 pressures tend to be interdependent (Davidowitz et al., 2016, Bochdanovits & Jong, 2003), yet
78 also – despite constraining correlations among traits – there is potential for independent
79 evolutionary change (Beldade & Brakefield, 2002, Frankino et al., 2005). To date, however,
80 there has been little emphasis on how multiple selection pressures influence life histories as a
81 whole. In particular, to the best of our knowledge, no study to date has combined two selection
82 regimes experienced at different stages across an organism's lifespan.
83
84 Here we combine variation in available nutrition during development with classical selection for
85 early or late reproduction during adulthood in a single fully-factorial EE design, using the fruit
86 fly, *Drosophila melanogaster* (Fig. 1a). Empirical work suggests that the two selection regimes
87 might exert opposing selection pressures, which will have to be integrated into the life history.
88 For example, adaptation to a poor quality diet generally selects for faster development coupled
89 with smaller adult size and decreased fecundity (Bochdanovits & Jong, 2003, Kolss et al., 2009),
90 whereas longer lifespan (the typical response to selection on increased age at reproduction) is
91 generally correlated with longer developmental time and larger size (Lints, 1978, Economos,
92 1980, Promislow, 1993, Khazaeli et al., 2005, but see Zwaan et al., 1991).
93
94 Our experimental design allows us to address several fundamental problems, including the
95 question of whether adaptation to environmental variation in each stage occurs independently.
96 For example, will a lower calorie developmental diet constrain the ability to extend lifespan in
97 response to selection on age at reproduction, or will lifespan extension be achieved at the
98 expense of other traits? To address this issue we assess the evolutionary responses of several life-

history traits. These include larval survival, developmental time, and adult weight, all of which have previously been found to evolve in response to larval acquisition (e.g. Bochdanovits & Jong, 2003, Kolss et al., 2009), as well as adult lifespan and fecundity, the two traits that commonly trade off in response to selection on age at reproduction (Luckinbill et al., 1984, Rose, 1984). Furthermore, we assay traits over multiple generations and in multiple larval dietary environments to gain insight into the temporal dynamics of evolution, and the evolution of phenotypic plasticity.

Materials and Methods

Design of the experimental evolution experiment

We combined three levels of larval diet (0.25, 1.0 and 2.5) with two ages at reproduction (Early and Late) in a fully factorial design (Fig.1a, inset). The larval diets differed only in sugar and yeast content with the 0.25 and 2.5 diets containing 25% and 250% as much sugar and yeast as the 1.0 diet, respectively (Table S1). These diets were chosen to fall within the range typically applied in studies of diet and life history in *D. melanogaster* (Zajitschek et al., 2016, Magwere et al., 2004, Lee et al., 2008). The early (E) and late (L) reproducing populations had generation times of 14 and 28 days respectively, thus adults laid eggs for the subsequent generation roughly two to four days post-eclosion in the Early (E) lines, and 16 to 18 days post-eclosion in the Late (L) lines (Fig. 1a). For each combination of larval diet and age at reproduction, we established four independent replicate lines (3 larval diets x 2 ages at reproduction x 4 replicate lines = 24 lines total; Fig. 1a, inset). All lines were maintained on the 1.0 diet as adults both in the course of evolution and in all experiments. We refer to the EE lines by their larval diet (0.25, 1.0, 2.5), their age at reproduction (E, L), or the combination of the two (0.25-E, 0.25-L, 1-E, 1-L, 2.5-E and 2.5-L) throughout. Since the diet and age-at-reproduction conditions of the 1-E lines mimic those of our standard laboratory maintenance regime, their responses can be considered representative of the baseline response both in terms of plasticity and the evolutionary response across generations. Lines were maintained

125 throughout under standard laboratory conditions (25°C, 65% relative air humidity, 12 h:12 h light
126 : dark cycle).
127

128 **Generating the starting population and initiating experimental evolution**

129 To ensure ample standing genetic variation the EE populations were derived from six populations
130 of flies collected along a latitudinal gradient across Europe (Fig. 1b). These populations were
131 maintained in the laboratory for 40 generations to allow for laboratory adaptation and then
132 combined into a single panmictic, genetically diverse baseline population, the starting ("S")
133 population, using a multi-generation crossing scheme (Fig. 1c; see May et al., 2015 for full
134 details of the crossing scheme). This scheme was employed to minimize linkage disequilibrium
135 and to ensure equal contributions of the component populations to the final "S" population. After
136 crossing, the "S" population was maintained under standard laboratory conditions for a further 10
137 generations at a population size of ~ 4000 individuals.
138

139 To initiate EE, eggs were collected from the "S" population into large glass bottles (500 mL
140 volume) filled with 65 mL of the respective larval diets. Two bottles of ~ 1000-2000 eggs were
141 collected per replicate line and allowed to develop to adulthood. For each larval diet four lines
142 were randomly assigned to the early (E) and late (L) reproduction regimes. Ten days after egg
143 laying (Monday) we transferred all newly eclosed adults into fresh bottles containing the 1.0 diet.
144 Populations were then transferred to fresh bottles of 1.0 medium every Monday, Wednesday, and
145 Friday until their respective ages at reproduction. Since larval diet affected developmental time,
146 not all adults from all lines had emerged by day 10, so on days 12 and 14 any additional late-
147 eclosing adults from the developmental bottles were added to the adult population bottles to
148 mitigate truncation selection on developmental time (Fig. 1a). Very few flies eclosed after day
149 12.
150

The day before populations reached their respective ages at reproduction, 1/16 of a teaspoon of dry yeast (Fermipan Red Instant dry bakers yeast) was added to each bottle to stimulate females to lay eggs. The following day females were transferred to fresh bottles containing their evolutionary larval diets and allowed to lay eggs. A test-tube cap containing dry yeast mixed with water was suspended in the bottle and removed when egg laying was complete so as not to modify yeast levels in the developmental diet. To control egg densities, bottles were visually inspected, and adult flies were removed from bottles when egg density was between ~ 1000-2000 eggs, typically over a period of two to four hours. Every generation, both replicate bottles within a line were mixed. Overall, population size was ~ 2000 to 4000 adult flies per replicate line over the course of EE.

Assessing changes in life history traits over the course of evolution

We measured four key life-history traits: egg-to-adult development time, mated female fecundity, mated lifespan and adult wet weight. We assayed these traits across eight independent phenotyping sessions, ranging from the beginning of EE up to generations 38 and 19 for E and L lines, respectively. Figure 1d provides an overview of each phenotyping session (P1-P8), including the elapsed generations of EE, the lines included, the traits measured, and the larval conditions under which flies were raised (i.e., assay environment). We deliberately chose larval diets that had negligible effects on larval survival to avoid population bottlenecks and strong viability selection. Larval survival ranged from 80-95% across evolutionary larval diets and assay conditions in all but one phenotyping session (Supplementary Figure 1) and did not show any systematic variation across selection regimes (Table 2 and Supplementary Figure 1), suggesting that larval survival was not under selection.

Whenever possible we measured the responses to selection in all lines and used all three larval assay diets. However, the scale of our design imposed some logistical constraints. In some phenotyping sessions, we monitored the progress of adaptation on the 1.0 larval assay diet only, while in others we raised larvae on all three diets. In all cases, we first allowed lines to develop

for one generation on the 1.0 diet to avoid potential maternal effects. Larvae developed at a density of 70 eggs per vial, with 6 mL of food per vial. For each line, eggs were collected from petridishes and randomized across assay diets.

We assessed development time and survival from egg to adult in all eight phenotyping sessions (Fig. 1d; n=5 vials per combination of line and assay diet). We scored developmental time until no new flies emerged over a period of 48 hours and then summed across the resulting adults to obtain a measure of egg-to-adult survival (proportion viability). While using vials allowed easier standardization of egg densities and more accurate counting of eclosing adults, development took ~ 24 hours longer in vials than in the EE population bottles.

Mated lifespan and fecundity were assessed on the evolutionary larval diet and on the 1.0 diet The size of this experiment necessitated two assays (Fig. 1d): in the first round, we tested all lines adapted to 0.25 or 1.0 larval food on these two diets (P7), and in the second round we tested all lines adapted to 1.0 and 2.5 larval diet across all three larval diets (P8). The 1.0 lines served as a reference to facilitate comparisons between the 0.25 and 2.5 lines and to monitor consistency of responses across both assays. For mated lifespan, we housed flies at a density of three males and three females per vial (n=10 vials per combination of line and larval diet). Flies were transferred to fresh vials and survival was scored every Monday, Wednesday, and Friday.

Mated fecundity was measured over three time spans: Early (days 2-4 of adulthood), Late (days 18-21) and Post-selection (days 25-28) with the Early and Late time points overlapping the ages at reproduction of the E and L lines. In the first assay (P7), we maintained a single male-female pair per vial (n=15 vials per line and larval food combination), while in the second assay (P8) we maintained two males and two females per vial (n=10 vials per line and larval food combination). Eggs were allowed to develop to adulthood and emerging adults were counted to score fecundity. Sperm depletion was prevented by replacing dead males with new males from the same experimental conditions.

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207 Wet weight of adult males and females raised on the 1.0 assay diet was obtained in generations
208 144 and 73 of the E and L lines respectively. All 24 EE populations were reared in small bottles
209 (200 mL) with 25 mL 1.0 food at a density of 600-800 eggs per bottle (Clancy & Kennington,
210 2001). After eclosion, males and females were housed together until weighing (i.e., they were
211 mated). Weight was measured at two time points chosen to mimic the conditions of the EE
212 procedure: 14 days after egg laying (~4-5 days after emergence) and 28 days after egg laying
213 (~18-19 days after emergence). The weight of the flies was measured on an ultramicro balance
214 (Sartorius Cubis Ultramicro Balance MSE) using batches consisting of two flies each (n=10).
215 Prior to the assay, all populations were first reared for two generations on 1.0 medium. This
216 assay was performed later than the other life-history assays, however, the results were consistent
217 with an interim measurement made on a subset of the lines at ER generation 100 and LR
218 generation 50 (data not shown).

219

220 **Statistical analysis**

221 All statistical analyses were performed in R version 3.2.0 (R Development Core Team, 2005). We
222 fitted a separate model for each trait within each phenotyping session. In each model we included
223 evolutionary dietary regime, age at reproduction, assay diet, sex (where applicable), and their
224 interactions as explanatory variables. We used mixed-effects models to accommodate the random
225 effect of replicate line nested within a selection regime. Both developmental time and mated
226 longevity were analyzed using mixed-effects Cox regression (proportional hazards) models
227 (coxme package; Therneau, 2015), while larval survival and fecundity were analyzed with
228 generalized linear mixed models (GLMM) with binomial and Poisson error distributions,
229 respectively (lme4 package; Bates et al., 2015). Weight was analyzed using a linear mixed-effects
230 model with a normal distribution. In the statistical tables (see below), we report the χ^2values of the
231 effect of each factor in the full model as obtained by Analysis of Deviance (car package; Fox &
232 Weisberg, 2010). We performed further model simplification by sequentially dropping non-

significant terms from the model and using a χ^2 test to compare models. To control for multiple comparisons we applied the sequential Holm–Bonferroni correction method to each fitted model (Holm, 1979) and the Tukey's range test for all post-hoc comparisons among means (Tukey, 1949).

For each trait assessed across multiple assay diets we fitted an additional model for the 1-E lines alone (unselected control lines) to determine the baseline plastic response. An inconsistent response of the 1-E lines across generations might indicate that the trait in question is sensitive to slight differences in developmental conditions; in this case, differences among lines might be highly dependent on variations in assay conditions and may thus not reflect robust evolutionary responses (cf. Ackermann et al. 2001).

Results

Developmental time depends on selection regime by assay diet interactions

Over the course of evolution, assay diet was the most important factor influencing developmental time (Table 1; Fig. 2). The 0.25, and to a lesser extent, 2.5 assay diet consistently increased developmental time relative to the 1.0 diet across all EE lines and phenotyping sessions (Fig. 2 $p<0.00001$ in all but one contrast). However, it is noteworthy that the mean duration of development on each of the three assay diets fluctuated greatly across phenotyping sessions (Fig. 2). Such variation is not uncommon in repeated measures of developmental time (e.g. Zwaan et al., 1995). To account for this, we plotted both the absolute values of developmental time per assay diet across phenotyping assays (Fig. 3 a-c) and relative to the mean of the 1-E lines (Fig. 3d-f).

In the early generations of EE (P1-P5) no consistent changes in developmental time were observed (Fig. 3). However, from P6 (E and L generations 30 and 15, respectively) onwards a consistent three-way interaction emerged between evolutionary larval diet, age at reproduction, and assay diet for the 0.25-E and 0.25-L lines (Table 1; Fig. 3). Both sets of lines evolved

substantially more rapid development on the 0.25 assay diet as compared to the 1-E lines (Fig. 3b,d). For the 0.25-E lines this effect was already present in P5 (P5 through to P8; all p-values <0.001), while for the 0.25-L lines it became apparent from P6 onwards (P6 through to P8; all p-values <0.001; Fig. 3b,e), although there was a trend for the magnitude of the effect to be smaller for the 0.25-L than 0.25-E lines (P5: p<0.0001; P6: p=<0.01; P7: p=0.33; P8: p=0.26). By contrast, on the 1.0 and 2.5 assay diets the responses of the 0.25-E and 0.25-L lines were not consistent across phenotypings (Fig. 3a,c,d,f). Relative to the strength and consistency of the response of the 0.25-E and 0.25-L lines, the 1-L and 2.5-E and 2.5-L lines did not show substantial or consistent changes in developmental time, suggesting that these regimes did not impose strong selection on the length of development.

Selection on age at reproduction increases lifespan across dietary selection regimes

We found that selection for increased age at reproduction increased lifespan in all lines and across all assay diets (Fig. 4; all p-values <0.03). However, the magnitude of the effect was dependent on sex and the evolutionary dietary regime for 0.25 lines and evolutionary dietary regime and assay diet for 2.5 lines, suggesting that adaptation to different levels of laerval acquisition can modify the response to selection on lifespan (Table 3).

For the 0.25-E and 0.25-L lines males and females had inverse responses to selection relative to the 1-E and 1-L lines (Table 3; Fig. 4a,b). In females, the lifespan of the 0.25-L lines was indistinguishable from that of 1-L lines (p=0.94), but 0.25-E lines had greater lifespans relative to the 1-E lines (p<0.0001) (Fig. 4a). In males, the exact inverse response was observed: while the 0.25-E and 1-E lines had similar lifespans (p=0.96), the lifespan extension of 0.25-L lines was less than that of the 1-L lines (p=0.01; Fig. 4b). These effects were consistent across both the 0.25 and 1.0 assay diets.

For the 2.5-E and 2.5-L lines, lifespan evolved in a similar manner in both sexes, but was dependent on assay diet (Table 3 and Figure 4c,d). Under 0.25 and 1.0 assay conditions, the

lifespan of the 2.5L lines did not differ from the 1-L lines (add p-value for both sexes), however, under 2.5 assay conditions 2.5-L flies evolved significantly shorter lifespans than 1-L lines in males (p=0.002, Fig. 4d) and nearly significantly shorter lifespans in females (p=0.08, Fig. 4c). The 2.5E lines showed an inverse pattern: lifespan on the 0.25 and 1.0 assay diets was generally higher for 1-E lines than for 2.5-E lines, whereas males and females of the 2.5-E lines outlived 1-E flies on the 2.5 assay diet (Fig. 4c,d; Males: on 0.25 and 1.0 diet 1-E>2.5-E, p=0.003 and 0.004, respectively; under 2.5 assay conditions 1-E=2.5-E, p=0.66. Females: on 0.25 assay diet 1-E=2.5-E, p=0.42; on 1.0 assay diet 1-E>2.5-E lines, p=0.02; and under 2.5 assay diet 1-E<2.5-E lines, p=0.01).

Fecundity is highly variable across phenotyping sessions

Because it was not possible to measure lifespan and fecundity for all lines at the same time, we used the 1-E and 1-L lines as a standard across the two replicate phenotyping sessions (see Materials and Methods). For mated fecundity, the plastic response of the 1-E lines to assay diet differed between the two phenotyping sessions (Table 2, Fig. 5). In the first phenotyping (P7) 1-E flies raised on the 0.25 assay diet had lower fecundity than those raised on the 1.0 assay diet at all three ages (Fig. 5a-c; all p-values<0.001). In the second assay (P8), the same effect was observed at early and post-selection ages (all p-values<0.001), but reversed at the late reproduction time point (p<0.001; Fig 5d, f). Furthermore, the difference between the 1-E and 1-L lines on the 1.0 assay diet was also inconsistent between assays P7 and P8 (Fig. 6). In P7 the E lines reproduced more than the L lines at the “Mid” time point and less at the “Late” time point, while in P8 the opposite pattern was observed (Fig.6, both p-values <0.003). Thus, while the GLMM’s indicated that fecundity at all ages was affected by interactions between diet regime, age at reproduction regime, and assay conditions (Table 4), the lack of consistency of the 1-E and 1-L lines hampers the interpretation of the evolutionary significance of these effects.

Adult weight

313 Adult weight evolved in response to the selection regimes in a sex- and age-dependent manner
314 (Fig. 7a-d). The largest effects of the EE regimes occurred in young flies (4-5-days post-
315 eclosion): in both sexes adaptation to later ages-at-reproduction led to larger adult size (Females:
316 $F_{1,24}$=8.4, p =0.01; Fig. 7a; Males: $F_{1,24}$=43.7, p=<0.0001; Fig.7b), and adaptation to the 0.25
317 larval diet decreased adult weight relative to 1.0 and 2.5 adapted lines (Females:$F_{2,24}$=10.9, p
318 =0.001; Fig. 7a; Males: $F_{2,24}$=29.6, p=<0.0001; Fig.7b; all pair-wise p-values <0.01). In females,
319 there was a marginal interaction between age at reproduction and evolutionary dietary regime
320 ($F_{2,24}$=2.7, p =0.09, Fig. 7a). While the 0.25-L and 1-L lines both evolved increased weights
321 relative to the 0.25-E and 1-E lines, the weight of the 2.5-E and 2.5-L lines did not differ (2.5-L =
322 2.5-E, p=1.0). At 18-19 days post-eclosion the effects of evolutionary regime became much
323 smaller and differed between the sexes. In males, the effect of EE regime was largely absent,
324 except in 0.25-E lines, which continued to weigh less than all other lines (all pairwise p-values
325 <0.003; Fig. 7d), while in females, only evolutionary dietary regime remained significant
326 ($F_{2,24}$=9.1, p=0.001, Fig. 7c), with weight increasing with increasing evolutionary larval diet (all
327 pairwise p-values <0.05). We also found large effects that were independent of the evolutionary
328 regimes: males weighed less than females (Sex: $F_{1,936}$=11644, p=<0.0001) and, while females
329 gained weight with age, males tended to lose or maintain the same weight (Sex x Age:
330 $F_{1,936}$=314.3, p=<0.0001; Fig.7).
331
332 **Discussion**
333 How different selection pressures interact to affect life-history adaptation is an unresolved
334 question. By utilizing the combined strength of extensive replication, multiple assay
335 environments, and assessment of evolution across multiple generations we were able to
336 discriminate between transient and consistent effects of adaptation to larval diet and age at
337 reproduction. We discuss our main findings in the light of theoretical predictions and previous
338 work.
339
340 **Adaptive responses reflect the influence of both selection regimes**

For both selection regimes we observed similar changes in life history traits to those observed in previous, univariate studies. That is, adaptation to increased age at reproduction increased lifespan across all evolutionary dietary regimes and in both sexes (Luckinbill et al., 1984, Rose, 1984, Partridge & Fowler, 1992), while selection on the 0.25 larval diet resulted in faster development (Fig. 3b,e), decreased adult weight (Fig. 7), and potentially lower fecundity (Fig.5a-c), again, in keeping with previous univariate selection experiments (Kristensen et al., 2010, Kolss et al., 2009). However, in both cases, we found that the addition of a second regime modified the magnitude of the responses. Thus the extent of the increase in lifespan imposed by selection for later age at reproduction was dependent on dietary regime (Fig. 4) and conversely, the changes in weight, length of development and potentially fecundity seen in the 0.25-E lines were modified by adding selection for late reproduction. Thus, while the two regimes continue to select for similar adaptive responses, the overall magnitude of the response depends on the interplay with the selection pressure experienced in the other life stage.

Fecundity: significant but inconsistent responses

Previous EE designs selecting on later age at reproduction also found inconsistent responses of fecundity across generations (Leroi et al., 1994a), or marked sensitivity to environmental variation (Leroi et al., 1994b). However, we observed strongly significant effects of both age at reproduction and evolutionary dietary regime in both phenotyping sessions (Table 4). For example, 0.25-E lines appeared to have decreased fecundity relative to 0.25-L, 1-E and 1-L lines at all ages (Fig. 5a-c), a response that is consistent with their lower body weight and faster development (Fig. 3b,e and Fig. 7). Given the large replication of our design (i.e., independent replicate populations per EE treatment) it is plausible that these responses represent adaptive responses to poor nutrition.

However, what hampers firm conclusions about fecundity are the inconsistent phenotypes of the 1.0 line females across the P7 and P8 sessions (Fig. 6). The slightly different assay conditions

368 between the two phenotyping sessions (one male and one female per vial in P7 vs. two females
369 and two males per vial in P8) present one potential cause as females are known to adjust their
370 fecundity based on density (e.g. Barker, 1973; this study compares the difference between vial
371 densities of 5 and 50 females or more). Slight changes in environmental conditions (e.g., note the
372 considerably faster development in P8 relative to P7; Fig. 2) might also have affected overall
373 patterns of fecundity. Clearly, further experiments (e.g., tracking fecundity across the entire
374 reproductive span) are necessary to clarify this issue.
375

376 **Does adult body size drive patterns of life history adaptation?**

377 In many studies body size correlates positively with developmental time, lifespan, and fecundity
378 (see above and Robertson, 1957, Hillesheim & Stearns, 1992, Honěk, 1993, Zwaan et al., 1995,
379 Prasad et al., 2001). Our results also showed such correlations; for instance, selection for late life
380 reproduction extended lifespan and increased adult weight for males and females alike. However,
381 these correlations are unlikely to constrain the evolution of the life history adaptations, but will
382 rather modulate them. For instance, while selection for late reproduction consistently increased
383 lifespan for the 0.25 lines, these lines also sped up their development and reduced their weight
384 relative to the 1 and 2.5 lines. Furthermore, the fact that the differences in body weight between
385 early and late life populations were large in early life, but disappeared later in life (at the time of
386 actual selection for the late lines) for 1 and 2.5 but not 0.25 lines (Fig. 7), suggests that body size
387 evolved for a different reason in these lines relative to the 0.25 lines. For instance, increased
388 body size as a response to late life reproduction in the 0.25 lines may serve to increase fecundity
389 in the face of decreased adult weight as an adaptive response to the larval nutritional condition,
390 while in the 1 and 2.5 lines increased body size it may be related to increasing lifespan.
391

392 **Conclusions**

393 Our results suggests that adaptation during one life stage may be contingent on the selection
394 pressures experienced in other stages, and that adaptation to two different selection pressures can

lead to different life history strategies to those found when adapting to only one selection pressure at a time. In particular, the dependence of lifespan extension on evolutionary developmental diet suggests that developmental acquisition can be an important factor influencing longevity. While there is still relatively little empirical work on adaptation to multiple or opposing selection pressures (but see: Lankau, 2007, Tarwater & Beissinger, 2013), their prevalence in nature means that a better understanding can further our understanding of evolution under natural conditions (reviewed in Schluter et al., 1991). Indeed, the idea that opposing selection pressures constrain trait evolution is one of the hypotheses put forward to explain why, despite strong consistent directional selection on many traits, there is often little change in trait means across generations in natural populations (Merilä et al., 2001, Kingsolver & Diamond, 2011, Siepielski et al., 2011). Given that multiple selection pressures are likely the norm rather than the exception in nature, our findings suggest that trade-offs should be considered not only between traits within an organism, but also between adaptive responses to differing selection pressures.

410 **References**

411 Baldal, E. A., Brakefield, P. M. & Zwaan, B. J. 2006. Multitrait evolution in lines of *Drosophila*
412 *melanogaster* selected for increased starvation resistance: the role of metabolic rate and
413 implications for the evolution of longevity. *Evolution* **60**: 1435-1444.

414 Barker, J. S. F. 1973. Adult population density, fecundity and productivity in Drosophila
415 melanogaster and Drosophila simulans. *Oecologia* **11**: 83-92.

416 Bates, D., Maechler, M., Bolker, B. & Walker, S. 2015. Fitting linear mixed-effects models using
417 lme4. *Journal of Statistical Software* **67**: 1-48.

418 Beldade, P. & Brakefield, P. M. 2002. The genetics and evo-devo of butterfly wing patterns.
419 *Nature Reviews Genetics* **3**: 442-452.

420 Bochdanovits, Z. & Jong, G. d. 2003. Experimental evolution in *Drosophila melanogaster*:
421 Interaction of temperature and food quality selection regimes. *Evolution* **57**: 1829-1836.

422 Bubliy, O. A. & Loeschcke, V. 2005. Correlated responses to selection for stress resistance and
423 longevity in a laboratory population of Drosophila melanogaster. *Journal of*
424 *Evolutionary Biology* **18**: 789-803.

425 Chippindale, A. K., Chu, T. J. & Rose, M. R. 1996. Complex trade-offs and the evolution of
426 starvation resistance in *Drosophila melanogaster*. *Evolution*: 753-766.

427 Clancy, D. J. & Kennington, W. J. 2001. A simple method to achieve consistent larval density in
428 bottle cultures. *Drosophila Information Service* **84**: 168-169.

429 Davidowitz, G., Roff, D. & Nijhout, H. F. 2016. Synergism and antagonism of proximate
430 mechanisms enable and constrain the response to simultaneous selection on body size
431 and development time: An empirical test using experimental evolution. *The American*
432 *Naturalist* **188**: 499-520.

433 de Jong, G. & van Noordwijk, A. J. 1992. Acquisition and allocation of resources: Genetic (co)
434 variances, selection, and life histories. *The American Naturalist* **139**: 749-770.

435 Economos, A. 1980. Taxonomic differences in the mammalian life span-body weight
436 relationship and the problem of brain weight. *Gerontology* **26**: 90-98.

437 Fox, J. & Weisberg, S. 2010. *An R companion to applied regression*. Sage.

438 Frankino, W. A., Zwaan, B. J., Stern, D. L. & Brakefield, P. M. 2005. Natural selection and
439 developmental constraints in the evolution of allometries. *Science* **307**: 718-720.
440 Hillesheim, E. & Stearns, S. C. 1992. Correlated responses in life-history traits to artificial
441 selection for body weight in *Drosophila melanogaster*. *Evolution* **46**: 745-752.
442 Hoffmann, A. A., Hallas, R., Anderson, A. R. & Telonis-Scott, M. 2005. Evidence for a robust
443 sex-specific trade-off between cold resistance and starvation resistance in *Drosophila*
444 *melanogaster*. *Journal of Evolutionary Biology* **18**: 804-810.
445 Holm, S. 1979. A simple sequentially rejective multiple test procedure. *Scandinavian journal of*
446 *statistics*: 65-70.
447 Honěk, A. 1993. Intraspecific variation in body size and fecundity in insects: a general
448 relationship. *Okios* **66**.
449 Kawecki, T. J., Lenski, R. E., Ebert, D., Hollis, B., Olivieri, I. & Whitlock, M. C. 2012.
450 Experimental evolution. *Trends in Ecology & Evolution* **27**: 547-560.
451 Khazaeli, A. A., Van Voorhies, W. & Curtsinger, J. W. 2005. The relationship between life span
452 and adult body size is highly strain-specific in *Drosophila melanogaster*. *Experimental*
453 *Gerontology* **40**: 377-385.
454 Kingsolver, J. G. & Diamond, S. E. 2011. Phenotypic selection in natural populations: what
455 limits directional selection? *The American Naturalist* **177**: 346-357.
456 Kirkwood, T. B. L. & Holliday, R. 1979. The evolution of ageing and longevity. *Proceedings of*
457 *the Royal Society of London B: Biological Sciences* **205**: 531-546.
458 Kirkwood, T. B. L. & Rose, M. R. 1991. Evolution of Senescence: Late Survival Sacrificed for
459 Reproduction. *Philosophical Transactions of the Royal Society of London B: Biological*
460 *Sciences* **332**: 15-24.
461 Kolss, M., Vijendravarma, R. K., Schwaller, G. & Kawecki, T. J. 2009. Life-history
462 consequences of adaptation to larval nutritional stress in Drosophila. *Evolution* **63**: 2389-
463 2401.

464 Kristensen, T. N., Overgaard, J., Loeschcke, V. & Mayntz, D. 2010. Dietary protein content
465 affects evolution for body size, body fat and viability in *Drosophila melanogaster*.
466 *Biology Letters*.
467 Lankau, R. A. 2007. Specialist and generalist herbivores exert opposing selection on a chemical
468 defense. *New Phytologist* **175**: 176-184.
469 Lee, K. P., Simpson, S. J., Clissold, F. J., Brooks, R., Ballard, J. W. O., Taylor, P. W., Soran, N.
470 & Raubenheimer, D. 2008. Lifespan and reproduction in Drosophila: new insights from
471 nutritional geometry. *Proceedings of the National Academy of Sciences* **105**: 2498-2503.
472 Leftwich, P. T., Nash, W. J., Friend, L. A. & Chapman, T. 2016. Adaptation to divergent larval
473 diets in the medfly, *Ceratitis capitata*. *Evolution* **71**: 289-303.
474 Leroi, A. M., Chen, W. R. & Rose, M. R. 1994a. Long-term laboratory evolution of a genetic
475 life-history trade-off in *Drosophila melanogaster*. 2. Stability of genetic correlations.
476 *Evolution* **48**: 1258-1268.
477 Leroi, A. M., Chippindale, A. K. & Rose, M. R. 1994b. Long-term laboratory evolution of a
478 genetic life-history trade-off in *Drosophila melanogaster.* 1. The role of genotype-by-
479 environment interaction. *Evolution* **48**: 1244-1257.
480 Lints, F. A. 1978. *Genetics and ageing*. S. Karger.
481 Luckinbill, L. S., Arking, R., Clare, M. J., Cirocco, W. C. & Buck, S. A. 1984. Selection for
482 delayed senescence in *Drosophila melanogaster*. *Evolution* **38**: 996-1003.
483 Magwere, T., Chapman, T. & Partridge, L. 2004. Sex Differences in the Effect of Dietary
484 Restriction on Life Span and Mortality Rates in Female and Male Drosophila
485 Melanogaster. *The Journals of Gerontology Series A: Biological Sciences and Medical*
486 *Sciences* **59**: B3-B9.
487 May, C. M., Doroszuk, A. & Zwaan, B. J. 2015. The effect of developmental nutrition on life
488 span and fecundity depends on the adult reproductive environment in Drosophila
489 melanogaster. *Ecology and Evolution* **5**: 1156-1168.
490 Merilä, J., Sheldon, B. & Kruuk, L. 2001. Explaining stasis: microevolutionary studies in natural
491 populations. *Genetica* **112**: 199-222.

492 Partridge, L. & Fowler, K. 1992. Direct and correlated responses to selection on age at
493 reproduction in *Drosophila melanogaster*. *Evolution* **46**: 76-91.
494 Prasad, N., Shakara, M., Anitha, D., Rajamani, M. & Joshi, A. 2001. Correlated responses to
495 selection for faster development and early reproduction in Drosophila: the evolution of
496 larval traits. *Evolution* **55**: 1363-1372.
497 Promislow, D. E. 1993. On size and survival: progress and pitfalls in the allometry of life span.
498 *Journal of gerontology* **48**: B115-B123.
499 R Development Core Team (2005) A language and environment for statistical computing. pp. R
500 Foundation for Statistical Computing, Vienna, Austria.
501 Robertson, F. W. 1957. Studies in quantitative inheritance XI. Genetic and environmental
502 correlation between body size and egg production in *Drosophila melanogaster*. *Journal*
503 *of Genetics* **55**: 428-443.
504 Roff, D. A. 1992. *Evolution of life histories: theory and analysis*. Springer Science & Business
505 Media.
506 Roff, D. A. 2001. *Life History Evolution* Sinauer Associates, Sunderland.
507 Rose, M. R. 1984. Laboratory evolution of postponed senescence in *Drosophila melanogaster*.
508 *Evolution* **38**: 1004-1010.
509 Schluter, D., Price, T. D. & Rowe, L. 1991. Conflicting selection pressures and life history trade-
510 offs. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **246**: 11-
511 17.
512 Siepielski, A. M., DiBattista, J. D., Evans, J. A. & Carlson, S. M. 2011. Differences in the
513 temporal dynamics of phenotypic selection among fitness components in the wild.
514 *Proceedings of the Royal Society of London B: Biological Sciences* **278**: 1572-1580.
515 Stearns, S. C. 1992. *The Evolution of Life Histories*. Oxford University Press, Oxford.
516 Tarwater, C. E. & Beissinger, S. R. 2013. Opposing selection and environmental variation
517 modify optimal timing of breeding. *Proceedings of the National Academy of Sciences*
518 **110**: 15365-15370.
519 Therneau, T. M. (2015) coxme: Mixed Effects Cox Models. pp.

520 Tukey, J. W. 1949. Comparing individual means in the analysis of variance. *Biometrics*: 99-114.

521 Van Noordwijk, A. J. & de Jong, G. 1986. Acquisition and allocation of resources: their
522 influence on variation in life history tactics. *American Naturalist*: 137-142.

523 Zajitschek, F., Zajitschek, S. R., Canton, C., Georgolopoulos, G., Friberg, U. & Maklakov, A. A.
524 (2016) Evolution under dietary restriction increases male reproductive performance
525 without survival cost. In: *Proc. R. Soc. B*, Vol. 283. pp. 20152726. The Royal Society.

526 Zwaan, B., Bijlsma, R. & Hoekstra, R. 1995. Artificial selection for developmental time in
527 *Drosophila melanogaster* in relation to the evolution of aging: direct and correlated
528 responses. *Evolution*: 635-648.

529 Zwaan, B. J. 1999. The evolutionary genetics of ageing and longevity. *Heredity* **82**: 589-597.

530 Zwaan, B. J., Bijlsma, R. & Hoekstra, R. F. 1991. On the developmental theory of ageing. I.
531 Starvation resistance and longevity in *Drosophila melanogaster* in relation to pre-adult
532 breeding conditions. *Heredity* **66**: 29-39.

533

534 **Tables**

535 **Table 1: Summary of GLMMs (Chi-square values) for the effect of assay diet (A),**

536 **evolutionary dietary regime (D) and evolutionary age at reproduction (R) on larval survival**

537 **and developmental time across phenotyping sessions.** Significance is indicated by: * =P<0.05,

538 **=P<0.01, ***=P<0.001.

539

Phenotyping	EE Diet (D)	EE Repro (R)	Assay diet (A)	D*R	D*A	R*A	D*R*A
Larval survival							
P1	0.26	0.05	---	0.24	---	---	---
P2	0.30	0.85	---	0.37	---	---	---
P3	0.71	0.02	6.6	0.57	1.50	0.00	0.66
P4	0.35	0.01	1.24	---	---	---	---
P5	13.06**	3.94	0.17	0.32	25.24***	0.46	0.85
P6	5.23.	2.49	42.69***	2.73	5.99	38.86***	12.91
P7	0.00	0.00	3.23	0.12	3.02	0.74	0.51
P8	1.45	0.04	8.07	0.62	2.68	0.43	3.66
Developmental time							
P1	2.07	27.58***	---	0.96	---	---	---
P2	4.14	0.63	---	0.68	---	---	---
P3	0.46	15.74***	20617.4***	0.34	140.61***	64.27***	72.31***
P4	3.86	2	----	0.97	----	----	----
P5	0.18	4.30	4830.06***	2.44	25.13***	244.81***	41.63***
P6	8.06	2.02	12746.57***	11.57**	76.11***	311.47***	48.51***
P7	6.90	0.18	8506.83*** 23285***	1.06	22.29***	34.18***	78.39***
P8	7.64	13.36***		1.21	181.40***	758.70***	46.53***

540

Table 2: Summary of GLMMs (Chi-square statistics, degrees of freedom, and their significance) for the effect of assay diet on larval survival, developmental time, lifespan, and fecundity on 1-E lines across phenotyping sessions. Where there was a significant effect of assay diet (i.e. plasticity for the response to assay diet) we report the outcomes of pairwise post-hoc comparisons between assay diets (p-values). Where several models were fit per trait we indicate the subset analyzed (Subset).

			Effect Assay diet			Post-hoc contrasts		
Phenotyping	Generation	Subset	Chi square	df	p-value	P:C	P:R	R:C
Larval survival								
P3	7	---	1.59	2	0.45	---	---	---
P5	12	---	6.18	1	0.01	0.01	---	---
P6	30	---	0.42	2	0.81	---	---	---
P7	32	---	0.44	2	0.80	---	---	---
P8	38	---	5.46	2	0.07	---	---	---
Developmental time								
P3	7	---	1878.70	2	<0.0001	<0.0001	<0.0001	<0.0001
P5	12	---	1090.70	1	<0.0001	<0.0001	---	---
P6	30	---	2648.30	2	<0.0001	<0.0001	<0.0001	<0.0001
P7	32	---	2303.30	2	<0.0001	<0.0001	<0.0001	<0.0001
P8	38	---	4212.50	2	<0.0001	<0.0001	<0.0001	<0.0001
Fecundity								
P7	32	Early	12.904	1	<0.0001	0.0001	---	---
		Mid	570.12	1	<0.0001	<0.0001	---	---
		Late	392.35	1	<0.0001	<0.0001	---	---
P8	38	Early	251.46	2	<0.0001	<0.0001	<0.0001	0.79
		Mid	225.24	2	<0.0001	<0.0001	<0.0001	<0.0001
		Late	65.824	2	<0.0001	<0.0001	0.42	<0.0001
Lifespan								
P7	32	F	16.015	1	<0.0001	<0.0001	---	---
		M	32.831	1	<0.0001	<0.0001	---	---
P8	38	F	55.467	2	<0.0001	<0.0001	<0.0001	0.23
		M	46.134	2	<0.0001	<0.0001	<0.0001	0.45

549 **Table 3: Summary of GLMMs (Chi-square values) for the effect of assay diet (A),**

550 **evolutionary dietary regime (D) and evolutionary age at reproduction (R) on lifespan**

551 **across phenotyping sessions.** Significance of Chi-square values are indicated by *: * =P<0.05,

552 **=P<0.01, ***=P<0.001.

	Phenotyping	
Factor	P7	P8
Evo Diet (D)	0.26	8.45*
Evo Repro (R)	15.66***	28.63***
Assay Diet (A)	3557.81***	3761.66***
Sex (S)	0.01	46.47***
D*R	4.13*	0.49
D*A	2.08	9.73**
R*A	0.02	0.55
S*D	12.69***	10.34**
S*R	1.63	16.94***
S*A	0.00	2.62
D*R*A	0.27	20.99***
R*A*S	0.44	0.59
S*D*R	4.43*	0.01
S*D*A	1.68	0.01
S*D*R*A	1.59	0.22

553

554

555 **Table 4: Summary of GLMMs (Chi-square values) for the effect of assay diet (A),**
556 **evolutionary dietary regime (D) and evolutionary age at reproduction (R) on fecundity at**
557 **early, mid and late ages across phenotyping sessions.** Significance of Chi-square values are
558 indicated by *: * =P<0.05, **=P<0.01, ***=P<0.001.

Phenotyping	Age	Evo Diet (D)	Evo Repro (R)	Assay Diet (A)	D*R	D*A	R*A	D*R*A
P7	Early	1.00	1.85	176.80***	4.45	35.26***	1.18	5.47
	Mid	4.69	0.49	1383.33***	8.14*	8.02*	20.00***	0.72
	Late	1.5	7.5*	2154.05***	0.7135	1.84	76.34***	7.62*
P8	Early	0.20	1.60	892.25***	0.77	9.05	0.49	8.05
	Mid	0.62	0.89	364.66***	10.20**	87.46***	43.04***	132.38***
	Late	0.29	7.54*	204.34***	0.934	84.75***	64.578***	90.37***

559

560

561 **Figure Legends**

562 **Figure 1. Experimental overview.** (a) Experimental evolution design. Four replicate
563 populations were established per combination of larval diet and age at reproduction (4 replicate
564 lines * 3 larval diets * 2 ages at reproduction = 24 lines in total). The main panel traces a
565 generation of EE for a single 2.5SY-E (top) and 2.5SY-L (bottom) line. (b) Collection sites
566 across Europe of the six populations that contributed to the "S" starting population. (c) A brief
567 description of the multi-generation crossing scheme used to cross the six populations in (b) to
568 generate the mixed "S" population used for experimental evolution. (d) Overview of traits
569 assayed in each phenotyping session (8 in total, labeled P1 through P8). Inclusion of lines and
570 assay diets in a phenotyping is diagrammed using filled (included) vs. unfilled boxes (not
571 included).. Briefly, the first column of boxes indicates the experimental evolution lines included,
572 while the second, third and fourth columns indicate the assay diets on which they were assessed
573 (key in inset box). In all cases, both the early (E) and late (L) reproducing lines were included.
574 Thus, for example, in P4 (Generation 10 & 5 of E and L lines respectively) the 0.25-E, 0.25-L, 1-
575 E, 1-L, 2.5-E, and 2.5-L lines were all included (first column all filled), but only assayed under
576 the 1.0 assay diet. It is noteworthy that there is a relatively large generation gap between sessions
577 P1 through P5 and P6 through P8.
578
579 **Figure 2. Developmental time from egg to adult (y-axis) across phenotyping sessions (x-**
580 **axis) by assay diet (0.25: beige square; 1.0 : pink circle; 2.5: purple triangle).** Not all
581 phenotyping sessions included all three assay diets. Each point represents taking the mean of the
582 average developmental time for each of the lines included in the assay. For example, if all 24
583 lines were included in the assay the mean developmental time was calculated per line and then
584 the mean and standard error of these 24 values was calculated.
585
586 **Figure 3. Developmental time from egg to adult (y-axis) across phenotyping sessions (x-**
587 **axis) for the 1.0 assay diet (a,d), the 0.25 assay diet (b,e) and the 2.5 assay diet (c,f). (a-c)**
588 **represent the observed developmental times while (d-f) are the developmental times relative**

589 **to the mean of the 1-E lines.** Each point represents taking the mean and standard error of the
590 average developmental time for each of the four lines**.**
591
592 **Figure 4. Lifespan (y-axis) across assay diets (x-axis) and phenotyping sessions P7 (a, b) and**
593 **P8 (c, d) for females (a, c) and males (b, d). Lifespan is expressed as days from adult**
594 **eclosion.** All error bars are standard errors of the mean across replicate lines.
595
596 **Figure 5. Reaction norms of realized early (a,d), late (b,e) and post-selection (c,f) female**
597 **fecundity (y-axis) across assay diets (x-axis) and phenotyping sessions P7 (a:c) and P8 (d:f).**
598 All error bars are standard errors of the mean across replicate lines.
599
600 **Figure 6. Inconsistencies in fecundity of 1.0 lines across phenotyping sessions.** All error bars
601 are standard errors of the mean across replicate lines.
602
603 **Figure 7. Body weight (mg) of female (a,c) and male (b,d) flies raised on the 1.0 assay diet at**
604 **young (~ 4-5 days old) and old (~18-19 days old) ages.** All error bars are standard errors of the
605 mean across replicate lines.
606

Figure 1

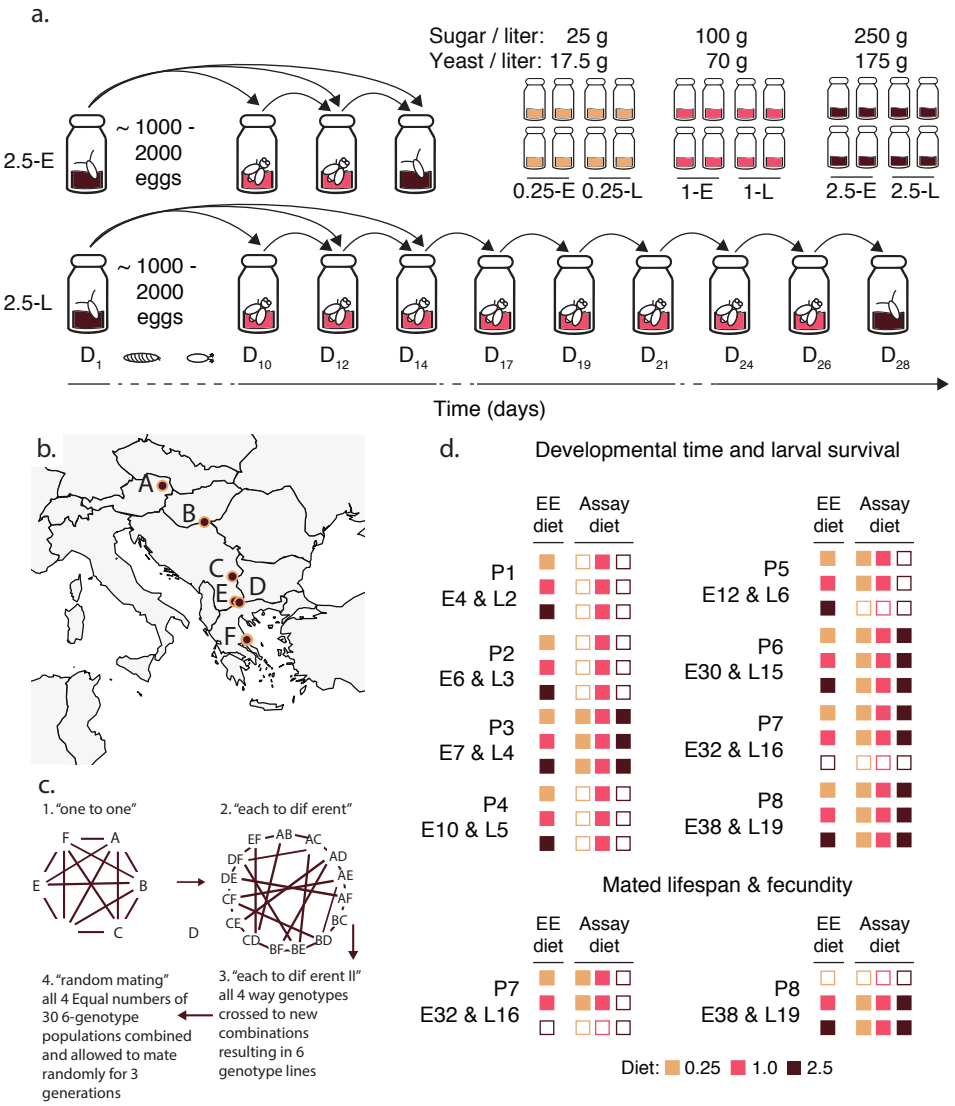

Figure 2

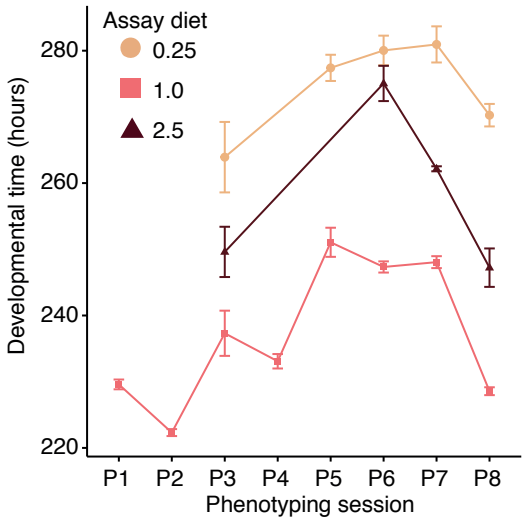

Figure 3

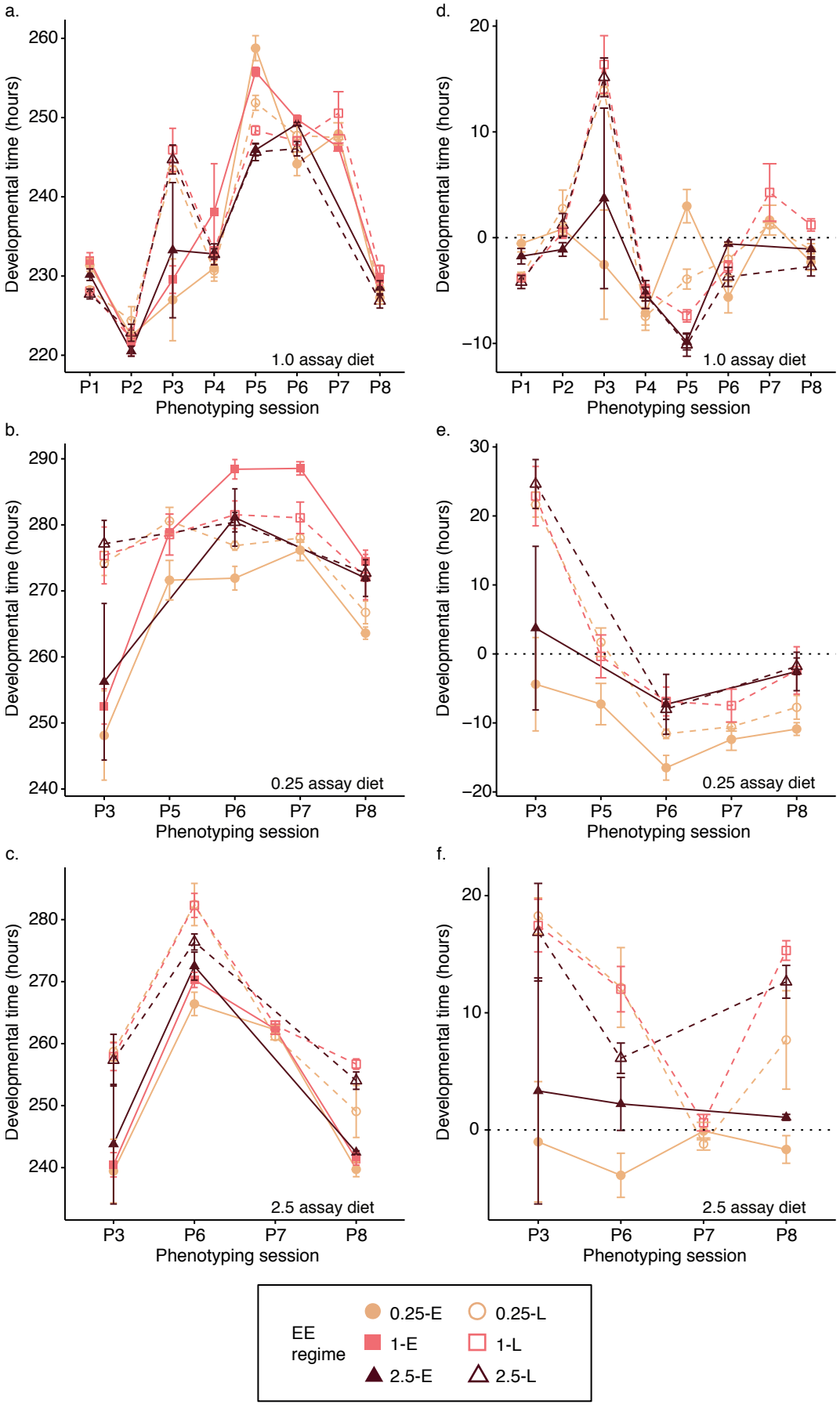

Figure 4

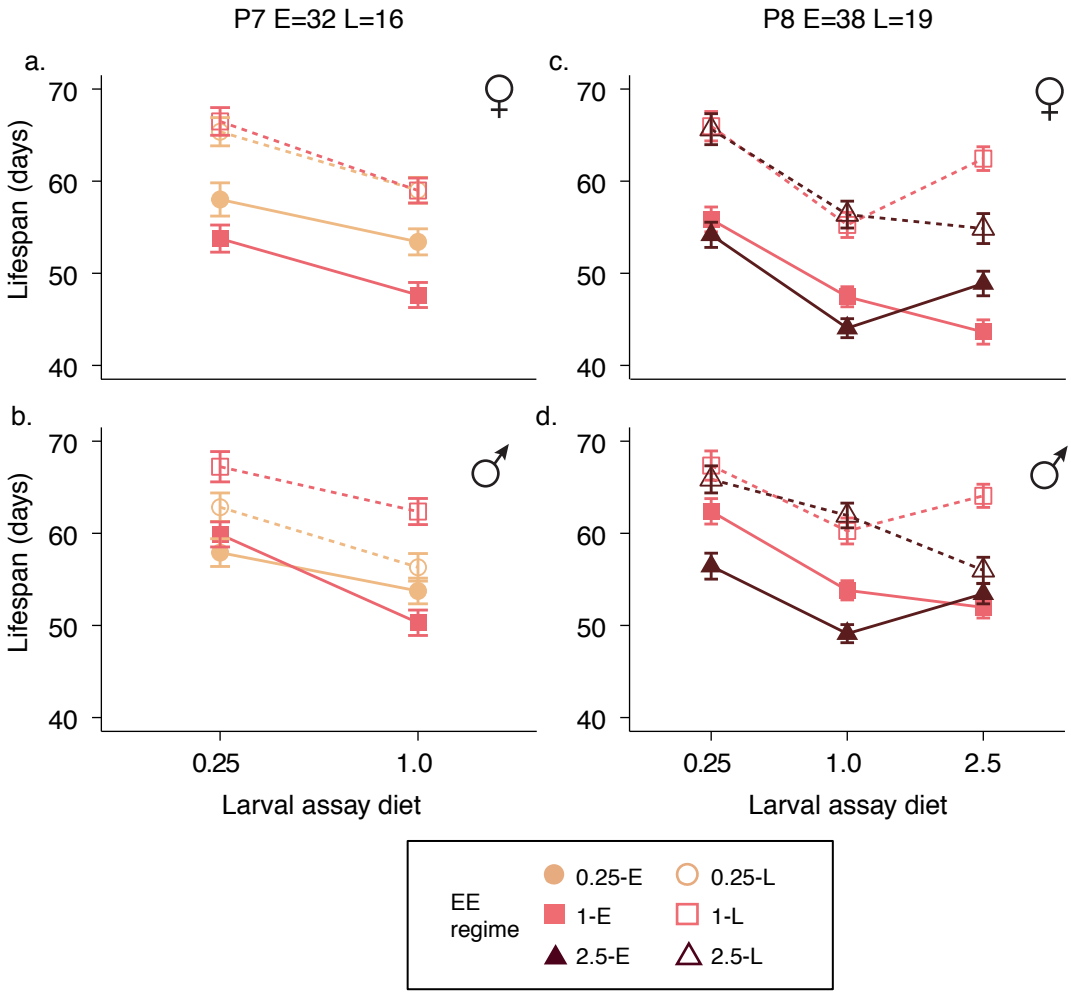

Figure 5

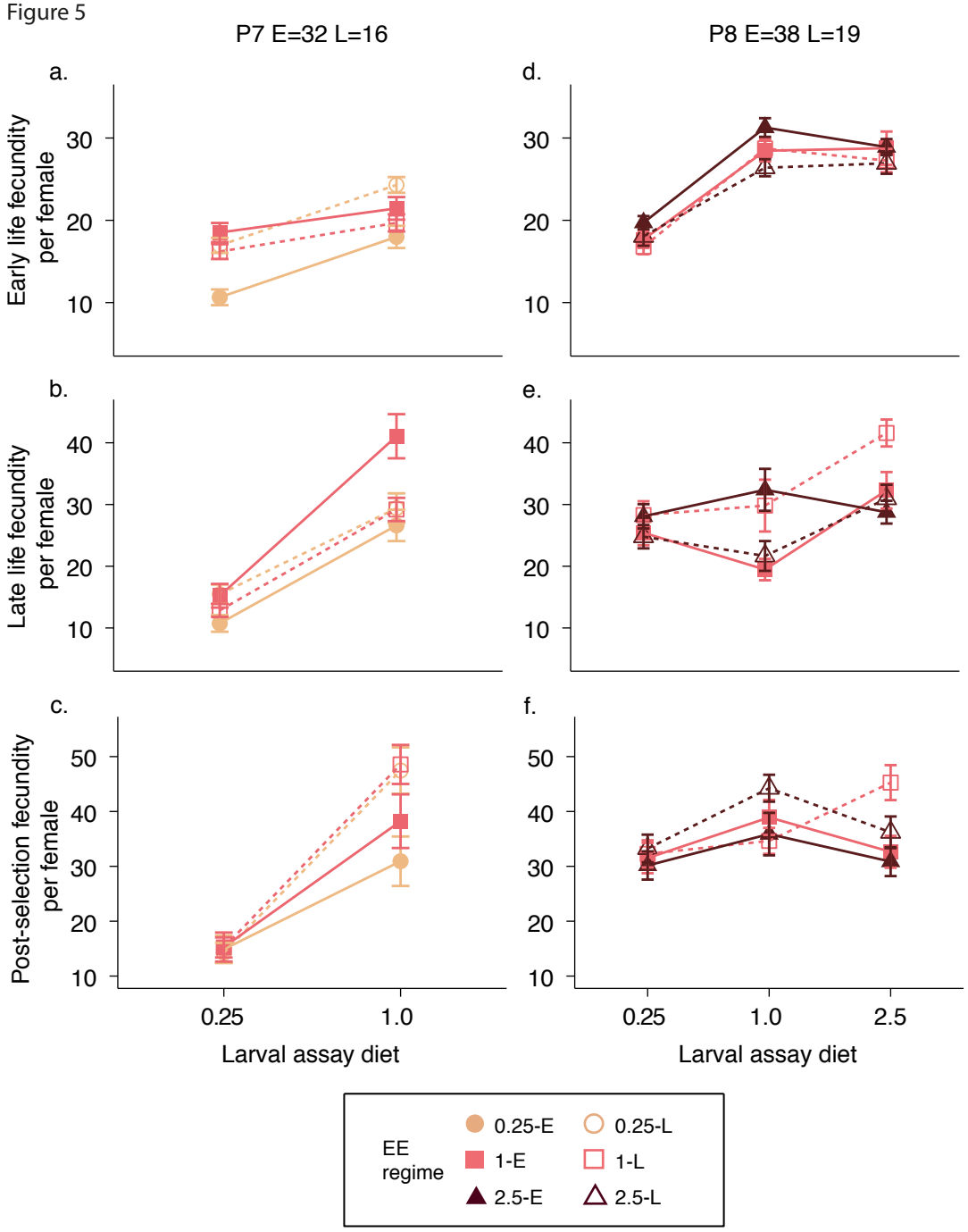

Figure 6

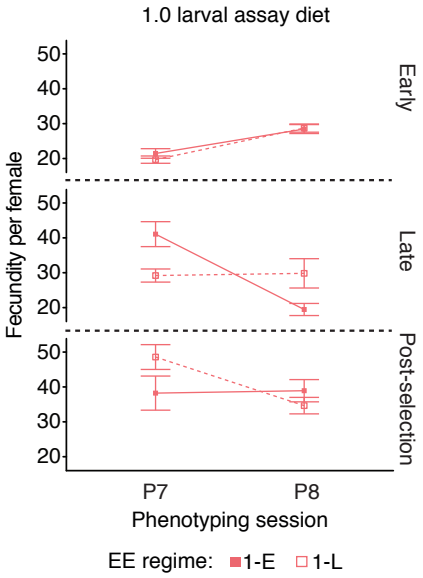
1.0 larval assay diet
Fecundity per female
50
40
30
20
Early
Late
Post-selection
P7
P8
Phenotyping session
EE regime: 1-E 1-L

Figure 7

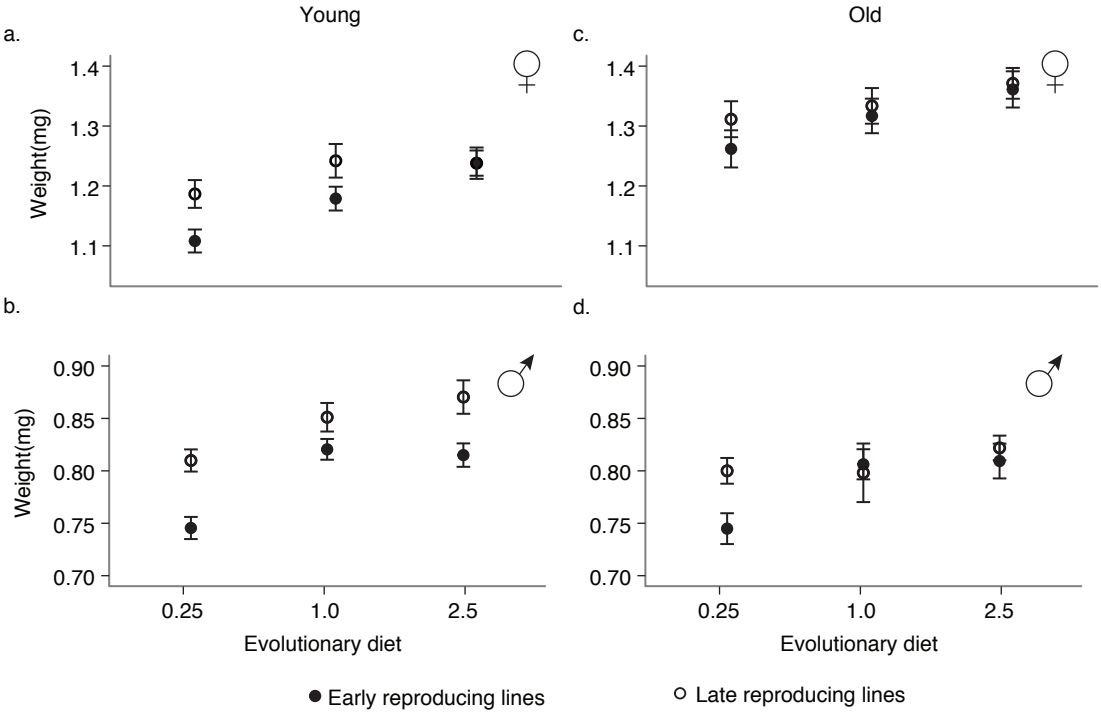

Figure 8