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Author: Heuvel, Joost van den
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Linking life history trait and transcriptome variation using cyclical nutritional environments in *Drosophila melanogaster*

Joost van den Heuvel*, Jelle Zandveld*, Maarten Mulder, Agnieszka Doroszuk, Paul M. Brakefield, Thomas B. L. Kirkwood, Daryl P. Shanley, Bas J. Zwaan

* These authors contributed equally to this work

“I’m the yo-yo man, always up and down, so take me to the end of, your tether”

McCulloch, Sergeant, Pattinson, de Freitas
Abstract

Phenotypic plasticity is a central concept in the study of life history evolution. For instance, most organisms respond plastically to variation in nutrition availability. Although many organisms experience variability of nutrition, little is known about different patterns of variation that affect life history traits. In this study we compare fruit flies fed a constant high or constant low diet with flies that were fed a ‘yoyo diet’, in which diet alternated between high and low levels within each week. We measured egg production and weight of individually housed virgin flies longitudinally and recorded their lifespan. Surprisingly, flies fed a low diet produced most eggs and were the shortest lived. The yoyo flies similarly produced most eggs during the low level diet phase. In contrast, the lifespan of the yoyo flies was slightly lower than the flies on constant high, but significantly higher than flies on constant low diet. We measured gene expression of flies on two consecutive time points for low control flies, high control flies and low and high yoyo flies on both the high and low nutritional environment. Consistent with the life history responses, flies on high diet showed a ‘dietary restriction’ type of expression of effector genes. Life history traits and the transcriptome of the flies show a very plastic response on short time scales, indicating the importance of this plasticity with respect to the type of variation of food experienced by flies in nature.

**Keywords:** plasticity, gene expression, diet, life history traits
Introduction

Phenotypic plasticity is the ability of a genotype to produce alternative phenotypes in different environments (PIERSMA and DRENT 2003; WEST-EBERHARD 2003) and is a very important concept in the study of life history evolution (ROFF 1992; STEARNS 1992; FLATT and HEYLAND 2011). Although phenotypic plasticity is often viewed as adaptive (STEARNS and KOELLA 1986), whether this is true is rarely tested (VAN DEN HEUVEL et al. 2013; chapter 2, but see for individual traits under laboratory conditions PARTRIDGE et al. 1994; FISCHER et al. 2003). If fact, phenotypic plasticity can be maladaptive if the phenotype does not match the requirements of the changed environment and the likelihood of this occurring critically depends on how predictable the environment is (REED et al. 2010). Both the evolution (ROFF 1992; STEARNS 1992) and plasticity of life history traits are greatly influenced by resource availability. For instance, organisms might develop into distinct phenotypes (MARET and COLLINS 1997; FRANKINO and PFENNIG 2001; BENTO et al. 2010) depending on resource availability, which in turn leads to variable food utilization (PFENNIG and MCGEE 2010). Such plastic responses may be subtle and continuous in nature. This may be reflected in results from laboratory experiments. For instance, in some species lifespan increases trough mild dietary restriction (McCay et al. 1935; Klass 1977; Muller et al. 1980; Weindruch et al. 1986; CHIPPINDALE et al. 1993), while other species respond differently (CAREY et al. 2002; COOPER et al. 2004; BECK 2007; KASUMOVIC et al. 2009). This variation between species and populations within species may reflect the degree of laboratory adaptation (NAKAGAWA et al. 2012), but, alternatively, evolutionary explanations might be able to account for the species and population-specific responses to dietary restriction.

Individuals acquire resources from their environment that they allocate to the many diverse functions such as growth, development, egg production and maintenance and repair, and past selection has resulted in an allocation pattern that maximizes fitness (CALOW and TOWNSEND 1981; CALOW 1987; SIBLEY and CALOW 1987). This framework of acquisition and allocation has been central for the development of the theory of life history evolution (ROFF 1992; STEARNS 1992; FLATT and HEYLAND 2011) and can for instance explain variation in lifespan between species (KIRKWOOD 1977; KIRKWOOD and HOLLIDAY 1979; KIRKWOOD and AUSTAD 2000). Importantly, combining genetic variation in acquisition and allocation can lead to trait correlations that vary between positive and negative (VAN NOORDWIJK and DE JONG 1986).

The resource acquisition and allocation framework (CALOW and TOWNSEND 1981; VAN NOORDWIJK and DE JONG 1986; CALOW 1987; SIBLEY and CALOW 1987) has been very helpful to understand the relationship between food content and various life history traits (BOGGS 2009). Using this framework, for instance, it can be explained why it is adaptive for mice to extend lifespan upon dietary restriction by increasing maintenance and repair at the cost of reproduction at intermediate food levels (SHANLEY and KIRKWOOD 2000). Similarly, in flies it has been shown that relatively more energy is invested in protein turnover compared to reproduction in dietary restriction regimes compared to fully fed individuals. In contrast, total energy spent on protein turnover, which can serve as an indicator for maintenance, was higher in fully fed flies (TATAR 2011). Animals with a reduced energy intake may also produce less damaging molecular species because of a reduction in metabolic rate (MCCARTER and MCGEE 1989), and therefore, total maintenance and repair of DNA and protein turnover must be seen in a relative amount, not only to total intake, but also compared to how much cellular damage is inflicted. Furthermore, additional acquisition of specific amino acids may increase or reproduction (GRANDISON et al. 2009) without affecting lifespan, which may indicate that organisms do not always fully use all
 resource in maximizing these two traits. These results indicate that the evolutionary framework of acquisition and allocation can be used to explain why relationships between life history traits vary between food levels, but mechanistic extensions to this framework are needed to explain specific outcomes such as those found in specific cases such as the fruit flies.

The ISS / Tor pathway is commonly regarded to play an important role in regulating growth (Nijhout 2003), reproduction (Harshman and Zera 2007), metabolism (Saltiel and Kahn 2001), and ageing (Zoncu et al. 2011). For instance, life history traits and insulin signaling are affected in a similar fashion by genetic manipulation of upstream regulators of insulin signaling and caloric restriction (Al-Regaiey et al. 2005). Furthermore, genetic manipulation of genes within the insulin signaling pathway affects lifespan in a wide variety of organisms such as mice (Brown-Borg et al. 1996; Holzenberger et al. 2003; Selman et al. 2008; Selman et al. 2011), flies (Clancy et al. 2001; Tatar et al. 2001; Tu et al. 2002; Hwangbo et al. 2004; Bai et al. 2012), and nematodes (Kenyon et al. 1993). Even more so, natural variations in allele frequencies in these pathways covary with life history parameters in a latitudinal cline of D. melanogaster (Fabian et al. 2012). Such conserved signaling pathways (Barbieri et al. 2003; Alic and Partridge 2011) are candidate regulators of the response of many downstream genes via transcription factors (Alic et al. 2011). Although these pathways are conserved, it is difficult to find common genes in these pathways that are regulated by food in a similar way in different species. Rather, a typical response to food at the transcription level is described by common biological processes such as (energy) metabolism, stress and immune response, regulation of transcription, cell growth, apoptosis and signal transduction (Han and Hickey 2005), and are ultimately linked to the expression of groups of genes that represent these processes (McElwee et al. 2007). The up- or down-regulation of these processes in response to diet can be seen as the mechanism by which energy acquisition and allocation of food alters life history traits such as reproduction and longevity.

As noted above, the response of life history traits to different diets, if adaptive, is ultimately an evolved plastic response to cope with variation in the natural environment. Such environmental variation occurs, for instance, in a seasonal fashion and organisms respond to that variation by different modes of plasticity, between generations (Windig et al. 1994), or between seasons within generations (Gorman and Zucker 1995). However, the response to diet in many experiments, including the ones cited above, is often investigated in a constant environment, where subjects are given constant levels of diet. How organisms respond to cycles of resource variation is not well described. It is known that fruit flies (Drosophila melanogaster) when they are exposed to a food shift once in their life, respond to food variation quickly (Mair et al. 2003). It is unknown however, how individuals respond to continuous varying nutrition.

Therefore we set out to investigate how continued cyclic variation between high and low nutrition levels affects multiple life history traits using longitudinal life course measurements of individually housed D. melanogaster females. Furthermore, we combine the trait measurement with the gene expression of flies on high and low diet in constant and variable environments. Our aims are to assess, (1) how life history traits change when variation in food is continued throughout the lifespan of the flies, (2) to what degree the phenotypes are comparable to control flies fed constant nutrition levels and (3) to what degree the transcriptome of the cyclic flies on high or low diet compares to the constant controls on the same nutrition levels. This comparison will allow the identification of the genes that mediate the responses to diet shifts, and potentially also the genes that regulate them. Therefore, on the one hand, this study is important from an evolutionary perspective, by analyzing energy allocation strategies; on the other hand the results will advance our mechanistic understanding of how variation affects life history traits.
Methods

Diet

We used three different nutritional environments, indicated by 1x (low), 2x (intermediate) and 5x (high) medium. The nutritional environments varied in sugar (50, 100 and 250 gram per liter in 1x, 2x and 5x medium, respectively) and yeast (35, 70, 175 gram per liter in 1x, 2x and 5x medium, respectively). Furthermore the medium contains agar (20 gram per liter) nipagine (15 ml of 100 g 4-methyl hydroxy benzoate per liter alcohol) and propionic acid (3 ml per liter).

Flies

Flies were collected in the summer of 2008 at six locations forming a transect ranging from Vienna to Athens. The flies we mixed and reared in population bottles with population numbers of at least 300 individuals per generation on 1x medium for 50 generations before the experiments were started (as in chapter 4). To prevent larval and maternal effects on adults, we kept the flies for at least three generations on intermediate nutrition. The larvae were reared in vials with 6 ml of intermediate diet, with a density of 50 eggs per vial. After eclosion, each fly was randomly put in either a low or high nutrition vial (6 ml of medium throughout the experiment) without using anesthesia. The single housed flies were all checked for sex and vials were inspected for fertilized eggs in the first three days. Accidental males and fertilized females were removed from the experiment. Therefore, all flies in this experiment were single housed, virgin female flies.

Adult diet treatment

Four adult diet treatments were used (see Figure 1). One group of flies was maintained on low diet throughout the experiment (low control, LC). A second group of flies was maintained on high diet throughout the experiment (high control, HC). A third group was transferred between vials of high and low diet every half week. Because the initial vial of this group of flies contained high diet, we call this group the high yoyo group (HY). A fourth treatment group started at the low diet medium (low yoyo group, LY), to control for the effect of first diet source level. For every treatment we started with 65 flies, and after the first transfer moment the number of days they were in a vial varied between 3 and 4, to keep in synchrony with the week, making sure that both yoyo treatments in total had the same number of days on a specific nutrition level throughout the experiment.

Trait Measurements

Before we transferred a fly to a new vial, the flies were anesthetized with ice (a maximum of 5 minutes) and individually weighed on a scale to the nearest 0.01 mg (Sartorius). After weighing, flies almost always directly revived in the new vial. Every vial was kept to count the number of eggs laid by each female. Every day we checked for survival, and flies were censored when they were found dead between the stopper, accidently crushed or escaped from scale because of insufficient anesthesia.

Microarray experiment

For the microarray experiment a new cohort of flies was set up. The flies received the same treatment as those for life history assessment, with the exception that after being anesthetized,
they were not weighed. Flies were sacrificed at two time points in the experiment: half of them from the 8th vial they were in during adulthood, and the other half after an additional 4 days later from the 9th vial (see Figure 1). Therefore the flies were on average 35 days old at the first time point 39 days old at the second time point. We did this in order to test whether the gene expression in the yoyo flies changed when they were changed from high to low for the low yoyo treatment and to compare that with the high yoyo treatment and controls. We sacrificed all the flies of the gene expression assay at similar times during the day (at noon) and for all groups the flies had spent four days in their food vial. We transferred flies to 2 ml eppendorf tubes (4 flies per tube) and snap froze them using liquid nitrogen. After this, flies were kept in -80 degrees celcius before RNA isolation. RNA was isolated using the TRIZOL method after which we used the RNAeasy from Quiagen to cleanup the samples. For both steps manufactures’ protocols were used. In total 64 samples were isolated, and the 32 samples with the highest RNA yield were used for microarrays. In total there are 8 treatment groups with 4 replicates per treatment. All 32 samples passed quality control and microarray measurements took place at Service XS in Leiden (http://www.servicexs.com) using the Affymetrix Drosophila genome 2.0 array.

Figure 1. Schematic overview of the setup of the experiment. The four food treatments vary in when they get high (H) or low (L) food (as indicated by the horizontal solid line on the dashed lines). The high control (HC) treatment flies are on high food throughout the experiment. The low control (LC) treatment flies are on low food throughout the experiment. The high yoyo (HY) treatment flies are on high food for halve a week, then transferred to low food, then to high again and so forth throughout the experiment. The low food yoyo flies differ only in the starting level of food, which is low. The number of days they are on the food is 4, 3, 3, 4, 4, 3, 3 and so forth which means that both yoyo treatments have had the same number of day on high and low food after 4 transfers. After every transfer the number of eggs and weight is measured. Every day survival is checked. In a second cohort set up for microarrays, a group of flies is sacrificed while in the 8th vial, a second group while in the 9th vial as indicated by μArray T 1 and μArray T 2.
Statistics

To establish the effect of dietary treatment on survival a Cox proportional hazard test (Cox 1972) using nutrition treatment as a factor. For weight, we fitted a repeated measure ANOVA where we used nutrition and time as fixed factors and individual as a random factor. We used the R package languageR to calculate appropriate p values (Baayen 2008). For the egg data we did the same, but then we used a GLMM with Poisson error distribution since number of eggs was not a normally distributed variable (Zuur et al. 2009).

The microarray data were normalized and summarized using the robust multi-array average (RMA) procedure (Bolstad et al. 2003), as implemented in Bioconductor, R (Gentleman et al. 2005; Hahne et al. 2008). We used MAANOVA to calculate permuted and adjusted FDR values using F test statistics. We used a cutoff value of 0.05 to call a difference significant. Gene expression was also correlated (Pearson) with number of eggs per sample. Again the p values were FDR adjusted. Then for all analyses we used the DAVID tool (Dennis et al. 2003) to look for overrepresented GO terms (gene enrichment analysis). We tested for 16 different contrasts in total (see Table 1). First we tested for food effect in all samples (high food contrasted against low food), and then we did the same for only the control treatment samples and the yoyo treatment samples. Similarly, we tested the effect of time, contrasting the first time point against the second. Thirdly, we contrasted the control samples against the yoyo samples, and, fourthly, the yoyo high start samples against yoyo low start samples. It needs to be noted, that the comparisons between specific groups contain different number of replicates (see Table 1), resulting in differences in statistical power between the analyses. Therefore, the absolute number of differently expressed genes are not always directly comparable, as the might reflect the number of samples, rather than the effect of the treatments.

To improve our inferences we also compared our results to results of other microarray experiments. The experiments were chosen to represent a wide variety in types of manipulations. In one study flies that were selected for higher starvation resistance, which were also long-lived compared to control flies were used (Doroszuk et al. 2012). Flies were kept on different adult food conditions and sacrificed at middle (90% alive) and old age (10% alive). The second study used flies that were deprived of food for different number of days and several body parts were used (Farhadian et al. 2012). Thirdly, we used data from an experiment that used both genetic manipulation and dietary restriction to increase the lifespan of flies and measured flies at young (10 days) and middle age (30 days, roughly 90% alive) (Bauer et al. 2010). Lastly, we used data from the study of (Pletcher et al. 2002), for which flies were sacrificed at many different ages varying form very young to very old, but also comparable to our study over a very short time period. Our study and all other studies mentioned here measured gene expression using the Genome 2.0 Affymetrix microarray, with the exception of the study of Pletcher et al. (2002) which used the Affymetrix roDROMEGa array (Pletcher et al. 2002). The question answered by this comparison is whether genes affected in our study have similar relationships with traits in other studies. From our GO enrichment study, we selected the seven most enriched GO terms for the list of genes that were up regulated on high food (comparison 1, Table 3), seven most enriched GO terms for the list of genes that were down regulated on high food, seven most enriched GO terms for the list of genes that were upregulated on the first time point and two most enriched GO terms for the list of genes that were down regulated on the first time point. Therefore we used the genes represented by the probes significant in our study and belonging to one of the 23 most enriched
GO terms (see Appendices C1, C2, D1, D2, E1, E2, F1, F2 for GO term enrichment results, G1 for results of this comparison, G2 for lists of probes). These enriched GO terms were represented by probe sets in our study. The average expression of the probe sets per GO term was calculated per sample for the four other studies to compare the expression with our study. This method allows to us to answer the question whether our genes that represent enriched GO terms in our study are also up or down regulated in these other studies.

Table 1. Number of significant genes up and down regulated in specific contrasts tested. Four types of contrasts are tested, high against low food samples, first against second time point, control against yoyo samples and yoyo high start against yoyo low start using different sets of samples as indicated in the left column.

<table>
<thead>
<tr>
<th>Food: high food vs low food</th>
<th>Samples</th>
<th>Genes significant</th>
<th>Up on high</th>
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</thead>
<tbody>
<tr>
<td>All samples (32)</td>
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<td>3150</td>
<td>2796</td>
<td></td>
</tr>
<tr>
<td>Control samples (16)</td>
<td>3914</td>
<td>2341</td>
<td>1573</td>
<td></td>
</tr>
<tr>
<td>Yoyo samples (16)</td>
<td>2722</td>
<td>1490</td>
<td>1232</td>
<td></td>
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<tr>
<td>Yoyo low start (8)</td>
<td>2004</td>
<td>1155</td>
<td>849</td>
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<th>Genes significant</th>
<th>Up in first</th>
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</thead>
<tbody>
<tr>
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<td>2848</td>
<td>1576</td>
<td></td>
</tr>
<tr>
<td>Control samples (16)</td>
<td>5251</td>
<td>3425</td>
<td>1826</td>
<td></td>
</tr>
<tr>
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<td>0</td>
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<td>1642</td>
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<td>Low food (16)</td>
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<td>26</td>
<td>48</td>
<td></td>
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<tr>
<td>yoyo on low food vs control on low food (16)</td>
<td>747</td>
<td>483</td>
<td>264</td>
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<td>yoyo on high food vs control on high food (16)</td>
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<th>Samples</th>
<th>Genes significant</th>
<th>Up for yoyo low</th>
<th>Down for yoyo low</th>
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<tr>
<td>Yoyo low start on high food vs yoyo high start on high food (8)</td>
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Results

A schematic overview of the most important results is given in Figure 2. This figure can be used as a guide to the whole results section which contains many details.

**Figure 2.** Schematic overview of the results. A. Survival is lowest for the low control line, and highest for the high control line. The low and high yoyo lines are significantly different from the low control, but only the high yoyo line has a significant lower survival rate compared to the high control. B. In time the weight of flies decreased, but with a similar amount between treatments and food types. Flies in different food thus do not differ in weight. C. Egg production is higher at the low food level when all samples are pooled. D. Egg production is higher for the control flies on low food compared to the ones on high food. E. Egg production is higher for yoyo flies when they are on low food. F. PC1 and PC2 of the PCA on gene expression of all genes can separate the low (L) and high (H) food samples. G. Number of eggs counted per sample correlates negatively with PC1, the low food samples overlap slightly with the high food samples. H. Number of differently expressed probes on the y axis dependent on the food contrast. If all (ALL) samples are taken, 5,946 probes are tested to be significantly differently expressed. Within the control sample (C) and yoyo samples (Y) this is less. More genes are differently expressed between high and low food when this was compared within the low yoyo treatment (LY) compared to the high yoyo treatment (HY). I. Number of differently expressed probes on the y axis dependent on the time contrast pooled for all samples (ALL), control samples (C), yoyo samples (Y), high food samples (H) and low food samples (L). The highest number of probes differently expressed was found in the control contrast (C). There is a big difference in number of genes expressed differently when the control contrast is compared to the yoyo contrast and when the high food contrast is compared to the low food contrast. J. Number of differently expressed probes on the y axis dependent on the control against yoyo treatments contrast pooled for all samples (ALL), low food samples (L) and high food samples (H). A very low number of probes is found differently expressed when all samples were pooled. A much larger number of probes were differently expressed the low food samples were tested compared to high food samples (0 probes differently expressed) and to all samples pooled.
**Phenotypes**

The high control flies (HC) had a higher survival compared to the low control flies (LC) ($p<0.05$). Survival was significantly higher for both yoyo treatment fly groups compared to the low control ($p<0.05$), while only the flies that started on high diet (HY) where significantly different from the high control (HC). Because the flies of the yoyo treatments did not have significantly different survival rates, this all indicates that survival of the yoyo flies resembled the high control more closely than the low control flies (see fig 2A, 3A). Weight was greatly affected by time ($p<0.05$), but surprisingly not by diet (fig. 2B, 3B). For all diet treatment groups, weight decreased with time. Flies laid more eggs on low diet, both for the control at the early time points and when the yoyo lines are compared to each other, throughout life (fig.2C-E, 3C). This means that yoyo flies increased their egg production with diet changes within 4 days when changed from high to low diet. When the total number of eggs in the first five weeks was regressed on the lifespan of flies surviving the first five weeks, the number of eggs correlated negatively with lifespan (fig. 4A, $p<0.05$). However, when this analysis was performed per line, this relationship disappeared (fig 4B). Therefore, this indicates that the negative relationship was more dependent on average differences between groups in lifespan and egg production than on differences between flies on similar treatments (fig. 4C).

![Figure 3A](cumulative_survival_per_line.png)

**Figure 3A.** Survivorship for the four different food treatments lines (green indicates low control, red high control, black low yoyo, blue high yoyo). Y axis represents proportion of flies alive.

![Figure 3B](average_weight_per_food_treatment.png)

**Figure 3B.** Average weight dependent on food treatment and time (measurement). The first 20 measurements are shown. At the first measurement flies are 4 days old, at the last in this figure 70 days.
Figure 3C. The average number of eggs for different food treatments and at different time.

Figure 4A. Early reproduction and lifespan in days for all individuals that survived beyond the first five weeks.

Figure 4B. Relationship between early reproduction and lifespan for individuals colored by food treatment.
Gene expression

Overview Microarrays

A PCA on all summarized probe sets indicated that samples grouped well together using diet as separating treatment (Fig 2F, 5A). Yoyo flies are separated within the low diet cloud from the control, while this is not true for the high diet samples (5B). Sampling time also has an effect. Samples taken at the second time point displayed average lower value on the PC2. PC1 related well with the number of eggs counted in the vials at the time of sacrifice (fig 2G, 5C). Our main interest is whether yoyo flies show a yoyo specific gene expression pattern or whether they are alike the control lines and like the phenotypes, gene expression relatively moves from a low diet to a high diet type of variation when moved from low to high diet. Indeed in general the PCA indicates that when yoyo flies are transferred from low to high diet they alter their gene expression from a low diet expression type (indicated in Figure 5A) to a high diet expression type. This indicated that the phenotypes, which change within the 4 days from a relatively low diet phenotype to a high diet phenotype (when compared to the control flies), were matched by the general variation pattern of the gene expression which also resemble relatively the control flies on the respective diet. Even more so, like the phenotypes, the yoyo flies are more like the high control when on high diet, but less like the low control flies, when on low diet (see figure 5B). Lastly, when the egg number per sample was related to the variation in PC1 per sample, a negative relationship appeared, further indicing the relationship between phenotypes and gene expression in this study (see Fig. 5C). This general pattern can also be appreciated from the number of probe sets significantly affected in the various contrasts tested (Table 1), which will be described in detail below.
Figure 5a. Variation in gene expression along PC1 and PC2. Samples are colored by the food they were on before sampling.

Figure 5b. PC1 and PC2 plotted samples, indicated per line and timing of sacrifice.

Figure 5c. PC1 and the number of eggs laid before sacrifice. Again line and time at sacrifice indicated.
Diet effect

First the difference in gene expression between samples on low and high diet was studied. Diet affected a large number of probe sets. When all samples were grouped together per diet, 3150 probe sets where significantly up-regulated on high diet, while 2796 where down-regulated on high diet (Table 1). In a similar analysis, but with only control diet samples, 2341 probe sets were up-regulated, while 1573 were down-regulated on high diet. For the yoyo diet samples these were 1490 and 1232 respectively. Since we are interested in whether diet affects gene expression in control samples and yoyo samples in a similar direction, we calculated the overlap between these probe set lists. There was a large overlap in genes up- or down-regulated on high diet compared to low diet in all samples, the control samples and the yoyo samples (989 probe sets, see fig. 6A). Probe sets that were only significantly up- or down-regulated in the full comparison (566 up, 903 down), showed an expression difference in a similar (but not significant) direction in the control and yoyo samples only. Probe sets that were only significantly affected in control and full (1191 up, 740 down) or only in yoyo and full (404 up and 496 down) all showed relatively similar expression patterns in the yoyo and control comparison respectively. On the contrary, genes which are only up- or down-regulated in the control group show on average a reversed pattern in the yoyo group, and vice versa, but this is only significant for a small number of genes (11 and 6 probes, fig 6A). Biologically this means that there are a very few genes that are significantly differently affected by nutrition between control and yoyo samples. For a gene ontology enrichment analysis we used all the genes that are up- or down-regulated (3150 probe sets up, 2796 down) for the full comparison. From these set of genes that are up-regulated on high food are associated with neuronal response, post embryonic development, neuropeptide signaling, ion transport and G protein coupled receptor signaling. Genes that are down-regulated on high food are associated with membrane organization and inter- and intra-cellular transport of macromolecules, sugar metabolism and catabolism and reproduction. More genes were differentially expressed between food types for flies of the yoyo low treatment compared to flies that started high (fig. 6B). The complete gene enriched results are given in appendix C1 and C2 for up- and down-regulated genes respectively. These results, especially the overrepresentation with reproduction genes, relate well with phenotypic data. Reproduction is lower on high diet, while genes down-regulated on high diet show an overrepresentation for the biological process reproduction. Moreover, in other studies these genes show a similar correlation with phenotypes (see Appendix G1 & G2).
Age effect

Despite the fact that the time points over age were separated only by four days, the effects age were pronounced (fig. 6C, also see Appendices D1 and D2). When all the samples were grouped and the two time points where compared, 2848 probe sets were down-regulated in the later age class, while 1576 were up-regulated at a later age (see table 1). In control samples the effect of age is pronounced (see Fig. 5B); 3425 probe sets are down-regulated, while 1826 are up-regulated at the later age class. Interestingly, no such effect is measured in yoyo flies, where 0 probe sets are either up- or down-regulated (see table 1). Upon inspection of the PC analysis plot, time did seem to have a similar effect in the yoyo samples compared to the control samples (lower values PC2, see Fig. 5B) but these changes are not significant. The effect of time was much larger on high diet than on low diet; 2919 probe sets were down-regulated at the later age, while 1643 were up-regulated on high diet, compared to 0 on either up- or down-regulated on low diet. Compared to the association of probe sets differently affected by diet, the association with
specific biological processes is much more apparent. For the gene ontology analysis, the 2415 and 938 (see figure 6C) that were up and down regulated in all, the control, and the high diet samples were used. Genes that are down-regulated in the second age class are associated with DNA metabolism, repair and replication, cell cycle, cytoskeleton and chromosome organization and transcription and many other processes (see Appendix D2). Genes that are up-regulated in the second age class are associated with oxidation reduction, proteolysis and fatty acid metabolism (see Appendix D1). Again, this resembles age effects in other studies, which used much larger differences in time between age classes (see Appendix G1 & G2).

**Overlap diet and age effect**

In the above described results, there was no age effect in the yoyo flies. Because in these samples age and food changes between time points simultaneously, the overlap in diet and age effects are studied. A large number of genes are differently expressed between high and low diet samples and between age classes, hence, it is expected that there is overlap between these sets of genes. There is an association with genes that are down-regulated on high food and down-regulated at a later age (712 probes, chi-square, p<0.05). This consisted of a bit more than a quarter of the genes differently expressed between food types and between time points (see table 2). Since also a very large group of probe sets did not overlap between the age and diet effect, there would have been a considerable number of probe sets that could have been affected in the yoyo samples by age, without being affected by diet. This was not the case.

<table>
<thead>
<tr>
<th></th>
<th>High food</th>
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<tbody>
<tr>
<td></td>
<td>3150 up</td>
</tr>
<tr>
<td></td>
<td>2796 down</td>
</tr>
</tbody>
</table>

**Table 2.** Overlap of genes of food comparison and time comparison.

<table>
<thead>
<tr>
<th></th>
<th>High food</th>
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</thead>
<tbody>
<tr>
<td>First time</td>
<td></td>
</tr>
<tr>
<td>All samples</td>
<td></td>
</tr>
<tr>
<td>2848 up</td>
<td>213</td>
</tr>
<tr>
<td>1576 down</td>
<td>427</td>
</tr>
<tr>
<td>First time</td>
<td></td>
</tr>
<tr>
<td>All, high food and control samples</td>
<td></td>
</tr>
<tr>
<td>2415 up</td>
<td>113</td>
</tr>
<tr>
<td>938 down</td>
<td>318</td>
</tr>
</tbody>
</table>

**Expression differences between control and yoyo flies**

A small number of genes (74) are differentially expressed between yoyo flies and control flies when all samples are used for comparison (see table 1). Only three of these showed a similar expression between the two control fly groups (high and low constant) and therefore a consistent difference between control and yoyo samples. More genes are differently expressed between control and yoyo samples on low diet (747), but 0 on high food. These genes up-regulated for yoyo treatments are associated with odorant binding proteins and abiotic and visual perception, possibly indicated an increased perception of the environment in yoyo flies. These processes are often indicated in studies considering responses to nutrition (PLETCHER et al. 2002; LIBERT et al. 2007; DOROSZUK et al. 2012). Some up-regulated genes are also associated with the breakdown
of proteins, while the breakdown of large carbohydrate molecules is down regulated, indicating a different metabolism in yoyo flies compared to control flies on low diet (see Appendix E1 & E2). Both the survival and the reproduction at the time of sampling of the yoyo samples resembled the high control flies when on high diet, compared to the low control flies on low diet. This is therefore associated with the difference in number of probe sets differently expressed.

Variation in gene expression within yoyo samples

Both in survival and reproduction the two different yoyo fly groups were very comparable. Therefore, small gene expression differences, if any, are expected between these two groups of flies. Indeed, 0 probe sets are differently expressed between the 8 samples of flies that started life on high diet compared to those that started on low diet. Also, 0 differently expressed probe sets were found between these samples when only high or low diet samples were tested (see Table 1).

Gene expression associations with number of eggs

To further establish a relationship between phenotypic traits and gene expression pattern we related the number of eggs per sample with the gene expression per probe set. Processes which are significantly overrepresented in the group of genes that significantly relate to number of eggs, might be different from the groups of genes related to diet or age. More genes are negatively correlated with number of eggs than positively correlated to number of eggs. Furthermore, biological processes are much stronger overrepresented in the group of genes that are negatively correlated with number of eggs. Processes that are negatively correlated with number of eggs are similar to the processes that are up-regulated on high diet, while the genes that are up-regulated with number of eggs strongly resemble the genes that are down-regulated on high food. This is according to the egg number phenotype in the different diet groups as on high diet fewer eggs were produced. Processes that were negatively related to number of eggs are neuropeptide signaling, cell cycle and translation, spindle and cytoskeleton organization and ion and cation transport (see Appendix F2). Processes which were positively correlated to the number of eggs are reproduction, protein localization, sugar metabolism, exocytosis, nucleotide metabolism and transport and oxidation reduction and GTP-ase activity (see Appendix F1). No additional biological processes were found using egg number compared to the diet contrast. Most of the very significant overrepresented biological processes found in the GO analysis with diet effects were found using egg number as a covariate, but, less significantly so (compare Appendix C1 & C2 with F1 & F2).

Discussion

In this study we set out to investigate the effect of cyclic nutrition variation that is sustained throughout the life of a fly on life history traits and gene expression. Egg production varied with diet level, increasing at low diet, which is relatively similar to the differences between control fly level egg production. Survival of the yoyo flies was intermediate of the two control groups, but more equal to the high control fly survival. The expression of a large part of the genes in the genome showed a plastic response and also here expression of yoyo flies on high diet resembled that of the control high diet flies. A difference in gene expression between yoyo flies and control
was only found on low diet. This aligns well with the fact that in survival the yoyo treatment flies resemble the high constant treatment flies more than the low constant treatment flies. Furthermore, the relationship of the phenotypes, especially egg production, related in a similar way to the gene expression profile when compared to other gene expression studies, although the relationship between diet and life history traits was different.

**Phenotypes of control flies**

Probably the most unexpected result of our study was a higher egg production on low diet levels. At the same time, lifespan was higher at the high diet. It has been demonstrated that when the yeast and sugar components of food are varied independently, reproduction is increased at higher levels of yeast, but egg production negatively affected by increasing sugar levels (Skorupa et al. 2008). Therefore, the observation that on high food levels of reproduction is decreased may be the resultant of the high sugar content which the effect cannot be outweighed by the concomitant increase in the yeast level. In contrast, lifespan is more comparable with previous published studies with means of 56 days on low diet and 66 on high diet. Importantly, lifespan for the yoyo treatment resembled the value for the constant high group more, indicating that the high food level had a larger effect on the lifespan than the low die.

In this study we kept flies as single virgin females. Single flies have a much lower food intake than flies housed in groups (Wong et al. 2009 and Appendix B, feeding essay this study). A possible explanation for the difference in reproduction might thus be that with lower diet intake (single flies) sugar has a much larger effect than when flies are held in groups and have a higher food intake. Since sugar has rather a negative effect on reproduction at the levels we used (Skorupa et al. 2008) this might explain that relatively, at the higher food level reproduction decreased, while at the lower level it increased (see above). In another species the effects of sugar and yeast components of diet vary with feeding rate (Fanson et al. 2012).

**Phenotypes yoyo flies**

The yoyo flies always produced more eggs while feeding on low diet than while placed on high diet. The flies showed plasticity towards the phenotypes of the controls, resembling the response of the high and low diet control flies. At the same time, lifespan of the yoyo flies resembled the value of constant high group, indicating that the high diet level had a larger effect on the lifespan than the low diet level. The egg production variation for the yoyo lines showed the same plasticity pattern throughout the life of the flies. This indicates that plasticity itself is not affected by ageing, while the reproduction level itself is.

It has been shown before that mortality changes fast in response to food in *Drosophila* (Mair et al. 2003). In our experiment, there also seemed to be a trend towards higher mortality on low food within the yoyo treatment, but this was not significant. The egg production, and to a lesser extent the mortality phenotypes, shows that the flies cope with variation in the environment using a complete responsive or direct plasticity (West-Eberhard 2003) in which the phenotypes resembled that of the flies that were fed a constant diet.

**Trade offs**

With all the pooled data, we found a negative relationship between early egg production (first five weeks) and lifespan. When the relationship was investigated within treatment groups,
for three food treatments it was positive, only for one negative. This indicates that the initial found trade-off was mainly due to differences between food treatment groups, especially between the low control and other treatments. The lack of trade-offs within treatments may indicate that the mechanism underpinning the relation between reproduction and lifespan for individual flies is not resource allocation. Rather, individuals can increase reproduction without a cost in lifespan because these individuals either acquire more resource or metabolize resource more efficiently and therefore can invest in both, which is in line with evolutionary quantitative genetic (Van Noordwijk and De Jong 1986; De Jong and Van Noordwijk 1992) and physiological evolutionary models (Calow and Townsend 1981; Calow 1987; Sibly and Calow 1987). Differences that lead to trade-offs are especially present between groups of organisms that differ greatly in treatment or genotype, rather than between organisms within treatments or genotype. The advantage of monitoring flies separately is that we could identify these differences in trade-offs within and between treatments. The differences between individual flies are quite considerable with respect to egg production, which is something that is masked in experiments where flies are not housed individually.

**Gene expression response on diet**

Similar to the yoyo phenotypes, the gene expression patterns showed considerable plasticity in response to diet. In the PCA the high diet samples clustered together (see fig. 5a) and separated completely from the low diet samples. This indicates that the general expression patterns in yoyo flies changed from a typical high diet expression profile towards a low diet expression profile. In addition, our analysis showed that there was basically no transcriptional signature of fluctuating environment that would be independent of specific food levels. This was clear from the general comparison between the control and yoyo treatments. From 74 probe sets that were significantly affected most showed very different expression between the two controls, of which one usually was very similar to the yoyo treatments.

When we focused on the differences between the control and yoyo treatments within diet levels, we found that under low diet the differences were considerable (table 1). The genes that were up-regulated in yoyo flies were associated with learning and external stimulus compared to the control treatments. This seems to match predictions since the yoyo flies were exposed to a variable environment. It is interesting that a possible difference between organisms in a variable diet compared to a constant diet environment is expressed in one type of nutrition, rather than in both. One of the reasons that we did not find a difference between the high control flies and the yoyo flies when on high diet might be that the variation within the high diet control treatment group was very large, within and between time points, where the low diet control treatment samples showed a relatively low variation between time points. Another, more biological explanation could be that a difference between past experience is only expressed when organisms are more challenged, i.e. in our experiment, when they are on low diet nutritional resource availability forces the flies to make allocation decisions. These results concerning gene expression similarity of the yoyo flies to the high diet control align well with the survival differences found, where the yoyo flies had a survival resembling those of the high control flies.

Besides quantifying the plasticity in gene expression, biological processes that are up- or down-regulated in our study are also of interest. Flies lived longer on high diet, while they reproduced more on low food, which is represented in the gene expression differences. Biological
processes which were up-regulated on high diet where associated with external stimulus, cell cycle and cytoskeleton organization and neuropeptide signaling, while processes that were down-regulated on high diet were associated with reproduction, sugar metabolism and transport of macromolecules. Interestingly, similar GO categories were shown to be down-regulated in treatments that increase lifespan in different species (McElwee et al. 2007). Therefore, the increased lifespan and reduced reproduction in the phenotypes of this study could be traced back to gene expression of typical processes that are normally associated with these phenotypes and therefore also indicate this negative relationship on the transcriptome level. Also other gene expression studies using flies (Pletcher et al. 2002; Bauer et al. 2010; Doroszuk et al. 2012; Farhadian et al. 2012) support these patterns. We therefore conclude that genes which we found down regulated on high food seemed to be normally down regulated on DR food, except for monosaccharide metabolic processes. Figure 7 lists the groups of genes that were differently expressed and how they might be related to each other.

Figure 7. Overview of how diet affects groups of genes with specific biological processes which are known to influence lifespan and egg production. One of the groups of genes affected by nutrition contains neuropeptides signaling proteins, which are known to influence several functions in many organisms. One of these processes is circadian rhythm (with genes such as takeout and clock), which is also affected by nutrition. Neuropeptides are expressed largely in the central nervous system (brain / head). One group of genes which where enriched as a GO term was mitotic spindle elongation, which contains ribosomal proteins such as S6 kinase and several eukaryotic initiation factors, known to influence processes such as (cellular) stress response and sugar metabolism, a biological function which was also affected by nutrition. This again aligns well with the function of some neuropeptides to regulate hemolymph carbohydrate content. Intra cellular and macro molecular transport are both associated with vesicle mediated transport, which are three of the down regulated groups of genes. These downregulated groups of genes might be associated with G protein coupled receptors and GTP-ase subfamily genes which are up regulated in the CNS, and known in many dietary restriction studies as perception of light stimulus genes, which are not solely regulated by visual perception. Although neuropeptides play critical roles in regulation various aspects of insect life histories, none of the upregulated neuropeptides are directly linked with reproduction. Gamete formation, cell cycle and chromosome organization are related to egg production in Drosophila. Neuropeptides found to be differently expressed regulate food intake, fat metabolism and other functions, while for instance allatostatins in other species of insects do regulate egg production more directly, via the regulation of juvenile hormone. The other biological processes (mitotic spindle elongation, circadian rhythm, sugar metabolism and transport) are known to be often differently expressed when long – and short – lived organisms are compared. In total, this summarizes a model of how genes with different biological processes relate diet with lifespan and egg production.
The effect of age on gene expression

The effect of age in our study was comparable to the effect of diet, and was more pronounced in the control treatment compared to the yoyo treatments and more in high diet compared to low diet treatment. In our study age classes only differed four days. Still, the processes which were down-regulated in our second age class were comparable to processes up regulated in young flies, or down regulated in older flies in other studies (see Appendix G1 & G2). Interestingly, when many time points are compared, results indicate that such gene expression differences do not consistently change with ageing, but rather vary a lot between time points that are close together (PLETCHER et al. 2002), except for genes related to reproduction, which are consistently down regulated on consecutive time points (see Appendix G1).

For GO categories enriched for genes down-regulated at the later age class the differences found between our time points are also found to be differently expressed when very young flies are compared to middle aged flies (BAUER et al. 2010) as well as when middle aged flies are compared to very old flies (DOROSZUK et al. 2012). The only case in which there is an association with age in our study as well as on smaller time differences (PLETCHER et al. 2002) and larger time differences (BAUER et al. 2010; DOROSZUK et al. 2012) was for oxidation and reduction genes of which cytochrome p450 genes are often involved. These genes where up-regulated with age and were found to be always expressed higher on average at later time points on low and high food in the study of (PLETCHER et al. 2002). Only in flies that are malnourished or starved, expression of oxidation and reduction genes was decreased at later time points (DOROSZUK et al. 2012; FARHADIAN et al. 2012), possibly indicating that when there is a severe energy constraint, these genes are down-regulated in flies with age. This also indicates that our low diet level does not pose too much of an energy constraint on our flies, which seems to be supported by the increase of egg production, often seen as a costly process. Overall, for many biological processes it is unknown whether they should increase or decrease with age. Studies such as (PLETCHER et al. 2002) do suggest many categories of genes show non-linear relationships between ageing and gene expression.

Summarizing, flies which were transferred between diet levels seemed to be very plastic in both the expression of phenotypes as well as the expression of a large part of the genome. The effect of time on gene expression could not be easily traced back to phenotypes, since our flies did not show a difference in any of the phenotypic traits in the same time interval. The direction of the response was similar to other studies, but most of these genes are not necessarily indicative for the ageing process, with a possible exception for genes related to female reproduction and oxidation and reduction genes. Differences between yoyo flies and controls were also only pronounced when flies were on low food. We found a clear relationship with the genes up or down regulated and the phenotypes. For instance, although lower egg counts on high food might have been unexpected, the up regulation of gene expression of female gamete generation and for instance eggs shell on low food matches this reproduction phenotype.

This study shows that flies cope with cyclical diet variation by changing the regulation of genes, resulting in rapid repeated changes in their phenotypes. Our model of yoyo fruit flies could be used to indicate which mechanisms play a role since the life history parameters (especially reproduction) do change quickly and towards the control fly phenotype. Neuropeptide signaling came up as one of the overrepresented group of genes and is therefore one of the candidate groups of genes because they are known to regulate many life history traits (ALSTEIN and NASSEL...
2010). The insulin signaling pathway genes are often associated with ageing phenotypes and the effect of dietary restriction. In our study, insulin signaling was not overrepresented in up- or down-regulated genes. Two reasons might be 1) insulin signaling mainly works on the post translational level or 2) insulin signaling can be up- or down-regulated by up-regulating some and down- regulating other genes. The latter should have led to an overrepresentation of the insulin pathway signaling when a GO enrichment analysis was done without separating up- or down-regulated genes (data not shown). Furthermore, insulin signaling pathway genes did not show coherent expression between several microarray experiments (see Appendix G1) and also the separate *Drosophila* insulin like peptides, which are part of the neuropeptide group, did not show differential expression levels in our study.

Our results indicate that flies have evolved a mechanism by which they can maintain plasticity throughout life in egg production. Egg production is significantly up or down regulated within three days. This indicates that flies respond very quickly and that reproduction throughout life is plastic. If these responses can be put into ecological context this might indicate that flies are adapted to be able to continuously respond to new nutritional situations and that they have evolved a way to act upon these variable diet conditions. This could suggest that for a fly it is optimal to be able to shift the suite life history traits on a very short time scale, which might resemble the type of variation experienced in nature.

**Acknowledgements**

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Overview Appendices
(available upon request: jvdh.science@gmail.com)

Appendix A
List of fdr adjusted permutated p values for the sixteen contrasts (columns c1 to c16).

Appendix B
Feeding essay of repeatedly measured flies in vial with densities of 1, 5 and 10 and fod levels of 1x, 2x and 5x medium. 3 replicate vails were used, all measured 21 times.

Appendix C1
DAVID enrichment analysis using the genes up-regulated on high food with a cutoff value of p=0.05 (c1, 3150 probes)

Appendix C2
DAVID enrichment analysis using the genes down-regulated on high food with a cutoff value of p=0.05 (c1, 2796 probes)

Appendix D1
DAVID enrichment analysis using the genes up-regulated on first time points with a cutoff value of p=0.05 (significant in c6, c7, and c9, 2415 probes)

Appendix D2
DAVID enrichment analysis using the genes down-regulated on first time points with a cutoff value of p=0.05 (significant in c6, c7 and c9, 938 probes)

Appendix E1
DAVID enrichment analysis using the genes up-regulated in yoyo low food contrast between yoyo and control (significant in c12, 483 probes)

Appendix E2
DAVID enrichment analysis using the genes down-regulated in yoyo low food contrast between yoyo and control (significant in c12, 264 probes)

Appendix F1
DAVID enrichment analysis using the genes that were positively related to egg number with a cut off value of 0.05

Appendix F2
DAVID enrichment analysis using the genes that were negatively related to egg number with a cut off value of 0.05

Appendix G1
Average gene expression from four other studies of most enriched gene ontology terms of this study

Appendix G2
List of probes that were significant in most enriched sets of genes from the gene ontology enrichment analysis that were used to compare with other data sets.
References


