Targeting environmental and genetic aspects affecting life history traits

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Chapter 3

Multi-trait evolution in lines of *D. melanogaster* selected for increased starvation resistance; the role of metabolic rate and implications for the evolution of longevity.

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Multi-trait evolution in lines of *D. melanogaster* selected for increased starvation resistance; the role of metabolic rate and implications for the evolution of longevity.

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Abstract

Starvation resistance is a trait often associated with longevity. Animals with increased longevity frequently show elevated starvation resistance and *vice versa*. Consequently, both life history traits are thought to share genetic and physiological mechanisms, such as increased fat content and lowered metabolic rate. Here, we present results from 20 generations of selection on *D. melanogaster* for increased starvation resistance at the time of adult eclosion. We observe that starvation resistance can be the result of more than one mechanism, all associated with an increase in fat resources. In general, metabolic rate is lowered under starved conditions relative to fed conditions. Metabolic rate in the starvation resistant lines is generally higher than in control lines under starved conditions. Starvation resistant flies are able to sustain a higher metabolic rate for a longer period of time when food is unavailable. This implies depletion of the increased fat reserves. However, longevity was not consistently affected by selection for increased starvation resistance. Similarly, paraquat resistance differed between selection lines and did not associate with starvation resistance, but rather with longevity. The results are discussed in relation to previous reported results on starvation resistance and its relation with mechanisms of ageing and longevity.

Keywords

Ageing, starvation resistance, longevity, fat content, metabolic rate, genetic correlation, complex trait

Introduction

Increased stress resistance is often observed in *Drosophila melanogaster* lines selected for increased longevity (Service et al. 1985; Service 1987; Leroi et al. 1994b; Force et al. 1995; Harshman et al. 1999b), in mutants (Lin et al. 1998) and in phenotypic manipulation experiments (e.g. Bouletreau-Merle and Fouillet 2002; Zwaan et al. 1991). This association also holds in other species that have been analyzed, such as mice and nematodes (Longo and Fabrizio 2002). Similarly, selection for increased adult starvation resistance has been shown to increase longevity (Rose et al. 1992; Chippindale et al. 1996, yet see Bubliy and Loeschcke 2005). Selection on shorter longevity also results in decreased starvation resistance (Zwaan et al. 1995b). These results strongly suggest that starvation resistance shares, at least in part, genetic regulatory mechanism with longevity. Moreover, the selection experiments indicated the presence of standing genetic (co)variation in natural populations. There are strong positive correlations between starvation resistance and longevity as opposed to the negative correlations both traits display with reproductive output. This indicates the presence of a trade-off between maintenance and reproduction, or between the soma and the germ line as has been proposed in the disposable soma theory (Kirkwood 1977; Kirkwood and Holliday 1979). This theory overlaps with the theory of antagonistic pleiotropy (Williams 1957), where advantages of early life traits are considered to be disadvantageous later in life (Kirkwood and Rose 1991). In pursuit of the validity of this latter theory, reproduction late in life was found to increase longevity considerably (Rose 1984). However, the correlated responses to selection can disappear when selection is either relaxed for longevity (Vermeulen and Bijlsma 2006), or continued over long periods in the case of correlations between starvation resistance and longevity (Archer et al. 2003; Phelan et al. 2003). The reasons for this can be diverse and may include genotype-by-environment effects and changed selection regimes. It does, however, emphasize that the correlations among the life history traits of interest are not as stable over evolutionary time as was previously thought.

Here, we report on selection for increased starvation resistance directly from adult eclosion. We consider development to be an important period where the physiology of an organism is determined (Zwaan et al. 1995a; Tu and Tatar 2003; Zwaan 2003; Baldal et al. 2005; Brakefield et al. 2005). We intended selection to be as independent as possible from interfering factors such as adult feeding behaviour. Therefore, we established lines by selecting flies for increased starvation resistance directly from eclosion onwards. In this way, the soma that was built during the pupa stage had to be adapted to the starvation condition, without any interference of secondary traits. *D. melanogaster* lines were selected for increased starvation resistance for 20 generations. Direct responses were monitored during selection, while in addition correlated responses were examined after selection had stopped. These responses included longevity, paraquat stress, fat content, and body weight. We also examined the effects of selection for increased starvation resistance on metabolic rate. A novelty here was the assessment of metabolic rate in starvation resistant lines under both fed and starved conditions. This study also provides a novel approach to research into life history traits by analysing supposedly correlated traits with a principal component analysis. Our results suggest that there are at least two genetic solutions to the environmental challenge of starvation resistance. One of those involves increased fat reserves, resistance to other stress factors, increased longevity and, counter intuitively, increased metabolic rate under

starvation conditions. The other involves elevated metabolic rate under starved conditions and increased fat reserves. The evolutionary implications of these physiological findings are in line with the earlier finding that correlations among traits may shift over time. These results underpin the idea of genome flexibility and suggest that several evolutionary solutions to environmental challenges may arise from a genetically homogeneous population on the middle to longer term. Generally, the data presented here are in line with the disposable soma theory of ageing.

Materials and methods

Stock population

One hundred and eleven female *Drosophila melanogaster* were caught and used to found iso-female lines. Of these, 17 were collected in France by B.A. Pannebakker, 22 in Panama by K. van der Linde and C. Krijger (all from Leiden University), 4 from Groningen (The Netherlands; kindly provided by R. Bijlsma and A. Boerema from the University of Groningen), and 68 were collected in the Leiden area (The Netherlands). Flies were given standard medium (20 gr. agar, 9 gr. kalmus [kalmus consists of 10 parts (weight) acidum tartaricum, 4 parts ammonium sulphate, 1 part magnesium sulphate and 3 parts potassium phosphate], 10 ml. nipagin [100 grams of 4-methyl hydroxy benzoate per liter ethanol], 50 gr. saccharose and 35 gr. of granulated yeast per liter water) in either vials filled with 6 ml of medium or bottles filled with 24 ml of medium. Temperature was kept constant at 25ºC and RH at 50% under a 12/12 L/D regime. All procedures presented here took place at these conditions unless indicated otherwise. Lines were kept in the laboratory for 10 generations before the onset of the experiment, to reduce the potential influence of laboratory selection on our stocks while selecting for starvation resistance. Moreover, 10 generations would also allow for a considerable amount of recombination of the genomes to reduce effects of linkage disequilibrium giving rise to correlated responses. However, starvation resistance is considered to be a polygenic trait, so unless major genes are segregating in the population, such linkage disequilibrium effects are unlikely. For culturing, single pairs of flies from each iso-female line were pooled in a bottle, replicated 10 times. After these 10 generations, the 10 bottles were mixed for another 2 generations in 6 replicate bottles. In each of 4 replicates, selection for increased starvation resistance was then applied by using 120 individuals of each sex, 10 per vial (see next section). The remaining 2 replicate bottles were, except for the starvation treatment, kept under similar generation times and experimental conditions and became two control lines.

Selection procedure

Selection was performed by placing 240 individuals per replicate line in 24 vials containing 6 ml of agar medium (20 gr. agar, 9 gr. kalmus, 5 cc. nipagin per liter water) without food to induce starvation but to prevent desiccation. When 50-70% of the flies had died, the surviving cohort was given medium containing live yeast. Selection was performed on virgin males and females to ward off the possible

positive (Service 1989) and negative (Chippindale et al. 1993) effects of mating on starvation resistance. After 2 days the sexes were mixed and allowed to mate for one day in a bottle containing standard medium. In this way the surviving cohort yielded the P-generation of flies in each subsequent generation of selection. The next day vials containing medium were replaced by new ones to provide fresh breeding substrate. Larval density was controlled by the amount of time the flies laid eggs and the number of flies per vial. This was kept comparable over all treatments to produce similar optimal larval conditions without handling the eggs.

Our method of selection differed from that of Harshman et al. (1999a) who employed pre-defined starvation time selection points on flies that were approximately 7 days old. Apart from differences in the age of the flies, our selection procedure matched those of Rose et al. (1992) and Harshman and Schmid (1998). Rose et al.'s studies started selection for starvation resistance at 14 days from the egg stage (personal communication), which is effectively 3 to 4 days of adult age and thus a little older than our flies. This critically allows adult feeding to be a factor in the response to selection. Harshman and Schmid (1998) selected mated females that were 4 to 6 days old in a similar way. As mentioned in the introduction, we aimed to select on the pre-adult phase and prevented interference from adult behavioural traits.

Starvation assays during selection procedure

Starvation resistance was assayed in each generation by putting 3 additional replicates of 10 virgin flies of each sex within 8 hours after eclosion in vials containing 6 ml agar medium (20 gr. agar 9 gr. kalmus, 5 ml. propionic acid, 5 ml. nipagin per liter water . The vials were checked daily for living flies, dead individuals were removed immediately to prevent the living flies from feeding on corpses or body fluids (but see Huey et al. 2004). Immobile flies were checked for death by physical stimulation.

Starvation assays after selection

Starvation resistance at 0, 7 and 21 days after eclosion was measured in virgin flies. Flies were kept at a density of 100 individuals in a half pint bottle on standard medium until the onset of the starvation experiment. Starvation resistance was measured in 100 flies of each sex and each line in vials each containing 10 flies. Starvation resistance from day 0 onwards was measured starting within 8 hours of eclosion, as above.

Paraquat resistance

Paraquat resistance was measured in virgin flies collected within 8 hours of eclosion in vials of 10 flies, with sexes separated. Flies were given agar plugs (20gr agar per liter water) to provide moisture and 500µl of a 5% sucrose, 30mM paraquat (methyl viologen, M2254, Sigma Aldrich) solution on 5 filtration papers (1 by 1 cm, as in (Vermeulen et al. 2006) and the flies were checked for survival twice a day).

Longevity assays

After selection had finished, longevity was measured for 100 virgin flies of each sex of each line, 5 per vial. Flies were checked daily for survival. Vials were changed every week to minimize death by other than intrinsic causes. During the selection procedure longevity was measured every third generation in a small sub-sample of 30 individuals per sex of each line.

Fat content and dry weight

Dry weight and fat content were measured in virgin flies harvested within 8 hours of eclosion. Only live flies were analyzed for fat content. For each line and sex, 10 replicates of 5 flies were weighed. The five individuals of each line and replicate were isolated and stored at –80ºC until further analysis. The flies were dried at 60ºC for 24h and then weighed on a Sartorius® ultra microbalance to determine dry weight. Fat was extracted by adding 1 ml of diethyl ether under continuous shaking (200 rpm) for 24 hours. The flies were then dried for 24h at 60ºC and re-weighed. The fat-free dry weight value was subtracted from the dry weight value. Relative fat content was calculated by dividing absolute fat content by dry weight (Zwaan et al. 1991; Baldal et al. 2005).

Metabolic rate

For each sex of each line, seven groups of 5 virgin flies were assayed for CO_2 production at 25ºC and under continuous light. The flies were first put either on starvation or on standard medium for 4 days, after which they were anaesthetized on ice and weighed before being assayed. The flies were then assayed in a 16-channel respirometer (Li-6251 CO_2 analyzer of Li-Cor) containing a small piece of agar medium to prevent the flies from dehydrating. Two channels were left empty as independent controls in each experiment. For each channel, 5 runs of respirometry were performed. The first 2 of these runs were discarded in each case because they generally showed elevated CO_2 levels due to the experimenter's exhaling when the flies were put into the channels by aspiration. The data thus comprises 3 separate runs of 7 groups of 5 flies per sex per line per feeding condition. Data were acquired and analyzed using the program Sable. The metabolic rate observed in the experiments was divided by the wet weight determined just before the experiment to get an accurate measurement of the mass specific metabolic rate.

Statistics

All data were analyzed using JMP 5.0.1 statistical software. The realized heritability was estimated by regression analysis of the response over the first 17 generations. After generation 17 the response showed a plateau, indicating that starvation resistance would not increase with further selection. Differences among the lines where tested using a regression model on the selection response with line and sex as independent factors, and cumulative selection differential as a covariant. Longevity data were analyzed using a Cox proportional hazard analysis because it

considers the shape of the curve rather than reducing it to an average with standard deviation as ANOVA tends to do. The data can therefore be compared more accurately. Data for other responses to stress, fat content and dry weight were analyzed using ANOVA unless indicated otherwise. Significant differences between groups were determined *post hoc* using Tukey tests. Vials were nested in the factor sex in each analysis. However, no vial effects were found throughout the experiment and thus this factor was later discarded from the analysis. For each analysis we performed a test with the replicate lines nested as a random factor in the factor "selection" (i.e. starvation resistance selected versus control), this was done to test the selected and control lines as replicates. This conservative way of testing was used to make sure that factors of large effect were found only on the basis of robust tests instead of high numbers of replication.

Averages of starvation resistance from eclosion, relative fat content, longevity, metabolic rate under starved conditions and paraquat resistance were determined for each sex of each line. These averages were arranged in a matrix consisting of each sex of each line (12 rows) and the 5 traits as described above (5 columns). To infer common principles from the large amount of data, we performed a PCA using Minitab 14. Although the power of this principal component analysis is limited because averages are analysed, it can reveal patterns in the data. The sexes were regarded separately, because they reveal dissimilar patterns, and function as a semi-independent replicate.

Results

Response to selection

Artificial selection for increased starvation resistance yielded a positive response in both sexes (figure 1). Absolute starvation resistance increased from ~4.5 to ~7.5 days in the selected lines. The resulting scaled SR of 1.6 –1.9 times the average of the controls underlines the difference between selection and control lines (figure 1). All realized heritabilities were significantly larger than zero, except for line SR2 in females (table 1). Covariance analysis revealed that there was a highly significant effect of line ($F_{3,120}$=4.61, P=0.0043) and cumulative selection differential (csd) ($F_{1,120}$=156, P<0.0001) and a significant line*csd interaction ($F_{3,120}$=4.43, P=0.0055). The latter result indicates that not all selection lines responded to selection in a similar way. Indeed, for both sexes lines SR1 and SR2 have lower heritabilities than lines SR3 and SR4 (table 1). The factors sex ($F_{1,120}$=1.8, P=0.18), line*sex ($F_{1,120}$=0.05, P=0.99), and line*sex*csd ($F_{1,120}$=0.22, P=0.88) were not significant, but the sex*csd was ($F_{1,120}$=9.26, P=0.0029). This indicates that there were considerable differences in the response of the sexes heritability depending on the cumulative selection differential, and thus that the sexes heritabilities could not be pooled.

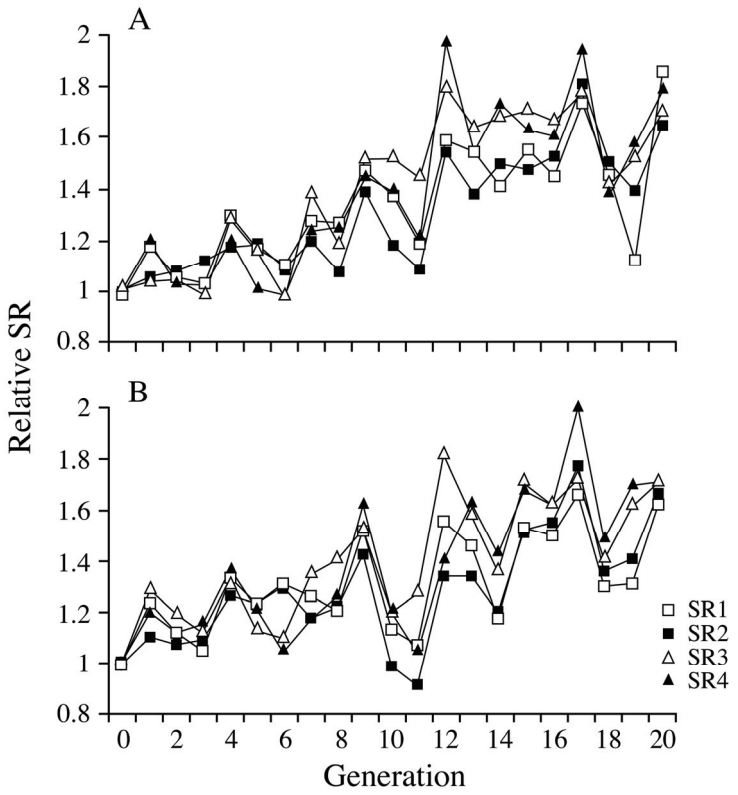

Figure 1. Changes in starvation resistance over 20 generations of selection in the 4 selected lines as scaled to the average of both control lines for males (A) and females (B) separately.

Table 1. Realized heritabilities for each sex of 4 starvation resistant lines after 20 generations of selection. The lower value gives the t- and P-values that indicate whether the cumulative selection differential is significantly different from 0.

Line	Males	Females
line SR1	0.26; t=6.15, P<0.0001	0.18; t=3.03, P=0.0085
line SR2	0.28; t=5.03, P=0.0001	0.12; t=1.73, P=0.10
line SR3	0.49; t=7.21, P<0.0001	0.31; t=4.93, P=0.0002
line SR4	0.45; t=5.44, P<0.0001	0.29; t=3.37, P=0.0042

Starvation resistance after selection

The lines selected for increased starvation resistance showed a clear effect of the selection procedure. In an overall analysis of starvation resistance after eclosion, with the factor line nested in the factor selected versus unselected lines, the selected lines performed significantly better than their controls ($F_{1,4}$=130, P=0.0003). In this analysis we also observed a significant line*sex interaction ($F_{4,1187}$=7.87, P<0.0001). Starvation resistance showed a significant age*line*sex interaction ($F_{10,3552}$=5.54, P<0.0001). The estimates were higher in lines SR3 and SR4 and in females (figure 2). All other factors (age, line, sex and their interactions) in this full factorial test were also highly significant (analysis not shown). Therefore we examined differences by age class, and all factors remained highly significant (P<0.0001). The line*sex interactions were significant (eclosion: $F_{5,1187}$=6.44, P<0.0001; day 7: $F_{5,1181}$=5.35, P<0.0001; day 21: $F_{5,1184}$=5.75, P<0.0001), indicating that the response of the sexes differed among lines.

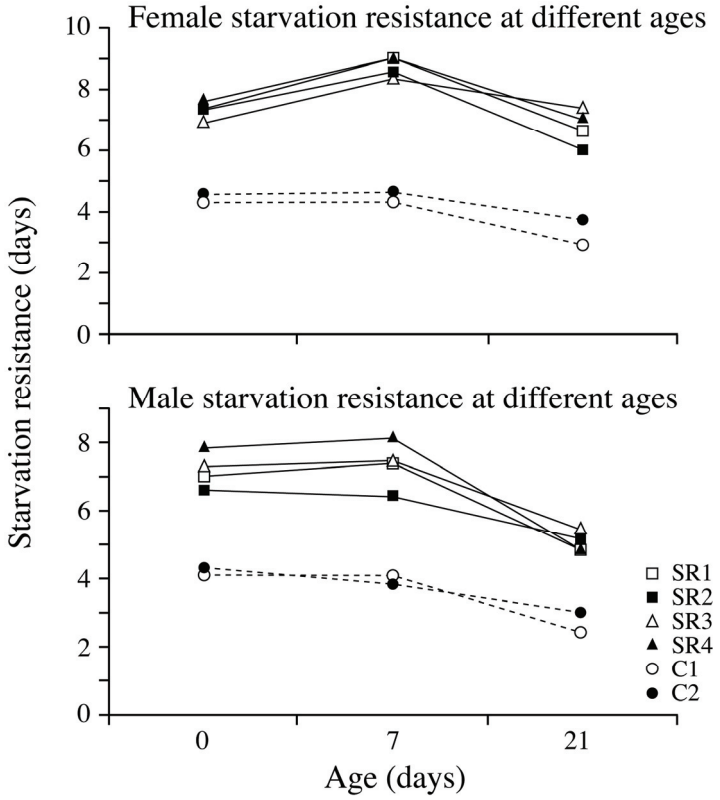

Figure 2. Average starvation resistance of males and females of each of the lines at three adult ages after 20 generations of selection. Standard error bars fell within the area of the symbols in the graph and were left out for clarity.

We also tested lines separately at the different ages and observed that all selected lines showed significant age*sex interactions (line SR1: $F_{2,590}$=8.33, P=0.0003; line SR2: $F_{2,591}$=8.46, P=0.0002; line SR3: $F_{2,592}$=14.19, P<0.0001; line SR4: $F_{2,593}$=14.09, P<0.0001), whereas control lines showed significant age and sex effects (all P<0.0001), but marginal age*sex interaction (C1: $F_{2,592}$=1.46, P=0.23; C2: $F_{2,594}$=3.27, P=0.04). Thus the sexes behave similarly over time in both control lines. When the sexes were analyzed separately all lines showed a highly significant effect of age (P<0.001). Starvation resistance is higher in selected lines than in control lines at all times. *Post hoc* Tukey testing on starvation resistance directly after eclosion showed a significantly higher position for line SR4 in males, whereas line SR2 males had a significantly lower starvation resistance than the other selected lines.

Dry weight and fat content

We found significant effects of selected versus unselected lines ($F_{1,4}$=21.4, P<0.01) and of line ($F_{4,4}$=27.9, P=0.0035). The latter indicates that there are differences among lines in the selected and/or unselected groups. A significant line*sex interaction ($F_{5,108}$=21.9, P<0.0001) was found for dry weight, indicating that the pattern of sexual dimorphism in dry weight differs across lines. Lines differed significantly (figure 3; females: $F_{5,54}$=60.1, P<0.0001; males: $F_{5,54}$= 26.4, P<0.0001) when sexes were analyzed separately. *Post hoc* Tukey testing showed that selected lines have a consistently higher dry weight than controls. Fat-free dry weight showed similar results (overall: $F_{5,108}$=19.4, P<0.0001; females: $F_{5,54}$=24.2, P<0.0001; males: $F_{5,54}$= 13.3, P<0.0001), although in this case there were no differences between selection and control lines. Absolute fat content showed a similar pattern (overall: $F_{5,108}$=8.3, P<0.0001; females: $F_{5,54}$=123, P<0.0001; males: $F_{5,54}$= 97.1, P<0.0001) with *post hoc* Tukey testing revealing marked differences between selected and control lines. An overall test of only selected versus unselected lines for their relative fat content showed that selected lines had higher relative fat contents than controls ($F_{1,118}$=245, P<0.0001). This is confirmed by examining relative fat content which revealed no line*sex interaction ($F_{5,108}$=1.6, P=0.17) but did reveal significant differences between lines ($F_{5,108}$=230, P<0.0001) and sexes ($F_{1,108}$=169, P<0.0001). Thus relative fat content showed similar patterns in both sexes in each line. When the sexes were analyzed separately, the differences among the lines remained (females: $F_{5,54}$=103, P<0.0001; males: $F_{5,54}$= 131, P<0.0001), including the divergence between selected and control lines. Thus, lines selected for starvation resistance showed increased absolute and relative fat content, while fat-free dry weight was unaffected relative to the control lines. The increased weight of the starvation resistant lines is therefore, to a substantial degree, accounted for by an increased fat content.

Paraquat resistance

We did not observe a significant effect of selection on paraquat resistance when selected lines were tested against unselected lines in the nested ANOVA ($F_{1,4}$=4.98, P=0.09). However, paraquat resistance showed significant differences between lines, when the factor line was taken as an independent factor ($F_{5,1161}$=27,

P<0.0001; figure 3). The sexes showed no difference ($F_{1,1161}$=0.85, P=0.37), whereas the line*sex interaction factor was significant ($F_{5,1161}$=5.73, P<0.0001). A Tukey test revealed a clustering of lines SR1 and SR2 as the most paraquat-resistant group, followed by lines SR3, SR4 and C2. Line C2 was also clustered together with C1. Thus lines SR1 and SR2 have significantly higher paraquat resistance, indicating that selection for starvation resistance may, but does not necessarily, increase paraquat resistance.

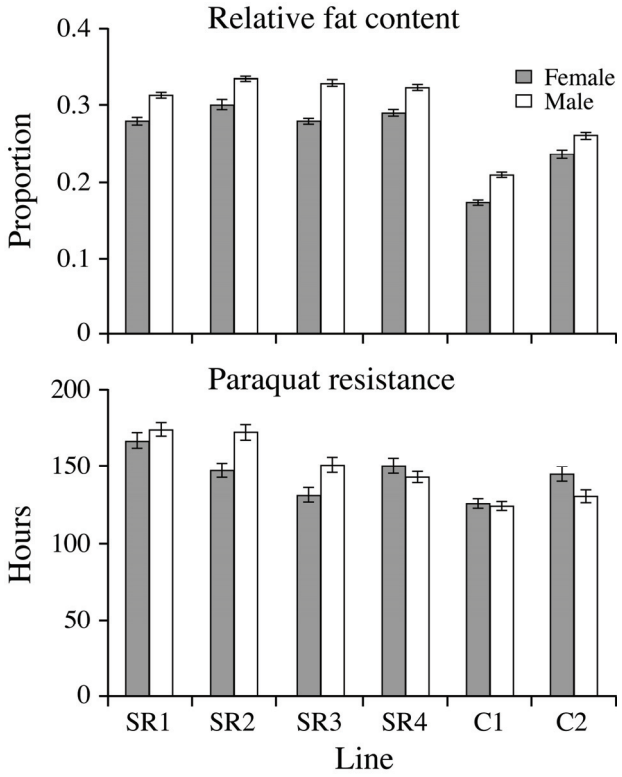

Figure 3. Relative fat content and paraquat resistance for each sex of each line (means with standard error bars).

Longevity

The longevity measurements during selection revealed no consistent association with the increase in starvation resistance (data not shown). However, lifespan measurements of larger cohorts after selection showed that selected lines were significantly longer lived than their controls (X^2_1=17.3, P<0.0001, figure 4) when the factor line was nested in the factor “selection”. No random effects could be added in this analysis, since Cox’ proportional hazards model does not allow these. Longevity

was significantly different among lines (X^2_5=24.3, P=0.0002) and sexes (X^2_1=17.4, P<0.0001), and showed a significant line*sex interaction (X^2_5=17.0, P=0.0045; figure 4). In the analysis of the sexes separately, females showed no significant effect of the factor line (X^2_5=7.14, P=0.21), whereas males did (X^2_5=39.3, P<0.0001).

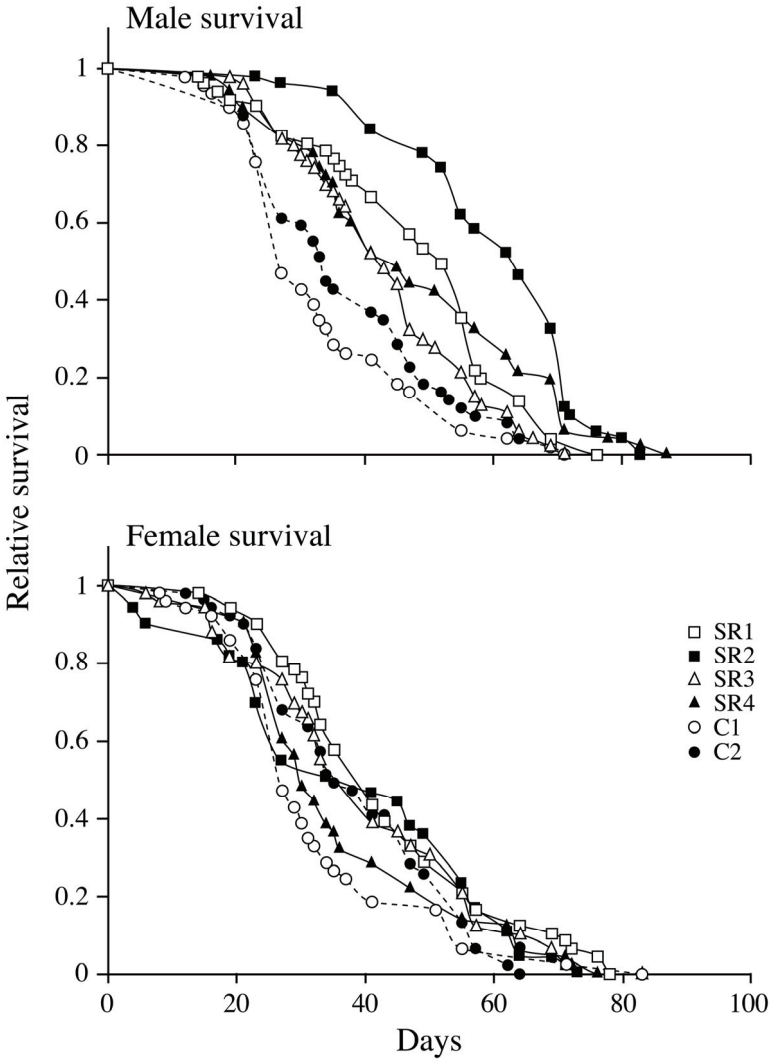

Figure 4. Mortality curves of selected and control lines.

Risk ratios indicated that line SR2 males have the lowest mortality, followed by lines SR4, SR1, C2, C1 and SR3. Examining significant differences among lines identifies three groupings: line SR2 on its own, with the lowest mortality; lines SR4 and SR1; and a cluster of lines C2, C1 and SR3 with the highest mortality. A longevity test associated with another experiment on these lines also showed this replicate specific longevity effect (a superior longevity of lines SR1 and SR2, Baldal et al. in prep).

Differences in metabolic rate among conditions

In the overall full factorial analysis the factor "condition" (for whether or not the animals had been starved for three days) was significant ($F_{1,469}$=462, P<0.0001); fed flies had a higher metabolic rate than starved flies (see figure 5). All other factors were also significant (P<0.0001). When each line was tested separately for effects of sex on metabolic rate, all showed significant differences, with males having higher metabolic rates per weight unit. Testing sexes separately revealed that in most cases fed flies had significantly higher metabolic rates than starved flies. Yet, for females of lines SR1 and C2 no significant difference was found ($F_{1,40}$=1.3, P=0.27; $F1_{,40}$=3.5, P=0.07, respectively). For those of lines SR2 and SR4, metabolic rate had even increased during starvation relative to fed conditions ($F_{1,37}$=9, P=0.005; $F_{1,40}$=4.3, P=0.045, respectively).

Metabolic rate of fed flies.
We observed no difference between selected flies and their controls in metabolic rate during feeding ($F_{1,4}$=0.0026, P=0.96). Fed flies revealed significant effects of the factors line ($F_{5,236}$=6.2, P<0.0001), sex ($F_{1,236}$=305, P<0.0001) and their interaction factor ($F_{5,236}$=5.1, P=0.0002). Tukey ranking among lines varied between the sexes.

A highly significant effect of line was observed in males ($F_{5,116}$=7.6, P<0.0001), but not in females ($F_{5,120}$=1.6, P=0.17). The following clusters were identified by *post hoc* Tukey testing: line SR3, line SR2 and C1 with the highest metabolic rate; C1, line SR4 and C2; and line SR4, C2 and line SR1. Lines that fall in the same cluster are not significantly different.

Even though males show significant differences among lines, there is no consistent pattern in metabolic rate among starvation resistant and control lines when fed. The two groups (i.e. SR1,2, and SR3,4) in the starvation resistant lines, as identified in the life history characteristics, are not observed in the analysis of metabolic rate.

Metabolic rate of starved flies.
Metabolic rate of starved flies was not significantly different in selected lines compared to control lines ($F_{1,4}$=4.28, P=0.11). Among starved flies we found a significant effect of line ($F_{5,233}$=48.8, P<0.0001) and a significant line*sex interaction factor ($F_{5,233}$=7.9, P<0.0001). We did not find any differences in metabolic rate between the sexes when the flies were starved ($F_{1,233}$=0.12, P=0.72). We identified three clusters in *post hoc* testing: SR2, SR4 and SR1 with the highest metabolic rate; C2 and SR3; and C1. This finding is surprising since one would *a priori* expect

the selected lines to be more effective in down scaling their metabolic rate under starved conditions (see discussion).

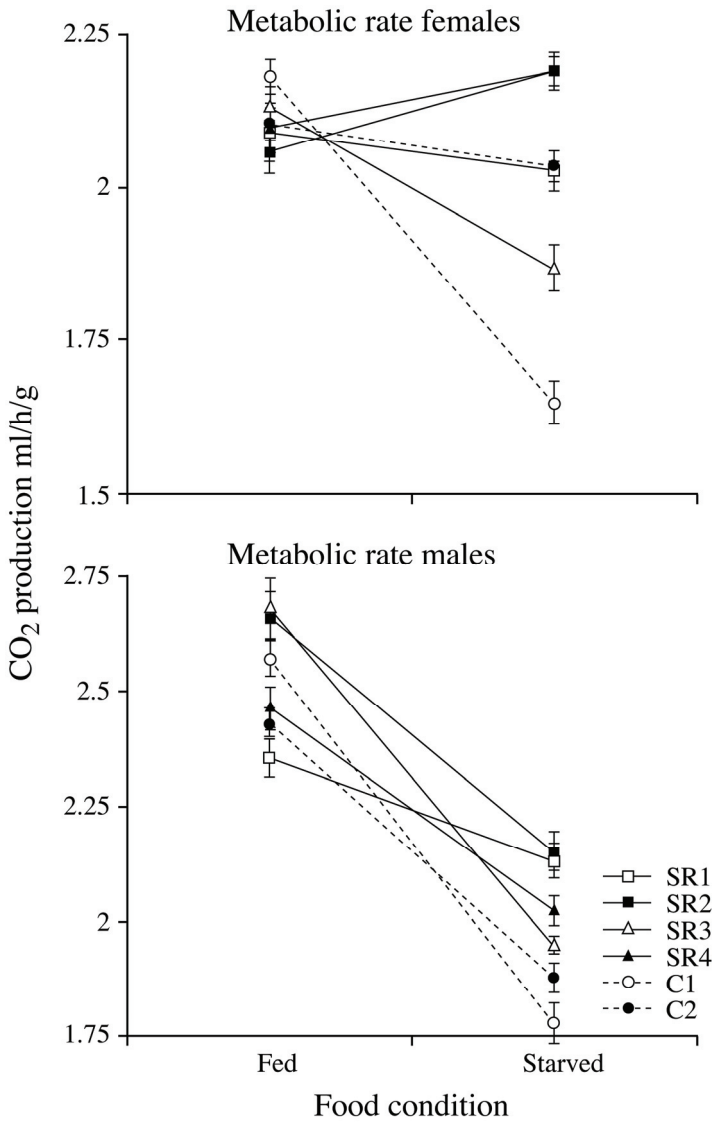

Figure 5. Average CO_2-production in ml per hour per gram of body weight of each sex of each line in both starved and fed conditions. Open symbols represent female data, dotted lines represent control lines, each line has a unique symbol-line structure.

Principal component analysis

We performed a principal component analysis on the averages of starvation resistance from eclosion, relative fat content, longevity, metabolic rate under starved conditions and paraquat resistance for each sex of each line. Three principal components captured nearly 95% of the variation (see table 2 for the traits and figure 6 for the objects). The first one (PC1) explained 72.7% of the variation and showed an effect of selected versus unselected lines (figure 6). All trait loadings on PC1 were similar (see table 2). In PC2, which accounted for 13.6% of all variation, longevity and starvation resistance were contrasted (see vectors table 2). PC3, which explained 7.9% of the variation present, revealed that paraquat resistance and metabolic rate contrasted with starvation resistance, relative fat content and longevity (see vectors table 2). Clustering of data in the PCs is shown in figure 6. PC1 shows separate clusters of selected (full line) and control lines (small dashed line) (all upper graph). PC2 shows a separation of the males (large dashed-dotted line) and females (full line) of the selected lines, and shows a single group for the control lines (small dashed line)(all lower graph). PC3 shows separation between the SR1-SR2 cluster (full line), the SR3-SR4 cluster (large dashed-dotted line) and the control lines (small dashed line)(all middle graph). Thus, this analysis on lines and sexes confirms patterns described for the individual traits and sex differences, and relates the different traits showing two distinct ways in stress adaptation.

Table 2. Trait weightings from PCA for data of starvation resistance from eclosion (SR), relative fat content (RFC), longevity (L), metabolic rate under starved conditions (MR) and paraquat resistance (PR). PC1 = first principal component, PC2 the second and PC3 the third. Bold characters indicate points of attention.

Trait	**PC1** (72.7%)	**PC2** (13.6%)	**PC3** (7.9%)
SR	-0.432	**0.541**	0.414
RFC	-0.487	0.106	0.398
L	-0.395	**-0.749**	0.299
MR	-0.454	0.274	**-0.588**
PR	-0.463	-0.245	**-0.484**

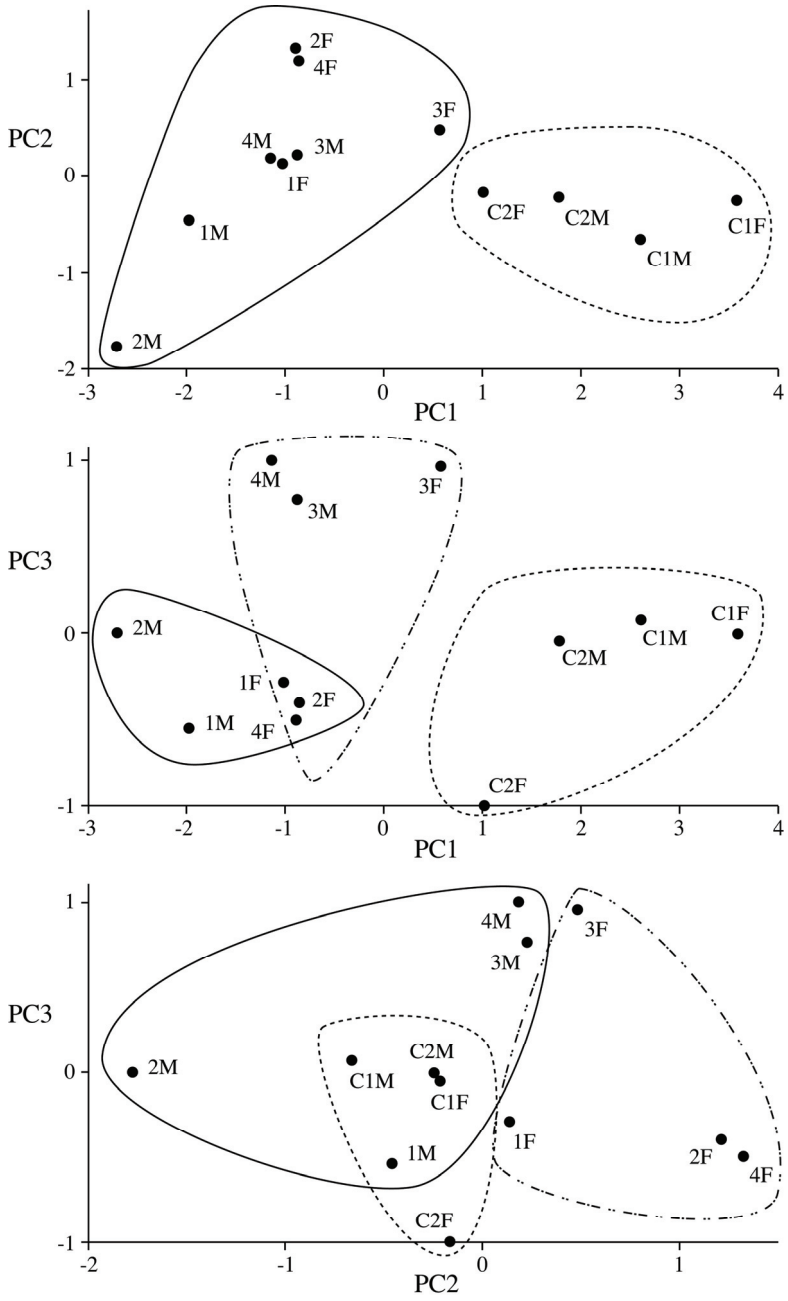

Figure 6. PCA across selection lines (1-4) and controls (C1-C2) for the variables of: starvation resistance from eclosion (SR), relative fat content (RFC), longevity (L), metabolic rate under starved conditions (MR) and paraquat resistance (PR). M = male; F = female.

Discussion

Starvation resistance

Selection for increased starvation resistance over 20 generations produced a significant and substantial response. The realized heritabilities of the different lines and the covariance analysis show a significant difference between lines SR1and SR2, and lines SR3 and SR4; the latter showing more rapid responses although this is scarcely apparent in the overall responses (figure 1). The heritabilities of lines SR3 and SR4 seem to be characteristic of morphological and physiological characters. The values of lines SR1 and SR2 fall within the range of life history traits (Mousseau and Roff 1987). Heritabilities for starvation resistance in the sexes of all lines presented here are lower than the starvation resistance heritability average presented in the study of Hoffmann (2000), which is possibly the result of our specific selection regime. The response to selection on starvation resistance directly after eclosion affects starvation resistance at other ages after feeding. Starvation resistance at 7 and 21 days after eclosion was significantly higher in selection lines than in control lines. Thus, the condition of starvation resistance is stable with age and can be considered characteristic of these selected lines throughout adult life. It is also, at least partly, independent of adult feeding history. Starvation resistance in selection line females tends to increase up to day 7, whilst the males show a progression of starvation resistance over time similar to control lines but at a higher level. Our flies show a decline in starvation resistance after day 21 in both sexes of all lines, maintaining the differences between selected and control lines.

Fat content and body weight

Harshman et al. (1999a) found that selection for increased starvation resistance led to an increase in lipid storage and higher body weight. Their table 4 suggests that protein content did not change under selection for increased starvation resistance. This indicates that fat free dry weight did not change, and thus that the increase in weight is contributed solely by increased fat reserves. Similarly, the present study found an increase in the lipid reserves, but no increase in fat-free dry weight. This is also consistent with the finding of Chippindale et al. (1996) that larval lipid acquisition was important in the evolution of starvation resistance. Baldal et al. (2005) showed that fat content is correlated with, but not necessarily a causal determinant of, starvation resistance. In this study, the evidence supports an important contribution of fat content to the physiological mechanisms underlying increased starvation resistance.

Paraquat resistance

Two out of four lines selected for increased starvation resistance showed significantly enhanced paraquat resistance relative to controls. The absence of a more uniform effect suggests that there is no consistent association between increased starvation resistance and increased paraquat resistance. The increase in paraquat resistance in two out of four starvation resistant lines should thus be considered a side-effect. We speculate that the correlated response in paraquat

resistance depends on where in the pathway selection took place. This implies pleiotropic effects of mechanisms upstream in the pathway.

Longevity

At standard food conditions, no significant differences in longevity were found among females (cf. Harshman et al. 1999b). Males of the selected lines tended to be longer-lived than control lines. This exemplifies that longevity is a complex trait depending on many conditions, including sex. Longevity is also highly sensitive to environmental variation (e.g. Baldal et al. 2005) and is associated with several genetic mechanisms that also influence other traits. Lines SR1 and SR2 showed clear positive differences in longevity from the control lines and had relatively low heritabilities for starvation resistance. It is noteworthy that the lines with increased longevity also have increased paraquat resistance. In PC2 (13.6%) longevity and starvation resistance are effectively contrasted (see table 2). We speculate that this may be because longevity and starvation resistance are not exactly the same traits and can be founded on the same resources, causing them to show a trade off.

Metabolic rate

Our data indicated a difference in metabolic rate between starved and non-starved flies. In all male, and two female tests, metabolic rate was higher under fed conditions than under starved conditions. This is expected because in facing food shortage, resource utilisation will be rationed (Djawdan et al. 1997; 1998; Harshman et al. 1999a; Harbison et al. 2004). Although differences occurred among lines, there was no consistent separation between metabolic rate of control lines and selected lines under fed conditions. Harshman and Schmid (1998) did not find a correlated response in metabolic rate with increased starvation resistance after 4 days of feeding on banana molasses medium. Hulbert et al. (2004) found no association of lifespan and mass-specific oxygen consumption under normal feeding conditions. In our experiments, we also found no striking differences among lines after 4 days of feeding. Thus, there is no indication that starvation resistant flies have a different metabolic rate than control lines when fed.

Three selected lines showed higher metabolic rates than controls under starved conditions. The starvation selected animals have elevated fat content relative to the controls. Fat is metabolically inactive, but is taken into account for wet weight. The mass specific metabolic rate (MSMR) is calculated per unit of wet weight. The MSMR of the starvation selected animals also takes the fat content into account. For a clear-cut comparison, the MSMR of the different lines should leave out the differing fat proportions. This would lead to the conclusion that MSMR of the starvation selected lines is actually even higher than it has been estimated now. MSMR of the starvations selected flies is thus higher than that of the control flies. Evidently, the metabolic rate of flies selected for starvation resistance changes when they are starved, but the pattern relative to the controls differs from our expectations.

The respiratory coefficient (RQ) is the coefficient of the amount of CO_2 produced over the amount of O_2 consumed. It is known that RQ is dependent on the resource that is being used. Metabolising carbohydrates generates a higher RQ than metabolizing lipids. Thus, a change in either the O_2 uptake or the CO_2 output may indicate a switch of resources rather than a difference in metabolic rate. Harshman et al. (1999a) assayed their flies' metabolic rate at 4 to 7 days after eclosion by measuring O_2 consumption. Where our starvation resistant flies under fed conditions showed no response in their CO_2 production, those of Harshman et al. showed a significant reduction in their use of oxygen. Combining this would lead to a higher RQ for the starvation resistant flies, implying that animals under normal feeding conditions rely mainly on the burning of carbohydrates for their energy.

Harshman et al. (1999a) found that after 28 hours of starvation, intermediary metabolic enzymes involved in carbohydrate degradation showed a decreased activity. So, during starvation fewer carbohydrates are burned and thus, the RQ should go down. We found a strong decrease in CO_2 production for male flies under starved conditions (figure 5a) relative to under fed conditions, and a variety of responses in females (figure 5b). If oxygen intake were to remain similar or increase, RQ is dramatically lowered, implying that starvation resistant flies rely heavily on burning fat under starved conditions. When oxygen consumption is also lowered and RQ changes little, flies could still rely on carbohydrate metabolism. However, the latter possibility is unlikely, considering the finding of Harshman et al.(1999a) that carbohydrate catabolic enzymes show reduced activity. Based on this reasoning, we conclude that during starvation flies most probably rely on burning lipids rather than carbohydrates as an energy source.

We examined CO_2 production in flies that were starved for 4 days. At that time, mortality risks of the flies of the control groups are significantly higher than those of the starvation resistant lines. In this respect, one may argue that physiologically, the control lines can not then be compared to the starved lines. We acknowledge this point and reason that lowered metabolic rate in starved flies may indicate the near depletion of the main resource, fat. The high metabolic rate in the selected flies thus reflects their increased fat content

Principal component analysis

Considering the traits overall, we observe that two groups of lines have formed as a result of selection for increased starvation resistance. The first principal component reveals a clear contrast between the control lines and the selected lines (figure 6, upper graph). PC2 tends to separate males and females of the selected lines, and also tends to group the control lines (figure 6, lower graph). PC3 shows separation between the SR1-SR2 cluster and the SR3-SR4 cluster overlapping with the control lines, which becomes especially clear in combination with PC1 (figure 6, middle graph). Again, this is consistent with the existence of more than one solution of how to cope with starvation conditions. This parallels the interpretations of other authors working on longevity (Harshman et al. 1999b; Arking et al. 2000; Archer et al. 2003; Phelan et al. 2003). However, these conclusions were based on longer periods after selection. Our results indicate two trajectories during selection.Thus, we conclude that though altering longevity yields many correlated responses, selection for starvation resistance does not necessarily alter other life history traits. These

findings may be related to our selection regime, which focuses on the important larval and pupae stages, without interference of the adult feeding behaviour and physiology.

Implications for evolutionary theory

The finding that starvation resistance and longevity do not necessarily co-vary shows that the trade offs that are often found between these traits do not always apply. We therefore should be cautious in the future to infer unitary evolutionary relationships from physiological trade offs.

The nature of the traits we measured is best put into the framework of the disposable soma theory of ageing. Starvation resistance and longevity are both soma related and are supposed to counter balance the germ line related traits. Since starvation resistance and reproduction are thought to rely on the same resource, one would expect a conflict. Since there is a substantial fat reserve, both allocation to the reproductive apparatus and starvation resistance may be relatively high. This conflict in the allocation between the “soma side” and the “germ line side” is consistent with the disposable soma theory. The fact that the allocation may yield a difference between the sexes fits as well, since differences may be present in sex-specific allocation. The fact that high allocation to the reproductive apparatus would lead to a reduction, or at least not to an extension, of life span is fundamental to the disposable soma theory. We will explore these issues further using different environments and gene expression analysis in relevant environments.

We have shown that in evolution there may be more than one solution to the environmental challenge of starvation resistance. Relative starvation resistance differences seem to be stable over time. We also showed that metabolic rate is neither associated with longevity, nor with starvation resistance. Principal component analysis provides a powerful way of analysis in life history theory and can uncover novel insights.

Our data suggest two distinct patterns associated with increased starvation resistance. The first is associated with high heritability, suggesting a rapid response to selection and a considerable standing genetic variation allowing increased fat content. The second response is using genetic mechanisms that are less variable (lower heritability) and increased fat content, increased paraquat resistance and longevity. Neither of the patterns are associated with a change in metabolic rate.