Linking growing up and getting old

plastic and evolutionary effects of developmental diet on adult phenotypes and gene expression in the fruit fly

Christina M. May

THESIS COMMITTEE

Promotor
Prof. Dr. B. J. Zwaan
Professor of Genetics
Wageningen University

Co-promotor
Dr. A. J. M. Debets
Associate Professor
Laboratory of Genetics

Other Members
Prof. Dr. Paul Brakefield, University of Cambridge
Prof. Dr. Marcel Dicke, Wageningen University
Prof. Dr. Thomas Flatt, University of Lausanne
Dr. IR. Jan Kammenga, Wageningen University

This research was conducted under the auspices
of the C.T. de Wit Graduate School for Production
Ecology and Resource Conservation

Linking growing up and getting old

plastic and evolutionary effects of developmental diet on adult phenotypes and gene expression in the fruit fly

Christina M. May

THESIS
Submitted in fulfilment of the requirements for the degree of doctor at Wageningen University by the authority of the Rector Magnificus Prof. Dr. A. P. J. Mol, in the presence of the Thesis Committee appointed by the Academic Board to be defended in public on Wednesday 22 June 2016 at 4.00 p.m. in the Aula

Christina M. May
Linking growing up and getting old:
plastic and evolutionary effects of developmental
diet on adult phenotypes and gene expression
in the fruit fly

PhD thesis; Wageningen University, Wageningen, NL (2016)
With references, with summary in English

ISBN 978-94-6257-763-3
DOI: 10.18174/376517

Table of Contents

THE FLY SAT UPON
OF THE CHARIOT
SAID, WHAT A

THE AXLE TREE WHEEL AND DUST DO I RAISE!

–Aesop

1

Introduction

INTRODUCTION

In this introduction, I provide a general context for the work presented in this thesis, which addresses the effects of developmental diet on adult life history traits and gene expression in the fruit fly, *Drosophila melanogaster*. I begin by giving a short introduction to the concepts of life history evolution and (developmental) phenotypic plasticity, and the approaches used to study them, followed by a brief introduction to the mechanisms whereby developmental diet may affect adult phenotypes. I then address the special case of ageing in life history evolution, and the theories put forward to explain the link between developmental conditions and late life health and lifespan. Finally, I give a general introduction to the model used in this thesis, *Drosophila melanogaster*, and a short synopsis of the subsequent chapters.

The struggle for existence

In 1826, the English cleric and political economist Thomas Robert Malthus observed, rather poetically:

> *"Through the animal and vegetable kingdoms, Nature has scattered the seeds of life abroad with the most profuse and liberal hand; but has been comparatively sparing in the room and the nourishment necessary to rear them. The germs of existence contained in this spot of earth, if they could freely develop themselves, would fill millions of worlds in the course of a few thousand years. Necessity, that imperious all-pervading law of nature, restrains them within the prescribed bounds. The race of plants and the race of animals shrink under this great restrictive law; and man cannot by any efforts of reason escape from it."*
>
> **-Malthus 1826**

In less elegant, but more concise words: life (i.e. animals, plants, microorganisms) increases at a faster rate than the resources needed to sustain it. As a consequence, not all individuals can survive and reproduce. While Malthus was primarily concerned with the dire consequences of human population growth outpacing resource availability, his idea was instrumental in the formulation of the theory of evolution by natural selection. Both Darwin and Wallace realised that because there are not enough resources for all individuals to survive, any heritable variation that increases the ability of an individual to contribute offspring to the next generation relative to others will be selected for and thus increase in frequency (Darwin & Wallace 1858).

From an evolutionary perspective then, the imperative of life is to reproduce. To meet this imperative, organisms have devised a bewildering array of strategies

- ranging from the "live fast - die young" approach of mayflies and rock stars to the "slow-and-steady wins the race" approach of tortoises and Buddhist monks. Each strategy is a different solution to the problem of how best to leave behind the most offspring. For some species or individuals, it means investing considerably in building and maintaining a high-quality body in order to raise a few, high quality offspring. For others it means investing very little in a high-quality body, but a lot in very many, lower quality offspring. These different modes of living can be more precisely defined as life histories: the sequence of events related to survival and reproduction that occur from birth through death (Roff 2001; Stearns 1992). The traits that contribute the most to fitness, such as frequency and age at reproduction, size, and lifespan are termed life history traits (Roff 1992).

Life history theory seeks to explain how the wide array of life history strategies observable in nature has evolved. Limited resources not only lead to a "struggle for survival" between individuals, but they also lead to a "struggle for investment" within an individual. The "struggle for investment" is conceptualised in the idea of trade-offs, a fundamental concept in life history theory. Because resources are limited, individuals cannot maximise all traits at once and thus, increased allocation of resources to one trait, such as reproduction, will by necessity lead to decreased allocation to another, such as lifespan (Stearns 1992). Acquisition-allocation theory seeks to understand how these allocation decisions are made in order to optimise an individuals fitness with a limited amount of resources (Sibley & Calow 1986), and also to explain the surprising lack of trade-offs observed in some instances (de Jong & van Noordwijk 1992; Roff & Fairbairn 2007; Van Noordwijk & de Jong 1986). More recent work has begun to characterise the genetic and physiological pathways underlying trade-offs (Braendle et al. 2011). For example, molecular genetic approaches have revealed that underlying signalling processes, rather than energy allocation *per se* may underpin the lifespan-reproduction trade-off in the worm, *C. elegans* (Barnes & Partridge 2003). This new aspect of life history theory is only beginning to be explored, but argues that a mechanistic approach can offer a more complete understanding of life history evolution (Braendle et al. 2011). It is important to note that while generally, the limiting resource being referred to in the context of trade-offs is food (e.g. Agrawal et al. 2010), trade-offs can also result from the limitation of other resources such as space and time, from underlying physiological constraints, or from genes with antagonistic-pleiotropic effects (Hoffmann 2014; Stearns 1992; Zera & Harshman 2001).

Plasticity, reaction norms, and development

Ultimately, life history traits are determined by the interaction of the genotype with the environment. One of the most important mechanisms whereby organisms can cope with environmental variation is via phenotypic plasticity

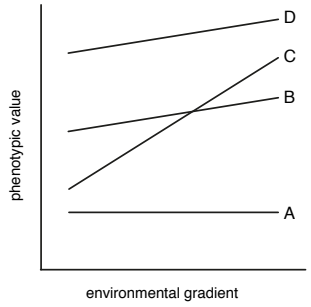
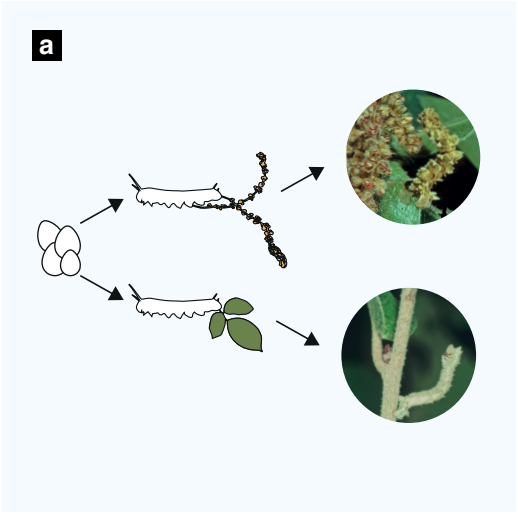
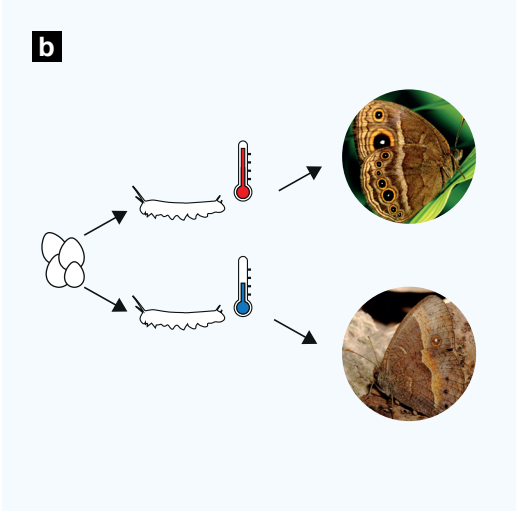


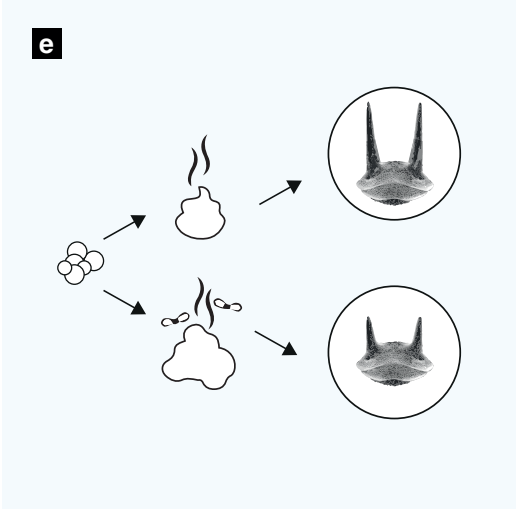
Figure 1: Reaction norms of four different genotypes across a hypothetical environmental gradient. Genotype A exhibits no variation across environments and is therefore canalized for this phenotype, while genotypes B, C and D display varying degrees of phenotypic plasticity (PP) for this trait. Genotypes B and D share the same slope and thus possess the same degree of plasticity, however, both Genotypes B and D are considerably less plastic than Genotype C, whose reaction norm has a much steeper slope. Such differences in the degree of plasticity between genotypes are termed genotype by environment (G by E) interactions.

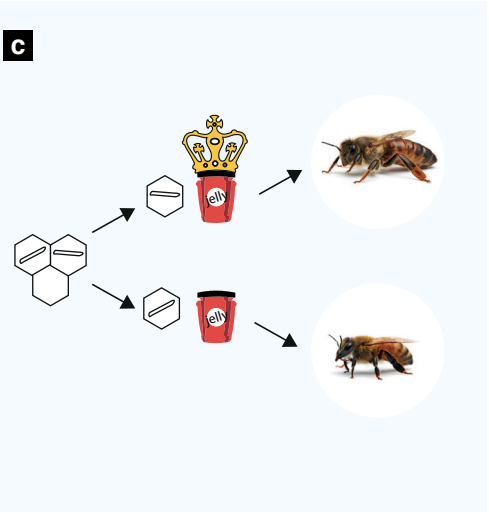
(West-Eberhard 2003) defined as the ability of a single genotype to produce multiple phenotypes in response to environmental variation (Schlichting & Pigliucci 1998; Schlichting & Smith 2002). Phenotypic plasticity is often visualised through the use of reaction norms where phenotypic variation (y-axis) is plotted as a function of environmental variation (x-axis) for a single genotype (Fig. 1; Schlichting & Pigliucci 1998). The extension of this approach to plotting the reaction norms of several genotypes in a single plot can reveal variation between genotypes in the degree of environmental plasticity they possess. Genotypes that are unable to respond to environmental variation are said to be canalised and exhibit flat reaction norms (Waddington 1942), while genotypes that are plastic are capable of responding to environmental variation and have non-flat reaction norms (Fig. 1). When the slope of the reaction norm differs between two genotypes this is termed a genotype by environment interaction (G by E) and indicates a genetically determined difference in the degree of phenotypic plasticity (Stearns 1992).

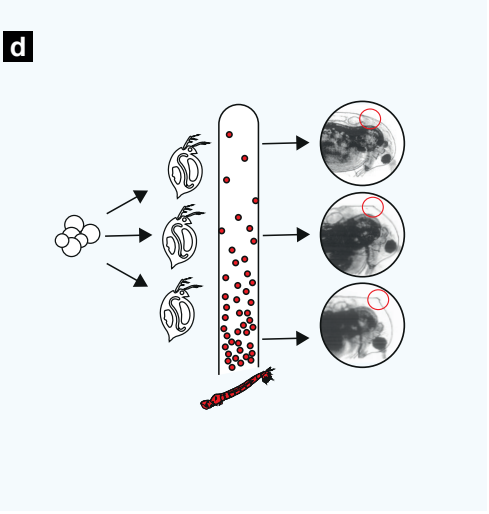
Phenotypic plasticity can occur at all stages of life, however a particularly important stage is during development. Cases in which environmental cues lead to discrete switches between two distinct phenotypes, termed polyphenisms, provide especially striking examples of the ability of a single genotype to produce different phenotypes in response to environmental variation (for examples of well-characterised polyphenisms see Fig. 2). Polyphenisms are common in insects (Simpson et al. 2011), and in many cases evolve in response to seasonal environmental variation (Brakefield & Zwaan 2011). In fact, theory predicts that they are most likely to evolve in species with short generation times relative to the frequency of variation in the environmental cue that induces the polyphenisms (Nylin & Gotthard 1998; Rickard & Lummaa 2007). By and large, however, most cases of developmental phenotypic plasticity are not as discrete


a


b


e


c
jelly
jelly


d

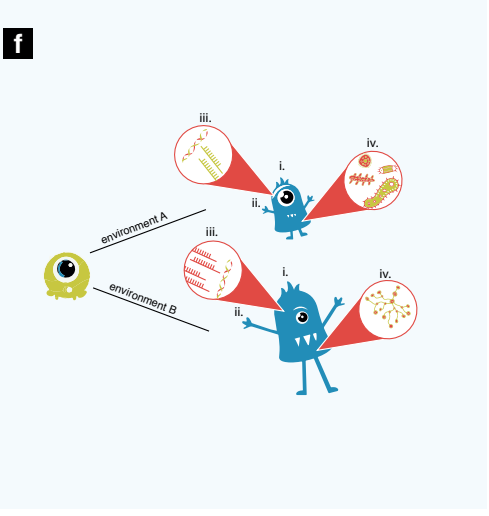

f
iii.
iv.
i.
ii.
environment A
iii.
i.
iv.
environment B
ii.

Figure 2: Discrete (a, b, c) and continuous (d, e, f) examples of phenotypic plasticity (PP). (a) The two morphs of the caterpillar stage of the moth species *Nemoria arizonaria* are so distinct they were long considered separate species. However, experiments have shown that the dramatic difference is driven by developmental diet: the spring form of the caterpillar feeds and looks like flowers of the oat catkin, while the fall form feeds on leaves and resembles a twig (Greene 1989). Photo courtesy of Erik Greene. (b) The butterfly *Bicyclus anynana*, an east-African species living in a seasonal environment (Brakefield et al. 2009) provides a particularly well characterized example of PP. In response to temperature cues reflecting the two seasonal environments the butterfly typically encounters, the caterpillar morphs into two very distinct adult phenotypes. Strikingly, these two phenotypes differ not only in external morphology such as eye spot size, but they also embody two completely distinct life history strategies within a single species: the wet season form adapts a live fast, die young strategy of rapid sexual maturity, high reproduction and short lifespan, while the dry season form delays reproduction, has fewer offspring and a long lifespan (Brakefield et al. 2007; Brakefield & Reitsma 1991). Excitingly, the physiological and molecular mechanisms underlying this switch are starting to be unravelled (Oostra et al. 2011). Photo adapted from the bicyclus.org website. (c) Caste determination in honeybees (*Apis mellifera*) provides one the most striking examples of adaptive developmental plasticity (Moczek and Snell-Rood 2008). Genetically identical larvae give rise to either long-lived, highly fecund queens, or short lived, usually non-reproductive workers. This difference is determined by the diet received during development: future queens receive royal jelly, while workers receive plain food. Interestingly, the difference between the two morphs can be quite reliably recapitulated by modulating DNA methylation during development (Kucharski, Maleszka et al. 2008) highlighting epigenetic change as a direct link between nature and nurture. (d) Continuous developmental plasticity is found in the water flea, *Daphnia pulex*. *D. pulex* grows protective neck spines in response to chemical cues signalling predators - the stronger the chemical cue, the more neck spines (Tollrian 1993). Furthermore, the degree of responsiveness to predator cues differs between genotypes, providing an excellent example of a genotype by environment interaction (Spitze 1992) Photo adapted from Tollrian, 1993. (e) Developmental diet affects the size of the horns of the horned beetle, *Onthophagus acuminatus*, relative to body size. Males reared under poor diets have larger horns at at given body size than their brothers raised on good diets (Emlen 1997). The drawings of the beetle horns are a fictional representation of the difference and serve only to illustrate the concept of allometry, not the actual magnitude of the effect observed, since no pictures were available in Emlen et al., (1997). (f) Potential effects of variation in developmental conditions on adults of a hypothetical organism. The individual raised in environment B is larger (i), has relatively longer arms (ii), different levels of gene expression (iii) and a distinct and less dense microbiota than the individual raised in environment A. Furthermore, it is important to note that the differences in gene expression (iii) can reflect not only long-term changes in epigenetic regulation of the genome, but also the combined effects of the relative sizes of different tissues (iii) and the structure and function of the microbiome (iv).

as those observed in polyphenisms. Continuous responses to developmental environmental variation can be adaptive and result in increased adult fitness (see the example of predator induced neck spine formation in *Daphnia pulex* in Fig. 2d), however, there is also great potential for variation in the developmental diet to have negative effects on fitness. For example, in order to survive an extremely poor developmental environment an individual may need to make compromises that decrease its overall fitness as an adult relative to an individual that developed under optimal conditions. Such responses are called "scarring" or "silver-spoon" effects (Brakefield & Zwaan 2011; Grafen 1988; Rickard & Lummaa 2007), the latter name coming from the English expression "to be born with a silver spoon in ones mouth". It is important to realise that even adaptive responses can decrease fitness when they are expressed in the wrong environment. For example, in response to chemical cues emitted by its main predator, the snail *Physa virgata* develops a rounder shell and reduces growth rate, thereby decreasing its risk of predation. However, the snail cannot distinguish between the chemical cues emitted by its predator and a closely related non-predatory species and induces the response indiscriminately, thereby suffering the costs of decreased growth with none of the benefits of decreased predation risk (Langerhans & DeWitt 2002). Thus it is important to note that whether the response to the developmental environment increases fitness or not depends on both the nature of the variation in developmental conditions, and the adult environment in which it is expressed (DeWitt et al. 1998; Ghalambor et al. 2007).

Mechanisms and outcomes of developmental plasticity

As illustrated above, developmental plasticity can lead to extensive variation in adult phenotypes. However, the mechanisms that underlie this variation are only beginning to be explored (Braendle et al. 2011; Oostra et al. 2011). Ultimately, responses to environmental variation during development must be mediated by changes in gene expression during development (Beldade et al. 2011). If one accepts the definition of epigenetic change as "mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence" (Russo et al. 1996), then the ability of a single genotype to change its gene expression in response to different environments must be driven by underlying changes in epigenetic regulation (Burdge & Lillycrop 2010). A suggestive example comes from the study of caste determination in honeybees, *Apis mellifera* (reviewed in Moczek & Snell-Rood 2008). Depending on developmental diet genetically identical individuals develop into either fertile queens or sterile workers: future queens are fed royal jelly, while future workers are fed a plainer diet (Fig. 2c). Kucharski et al., (2008) showed that the methylation of many genes differs between queens and workers during development, and strikingly, that it is possible to generate queens from a worker diet simply by artificially decreasing levels of DNA

methylation during development. This illustrates the ability of epigenetic changes to induce alternate phenotypes in response to environmental variation - in essence linking nature to nurture - and represents an exciting new frontier in the understanding of environmentally mediated effects on development.

After responses to the developmental environment are effected, there are at least three (non-mutually exclusive) routes whereby they can effect the adult phenotype: 1) epigenetic changes induced during development can persist and continue to affect gene expression into adulthood, 2) the overall form of the body (size or shape) can be affected, or 3) the microbiota associated with the individual can be affected (illustrated schematically in Fig. 2f). In the following paragraphs I will provide some examples of these effects, focusing mostly on responses to variation in developmental diet.

The idea that persistent changes in the epigenome induced by developmental diet continue to affect adult traits has gained a particular hold in the field of human epidemiology (Burdge 2007). The finding that individuals exposed to famine *in utero* have different methylation levels of the imprinted gene insulin-like growth factor II (IGF2) (Heijmans et al. 2008) and other genes nearly 60 years later (Tobi et al. 2014), as well as increased risk of developing unfavourable metabolic traits such as high LDL cholesterol and type II diabetes (Lumey et al. 2011) has resulted in the hypothesis that long term effects of developmental diet in humans may be mediated by changes in the epigenome (Burdge 2007; Burdge & Lillycrop 2010). While epigenetic mechanism are undoubtedly important regulators of gene expression and environmental responses, it is not yet clear whether the epigenetic changes induced in response to developmental conditions still play a functional role in adulthood. It is important to consider the alternate hypothesis: that they represent a non-functional marker that serves as a memory of the response effected during development. For example, many of the differentially methylated genes identified so far in humans play a role in the insulin signalling pathway (Heijmans et al. 2008). This pathway is a primary regulator of nutrient-dependent growth during development (Shingleton 2011) and thus epigenetic changes may reflect changes made in these pathways to accommodate poor nutrition during development and growth to adulthood.

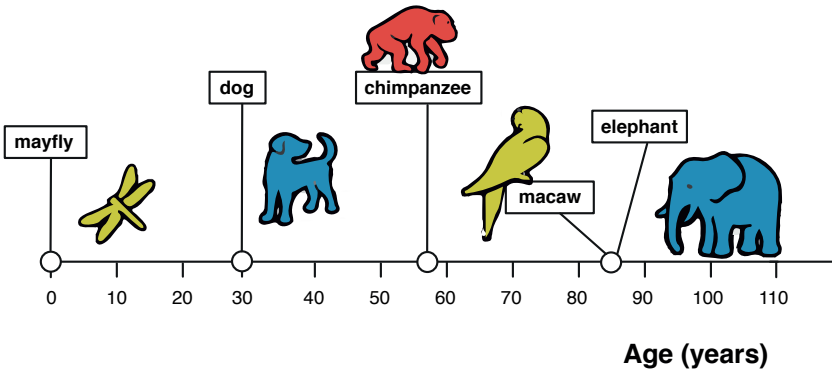
A better characterised long-term effect of developmental conditions on phenotypes is through changes in the overall size and shape (i.e. allometry) of the resulting adult. In almost all animals, reduced nutrition during development restricts final adult size (Shingleton 2011) - a relationship that appears quite intuitive when growth is put in the context of converting nutrients to tissue: less nutrients equals less tissue. These changes in overall body size can then go on to effect many fitness related traits in adulthood. In insects, for example, a poor diet almost invariably leads to smaller ovaries, and by extension, lower

reproductive capacity (Bergland 2011). In addition to influencing overall size, developmental diet can influence the size of tissues relative to the whole body. In the horned beetle, for example, the size of the horn relative to the body depends on developmental diet: individuals raised on low quality diets have larger horns at a given body size than siblings raised on a high quality diet (Emlen 1997; Fig. 2e). In both cases, the changes induced during development are more or less fixed upon adulthood and thus may continue to affect fitness throughout the lifespan.

The effect of the microbiome on phenotypes has seen a great upsurge in interest in recent years, adding a third dimension to the genotype-phenotype relationship. In fact, the concept of the hologenome proposes that an individuals fitness and capacity to respond to the environment is determined not only by its own genome, but also by the genomes of all of the symbionts it is associated with (Zilber-Rosenberg & Rosenberg 2008). Variation in the microbiome has important effects on health and fitness in humans and many other organisms (Broderick et al. 2014; Clemente et al. 2012; Wong et al. 2014), affecting such diverse traits as ageing (Heintz & Mair 2014) and mate selection (Sharon et al. 2010). Importantly, the microbiome is often established during development and its composition is affected by the conditions experienced (Mueller et al. 2015), thus, changes in the microbiome provide another route whereby developmental conditions can affect adult traits.

Approaches to studying life histories

Historically, a range of theoretical and empirical approaches have been applied to understanding the role of the environment in determining phenotypes. These include theoretical modelling, comparative phylogenetic studies, studies of evolution in the field (e.g. mark-recapture studies), environmental manipulation, studies of gene expression, and experimental evolution (EE). EE in particular has proven a powerful tool for understanding life history evolution as it allows the experimenter to impose carefully controlled conditions



and observe evolutionary responses in real time (Kawecki et al. 2012). This approach can yield theoretical as well as practical insights. For example, EE has been used to test the hypothesis that high extrinsic mortality should lead to decreased lifespan. By subjecting fruit fly populations to different levels of external mortality, Stearns et al., (2000) showed that, as predicted, populations adapted to high extrinsic mortality evolved higher intrinsic mortality and therefore shorter lifespans. Additional EE studies have also yielded insights into how organisms adapt to environments with varying temperatures (Bennett & Lenski 1993), developmental diet quality (Kolss et al. 2009; Vijendravarma et al. 2015), or competition levels (Santos et al. 1997). More practically, EE has been used to understand and prevent the evolution of antibiotic resistance in microbes (Palmer & Kishony 2013). Importantly, the recent decline in the costs of sequencing have allowed an extension of the EE approach, termed "evolve and resequence" (E and R) to address the genomic changes underlying adaptation (Long et al. 2015). E and R provides a more ecologically relevant complement to mutant studies, by revealing the actual genes and pathways accessible to evolution in natural populations.

While there are many advantages to EE, one of the main disadvantages is the labor-intensive nature of the experiments, particularly when using eukaryotes. This has lead to most experiments having relatively simple designs with little or even no replication. However, organisms live in complex environments, and as articulated in life history theory, they often need to balance investment in competing interests. Thus there is a need for experimental evolution designs with high replication that employ more than one selection pressure. EE of fruit flies to variation in temperature and larval food quality simultaneously (Bochdanovits & Jong 2003) showed that responses to both selection pressures simultaneously lead to different adaptations than adaptation to each of the selection pressures independently. Thus, applying two selection pressures simultaneously can yield more realistic insight into the evolution of life histories. Of particular interest would be designs that vary conditions during

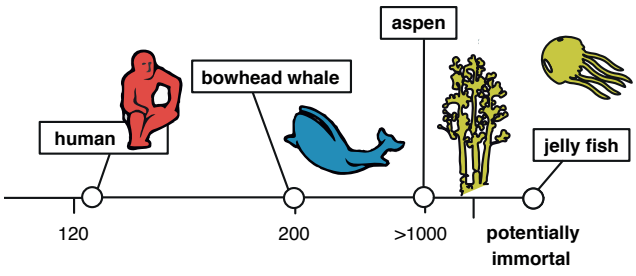


Figure 3: Examples of approximate maximum lifespan across animal species. Some species, such as the jellyfish *Turritopsis dohrnii* (far right) retain the ability to revert to juvenile polyps from mature individuals and thus do not experience a decline in function with time (Piraino, Boero et al. 1996). This implies an absence of aging and has lead to the proposal that they are potentially biologically immortal.

both developmental and adult life stages, offering insight into how the differing demands of growth *versus* reproduction are reconciled.

Studies of gene expression have also been instrumental in allowing a more mechanistic understanding of life histories. For example, they have provided some powerful examples of how trade-offs may be mediated at the genetic level via genes with antagonistic pleiotropic effects (see Bochdanovits & de Jong 2004; Festucci-Buselli et al. 2005). Furthermore, they provide a read-out, in real time, of the physiological status of an individual. Often, experimenters are interested in which specific genes are expressed differently (i.e. when classifying tumours), however, a more broad scale approach to identify the overall magnitude of the effects of different treatments on gene expression can also be insightful, especially in manipulations where this has not yet been classified. For example, the negative effects of poor developmental conditions in humans are often hypothesised to be due to long-term epigenetic changes that continue to regulate gene expression throughout adulthood (Burdge et al. 2007; Burdge & Lillycrop 2010). However, to date, there have been no large studies that assess the consequences of developmental diet for adult gene expression across the lifespan.

The special case of ageing in life history evolution

One of the most important fitness related traits is lifespan, and as such, ageing has received special treatment in the study of life histories. The existence of ageing, defined in this thesis as "a persistent decline in the age-specific fitness components of an organism due to internal physiological degeneration" (Rose 1991), long posed a puzzle for evolutionary biologists. By definition ageing decreases fitness, therefore it should be opposed by natural selection. However, species-specific lifespans (Fig. 3) imply that genetic variation exists for ageing, and that natural selection has played a role in determining the rate of ageing (Zwaan 1999). The solution to this puzzle stemmed from the realisation that even a potentially immortal organism cannot escape external forces such as predation or disease indefinitely, therefore, the likelihood that an individual will live to a certain age and reproduce declines with time. Without reproduction, there is nothing for selection to act upon, resulting in a "selection shadow" in late life (Fig. 4a).

The first evolutionary theory of ageing proposed that the existence of a selection shadow would allow the accumulation of late acting deleterious mutations whose negative effects could only be weakly opposed by selection, while deleterious mutations that occur early in life would be eliminated by the strong force of selection at those ages (Medawar 1952). This became known as the mutation accumulation theory of ageing. In 1957, Williams proposed a second mechanism through which ageing could evolve. He argued that alleles that

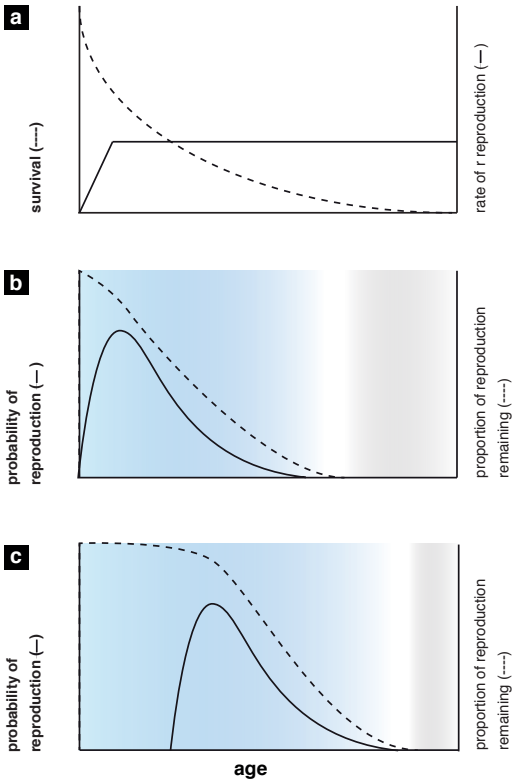


Figure 4: The evolutionary explanation of aging (adapted from Zwaan 1999). (a) Survival (lx) and reproductive rate (mx) and (b,c) the probability of reproduction and proportion of reproduction remaining for an individual in a hypothetically non-ageing population. The probability of reproduction at age x is the product of lx and mx . The proportion of reproduction remaining can be roughly interpreted as the strength of natural selection (green shading) at age x. It can be seen that even in the absence of aging, the strength of natural selection declines with age resulting in a selection shadow (grey shading) when the strength of natural selection reaches 0. Genes that have negative effects on fitness in the selection shadow cannot be selected against, and will be actively selected for if they increase fitness in early life. (c) if reproduction is shifted to later ages (an approach taken in many experimental evolution studies), then the strength of natural selection at later ages also increases (green shading), and the selection shadow is shifted to the right (grey shading).

increased fitness early in life but decreased fitness late in life could nevertheless be selected for because the strength of selection is much stronger early in life. This became known as the antagonistic pleiotropy theory of ageing (Williams 1957). In a sense, however, the arguments used in these two theories, while hugely influential, contain an element of circular reasoning. Namely, they both assume that biological ageing already exists. For example, for a mutation to have a negative effect "late" in life that is less efficiently selected against, there must already be a late life. Thus, while these hypotheses describe the types of genes that could contribute to ageing and how they would avoid elimination by natural selection, they do not explain its initial evolution.

A more recent evolutionary theory of ageing also relies on the concept of the selection shadow, but circumvents the circular aspects of the older theories. This theory is called the disposable soma theory of ageing (Kirkwood 1977) and is based on the observation that organisms are constantly acquiring damage at the cellular (and organismal) level: DNA replication errors occur, proteins get damaged, and free-radicals run rampant. Mechanisms to repair or prevent this damage exist, however, their deployment is energetically costly, and as frequently mentioned in this chapter, resources are generally limiting. Because an individual will eventually die from causes outside its control the soma

only needs to stay in good shape for as long as an organism can reasonably be expected to live, thus a balance should be achieved between investment in reproduction and investment in repair mechanisms (Kirkwood 1977; Kirkwood & Holliday 1979). Importantly it adds a mechanistic basis to the understanding of ageing - the rate of ageing is determined by the balance between investment in repair mechanisms and investment in early reproduction (Zwaan 1999), and it does not require the pre-existence of biological ageing, as is the case for the mutational accumulation and antagonistic pleiotropy theories of ageing. The disposable soma theory predicts that the exact balance that is struck between investment in repair and investment in reproduction will depend on the ecology of a species, and especially the extrinsic risk of mortality: organisms with low risk of external mortality (e.g. whales, tortoises, birds; Fig. 3) should evolve relatively longer lifespans, coupled with relatively lower rates of reproduction.

Experimental approaches to understanding ageing

Experimental approaches have contributed greatly to the understanding of ageing. Experimental evolution/artificial selection approaches in particular have provided some of the strongest evidence for the evolutionary theories of ageing. A series of elegant experiments showed that by adapting fruit flies to later ages at reproduction, in essence shifting the selection shadow towards later ages (Fig. 4b), lifespan extension can be achieved, usually with a concomitant decrease in early fecundity, as predicted by the disposable soma theory (Luckinbill et al. 1984; Partridge & Fowler 1992; Rose 1984). Later experiments showed that lifespan extension could also be achieved by selecting on lifespan directly (Zwaan et al. 1995). Another experimental approach to extend lifespan emerged in the 1930's when it became clear that underfeeding without malnutrition, termed caloric or dietary restriction (CR/DR) can extend lifespan, usually at the expense of fecundity, across a range of organisms from yeast to mice (McCay et al. 1935; Nakagawa et al. 2012), and possibly even primates (Colman et al. 2009). This effect is thought to represent an adaptive strategy whereby resources are diverted away from reproduction towards survival to remain alive until conditions more favourable to reproduction return (Holliday 1989; Kirkwood & Shanley 2005; but see Adler & Bonduriansky 2014), and highlights the importance of available resources in determining lifespan. In addition, in the 1980's it was discovered that mutations in single genes could recapitulate the lifespan extending effects of DR in model organisms (Friedman & Johnson 1988; Kenyon et al. 1993; Klass 1983). Interestingly, the genes that affect lifespan tend to share some commonalities: in general, their effects on lifespan are relatively conserved across model organisms and they are often involved in the insulin signalling (IIS) and target of rapamycin (TOR) pathways (Kuningas et al. 2008). The TOR and IIS signalling pathways both act to match the activity of energy consuming processes such as reproduction and growth to the actual nutritional status of the individual (Gems & Partridge 2013). While

the IIS signalling pathway has both intra- and extra-cellular components and coordinates the response to nutrients across the organism, the TOR pathway controls intra-cellular nutrient status only (Evans et al. 2011). Both pathways respond to low energy situations by shifting metabolism away from growth towards cellular repair and maintenance (Fontana et al. 2010).

As a whole, each of these interventions suggest that lifespan is regulated by tuning the balance between investment in the soma (e.g. repair mechanisms) and investment in fecundity, as predicted by the disposable soma theory (Kirkwood 1977; Kirkwood & Rose 1991). Furthermore, this balance also appears to be regulated by either actual (DR) or perceived (mutations in IIS/TOR signalling) nutrition, highlighting the important role of resources acquisition in lifespan determination. An important note, however, is that for each of these interventions, the magnitude of the effect can differ greatly between the sexes. For example, the difference in the effect of DR between the sexes can be as large as the differences observed between species (Magwere et al. 2004). This highlights the need to consider both sexes when studying interventions that affect lifespan and furthermore suggests that the optimal balance between investment in the soma and investment in reproduction likely differs between the sexes.

Applying evolutionary thinking to humanageing in our contemporary society

Evolutionary thinking has also been applied to try to understand human ageing in contemporary society, and in particular to explain the increased rate of type II diabetes, obesity, and heart disease in modern human populations. In this context, two main theories have emerged: the thrifty genotype and the thrifty phenotype hypothesis (Barker et al. 1989; Neel 1962). The thrifty genotype hypothesis (Neel 1962) suggests that modern conditions of affluent nutrition do not match the purported cycles of feast and famine that were the norm for most of the evolutionary history of our species. Thus adaptations made during our evolutionary history to survive these cyclical conditions such as a tendency to store excess calories as fat are maladaptive in many contemporary societies, where periods of famine no longer occur. While this hypothesis is attractive, it relies on assumptions about our evolutionary history that are difficult to test (Bouchard, Zwaan 2003).

The second theory, termed the thrifty phenotype hypothesis, highlights development as an important period for determining adult health. It arose from a large epidemiological study done by Barker et al., (1989) which found that low birth weight in humans is strongly associated with an increased risk of developing heart disease in adulthood. Barker et al., (1989) proposed that this effect was mediated by changes made to the insulin-signalling pathway (IIS) *in*

utero in order to survive a poor developmental environment. Later on, it was proposed that this response was adaptive in nature. It was suggested that the developing foetus interprets the poor developmental environment as a cue that predicts a poor post-natal environment and thus alters its phenotype not in order to survive current conditions, but rather to increase its fitness in adulthood. This is called the predictive adaptive response (PAR) hypothesis (Bateson et al. 2014; Gluckman & Hanson 2004). This theory shares many similarities with the polyphenisms discussed earlier, including the prediction that mismatches between the predicted and actual environment can negatively affect fitness. However, most of the theory on polyphenisms and other examples of adaptive developmental plasticity suggests that such responses are only likely to evolve when the generation time of the species is relatively short relative to the cycle of the environmental cue which induces the adaptation (Nylin & Gotthard 1998). Given that human development and fecundity are separated by decades, it is difficult to imagine that a cue reflecting environmental variation during development will retain predictive value until the time at which reproduction occurs. Indeed, this argument has formed the crux of many arguments against predictive adaptive responses in humans (Rickard & Lummaa 2007; Wells 2012). A more plausible alternative is that PARs increase fitness in early life (i.e infancy and childhood). Infancy and development are much more closely linked temporally, thus a prediction made during development is much more likely to remain accurate. Furthermore, infancy and childhood traditionally are phases of high external mortality, thus adaptations that increase fitness at this age are likely to be under strong selection, even if they decrease fitness at later ages.

Finally, it is important to bear in mind that the effects of developmental conditions on human health need not be adaptive. As in other organisms, suboptimal developmental conditions may simply result in scarring or silver spoon effects (Brakefield et al. 2005; Grafen 1988). This is simple to imagine given the following dichotomy: dying during development yields zero fitness, while surviving, even in a compromised fashion, may eventually yield some fitness.

Differentiating between these alternative hypotheses has proven difficult. The necessary manipulations of both developmental and adult environments are obviously not possible in humans. Given that some level of conservation is expected at the level of evolutionary driving forces and the actual evolved mechanisms, as has been observed in the study of DR and lifespan-extending mutations (Gems & Partridge 2013; Nakagawa et al. 2012), it is useful to turn first to model organisms to unravel the evolutionary and mechanistic processes underlying the effects of developmental conditions on late life health. Such an approach has been taken by the IDEAL (Integrated research on Developmental

determinants of Ageing and Longevity) consortium of which the work contained in this thesis forms a small part. IDEAL is an EU funded FP7 project comprising researchers from fourteen universities across Europe. Using a broad range of model species (fruit flies: this thesis, nematodes, fish, frogs, mice, birds *et cetera*) and human cohorts IDEAL aims to address the mechanisms and consequences of developmental conditions for late life health. By combining research on more pliable model species with well-characterized human cohorts IDEAL links developmental conditions to late-life health in a broad phylogenetic and evolutionary framework.

Drosophila melanogaster: model organism of choice for studies of ageing, diet and evolution

In this thesis I use the holometabolous insect *Drosophila melanogaster* - better known as the fruit fly - as a model for understanding the plastic and evolutionary effects of developmental diet on life history traits. Its life cycle passes through several distinct stages starting from an egg, through three larva stages, a pupal stage, and a final adult (imago) stage. The egg stage lasts about 24 hours and ends with the hatching of the first instar larva. The progression through all three larval instars lasts approximately four days at 25 degrees Celsius and is a period of near constant feeding and growth. In fact, larval size increases approximately 100 fold during this time. Advancement through these instars and into the next stage, pupation, is regulated by hormonal changes (Nijhout 1998; Shingleton 2011). After reaching a critical size during the third instar the larva pupates and metamorphoses into the adult form. During pupation, nearly all larval tissues are histolysed, and the adult structures are formed from the imaginal discs and histoblasts, two sets of cells that were present in undifferentiated form throughout the larval stage. The imaginal discs give rise to the epidermal structures of the adult body such as the legs, eyes, mouthparts *et cetera*, while the histoblasts give rise to internal structures such as the gut and other organs. After remodeling is complete the adult emerges, and within eight to twelve hours becomes capable of reproduction. Flies typically live about a week as adults in the wild, but can live more than 12 weeks under laboratory conditions. During this time females are capable of very high levels of reproduction, producing up to 2000 offspring in their lifetime.

Several features of this life history make flies excellent models: they reproduce early and often, their lifespan is short enough to study in the lab, and their holometabolic life cycle allows the manipulation of developmental and adult conditions independently. Many researchers have benefited from these advantages, and in the process have generated a wide array of genetic and genomic tools, making the fruit fly one of the most tractable organisms for unraveling underlying mechanisms. Furthermore, research into dietary and genetic lifespan determinants have shown an unexpected degree of

conservation between fruit flies and other organisms in the genes and pathways that underlie variation in lifespan (Kuningas et al. 2008; Partridge & Gems 2002), suggesting that there is some scope for extending findings relating to diet and lifespan from *Drosophila* to more ecologically, economically or personally (i.e. humans) relevant species.

Aims and outline of this thesis

The general aim of this thesis is to increase our understanding of how developmental diet influences life histories in the fruit fly. Within this broad mandate, I seek to address three main questions: first, to what extent does the phenotypic effect of developmental diet depend on the adult environment, second, does variation in developmental diet have consequences for gene expression across the lifespan and finally, how do life histories evolve in response to variation in developmental diet? All three of these questions are important from a general biological perspective, but they also each bear on the observations and hypotheses formulated to explain the link between diet, development and human ageing. By addressing the nature of this link first in the fruit fly, as has been done for countless other questions, it is hoped that the findings from this thesis can also be used to enhance our evolutionary and mechanistic understanding of human ageing.

To address the three questions outlined above, I apply two different approaches. The first approach, applied in **Chapters 2, 3, and 4**, involves raising flies on different concentrations of sugar and yeast as larvae and assessing the effects on either life history traits (**Chapters 2 & 4**) or gene expression (**Chapters 3 & 4**) across a range of adult conditions. Ultimately the goal of these chapters is to gain a detailed insight into the consequences of developmental and adult diet for both life history traits and gene expression within a single generation, i.e. to categorise the degree and nature of plasticity. The second approach, applied in **Chapter 5**, uses experimental evolution as a tool to unravel how life histories can evolve in response to the combination of variation in developmental diet and variation in age at reproduction during adulthood. This approach has two main aims: first, to assess how developmental diet influences life history evolution, and second, to determine how larval diet availability interacts with selection on age at reproduction. Combining selection pressures in two different life stages provides not only a more realistic picture of life history evolution, but also can indicate how life histories reconcile selection pressures experienced in two very different life stages.

Chapter 2 provides an initial assessment of the phenotypic effects of the three larval diets used throughout the thesis. These three diets differ by an order of magnitude in the amount of sugar and yeast (protein and carbohydrate sources) that they contain and thus represent drastically different developmental

environments. The phenotypes measured include larval and adult weight, the length of development from egg to adult, larval survival, and virgin longevity. A second set of experiments also addresses how these developmental diets interact with variation in the reproductive potential of the adult environment (manipulated by adding either yeast or yeast and a male to the environment) to influence two of the most important life history traits: lifespan and fecundity. Ultimately the goal of this chapter is to gain a general understanding of the life history consequences of these diets, before embarking on more detailed transcriptional analyses (**Chapters 3 and 4**).

Chapter 3 assesses the relative contributions of developmental and adult diets to transcriptional variation in whole body samples of one-day-old adult male and female flies. I use a full factorial design combining three larval and three adult diets (9 treatments total). After eclosion adults are immediately transferred to one of the three adult diets and samples are collected 24 hours later. This design allows the magnitude of larval diet effects in early life to be assessed, as well as the rapidity and magnitude of the response to the adult diet. In fact, a first goal is to address how much of the variation in expression is attributable to developmental and adult diet respectively. This is estimated by applying Principle Components Analysis. A second goal is to identify and characterise the genes whose expression is affected by larval diet, adult diet or both. To do so I use WGCNA (Weighted Gene Correlation Networks Analysis) to identify modules of co-expressed genes whose expression is affected by larval or adult diet. These modules are then assessed for evidence of tissue-specific function, association with particular transcription factors and enrichment for Gene Ontology terms. Overall this study gives a snapshot of the processes driving transcriptional variation in very young flies and in particular the relative roles of larval and adult diet therein.

Chapter 4 combines phenotypic and transcriptomic approaches to look at the long-term effects of developmental diet in adulthood. Using the same full factorial approach used in **Chapter 3**, I assess virgin and mated lifespan and fecundity. Furthermore, on a subset of flies from the virgin lifespan cohort we measure gene expression at middle and old age. The aim of this study is two fold: first to determine the nature of the interaction between developmental and adult diet in determining phenotypes, particularly with respect to existing theories such as the silver spoon and predictive adaptive response hypothesis. Second, using an ANOVA approach combined with K-means clustering we aim to identify the number and type of genes affected by larval diet across the lifespan in each sex. To date, very few studies have addressed long-term effects of developmental diet on the transcriptome, although it is often assumed that this is an important mechanism mediating phenotypic effects. Thus here

I aim to provide insight into the magnitude and nature of long-term effects of developmental diet on gene expression in the fruit fly.

In **Chapter 5**, I use experimental evolution to assess how life histories evolve in response to environmental variation experienced during development and during adulthood. To do so I employ a well-replicated full factorial design consisting of three larval diets and two ages at reproduction. Evolution in response to age at reproduction and variation in larval diet have both been independently assessed previously, however, how the two selection pressures interact is unknown. Looking at both together gives a more realistic and ecologically relevant portrait of life history evolution, as organisms are generally faced with environmental variation in more than one factor and more than one stage. Furthermore, it gives increased insight into how developmental acquisition-allocation decisions are made given different selection pressures in adulthood. In order to differentiate between transient and long-term effects I measure several key life history traits repeatedly across several generations including developmental time, larval survival, fecundity, and lifespan. Furthermore, I generally assess the responses on all three larval diets, revealing whether changes in plasticity are involved in adaptation.

In the general discussion in **Chapter 6**, I synthesise the findings across the experimental chapters, and address how they relate to each other. Furthermore, I will discuss some more general points that emerge from considering the experiments as a whole. Finally, I will address future directions suggested by this thesis, as well as the potential insights that can be gained from this work in the context of theories linking developmental conditions to late life health in humans.

REFERENCES

Adler MI, Bonduriansky R (2014) Why do the well-fed appear to die young? BioEssays **36**, 439-450.

Agrawal AA, Conner JK, Rasmann S (2010) Tradeoffs and negative correlations in evolutionary ecology. In: Evolution After Darwin: The First 150 Years (ed. M. Bell WE, D. Futuyama, J. Levinton), pp. 243-268. Sinauer Associates, Sunderland, MA.

Barker D, Winter P, Osmond C, Margetts B, Simmonds S (1989) Weight in infancy and death from ischaemic heart disease. *Lancet* **2**, 577-580.

Barnes AI, Partridge L (2003) Costing reproduction. *Animal Behaviour* **66**, 199-204.

Bateson P, Gluckman P, Hanson M (2014) The biology of developmental plasticity and the Predictive Adaptive Response hypothesis. *The Journal of Physiology* **592**, 2357-2368.

Beldade P, Mateus ARA, Keller RA (2011) Evolution and molecular mechanisms of adaptive developmental plasticity. *Molecular Ecology* **20**, 1347-1363.

Bennett AF, Lenski RE (1993) Evolutionary adaptation to temperature II. Thermal niches of experimental lines of *Escherichia coli*. *Evolution* **47**, 1-12.

Bergland A (2011) Mechanisms of nutrient-dependent reproduction in dipteran insects. In: Mechanisms of Life History Evolution: the genetics and physiology of life history traits and trade-offs. (eds. Flatt T, Heyland A), pp. 127-136. Oxford University Press.

Bochdanovits Z, de Jong G (2004) Antagonistic pleiotropy for life–history traits at the gene expression level. *Proceedings of the Royal Society of London B: Biological Sciences* **271**, S75-S78.

Bochdanovits Z, Jong Gd (2003) Experimental evolution in *Drosophila melanogaster*: Interaction of temperature and food quality selection regimes. *Evolution* **57**, 1829-1836.

Braendle C, Heyland A, Flatt T (2011) Integrating mechanistic and evolutionary analysis of life history variation. *Mechanisms of life history evolution: The genetics and physiology of life history traits and trade-offs*, 3-10.

Brakefield P, Zwaan B (2011) Seasonal polyphenisms and environmentally induced plasticity in the Lepidoptera: The coordinated evolution of many traits on multiple levels. In: *Mechanisms of Life History Evolution. The genetics and physiology of life history traits and trade-offs*, p. 512. Oxford University Press.

Brakefield PM, Gems D, Cowen T, *et al.* (2005) What are the effects of maternal and pre-adult environments on ageing in humans, and are there lessons from animal models? *Mechanisms of Ageing and Development* **126**, 431-438.

Broderick NA, Buchon N, Lemaitre B (2014) Microbiota-induced changes in *Drosophila melanogaster* host gene expression and gut morphology. In: *mBio*, pp. e01117-01114.

Burdge GC, Hanson MA, Slater-Jefferies JL, Lillycrop. KA (2007) Epigenetic regulation of transcription: a mechanism for inducing variations in phenotype (fetal programming) by differences in nutrition during early life? *British Journal of Nutrition* **97**, 1036-1046.

Burdge GC, Lillycrop KA (2010) Nutrition, epigenetics, and developmental plasticity: Implications for understanding human disease. *Annual Review of Nutrition* **30**, 315-339.

Clemente Jose C, Ursell Luke K, Parfrey Laura W, Knight R (2012) The impact of the gut microbiota on human health: An integrative view. *Cell* **148**, 1258-1270.

Colman RJ, Anderson RM, Johnson SC, *et al.* (2009) Caloric restriction delays disease onset and mortality in rhesus monkeys. *Science* **325**, 201-204.

Darwin C, Wallace A (1858) On the tendency of species to form varieties; and on the perpetuation of varieties and species by natural means of selection. *Journal of the Proceedings of the Linnean Society of London. Zoology* **3**, 45-62.

de Jong G, van Noordwijk AJ (1992) Acquisition and allocation of resources: genetic (co) variances, selection, and life histories. *The American naturalist* **139**, 749-770.

DeWitt TJ, Sih A, Wilson DS (1998) Costs and limits of phenotypic plasticity. *Trends in Ecology & Evolution* **13**, 77-81.

Emlen DJ (1997) Diet alters male horn allometry in the beetle *Onthophagus acuminatus* (Coleoptera: Scarabaeidae). *Proceedings of the Royal Society of London B: Biological Sciences* **264**, 567-574.

Evans DS, Kapahi P, Hsueh WC, Kockel L (2011) TOR signaling never gets old: Aging, longevity and TORC1 activity. *Ageing research reviews* **10**, 225-237.

Festucci-Buselli R, Carvalho-Dias A, Oliveira-Andrade D, *et al.* (2005) Expression of Cyp6g1 and Cyp12d1 in DDT resistant and susceptible strains of *Drosophila melanogaster*. *Insect Molecular Biology* **14**, 69-77.

Fontana L, Partridge L, Longo VD (2010) Extending healthy life span-from yeast to humans. *Science* **328**, 321-326.

Friedman DB, Johnson TE (1988) A mutation in the age-1 gene in *Caenorhabditis elegans* lengthens life and reduces hermaphrodite fertility. *Genetics* **118**, 75-86.

Gems D, Partridge L (2013) Genetics of longevity

REFERENCES

in model organisms: debates and paradigm shifts. *Annual review of physiology* **75**, 621-644.

Ghalambor CK, McKay JK, Carroll SP, Reznick DN (2007) Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. *Functional Ecology* **21**, 394-407.

Gluckman PD, Hanson MA (2004) The developmental origins of the metabolic syndrome. *Trends in Endocrinology and Metabolism* **15**, 183-187.

Grafen A (1988) On the uses of data on lifetime reproductive success. In: *Reproductive success* (ed. Clutton-Brock TH), pp. 454-471. University of Chicago Press.

Heijmans BT, Tobi EW, Stein AD, *et al.* (2008) Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proceedings of the National Academy of Sciences* **105**, 17046-17049.

Heintz C, Mair W (2014) You are what you host: microbiome modulation of the aging process. *Cell* **156**, 408-411.

Hoffmann A (2014) Evolutionary limits and constraints. The Princeton Guide to Evolution. New Jersey: Princeton University Press.

Holliday R (1989) Food, reproduction and longevity: Is the extended lifespan of calorie-restricted animals an evolutionary adaptation? *BioEssays* **10**, 125-127.

Kawecki TJ, Lenski RE, Ebert D, *et al.* (2012) Experimental evolution. *Trends in Ecology & Evolution* **27**, 547-560.

Kenyon C, Chang J, Gensch E, Rudner A, Tabtiang R (1993) A *C. elegans* mutant that lives twice as long as wild type. *Nature* **366**, 461-464.

Kirkwood TB (1977) Evolution of ageing. *Nature* **270**, 301-304.

Kirkwood TBL, Holliday R (1979) The evolution of ageing and longevity. *Proceedings of the Royal Society of London B: Biological Sciences* **205**, 531-546.

Kirkwood TBL, Rose MR (1991) Evolution of senescence: Late survival sacrificed for reproduction. *Philosophical Transactions of the Royal Society of London B: Biological Sciences* **332**, 15-24.

Kirkwood TBL, Shanley DP (2005) Food restriction, evolution and ageing. *Mechanisms of Ageing and Development* **126**, 1011-1016.

Klass MR (1983) A method for the isolation of longevity mutants in the nematode *Caenorhabditis elegans* and initial results. *Mechanisms of Ageing and Development* **22**, 279-286.

Kolss M, Vijendravarma RK, Schwaller G, Kawecki TJ (2009) Life-history consequences of adaptation to larval nutritional stress in drosophila. *Evolution* **63**, 2389-2401.

Kucharski R, Maleszka J, Foret S, Maleszka R (2008) Nutritional control of reproductive status in honeybees via DNA methylation. *Science* **319**, 1827-1830.

Kuningas M, Mooijaart SP, Van Heemst D, *et al.* (2008) Genes encoding longevity: from model organisms to humans. *Aging Cell* **7**, 270-280.

Langerhans RB, DeWitt TJ (2002) Plasticity constrained: over-generalized induction cues cause maladaptive phenotypes. *Evolutionary Ecology Research* **4**, 857-870.

Long A, Liti G, Luptak A, Tenaillon O (2015) Elucidating the molecular architecture of adaptation via evolve and resequence experiments. *Nat Rev Genet* **16**, 567-582.

Luckinbill LS, Arking R, Clare MJ, Cirocco WC, Buck SA (1984) Selection for delayed senescence in *Drosophila melanogaster*. *Evolution* **38**, 996-1003.

Lumey LH, Stein AD, Susser E (2011) Prenatal famine and adult health. *Annual review of public health* **32**, 10.

Magwere T, Chapman T, Partridge L (2004) Sex differences in the effect of dietary restriction on life span and mortality rates in female and male *Drosophila melanogaster. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences* **59**, B3-B9.

Malthus TR (1872) An essay on the principle of population; or, a view of its past and present effects on human happiness; with an inquiry into our prospects respecting the future removal or mitigation of the evils which it occasions Reeves and Turner, London.

McCay CM, Crowell MF, Maynard LA (1935) The effect of retarded growth upon the length of life span and upon the ultimate body size. *Nutrition (Burbank, Los Angeles County, Calif.)* **5**, 155-172.

Medawar PB (1952) An unsolved problem of biology College.

Moczek AP, Snell-Rood EC (2008) The basis of beeing different: the role of gene silencing in plasticity. *Evol Dev* **10**, 511-513.

Mueller NT, Bakacs E, Combellick J, Grigoryan Z, Dominguez-Bello MG (2015) The infant microbiome development: mom matters. *Trends in molecular medicine* **21**, 109-117.

Nakagawa S, Lagisz M, Hector KL, Spencer HG (2012) Comparative and meta-analytic insights into life extension via dietary restriction. *Aging Cell* **11**, 401-409.

Neel JV (1962) Diabetes mellitus: a "thrifty" genotype rendered detrimental by "progress"? *American journal of human genetics* **14**, 353 - 362.

Nijhout HF (1998) *Insect hormones* Princeton University Press.

Nylin S, Gotthard K (1998) Plasticity in Life-History

Traits. *Annual Review of Entomology* **43**, 63-83.

Oostra V, de Jong MA, Invergo BM, *et al.* (2011) Translating environmental gradients into discontinuous reaction norms via hormone signalling in a polyphenic butterfly. *Proceedings of the Royal Society of London B: Biological Sciences* **278**, 789-797.

Palmer AC, Kishony R (2013) Understanding, predicting and manipulating the genotypic evolution of antibiotic resistance. *Nat Rev Genet* **14**, 243-248.

Partridge L, Fowler K (1992) Direct and Correlated responses to selection on age at reproduction in *Drosophila melanogaster*. *Evolution* **46**, 76-91.

Partridge L, Gems D (2002) Mechanisms of aging: public or private? *Nature Reviews Genetics* **3**, 165-175.

Piraino S, Boero F, Aeschbach B, Schmid V (1996) Reversing the life cycle: medusae transforming into polyps and cell transdifferentiation in *Turritopsis nutricula* (Cnidaria, Hydrozoa). *The Biological Bulletin* **190**, 302-312.

Rickard IJ, Lummaa V (2007) The predictive adaptive response and metabolic syndrome: challenges for the hypothesis. *Trends in Endocrinology Metabolism* **18**, 94-99.

Roff DA (1992) Evolution of life histories: theory and analysis Springer Science & Business Media.

Roff DA (2001) *Life History Evolution* Sinauer Associates, Sunderland.

Roff DA, Fairbairn DJ (2007) The evolution of trade-offs: where are we? *Journal of Evolutionary Biology* **20**, 433-447.

Rose MR (1984) Laboratory evolution of postponed senescence in *Drosophila melanogaster*. *Evolution* **38**, 1004-1010.

Rose MR (1991) Evolutionary biology of aging/ Michael R. Rose Oxford University Press, New York.

Russo VE, Martienssen RA, Riggs AD (1996) *Epigenetic mechanisms of gene regulation* Cold Spring Harbor Laboratory Press.

Santos M, Borash DJ, Joshi A, Bounlutay N, Mueller LD (1997) Density-dependent natural selection in *Drosophila*: evolution of growth rate and body size. *Evolution*, 420-432.

Schlichting C, Pigliucci M (1998) Phenotypic evolution: A reaction norm perspective Sinauer.

Schlichting CD, Smith H (2002) Phenotypic plasticity: linking molecular mechanisms with evolutionary outcomes. *Evolutionary Ecology* **16**, 189-211.

Sharon G, Segal D, Ringo JM, *et al.* (2010) Commensal bacteria play a role in mating preference of *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences* **107**, 20051-20056.

Shingleton A (2011) Evolution and the regulation of growth and body size. *Mechanisms of life history evolution. Oxford University Press, Oxford*, 43-55.

Sibley R, Calow P (1986) Physiological ecology of animals. An evolutionary approach. Blackwell Scientific Publications, Oxford and London.

Simpson Stephen J, Sword Gregory A, Lo N (2011) Polyphenism in insects. *Current Biology* **21**, R738-R749.

Stearns SC (1992) *The Evolution of Life Histories* Oxford University Press, Oxford.

Stearns SC, Ackermann M, Doebeli M, Kaiser M (2000) Experimental evolution of aging, growth, and reproduction in fruitflies. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 3309-3313.

Tobi EW, Goeman JJ, Monajemi R, *et al.* (2014) DNA methylation signatures link prenatal famine exposure to growth and metabolism. *Nat Commun* **5**.

Van Noordwijk AJ, de Jong G (1986) Acquisition and allocation of resources: their influence on variation in life history tactics. *American Naturalist*, 137-142.

Vijendravarma RK, Narasimha S, Chakrabarti S, *et al.* (2015) Gut physiology mediates a trade-off between adaptation to malnutrition and susceptibility to food-borne pathogens. *Ecology Letters* **18**, 1078-1086.

Waddington CH (1942) Canalization of development and the inheritance of acquired characters. *Nature* **150**, 563-565.

Wells JCK (2012) A critical appraisal of the predictive adaptive response hypothesis. *International Journal of Epidemiology* **41**, 229-235.

West-Eberhard MJ (2003) Developmental plasticity and evolution Oxford University Press.

Williams GC (1957) Pleiotropy, natural selection, and the evolution of senescence. *Evolution* **11**, 398-411.

Wong AC-N, Dobson AJ, Douglas AE (2014) Gut microbiota dictates the metabolic response of *Drosophila* to diet. *The Journal of Experimental Biology* **217**, 1894-1901.

Zera AJ, Harshman LG (2001) The physiology of life history trade-offs in animals. *Annual Review of Ecology and Systematics* **32**, 95-126.

Zilber-Rosenberg I, Rosenberg E (2008) Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. *FEMS microbiology reviews* **32**, 723-735.

Zwaan B, Bijlsma R, Hoekstra RF (1995) Direct selection on life span in *Drosophila melanogaster*. *Evolution* **49**, 649-659.

Zwaan BJ (1999) The evolutionary genetics of ageing and longevity. *Heredity* **82**, 589-597.

The effect of developmental nutrition on lifespan and fecundity depends on the adult reproductive environment in *Drosophila melanogaster*

Christina M. May[1], Agnieszka Doroszuk[1,2] and Bas J. Zwaan[1]

1. Laboratory of Genetics, Wageningen University, Wageningen
2. Current affiliation: Rijk Zwaan, the Hague, the Netherlands

ABSTRACT

Both developmental nutrition and adult nutrition affect life-history traits; however, little is known about whether the effect of developmental nutrition depends on the adult environment experienced. We used the fruit fly to determine whether life-history traits, particularly life span and fecundity, are affected by developmental nutrition, and whether this depends on the extent to which the adult environment allows females to realize their full reproductive potential. We raised flies on three different developmental food levels containing increasing amounts of yeast and sugar: poor, control, and rich. We found that development on poor or rich larval food resulted in several life-history phenotypes indicative of suboptimal conditions, including increased developmental time, and, for poor food, decreased adult weight. However, development on poor larval food actually increased adult virgin life span. In addition, we manipulated the reproductive potential of the adult environment by adding yeast or yeast and a male. This manipulation interacted with larval food to determine adult fecundity. Specifically, under two adult conditions, flies raised on poor larval food had higher reproduction at certain ages – when singly mated this occurred early in life and when continuously mated with yeast this occurred during mid-life. We show that poor larval food is not necessarily detrimental to key adult life-history traits, but does exert an adult environment-dependent effect, especially by affecting virgin life span and altering adult patterns of reproductive investment. Our findings are relevant because (1) they may explain differences between published studies on nutritional effects on life-history traits; (2) they indicate that optimal nutritional conditions are likely to be different for larvae and adults, potentially reflecting evolutionary history; and (3) they urge for the incorporation of developmental nutritional conditions into the central life-history concept of resource acquisition and allocation.

INTRODUCTION

Nutrition is a primary determinant of lifespan, the rate of ageing, and reproductive capacity (Chippindale et al. 1993; Fontana et al. 2010; Good & Tatar 2001; Walker et al. 2005; Weindruch & Walford 1982) and as such its relationship to life history has been studied extensively. The bulk of this research has focussed on the impact of adult nutritional quantity and quality, leading to important insights in the field of gerontology. For instance, the discovery of lifespan extension upon dietary restriction across many different animal species has resulted in a booming field concerned with characterizing the mechanism and specific nutrient dependencies of the effect (Austad 1989; Chippindale et al. 1993; Grandison et al. 2009; Weindruch & Walford 1982). However, a growing body of evidence suggests that developmental nutrition can also impose far-reaching effects on adult traits, including lifespan and fecundity (Barrett et al. 2009; Brakefield et al. 2005; Cleal et al. 2007; Dmitriew & Rowe 2011; Gluckman et al. 2008; Gluckman & Hanson 2004; Joy et al. 2010).

25 years ago, Barker et al., (1989) found that human infants with low birth weights had higher adult mortality from cardiovascular disease. In this case low birth weight was regarded as a proxy for malnutrition in utero. This finding has since been confirmed in many other epidemiological studies, which have tied under-nutrition in utero to an increased risk of traits associated with the metabolic syndrome - a disorder of energy storage which increases the risk of heart disease and type II diabetes (Barker et al. 1989; Leon et al. 1998). In mammalian models for these observations, both inadequate or excessive developmental nutrition has been shown to increase the incidence of traits of the metabolic syndrome, including decreased glucose tolerance, obesity and diabetes (Barker & Thornburg 2013; George et al. 2012; Painter et al. 2005). In some cases this has also resulted in increased mortality rates (Aihie Sayer et al. 2001; Ozanne & Hales 2004). In order to interpret these effects in relation to ecological and evolutionary theory (Van den Heuvel et al. 2013), and to quantify the epidemiological consequences for health, the effects of variation of the developmental environment in concert with the adult environment should be assessed. However, given the long lifespan and cost of upkeep of mammalian models large factorial designs considering multiple life history traits across different environments quickly become infeasible.

Studies using more tractable insect models have shown that poor nutrition during development generally results in detrimental fitness effects including decreased size, fecundity and lifespan (Barrett et al. 2009; Bauerfeind et al. 2009; Blanckenhorn 2006; Boggs & Freeman 2005; Colasurdo et al. 2009; Dmitriew & Rowe 2011; Kaspi et al. 2002; Kolss et al. 2009; Zajitschek et al. 2009; Zwaan et al. 1991). In fact, it is often assumed that poor larval food inevitably leads to

Figure 1: *Drosophila melanogaster*

detrimental effects in the adult. Several recent studies, however, suggest that the effect of the developmental environment depends on the specific adult environment experienced (Allen & Marshall 2013). For example, Adler et al., (2013) highlighted the context-dependence of the effect of larval food on adult lifespan in the neriid fly *Telostylinus angusticolus* – when housed in same-sex groups, males raised on calorically rich larval food lived longer than females, however, this difference disappeared in mixed sex groups. A similar interaction with housing conditions was shown for adult nutrition, where the extent of lifespan changes in response to nutrition in male fruit flies depended on whether or not the flies were kept in mixed sex groups (Zajitschek et al. 2013). Because increasing reproduction often comes at the expense of lifespan (Flatt 2011; Harshman & Zera 2007; Kenyon 2010), it is important to know how nutritional manipulations affect longevity in environments with differing reproductive potentials (i.e. the extent to which females can reach their full reproductive potential). Indeed, the reproductive potential of the environment, and the differing costs associated with achieving that potential after development on foods differing in quality as larvae, might be the driving force behind some of the interactions between larval and adult nutritional environment.

Mechanistic links between diet and ageing have often been explored using Drosophila melanogaster as a model organism (Mair et al. 2005; Min et al. 2007). To our knowledge, only one study has addressed the effect of developmental nutrition on both adult longevity and fecundity in Drosophila. It is important to know how both of these traits respond since ageing is characterized by both accelerating mortality rates with time, and by an associated decline in offspring production (Kirkwood & Rose 1991; López-Otín et al. 2013). Tu and Tatar (2003) deprived third instar larvae of yeast and found that they displayed decreased fecundity but no concomitant change in longevity as adults. Applying yeast deprivation to third instar larvae only, however, is likely to cause different effects compared to limitation across the whole developmental period (Danielsen et al. 2013), a methodology more comparable to approaches taken in other species when evaluating the effects of adult nutrition. It is also important to note that research in insects on developmental nutrition concerns primarily the effects of underfeeding, and the effects of overfeeding are less well-known, although it has been shown in mammals that the effects of over and under-feeding could be phenotypically similar (Ford & Long 2011).

In this study, we address the effect of under- and over-nutrition of D. melanogaster (Fig. 1) during the entire juvenile stage on longevity, fecundity, and other life history traits. We combine these larval nutritional manipulations with three adult reproductive environments (singly mated, SM; singly mated with yeast, SMY; and continuously mated with yeast, CMY) in a full-factorial design in order to determine whether adult environment modulates the effects of developmental nutrition. Generally, the addition of yeast increases fecundity in Drosophila (Bass et al. 2007), while the presence of a male allows females to reach higher reproductive potentials by preventing sperm depletion (Kaufman 1942), despite shortening lifespan (Partridge et al. 1987). We hypothesize that the detrimental effects of developmental under or over-nutrition will be highest in the most reproductively conducive adult environment, as presumably both the under and over-fed flies are not able to make full use of the reproductive potential of the environment, or will pay a greater cost in terms of lifespan for doing so.

MATERIALS & METHODS

Drosophila stock and culturing

The stock population originated from wild populations collected in 2006 from six locations across Europe. To ensure that the genetic variation of the original wild population was equally represented in the stock, we performed four rounds of crosses among the six component populations (Appendix 1), ensuring that the effects of developmental nutrition are unlikely to be genotype specific. The stock population has been maintained in the laboratory for more than forty generations under standard laboratory conditions (25°C, 65% humidity, 12h:

12h light: dark cycle, 14 day generation time, and a standard control diet (1x) of 70 grams yeast (Fermipan Instant Yeast Red Label), 100 grams sugar, 20 grams agar, 15 mL nipagin and 3 mL propionic acid per litre of water) at a population size of approximately 2000 individuals.

Larval diet

Eggs collected from four-day-old adults of the stock population were transferred to vials filled with 7 mL of media (100 eggs/vial, 75 mm vial diameter). Larvae were raised on media where yeast and sugar content was manipulated to obtain diet treatments representing poor, control and rich food levels. The concentrations of yeast and sugar were relative to those of the standard medium: we used 0.25x concentration for the poor food, 1x for control and 2.5x for the rich food treatments (Appendix 2). Amounts of agar, nipagin and propionic acid remained unchanged across all food levels.

Experimental set-up

Two cohorts of flies were raised on the three larval food levels. In the first cohort, development time, survival from egg to adult, larval weight, adult weight, egg weight and virgin survival were assessed. In this cohort, all adult flies were maintained on the control medium. In the second cohort, female survival and fecundity were assessed in three adult reproductive environments: singly mated flies on control medium (SM), singly mated flies on control medium with yeast supplement (SMY), and continuously mated flies on control medium with yeast supplement (CMY). The full factorial setup in the second cohort allowed for the estimation of the relative importance of developmental food conditions and adult reproductive environment on lifespan and reproduction.

Developmental time and larval survival

Developmental time and larval survival were assessed for 400 individuals per food level (four vials of 100 eggs each). The number of newly eclosed flies was recorded every hour between 8:00 am and 5:00 pm from the first day of eclosion until no new flies had eclosed for more than five hours.

Larval weight, adult weight and egg weight

Larvae were extracted from the medium four days post egg-laying following Bochdanovits and de Jong (2003) and weighed in groups of three (n=15) to obtain both wet (fresh) and dry weight (dried for 24 hours at 65°C). Adult flies were weighed in unisex groupings of three individuals, one day after eclosion. Weight was measured for 48 flies per treatment (12 groups of 3 flies each). After wet weight was obtained, flies were dried in an oven at 65°C for 72 hours, and then re-weighed to obtain dry weight.

After development on the different larval foods, adult females were maintained as virgins on control food for two days at a density of 10 females per vial. They were then placed on agar plates with yeast to stimulate egg-laying for 3 hours. Eggs were collected and weighed on a Sartorius ultra-micro balance in groups of 20 per larval food (n=8) to obtain wet weight, then dried for 24 hours at 65°C in an oven and re-weighed to obtain dry weight. All weights were obtained with a Sartorius ultra-micro balance accurate to the nearest 0.1 µg.

Virgin survival

To measure adult virgin survival, flies were sexed under mild CO_2 anaesthesia between the third and fourth hour after eclosion. Emergence of flies was synchronized by staggering egg collection days. Flies were maintained in unisex groups of five individuals per vial, per sex and per larval food level (n=20) and transferred to fresh media weekly. Survival was recorded every second day.

Reproduction and survival of mated flies

After eclosing, flies raised on different larval treatments were kept separately in mixed-sex groups in 250 mL bottles with standard medium for 48 hours to allow time for mating. Flies were then sexed under mild CO_2 anaesthesia and females were transferred to one of three adult treatments: singly mated (SM), singly mated with yeast (SMY), or continuously mated with yeast (CMY). The yeast supplement consisted of 20-30 grains of yeast added to the surface of the medium. Females were housed individually or with a single male and transferred to fresh medium every second day. At this time survival was scored, yeast supplement was reapplied and any dead males were replaced by individuals from the same cohort. Previously inhabited vials were retained until the eggs developed into adults. These offspring were counted, giving an accurate measure of realized female fecundity. This regimen was maintained until all females had died.

Statistical Analysis

Wet and dry weight of eggs, larvae and adults were analysed using ANOVAs with larval food as the independent variable. Post hoc determination of differences between larval food treatments was done using the Tukey HSD test. Survival from egg to adult was analysed as binomial data with a generalized linear mixed models approach designating larval food as a fixed factor and vial as a random variable nested within the food treatment. Egg to adult development time and adult virgin survival were analysed using Cox proportional hazards with larval food treatment as the independent variable.

Adult mated survival was analysed using Cox proportional hazards, with larval food treatment and adult reproductive environment as independent variables. Fecundity was analysed using a repeated-measures ANOVA which estimated

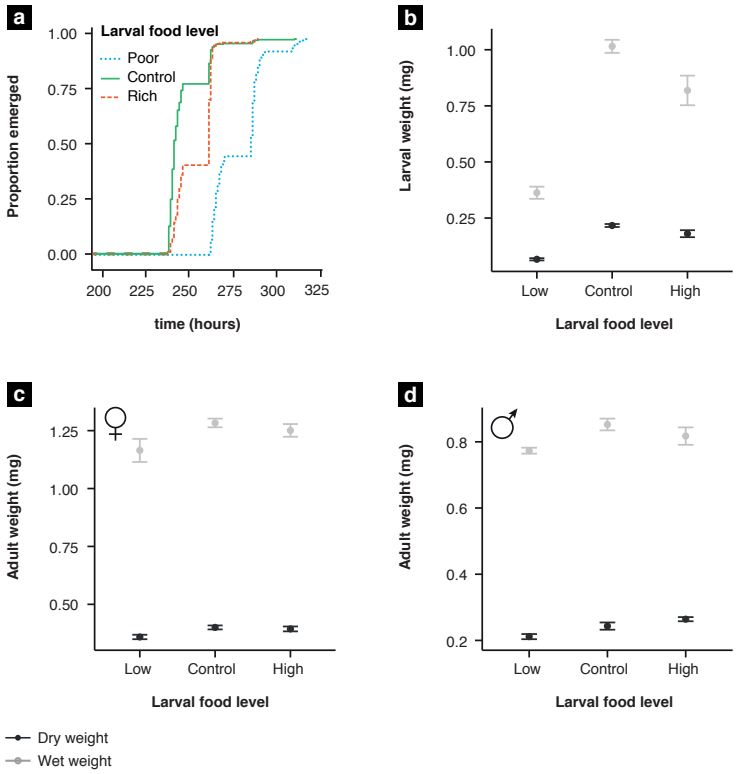


Figure 2: The effect of larval food level on development time (a), larval wet and dry weight (b) adult female wet and dry weight (c) and adult male wet and dry weight (d). All error bars are standard errors of the mean (SE).

both between and within-subjects effects. Between-subjects effects address the effect of larval food and adult reproductive environment on total fecundity, while within-subjects effects assess whether larval food or adult reproductive environment affect patterns of reproduction over time. To disentangle significant interactive effects in the model between time and adult reproductive environment, we performed independent ANOVA's for each 48-hour period for each adult reproductive environment. To correct for multiple comparisons we used the Bonferroni correction. All statistical analysis was performed in JMP statistical software (v.9.0.0) and in R (v. 3.0.1; R Core Team 2013).

RESULTS

Effects of larval nutrition on larvae and young adults

Larvae raised on poor and rich food showed a delay in development compared to the control food level (Cox PH model: χ^2=553.164, p <0.0001, Fig. 2a). While for the larvae raised on rich media the delay was only about eight hours, larvae

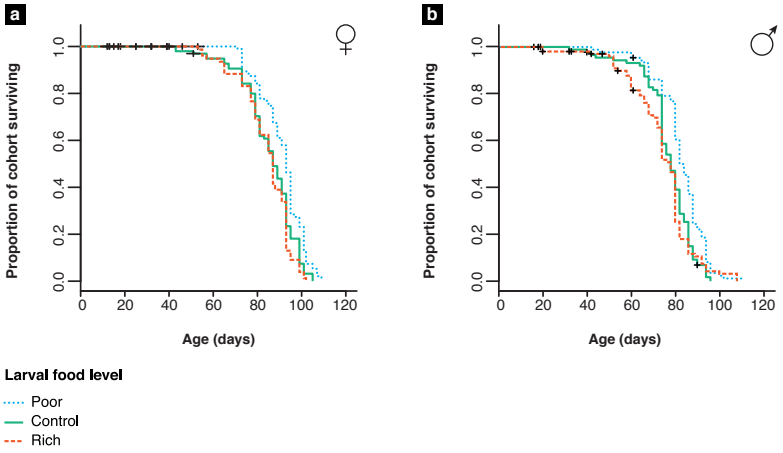


Figure 3: The effect of larval food level on virgin adult female (a) and male (b) survival. Rearing on poor larval food increases longevity of both male and female flies, while flies raised on control and rich food as larvae show similar adult lifespans in both sexes.

on poor food took 34 hours longer to develop on average (Fig. 2a). In addition, larvae feeding on rich and poor food showed significantly lower survival compared to the control treatment (Generalized Linear Model, best fit model: AIC=1014, z=3.42, p=0.0006, mean survival from egg to adult ± SEM: Poor = 80 ± 3%, Control = 89 ± 1%, Rich = 80 ± 3%).

Larval weight at four days post hatching was strongly affected by larval food level (ANOVA: $F_{2, 42} = 56.6690$, $p < 0.0001$ and $F_{2, 42} = 59.4345$, $p < 0.0001$ for wet and dry weight, respectively, Fig. 2b). Both poor and rich food raised larvae were lighter than control larvae (Tukey HSD: $p<0.001$ for poor and rich raised larvae respectively, Fig.2b), however the effect was much stronger on poor food raised larvae, which were 65% lighter than controls.

Both male and female flies developing on a poor food diet weighed significantly less as adults than those raised on control and rich larval food (ANOVA: $F_{2,78} = 9.641$, $p = 0.006$ and $F_{2,70}=21.273$, $p <0.001$ for wet and dry weight, respectively; Fig. 2c, d), which means that the longer period of larval growth did not compensate entirely for the adverse effects of poor food on body mass. Interestingly, there was no difference in adult size between flies raised on rich and control food levels (Tukey HSD: $p = 0.907$ and $p=0.277$ for wet and dry weight respectively, Fig. 2c, d), indicating that flies raised on rich food were able to compensate for their larval weight differential, perhaps via their slightly

Table 1: Tests of between-subjects effects on overall fecundity across the lifespan. GG: Greenhouse-Geisser corrected p-value.

Source of variation	d.f.	Sum of squares	Mean square	F	P value (GG)
Larval food level (LFL)	2	25608	12804	2.502	0.085
Adult reproductive environment (ARE)	2	2963847	1481923	289.531	<0.001
LFL x ARE	4	46088	11522	2.251	0.066
Error	161	824055	5118		

Table 2: Tests of within-subjects effects on patterns of reproduction over time. GG: Greenhouse-Geisser corrected p-value.

Source of variation	d.f.	Sum of squares	Mean square	F	P value (GG)
Time	5.357	2010850	375383	223.964	<0.001
Time x larval food level (LFL)	10.714	37545	3504	2.091	0.02
Time x adult reproductive environment (ARE)	10.714	1319910	123200	73.504	<0.001
Time x LFL x ARE	21.427	67144	3134	1.87	0.01
Error (time)	862.44	1445533	1676		

increased development time. It is worth noting though, that this compensation may not have been complete, as there still appears to be a trend towards lower weight in rich-raised flies. Both sexes showed similar responses to larval food (ANOVA: $F_{2,78} = 0.332$, p=0.72 and $F_{2,70} = 0.949$, p=0.392 for wet and dry weight, respectively) and, as expected, females were heavier than males, irrespective of larval food conditions (ANOVA: $F_{1, 78} = 314.883$, $p < 0.0001$ and $F_{1, 70} = 347.38$, $p < 0.0001$ for wet and dry weight, respectively).

Eggs laid by females raised on poor food had a higher wet weight than those of other treatments (ANOVA: $F_{2, 21} = 4.253$, p=0.0281, mean wet weight (mg) ± SEM: Poor = 0.220 ± 0.003, Control: 0.165 ± 0.014, Rich: 0.2006 ± 0.018), however, no difference was observed when the eggs were dry (ANOVA: $F_{2, 21} = 1.322$, p =0.288, mean dry weight (mg) ± SEM: Poor = 0.055 ± 0.004, Control: 0.047 ± 0.003, Rich: 0.055 ± 0.003), indicating a similar resource investment in egg production. Overall, while our results show that larvae and young adults suffer what have classically been considered detrimental effects of poor nutrition such as increased development time and decreased adult weight, the effects of rich nutritional levels are less pronounced.

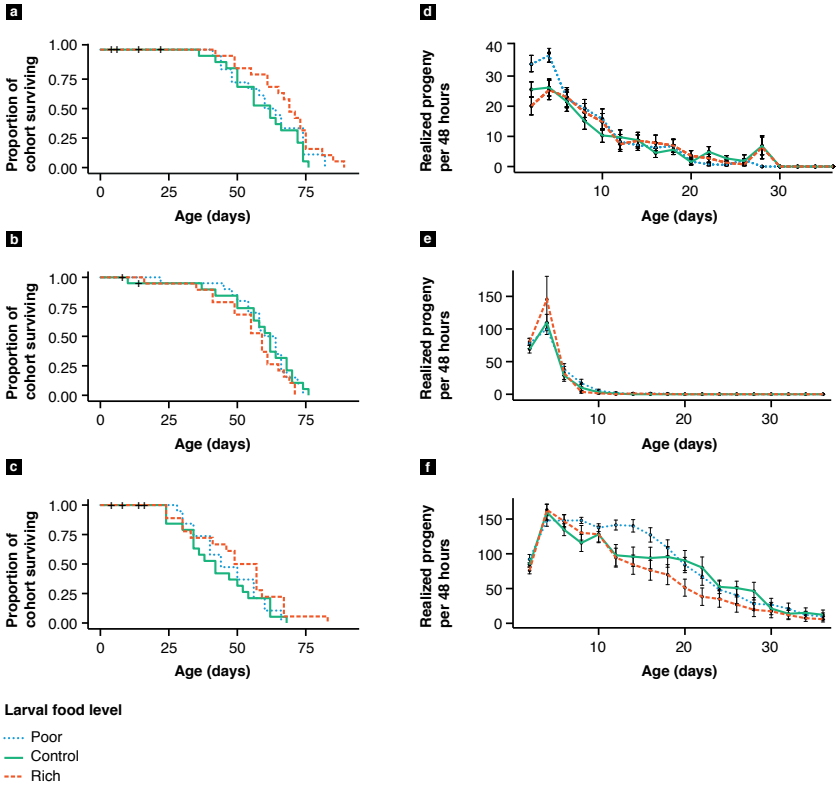


Figure 4: The effect of larval food conditions and adult reproductive environment on mated longevity (a:c) and fecundity over time (d:f). Longevity is shown under (a) singly mated conditions (SM), (b) continuously mated conditions (CM) and (c) continuously mated with yeast (CMY) conditions. Fecundity is also shown for (d) SM, (e) CM, and (f) CMY conditions. In contrast to virgin longevity, there are no differences in mated longevity between larval food levels within an adult condition (a:c). However, adult conditions do profoundly affect lifespan, with lifespan decreasing dramatically from SM (a) to SMY (b) to CMY conditions (c). Maximum reproductive rate and total reproduction occur when flies were continuously mated with yeast (f), while adding yeast alone (e) only increases maximum reproductive rate but not total reproduction relative to singly mated flies (d). In addition, flies raised on poor larval food have higher early reproduction when singly mated (d) and higher mid-reproductive span reproduction when continuously mated with yeast (f) relative to flies raised on rich food.

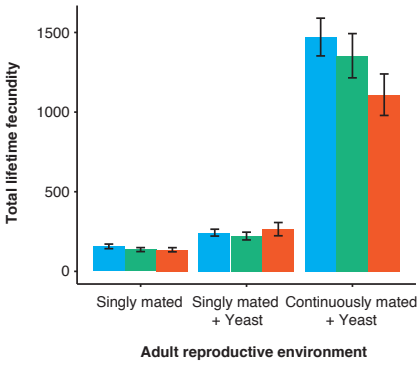


Figure 5: Total lifetime fecundity as a function of larval food level and adult reproductive environment. Across larval diets, the continuous mating with added yeast (CMY) condition results in significantly higher lifetime fecundity than single mating (SM), or single mating with yeast (SMY) conditions. While there is no significant main effect of larval food on total lifetime fecundity, there is a trend towards an interaction between larval and adult conditions caused by the tendency for flies raised on poor larval food to have slightly higher lifetime fecundity than rich larval food raised flies under CMY conditions (Bonferroni post-hoc test, p=0.10).

Effects of larval nutrition on virgin longevity

Females were longer lived than males (Cox PH model: $\chi^2 = 74.739$, $p < 0.0001$), but both sexes showed a similar response to larval treatments. Remarkably, flies raised on poor food actually lived 7 and 8% longer on average than those raised on control and rich medium respectively (Cox PH model: $\chi^2 = 28.8517$, $p < 0.0001$; Fig. 3a, b). This translates into an increase in lifespan of about 6 days on average, relative to the control, while flies raised as larvae on control and rich food did not differ in lifespan ($p = 0.73$; Fig. 3a, b).

Effects of larval nutrition and reproductive environment on mated longevity

There was a profound effect of reproductive environment on longevity (Cox PH model: $\chi^2 = 34.955$, $p < 0.0001$, Fig. 4a-c). Longevity decreased stepwise relative to singly mated females; adding yeast decreased longevity by approximately 8% while adding yeast and allowing continuous mating decreased average longevity by approximately 30% (Fig. 4a-c). In contrast to virgin longevity, mated longevity was not influenced by larval food level (Cox PH model: $\chi^2=2.3087$, $p= 0.315$).

Effects of larval nutrition and reproductive environment on reproduction

The adult reproductive environment profoundly affected both total reproduction (Table 1, Fig. 5) and patterns of reproduction over time (Table 2, Fig. 4d-f). The strongest effect was seen when flies were continuously mated with added yeast (CMY) – this treatment resulted in much higher lifetime reproduction (nine and six times higher than females experiencing single mating or single mating plus yeast conditions respectively; Bonferroni post-hoc test: $p<0.001$ for both, Fig. 4d-f). CMY also increased the maximum rate of reproduction achieved per

Table 3: ANOVA's of total fecundity per time point and adult reproductive environment. Only significant results shown.

		ANOVA results			Means ± SE			Post hoc tests		
Age (days)	**Adult reproductive environment**	**F**	**d.f.**	**P**	**Poor**	**Control**	**Rich**	**Poor vs. Control**	**Poor vs. Rich**	**Control vs. Rich**
2	Singly mated	6.48	2,53	0.003	34.1 ± 2.8	25.1 ± 2.6	19.7 ± 3.1	0.074	0.002	0.373
	Continuous + yeast	4.33	2,53	0.018	96.7±5.5	84.5±3.1	80.3±3.2	0.0957	0.0182	0.7467
4	Singly mated	4.8	2,53	0.012	37.1 ± 2.5	25.8±2.9	25.6±3.4	0.0262	0.0239	0.9992
12	Continuous + yeast	4.03	2,53	0.024	141.4±7.1	102.9±15.4	93.1±14.3	0.0885	0.0267	0.8501
14	Continuous + yeast	5.61	2,53	0.006	140.2±8.9	95.8±13.9	83.3±14.6	0.04	0.0071	0.7667
16	Continuous + yeast	3.64	2,53	0.033	127.2±10.3	93.9±15.5	76.3±14.3	0.1956	0.0282	0.6303

48-hour period, with average fecundity from ages two to four days of 161.54 ±4.76 SE relative to SMY (109.41±6.37) and SM (29.38±1.83) (ANOVA days 2 to 4: $F_{2, 162} = 188.43$, $p < 0.001$). In addition CMY flies continued reproducing until day 36 of adult life while in SMY and SM flies all reproduction had ceased by days 20 and 26 respectively (Fig. 4d-f). In contrast, the difference between SM and SMY was subtler; while they did not differ in total lifetime fecundity (Bonferroni post-hoc test: p=0.43, Fig. 5), their patterns of reproduction across life were different. (Fig. 4d-f) Adding yeast to singly mated females resulted in a rapid burst of reproduction early in life followed by a quick (near) cessation of reproduction (Fig. 4e). In contrast, singly mated females without yeast did not reach a similarly high peak of early reproduction, but their reproduction was spread out across the lifespan (Fig. 4d).

There was also a near significant interactive effect between the larval food environment and adult reproductive environment on total fecundity (Table 1, Fig. 5), as flies raised on poor food had slightly higher lifetime reproduction than those raised on rich food in the CMY condition (Bonferroni post-hoc test, p=0.10). Indeed, contrary to expectation, flies raised on the lowest food as larvae did not show compromised reproduction in any adult reproductive environment.

In addition, larval food modified patterns of reproduction across time depending on the adult reproductive environment (Table 2, Fig. 4d-f). In order to break down this interaction we performed individual ANOVA's per adult reproductive environment on each 48 hour time period in which reproduction was measured. This showed that under singly mated conditions, females raised on poor food

had higher early reproduction than those raised on rich food (days one to four of adult life, Table 3), while control and rich-raised flies did not differ. For the rest of the lifespan the rate of reproduction of poor raised flies was similar to the other larval treatments. When flies were singly mated with yeast, the differences between larval food levels in patterns of reproduction across the lifespan disappeared (Repeated Measures ANOVA: $F_{2.5,\ 137.4}$=0.749, p=0.588). However, when under the CMY (continuous mating + yeast) condition, again poor flies showed an increase in reproduction relative to flies raised on rich food, while control and rich-raised flies did not differ from each other. Notably, the increase in fecundity of poor-raised flies appeared later on in life, from days 12 to 16 of life (Table 3). Thus it appears that not only does larval food alter patterns of reproduction, and therefore the adult life history of the fly, but this effect is also dependent on the adult reproductive environment.

DISCUSSION

Effects of larval nutrition on larvae and young adults

A wide array of insect literature has found that calorically poor food during development leads to increased development time, and decreased adult weight (Colasurdo et al. 2009; Dmitriew & Rowe 2011; Kaspi et al. 2002; Kolss et al. 2009), which agrees with our finding that developing on poor food decreases larval and adult weight while increasing development time. Although the literature on overfeeding during development in insects is rather sparse, existing studies suggest that high-protein diets accelerate larval development while high sugar levels can cause growth inhibition and development of "hallmarks" of type-2-diabetes (Danielsen et al. 2013; Pasco & Léopold 2012). In our study a high-protein-high-sugar diet resulted in a moderate increase of development time, and a decrease of larval but not adult weight. It appears that flies raised on rich developmental nutrition may use an increase in development time to overcome the challenges of over-feeding, ultimately resulting in flies phenotypically indistinguishable from control flies in all other adult traits we assayed, but with lower fecundity than poor-raised flies at certain ages.

As found previously by Prasad et al., (2003) and Vijendravarma et al., (2010), flies raised on poor food as larvae laid significantly heavier eggs than those raised on control food, despite being smaller adults. Vijendravarma et al., (2010) hypothesized that this was due to enhanced maternal egg provisioning. Our results showed that the increased egg weight in poor-raised females was due to increased water content of the eggs; the dry weight of eggs did not differ between the control and poor raised females. This increased water content could be caused simply by increased allocation of water by the poor raised flies, or by a change in the allometry of the different components of the egg. Whether either of these mechanisms is beneficial to the offspring is unclear, but merits further testing.

Effects of larval nutrition on virgin longevity

While several traits responded as expected to larval nutrition, virgin longevity was a notable exception in our experiment. Flies raised on rich food as larvae showed no difference relative to control in terms of lifespan, while, flies of both sexes raised on poor food displayed a 7% increase in lifespan relative to control (Fig. 2a-b). While this increase may seem rather modest, it is by no means negligible and indicates a far-reaching effect of larval nutrition on lifespan. In fact, this extension falls within the range of lifespan extension achieved by induction of dFOXO (Hwangbo et al. 2004), a key gene in the insulin-signalling pathway (Giannakou & Partridge 2007).

Of the few studies that have applied restricted nutrition to Drosophila during development and consequently measured longevity, only one has shown an increase in lifespan. Zwaan et al., (1991) found that adult lifespan was increased in flies that had been transferred as larvae onto agar-only medium after 60 hours of development. In contrast, Tu and Tatar (2003) found that removing yeast in the third instar did not affect adult longevity. It seems rather likely that reduction of both yeast and sugar, as done by Zwaan et al., (1991) and in our experiments, would have considerably different effects to reducing only yeast. In fact, for adult Drosophila it has been shown that the ratio of carbohydrate to protein (i.e. sugar to yeast) is often very important in determining adult longevity (Lee et al. 2008). In addition, studies on Drosophila larvae have indicated that different relative protein and sugar contents of developmental food can cause long term alterations in insulin signalling with possible effects on adult traits (Danielsen et al. 2013; Pasco & Léopold 2012). In these studies, high sugar diets induced delayed eclosion, smaller body size and a type-2-diabetes-like phenotype in adults. In the Tu and Tatar (2003) study the lack of any protein in the diet but ample sugar caused several of those effects, but the demographic patterns of ageing remained normal. Perhaps decreasing both sugar and yeast in a balanced way, as in our study and that of Zwaan et al., (1991) could induce other types of long term metabolic changes resulting in a long-lived phenotype. Indeed, body composition (most notably, relative fat content) of the adults was significantly affected by the larval developmental environment in the Zwaan et al, (1991) study.

Effects of larval nutrition and reproductive environment on mated longevity and reproduction

In contrast to virgin flies, the lifespan of mated flies was not affected by larval food, regardless of the adult reproductive environment. Interestingly, this closely parallels the response to selection for lifespan observed by Zwaan et al., (1995) wherein increases in lifespan in response to selection were observed in virgin but not mated flies. One possible explanation is that the lifespan shortening effects of reproduction make lifespan differences more difficult to detect.

In mated flies, the adult reproductive environment acted as the main determinant of mated lifespan and fecundity. This is not a novel finding, in fact, an increase in fecundity with added yeast and added males coupled with a concomitant decrease in longevity is well documented (Bass et al. 2007; Kaufman 1942; Partridge et al. 1987). However, these adult reproductive environments were included in our experiment in order to determine whether or not the effect of developmental environment depended on the adult reproductive environment, and in this sense they proved very instructive. Specifically, we had hypothesized that the negative effects of poor or rich developmental food would be more pronounced in the adult environments in which reproduction was most favoured (added yeast and males). This proved to be incorrect as neither poor nor rich raised flies suffered significantly decreased longevities or fecundities relative to the control in either of these situations (Fig. 4).

Across insects, adult size is quite strongly correlated with fecundity (Honek 1993). In a meta-analysis of 68 insect species, Honek (1993) found that for every one percent increase in body mass, median fecundity increased by 0.95%. In our experiment flies raised on poor food were 9.3 and 10.8% smaller than rich and control raised flies respectively (Fig. 2c-d). However, at no point did they display decreased fecundity. Rather, when singly mated without yeast, poor raised females had higher reproduction early in life, and when continuously mated with yeast poor raised females had increased reproduction in the middle of the reproductive span (Table 3). No such differences existed in the SMY condition, likely because the high rates of reproduction afforded by the added yeast resulted in consistent sperm depletion across treatments.

The mechanisms responsible for the increased virgin lifespan and age-specific fecundity of poor raised flies remain speculative. One potential mechanism is by "viability" selection, as flies raised on poor and rich food have significantly lower larval survival than controls. However this seems unlikely, as despite both treatments resulting in similar larval survival, only the poor raised flies have increased lifespan. Two more likely alternative mechanisms are stress-response hormesis or the induction of a thrifty phenotype.

Stress-response hormesis refers to the phenomenon whereby exposure to a mild stressor increases future resistance to stress (Gems & Partridge 2008; Shore & Ruvkun 2013), usually via induction of chaperone proteins such as those involved in heat shock. In fact, in C. elegans brief thermal stress increases lifespan, and the increase is greater the earlier the stress is applied (Olsen et al. 2006). In our experiment it is possible that decreased nutrition during early development acts as a hormetic, increasing the robustness of the organism. However, this does not appear to hold true for flies raised on rich food; while

these larvae do show some indicators of stress during development, they do not exhibit increased lifespan or reproduction.

The thrifty phenotype hypothesis (Hales & Barker 1992) proposed that nutritionally poor developmental conditions induce a metabolically thrifty metabolism to survive development, but that this metabolic phenotype can be detrimental later in life. In our case it is possible that the poor developmental nutrition does indeed impose a change in metabolism, potentially to a more energetically efficient "thrifty" metabolism while the rich larval food results in the opposite. There is, however, one main difference to the thrifty phenotype hypothesis as proposed by Hales and Barker for humans – humans experiencing poor developmental nutrition show negative consequences of adequate nutrition *post-utero* including an increased risk of developing the metabolic syndrome (Danielsen et al. 2013; Gluckman et al. 2007; Painter et al. 2005). In our flies this does not appear to be the case. This could be due to differences in the way metabolism influences disease risk in flies as compared to humans, or possibly that the adverse effects in adult flies are induced only in the case of a larval diet dominated by sugars.

In keeping with these hypotheses, one could also consider more proximate explanations of higher reproduction in young adults raised on poor food.
A recent study by Aguila et al., (2013) reports that programmed cell death of larval fat cells in the adult is important for female reproduction. The authors report that in two-day-old adults more than half of the nutrients acquired by the ovaries are dependent on the death of fat cells, and that if programmed cell death is inhibited, ovarian development is delayed. One could imagine that normal levels of programmed cell death in larvae reared on poor food, and therefore with less larval fat, would result in a higher relative efficiency of programmed cell death in this tissue, and consequently facilitate ovarian nutrient acquisition and faster ovarian development. Alternatively, development on poor food could have resulted in adults able to mobilize larval fat to the ovaries more efficiently.

Relevance for life history theory: resource acquisition and allocation

Our results indicate that the optimal nutritional conditions for fruit flies differ across the lifespan. In particular, less nutrient-rich larval diets may be beneficial for adult fitness, at least for females. This may reflect the evolutionary history of this insect in nature, where larval conditions may be substantially poorer on average than the adult ones.

Inspired by observations such as those of Hales and Barker (2001), several adaptive explanations have been put forward. They include a role of the developmental environment as a predictor of the (nutritional) status of the

impending adult environment. Natural selection could have favoured genotypes that would adjust their physiological, metabolic, and/or life history phenotypes to ensure a good match with the early and/or late life adult environment (for instance Predictive Adaptive Responses; Bateson et al. 2004; Gluckman et al. 2007; see also Van den Heuvel et al. 2013).

It is tempting to interpret our results in the light of such hypotheses. Poor-raised flies have increased early life reproduction when singly mated, which could be an immediate response to an anticipated poor adult environment. As indicated earlier, this effect could have been masked in the added-yeast treatment because of additional resource availability and a rapid depletion of sperm in females. Similarly, the increased reproductive output in the CMY-treated flies during mid-life may be an indication of increased willingness of poor-raised females to re-mate, and/or an increased allocation during development to reproduction.

The reported effects argue that the role of the larval and adult environment in resource acquisition and allocation should be explicitly incorporated in theoretical models and experimental studies of life history evolution. Further work to determine the mechanism by which the flies raised on poor food extend their virgin lifespan and increase fecundity under certain adult conditions can help to understand this response. This could include studies of gene expression, metabolic rate and stress resistance across the lifespan to determine whether these are also lastingly affected by developmental environment. In addition, the creation of artificial selection or experimental evolution lines adapted to different larval nutritional environments may help to clarify to what extent the plastic effects of developmental food are adaptive (May et al., unpublished data).

Conclusions

This study shows that while larval over-feeding in Drosophila appears to have minimal effects on lifespan and fecundity, larval under-feeding can dramatically affect life history traits across a developmental boundary. In addition, this effect depends on the reproductive environment in which the traits are expressed. In contrast to expectation, larval underfeeding extends adult virgin longevity, does not affect mated-longevity and increases fecundity at certain ages. We propose that this could occur in two separate, though not mutually exclusive ways – either by the induction of stress response hormesis, producing hardier flies, or via the induction of an altered metabolism which gives the flies a general advantage as adults in these environments. Our results urge for a more explicit incorporation of the developmental environment in life history theory. Further experiments are suggested to determine the metabolic rate and stress resistance of the flies raised on poor food, as well as to determine potential

differences in gene expression between flies raised on poor or rich food. A more clear understanding of the lifespan advantage gained by development on poor food in the exceedingly tractable model organism Drosophila may be instrumental in determining new areas to explore in the human-oriented field of developmental nutrition.

ACKNOWLEDGEMENTS

We thank Kees Koops for assistance with laboratory work. This work was supported by the European Union's FP6 Programme (Network of Excellence LifeSpan FP6/036894) and the EU's FP7 Programme (IDEAL FP7/2007-2011/259679).

REFERENCES

Adler MI, Cassidy EJ, Fricke C, Bonduriansky R (2013) The lifespan-reproduction trade-off under dietary restriction is sex-specific and context-dependent. *Experimental Gerontology* **48**, 539-548.

Aguila JR, Hoshizaki DK, Gibbs AG (2013) Contribution of larval nutrition to adult reproduction in *Drosophila melanogaster*. *The Journal of Experimental Biology* **216**, 399-406.

Aihie Sayer A, Dunn R, Langley-Evans S, Cooper C (2001) Prenatal exposure to a maternal low protein diet shortens life span in rats. *Gerontology* **47**, 9-14.

Allen RM, Marshall DJ (2013) Phenotypic links among life-history stages are complex and context-dependent in a marine invertebrate: interactions among offspring size, larval nutrition and postmetamorphic density. *Functional Ecology* **27**, 1358-1366.

Austad SN (1989) Life extension by dietary restriction in the bowl and doily spider, *Frontinella pyramitela*. *Experimental Gerontology* **24**, 83-92.

Barker D, Thornburg K (2013) The obstetric origins of health for a lifetime. *Clinical obstetrics and gynecology* **56**, 511-519.

Barker D, Winter P, Osmond C, Margetts B, Simmonds S (1989) Weight in infancy and death from ischaemic heart disease. *Lancet* **2**, 577-580.

Barrett ELB, Hunt J, Moore AJ, Moore PJ (2009) Separate and combined effects of nutrition during juvenile and sexual development on female life-history trajectories: The thrifty phenotype in a cockroach. *Proceedings of the Royal Society B: Biological Sciences* **276**, 3257-3264.

Bass TM, Grandison RC, Wong R, et al. (2007) Optimization of dietary restriction protocols in *Drosophila*. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences **62**, 1071-1081.

Bateson P, Barker D, Clutton-Brock T, *et al.* (2004) Developmental plasticity and human health. *Nature* **430**, 419-421.

Bauerfeind S, Perlick J, Fischer K (2009) Disentangling environmental effects on adult life span in a butterfly across the metamorphic boundary. *Experimental Gerontology* **44**, 805-811.

Blanckenhorn WU (2006) Divergent juvenile growth and development mediated by food limitation and foraging in the water strider *Aquarius remigis* (Heteroptera: Gerridae). *Journal of Zoology* **268**, 17-23.

Bochdanovits Z, de Jong G (2003) Temperature dependent larval resource allocation shaping adult body size in *Drosophila melanogaster*. *Journal of Evolutionary Biology* **16**, 1159-1167.

Boggs CL, Freeman KD (2005) Larval food limitation in butterflies: Effects on adult resource allocation and fitness. *Oecologia* **144**, 353-361.

Brakefield PM, Gems D, Cowen T, *et al.* (2005) What are the effects of maternal and pre-adult environments on ageing in humans, and are there lessons from animal models? *Mechanisms of Ageing and Development* **126**, 431-438.

Chippindale AK, Leroi AM, Kim SB, Rose MR (1993) Phenotypic plasticity and selection in *Drosophila* life-history evolution. I. Nutrition and the cost of reproduction. *Journal of Evolutionary Biology* **6**, 171-193.

Cleal JK, Poore KR, Boullin JP, *et al.* (2007) Mismatched pre- and postnatal nutrition leads to cardiovascular dysfunction and altered renal function in adulthood. *Proceedings of the National Academy of Sciences* **104**, 9529-9533.

Colasurdo N, Gélinas Y, Despland E (2009) Larval nutrition affects life history traits in a capital breeding moth. *Journal of Experimental Biology* **212**, 1794-1800.

Danielsen ET, Moeller ME, Rewitz KF, Ann ER, Michael BOC (2013) Chapter two - Nutrient signaling and developmental timing of maturation. In: *Current Topics in Developmental Biology*, pp. 37-67. Academic Press.

Dmitriew CM, Rowe L (2011) The effects of larval nutrition on reproductive performance in a food-limited adult environment. *PloS one* **6**.

Flatt T (2011) Survival costs of reproduction in *Drosophila*. *Experimental Gerontology* **46**, 369-375.

Fontana L, Partridge L, Longo VD (2010) Extending Healthy Life Span—From Yeast to Humans. *Science* **328**, 321-326.

Ford SP, Long NM (2011) Evidence for similar changes in offspring phenotype following either maternal undernutrition or overnutrition: potential impact on fetal epigenetic mechanisms. *Reproduction, Fertility and Development* **24**, 105-111.

Gems D, Partridge L (2008) Stress-response hormesis and aging:"that which does not kill us makes us stronger". *Cell Metabolism* **7**, 200-203.

George LA, Zhang L, Tuersunjiang N, et al. (2012) Early maternal undernutrition programs increased feed intake, altered glucose metabolism and insulin secretion, and liver function in aged female offspring.

Giannakou ME, Partridge L (2007) Role of insulin-like signalling in *Drosophila* lifespan. *Trends in Biochemical Sciences* **32**, 180-188.

Gluckman P, Hanson M, Cooper C, Thornburg K (2008) Effect of in utero and early-life conditions on adult health and disease. *The New England journal of medicine* **359**, 61-73.

Gluckman PD, Hanson MA (2004) The

REFERENCES

developmental origins of the metabolic syndrome. *Trends in Endocrinology and Metabolism* **15**, 183-187.

Gluckman PD, Hanson MA, Beedle AS (2007) Early life events and their consequences for later disease: A life history and evolutionary perspective. *American Journal of Human Biology* **19**, 1-19.

Good TP, Tatar M (2001) Age-specific mortality and reproduction respond to adult dietary restriction in *Drosophila melanogaster*. *Journal of Insect Physiology* **47**, 1467-1473.

Grandison RC, Piper MDW, Partridge L (2009) Amino-acid imbalance explains extension of lifespan by dietary restriction in *Drosophila*. *Nature* **462**, 1061-1064.

Hales C, Barker D (1992) Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. *Diabetologia* **35**, 595-601.

Hales CN, Barker DJP (2001) The thrifty phenotype hypothesis. *British Medical Bulletin* **60**, 5-20.

Harshman LG, Zera AJ (2007) The cost of reproduction: the devil in the details. *Trends in Ecology and Evolution* **22**, 80-86.

Honek A (1993) Intraspecific variation in body size and fecundity in insects: a general relationship. *Oikos* **66**, 483-492.

Hwangbo D, Gershman B, Tu M, Palmer M, Tatar M (2004) *Drosophila* dFOXO controls lifespan and regulates insulin signalling in brain and fat body. *Nature* **429**, 562 - 566.

Joy TK, Arik AJ, Corby-Harris V, Johnson AA, Riehle MA (2010) The impact of larval and adult dietary restriction on lifespan, reproduction and growth in the mosquito *Aedes aegypti*. *Experimental Gerontology* **45**, 685-690.

Kaspi R, Mossinson S, Drezner T, Kamensky B, Yuval B (2002) Effects of larval diet on development rates and reproductive maturation of male and female Mediterranean fruit flies. *Physiological Entomology* **27**, 29-38.

Kaufman BP, and Demerec, M. (1942) Utilization of sperm by female *Drosophila melanogaster*. *The American naturalist* **76**, 445-469.

Kenyon C (2010) A pathway that links reproductive status to lifespan in *Caenorhabditis elegans*. *Ann. NY Acad. Sci.*, 156-162.

Kirkwood TBL, Rose MR (1991) Evolution of senescence: Late survival sacrificed for reproduction. *Philosophical Transactions of the Royal Society of London B: Biological Sciences* **332**, 15-24.

Kolss M, Vijendravarma RK, Schwaller G, Kawecki TJ (2009) Life-history consequences of adaptation to larval nutritional stress in drosophila. *Evolution* **63**, 2389-2401.

Lee KP, Simpson SJ, Clissold FJ, *et al.* (2008) Lifespan and reproduction in *Drosophila*: new insights from nutritional geometry. *Proceedings of the National Academy of Sciences* **105**, 2498-2503.

Leon D, Lithell H, Vâgerö D, *et al.* (1998) Reduced fetal growth rate and increased risk of death from ischaemic heart disease: cohort study of 15 000 Swedish men and women born 1915-29. *BMJ (Clinical research ed.)* **317**, 241-245.

López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G (2013) The hallmarks of aging. *Cell* **153**, 1194-1217.

Mair W, Piper MDW, Partridge L (2005) Calories do not explain extension of life span by dietary restriction in *Drosophila*. *PLoS Biol* **3**, e223.

Min KJ, Flatt T, Kulaots I, Tatar M (2007) Counting calories in *Drosophila* diet restriction. *Experimental Gerontology* **42**, 247-251.

Olsen A, Vantipalli M, Lithgow G (2006) Lifespan extension of *Caenorhabditis elegans* following repeated mild hormetic heat treatments. *Biogerontology* **7**, 221-230.

Ozanne SE, Hales CN (2004) Lifespan: Catch-up growth and obesity in male mice. *Nature* **427**, 411-412.

Painter RC, Roseboom TJ, Bleker OP (2005) Prenatal exposure to the Dutch famine and disease in later life: An overview. *Reproductive Toxicology* **20**, 345-352.

Partridge L, Green A, Fowler K (1987) Effects of egg-production and of exposure to males on female survival in *Drosophila melanogaster*. *Journal of Insect Physiology* **33**, 745-749.

Pasco M, Léopold P (2012) High sugar-induced insulin resistance in *Drosophila* relies on the lipocalin Neural Lazarillo. *PloS one* **7**.

Prasad N, Shakarad M, Rajamani M, Joshi A (2003) Interaction between the effects of maternal and larval levels of nutrition on pre-adult survival in *Drosophila melanogaster*. *Evolutionary Ecology Research* **5**, 903-911.

Shore D, Ruvkun G (2013) A cytoprotective perspective on longevity regulation. *Trends in cell biology* **23**, 409-420.

Tu MP, Tatar M (2003) Juvenile diet restriction and the aging and reproduction of adult *Drosophila melanogaster*. *Aging Cell* **2**, 327-333.

Van den Heuvel J, Saastamoinen M, Brakefield PM, *et al.* (2013) The predictive adaptive response: modeling the life-history evolution of the butterfly*Bicyclus anynana*in seasonal environments. *The American naturalist* **181**, E28-E42.

Vijendravarma RK, Narasimha S, Kawecki TJ (2010) Effects of parental larval diet on egg size and offspring

REFERENCES

traits in *Drosophila*. *Biology letters* **6**, 238-241.

Walker G, Houthoofd K, Vanfleteren JR, Gems D (2005) Dietary restriction in *C. elegans*: From rate-of-living effects to nutrient sensing pathways. *Mechanisms of Ageing and Development* **126**, 929-937.

Weindruch R, Walford RL (1982) Dietary restriction in mice beginning at 1 year of age: effect on life-span and spontaneous cancer incidence. *Science* **215**, 1415-1418.

Zajitschek F, Hunt J, Jennions MD, Hall MD, Brooks RC (2009) Effects of juvenile and adult diet on ageing and reproductive effort of male and female black field crickets, T*eleogryllus commodus*. *Functional Ecology* **23**, 602-611.

Zajitschek F, Zajitschek SK, Friberg U, Maklakov A (2013) Interactive effects of sex, social environment, dietary restriction, and methionine on survival and reproduction in fruit flies. *Age* **35**, 1193-1204.

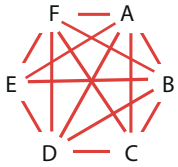
Zwaan B, Bijlsma R, Hoekstra RF (1995) Direct selection on life span in *Drosophila melanogaster*. *Evolution* **49**, 649-659.

Zwaan BJ, Bijlsma R, Hoekstra RF (1991) On the developmental theory of ageing. I. Starvation resistance and longevity in *Drosophila melanogaster* in relation to pre-adult breeding conditions. *Heredity* **66**, 29-39.

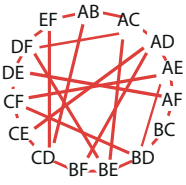
APPENDICES

Appendix 1. Derivation of the stock population.
The stock population was derived from 6 wild-caught European populations. The crosses were designed to create a stock population with a high level of genetic polymorphism and to prevent uneven contribution of genetic variation from the initial populations.

Initial component populations:
A: Vienna (Austria; N 48˚ 14.733'; E 016˚ 16.024')
B: Palic (northern part of Serbia; N 46˚ 05.910'; E 019˚ 45.649')
C: Predijane (southern part of Serbia; N 42˚ 52.609'; E 022˚ 05.242')
D: northern Macedonia (N 41˚ 24.290'; E 022˚ 17.951')
E: Dorjan (southern Macedonia; N 41˚ 20.480'; E022˚ 26.575')
F: Pournis (Greece; N 39˚ 08.443'; E 023˚ 17.181'
Crossing Scheme



1 First crossing round: "one to one"
- Populations were crossed with each other inpairs (15 combinations)
- In each cross, 100 females of one component population and 100 males of other component populations were mixed
- Crosses were performed reciprocally (30 crosses in total
- Crosses deliver "2-genotype populations": AB, AC, AD, etc.



2 Second crossing round: "each to a different"
- 2-genotype populations were crossed to other
- 2-genotype populations derived from different component populations (15 combinations)
- perform reciprocally, 30 crosses
- Crosses deliver "4-genotype populations": ABCD, ABEF, ADBF, ADCE etc.

3 Third crossing round: "each to a different II"
- 4-genotype populations were crossed to other
- 4-genotype populations derived from different component populations
- performed reciprocally, 30 crosses
- Crosses deliver 30 "6-genotype populations": ABCDEF

4 Fourth crossing round: "Random mating"
- 30 "6-genotype populations" ABCDEF were mixed in equal proportions.
- The mixed base population was subsequently divided into 4 replicate mixed 6-genotype populations
- The populations were allowed to mate randomly for 3 generations to prevent linkage disequilibrium

Appendix 2. Diet recipes
Diet composition per litre of water

Diet composition	Low (0.25x)	Control (1x)	High (2.5x)
Yeast*	17.5g	70g	175g
Sugar †	25g	100g	250g
Agar	20g	20g	20g
Nipagin solution	15mL	15mL	15mL
Propionic acid	3mL	3mL	3mL

*Fermipan Red Label instant yeast
†Suiker Unie Granulated Sugar Extra Fine

The transcriptome in transition: Global gene expression profiles of young adult fruit flies depend more strongly on developmental than adult diet

Christina M. May[1], Agnieszka Doroszuk[1,2] and Bas J. Zwaan[1]

1. Laboratory of Genetics, Wageningen University, Wageningen
2. Molecular Epidemiology, Leiden University Medical Center, Leiden
3. Delft Bioinformatics Lab, Delft University of Technology, Delft

ABSTRACT

It is often proposed that the effects of developmental diet on adult phenotypes are mediated by long-term changes in gene expression. However, no studies to date have addressed the overall magnitude of the effect of developmental diet on the entire adult transcriptome, and how it compares to the effect of adult diet. Here, we use a full-factorial design to address how three different larval and adult diets interact to affect gene expression in one day-old adult fruit flies (*Drosophila melanogaster*) of both sexes. We find that in both sexes the biggest contributor to transcriptional variation is larval, and not adult diet. The magnitude of this effect is especially large in females but relatively small in males. Furthermore, the effect of increasing caloric content of the larval diet on gene expression was not linear, suggesting that calories *per se* do not drive global patterns of gene expression variation early in life. To address the biological significance of the observed patterns of variation, we further characterised gene expression by applying Weighted Gene Correlation Networks Analysis (WGCNA) to identify modules of co-expressed genes whose expression was affected by larval or adult dietary conditions. In females, larval diet affected gene expression by modulating the expression of two large strongly negatively correlated modules which appeared to reflect relative investment into reproduction versus non-reproduction related processes. In males, modules affected by larval and/or adult diet appeared to relate primarily to nutrient sensing and metabolic functions, and contained probes highly expressed in the gut and fat body. The gut and fat body are among the most important nutrient sensing tissues, and are also the only tissues for which it is well described that they are not completely histolysed during pupation, suggesting that effects in male flies may be mediated by the carry-over of these tissues into young adulthood. Our results show that developmental diet can have profound effects on gene expression in early life and suggest further work is necessary to determine the extent to which such effects persist across the lifespan, and how they relate to phenotypes. This would be important for understanding the commonly observed link between developmental conditions and late-life patterns of ageing and health.

INTRODUCTION

The environment experienced during development can have important consequences for the adult phenotype of an individual (Schlichting & Pigliucci 1998). Frequently, individuals must develop under conditions of nutritional scarcity, or, less commonly, nutritional excess. Development under such conditions can affect the resulting phenotype via a variety of mechanisms (reviewed in Lindström 1999; Monaghan 2008) including directly constraining the overall size or quality of the resulting adult (so-called "silver-spoon" effects; Grafen 1988), changing the relative allocation to different tissues or processes (e.g. Emlen 1997; Lanet & Maurange 2014), and/or by acting as a cue predicting the likely quality of the adult environment, allowing individuals to adjust their phenotypes for better performance under these conditions (Bateson et al. 2014; so-called Predictive Adaptive Responses: Gluckman et al. 2007). In general, each one of these mechanisms is considered independently, but it is more likely that the adult phenotype will reflect the combined action of some or all of these effects.

Importantly, regardless of the mechanisms, such developmental influences on adult phenotypes are expected to result in gene-expression changes at the level of the whole organism. In fact, in the case of predictive adaptive responses (PARs) it is hypothesised that gene expression changes per se may be a principal cause of long-term phenotypic effects (Burdge 2007; Burdge & Lillycrop 2010). This hypothesis comes from the observation that low birth weight in humans (a proxy for poor nutrition in utero), is associated with greater risk of metabolic disease in late life (Barker et al. 1993; Barker et al. 1989), an effect which appears to be exacerbated by a plentiful adult diet (Gluckman & Hanson 2004; Ravelli et al. 1998). It is proposed that through epigenetic changes, individuals modulate the long-term expression of genes, particularly metabolic genes, in order to be better adapted to the predicted adult environment (Burdge 2007; Burdge & Lillycrop 2010; Gluckman & Hanson 2004). However, if the developmental cue turns out to be wrong, as is proposed to be the case in humans who develop in poor intrauterine environments and subsequently experience conditions of plenty as adults, the individual's gene expression profile will be mismatched with its environment, potentially leading to metabolic disease.

Despite the widespread incidence of variation in the quality of the developmental environment, and the fact that phenotypic effects will likely be reflected in gene expression, relatively few studies have addressed the extent to which developmental diet influences gene expression in adulthood. Studies in rats have shown changes in the expression of genes related to metabolism in adult offspring of protein-restricted mothers (Bertram et al. 2001; Lillycrop et al. 2005; Maloney et al. 2003), while a study in fruit flies has shown that high

protein larval diets increase the expression of some immune genes in young adults (Fellous & Lazzaro 2010). These candidate gene approaches suggest that developmental diet does play a role in adult gene expression, however to our knowledge, no study has addressed the global effect on the transcriptome, nor if and how it depends on the adult environment. Understanding the global variation in gene expression induced by variation in developmental diet can provide insight into which of the above mentioned mechanisms plays a role, and/or which tissues and biological functions are the most affected.

Here we use the fruit fly, Drosophila melanogaster, to determine to what extent developmental diet affects whole-body, whole-genome, gene expression in one-day old adult male and female flies, and how this interacts with different adult dietary conditions. We choose one-day old flies in order to establish a baseline effect of developmental diet on adult gene-expression. Because this study is the first to address this question in Drosophila, there are no concrete a priori hypotheses concerning which tissues or mechanisms may be involved, thus we employ a whole body, systemic approach to provide a first indication of the magnitude of the effect and the tissues and mechanisms that may play a role. Such an approach has previously been productively applied to the study of transcriptional responses to dietary restriction (DR) in adults, identifying a global down-regulation of cell-cycle, metabolic and reproduction related genes in response to DR and implicating these processes as potentially important mechanisms mediating the effect of DR (Pletcher et al. 2002).

To address the effects of developmental and adult diet on gene expression we first assess broad-scale patterns of expression variation as obtained from Principle Component Analysis (PCA). Subsequently, we use Weighted Gene Co-expression Network Analysis (WGCNA; Langfelder & Horvath 2008) to identify modules of co-expressed genes whose expression is affected by larval and/or adult dietary conditions. There are several reasons to choose WGCNA over traditional differential expression approaches: first, it is unbiased, as modules are detected on the basis of expression only, with no information on treatments; second, it alleviates the problem of multiple testing associated with differential expression analysis by reducing the number of comparisons from tens of thousands of genes down to the number of modules, and, third, networks (and by extension modules) likely more accurately reflect the biological relationships between genes as genes do not act in isolation, but rather interact in hierarchically structured regulatory networks (Zhang & Horvath 2005). As such, co-expression often reflects co-regulation (Allocco et al. 2004), or tissue-specific function (Boutanaev et al. 2002), thus modules can provide biologically relevant insight into the tissues and processes underlying gene expression differences. To date, WGCNA has been productively applied to identify co-

expression modules that are highly correlated to biological traits and functional pathways in multiple species (Hilliard et al. 2012; Xue et al. 2013), including identifying tissue-specific expression profiles that would be difficult if not impossible to identify by more traditional differential expression analysis (e.g. Oldham et al. 2006). After identifying modules of co-expressed genes affected by dietary conditions, we use several external data sets to annotate the modules and address whether there is evidence for specific functions (Gene Ontology), tissue-specific roles (FlyAtlas and FlyGut databases), or co-regulation of the modules by specific transcription factors (DroID Database). The combined results are discussed in the context of theories and hypotheses linking early life conditions to late life health and the possible role of epigenetic mechanisms there-in.

MATERIALS AND METHODS:

Fly Stocks and Experimental Design

We used the laboratory stock population described in May et al., (2015). This population has been maintained in the laboratory for more than 60 generations under standard laboratory conditions (25°C, 65% humidity, 12:12h light : dark cycle, 14 day generation time, and a standard control diet (C) of 70 grams yeast, 100 grams sugar, 20 grams agar, 15 mL nipagin and 3 mL propionic acid per litre of water).

To address how developmental and adult diet interact to affect the transcriptome of one day old-flies we raised flies on three different diets as larvae and switched them across these same three diets as adults. These diets were the standard laboratory diet, hereafter referred to as control (C), a poor diet (P), and a rich diet (R). The poor and rich diets contained 25% and 250% as much sugar and yeast as the control diet respectively. For the experiment, we raised flies on these three diets as larvae at a density of 100 eggs per vial (6mL medium) and immediately after eclosion randomly distributed the resulting adults across these three diets again in a full factorial design. This resulted in nine combinations of larval and adult diet, three experiencing the same diet during both larval development and adulthood and six experiencing a novel adult diet in the first twenty-four hours of adult life (same diet: PP,CC,CR; diet transition: PC,PC,CP,CR,RP,RC). To obtain virgin flies we sexed the adults immediately after the cuticle had hardened (~ three hours post eclosion) and maintained them at a density of 10 adults per vial. Twenty-four hours after eclosion, we flash froze the flies in groups of five for gene expression analysis (25 flies per combination of larval food, adult food and sex) and used these samples to obtain systemic, whole-body and whole-genome gene expression.

RNA extraction, hybridisation and data pre-processing

We extracted RNA from four replicates per combination of sex, larval diet, and adult diet (4 replicates x 2 sexes x 3 larval diets x 3 adult diets = 72 arrays), using the Machery Nagel Nucleospin II kit (Machery and Nagel). For each sample we homogenised five whole flies together in order to minimise the effect of random variation between individuals. Biotin labelling, cRNA synthesis, hybridisation to Affymetrix Drosophila 2.0 GeneChips and array readouts were performed by ServiceXS (www.servicexs.com).

Prior to analysis we used several quality control measures to exclude outlier arrays or arrays of insufficient quality from the dataset. Based on these results we excluded ten female samples and four male samples from further analysis, randomly distributed over the nine nutritional groups. The large number of female arrays excluded is due to our very stringent quality control measures. Subsequently we performed background adjustment, quantile normalisation and summarisation using the robust multi-array average (RMA) algorithm (Irizarry et al. 2003). When normalising males and females together, 96% of the variation in expression was due to sex, so we chose to normalise male and female samples separately to emphasise the effects of larval and adult diet rather than ubiquitous and well-documented sex-specific differences (e.g. Ayroles et al. 2009). We performed all subsequent analysis steps separately for each sex. The data quality check and all other analyses were performed using R (version 3.0.0) and Bioconductor (Gentleman et al. 2004). To gain an understanding of the broad patterns of variation in the data we applied principal components analysis to the normalised expression data (PCA; Pearson 1901).

Weighted Gene Correlation Network Analysis

After normalising the data we applied WGCNA to the expression data for each sex, using the default settings for detection of signed modules (Langfelder & Horvath 2008). Implementation of WGCNA is freely available in the WGCNA R package. In brief, WGCNA follows the following steps. First, expression correlations are determined for all pairs of genes and weighted by the connection strength. These correlations are then clustered using hierarchical clustering, and modules are defined as branches of the clustering tree. The expression profile of each module can be summarised by its eigengene (E) which is defined as the first principal component of the expression matrix. The eigengene can be thought of as a weighted average expression profile and thus for a particular sample, the value of its eigengene is representative of the overall expression of the module (Langfelder & Horvath 2008). For each module, eigengenes can be compared across samples to detect significant correlations between treatments and module eigengenes (i.e. expression). By convention, modules are named by colours, with unassigned genes grouped together in the “grey” module.

Annotating modules with external information

After performing module discovery using WGCNA we applied several additional analysis steps to characterise the modules. First, for each module we fit an ANOVA model that partitioned the variance in module eigengene expression explained by larval diet (L), adult diet (A), and their interaction (L by A). We set a false discovery rate (FDR) threshold of 0.001 (FDR; Benjamini & Hochberg 1995) and focused the remaining analysis on the modules that showed a significant effect of dietary treatment on their eigengene value (as explained above, this is roughly equivalent to expression).

We next submitted the probe list associated with each module to DAVID (Database for Annotation, Visualization and Integrated Discovery; Huang et al. 2008) for gene-ontology enrichment analysis focusing on GO FAT terms. GO FAT terms exclude broader GO terms and eliminate term redundancy. To facilitate interpretation, we submitted lists of more than 20 terms to the REVIGO online tool which uses semantic similarity to reduce large lists of terms to a representative subset of terms (REduce and VIsualize Gene Ontology: Supek et al. 2011).

To address whether modules showed evidence of tissue-specific expression profiles we used two external data sets: FlyAtlas (Chintapalli et al. 2007) and FlyGut (Buchon et al. 2013). FlyAtlas contains tissue-specific gene expression data for both larval and adult tissues of the fruit fly, while FlyGut contains region-specific expression patterns in the gut. For each of our modules, we calculated the median expression across all of its probes in each tissue available in the FlyAtlas database and in each gut region available in the FlyGut database. This provides a simple read-out of the average expression levels of the module in different tissues or gut-regions.

To address whether there was evidence that modules were co-regulated by certain transcription factors (TFs) we used the Drosophila Interactions Database (DroID; Murali et al. 2010), a merged dataset of empirically validated interactions between TFs and genes from the RedFly (Halfon et al. 2008) and modENCODE databases (Roy et al. 2010). We first filtered the data set to exclude transcription factors that are known to interact with less than five genes. We then performed a Fisher's exact test to identify modules enriched for genes known to be regulated by certain transcription factors. We considered a module enriched for probes binding a certain transcription factor at an odds-ratio of greater than 1.5 fold and p-value of <0.05, although at this threshold, not all interactions remained significant after stringent correction for multiple testing.

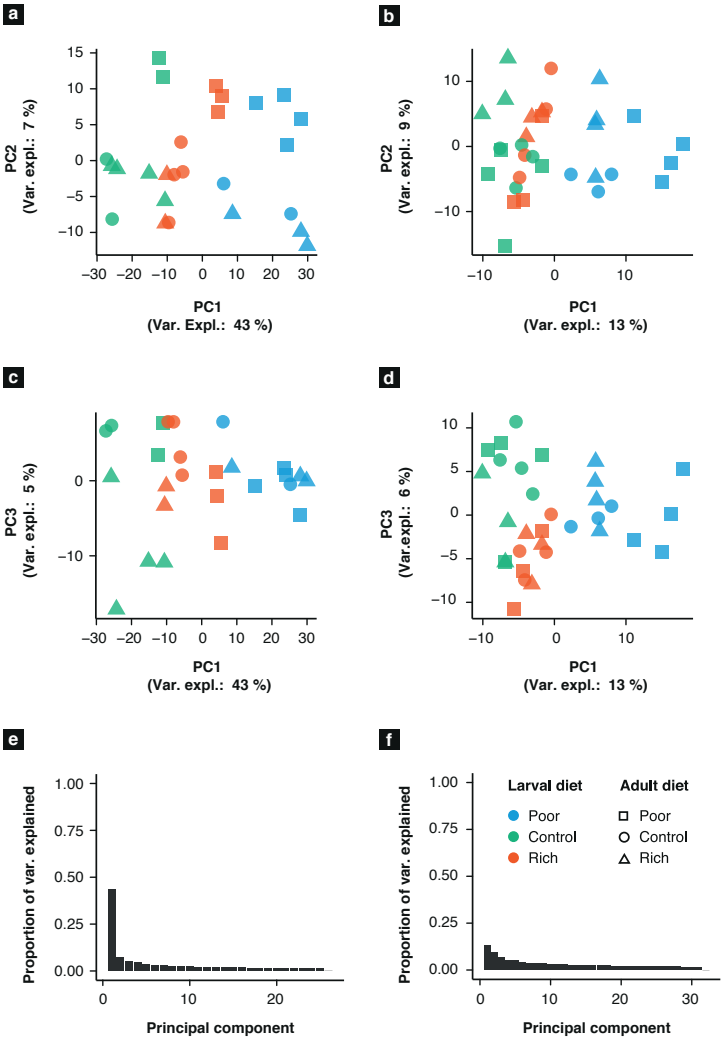


Figure 1: (a and b) Plot of PC1 versus PC2 in females (a) and males (b). In both sexes, PC1 separates samples by their larval diets, and flies raised on the rich larval diet fall in-between those raised on poor and control. (c and d) Plot of PC1 versus PC3 in females (c) and males (d). In females (c), PC3 separates three out of four CR samples from the rest while in males (d), the plot of PC1 versus PC3 more clearly divides samples based on their larval diets. (e and f) Plot of proportion of variation explained across principal components in females (e) and males (f). PC1 explains more than three times as much variation in females (e) than in males (f).

RESULTS

Expression in one-day old flies more tightly associated with larval than adult diet

PC1 clearly groups flies by their larval diets in both sexes (Females: Fig. 1a; Males: Fig. 1b), but explains three time more variation in females than in males (Females: Fig. 1e; Males: Fig. 1f). In both sexes, PC1 separates flies raised on the poor larval diet from those raised on the control larval diet, while flies raised on the rich larval diet fall in between (Fig. 1a,b). The separation between flies raised on the rich and control diet is distinct in females (Fig. 1a), but there is some overlap in males (Fig. 1b). However, when plotting PC1 versus PC3 for males, flies raised on the rich larval diet form a very distinct cluster (Fig. 1d).

In females, and to a lesser extent, males, PC2 segregates samples by adult diet, and, in contrast to PC1, explains a similar amount of variation in both sexes (females: 7%, males: 9%). In females, PC2 separates flies transferred to the poor adult diet upon eclosion (squares) from the rest (Fig. 1a), suggesting a rapid transcriptional response to the poor adult diet across larval diets. In males, there is a trend towards flies transferred to the rich diet as adults (triangles) having high values on PC2 and flies transferred to the poor diet as adults (circles) having low values (Fig. 1b). Notably, the most extreme values for PC1 in males are observed for flies both raised on and subsequently transferred to the poor adult diet (blue squares; Fig. 1b).

WGCNA analysis reveals large sex-specific differences in module size and membership

In females, WGCNA identified two very large co-expression modules consisting of 5509 (blue) and 5695 (turquoise) probes respectively, while 7565 (grey) genes were unassigned (Fig. 2a). The eigengenes of these two modules were nearly perfectly negatively correlated ($r^2=-0.96$, $n=26$, $p<0.0001$; Fig. 2b), thus increased expression of one module implies decreased expression of the other. In males, we identified 22 co-expression modules ranging in size from 41 to 3,805 probes (Fig. 2c). A total of 15,082 probes (80% of total) were assigned to a module while 3,687 background genes were unassigned (grey module). Nearly all modules possessed significant GO annotation and enrichment for binding of specific transcription factors (Appendix 1), as well as distinct tissue-specific expression profiles in the FlyAtlas and FlyGut databases (Fig. 3). Furthermore, modules that have positively correlated eigenvalues tend to possess very similar tissue-specific expression profiles, suggesting that WGCNA identified modules of genes that act in particular tissues. For example, the red, yellow, black and cyan male modules cluster together in the hierarchical clustering dendrogram and have highly positively correlated eigengene values (Fig. 2c,d). They are all nearly exclusively expressed in the adult testes in FlyAtlas (Fig. 3e). Furthermore, the data in FlyGut and FlyAtlas agree with each other: modules

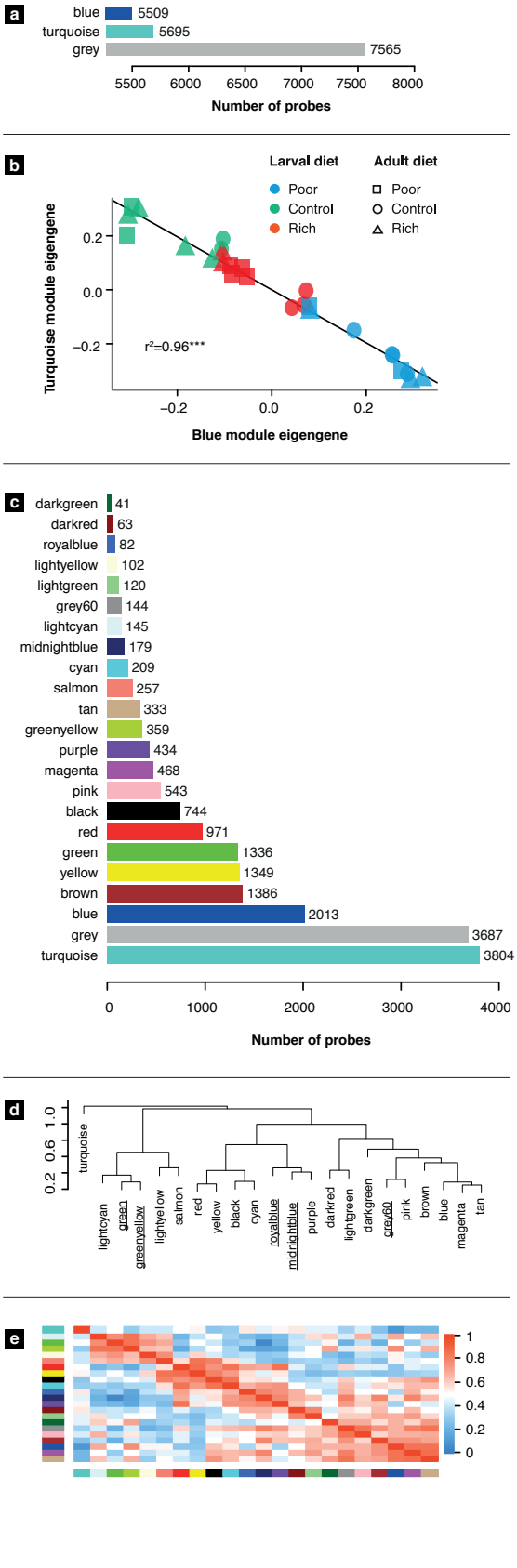
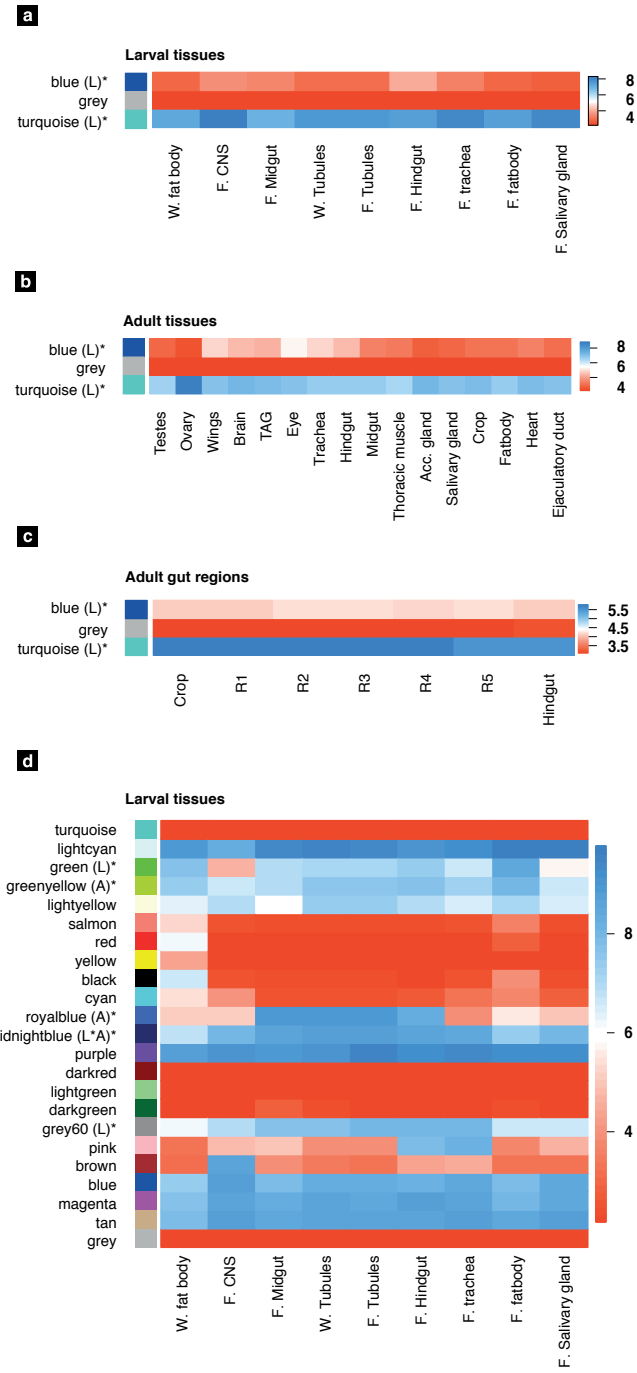


Figure 2: Overview of modules detected by WGCNA (a) Module sizes in females (b) Module eigengenes of the female blue module plotted against eigengenes of the female turquoise module. Eigengenes of the two modules are nearly perfectly negatively correlated (r2=0.96). Colour indicates larval diet and shape indicates adult diet (Legend in figure). (c) Module sizes in males (d) Hierarchical clustering dendrogram of male module eigengenes (labeled by their colors). (e) Heatmap plot of the adjacencies in the eigengene network. Each row and column in the heatmap corresponds to one module eigengene (labeled by color). A value of 1 (red) represents high adjacency (perfect positive correlation), while 0 (blue) represents low adjacency (perfect negative correlation). Modules with high adjacency tend to have similar tissue-specific expression profiles in the FlyAtlas and FlyGut datasets. For both sexes, the “grey” module contains probes that were unassigned to a module.


a
Larval tissues
blue (L)*
grey
turquoise (L)*
8
6
4
W. fat body
F. CNS
F. Midgut
W. Tubules
F. Tubules
F. Hindgut
F. trachea
F. fatbody
F. Salivary gland
b
Adult tissues
blue (L)*
grey
turquoise (L)*
8
6
4
Testes
Ovary
Wings
Brain
TAG
Eye
Trachea
Hindgut
Midgut
Thoracic muscle
Acc. gland
Salivary gland
Crop
Fatbody
Heart
Ejaculatory duct
c
Adult gut regions
blue (L)*
grey
turquoise (L)*
5.5
4.5
3.5
Crop
R1
R2
R3
R4
R5
Hindgut
d
Larval tissues
turquoise
lightcyan
green (L)*
greenyellow (A)*
lightyellow
salmon
red
yellow
black
cyan
royalblue (A)*
midnightblue (L*A)*
purple
darkred
lightgreen
darkgreen
grey60 (L)*
pink
brown
blue
magenta
tan
grey
8
6
4
W. fat body
F. CNS
F. Midgut
W. Tubules
F. Tubules
F. Hindgut
F. trachea
F. fatbody
F. Salivary gland

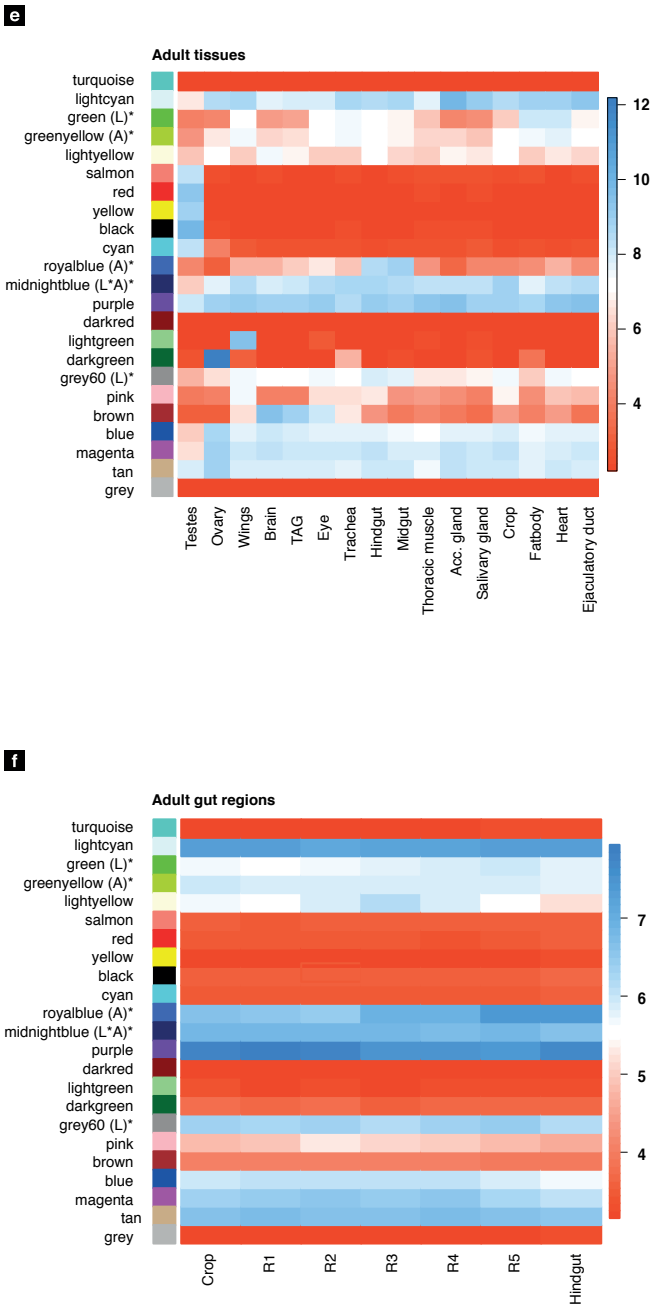


Figure 3: Heatmaps of median expression of female and male modules per tissue in FlyAtlas and FlyGut tissue-specific gene expression databases. (a,b,c) Median expression of female modules per tissue in (a) larval tissues in FlyAtlas, (b) adult tissues in FlyAtlas and (c) across gut regions in FlyGut. (d,e,f) Median expression of male modules per tissue in (d) larval tissues in FlyAtlas, (e) adult tissues in FlyAtlas and (f) across gut regions in FlyGut. Abbreviations: W: wandering, F: feeding, CNS: central nervous system, Acc. gland: accessory gland. For larval tissues "W." refers to tissues collected during the wandering larval stage (late third instar) and "F." refers to tissues collected while third instar larvae were still feeding. For FlyGut data (c, f) R1 through R5 refer to five sequential regions of the gut which are delineated by the combination of (i) an anatomical constriction between one region and the next, (ii) changes in histology and (iii) changes in gene expression (Buchon et al. 2013). Modules are shown in the same order as they appear in the hierarchical clustering dendrogram. Modules whose expression is affected by larval or adult diet are indicated by a "*" and annotated with whether they are affected by larval diet (L), adult diet (A), or their interaction (L*A).

Table 1: q-values for per-module ANOVAs assessing the effect of larval diet (L), adult diet (A) and their interaction (L*A) on module eigengene values. Modules with q-values below the significance cut-off (0.001) are indicated by a " * ".

	q-value		
Module	**Larval diet (L)**	**Adult diet (L)**	**L*A**
Female			
blue*	<0.001	0.024	0.490
turquoise*	<0.001	0.320	0.680
grey	0.690	0.035	0.680
Male			
turquoise	0.720	0.440	0.073
lightcyan	0.440	0.021	0.160
green*	<0.001	0.011	0.110
greenyellow*	0.011	<0.001	0.760
lightyellow	0.043	0.230	0.730
salmon	0.021	0.440	0.600
red	0.440	0.036z	0.150
yellow	0.160	0.210	0.440
black	0.140	0.720	0.210
cyan	0.300	0.290	0.160
royalblue*	0.051	<0.001	0.790
midnightblue*	<0.001	<0.001	<0.001
purple	0.078	0.074	0.440
darkred	0.011	0.360	0.500
lightgreen	0.022	0.410	0.810
darkgreen	0.410	0.033	0.760
grey60*	<0.001	0.003	0.440
pink	0.021	0.240	0.240
brown	0.005	0.730	0.083
blue	0.530	0.079	0.530
magenta	0.120	0.710	0.600
tan	0.130	0.400	0.700
grey	0.720	0.180	0.570

with low expression in gut tissues in FlyAtlas tend to have low expression across gut regions in FlyGut and vice versa (Fig. 3).

We next assessed which modules were affected by larval or adult diet. ANOVA analysis of module eigengenes showed that the expression of both female modules and five out of 22 male modules were affected by dietary conditions (Table 1), thus we focus our remaining analysis on these modules.

Gene expression in young adult females is driven by larval diet

In females, the eigengene value of both modules (i.e. expression) was strongly affected by larval diet (Table 1). In both modules, the poor and control raised flies form the two extremes of expression, while the rich raised flies fall in between, similar to the differences observed for the PCA analysis (Fig. 1). The blue module is up-regulated in poor raised flies (Fig. 4a), and the turquoise module is down-regulated (Fig. 4b), consistent with the tight negative correlation of the module eigengene values (Fig. 2b). Finally, while not reaching significance (Table 1), the poor adult diet affects expression in the same direction as the poor larval diet for both modules, suggesting that whether experienced as a larva or adult, the poor diet has similar effects on gene expression.

Both modules have extensive GO enrichment (Blue module: 373 terms; Turquoise module: 564 terms; Appendix 1) with only seven terms overlapping between the two lists, suggesting they represent distinct processes. Summarisation with REVIGO (Supek et al. 2011) shows the blue module is enriched for a broad spectrum of terms including mesoderm development, detection of external stimuli, leg and limb morphogenesis, energy derivation by oxidation of organic compounds, and generation of precursor metabolites and energy (Fig. 5a), while the turquoise module is annotated with terms related to cell division, cell cycle, oogenesis and reproduction (Fig. 5b).

The tissue-specific expression profiles of the two modules are broadly consistent with their GO annotation and negative eigengene correlation: the turquoise module has relatively high overall expression (Fig. 3a:c), and is particularly highly expressed in the adult ovary (Fig. 3b), consistent with its enrichment for cell cycle and reproduction related GO terms, while the blue module has relatively low median expression overall (Fig. 3a:c) and in the ovary in particular (Fig. 3b), consistent with its lack of reproduction and cell-cycle related GO terms. Assessment of enrichment of TF binding probes found enrichment for 19 transcription factors in the turquoise module, but none in the blue module (Table 2). These 19 TFs show similar tissue-specific transcription patterns to the turquoise module overall (i.e. they are highly expressed in general, and especially in the ovary; Appendix 2a:c) and also share very similar GO annotation with the turquoise module, with 89 of the 92 terms

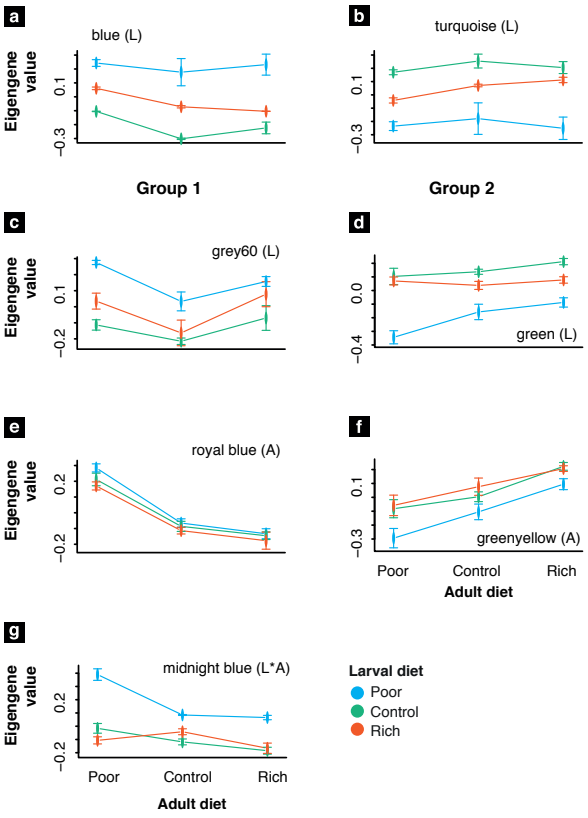


Figure 4: (a, b) Module eigengenes of blue (a) and turquoise (b) female modules. Both modules are significantly affected by larval diet. (c,d) Module eigengenes of grey60 (c) and green (d) male modules. The eigengene values of both modules are significantly affected by larval diet. (e,f) Module eigengenes of royal blue (e) and green yellow (f) male modules. The eigengene values of both modules are significantly affected by adult diet. (g) Module eigengenes of the midnight blue module (g). Expression of the midnight blue module is significantly affected by the interaction between larval and adult diet. Male modules can be divided into two groups based on their expression correlation: Group 1 (c,e,g) and Group 2 (d, f). Within a group eigengene expression is highly correlated, but across groups, eigengene expressions are negatively correlated. All error bars are 95% confidence intervals.

enriched for the TF list also enriched in the turquoise module. Furthermore, REVIGO summary of these 92 terms shows a similar emphasis on reproduction, oogenesis, and early embryonic development (Appendix 2d).

Taken together, these results show that gene expression in one day old female flies is driven by relative expression levels of two very large modules, and that the balance between them is determined by larval diet. Furthermore, annotation of the modules suggests that they represent gene expression related to reproduction and cell-cycle related processes (turquoise) versus non-reproduction related processes (blue; Table 3). The poor larval diet shifts the balance toward higher expression of the blue, non-reproduction related module, while the control larval diet does the opposite. This suggests larval diet may determine the degree to which females have the potential to engage in reproduction related gene expression in early life.

Male modules affected by diet fall into two distinct groups

In males we found significant effects of diet in five out of 22 modules (Table 1). Thus, in contrast to females, a significant proportion of gene expression in young males is independent of dietary conditions. Of the five modules

affected, two were affected by larval diet (grey60, green), two were affected by adult diet (royal blue, green yellow), and one was affected by the interaction between larval and adult diet (midnight blue). The modules could be broadly divided into two groups whose eigengenes were highly positively correlated within groups, but negatively correlated across groups (Fig. 2d). The first group consists of the royal blue, midnight blue and grey60 modules (Group 1), and the second group consists of the green and green yellow modules (Group 2). Within a group, there is a degree of similarity in module expression profiles, though they differ in whether they are more strongly affected by larval or adult diet, while across groups expression profiles are roughly inverse (Fig. 4 c-g).

The modules in Group 1 are most highly expressed in flies either raised on (grey60, midnight blue) or transferred to (royal blue, midnight blue) the poor diet (Fig. 4c,e,g). The midnight blue module shows an interaction between these two effects as it is up-regulated in flies raised on the poor larval diet, but this effect is especially pronounced when combined with transfer to the poor adult diet (Fig. 4g). The modules in Group 2 are down-regulated in flies raised on (green) or transferred to (green-yellow) the poor diet (Fig. 4d,f). In both groups, the differences between the rich and control diets depends on whether the module is affected by larval or adult diet. For the two modules affected by larval diet (grey60, green), flies raised on the rich diet have intermediate expression between poor and control raised flies, similar to the pattern observed for the modules affected by larval diet in female flies (Fig. 4c,d). For the two modules affected by adult diet (royal blue, midnight blue) the effect of adult diet tends to be linear, thus expression either decreases (green yellow) or increases (royal blue) with increasing adult diet (Fig. 4e,f). For the interacting module, midnight blue, there is no consistent difference between the flies raised on or transferred to the control and rich diet, suggesting that this module is driven by changes in expression particular to the poor diet (Fig. 4g).

GO analysis of key male modules suggests roles in nutrient sensing and metabolism

Each of the five modules possess enrichment for GO terms (Appendix 1). The two modules affected by larval diet, grey60 and green, possess the most extensive annotation. The grey60 module, up-regulated in flies raised on the poor larval diet (Fig. 4c), is enriched for 21 terms, and REVIGO summary of these identifies three main functional categories: oxidation-reduction process, generation of precursor metabolites and energy, and cellular respiration (Fig. 5c). The green module, which is down-regulated in poor raised flies (Fig. 4d), is significantly enriched for 75 terms related to metabolism (hexose metabolic process, amine biosynthetic process, cofactor metabolic process et cetera) and defence response (Fig. 5d). By contrast, the two modules affected by adult diet show relatively little annotation: the royal blue module, which is strongly up-

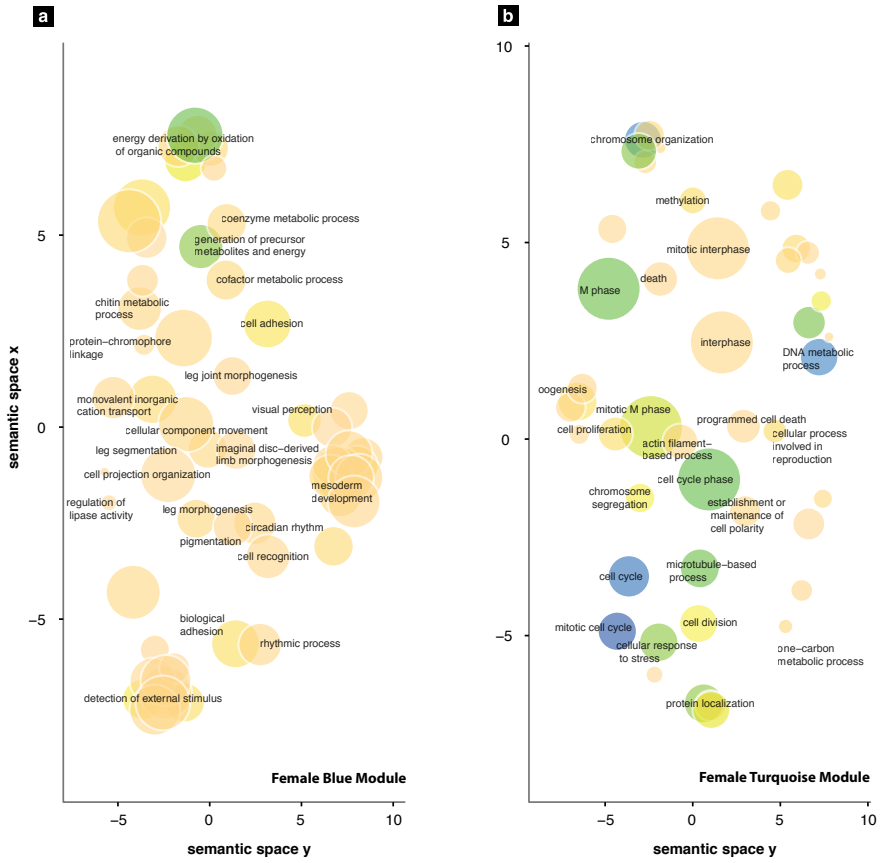
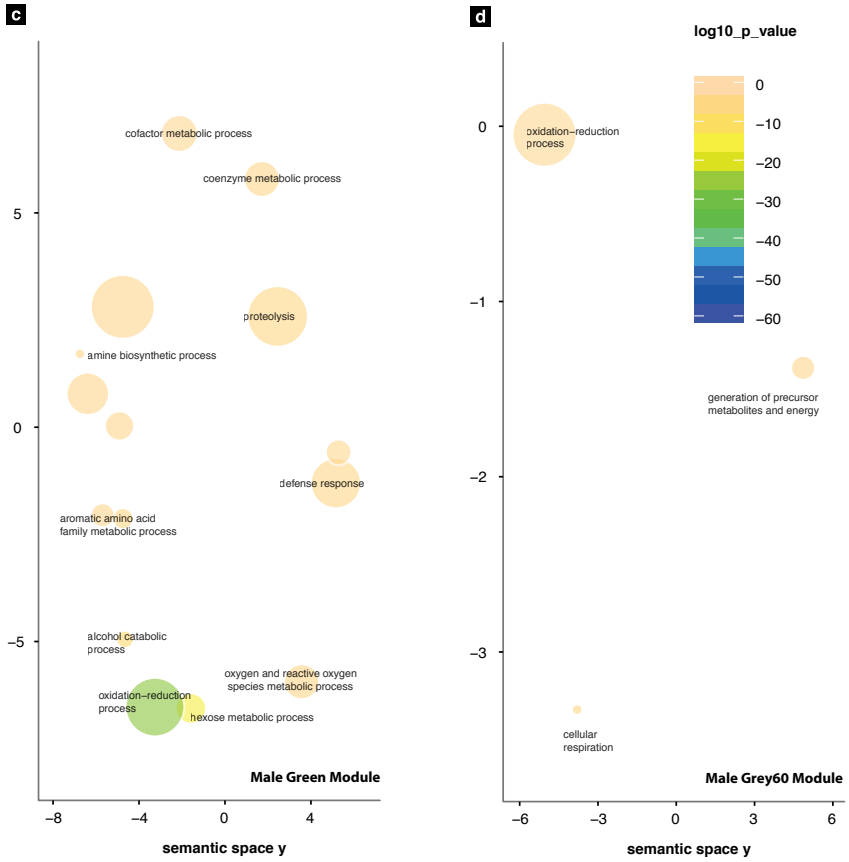


Figure 5: Summarization of module GO annotation using REVIGO. Representative GO terms summarizing (a) the 373 GO terms significantly associated with the blue female module, (b) the 564 GO terms associated with the turquoise female module, (c), the 75 GO terms associated with the green male module and (d) the 21 terms associated with the grey60 male module. Each scatterplot shows the cluster representatives (i.e. terms remaining after the redundancy reduction) in a two dimensional space derived by applying multidimensional scaling to a matrix of the GO terms' semantic similarities. Bubble size indicates the frequency of the GO term in the underlying GO database; more general terms have larger bubbles. Bubble colour indicates the significance of the enrichment in the DAVID analysis (log10 p-value).



regulated in flies transferred to the poor adult diet (Fig. 4e), is solely enriched for the term alkaline phosphatase activity and contains five out of the 13 putative Drosophila alkaline phosphatases. The green yellow module, whose expression increases linearly with increasing adult diet (Fig. 4f), is annotated with the two general terms proteolysis and peptidase activity. These terms are also enriched in the green module, which falls in the same group. Finally, the midnight blue module is annotated with the general terms “response to nutrients” and “response to extracellular stimulus”.

Of the 101 GO terms enriched in all five male modules, none overlapped with the terms enriched for the female turquoise reproduction and cell-cycle related module, but nearly 60% (58/101) overlapped with the female blue module. This suggests that the response to diet in males is not directly related to reproduction and cell-cycle related processes, and furthermore, processes that show more subtle regulation in males are subsumed in females into the blue “non-reproduction” module.

Module	TF	OR	p value	q value	# genes in module	# genes regulated by TF	overlap
Female							
turquoise	BEAF-32	1.54	<0.0001	<0.0001	4771	6597	4200
	CBP	1.79	<0.0001	<0.0001	4771	1291	953
	cnc	1.54	<0.0001	<0.0001	4771	440	279
	disco	1.53	<0.0001	<0.0001	4771	1481	938
	E2f2	1.54	<0.0001	<0.0001	4771	2251	1436
	GATAe	1.60	<0.0001	<0.0001	4771	647	428
	jumu	1.82	<0.0001	<0.0001	4771	920	690
	kn	1.52	<0.0001	<0.0001	4771	497	312
	lin-52	1.53	<0.0001	<0.0001	4771	2161	1366
	Med	1.52	<0.0001	<0.0001	4771	4974	3116
	mip120	1.61	<0.0001	<0.0001	4771	4943	3286
	Myb	1.55	<0.0001	<0.0001	4771	5589	3583
	pho	1.56	<0.0001	<0.0001	4771	2978	1921
	phol	1.64	<0.0001	<0.0001	4771	3713	2508
	Snr1	1.82	<0.0001	<0.0001	4771	289	217
	TfIIB	1.67	<0.0001	<0.0001	4771	2661	1835
	trx	1.52	<0.0001	<0.0001	4771	6591	4137
	ttk	1.75	<0.0001	<0.0001	4771	453	328
	Ubx	1.72	<0.0001	<0.0001	4771	2251	1603
Male							
royalblue	bap	6.87	0.004	0.027	70	96	4
	bin	2.48	0.016	0.087	70	598	9
midnightblue	bap	3.21	0.042	0.179	150	96	4
	bin	2.06	0.011	0.062	150	598	16
	disco	1.56	0.033	0.149	150	1481	30
	Dsp1	1.69	0.009	0.056	150	1455	32
	E(z)	1.72	0.007	0.048	150	1521	34
	gro	1.65	0.035	0.155	150	1024	22
	hkb	1.68	0.020	0.107	150	1236	27
	Mef2	1.77	0.003	0.023	150	1699	39
	NELF-B	2.13	0.002	0.013	150	869	24
	Nelf-E	1.78	0.047	0.195	150	606	14
	slp1	1.84	0.026	0.125	150	669	16
grey60	inv	1.72	0.050	0.205	127	897	17
	run	1.95	0.024	0.119	127	654	14
greenyellow	gsb-n	1.64	0.022	0.111	309	594	26
	NELF-B	1.59	0.014	0.079	309	869	37
	tin	1.80	0.024	0.121	309	374	18

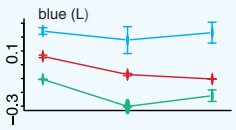
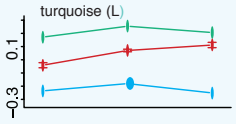
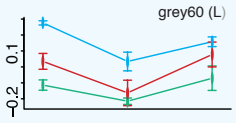
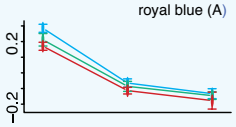
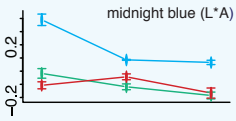
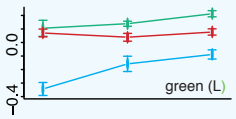
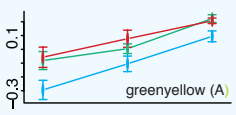
Table 2: Transcription-factor binding enrichments per module. Results of the hyper-geometric test for enrichment of probes known to bind particular transcription factors (TFs) per sex and module. For each significant TF we include the odds ratio (OR), the p-value and the FDR corrected q-value, the number of genes in the module (# module), the number of probes known to interact with the transcription factor (# TF), and the number of probes overlapping between the module and the probes modulated by the transcription factor (Overlap). We set a significance cut-off at OR >1.5 and p-value < 0.05. The full set of tests for all modules and all TFs can be found in Appendix 1.

Male modules show two distinct tissuespecific expression profiles

In addition to having highly correlated eigengenes (Fig. 2d), the two groups each possess similar tissue specific expression profiles (Fig. 3d:f). In larval tissues in FlyAtlas, the Group 2 modules (green, green yellow) which are down-regulated in response to poor larval or adult diet are highly expressed overall, and they are most highly expressed in the feeding and wandering fat body (Fig. 3d). By contrast, the Group 1 modules, which are up-regulated in response to poor larval or adult diet show generally high expression across tissues, particularly in the midgut, hindgut and malpighian tubules, but have their lowest expression in the feeding and wandering fat body (Fig. 3d). Expression of the royal blue module in particular is restricted to the midgut, hindgut and malpighian tubules in FlyAtlas. In adult tissues of FlyAtlas, the Group 2 modules (green and green yellow) show generally lower expression than in larval tissues, but are most highly expressed in the fat body, heart and trachea (Fig. 3e). The Group 1 modules are also generally less highly expressed in adult tissues than in larval tissues. Expression of the royal blue module in adult tissues is also constrained to the hindgut and midgut similar to its expression in larval tissues (Fig. 3e). The grey60 module shows a similar pattern, while the midnight blue module shows expression across all tissues, but a slight tendency for higher expression in the crop (Fig. 3e). Furthermore, two of the three modules (midnight blue, grey 60) have their lowest expression in the adult fat body (Fig. 3e). Finally, we looked at expression in the FlyGut database (Fig. 3f). Consistent with their relatively high expression in gut tissues, the Group 1 modules are relatively highly expressed across gut regions, while the Group 2 modules show intermediate expression. It is noteworthy that the royal blue module, whose expression is most strongly restricted to the gut and malpighian tubules, contains several alkaline phosphatases, which are known to be expressed almost exclusively in the midgut and malpighian tubules.

Transcription factor enrichment of key male modules

The Group 1 modules which were up-regulated in response to poor larval or adult diet were each enriched for probes binding TFs known to be involved in gut development (Table 2), which is consistent with their tissue-specific expression profiles. Both the royal blue and midnight blue module were enriched

Sex	Module	Size	ANOVA	Eigengene values across diets	Group	GO Terms	GO Terms
Female	Blue	5509	L			373	mesoderm development detection of external stimuli leg and limb morphogenesis energy derivation by ox. organic compounds gen. of precursor metabolites &
	Turqouise	5695	L			564	cell division cell cycle oogenesis reproduction
Male	Grey60	144	L		1	21	oxidation-reduction process generation of precursor metabolites and energy cellular respiration
	Royal blue	82	A		1	1	alkaline phosphatase activity
	Midnight blue	179	L*A		1	2	response to nutrients response to extracellular stimulus
	Green	1336	L		2	75	hexose metabolic process amine biosynthetic process cofactor metabolic process defence response
	Green yellow	359	A		2	2	proteolysis peptidase activity

for probes binding two related transcription factors (Table 2): bagpipe *(bap)* and biniou *(bin)*. Both of these transcription factors are important regulators of the development of the visceral musculature of the midgut (Azpiazu & Frasch 1993; Zaffran et al. 2001). The midnight blue module was also enriched for nine additional transcription factors (Table 2) which are enriched for GO terms related to mesoderm development/morphogenesis, heart development, formation of the primary germ layer, and regulation of transcription from RNA polymerase II (Appendix 2e). Finally, the grey60 module was enriched for the

Table 3: Summary of module annotations and biological interpretation as developed in the discussion

Expr. Larval tissues	Expr. Adult Tissues	Expr. Gut Regions	TF	Biological Interpretation
low expression overall	low expression overall, especially in ovary	low	0	These two modules appear to represent the modulation by larval diet of the expression of non-reproduction (blue) versus reproduction (turqousie) related processes. This could be due to changes in ovariole number, larval fat body size or both.
high expression overall, especially in CNS and trachea	high expression overall, especially in ovary	high	19 TFs; similar expression profiles and GO annotation to whole module (Appendix 3)	
high expression in mid and hindgut, malphigian tubules, trachea	highest expression in midgut, hindgut, crop and wings, some expression overall	intermediate to low	invected/runt - both involved in hindgut development	These three modules are highly expressed in the gut and malpighian tubules but not the fat body. Given that both of these tissues are known to persistthrough pupation into adulthood, one possiblity is that these module reflect the carry over of larval tissues into adulthood, and that larval diet affected their size or regulation.Furthermore, the effects of adult diet on these tissues may reflect adaptation to prevailing adult conditions.
high expression in mid and hindgut, malphigian tubules	high expression in hindgut and midgut, low elsewhere	high	bap and bin, regulators of development of visceral gut musculature	
high expression in mid and hindgut, malphigian tubules, trachea & salivary gland	high expression overall, highest in crop and ejaculatory duct	high	11 TFs including bap and bin, regulators of development of visceral gut musculature	
highest in wandering and feeding fat body, intermediate expression across gut tissues	highest expression in fat body and heart, intermediate expression across gut tissues	low	0	These two modules are highly expressed in the larval fatbody, another tissue that is known to persist through pupation into adulthood. Therefore, these modules may reflect larval diet induced changes in the larval fat body, and the green yellow module may reflect the break down of the larval fat body depending on adult dietary conditions.
highest in wandering and feeding fat body, intermediate expression across gut tissues	highest expression in fat body, heart, and wings intermediate expression across gut tissues	low	gooseberry neuro (gsb-n); NELF-B, tinman: each associated with different processes	

TFs *runt* and *invected* (Table 2), both of which are important in the embryonic development of the hindgut (Iwaki & Lengyel 2002; Nam et al. 2002).

Of the Group 2 modules, which were down-regulated in response to poor larval or adult diet, only the green yellow module was enriched for TF binding (Table 2). It was enriched for three seemingly unrelated TFs (Table 2): gooseberry-neuro *(gsb-n)*, Negative Elongation Factor Complex Member B *(NELF-B)*, and *tinman (tin)*. *gsb-n* is known to play an important role in the patterning of the

central nervous system (He & Noll 2013) while *NELF-B* is a component of the NELF complex which negatively regulates the elongation of transcription by RNA II polymerase (Wu et al. 2005). tinman is essential to the development of visceral and cardiac mesoderm during development (Azpiazu & Frasch 1993).

Taken together, the different annotations of the male modules result in the following summary. The GO annotation of the modules affected by diet shows that they are generally involved in metabolic processes or nutrient sensing (Table 3). Furthermore, modules with highly correlated eigengenes tend to have similar eigengene expression profiles and tissue-specific expression profiles in FlyAtlas. Modules up-regulated in response to the poor larval or adult diet tend to be highly expressed in the gut and malpighian tubules, and lowly expressed in the fat body, while modules down-regulated in response to the poor larval or adult diet tend to be more highly expressed in larval tissues, particularly in the larval fat body (Table 3). This suggests that the effects of both larval and adult diet on gene expression in males may be mediated by changes in the same tissues, the fat body, gut and malpighian tubules.

DISCUSSION

Here we show that both larval and adult diet affect the whole-body transcriptome of young adults, however, the effect of larval diet is considerably larger than that of adult diet, especially in females. Thus flies do not begin life with a clean slate, but rather, retain a considerable legacy of their developmental conditions in their whole-body transcriptome, setting the stage not only for early life (life history) differences in phenotypic trait values, but also for potential long-term effects of developmental diet on adult gene expression and late-life phenotypes. There was no linear relationship between increasing larval diet concentration and gene expression, as flies raised on the richest larval diet had intermediate expression values between flies raised on poor and control larval diets, suggesting that total calories per se do not determine gene expression levels (Fig. 1a,b). Furthermore, in females, there was a rapid transcriptional response to the poor adult diet (Fig. 1a).

By applying WGCNA, we were able to identify modules of co-expressed genes and test their association with larval or adult dietary conditions. We found that in females nearly all genes fall into one of two negatively correlated co-expression modules, the relative expression of which is dependent on larval diet. Annotation of these two modules (summarised in Table 3) suggests that they reflect relative investment into reproduction/cell-cycle related processes (turquoise module) versus non-reproduction/cell cycle related processes (blue module). Females raised on the poor larval diet have lower expression of the turquoise module (reproduction/cell cycle related processes) but higher expression of the blue module suggesting an important role for larval diet in

determining broad scale patterns of transcriptional variation in young females.

For males, we identified 22 modules, only five of which were affected by diet, either larval and/or adult. Annotation of the affected modules indicates their involvement in metabolism and nutrient sensing rather than reproduction or cell-cycle related processes, suggesting a very different role of diet in the male versus the female transcriptome. Furthermore, the modules affected by diet in males were highly expressed in the larval and adult gut, malpighian tubules and the fat body in FlyAtlas, suggesting that these important nutrient sensing tissues may play a role in modulating the effect of developmental diet on adult gene expression.

No linear correlation between increasing larval diet and gene expression

We found that development on the rich larval diet, rather than leading to the most extreme expression, actually lead to intermediate expression in both sexes (Fig. 1a,b). Therefore, there is no linear correlation between available calories during development and global gene expression profiles. We have previously shown that the effects of these diets on larval phenotypes follows a similar pattern: flies raised on the poor larval diet develop slower and are smaller as adults, while flies raised on the rich larval diet are intermediate between poor and control (May et al. 2015). This suggests that both the poor and rich larval diet may reflect sub-optimal developmental conditions, and that this may then also be reflected in the relative similarity of their gene expression profiles. Interestingly, the long-term phenotypic effects of the larval diets follow a different pattern: as larval diet increases adult lifespan and fecundity decreases (May et al. 2015). Thus the global patterns of gene expression patterns at the beginning of adult life do not appear to translate to patterns of life-span variation or fecundity. This suggests that either the effect is transient, or that more subtle patterns of gene expression variation are driving long-term effects of larval diet.

Larval diet determines relative investment in reproduction and cell-cycle related processes in females

Female fruit flies begin life primed for egg-laying and reproduction, being both most receptive to mating and most fecund within the first week of life (Fricke et al. 2013; Sgrò & Partridge 2000). We show here that this immediate and intense drive for reproduction appears to also translate to the transcriptome; while in males we could identify many different co-expression modules, the female transcriptome could only be broken down into two highly negatively correlated modules, one highly related to reproduction related processes (turquoise module) and one not (blue module). Furthermore, the relative expression of the two modules was strongly dependent on larval diet. This suggests that (i) at

the beginning of life, there is scope for a large effect of developmental diet on reproductive output, and (ii) that developmental diet can also have long-term effects on the transcriptome, a prerequisite for each of the hypotheses that link development to adult late-life phenotypes (e.g. silver spoon and PAR).

There are several plausible mechanisms by which larval diet may affect the extent of reproduction related gene expression. First, larval diet may determine the overall energy available to reproduction. In fruit flies, larval-derived carbon is known to contribute to egg production in the first week of adult life (Min et al. 2006), and furthermore, considerable energy for early reproduction comes from the larval fat body (Aguila et al. 2013) which dissociates during pupation and persists into early adulthood as free-floating fat cells (Aguila et al. 2013; Aguila et al. 2007; Nelliot et al. 2006). Thus, a poor larval diet may simply decrease the relative size of energy stores, and in so doing decrease overall reproduction related expression. However, such an effect is not likely to persist, as the larval fat body dissociates and after one week larval-derived carbon is no longer detectable in eggs (Min et al. 2006). Alternatively, larval diet may affect the relative size of reproductive tissues. Ovariole number is set during development, and females raised on nutritionally poor larval diets often have fewer ovarioles as adults, putting an upper limit on their potential fecundity (Hodin & Riddiford 2000; Tu & Tatar 2003). Thus, it is also plausible that the poor larval diet leads to fewer ovarioles, and a concomitant decrease in overall fecundity and reproduction related gene expression. However, given the (non-significant) trend towards decreased expression of the turquoise module observed in flies transferred to the poor larval diet as adults (whose ovariole number is set), ovariole number alone cannot account for the entire response. Thus it would be very informative to measure gene expression at later ages, to determine to what extent the effect of larval diet on the transcriptome persists.

Male responses to diet

In contrast to females, male gene expression could be decomposed into a substantially larger number of co-expression modules, most of which were unaffected by dietary conditions, suggesting less scope for early and long-term effects of developmental diet in males than in females. For the modules that were affected by diet, GO annotation showed that they were primarily annotated with terms related to metabolism and nutrient sensing, and furthermore, while they overlapped considerably with the female blue "non-reproduction" module, they did not overlap at all with the female turquoise "reproduction-related" module. This suggests a much smaller role for diet in reproduction related processes in males, and shows that processes that show more subtle regulation in males are subsumed in females into the dichotomy of "reproduction" versus "non-reproduction" related gene expression. In general, these patterns and our interpretation aligns well with the much lower direct

costs to reproduction in males than in females (i.e. sperm is much less "costly" than eggs).

We found that the male modules affected by diet could be divided into two groups based on the correlation of their eigengene values, and that modules in the same group tended to have both similar responses to diet and similar tissue specific expression profiles (Table 3). Group 1 (grey60, royal blue, midnight blue) consisted of modules up-regulated in response to the poor larval or adult diet, while Group 2 (green, green yellow) modules were down-regulated. When the expression of these two Groups in the larval tissues of FlyAtlas was compared there was a clear distinction between them: modules in Group 1 had their lowest expression in the larval feeding and wandering fat body, but high expression in the gut and malpighian tubules, while Group 2 had their highest expression in the larval feeding and wandering fat body. This distinction is observed to a lesser extent in adult tissues, suggesting that the effects of diet in males may go through the gut, malpighian tubules, and fat body. This is consistent with the GO annotation of the modules: for example, the midnight blue module (Group 1) is enriched for the term alkaline phosphatase (APH) activity and contains five of 13 known APHs (Pletcher et al. 2005), which are known to be expressed almost exclusively in the midgut and malpighian tubules, while the green module (Group 2) is annotated with terms related to metabolism (hexose metabolic process, amine biosynthetic process, cofactor metabolic process) and defence response, canonical functions of the insect fat body (Arrese & Soulages 2010; Zhang & Xi 2015).

It is becoming increasingly clear that some tissues are not histolysed during metamorphosis in *Drosophila melanogaster,* but rather persist into adulthood. These tissues include the visceral musculature of the gut (Klapper 2000), the larval fat body (Aguila et al. 2013; Aguila et al. 2007; Nelliot et al. 2006), and the malpighian tubules (Riddiford 1993). Thus one intriguing hypothesis for the relatively high expression of the male modules affected by larval diet in these tissues is that they simply represent the carry-over of these tissues from development into adulthood, and that the size and/or the regulation of the tissue has been affected by larval diet. For example, the green module, which is highly expressed in the larval fat body, is strongly down-regulated in flies raised on the poor larval diet, suggesting that they may have accumulated less fat during development. The modules affected by adult diet then could reflect modifications of the expression of these "carried over" tissues to adapt to the current adult conditions. For example, the green yellow module, which is also highly expressed in the larval fat body (but with less specificity than the green module), is strongly down-regulated in flies transferred to the poor adult diet. Because the adult fat body only forms several days after eclosion

(Aguila et al. 2007; Hoshizaki et al. 1995), one hypothesis suggested by the annotation of proteolysis and peptidase activity of this module is that the green yellow module may relate to the breakdown of the larval fat body, and that this process depends on the adult dietary conditions. It has been shown that inhibition of programmed cell death (PCD) of the larval fat body in adults increases starvation resistance (Aguila et al. 2007), thus it seems plausible that PCD of the larval fat body may also be inhibited in response to poor adult dietary conditions, and up-regulated in plentiful adult conditions where stores can easily be replenished. The two modules affected by adult diet and highly expressed in the gut (royal blue, midnight blue) may reflect the adaptation of the visceral musculature to adult dietary conditions, or adaptation of other regions of the gut. Regional patterning of the Drosophila gut is not complete until approximately three days after eclosion (Buchon et al. 2013), thus early adulthood may provide an optimal time to adjust the gut to prevailing dietary conditions.

The question then remains: why didn't we observe any evidence for a role of the gut in females? This could be because there is no such effect in females, or because we were unable to detect it. The latter explanation is simpler: because of the huge effect of reproduction versus non-reproduction related gene expression on the female transcriptome, more subtle changes may be obscured, especially at the level of the whole-body transcriptome. However, the former explanation is also plausible given recent findings that show that the male and female gut of Drosophila are fundamentally different, and that these differences may underlie observed differences between the sexes in the lifespan response to adult diet (Regan et al. 2016). Thus the male and female gut may also respond differently to developmental and early adult diet. Furthermore, it has recently been shown that at least in the female, the gut retains extensive plasticity in adult life, showing extensive remodelling in response to mating (Reiff et al. 2015), thus there may be no incentive for females to "remodel" their gut early in life, as we hypothesise may be the case in males.

CONCLUSIONS

Here we show that developmental diet continues to affect the whole-body transcriptome of young adult flies, especially of females. This finding is relevant for the potential of the flies to respond to early life environments and their capacity for reproduction. Moreover, there is scope for long-term effects of developmental diet on gene expression, which is necessary for all hypothesised mechanisms that link developmental conditions to late-life health and disease. In females, larval diet modulates the relative expression levels of reproduction versus non-reproduction related genes, while in males a large portion of the transcriptome is unaffected by dietary conditions, suggesting a lesser role for both larval and adult diet in affecting gene expression. The modules affected

by diet in males relate primarily to nutrient sensing and metabolism and show no evidence of the reproduction and cell-cycle related processes identified in females, however, their expression in external tissue specific data sets suggests a role for the gut and fat body in mediating the effects of diet in males. Given the oft cited hypothesis that long-term phenotypic effects of developmental diet may be mediated by changes in gene expression, these findings suggest that such effects are possible, and furthermore, at least in Drosophila, are likely to be largely sex specific.

ACKNOWLEDGEMENTS

We would like to thank Dennie Helmink, Marijke Slakhorst and Bertha Koopmaanschap for help with fly rearing and the set-up of the experiment. We would also like to thank Fons Debets and Jelle Zandveld for helpful discussions on the experimental design and interpretation of the results. This work was supported by the European Union's FP6 Programme (Network of Excellence LifeSpan FP6/036894) and the EU's FP7 Programme (IDEAL FP7/2007-2011/259679).

REFERENCES

Aguila JR, Hoshizaki DK, Gibbs AG (2013) Contribution of larval nutrition to adult reproduction in *Drosophila melanogaster*. *The Journal of Experimental Biology* **216**, 399-406.

Aguila JR, Suszko J, Gibbs AG, Hoshizaki DK (2007) The role of larval fat cells in adult *Drosophila melanogaster*. *Journal of Experimental Biology* **210**, 956-963.

Allocco DJ, Kohane IS, Butte AJ (2004) Quantifying the relationship between co-expression, co-regulation and gene function. *BMC Bioinformatics* **5**, 18-18.

Arrese EL, Soulages JL (2010) Insect fat body: Energy, metabolism and regulation. *Annual Review of Entomology* **55**, 207-225.

Ayroles JF, Carbone MA, Stone EA, *et al.* (2009) Systems genetics of complex traits in *Drosophila melanogaster*. *Nature Genetics* **41**, 299-307.

Azpiazu N, Frasch M (1993) tinman and bagpipe: two homeo box genes that determine cell fates in the dorsal mesoderm of *Drosophila*. *Genes Development* **7**, 1325-1340.

Barker DJP, Hales CN, Fall CHD, *et al.* (1993) Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): relation to reduced fetal growth. *Diabetologia* **36**, 62-67.

Barker DJP, Osmond C, Winter PD, Margetts B, Simmonds SJ (1989) Weight in infancy and death from ischaemic heart disease. *The Lancet* **334**, 577-580.

Bateson P, Gluckman P, Hanson M (2014) The biology of developmental plasticity and the Predictive Adaptive Response hypothesis. *The Journal of Physiology* **592**, 2357-2368.

Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society. Series B (Methodological)* **57**, 289-300.

Bertram C, Trowern A, Copin N, Jackson A, Whorwood C (2001) The maternal diet during pregnancy programs altered expression of the glucocorticoid receptor and Type 2 11–hydroxysteroid dehydrogenase: Potential molecular mechanisms underlying the programming of hypertension *in utero* 1. *Endocrinology* **142**, 2841-2853.

Boutanaev AM, Kalmykova AI, Shevelyov YY, Nurminsky DI (2002) Large clusters of co-expressed genes in the *Drosophila* genome. *Nature* **420**, 666-669.

Buchon N, Osman D, David Fabrice PA, *et al.* (2013) Morphological and molecular characterization of adult midgut compartmentalization in *Drosophila*. *Cell Reports* **3**, 1725-1738.

Burdge GC, Hanson, MA, Slater-Jefferies, JL, and KA Lillycrop. (2007) Epigenetic regulation of transcription: a mechanism for inducing variations in phenotype (fetal programming) by differences in nutrition during early life? *British Journal of Nutrition* **97**, 1036-1046.

Burdge GC, Lillycrop KA (2010) Nutrition, epigenetics, and developmental plasticity: Implications for understanding human disease. *Annual Review of Nutrition* **30**, 315-339.

Emlen DJ (1997) Diet alters male horn allometry in the beetle *Onthophagus acuminatus* (Coleoptera: Scarabaeidae). *Proceedings of the Royal Society of London B: Biological Sciences* **264**, 567-574.

Fricke C, Green D, Mills WE, Chapman T (2013) Age-dependent female responses to a male ejaculate signal alter demographic opportunities for selection. *Proceedings of the Royal Society B: Biological Sciences* **280**, 20130428.

Gluckman PD, Hanson MA (2004) Living with the past: evolution, development, and patterns of disease. *Science* **305**, 1733-1736.

Gluckman PD, Hanson MA, Beedle AS (2007) Early life events and their consequences for later disease: A life history and evolutionary perspective. *American Journal of Human Biology* **19**, 1-19.

Grafen A (1988) On the uses of data on lifetime reproductive success. In: *Reproductive success* (ed. Clutton-Brock TH), pp. 454-471. University of Chicago Press.

Halfon MS, Gallo SM, Bergman CM (2008) REDfly 2.0: an integrated database of cis-regulatory modules and transcription factor binding sites in *Drosophila*. *Nucleic Acids Research* **36**, D594-D598.

He H, Noll M (2013) Differential and redundant functions of *gooseberry* and *gooseberry neuro* in the central nervous system and segmentation of the *Drosophila* embryo. *Developmental Biology* **382**, 209-223.

Hilliard AT, Miller JE, Fraley ER, Horvath S, White SA (2012) Molecular microcircuitry underlies functional specification in a basal ganglia circuit dedicated to vocal learning. *Neuron* **73**, 537-552.

Hodin J, Riddiford LM (2000) Different mechanisms underlie phenotypic plasticity and interspecifc variation for a reproductive character in drosophilids (Insecta: Diptera). *Evolution* **54**, 1638-1653.

Hoshizaki DK, Lunz R, Johnson W, Ghosh M (1995) Identification of fat-cell enhancer activity in *Drosophila melanogaster* using P-element enhancer traps. *Genome* **38**, 497-506.

Huang DW, Sherman BT, Lempicki RA (2008) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protocols* **4**, 44-57.

Iwaki DD, Lengyel JA (2002) A Delta–Notch signaling

border regulated by Engrailed/Invected repression specifies boundary cells in the *Drosophila* hindgut. *Mechanisms of Development* **114**, 71-84.

Klapper R (2000) The longitudinal visceral musculature of *Drosophila melanogaster* persists through metamorphosis. *Mechanisms of Development* **95**, 47-54.

Lanet E, Maurange C (2014) Building a brain under nutritional restriction: insights on sparing and plasticity from *Drosophila* studies. *Frontiers in Physiology* **5**, 117.

Langfelder P, Horvath S (2008) WGCNA: an R package for weighted correlation network analysis. *BMC Bioinformatics* **9**, 559.

Lillycrop KA, Phillips ES, Jackson AA, Hanson MA, Burdge GC (2005) Dietary protein restriction of pregnant rats induces and folic acid supplementation prevents epigenetic modification of hepatic gene expression in the offspring. *The Journal of Nutrition* **135**, 1382-1386.

Lindström J (1999) Early development and fitness in birds and mammals. *Trends in Ecology & Evolution* **14**, 343-348.

Maloney CA, Gosby AK, Phuyal JL, *et al.* (2003) Site-specific changes in the expression of fat-partitioning genes in weanling rats exposed to a low-protein diet in utero. *Obesity research* **11**, 461-468.

May CM, Doroszuk A, Zwaan BJ (2015) The effect of developmental nutrition on life span and fecundity depends on the adult reproductive environment in *Drosophila melanogaster*. *Ecology and Evolution* **5**, 1156-1168.

Min K-J, Hogan MF, Tatar M, O'Brien DM (2006) Resource allocation to reproduction and soma in *Drosophila*: A stable isotope analysis of carbon from dietary sugar. *Journal of Insect Physiology* **52**, 763-770.

Monaghan P (2008) Early growth conditions, phenotypic development and environmental change. *Philosophical Transactions of the Royal Society B: Biological Sciences* **363**, 1635-1645.

Murali T, Pacifico S, Yu J, *et al.* (2010) DroID 2011: a comprehensive, integrated resource for protein, transcription factor, RNA and gene interactions for *Drosophila*. *Nucleic Acids Research*.

Nam S, Jin Y-H, Li Q-L, *et al.* (2002) Expression Pattern, regulation, and biological role of runt domain transcription factor, *run*, in *Caenorhabditis elegans*. *Molecular and Cellular Biology* **22**, 547-554.

Nelliot A, Bond N, Hoshizaki DK (2006) Fat-body remodeling in *Drosophila melanogaster*. *genesis* **44**, 396-400.

Oldham MC, Horvath S, Geschwind DH (2006) Conservation and evolution of gene coexpression networks in human and chimpanzee brains. *Proceedings of the National Academy of Sciences* **103**, 17973-17978.

Pletcher S, Macdonald S, Marguerie R, *et al.* (2002) Genome-wide transcript profiles in aging and calorically restricted *Drosophila melanogaster*. *Curr Biol* **12**, 712 - 723.

Pletcher SD, Libert S, Skorupa D (2005) Flies and their golden apples: the effect of dietary restriction on *Drosophila* aging and age-dependent gene expression. *Ageing research reviews* **4**, 451-480.

Ravelli ACJ, van der Meulen JHP, Michels RPJ, *et al.* (1998) Glucose tolerance in adults after prenatal exposure to famine. *The Lancet* **351**, 173-177.

Regan JC, Khericha M, Dobson AJ, *et al.* (2016) Sex difference in pathology of the ageing gut mediates the greater response of female lifespan to dietary restriction. *Elife* **5**, e10956.

Reiff T, Jacobson J, Cognigni P, *et al.* (2015) Endocrine remodelling of the adult intestine sustains reproduction in *Drosophila*. *Elife* **4**, e06930.

Riddiford LM (1993) Hormones and *Drosophila* development. *The development of Drosophila melanogaster* **2**, 899-939.

Roy S, Ernst J, Kharchenko PV, *et al.* (2010) Identification of functional elements and regulatory circuits by *Drosophila* modENCODE. *Science* **330**, 1787-1797.

Schlichting C, Pigliucci M (1998) Phenotypic evolution: A reaction norm perspective Sinauer.

Sgrò CM, Partridge L (2000) Evolutionary responses of the life history of wild-caught *Drosophila melanogaster* to two standard methods of laboratory culture. *The American naturalist* **156**, 341-353.

Supek F, Bošnjak M, Škunca N, Šmuc T (2011) REVIGO summarizes and visualizes long lists of gene ontology terms. *PloS one* **6**, e21800.

Tu MP, Tatar M (2003) Juvenile diet restriction and the aging and reproduction of adult *Drosophila melanogaster*. *Aging Cell* **2**, 327-333.

Wu C-H, Lee C, Fan R, *et al.* (2005) Molecular characterization of *Drosophila* NELF. *Nucleic Acids Research* **33**, 1269-1279.

Xue Z, Huang K, Cai C, *et al.* (2013) Genetic programs in human and mouse early embryos revealed by single-cell RNAsequencing. *Nature* **500**, 593-597.

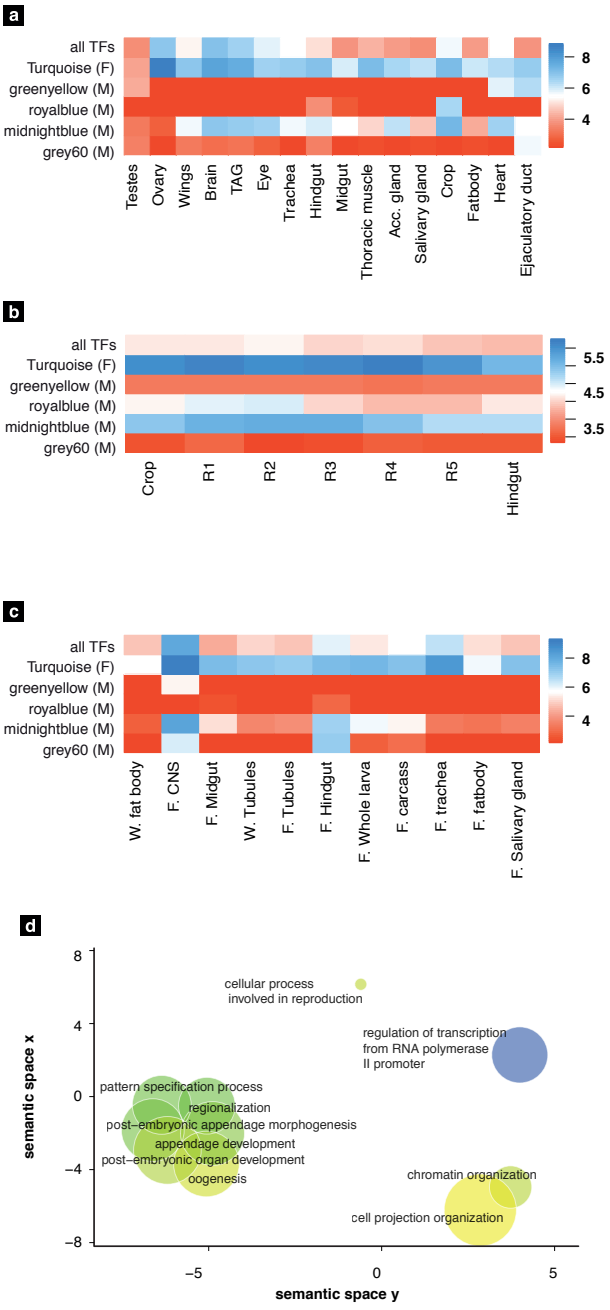
Zaffran S, Küchler A, Lee H-H, Frasch M (2001) biniou (FoxF), a central component in a regulatory network controlling visceral mesoderm development and midgut morphogenesis in *Drosophila*. *Genes Development* **15**, 2900-2915.

Zhang B, Horvath S (2005) A general framework

REFERENCES

for weighted gene co-expression network analysis. *Statistical applications in genetics and molecular biology* **4**.

Zhang Y, Xi Y (2015) Fat body development and its function in energy storage and nutrient sensing in *Drosophila melanogaster*. *Journal of Tissue Science Engineering* **2015**.

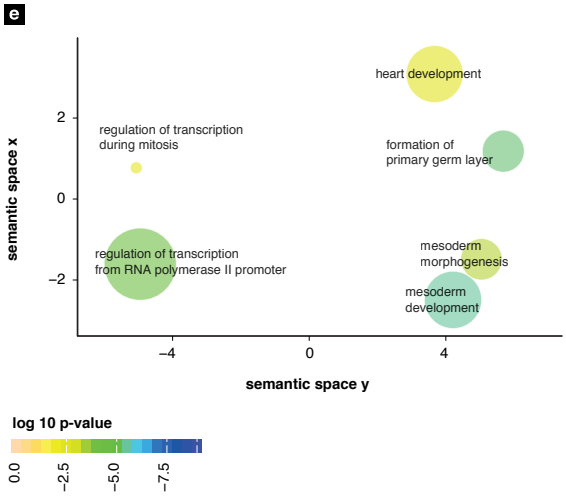

a
all TFs
Turquoise (F)
greenyellow (M)
royalblue (M)
midnightblue (M)
grey60 (M)
Testes
Ovary
Wings
Brain
TAG
Eye
Trachea
Hindgut
Midgut
Thoracic muscle
Acc. gland
Salivary gland
Crop
Fatbody
Heart
Ejaculatory duct
8
6
4
b
all TFs
Turquoise (F)
greenyellow (M)
royalblue (M)
midnightblue (M)
grey60 (M)
Crop
R1
R2
R3
R4
R5
Hindgut
5.5
4.5
3.5
c
all TFs
Turquoise (F)
greenyellow (M)
royalblue (M)
midnightblue (M)
grey60 (M)
W. fat body
F. CNS
F. Midgut
W. Tubules
F. Tubules
F. Hindgut
F. Whole larva
F. carcass
F. trachea
F. fatbody
F. Salivary gland
8
6
4
d
cellular process
involved in reproduction
regulation of transcription
from RNA polymerase
II promoter
pattern specification process
regionalization
post−embryonic appendage morphogenesis
appendage development
post−embryonic organ development
oogenesis
chromatin organization
cell projection organization
semantic space x
semantic space y
8
4
0
−4
−8
−5
0
5

APPENDICES

Appendix 1: Module membership, GO enrichment and TF enrichment
Excel File containing module membership of all probes for both sexes (Tab 1), significant GO terms for each module (Tab 2) and results of the hypergeometric test for transcription factor enrichment for each TF in each module (Tab 3).

Appendix 1 can be found in the folder associated with this chapter in the online Dropbox folder associated with this thesis at:
https://www.dropbox.com/sh/q37qolcfi6ib05m/AABBF-G1k3hlOGFK-WXUyCnqBa?dl=0

Appendix 2: Expression and annotation of enriched transcription factors
Expression profiles of TFs enriched per module in (a) adult FlyAtlas data, (b) FlyGut regions, and (c) larval FlyAtlas data. REVIGO analysis of GO terms associated with TFs enriched in (e) the female turquoise module and (f) the male midnight blue module. For (e) and (f) each scatterplot shows the cluster representatives (i.e. terms remaining after the redundancy reduction) in a two dimensional space derived by applying multidimensional scaling to a matrix of the GO terms' semantic similarities. Bubble size indicates the frequency of the GO term in the underlying GO database; more general terms have larger bubbles. Bubble colour indicates the significance of the enrichment in the DAVID analysis (log10 p-value).



The shadow of developmental past: Effects of developmental diet on adult lifespan and gene expression persist but are small compared to adult plasticity in the fruit fly

Christina M. May[1] and Bas J. Zwaan[1]

1. Laboratory of Genetics, Wageningen University, Wageningen

ABSTRACT

In nature, individuals must cope with nutritional variation during both development and adulthood, however most experiments manipulate diet in only a single life stage. Here we vary both by raising the fruit fly across three different developmental and adult diets. We then assess how dietary conditions during the two life stages interact to determine adult life history phenotypes and gene expression. We find that developmental and adult diet exert largely independent effects on adult life history traits and gene expression, and that adult diet has considerably more influence on both classes of traits. Yet, we do find a highly consistent effect of larval diet on virgin lifespan across adult diets in both sexes – as sugar and yeast content of the larval diet increases, adult lifespan decreases. Furthermore, for the first time, we show that a signature of larval diet is detectable in the fruit fly transcriptome throughout adult life. The expression of most of the genes affected showed no linear correlation with lifespan suggesting that their expression was unrelated to the life history phenotype. In contrast, consistent across the sexes, we identified a cluster of genes whose expression is negatively correlated with lifespan and that is enriched with terms related to transcription and translation, especially ribosomes. Whether this effect is causal or incidental is unclear, however, given several recent studies showing lifespan extension in response to decreased expression of ribosomal sub-units and other transcription and translation related proteins, these genes provide promising candidates for mediating the long-term effects of larval diet on lifespan we identify. We discuss how our observations relate to theories that link developmental conditions to late-life phenotypes, the epigenetic mechanisms underpinning this link, and the likelihood that gene expression differences caused by developmental exposure causally relate to the adult ageing phenotypes.

INTRODUCTION

The quality or quantity of available nutrition is a major factor affecting the life history of an organism (Roff 2001; Stearns 1992). For many years, the reigning paradigm for studying the effects of nutrition on life histories has been to manipulate diet quality or quantity in a single life stage. In this manner, pervasive effects of both developmental and adult diets on the phenotype have been identified (Skorupa et al. 2008; Solon-Biet et al. 2014; Tu & Tatar 2003). However, in natural settings, organisms are likely to experience nutritional variation across multiple life stages. Furthermore, the changes made in an earlier life stage can potentially influence the range of possible phenotypic responses in later stages (reviewed in Lindström 1999; Monaghan 2008). Given the current swift pace of global environmental change many organisms, including humans, are likely to encounter adult environments markedly different to those in which they developed, highlighting the importance of a more comprehensive understanding of how the developmental and adult diet interact (Chown et al. 2010).

Several models have been proposed to explain the potential interactive effects between developmental and adult diet in determining phenotypic variation. The silver spoon hypothesis proposes that developmental diet affects the overall quality of the individual independent of the adult environment experienced (Grafen 1988). Thus individuals that develop under poor conditions become poor quality adults with a disadvantage across adult environments, while the opposite is true of individuals that develop under good conditions. Such effects have been observed across a broad range of taxa including birds, insects and mammals (Lindström 1999; Madsen & Shine 2000; Monaghan 2008). Alternatively, the phenotypic effect of the developmental diet can depend on the adult environment. Individuals can make phenotypic changes to adapt to the current developmental environment which persist into adulthood, so-called "developmental programming" responses (Fernandez-Twinn & Ozanne 2006), or they can use cues perceived during development to make phenotypic changes to be well-adapted to the future adult environment – so-called "predictive adaptive responses" (PAR; Gluckman & Hanson 2004b; Gluckman et al. 2007). For example, adaptations made to increase fitness in a poor developmental environment (programming) or in preparation for a predicted poor adult environment (PARs) such as storing food as fat or maintaining high blood glucose levels will likely be beneficial in a poor quality adult environment, but detrimental in a high-quality one. Thus both PAR's and programming predict negative fitness effects in the case of mismatches between developmental and adult diets.

At a physiological level, developmental conditions can influence adult phenotypes by changing the overall size of the individual (Shingleton 2011),

by changing the relative investment into different tissues or functions (e.g. Emlen 1997; Jannot et al. 2007; Shingleton et al. 2009), and/or by permanently modulating patterns of gene expression. In all cases, these effects are expected to manifest themselves as changes in gene expression at the level of the whole organism. In fact in the case of predictive adaptive responses (PARs) it is often hypothesized that gene expression changes per se may be the principal cause of long-term phenotypic effects, rather than just a read-out of past changes (Burdge 2007; Burdge & Lillycrop 2010). However, despite the potential importance of gene expression changes in modulating long-term effects of developmental diet, the extent of the effect of developmental diet on the adult transcriptome has not yet been addressed. Candidate gene approaches have revealed that developmental effects on adult gene expression do persist into young adulthood; for example, in rats a restricted-protein maternal diet strongly induces the expression of PPAR- at six days of age (Lillycrop et al. 2005), while in fruit flies, high protein larval diets increase the expression of some immune genes in young adults (Fellous & Lazzaro 2010). Importantly, neither of these studies link the observed gene expression differences to phenotypic variation, nor do they indicate the overall magnitude of the effect on the transcriptome and whether such effects will persist past young adulthood, a key prediction of both the PAR and programming hypotheses.

To address these unanswered questions we use the fruit fly, Drosophila melanogaster, as a model for understanding the consequences of mismatches between developmental and adult diet for both adult phenotypes and gene expression. To do so we vary the caloric content of both developmental and adult diet 10-fold in a three-by-three full factorial design (Fig. 1). This approach has three main aims: the first is to classify the nature of the phenotypic relationship between developmental and adult diet in the fruit fly, which to our knowledge has not yet been assessed. The second is to determine the overall magnitude of the effect of developmental diet on gene expression in middle and old-aged flies and if and how this depends on the adult environment, and the third is to determine whether there is any discernible link between developmentally-induced changes in gene expression and the observed phenotypes. Because there is no a priori hypothesis about the tissues and functions that play a role, we choose to employ a similar approach to that taken to understand the transcriptional responses underlying dietary restriction (DR) by measuring gene expression profiles in whole bodies (e.g. Pletcher et al. 2002). Furthermore, while most studies of dietary effects on gene expression and phenotypes tend to use only one sex (in fruit flies: females; e.g. Bauer et al. 2010; Doroszuk et al. 2012; Pletcher et al. 2002), there is considerable evidence that at least the phenotypic responses to diet tend to differ between the sexes (Magwere et al. 2004). Thus here we address the transcriptional and phenotypic responses to diet in males and females.

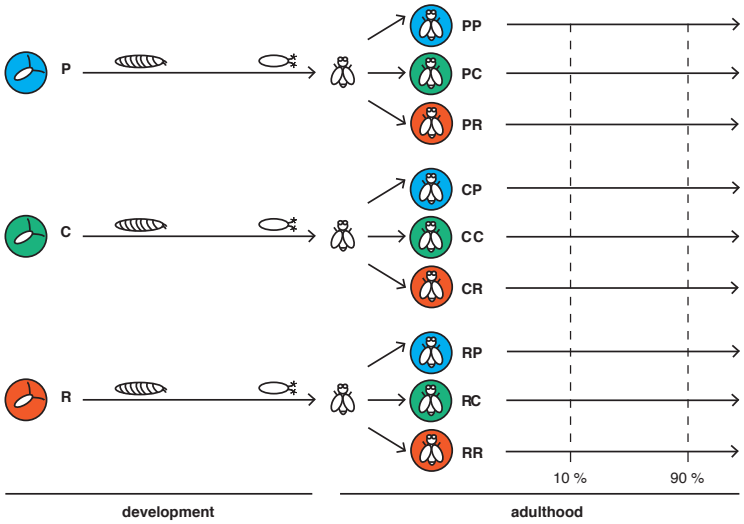


Figure 1: Experimental design. Eggs developed from larvae to adults under three diets, poor (P), control (C) and rich (R) which differed only in their concentrations of sugar and yeast. Emerging adults were immediately divided across these same three diets resulting in a total of nine different treatments. Gene expression was measured on virgin flies sampled when 10% (middle age) and 90% (old age) of the treatment cohort had died.

MATERIALS AND METHODS

Experimental Design

To test the effect of mismatches between developmental and adult diet on lifespan, fecundity and gene expression, we raised flies from the laboratory stock population (S), described in May et al., (2015), on three larval diets at a density of 100 eggs per vial. Upon emergence virgin adults were sexed and randomly distributed across these same diets in a three by three full factorial design (Fig. 1). The three diets, hereafter designated poor (P), control (C), and rich (R) differed only in the amount of sugar and yeast they contained. The control diet (C) was identical to the standard laboratory diet (70g yeast, 100g sugar, 20g agar, 15mL nipagine, and 3mL propionic acid per liter of water), while the poor (P) and rich (R) diet contained 25% and 250% as much sugar and yeast as the control diet, respectively.

In previous experiments we assessed the effects of these three diets on developmental traits (May et al. 2015). Flies raised on the poor larval diet develop more slowly and are smaller as adults than those raised on the control diet, while flies raised on the rich larval diet are intermediate between the two (May et al. 2015). Furthermore, under control adult dietary conditions flies raised on the poor adult diet had the longest virgin lifespan, while those raised on the rich diet had the shortest.

Mated lifespan and fecundity

To assess mated lifespan and fecundity, flies were maintained in groups of five males and five females per vial with 20 replicate vials per treatment. Survival was measured three times per week, while fecundity was scored biweekly over two time periods: early fecundity (days 1 to 7 of adult life), when peak fecundity occurs, and late fecundity (days 7 to end of reproduction) when fecundity is steadily decreasing (Novoseltsev et al. 2005; Robertson & Sang 1944; Sgro et al. 2000). Fecundity was scored as realized fecundity – i.e. the number of eclosing adults per vial.

Virgin lifespan and gene expression

To measure virgin lifespan and gene expression, we maintained 500 flies per combination of sex, larval diet and adult diet at a density of ten flies per vial (9000 flies total) and monitored their survival. When 10% and 90% of the cohort had died, 50 flies per combination of sex, larval diet and adult diet were flash-frozen for gene expression analysis.

We extracted RNA from whole bodies of five flies per replicate, with four replicates per combination of sex, larval diet, adult diet and age (4 replicates x 2 sexes x 3 larval diets x 3 adult diets x 2 ages = 144 arrays), using the Machery Nagel Nucleospin II kit (Machery and Nagel). Biotin labelling, cRNA synthesis, hybridization to Affymetrix Drosophila 2.0 GeneChips and array readouts were performed by ServiceXS (www.servicexs.com).

All statistical analyses were performed using R (R Core Team 2013). Virgin and mated lifespan were analyzed using Cox proportional hazard regression models and fecundity was analyzed using a general linear model (GLM) with a Poisson distribution.

Gene expression data pre-processing

Prior to normalization we assessed the quality of the arrays and identified outliers using the simpleaffy R package (Wilson & Miller 2005) and Principle Component Analysis (Pearson 1901). Based on these results we excluded two female and four male samples from further analysis due to insufficient quality. These excluded samples were evenly distributed and thus no experimental group contained fewer than three biological replicates. Subsequently we performed background adjustment, quantile normalization and summarization using the robust multi-array average (RMA) algorithm (Irizarry et al. 2003) on the remaining 138 samples. We found that if males and female samples were normalized together, 92% of the variation in expression was due to sex, so we chose instead to normalize male and female samples separately to emphasize the effects of diet and age rather than ubiquitous and

well-documented sex-specific differences (Ayroles et al. 2009). We performed all subsequent analysis steps separately for each sex using R (version 3.0.0) and Bioconductor (Gentleman et al. 2004).

Assessing the relative contribution of larval diet, adult diet, and age to variation in gene expression

To understand the major factors driving the variation in the transcriptomics data we applied principal components analysis (PCA; Pearson 1901) and principal variance components analysis (PVCA; Bushel 2013). PVCA is a supervised version of PCA that partitions the proportion of total variation attributable to treatment factors, and thus estimates the total variation in the gene expression data explained by larval diet, adult diet, age, and their interactions.

To understand the factors driving expression at a per probe level we fitted an ANOVA model to each expressed transcript following Ayroles et al., (2009). For each transcript the model partitioned the variation in expression between larval diet (L), adult diet (A) and age (T), as well as the interactions between these factors (L x A; L x T; A x T; L x T x A). We then filtered the data to obtain individual lists of genes affected by each of these factors at a False Discovery Rate of 0.05 (FDR; Benjamini & Hochberg 1995). For all main effect gene lists (L, A, T) we applied an additional filtering step to remove all genes that also showed a significant interaction with another factor. For example, the "L" gene list contains transcripts whose expression was significantly affected by larval diet at an FDR of 0.05, but with no significant interaction between larval diet and any other factors.

Assessing expression differences between larval diets

We next grouped probes affected by larval diet into clusters of genes showing similar expression profiles by applying K-means clustering to the z-score transformed expression values for each sex and for each of the effects (L, L*T, L*A, L*T*A). We then addressed whether the clusters showed any evidence of shared biological function by applying three additional analysis steps to each cluster: first we assessed enrichment of gene ontology (GO) terms using DAVID v6.7 (The Database for Annotation, Visualization and Integrated Discovery; Huang et al. 2008). We focussed on "GO FAT" terms, which eliminate term redundancy and increase specificity of gene ontology analysis. Second, we checked for over-representation of probes expressed only in particular tissues by using the FlyAtlas database which contains gene expression data from individual Drosophila tissues (Chintapalli et al. 2007). We filtered the FlyAtlas dataset to extract lists of genes that were exclusively expressed in a single tissue (Appendix 1) and then checked for over-representation of tissue-specific lists in each of the clusters using a hypergeometric test. Finally, we checked for significant overlap in cluster composition between the sexes using a hypergeometric test.

Table 1: Analysis of deviance for each phenotype, indicating the relative effect size of adult diet, larval diet and their interaction per sex relative to the null model with no factor effects. For Cox proportional hazard models and GLMs with Poisson distribution the chi-squared test is most appropriate.

Phenotype	Sex	Factor	Log likelihood	χ^2	df	p value
Virgin Lifespan	Female	Null model	-22555.16			
		Adult diet (A)	-21754.99	1600.34	2	<0.001
		Larval diet (L)	-21679.02	151.94	2	<0.001
		A * L	-21667.75	22.53	4	<0.001
	Male	Null model	-19930.17			
		Adult diet (A)	-19757.87	282.18	2	<0.001
		Larval diet (L)	-19898.96	62.42	2	<0.001
		A*L	-19747.86	20.01	4	<0.001
Mated Lifespan	Female	Null model	-4927.58			
		Adult diet (A)	-4894.87	65.42	2	<0.001
		Larval diet (L)	-4893.09	3.55	2	0.171
		A * L	-4886.69	12.79	4	0.012
	Male	Null model	-3919.84			
		Adult diet (A)	-3916.34	3.77	2	0.151
		Larval diet (L)	-3918.23	3.23	2	0.200
		A*L	-3911.46	9.74	4	0.045
Early Fecundity	Female	Adult diet (A)		3100.10	2	<0.001
		Larval diet (L)		94.98	2	<0.001
		A * L		4.49	4	0.343
Late Fecundity	Female	Adult diet (A)		8461.28	2	<0.001
		Larval diet (L)		91.94	2	<0.001
		A * L		53.53	4	<0.001

RESULTS

Adult diet is the primary determinant of adult phenotypes but is consistently and significantly influenced by larval diet

In both sexes, most of the variation in virgin lifespan was explained by adult diet (Table 1). For females, lifespan peaked on the control adult diet (Fig.2a), while for males, lifespan increased with increasing adult diet (Fig.2b). Larval diet, by contrast, explained a smaller, though still highly significant proportion of the variation in virgin lifespan (Table 1). Furthermore, it affected lifespan similarly in both sexes and consistently across adult environments (Fig. 2a; Fig. 2b). The rich larval diet decreased lifespan across adult diets, while the poor larval diet increased lifespan (Fig. 2a,b; p-values for all pairwise comparisons

between larval diets given in Appendix 1). In both sexes, there was a relatively weak interaction between larval and adult diet (Table 1) due to the fact that differences in lifespan between larval diets were smaller on the poor adult diet and not always significant (Appendix 1).

In a similar fashion to virgin lifespan, mated fecundity depended primarily on the adult diet (Table 1), both early in life (Fig. 2e) and late in life (Fig.2f). In the first week of life, adult diet and larval diet both affected fecundity independently but with opposing effects: as adult diet increased fecundity increased across all larval diets (all p values <0.0001), while as larval diet increased fecundity decreased across all adult diets (all p values <0.01,Fig. 2e). Thus, as was the case for virgin female lifespan (Fig. 2a), larval diet had a consistent effect on female fecundity across adult diets in early life. After the first week of adult life, however, the differences in fecundity between larval diets disappeared on the poor and control adult diets (all p-values >0.05), but persisted on the rich adult diet (all p-values <0.02; Fig.2f), resulting in a significant interaction between larval and adult diet in determining late fecundity (Table 1).

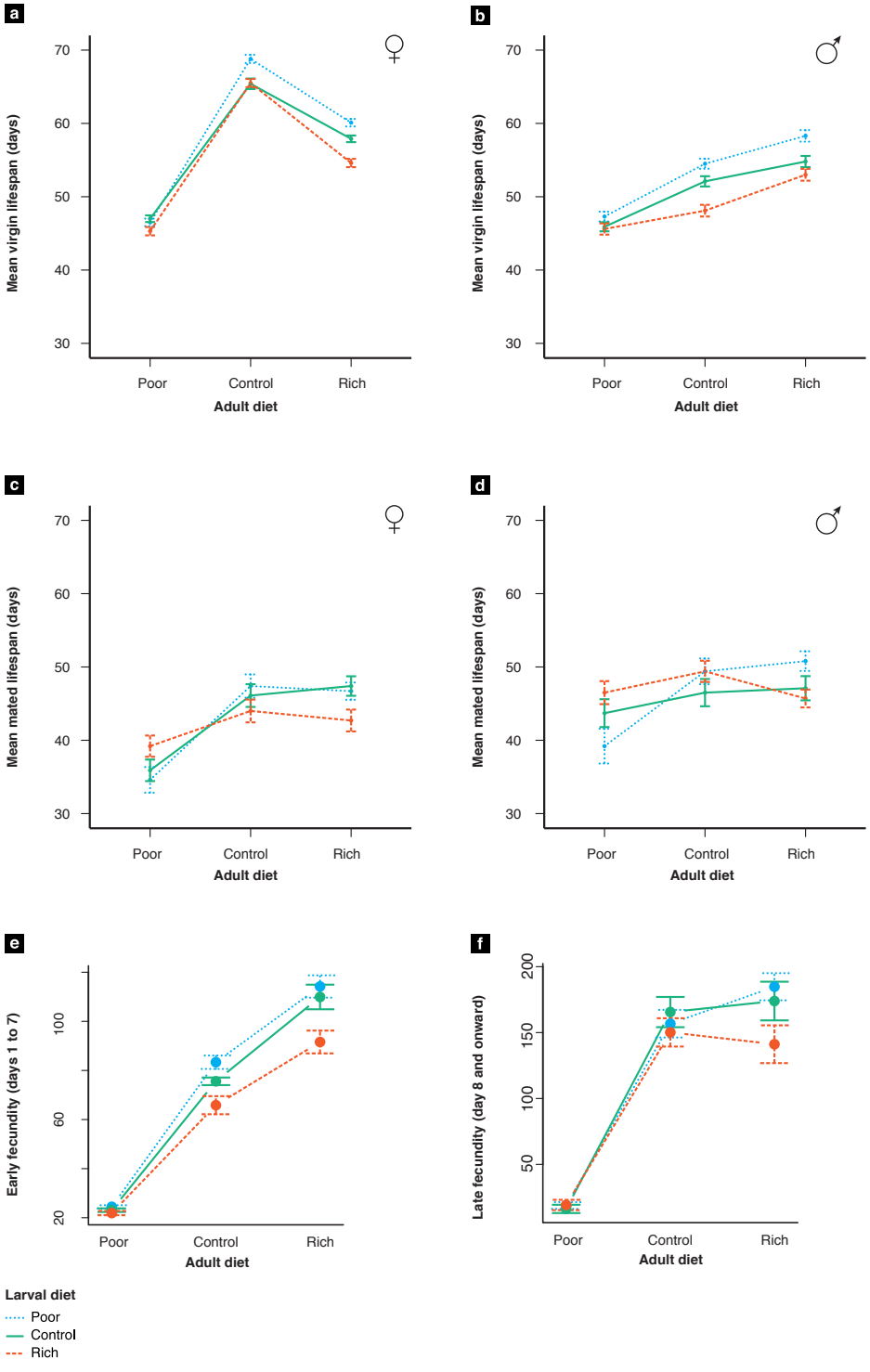
Mated lifespan was much less sensitive to larval and adult diet and showed considerably more variability (Table 1). In males, adult diet had no overall effect on lifespan, while in females the poor adult diet shortened lifespan (Females: Fig. 2c; Males: Fig. 2d). In addition, larval diet affected mated lifespan in each sex only under particular adult diets (Appendix 1). Under rich adult dietary conditions flies raised on the rich larval diet (RR) had decreased mated lifespan relative to PR and CR flies in both sexes (Females: Fig. 2c; Males: Fig. 2d; Appendix 1). In females they also had decreased lifespan under control adult conditions (Fig. 2c; Appendix 1).

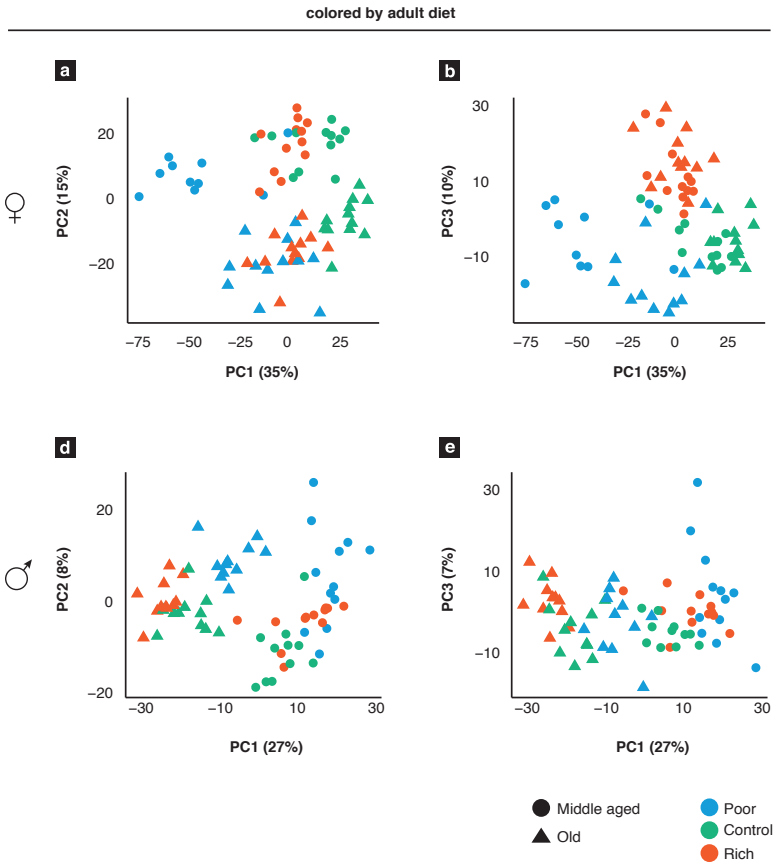
Overall, there was no phenotypic evidence that fitness was negatively affected by mismatches between developmental and adult environments, as proposed by the PAR and programming hypotheses. Rather, the superior lifespan and fecundity of flies raised on the poor larval diet across the adult diets is consistent with the silver spoon hypothesis.

Pervasive effects of adult diet and age, and more subtle effects of larval diet on adult gene expression variation

We observed that virgin lifespan depended predominantly on adult diet. However, within an adult diet treatment, larval diet consistently modulated the

Figure 2: Responses of adult life history traits to adult diet (along x-axis) and larval diet (indicated by colour coding). Mean female (a) and male (b) virgin lifespan. Mean female (c) and male (d) mated lifespan. Mean early (e) and late (f) mated female fecundity. All values are means ± standard error.


a
b
c
d
e
f
Mean virgin lifespan (days)
Mean mated lifespan (days)
Early fecundity (days 1 to 7)
Late fecundity (day 8 and onward)
Poor
Control
Rich
Adult diet
Larval diet
Poor
Control
Rich



overall magnitude of the response in both sexes. Having established that larval diet does indeed play a role in determining adult lifespan we next assessed whether it also had any effect on the transcriptome of middle- and old-aged virgin flies sampled from the same cohort.

We first evaluated how larval diet, adult diet, and age related to the largest principal components of gene expression variation. In both sexes, PCA revealed clear segregation between age classes (middle and old-age) and adult diets when plotting PC1 versus PC2 and PC1 versus PC3, but no clear segregation between larval diets (Fig. 3). In females, PC1 divided flies on the poor adult diet from those on control, while flies on the rich adult diet fell in between and did not form a distinct cluster (Fig. 3a). PC2 separated middle-aged flies (circles) from old flies (Fig. 3a), and PC3 separated flies on the rich adult diet from those on poor and control (Fig. 3b). In males, the largest component of variation (PC1) separated young flies (circles) from old flies (triangles) and within these age-classes also separated flies roughly by adult diet (Fig. 3d), while PC2 further separated flies living on the poor adult diet from the rest (Fig. 3d). When labeling samples by larval diet, no clear pattern emerged when plotting PC1

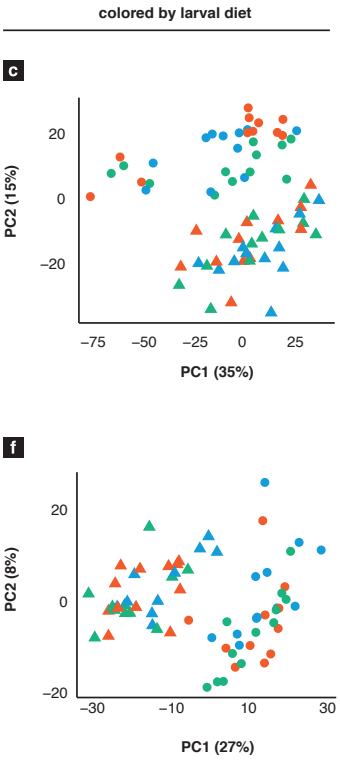


Figure 3: Global patterns of gene expression variation across the lifespan in response to larval diet, adult diet, and age. Scatterplots of PC1 versus PC2 (a, c, d, f) and PC1 versus PC3 (b, e) in females (top row) and males (bottom row). In (a, b, d and e) samples are coloured by adult diet and shape represents age, while in (c) and (f) colour indicates larval diet. In females PC1 roughly separates samples by adult diet (a), this is especially visible when plotted against PC3 (b). PC2 separates female samples by age (a). In males PC1 separates samples by age and within each age class also roughly separates samples by adult diet (d). PC2 in males further segregates flies living on the poor adult diet from the rest (d). When colouring PC1 versus PC3 by larval diet (c and f), no clear grouping is visible.

versus PC2 in females (Fig. 3c) and males (Fig. 3f), suggesting that larval diet does not play a role in the major components of transcriptional variation.

The ANOVA and PVCA analyses broadly confirmed the patterns observed in the PCA (Table 2). In both cases and both sexes, most of the variation in expression (PVCA) and significant probes (ANOVA) was due to adult diet and age. PVCA attributed 32.5 and 22.7% of the total observed variation in expression in females to adult diet and age respectively, and 15.5 and 35.6% in males, while ANOVA identified 4504 and 3589 probes affected by adult diet and age in females and 2688 and 6111 probes in males (Table 2). Thus the relative importance of adult diet and age is reversed between the sexes, the transcriptome being more responsive to adult diet in females and age in males (Table 2). However, in females there are considerably more probes showing an interaction between adult diet and age (TA; 3832 probes) than in males (TA; 806 probes) suggesting that the effect of age in females is more strongly contingent on the adult diet experienced.

Table 2: Overall effects of larval diet, adult diet, age, and their interaction on global patterns of transcriptional variation as determined by principal variance components analysis (PVCA) and ANOVA analysis. The "% of probes" category shows the percentage of probes affected as a fraction of the total number of probes affected by any factor in each sex and sums to more than 100% because many probes show effects of several factors (Males : 10164 probes; Females : 11695).

	Male			Female		
Factor	**PCAV (%)**	**Probes**	**% of probes**	**PCAV (%)**	**Probes**	**% of probes**
Larval diet(L)	4.4	1999	19.7	1.1	321	2.7
Adult diet(A)	15.5	2688	26.6	32.5	4504	38.5
Age(T)	35.6	6111	60.4	22.7	3589	30.7
LA	1.1	140	1.4	1.5	770	6.6
LT	0.8	240	2.4	0.7	170	1.5
TA	4.1	806	8.0	8	3832	32.8
LTA	-	288	2.8	-	464	4.0
Residual	38.6	-	-	33.5	-	-

ANOVA analysis also identified effects of larval diet on the transcriptome, both as a main effect and in interaction with the other treatments, though in both sexes the effect was considerably smaller than the main effects of adult diet or age (Table 2). In males, we identified 2,667 probes showing an effect of larval diet, either as a main effect or in interaction with other factors, while in females we identified 1725 probes. In males, the majority of probes showed a main effect of larval diet (1999 probes), while in females, the largest group of probes were those showing an interaction between larval diet and adult diet (770 probes; Table 2). Thus there is a persistent effect of developmental diet on the adult transcriptome into middle- and old age, though it is considerably smaller than the effects of adult diet and age, and furthermore, the degree of interaction with adult conditions and age is sex-dependent.

After quantifying the relative effect of larval versus adult diet on the transcriptome, our next aim was to address whether we could link the observed phenotypic responses to larval diet to the patterns of transcriptional variation. For each list of probes affected by larval diet (L) or its interaction with adult diet (LA), age (LT) or both (LTA), we applied K-means clustering to identify probes showing similar expression profiles. For each cluster we checked for overlap of cluster composition between the sexes, overlap with genes with tissue-specific expression profiles mined from FlyAtlas, and for significant gene ontology annotation. The results of all three of these analyses for each cluster are provided in Appendix 2 and 3. Given that the phenotypic effect of larval diet

Table 3: Gene ontology term annotation of male Cluster 9 and female Cluster 3. Italics indicate GO terms that are significant in both clusters. BP: Biological process; CC: Cellular component; MF: Molecular function.

Cluster	Category	GO ID	Term	P-value
C9 Male	BP	GO:0006396	RNA processing	0.000
	BP	GO:0034660	ncRNA metabolic process	0.000
	BP	GO:0034470	ncRNA processing	0.000
	MF	GO:0000166	nucleotide binding	0.000
	CC	GO:0031981	nuclear lumen	0.000
	MF	GO:0001882	nucleoside binding	0.000
	BP	GO:0016071	mRNA metabolic process	0.000
	MF	GO:0005524	ATP binding	0.000
	MF	GO:0032559	adenyl ribonucleotide binding	0.000
	BP	GO:0006397	mRNA processing	0.000
	MF	GO:0030554	adenyl nucleotide binding	0.000
	MF	GO:0001883	purine nucleoside binding	0.000
	BP	GO:0006399	tRNA metabolic process	0.000
	CC	GO:0070013	intracellular organelle lumen	0.000
	CC	GO:0043233	organelle lumen	0.000
	CC	GO:0031974	membrane-enclosed lumen	0.000
	MF	GO:0017076	purine nucleotide binding	0.000
	MF	GO:0032553	ribonucleotide binding	0.000
	MF	GO:0032555	purine ribonucleotide binding	0.000
	BP	GO:0022613	ribonucleoprotein complex biogenesis	0.002
	MF	GO:0003723	RNA binding	0.000
	BP	GO:0008380	RNA splicing	0.004
	BP	GO:0042254	ribosome biogenesis	0.004
	MF	GO:0004386	helicase activity	0.001
	CC	GO:0005730	nucleolus	0.003
	MF	GO:0008186	RNA-dependent ATPase activity	0.003
	MF	GO:0004004	ATP-dependent RNA helicase activity	0.003
	BP	GO:0006364	rRNA processing	0.012
	BP	GO:0016072	rRNA metabolic process	0.012
	BP	GO:0006360	transcription from RNA polymerase I promoter	0.021
	CC	GO:0005654	nucleoplasm	0.011
	BP	GO:0006418	tRNA aminoacylation for protein translation	0.022
	BP	GO:0043039	tRNA aminoacylation	0.022
	MF	GO:0016875	ligase activity, forming carbon-oxygen bonds	0.007
	MF	GO:0016876	ligase activity, forming aminoacyl-tRNA	0.007
	MF	GO:0004812	aminoacyl-tRNA ligase activity	0.007
	BP	GO:0043038	amino acid activation	0.023
	MF	GO:0016779	nucleotidyltransferase activity	0.007
	MF	GO:0034062	RNA polymerase activity	0.006
	MF	GO:0003899	DNA-directed RNA polymerase activity	0.006
	MF	GO:0003724	RNA helicase activity	0.006
	BP	GO:0035196	gene silencing by miRNA, production of miRNAs	0.026
	BP	GO:0035195	gene silencing by miRNA	0.028
	MF	GO:0008026	ATP-dependent helicase activity	0.013
	MF	GO:0070035	purine NTP-dependent helicase activity	0.013
	BP	GO:0000398	nuclear mRNA splicing, via spliceosome	0.047
	BP	GO:0000377	RNA splicing, via transesterification reactions with adenosine	0.047
	BP	GO:0000375	RNA splicing, via transesterification reactions	0.047
	MF	GO:0003729	mRNA binding	0.015
	MF	GO:0042624	ATPase activity, uncoupled	0.018
	MF	GO:0004540	ribonuclease activity	0.021
	MF	GO:0032549	ribonucleoside binding	0.029
	MF	GO:0003677	DNA binding	0.031
	MF	GO:0000287	magnesium ion binding	0.038
	MF	GO:0016887	ATPase activity	0.040
C3 Female	BP	GO:0009451	RNA modification	0.002
	CC	GO:0030529	ribonucleoprotein complex	0.001
	BP	GO:0034660	ncRNA metabolic process	0.004
	BP	GO:0034470	ncRNA processing	0.003
	BP	GO:0042254	ribosome biogenesis	0.006
	BP	GO:0022613	ribonucleoprotein complex biogenesis	0.027
	CC	GO:0022625	cytosolic large ribosomal subunit	0.020

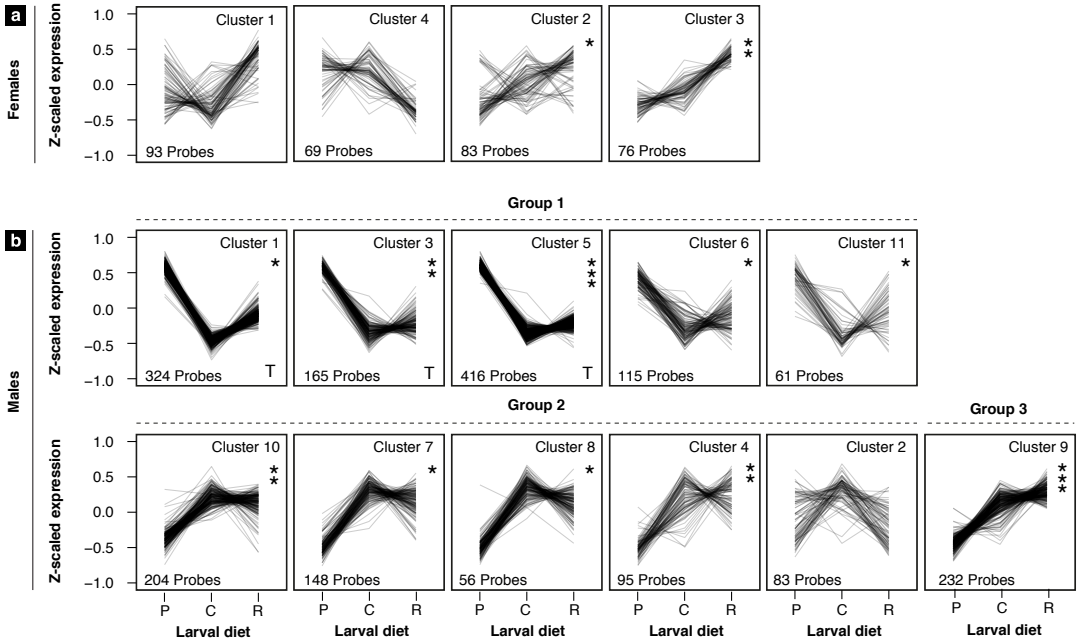


Figure 4: Expression profiles of clusters of probes affected by larval diet across adult diets and age classes in females (a) and males (b). In males (b) clusters can be roughly classified into three different groups showing similar expression profiles. Clusters in the first group (Group 1) are most highly expressed in poor-raised flies and least expressed in control-raised flies, while rich raised flies fall in between. They differ primarily in the tightness of co-expression, with rich-raised flies showing the most variation. Clusters 1, 3 and 5 in Group 1 are very significantly enriched for genes with testes-specific expression, and for testes-specific GO terms and contain nearly half of all probes affected by larval diet in males. The second group (Group 2) shows the inverse expression profile of Group 1 and clusters also differ primarily in the tightness of co-expression, especially in rich-raised flies. The last group (Group 3) consists of one cluster whose expression is positively correlated with increasing larval diet. This cluster significantly overlaps in cluster composition with female cluster 3 (a) as well as sharing a similar expression profile and GO enrichment for terms related to ribosomes and transcription and translation. Clusters of probes with similar expression profiles were identified using K-means clustering. * indicates number of significant GO terms associated with a cluster *:1 term, **: 2 to 9 terms,***: 10 or more terms.

was similar both across sexes and across larval diet, the simplest hypothesis is that genes related to the phenotypes would have similar expression profiles across adult diets and across ages: i.e. that they would fall into the "L" category. Indeed, given the consistent phenotypic responses it is very difficult to envision how such linear responses could be attributable to interactions between larval diet and adult diet and/or age (though this possibility cannot be definitively excluded). Thus we focus on the probes showing a main effect of larval diet (L), and provide the results for the probes showing an interaction (LT, LA, LTA) in Appendices 4, 5, and 6 respectively. However, it is noteworthy that nearly all of the clusters of probes showing interactive effects primarily differ in the response of poor raised flies to age, adult diet, or their interaction, suggesting that while they may be linked to the effects of the poor larval diet on lifespan, they likely do not explain the lifespan differences observed between control and rich-raised flies.

The expression of ribosome-related probes is positively correlated with larval diet in females

K-means clustering of the relatively small female "L" list (321 genes) identified 4 clusters (Fig. 4a). Clusters 1 and 4 were not associated with any significant GO enrichment nor did they show any evidence of enrichment of tissue-specific genes (Appendix 2 and 3). Cluster 1 had variable expression in poor, low expression in control, and high expression in rich-raised flies, while cluster 4 had roughly the opposite expression pattern (Fig. 4a). The two remaining clusters, Clusters 2 and 3, had expression profiles characterized by increasing expression with increasing larval diet (Fig. 4a). This pattern was particularly evident in cluster 3. Both clusters possessed significant gene ontology annotation related to ribosomes – cluster 2 (83 probes) was solely annotated with the term cytosolic ribosome while cluster 3 (76 probes) was annotated with the terms RNA modification, ribonucleoprotein complex, ncRNA metabolic process, ncRNA processing, ribosome biogenesis, ribonucleoprotein complex biogenesis, and cytosolic large ribosomal subunit (Table 3), and contained several sub-units of the 60s large ribosomal sub-unit (RpL3, RpL18, RpL7-like, RpL22 and RpL34a).

Larval diet affects expression of testes-specific genes across the lifespan in males

In males we identified 11 clusters of genes in the "L" list which could be grouped into three different expression profiles (Fig. 4b). The first group (Clusters: 1, 3, 5, 6, and 11) was representative of more than 50% of the probes affected by larval diet (1081 probes) and was characterized by high expression in poor raised flies, low expression in control raised flies, and intermediate expression in rich raised flies (Fig. 4b). The clusters differed primarily in the tightness of co-expression, especially in rich-raised flies. The second group (Clusters 10, 7, 8, 4 and 2) was

notable for having an inverse expression profile relative to Group 1 (Fig. 4b). These clusters also differed primarily in the tightness of co-expression, again, especially in rich-raised flies. The third group consisted of only a single cluster (Cluster 9) which was the only cluster to show a positive expression correlation with increasing larval diet (Fig. 4b).

In contrast to the females, which showed no tissue-specific enrichment, three of the male clusters (C1, C3, and C5 - all in Group 1) were highly enriched for probes exclusively expressed in the testes (hypergeometric test: all p-values <0.0001; Appendix 2), most differentially expressed in the testes relative to the whole body, and most highly expressed in the testes in the FlyAtlas data set (all p-values <0.01). This overlap also extends to the GO terms associated with these clusters as they overlap substantially with those enriched for probes highly expressed in the testes in FlyAtlas (C1: 100% overlap, C3: 67% overlap, C5: 96% overlap; Appendix 2). Thus a large proportion of the effect of larval diet in males is through changes in the expression of groups of genes with testes-specific expression and function, and may explain why we identified considerably less genes affected by larval diet in females. The two remaining clusters in Group 1 (Clusters 6 and 11) show no evidence of tissue-specific expression and are solely annotated with the GO terms neurological system process (C6) and (intracellular) non-membrane bound organelle (C11;Appendix 1). The second group of clusters (Clusters 10, 7, 8, 4 and 2; Fig. 4b), was representative of most of the remaining probes (818 probes). None of these clusters showed any significant overlap with genes with testes-specific expression (all p-values >0.94), nor with any other tissue. Furthermore, each of the clusters except cluster 2 possessed distinct GO annotation including terms related to mitochondrial ribosomes (C4), serine-type peptidase activity (C7), transcription (C8), and the break-down of peptidoglycan bonds and immune function (C10; all terms in Appendix 2) suggesting that these clusters represent an array of different processes.

The third expression pattern we identified consisted of one cluster (Cluster 9; Fig. 4b) that contained probes whose expression increased with increasing larval diet (Cluster 9; 232 probes, Fig. 4b). It showed no evidence of enrichment of tissue-specific probes (Appendix 2) and was annotated with 56 GO terms related to ribosome structure, function and regulation as well as to other aspects of transcription and translation including tRNA metabolic activity and RNA polymerase activity (Table 3), and contained sub-units of all three eukaryotic RNA polymerases' (RpII140, RpI135, RpIII128 and RpI1). The probe composition of this cluster overlapped significantly with that of female cluster 3 (Hypergeometric test; p<0.0001), which showed a similar response to larval diet. The overlapping genes were CDKAL1-like, CG32409, CG6769,

Notches (*nle*) and Elongator complex protein 2 (*elp2*). All five of these genes are either annotated with GO terms related to ribosome biogenesis and function (ribosome biogenesis: CG32409; ribosomal large subunit biogenesis: CG6769) or are known to play crucial roles in ribosome biogenesis (nle; Le Bouteiller et al. 2013; Ulbrich et al. 2009) or in transcription and translation (elp2; Glatt & Müller 2013; Otero et al. 1999) (CDKAL-1; Wei et al. 2011). Furthermore, of the seven GO terms associated with female cluster 3, four were also associated with male cluster 9. These terms were ncRNA metabolic process, ncRNA processing, ribosome biogenesis and ribonucleoprotein complex biogenesis (Table 3). Taken together, these results suggest that the up-regulation of genes involved in ribosome biogenesis and transcription and translation is a conserved effect of increasing larval diet in both sexes.

DISCUSSION

Phenotypic responses to developmental diet follow the silver spoon hypothesis in Drosophila

Our first aim in this study was to determine the nature of the relationship between developmental and adult diet in fruit flies, particularly with respect to the silver spoon (Grafen 1988), programming (Ozanne & Hales 2004) and predictive adaptive response hypotheses (Gluckman & Hanson 2004a): the former predicting that individuals raised in a low quality environment will be of relatively poorer quality and have a disadvantage across adult environments and the two latter hypotheses predicting that the effect of the developmental environment will depend on the degree of mismatch between predicted and actual adult conditions. Our finding that all larval diets show a similar response to adult dietary conditions, particularly in terms of virgin lifespan and early fecundity, is more consistent with the predictions of the silver spoon hypothesis. In fact, we see that flies raised on the different larval diets differ only in their average performance, with poor-raised flies tending to live longer (Fig. 2a,b) and reproduce more in early life (Fig. 2e), than those raised on the rich larval diet.

Importantly, adult diet explained considerably more variation than larval diet in terms of both phenotypes (Fig. 2) and gene expression (Fig. 3; Table 2). Thus while larval diet does result in long-term changes in phenotypes and gene expression, adult fruit flies retain extensive plasticity with respect to their ability to respond to adult diet. This suggests that the developmental environment is not a reliable predictor of adult conditions and thus there is no evolutionary advantage to inflexibly "setting" a phenotype to predicted adult conditions in fruit flies. Rather it is more likely that the effect of developmental diet on adult phenotypes is due to the persistence of changes that either increased fitness during development, or were unavoidable consequences of a (sub-optimal) developmental environment. Such explanations have also been proposed as

alternatives to the PAR hypothesis as applied to humans (Rickard & Lummaa 2007; Wells 2012), and a few studies have also shown that poor developmental conditions are detrimental regardless of adult conditions in some human cohorts (Hayward & Lummaa 2013; Hayward et al. 2013). In any case, the evolutionary history of humans and flies are very different, thus the absence of PARs in flies does not give any information about their role in humans, however, as has been shown for dietary restriction, the mechanisms underlying the effect may be similar, even if the adaptive significance of the response differs (e.g. van den Heuvel et al., submitted modelling paper).

Long-term effects of larval diet on the transcriptome

Despite the lack of evidence for PARs, we identified long-term effects of larval diet on the transcriptome suggesting that fruit flies are unable to, or do not benefit from, completely erasing the legacy of their developmental conditions. Given our use of whole flies, these changes could reflect change in tissue size, regulation or both (Harrison et al. 2015), and presumably some of them relate to the observed phenotypic differences. However, despite the relatively consistent effect of larval diet on adult phenotypes across adult environments and across sexes, most of the probes affected by larval diet show no linear relationship with the phenotypes, either because they are involved in an interaction with adult diet, age or both (Appendices 4 to 6), or because they showed a main effect of larval diet, but not in a direction consistent with a linear effect of diet. For example, ten of the eleven clusters of probes affected by larval diet in males were characterised by intermediate expression in rich-raised flies, while in terms of phenotypes, rich-raised flies are both most short-lived (males and females) and least fecund (females). This suggests that if the expression of these probes is related to lifespan then the relationship between their expression and lifespan is non-linear (e.g. Lebedeva et al. 2012; Meyer et al. 2014; Qu & Xu 2006). The ability to conclude this is a consequence of including three diets in our design. Had we included only two in a classical case-control analysis we would have reached contrasting conclusions on the relationship between gene expression and lifespan for these ten clusters. For example if we used poor and control only, it would have been logical to infer that the genes up and down-regulated in poor-raised flies relative to control contribute linearly to the phenotypic differences observed between the two, however, had we compared control to rich, we would have concluded that the relationship between expression levels and phenotypic values was in the opposite direction. The risk of such misguided interpretation in a two-factor design strongly warrants the inclusion of three or more environments, especially when establishing the causality between the life history (lifespan, ageing) phenotype and some measure of molecular and/or genetic variation is difficult.

Intriguingly, three of the male clusters with intermediate expression in rich

raised flies (Fig. 4b) were highly enriched for probes with testes-specific expression and annotation (C1, C3, C5), suggesting that the effects of larval diet on the male transcriptome may be largely through changes in the expression of testes-related genes. Whether these changes are related to the observed lifespan differences is unclear, however, if they are, it would imply a "U" shaped relationship between the expression of testes-related genes and lifespan in males. These changes in expression may reflect changes in relative testes size induced by larval diet, or changes in the regulation of testes-specific gene expression (Harrison et al. 2015). In either case, because we used whole bodies, a relative up-regulation of testes-specific genes would by definition mean a relatively smaller proportion of non-testes mRNA in the whole body pool. This is one potential explanation for the nearly perfect inverse expression profile of the Group 2 clusters relative to Group 1 (which contains C1, C3 and C5; Fig. 4b). These clusters may simply reflect the "down-regulation" of other processes as a result of up-regulation of testes specific genes. This seems especially plausible given the lack of similarly in GO annotation between the Group 2 clusters, suggesting that they represent a variety of functions (Appendix 2). In addition, several recent studies have shown that a calorically-poor developmental diet leads to a relatively larger genital arch in male flies (Shingleton et al. 2009; Tang et al. 2011). If this extends to other sexual traits it is possible that a similar response has occurred in the testes. Regardless of the mechanism, it is clear that larval diet, particularly the poor larval diet, disproportionately affects the expression of testes-specific genes across the lifespan in males.

Larval diet affects the expression of probes related to ribosomes and translation in both sexes across the lifespan

As mentioned previously, the consistent effect of larval diet on phenotypes across adult diets and in both sexes suggests that the simplest relationship between gene expression and phenotype would be through larval-diet induced changes in the expression of genes that also show similar responses across adult diets and in both sexes. Indeed, we identified a single cluster of probes showing a linear relationship with larval diet in both sexes (Females C3, Males C9; Fig. 4). Both of these clusters show increasing expression with increasing larval diet, across adult diets and age classes and are thus negatively correlated with the observed lifespan differences (Fig. 4). Furthermore, these clusters show significant overlap in probe composition and share similar GO annotation: the female cluster being enriched for terms relating to ribosomes and ncRNA processing (Table 2) and the male cluster also being enriched for terms related to ncRNA and ribosomes, as well as many other terms related to transcription and translation (Table 2). Given the ubiquitous and high expression of ribosomes and the essential roles of transcription and translation in cellular homeostasis, changes in the regulation of these processes across the lifespan may have important consequences for phenotypes, particularly

lifespan. Indeed, in model organisms (primarily flies, yeast, and worms) it has been shown that the down-regulation or knock out of ribosomal sub-units results in increased lifespan (Chiocchetti et al. 2007; Curran & Ruvkun 2007; Kaeberlein et al. 2005; Larson et al. 2012; McCormick et al. 2015; Steffen et al. 2008). The same is true of down-regulation of translation through the knock-out of various translation initiation factors (Chen et al. 2007; Curran & Ruvkun 2007; Hansen et al. 2007; Pan et al. 2007). Thus there is evidence that increased expression of ribosomes and transcription and translation machinery would decrease lifespan, consistent with the negative correlation between expression of the male and female clusters and adult virgin lifespan. Taken together this suggests that these clusters are attractive candidates for mediating the effect of larval diet on lifespan.

Intriguingly, a recent study has also found an effect of larval diet on the expression of ribosomes in early adulthood. Branco et al., (2014) subjected flies to development on medium supplemented with the environmental toxin BPA, high sugar, or both and measured whole-body transcription in adulthood. Intriguingly, they found that BPA alone, but especially in combination with high sugar during development lead to increased expression of many structural components of the ribosome in adulthood. Because BPA is a known toxin, the stronger response of the transcriptome in high sugar environments suggests that high sugar is also toxic (Branco & Lemos 2014). This suggests that the increasing levels of sugar and yeast used in our study may also reflect a form of toxicity, and that some legacy of this persists across the lifespan. In any case, regardless of the cause of the changes, we show that long-lasting developmental effects are visible at the transcriptome level, a prerequisite for long-term effects on adult phenotypes.

CONCLUSIONS

Here we addressed how developmental and adult diet affect adult phenotypes and gene expression in the fruit fly. We found that for the most part larval and adult diet exerted independent effects on the phenotype and on gene expression, and thus there was no evidence for Predictive Adaptive Responses operating in Drosophila melanogaster. Rather, the responses followed the silver spoon hypothesis which predicts that the effect of developmental conditions will be similar across adult conditions. Furthermore, adult diet explained considerably more variation in gene expression and phenotypes than larval diet, showing that flies retain extensive plasticity into adulthood, and suggesting that the long-term effects of developmental diet likely reflect the inability or lack of incentive to erase such effects, rather than an adaptive response. We do find that some genes retain a legacy of developmental diet in their expression into middle and old-age. Many of these genes show no linear correlation with the observed phenotypic responses, however, in both sexes, we identify a cluster of genes whose expression is negatively correlated with the observed

lifespan differences and that are enriched with terms related to transcription and translation, particularly with respect to ribosomes. Given several recent studies which show that the down-regulation of ribosomes and other aspects of transcriptional and translational machinery increases lifespan these genes provide promising candidates for mediating the long-term effects of larval diet on lifespan. As these processes are highly conserved across the tree of life our results may be relevant for other species as well, including for humans.

ACKNOWLEDGEMENTS

We would like to thank Dennie Helmink, Marijke Slakhorst and Bertha Koopmaanschap for help with fly rearing and the set-up of the experiment. We would also like to thank Fons Debets, Jelle Zandveld, Florien Gorter and Claudio Valero-Jimenez for helpful discussions on the experimental design and interpretation of the results. This work was supported by the European Union's FP6 Programme (Network of Excellence LifeSpan FP6/036894) and the EU's FP7 Programme (IDEAL FP7/2007-2011/259679).

REFERENCES

Ayroles JF, Carbone MA, Stone EA, *et al.* (2009) Systems genetics of complex traits in *Drosophila melanogaster*. *Nature Genetics* **41**, 299-307.

Bauer J, Antosh M, Chang C, *et al.* (2010) Comparative transcriptional profiling identifies takeout as a gene that regulates life span. *Aging*.

Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society. Series B (Methodological)* **57**, 289-300.

Branco AT, Lemos B (2014) High intake of dietary sugar enhances bisphenol A (BPA) disruption and reveals ribosome-mediated pathways of toxicity. *Genetics* **197**, 147-157.

Burdge GC, Hanson, MA, Slater-Jefferies, JL and KA Lillycrop. (2007) Epigenetic regulation of transcription: a mechanism for inducing variations in phenotype (fetal programming) by differences in nutrition during early life? *British Journal of Nutrition* **97**, 1036-1046.

Burdge GC, Lillycrop KA (2010) Nutrition, epigenetics, and developmental plasticity: Implications for understanding human disease. *Annual Review of Nutrition* **30**, 315-339.

Bushel P (2013) pvca: Principal Variance Component Analysis (PVCA).

Chen D, Pan KZ, Palter JE, Kapahi P (2007) Longevity determined by developmental arrest genes in *Caenorhabditis elegans*. *Aging Cell* **6**, 525-533.

Chintapalli VR, Wang J, Dow JAT (2007) Using FlyAtlas to identify better *Drosophila melanogaster* models of human disease. *Nat Genet* **39**, 715-720.

Chiocchetti A, Zhou J, Zhu H, *et al.* (2007) Ribosomal proteins Rpl10 and Rps6 are potent regulators of yeast replicative life span. *Experimental Gerontology* **42**, 275-286.

Chown SL, Hoffmann AA, Kristensen TN, *et al.* (2010) Adapting to climate change: a perspective from evolutionary physiology. *Climate research* **43**, 3.

Curran SP, Ruvkun G (2007) Lifespan Regulation by evolutionarily conserved genes essential for viability. *PLoS Genet* **3**, e56.

Doroszuk A, Jonker M, Pul N, Breit T, Zwaan B (2012) Transcriptome analysis of a long-lived natural *Drosophila* variant: a prominent role of stress- and reproduction-genes in lifespan extension. *BMC Genomics* **13**, 1-16.

Emlen DJ (1997) Diet alters male horn allometry in the beetle *Onthophagus acuminatus* (Coleoptera: Scarabaeidae). *Proceedings of the Royal Society of London B: Biological Sciences* **264**, 567-574.

Fellous S, Lazzaro BP (2010) Larval food quality affects adult (but not larval) immune gene expression independent of effects on general condition. *Mol Ecol* **19**.

Fernandez-Twinn DS, Ozanne SE (2006) Mechanisms by which poor early growth programs type-2 diabetes, obesity and the metabolic syndrome. *Physiology & Behavior* **88**, 234-243.

Gentleman R, Carey V, Bates D, *et al.* (2004) Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol* **5**, R80.

Glatt S, Müller CW (2013) Structural insights into Elongator function. *Current Opinion in Structural Biology* **23**, 235-242.

Gluckman PD, Hanson MA (2004a) The developmental origins of the metabolic syndrome. *Trends in Endocrinology & Metabolism* **15**, 183-187.

Gluckman PD, Hanson MA, Beedle AS (2007) Early life events and their consequences for later disease: A life history and evolutionary perspective. *American Journal of Human Biology* **19**, 1-19.

Grafen A (1988) On the uses of data on lifetime reproductive success. In: *Reproductive success* (ed. Clutton-Brock TH), pp. 454-471. University of Chicago Press.

Hansen M, Taubert S, Crawford D, *et al.* (2007) Lifespan extension by conditions that inhibit translation in *Caenorhabditis elegans*. *Aging Cell* **6**, 95-110.

Harrison PW, Wright AE, Zimmer F, *et al.* (2015) Sexual selection drives evolution and rapid turnover of male gene expression. *Proceedings of the National Academy of Sciences* **112**, 4393-4398.

Hayward AD, Lummaa V (2013) Testing the evolutionary basis of the predictive adaptive response hypothesis in a preindustrial human population. *Evolution, Medicine, and Public Health* **2013**, 106-117.

Hayward AD, Rickard IJ, Lummaa V (2013) Influence of early-life nutrition on mortality and reproductive success during a subsequent famine in a preindustrial population. *Proceedings of the National Academy of Sciences* **110**, 13886-13891.

Huang DW, Sherman BT, Lempicki RA (2008) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protocols* **4**, 44-57.

Irizarry RA, Hobbs B, Collin F, *et al.* (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* **4**, 249-264.

Jannot JE, Bruneau E, Wissinger SA (2007) Effects of larval energetic resources on life history and adult allocation patterns in a caddisfly (Trichoptera: Phryganeidae). *Ecological Entomology* **32**, 376-383.

REFERENCES

Kaeberlein M, Powers RW, Steffen KK, *et al.* (2005) Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients. *Science* **310**, 1193-1196.

Larson K, Yan S-J, Tsurumi A, *et al.* (2012) Heterochromatin formation promotes longevity and represses ribosomal rna synthesis. *PLoS Genet* **8**, e1002473.

Le Bouteiller M, Souilhol C, Beck-Cormier S, *et al.* (2013) Notchless-dependent ribosome synthesis is required for the maintenance of adult hematopoietic stem cells. *The Journal of Experimental Medicine* **210**, 2351-2369.

Lebedeva G, Yamaguchi A, Langdon SP, Macleod K, Harrison DJ (2012) A model of estrogen-related gene expression reveals non-linear effects in transcriptional response to tamoxifen. *BMC Systems Biology* **6**, 1-22.

Lillycrop KA, Phillips ES, Jackson AA, Hanson MA, Burdge GC (2005) Dietary protein restriction of pregnant rats induces and folic acid supplementation prevents epigenetic modification of hepatic gene expression in the offspring. *The Journal of Nutrition* **135**, 1382-1386.

Lindström J (1999) Early development and fitness in birds and mammals. *Trends in Ecology & Evolution* **14**, 343-348.

Madsen T, Shine R (2000) Silver spoons and snake body sizes: Prey availability early in life influences long-term growth rates of free-ranging pythons. *Journal of Animal Ecology* **69**, 952-958.

Magwere T, Chapman T, Partridge L (2004) Sex differences in the effect of dietary restriction on life span and mortality rates in female and male *Drosophila* Melanogaster. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences* **59**, B3-B9.

May CM, Doroszuk A, Zwaan BJ (2015) The effect of developmental nutrition on life span and fecundity depends on the adult reproductive environment in *Drosophila melanogaster*. *Ecology and Evolution* **5**, 1156-1168.

McCormick Mark A, Delaney Joe R, Tsuchiya M, *et al.* (2015) A comprehensive analysis of replicative lifespan in 4,698 single-gene deletion strains uncovers conserved mechanisms of aging. *Cell Metabolism* **22**, 895-906.

Meyer E, Aspinwall MJ, Lowry DB, *et al.* (2014) Integrating transcriptional, metabolomic, and physiological responses to drought stress and recovery in switchgrass (*Panicum virgatum L.*). *BMC Genomics* **15**, 1.

Monaghan P (2008) Early growth conditions, phenotypic development and environmental change. *Philosophical Transactions of the Royal Society B: Biological Sciences* **363**, 1635-1645.

Novoseltsev VN, Arking R, Carey JR, Novoseltseva JA, Yashin AI (2005) Individual Fecundity and Senescence in *Drosophila* and Medfly. *The journals of gerontology. Series A, Biological sciences and medical sciences* **60**, 953-962.

Otero G, Fellows J, Li Y, *et al.* (1999) Elongator, a multisubunit component of a novel RNA Polymerase II holoenzyme for transcriptional elongation. *Molecular Cell* **3**, 109-118.

Ozanne SE, Hales CN (2004) Lifespan: Catch-up growth and obesity in male mice. *Nature* **427**, 411-412.

Pan KZ, Palter JE, Rogers AN, *et al.* (2007) Inhibition of mRNA translation extends lifespan in *Caenorhabditis elegans*. *Aging Cell* **6**, 111-119.

Pearson K (1901) LIII. On lines and planes of closest fit to systems of points in space. *The London, Edinburgh, and Dublin Philosophical Magazine and Journal of Science* **2**, 559-572.

Pletcher S, Macdonald S, Marguerie R, *et al.* (2002) Genome-wide transcript profiles in aging and calorically restricted *Drosophila melanogaster*. *Curr Biol* **12**, 712 - 723.

Qu Y, Xu S (2006) Quantitative trait associated microarray gene expression data analysis. *Molecular biology and evolution* **23**, 1558-1573.

R Core Team (2013) R: A language and environment for statistical computing (ed. Computing RFfS), Vienna, Australia.

Rickard IJ, Lummaa V (2007) The predictive adaptive response and metabolic syndrome: challenges for the hypothesis. *Trends in Endocrinology & Metabolism* **18**, 94-99.

Robertson FW, Sang JH (1944) The ecological determinants of population growth in a *Drosophila* Culture. I. Fecundity of adult flies. *Proceedings of the Royal Society of London B: Biological Sciences* **132**, 258-277.

Roff DA (2001) *Life History Evolution* Sinauer Associates, Sunderland.

Sgro, M. C, Partridge L, Editor: Joseph T (2000) Evolutionary responses of the life history of wild & caught *Drosophila melanogaster* to two standard methods of laboratory culture. *The American naturalist* **156**, 341-353.

Shingleton A (2011) Evolution and the regulation of growth and body size. *Mechanisms of life history evolution. Oxford University Press, Oxford*, 43-55.

Shingleton AW, Estep CM, Driscoll MV, Dworkin I (2009) Many ways to be small: different environmental regulators of size generate distinct scaling relationships in*Drosophila melanogaster*. *Proceedings of the Royal Society B: Biological Sciences*.

REFERENCES

Skorupa DA, Dervisefendic A, Zwiener J, Pletcher SD (2008) Dietary composition specifies consumption, obesity, and lifespan in *Drosophila melanogaster*. *Aging Cell* **7**, 478-490.

Solon-Biet SM, McMahon AC, Ballard JW, *et al.* (2014) The ratio of macronutrients, not caloric intake, dictates cardiometabolic health, aging, and longevity in ad libitum-fed mice. *Cell Metabolism* **19**, 418-430.

Stearns SC (1992) *The Evolution of Life Histories* Oxford University Press, Oxford.

Steffen KK, MacKay VL, Kerr EO, *et al.* (2008) Yeast life span extension by depletion of 60S ribosomal subunits is mediated by Gcn4. *Cell* **133**, 292-302.

Tang HY, Smith-Caldas MSB, Driscoll MV, Salhadar S, Shingleton AW (2011) FOXO Regulates Organ-Specific Phenotypic Plasticity In *Drosophila*. *PLoS Genet* **7**, e1002373.

Tu MP, Tatar M (2003) Juvenile diet restriction and the aging and reproduction of adult *Drosophila melanogaster*. *Aging Cell* **2**, 327-333.

Ulbrich C, Diepholz M, Baßler J, *et al.* (2009) Mechanochemical removal of ribosome biogenesis factors from nascent 60S ribosomal subunits. *Cell* **138**, 911-922.

Wei F-Y, Suzuki T, Watanabe S, *et al.* (2011) Deficit of tRNA(Lys) modification by Cdkal1 causes the development of type 2 diabetes in mice. *The Journal of Clinical Investigation* **121**, 3598-3608.

Wells JCK (2012) A critical appraisal of the predictive adaptive response hypothesis. *International Journal of Epidemiology* **41**, 229-235.

Wilson C, Miller C (2005) Simpleaffy: a BioConductor package for Affymetrix Quality Control and data analysis. *Bioinformatics* **21**, 3683-3685.

Phenotype	Adult diet	Larval diet contrast	Hazard Ratio [95% CI]	p value
Male Virgin Lifespan	Poor	P:C	0.80 [0.68,0.94]	0.0056
		R:C	0.88 [0.75,1.03]	0.12
		R:P	1.10 [0.94,1.29]	0.24
	Control	P:C	0.71 [0.60,0.85]	0.0001
		R:C	1.33 [1.12,1.58]	0.0013
		R:P	1.87 [1.56,2.24]	<0.0001
	Rich	P:C	0.69 [0.59,0.80]	<0.0001
		R:C	1.11 [0.95,1.29]	0.198
		R:P	1.61 [1.37,1.89]	<0.0001
Female Virgin Lifespan	Poor	P:C	1.00 [0.86,1.16]	0.99
		R:C	1.37 [1.18,1.60]	<0.0001
		R:P	1.37 [1.17,1.60]	<0.0001
	Control	P:C	0.67 [0.57,0.78]	<0.0001
		R:C	1.33 [1.15,1.55]	0.0002
		R:P	1.99 [1.70,2.34]	<0.0001
	Rich	P:C	0.70 [0.60,0.81]	<0.0001
		R:C	1.43 [1.23,1.67]	<0.0001
		R:P	2.05 [1.75,2.40]	<0.0001
Male Mated Lifespan	Poor	P:C	1.07 [0.79,1.45]	0.65
		R:C	0.93 [0.69,1.25]	0.62
		R:P	0.86 [0.63,1.18]	0.36
	Control	P:C	0.73 [0.52,1.02]	0.062
		R:C	0.82 [0.59,1.15]	0.24
		R:P	1.13 [0.82,1.56]	0.46
	Rich	P:C	0.86 [0.62,1.19]	0.37
		R:C	1.49 [1.09,2.02]	0.011
		R:P	1.73 [1.26,2.37]	0.0007
Female Mated Lifespan	Poor	P:C	0.88 [0.66,1.16]	0.36
		R:C	0.78 [0.59,1/03]	0.084
		R:P	0.89 [0.68,1.18]	0.42
	Control	P:C	0.95 [0.72,1.25]	0.71
		R:C	1.42 [1.07,1.89]	0.016
		R:P	1.50 [1.11,2.01]	0.0076
	Rich	P:C	1.10 [0.82,1.48]	0.52
		R:C	1.50 [1.11,2.04]	0.0089
		R:P	1.37 [1.02,1.834]	0.039

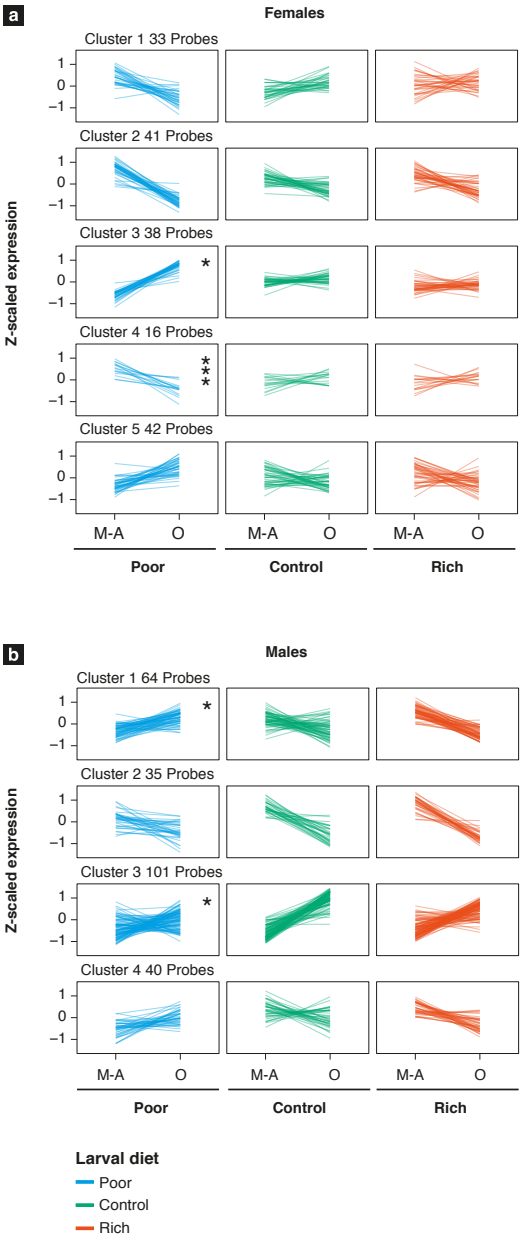
APPENDICES

Appendix 1. Pairwise contrasts between larval diets per adult diet for male and female virgin and mated lifespan (Table).

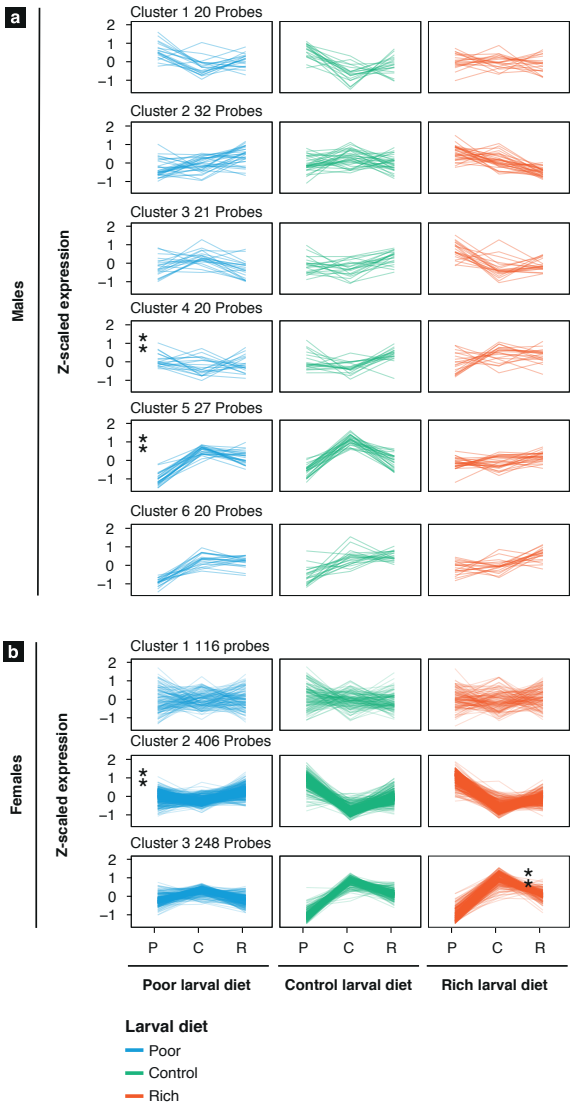
Appendix 2: Tabbed Excel File of results of ANOVA analysis per probe in females (Tab 1), Males (Tab 2). Cluster membership in both sexes (Tab 3). Tissue-specific gene lists mined from FlyAtlas for females (Tab 4) and for males (Tab 5). Significant GO term annotation of all clusters (Tab 6). Number of overlapping GO terms between tissue-specific gene lists and clusters in females (Tab 7) and males (Tab 8). This file can be found in the folder associated with this chapter at the following Dropbox link.

Appendix 3: Tabbed excel file of hypergeometric tests of overlaps in probe composition between male and female clusters (Tabs 1 to 4) and for overlap in cluster probe composition with tissue-specific probe lists mined from FlyAtlas (Tabs 5 to 12). This file can be found in the folder associated with this chapter at the following Dropbox link.

Appendices 2 and 3 can be found in the folder associated with this chapter in the online Dropbox folder associated with this thesis at:
https://www.dropbox.com/sh/q37qolcfi6ib05m/AABBFG1k3h-lOGFK-WXUyCnqBa?dl=0


a
Females
Cluster 1 33 Probes
Cluster 2 41 Probes
Cluster 3 38 Probes
Cluster 4 16 Probes
Cluster 5 42 Probes
Z-scaled expression
1
0
−1
*
***
M-A
O
Poor
Control
Rich
b
Males
Cluster 1 64 Probes
Cluster 2 35 Probes
Cluster 3 101 Probes
Cluster 4 40 Probes
Larval diet
Poor
Control
Rich

Appendix 4: Expression profiles of clusters of probes showing an interaction between larval diet and age in females (a) and males (b). Colour indicates larval diet (blue: poor, green: control, red: rich) and the x-axis indicates larval diet and age (M-A: middle-aged and O: old). We identified 5 clusters in females (a) and 4 clusters in males (b), with no significant overlap between the male and female clusters, and with little significant GO annotation. These clusters revealed that the interaction between larval diet and age in both sexes was primarily due to poor-raised flies showing attenuated (males) or opposite (females) changes in expression with increasing age relative to control and rich flies. In males, 3 of the clusters (C1,C2 & C4) were down-regulated with age in control and rich raised flies but up-regulated (C1 – proteasome complex, C4) or unchanging (C2– no annotation) in flies raised on the poor diet. The remaining cluster C3, was associated with the mitochondrial envelope and was down-regulated with age in control and rich-raised flies, but relatively constant in poor-raised flies. In females, all clusters showed relatively little change in expression with age in control and rich raised flies, but down (C1,C2, C4) or up-regulation (C3,C5) in poor raised flies. The only significant GO annotation was found for C3 (iron ion binding) and C4 (heme binding and terms relating to mitochondrial components and ATP synthesis). M-A: Middle-aged; O: Old age. Clusters of probes with similar expression profiles were identified using k-means clustering. * indicates number of significant GO terms associated with a cluster *:1 term, **: 2: to 9 terms,***: 10 or more terms.


a
Males
Z-scaled expression
Cluster 1 20 Probes
Cluster 2 32 Probes
Cluster 3 21 Probes
Cluster 4 20 Probes
Cluster 5 27 Probes
Cluster 6 20 Probes
b
Females
Cluster 1 116 probes
Cluster 2 406 Probes
Cluster 3 248 Probes
P C R
Poor larval diet
Control larval diet
Rich larval diet
Larval diet
Poor
Control
Rich

Appendix 5: Expression profiles of clusters of probes showing an interaction between larval diet and adult diet in males (a) and females (b). Colour indicates larval diet (blue: poor, green: control, red: rich) and the x-axis indicates adult diet (P: poor, C: control, R: rich). In contrast to the genes affected by larval diet alone (L), there were many more probes showing L*A effects in females (770) than in males (140). In males, these probes broke down into six small clusters, only two of which (Clusters 4 and 5) had significant annotation. Cluster 4, which was up-regulated on the control adult diet in rich-raised flies was annotated with responses to abiotic stimuli, particularly heat and oxygen, while cluster 5 which was down-regulated on the control adult diet in rich raised flies was associated with nucleobase metabolic processes. In females we identified three large clusters, two of which showed very distinct expression patterns and significant annotation. As with the “LT” clusters, these clusters were characterized by very similar expression patterns in control and rich raised flies, but distinct patterns in poor-raised flies – Cluster two was characterized by high expression on the poor adult diet for control and rich raised flies, but low expression in poor raised flies. It was strongly associated with visual perception, circadian rhythm, regulation of behaviour and metal ion transport. Cluster three was characterized by low expression on the poor adult diet for control and rich raised flies, but higher expression in poor raised flies. This cluster was associated with nucleotide binding and female reproduction. Clusters of probes with similar expression profiles were identified using k-means clustering. * indicates number of significant GO terms associated with a cluster *:1 term, **: 2: to 9 terms,***: 10 or more terms.

a
Males
Z-scaled expression
Pattern 1
Pattern 2
Pattern 3
Cluster 1 66 Probes
Cluster 4 36 Probes
Cluster 6 52 Probes
Cluster 3 48 Probes
Cluster 5 52 Probes
Cluster 2 34 Probes
*
*
P C R
Poor larval diet
Control larval diet
Rich larval diet
b
Females
Z-scaled expression
Cluster 1 132 Probes
Cluster 2 75 Probes
Cluster 3 57 Probes
Cluster 4 50 Probes
Cluster 5 150 Probes
**
***
M-A
O
Larval diet
Poor
Control
Rich

Appendix 6: Expression profiles of clusters of probes showing an interaction between larval diet (colour), adult diet (x-axis) and age in males (a) and females (b). Colour indicates larval diet (blue: poor, green: control, red: rich), the x-axis indicates adult diet (P: poor, C: control, R: rich) and the panels are split into middle age (M-A) and old-age (O). In males we identified six clusters of which only two possessed significant GO annotation – C4: endoplasmic reticulum and C5: post-mating behaviour. Clusters C1, C4 and C6 are characterized by distinct expression profiles for each larval diet across adult diets at middle-age, which are then inverted in old age, while clusters C3 & C5 are characterized by inversions in expression profiles across adult diets in age classes in rich-raised flies only. Finally, C2 shows distinct expression profiles in middle-age for P & C raised flies, but no clear expression pattern in other ages or in rich raised flies. In females, we identified five clusters each of which displayed distinct expression profiles and only two of which were annotated with GO terms (C3 and C5). As opposed to the males, we saw no evidence of inversion of responses to adult diet with increasing age. Cluster 3, which is associated with peptidase activity shows a distinct expression profile in old-aged flies raised on the rich larval diet, while cluster 5, which is associated with various terms relating to immune function and development, also shows a distinct expression profile in old-aged flies raised on the rich larval diet, as well as in middle-aged flies raised on the poor larval diet. Clusters of probes with similar expression profiles were identified using k-means clustering. * indicates number of significant GO terms associated with a cluster *:1 term, **: 2: to 9 terms,***: 10 or more terms.

Growing up and getting old: Life history traits reflect the interaction between developmental and adult selection regimes

Christina M. May[1], Joost van den Heuvel[1,2], Agnieszka Doroszuk[1,3], and Bas J. Zwaan[1]

1. Laboratory of Genetics, Wageningen University, Wageningen
2. Institute for Cell and Molecular Biosciences, Newcastle University
3. Current affiliation: Rijk Zwaan, the Hague, the Netherlands

ABSTRACT

Experimental evolution (EE) has proven a powerful tool for addressing how environmental factors influence life history evolution. In nature, individuals must optimise their life histories in response to multiple selection pressures experienced at different stages of their lives, however to date nearly all EE studies have applied only one selection pressure at a time within a single life stage. Here we assess the life history consequences of adaptation to three different diets during development in combination with selection for early or late reproduction during adulthood in a full factorial design in the fruit fly. We show that the two life stages do not act independently but rather interact to determine both developmental time and lifespan. Across all evolutionary diets, selection for later age-at-reproduction increases lifespan, however, the magnitude of the response is dependent on the sex, the evolutionary diet, and the experimental assay conditions. Developmental time also depends on the interaction between evolutionary larval diet, reproductive regime and assay conditions. For example, while a poor evolutionary larval diet selects for faster development from egg to adult, this effect is lessened when combined with selection for later age-at-reproduction. We also identify a positive correlation between developmental time and lifespan when lines are raised in the assay diet matching their evolutionary diet, but no consistent correlation in novel (i.e. non-evolutionary diet) assay conditions, consistent with quantitative genetic theory. Overall, we show that flies are not able to adapt to selection at two different life stages independently, but rather, must integrate both into their life history. Given that multiple selection pressures are likely the norm rather than the exception in nature, this finding argues that trade-offs should be considered not only between traits within an organism, but also between adaptive responses to differing selection pressures.

INTRODUCTION

One of the tenets of life history evolution is that individuals cannot optimise all fitness-related traits at once and thus trade-offs must exist between traits (Roff 2001; Stearns 1992). Often, underlying these trade-offs is the fact that individuals only have limited resources at their disposal and thus must make allocation decisions between competing functions (de Jong & van Noordwijk 1992; Van Noordwijk & de Jong 1986). To understand the trade-offs caused by limited resources, many studies have imposed variation in diet quantity and quality within a single generation and observed the consequences for phenotypes, - that is, they have addressed the plastic response.

Studies on plasticity have provided invaluable insight into the role of available resources in affecting life history traits (e.g. see the extensive literature on dietary restriction: Kirkwood & Shanley 2005; Mair et al. 2003; Nakagawa et al. 2012), however, there are some aspects of life history evolution that cannot be addressed in this framework. For instance, it is often not possible to discriminate between an adaptive response and a direct detrimental consequence of the environmental pressure (Schlichting & Pigliucci 1998; Stearns 1992). Furthermore, many responses may become fixed over evolutionary time and will therefore become effectively invisible in studies of plasticity (Stearns 1992). At evolutionary equilibrium, resource acquisition is predicted to be maximised within physical and physiological constraints. Thus at the genetic level variation for resource acquisition is expected to be low (positive alleles fixed, negative alleles in mutation-selection balance). One way to circumvent these issues is to use experimental evolution (EE). EE allows the experimenter to impose carefully controlled conditions and observe evolutionary responses in real time (Kawecki et al. 2012). EE can readily distinguish between adaptive and maladaptive responses and can identify the trade-offs or costs involved in adapting to particular environmental variables (Kolss et al. 2009).

Several different EE paradigms have been used to understand how resource availability affects life histories - in essence addressing how acquisition can influence allocation on an evolutionary time scale. These studies have often turned to the fruit fly Drosophila melanogaster as a model due to its short generation time and high fecundity. For example, a set of Drosophila selection lines adapted to chronic severe larval malnutrition evolved changes in both larval and adult life history traits (Kolss et al. 2009; Vijendravarma et al. 2015; Vijendravarma et al. 2013). Selection resulted in better larval performance on the poor diet, measured as higher larval survival and faster larval development, however, this appeared to come at the cost of adult fitness as the evolved lines exhibited both decreased adult size and early-life fecundity (Kolss et al. 2009). Notably this response is quite different from the evolutionary response to acute

starvation during adulthood. Rather than speeding up developmental time and decreasing size, as is the case for larval malnutrition, selection on starvation resistance in young adulthood generally selects for slower development and increased lipid accumulation, resulting in larger adult size (Baldal et al. 2006; Bubliy & Loeschcke 2005; Chippindale et al. 1996) (but see Hoffmann et al. 2005). This difference highlights the fact that similar selection pressures may select for different adaptations depending on when in life they are experienced.

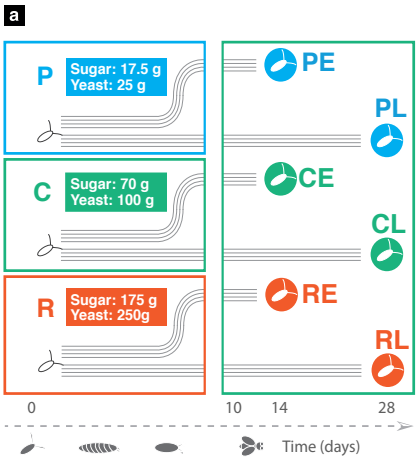
EE studies have also shed light on the evolution of ageing. Contemporary theories of ageing are under-pinned by the concept of the selection shadow: because even potentially immortal individuals will eventually be killed by forces outside their control (i.e. disease, predation et cetera), the likelihood that reproduction will occur decreases with time (Medawar 1952). Since natural selection must act through differential reproductive success, less reproduction with time also means a decline in the force of natural selection, ultimately resulting in a "selection shadow" in late life. Furthermore, staying alive requires the activation of costly repair mechanisms, which will take up some proportion of an individuals potentially limited resources. The disposable soma theory of ageing proposes that individuals should only invest enough in repair mechanisms to maintain a good soma for as long as they can reasonably be expected to live, and that a balance should be struck between investment in repair and investment in early reproduction (Kirkwood 1977; Kirkwood & Holliday 1979). Several EE studies have shown that by selecting flies for later ages-at-reproduction - in essence shifting the selection shadow to later ages - lifespan extension can be achieved (Luckinbill et al. 1984; Partridge & Fowler 1992; Rose 1984). In nearly all cases, decreased early or life-long fecundity is observed as a correlate of lifespan extension. Thus there appears to be a trade-off between lifespan extension and fecundity, as predicted by the disposable soma theory (Zwaan 1999).

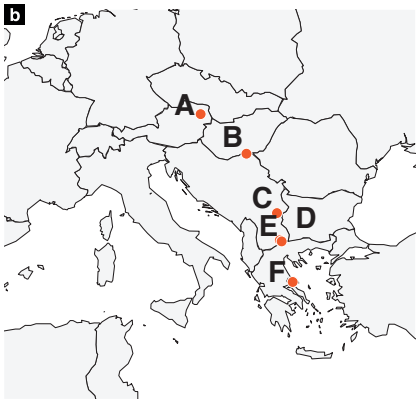
The EE experiments described above illustrate the complex nature of adaptation, including the frequent existence of trade-offs. Each of these designs, however, reflects the response to only one selection pressure at a time and within a single life stage. This represents an informative but simplified version of natural conditions, where organisms likely need to cope with several different selection pressures, and furthermore must balance the costs and benefits of adaptations in one stage such as development, with their consequences in another stage, such as adulthood. That the responses to different selection pressures are likely to be interactive and not independent has been shown in fruit fly lines adapted to both temperature and food quality stress simultaneously during development: the response to each selection pressure experienced independently was very different to that observed when the flies needed to accommodate both

pressures (Bochdanovits & Jong 2003). Thus, there is a need to address how life histories evolve in response to more than one selection pressure, and how selection within one life stage is weighed against selection in another.

In this article we combine two of the EE paradigms described above, selection on different levels of larval acquisition and selection on age-at-reproduction into a single full factorial EE experiment (Fig. 1a). In this way we address how the response to both selection pressures simultaneously differs from the response to each regime alone. Previous work suggests that there may be some conflict between the two selection regimes. For example, adaptation to a poor quality diet generally selects for faster development coupled with smaller adult size (Bochdanovits & Jong 2003; Kolss et al. 2009), while longer lifespan is generally (though not always; see Zwaan et al. 1991) correlated to longer developmental time and larger size (Economos 1980; Khazaeli et al. 2005; Lints 1978; Promislow 1993). Thus, for example lines adapted to a poor developmental diet and increased age-at-reproduction may experience a conflict between the two differing adaptive responses. Furthermore, combining these two specific EE regimes is especially relevant given on the one hand the theory on the role of resource acquisition and allocation in determining relationships between life history (fitness) traits, and on the other, how resource availability and allocation relates to the evolution of ageing.

We evolve flies on three different larval diets (Poor, Control and Rich) and at two ages-at-reproduction (Early and Late) in a full factorial design. To our knowledge, this is the first time an EE approach has been applied which combines selection pressures at two different life stages. Using this approach the main question we wish to address is how selection on different larval acquisition levels interacts with selection on age-at-reproduction to determine life history traits. Can flies adapt to environmental variation in each stage independently, or will the response affected in one stage also depend in the environment experienced in the other. For example, will a poor larval diet constrain the ability to extend lifespan in response to selection on age at reproduction, or will lifespan extension be achieved at the expense of other traits? Conversely, will a rich larval diet allow even greater lifespan extension, or will potential excess energy be re-allocated to reproduction? To address these questions we assess the responses of several life history traits across multiple generations. These include larval survival and developmental time, which showed strong evidence of adaptation in response to larval acquisition previously, and adult lifespan and fecundity, the two traits that commonly trade-off in response to selection on age-at-reproduction. In addition, we assess whether adaptation to one developmental diet and reproductive regime influences the response to other developmental diets (i.e. the evolution of plasticity). For example, does adaptation to the poor larval diet also change the response to other assay diets, or are the responses





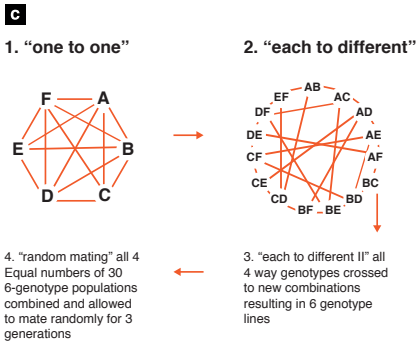


Figure 1: Overview of the experimental evolution regime (a) and the origins (b) and establishment (c) of the mixed population used to start experimental evolution. (a) Experimental evolution (EE) regimes: blue, green and red represent poor (P), control (C) and rich (R) diets respectively; each line represents a distinct evolutionary lineage. Eight replicate populations were established per larval diet and allowed to develop to adulthood on their respective diets. After eclosion all lines were transferred to the control diet. Four lines from each larval diet reproduced 14 days post egg-laying (E) and four reproduced at 28 days post egg laying (L) on their respective larval diet, resulting in four replicate lines each of PE,PL,CE,CL,RE and RL. This regimen was continued for the duration of EE. (b) Collection sites across Europe of the six starting populations from which the EE lines were derived. (c) A brief description of the multi-generation crossing scheme used to cross the populations in (b) to generate the mixed “S” population used for experimental evolution.

specific to the evolutionary environment in which they evolved. To address this we assay our lines across all three diets wherever possible. Therefore, our study has the potential to provide valuable insights into how fitness is affected by adaptations to selection pressures in two life stages and also how this has involved or affected phenotypic plasticity.

Materials and Methods:

Design of experimental evolution

In our experimental evolution (EE) regime we combine three levels of larval resource acquisition (Poor, Control and Rich) with two ages-at-reproduction (early and late) in a full factorial design. The three larval diets differ only in the amount of sugar and yeast that they contain (see Table S1 for recipes). Relative to the rich diet (R), the poor diet (P) and control diet (C) contain 10% and 40% of the sugar and yeast levels respectively. Early (E) and late (L) reproduction were set at 14 and 28 days post egg-laying respectively. For each combination of larval diet and age at reproduction we established four replicate lines (3 larval diets x 2 ages at reproduction x 4 replicate lines = 24 lines total; Fig. 1a). The control diet (C) combined with early age-at-reproduction (E) is identical to the standard conditions in our laboratory and thus the lines maintained under these conditions provide an unselected control and their responses to different assay conditions provide the baseline pattern of plasticity.

For clarity, we refer to the experimental evolution lines throughout by their larval diet abbreviation, (P, C, or R), their age at reproduction abbreviation (E, L) or the combination of the two (PE, PL, CE, CL, RE, RL). Assay diets (i.e. the conditions under which we phenotype the lines) are referred to by their full names (Poor, Control, and Rich).

Creating a genetically diverse starting population

In order to ensure ample genetic variation for selection to act upon the experimental evolution populations were derived from six populations of flies collected in a longitudinal gradient across Europe (Fig. 1b). These populations were maintained in the lab for 40 generations to allow laboratory adaptation and then combined into one genetically diverse population, the starting or "S" population, via a multi-generation crossing scheme (Fig. 1c; see May et al. 2015 for full details of the crossing scheme). This crossing scheme was employed to ensure that the S population was in linkage equilibrium and to ensure equal contributions of the individual component populations. After crossing, the "S" population was maintained under standard laboratory conditions for another ten generations at a population size of approximately 4000 individuals before experimental evolution was started.

Commencing Experimental Evolution

To begin experimental evolution, eggs were collected from the "S" population into large glass bottles (500mL volume) filled with 65 mL of the respective larval

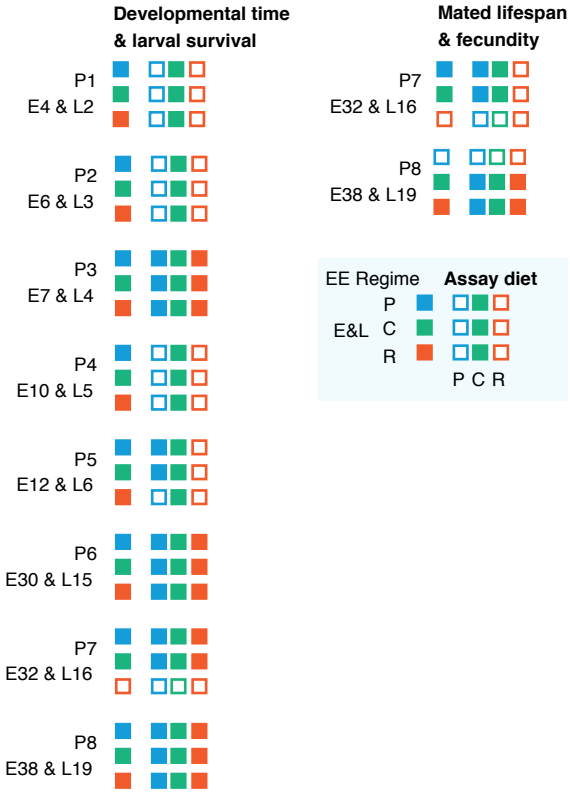


Figure 2: Overview of traits assayed in each phenotyping session. For each phenotyping session (8 in total, labeled P1 through P8) we diagram the traits measured in the columns (first column: Developmental time and larval survival, second column: Mated lifespan and fecundity) across phenotyping sessions and EE generations (rows). Developmental time and larval survival were assessed in all phenotypings sessions (P1 through P8) while mated lifespan and fecundity were only addressed in the later generations of evolution (P7 and P8). Because the size of our experiment meant that we could not always assess the responses of all lines in all assay environments, the exact lines and assay conditions included in each phenotyping session varied. This is diagrammed using the filled versus unfilled boxes, with filled boxes representing inclusion in the assay. Briefly, the first column of boxes indicates the evolutionary diet conditions included, while the second, third and fourth columns indicate assessment on the poor, control and rich assay diets respectively (the key can be found in the inset box). In all cases, both the early (E) and late (L) reproducing lines were included. Thus, for example, in P4 (Generation 10 & 5 of E and L lines respectively) the PE, PL, CE, CL, RE, and RL lines were all included (first column all filled) but only assayed under the control assay diet. It is noteworthy that there is a relatively large generation gap between phenotyping sessions P1 through P5 and P6 through P8.

diets. Two bottles of approximately 1000-2000 eggs were collected per replicate line and allowed to develop to adulthood. For each larval diet four lines were randomly selected to be the early reproducing populations (E) and four lines were selected to be the late reproducing populations (L). After emergence, all lines were maintained on the control food and in each generation both replicate bottles of a line were mixed to ensure that bottles did not become distinct populations. Overall population size was generally 2000 to 4000 flies per replicate line over the course of EE.

Assessing changes in life history traits over the course of evolution

We measured four key life history traits: length of development from egg to adult, survival from egg to adult, mated female fecundity, and mated lifespan. These assays were done across eight phenotyping sessions spanning multiple generations ranging from the very beginning of evolution to relatively advanced stages (Fig. 2). Figure 2 provides an overview of each phenotyping session, hereafter referred to as P1 through P8, including the elapsed number of generations of evolution, the lines included in the assay, the traits measured, and the actual larval conditions under which flies were raised, hereafter referred to as the assay environment.

Wherever possible we measured responses in all lines and used all three assay diets, however, the scale of our experimental evolution design imposed some logistical constraints on the breadth of these assessments. Therefore, in some phenotyping sessions we monitored the progress of selection in only a single assay environment (Control – the standard laboratory condition) while in others we assessed genotype by environment interactions (G by E) by raising larvae on all three assay diets. In all cases, we first allowed the lines to develop for one generation on control larval food, to avoid potential maternal effects. For all experiments larvae developed at a density of 70 eggs per vial, with 6mL of food per vial. Since we always analysed lines selected for early and late reproduction concomitantly, the E lines had always undergone roughly twice as many generations of selection as the L lines (Fig.2).

Assessing development time and larval survival

We assessed development time and survival from egg to adult in all eight phenotyping sessions (Fig. 2). This approach allowed us to address how the response changed over evolutionary time. We collected 5 vials of 70 eggs per line and assay diet. We scored development until no new flies emerged over a period of 48 hours and summed the resulting adults to obtain a measure of larval survival.

Assessing mated lifespan and fecundity

For lifespan and fecundity, we assayed mated flies on both their evolutionary diet and under control conditions. The size of this experiment necessitated splitting the experiment across two assays done on different generations (Fig. 2): in the first round all lines adapted to poor or control larval food were used and assayed under these two conditions (P7), while in the second round all lines adapted to control and rich larval diet were used and assayed across all three larval diets (P8). The control lines served as a reference to facilitate comparisons between the P and R lines.

We assessed fecundity at three ages: the ages at which early and late reproducing lines laid eggs for the next generation (Early, Age: 2 to 4 days and Late, Age: 18-21 days) and a few days after this (Post-Selection, Age: 25-28 days), an age that flies never reached in experimental evolution. There was a slight methodological difference between the two assays – in the first we maintained a single male-female pair per vial (n=15 vials per line and larval food combination) while in the second assay we maintained two males and two females per vial (n=10 vials per line and larval food combination). New males from the same experimental conditions replaced dead males, in order to ensure that females were not sperm-depleted. Rather than assessing egg numbers we counted the actual offspring resulting from the eggs, giving an accurate measure of realised fecundity.

To assess mated lifespan, flies were housed at a density of three males and three females per vial (n=10 vials per combination of line and larval diet). Flies were transferred to fresh vials and survival was scored every Monday, Wednesday and Friday.

Statistical analysis

All statistical analyses were performed in R (2005). We fit a separate model for each trait within each phenotyping session. In each model we included evolutionary diet, evolutionary age at reproduction, assay diet, sex (where applicable) and their interactions as explanatory variables. In addition, for each trait that was assessed under multiple assay diets we also fit a model for the CE lines alone to address the plastic response to variation in developmental diet. In order to accommodate the random effect of replicate line nested within the interaction between evolutionary diet and age at reproduction we used mixed effects models. Both developmental time and mated longevity were analysed using mixed effects Cox models (coxme package) while larval survival and fecundity were analysed with generalised linear mixed models (GLMM) with binomial and poisson distributions respectively.

In the tables, we report the Chi-squared values of the effect of each factor in the full model as obtained by Analysis of Deviance. We performed further model simplification by dropping non-significant terms from the model sequentially and using a Chi-squared test to compare models. Where significant interactions existed we also performed pairwise contrasts to determine which specific contrasts were significant.

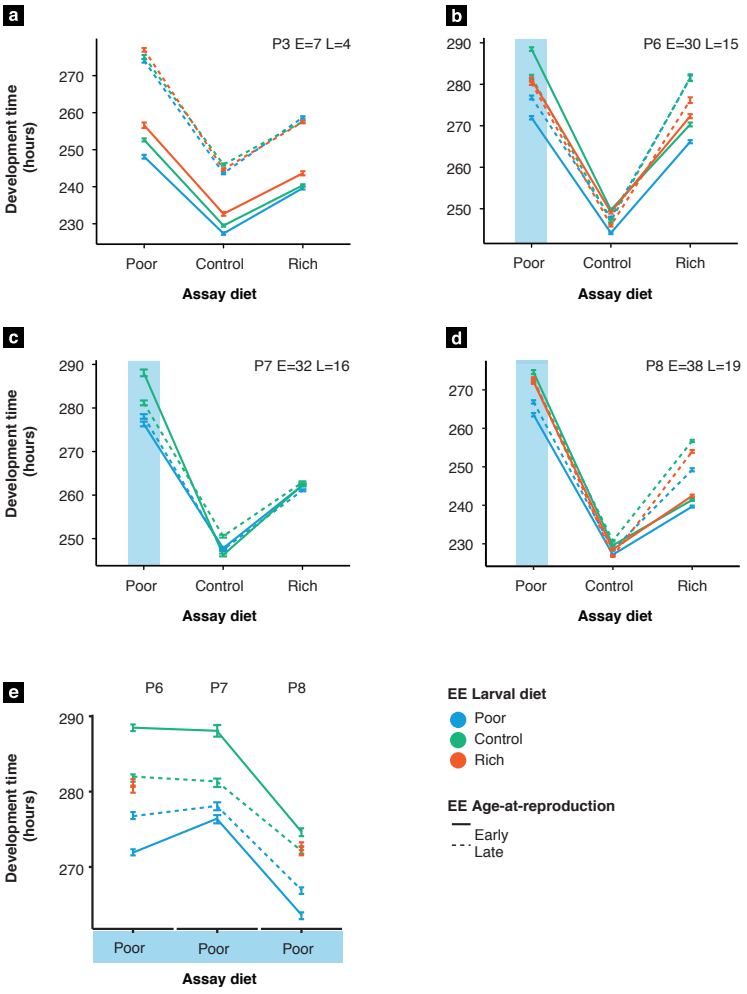


Figure 3: Developmental time from egg to adult (y-axis) across assay diets (x-axis) and phenotyping sessions for each combination of evolutionary diet (color) and age-at-reproduction (line type). Blue, green and red represent the poor (P), control (C) and rich (R) EE larval diets respectively. Solid and dashed lines represent early (E) and late (L) reproducing lines respectively. All error bars are standard errors of the mean across replicate lines. For simplicity, only phenotyping sessions carried out using all three assay diets are included: P3 (a), P6 (b), P7 (c) and P8 (d). (e) zooms in on the blue highlighted areas in (b), (c),z and (d) to emphasize the consistent decrease in developmental time of PE and PL lines when raised on the poor assay diet across the later generations of EE.

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

			Effect Assay diet			Post-hoc contrasts		
Phenotyping	**Generation**	**Subset**	**Chi-square**	**df**	**p-value**	**P:C**	**P:R**	**R:C**
Larval survival								
P3	7		1.59	2	0.45	---	---	---
P5	12		6.18	1	0.01	0.0129	---	---
P6	30		0.42	2	0.81	---	---	---
P7	32		0.44	2	0.80	---	---	---
P8	38		5.46	2	0.07	---	---	---
Developmental time								
P3	7		1878.70	2	<0.0001	<0.0001	<0.0001	<0.0001
P5	12		1090.70	1	<0.0001	<0.0001	---	---
P6	30		2648.30	2	<0.0001	<0.0001	<0.0001	<0.0001
P7	32		2303.30	2	<0.0001	<0.0001	<0.0001	<0.0001
P8	38		4212.50	2	<0.0001	<0.0001	<0.0001	<0.0001
Fecundity								
P7	32	Early	12.904	1	<0.0001	0.0001	---	---
		Mid	570.12	1	<0.0001	<0.0001	---	---
		Late	392.35	1	<0.0001	<0.0001	---	---
P8	38	Early	251.46	2	<0.0001	<0.0001	<0.0001	0.79
		Mid	225.24	2	<0.0001	<0.0001	<0.0001	<0.0001
		Late	65.824	2	<0.0001	<0.0001	0.42	<0.0001
Lifespan								
P7	32	F	16.015	1	<0.0001	<0.0001	---	---
		M	32.831	1	<0.0001	<0.0001	---	---
P8	38	F	55.467	2	<0.0001	<0.0001	<0.0001	0.23
		M	46.134	2	<0.0001	<0.0001	<0.0001	0.45

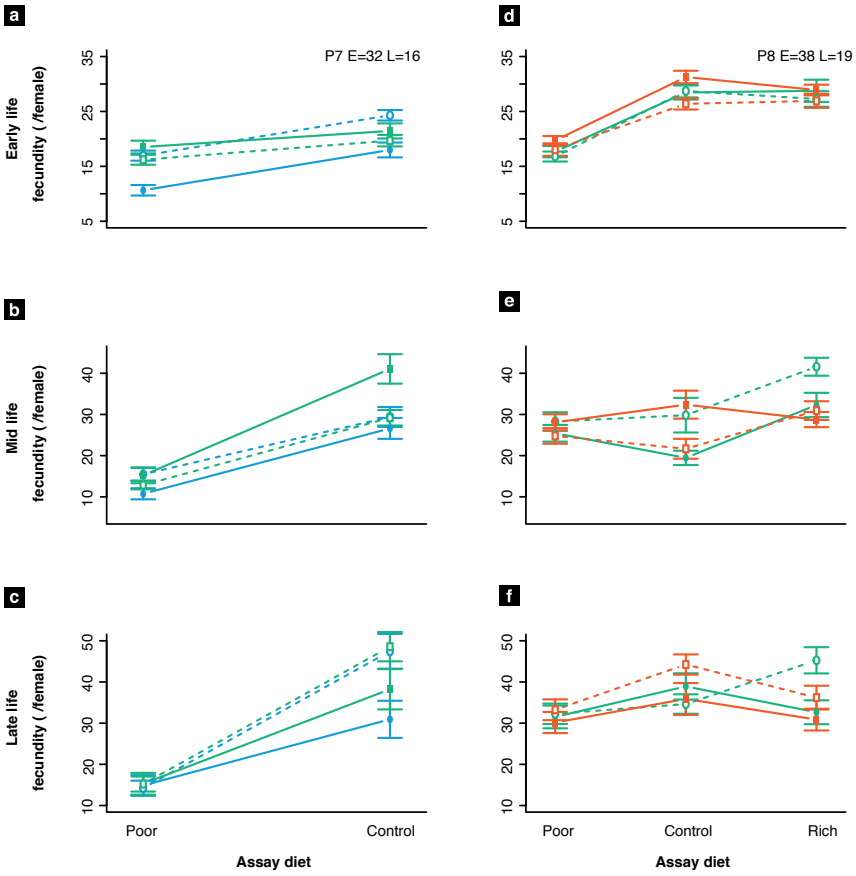


Figure 4: Realized female fecundity (y-axis) across assay diets (x-axis) and phenotyping sessions P7 (a:c) and P8 (d:f) for each combination of evolutionary diet (color) and age-at-reproduction (line type). Fecundity was assessed at the age when the E lines lay eggs for the next generation (a,d; Early life), when the late lines lay eggs for the next generation (b, e; Mid Life) and a few days after (c,f; Late life). Blue, green and red represent the poor (P), control (C) and rich (R) EE larval diets respectively. Solid and dashed lines represent early (E) and late (L) reproducing lines respectively. All error bars are standard errors of the mean across replicate lines. P7 (a,b,c) included all PE, PL, CE & CL lines raised on both poor and control assay diets while P8 (d,e,f) included all CE, CL, RE & RL lines raised on all three assay diets. While there is some evidence that PE lines have evolved lower fecundity at all ages (a:c) the inconsistency of the response of the CE lines across the two replicate phenotyping sessions make conclusive interpretations difficult (see Figure 5).

Table 2: Summary of GLMMs (Chi-square values) for the effect of assay diet (A), evolutionary diet (D) and evolutionary age-at-reproduction (R) on larval survival and developmental time across phenotyping sessions. * =P<0.05, **=P<0.01, ***=P<0.001.

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

Table 3: Summary of GLMMs (Chi-square values) for the effect of assay diet (A), evolutionary diet (D) and evolutionary age-at-reproduction (R) on fecundity at early, mid and late ages across phenotyping sessions. * =P<0.05, **=P<0.01, ***=P<0.001.

Phenotyping	Age	Evo Diet (D)	Evo Repro (R)	Assay Diet (A)	D*R	D*A	R*A	D*R*A
P7	Early	1.00	1.85	176.80***	4.45*	35.26***	1.18	5.47*
	Mid	4.69*	0.49	1383.33***	8.14**	8.02**	20.00***	0.72
	Late	1.5	7.5**	2154.05***	0.7135	1.84	76.34***	7.62**
P8	Early	0.2044	1.5996	892.25***	0.7745	9.05*	0.4933	0.7745*
	Mid	0.6249	0.8895	364.66***	10.20***	87.46***	43.04***	132.38***
	Late	0.2853	7.54**	204.34***	0.934	84.75***	64.578***	90.37***

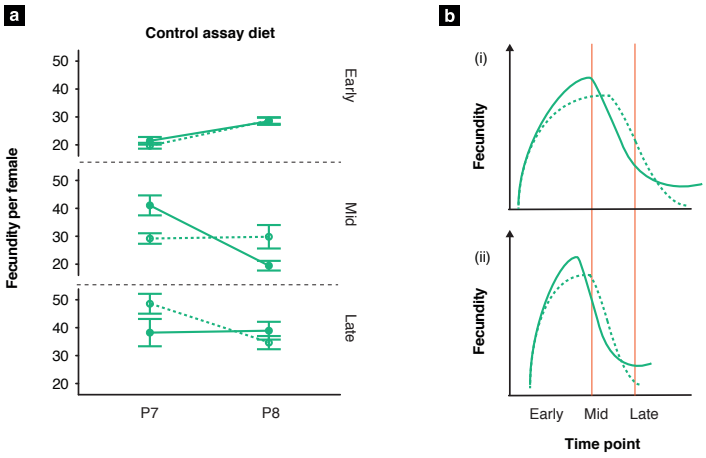


Figure 5: Inconsistencies in fecundity of control lines across phenotyping sessions (a) and a potential explanation (b). (a) Inconsistencies in fecundity of CE (yellow solid line) and CL lines (yellow dashed line) across phenotyping sessions P7 and P8. All error bars are standard errors of the mean across replicate lines. In P7 CE lines have higher fecundity than CL lines in midlife and lower fecundity in late life. In P8 this relationship is reversed as indicated by the crossing of the reaction norms at mid and late life across phenotyping sessions. (b) gives a potential explanation for the inconsistencies in fecundity across the phenotyping sessions. Both (i) and (ii) show similar patterns of fecundity (y-axis) across the lifespan (x-axis). In (ii) slight environmental variation (e.g. temperature, humidity) leads to overall compression of the fecund period, despite the relationship between early and late selected lines remaining similar across the lifespan. Because we assessed fecundity at three chronologically and not biologically determined time points, slight variation in the duration and magnitude of fecundity due to environmental variation between the two phenotyping sessions may have led us to sample at different parts of the curve (red lines), resulting in opposite observations for the difference between CE and CL lines between the two phenotyping sessions.

RESULTS

Experimental evolution does not affect larval survival

Overall, larval survival was very high across assay diets, ranging from 80 to 95% survival in all but one phenotyping session (Appendix 1). In fact, the plastic effect of assay diet on CE lines was insignificant in all but one (P5) out of five phenotyping sessions (Table 1). Given the lack of an effect of larval assay diet on survival in the control lines (CE), it is perhaps not surprising that there was also no consistent evolved response (Appendix 1). Across all eight phenotyping sessions we detected significant effects of evolutionary regime on larval survival in only three of them (P5, P7, and P8) however, the factors playing a significant role did not overlap (Table 2). For example in P6, L populations displayed a clear reduction in survival relative to E populations under rich assay conditions (Reproductive regime x Assay interaction: -2=39.86, p=<0.0001; Supplementary Fig. 1b), however, this response disappeared in both subsequent phenotyping

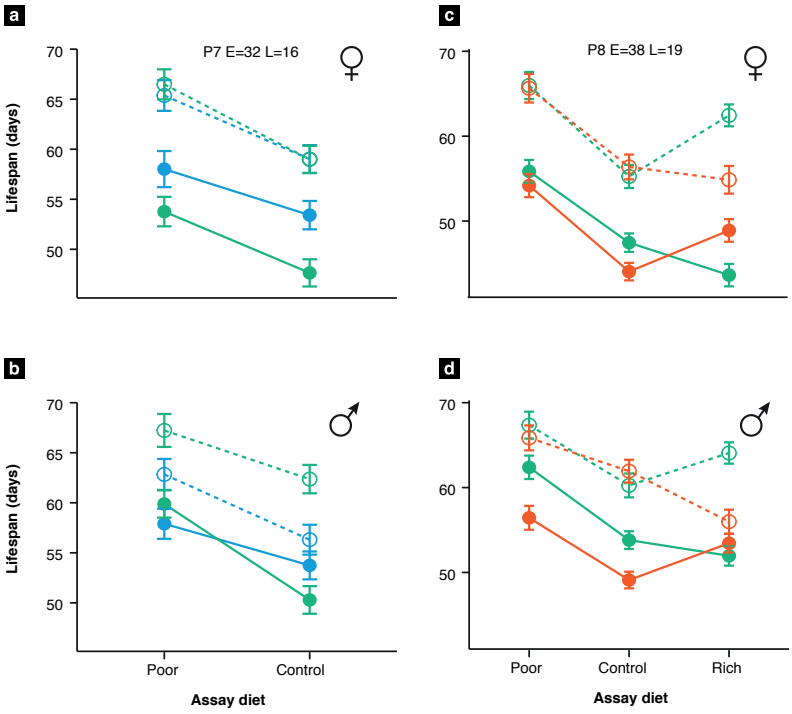


Figure 6: Lifespan (y-axis) across assay diets (x-axis) and phenotyping sessions P7 (a,b) and P8 (c,d) for females (a,c) and males (b,d) for each combination of evolutionary diet (color) and age-at-reproduction (line type). Blue, green and red represent the poor (P), control (C) and rich (R) EE larval diets respectively. Solid and dashed lines represent early (E) and late (L) reproducing lines respectively. All error bars are standard errors of the mean across replicate lines. P7 (a,b) included all PE, PL, CE & CL lines raised on both poor and control assay diets while P8 (d,e) included all CE, CL, RE & RL lines raised on all three assay diets. In contrast to fecundity, the response of CE and CL lines to the assay diets is qualitatively identical across the two phenotyping sessions. The response of lifespan to EE on the poor larval diet is sex dependent but consistent across assay diets (a,b). In females (a) PL lines obtain lifespans indistinguishable from CL lines, while PE lines evolve increased lifespans relative to the CE lines. In males (b), PL lines do not extend lifespan to the same degree as CL lines, while PE lines have similar lifespans to CE lines. The response of lifespan to the rich EE diet is similar between the sexes, but highly dependent on the assay diet and selection on age-at-reproduction (c,d). In both sexes, RL lines show similar lifespan extension to CL lines on both poor and control assay diets, however, in the assay diet matching their own evolutionary diet, RL lines have considerably shorter lifespans than CL lines. RE lines exhibit essentially the opposite pattern to RL lines, although the magnitude of the response differs between the sexes: on the poor (significant in males only) and control (significant in both sexes) assay diets they tend to have shorter lifespans than CE lines, while on the rich assay diet they appear to have evolved slightly longer lifespans (significant in females only).

sessions (Table 2, Supplementary Fig. 1c,d). The same pattern is true of all other sessions – while significant effects arise, they are not consistent across generations (Table 2). Thus it appears that while larval survival is sensitive to transient effects, there is no consistent response of larval survival to the two selection regimes or their interaction.

Emerging consistency: interactions between evolutionary diet, age-at-reproduction, and assay diet determine developmental time

Contrary to larval survival, development time showed consistent responses to assay conditions over time in CE lines (Fig. 3a-d): both the poor and, to a lesser extent, rich assay conditions increased developmental time relative to the control assay diet in all phenotypings (Table 1, all p-values <0.0001). However, across the early generations of evolution, in a manner similar to larval survival, developmental time showed few consistent effects of experimental evolution (Table 2). Starting from P5 (EG12 and LG6), however, a clear three-way interaction emerged (Table 2). PE lines evolved substantially quicker development on the poor assay food relative to CE and RE lines, and this effect persisted across all subsequent phenotyping sessions (all p-values <0.001; Fig.3e). There was also some evidence that they had evolved faster development under rich assay conditions in P6 (Fig. 3b) and P8 (Fig. 3d), however this effect was not present in P7 (Fig.3c). Notably, faster development on the poor assay diet also appeared to evolve in the PL lines, but this effect only reached significance in phenotyping sessions P6 and P8 and was in both cases less pronounced than for the PE lines (Fig. 3e).

RE and RL lines showed less evidence of adaptation than P lines, however some patterns emerged across the later phenotyping sessions in which they were assayed (P6 and P8; Fig. 3b,d). Under rich assay conditions, RL lines developed more slowly than RE lines (both p-values <0.01), however, this pattern was reversed under control conditions (i.e. RE slower than RL: p<0.0001 and p=0.08 respectively) and was completely absent under poor assay conditions (both p values >0.75). CE and CL lines showed a similar pattern (Fig. 3b,c,d): under rich assay conditions the L lines developed more slowly. This effect became smaller under control assay conditions, and reversed under poor assay conditions (Fig.3b). Overall, while the plastic response of unselected lines is very consistent across assays, the evolved response is different for each larval diet regime and is dependent on both the assay conditions and whether or not selection for late age-at-reproduction was also applied.

Fecundity shows extreme variability between phenotyping sessions and no consistent evidence of adaptation

Because it was not possible to measure lifespan and fecundity for all lines at once, we used the CE and CL lines as a standard across the two replicate

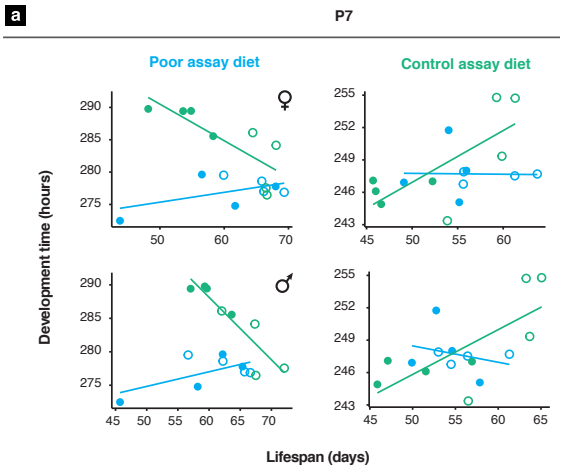
	Phenotyping	
Factor	P7	P8
Evo Diet (D)	0.26	8.45**
Evo Repro (R)	15.66***	28.63***
Assay Diet (A)	3557.81***	3761.66***
Sex (S)	0.01	46.47***
D*R	4.13*	0.49
D*A	2.08	9.73**
R*A	0.02	0.55
S*D	12.69***	10.34**
S*R	1.63	16.94***
S*A	0.00	2.62
D*R*A	0.27	20.99***
R*A*S	0.44	0.59
S*D*R	4.43*	0.01
S*D*A	1.68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

Table 4: Summary of GLMMs (Chi-square values) for the effect of assay diet (A), evolutionary diet (D) and evolutionary age-at-reproduction (R) on lifespan across phenotyping sessions. * =P<0.05, **=P<0.01, ***=P<0.001.

phenotyping sessions (see Materials and Methods). The plastic response of the CE lines to assay conditions differed between the two phenotyping sessions, despite a relatively short gap of only six generations (Table 1, Fig. 4). In the first phenotyping (P7) CE flies raised on the poor assay diet had lower fecundity than those raised on the control assay diet at all three ages (Fig. 4a:c; all p-values<0.001). In the second assay (P8), the same effect was observed at early and post-selection ages (all p-values<0.001), but reversed at the late reproduction time point (p<0.001; Fig 4d:f). This inconsistency is also reflected in the observed differences between the CE and CL lines on the control assay diet in the two phenotyping sessions (Fig. 5). While in P7 the L lines reproduced less than E lines at the "Mid" time point and more at the "Late" time point, the opposite effect was observed in P8 (Fig.5a, both p-values <0.003). Therefore, although fecundity at all ages appeared to be determined by interactions between evolutionary diet, evolutionary age-at-reproduction and assay conditions (Table 3) likely reflecting actual adaptive responses to the experimental evolution regimes, the lack of repeatability of the response of the CE and CL lines hampers the the interpretation of the evolutionary significance of the observed differences between EE lines.

Lifespan shows consistent effects across phenotyping sessions

Contrary to fecundity, the effect of assay diet on lifespan was qualitatively identical for the CE lines across phenotypings P7 and P8 (Fig. 6). In both



phenotyping sessions and for both sexes, development on the poor assay diet increased lifespan relative to the control assay diet for CE lines (all p-values <0.0001; Table 1). Furthermore, adding experimental evolution for late reproduction to the control larval diet regime (CL lines) did not change the plastic response to assay diet, as CL lines also showed extended lifespan when raised on the poor assay diet relative to the control assay diet (all p-values <0.001) however, selection for later age-at-reproduction increased lifespan overall in both phenotyping sessions, in both sexes, and across assay conditions (all p-values<0.01)(Fig. 6).

Next we examined how adaptation to either poor (PE, PL) or rich (RE, RL) evolutionary diet influenced lifespan. We found that in both sexes and across assay diets selection for later age-at-reproduction had been effective in increasing lifespan (i.e. PL>PE; RL>RE) (all p-values<0.03; Fig. 6). This was not a foregone conclusion, and shows that adaptation to age-at-reproduction is an important modifier of life history, even upon variation in larval acquisition levels. However, the magnitude of the effect was dependent on the evolutionary diet, indicating a modifying role of nutrition during development for determining lifespan (Table 4).

Adaptation to the poor larval diet results in sex-specific changes in longevity

While the CE and CL lines showed consistent responses to selection across the sexes, the response of PE and PL lines to selection was sex-specific relative to the control lines (Table 4; Fig. 6a,b). In essence, the two sexes showed a completely inverse pattern: in females PL lines showed lifespans indistinguishable from CL lines (p=0.94), but PE lines had evolved increased lifespans relative to the CE lines (p<0.0001) (Fig.6a), while In males, the exact inverse response was observed: PE adapted lines showed no lifespan differences relative to CE lines

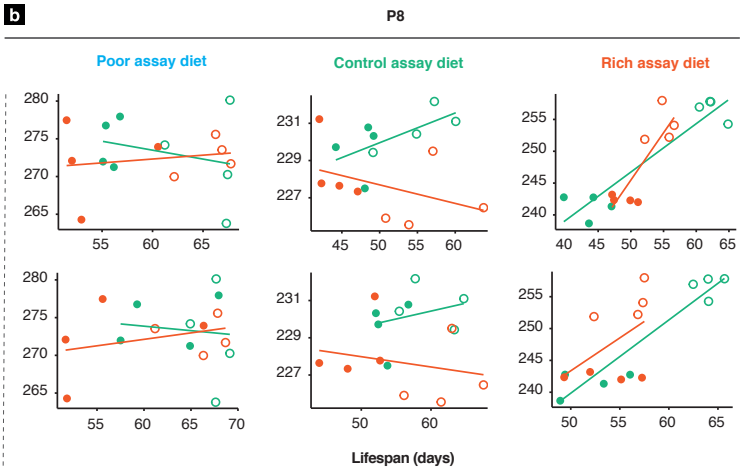


Figure 7: Regression of mean lifespan (x-axis) against mean developmental time (y-axis) per line, sex, and assay diet in P7 (a) and P8 (b). Blue, green and red represent the poor (P), control (C) and rich (R) EE larval diets respectively. Closed and open circles represent early (E) and late (L) reproducing lines respectively. In all cases, the slope of the regression of developmental time against lifespan is positive when lines are raised on the assay diet matching their evolutionary diet. Under other assay diets, the slope shows no consistent direction, being either positive, negative or flat. For example, for the flies adapted to the poor evolutionary diet (PE and PL lines) lifespan and developmental time are positively correlated on the poor assay diet, but show a neutral (females) or negative (males) slope on the control assay diet.

(p=0.96) but PL lines evolved shorter lifespan than CL lines (p=0.01) (Fig.6b). These effects were consistent across both the poor and control assay diets.

The effects of adaptation to rich larval diet on lifespan depend on assay conditions

In contrast to adaptation to the poor larval diet, adaptation to the rich larval diet led to similar responses in each sex, but differences in the magnitude of the response and strong interactions with the assay diet experienced (Table 4 and Figure 6). Under poor and control assay conditions, selection for late reproduction lead to identical increases in lifespan in RL lines to that observed for CL lines in both sexes, however, under rich assay conditions, RL flies evolved significantly shorter lifespans in males (p=0.002, Fig. 6d), and nearly significantly shorter lifespans in females (p=0.08, Fig. 6c) than CL lines. Selection on the rich larval diet and for early reproduction (RE) showed an essentially inverse pattern, with the sexes displaying similar responses that differed in magnitude (Fig. 6c,d). In males raised under poor and control assay conditions, RE lines evolved decreased lifespan relative to CE lines (p=0.003 and 0.004 respectively) while under rich assay conditions RE and CE lines did

not differ (p=0.66, Fig. 6d). In females, RE and CE lines did not differ under poor assay conditions (p=0.42), while under control assay conditions RE lines exhibited shortened lifespan (p=0.02), and under rich assay conditions RE lines had extended lifespan (p=0.01, Fig. 6c). This is in direct contrast to flies adapted to the poor diet (PE and PL) whose responses are consistent across both poor and control assay diets (Fig.6a,b).

Lifespan and developmental time correlate positively when flies develop in the evolutionary assay diet, but vary under the other assay diets

We also investigated the relationship between lifespan and development time in P7 and P8 by correlating lifespan and developmental time on a per line and per assay diet basis (Fig. 7). This revealed a very striking pattern – for every evolutionary diet, there was a positive correlation between lifespan and development time when flies experienced the same assay diet as their evolutionary diet (binomial test: p-value = 0.03125), however, this relationship broke down, or even reversed under the other assay diets (Fig. 7; binomial test: p-value = 0.39). As an example, refer to the positive slope of the correlation between lifespan and development time of the C lines (yellow lines) on the control assay diet relative to the negative slope of these same lines under the poor assay diet (Fig. 7a) Therefore, the positive correlation between lifespan and development time is specific to the evolutionary dietary conditions the lines experienced. These patterns held true for both sexes, despite the fact that their lifespans had evolved differently. Unfortunately we could not apply a similar analysis to fecundity versus lifespan or development time because of the lack of consistency of the fecundity measure across phenotyping sessions.

DISCUSSION

By combining extensive replication at the level of EE treatments with repeated phenotypings over multiple generations and across assay diets, we were able to gain a nuanced understanding of the evolutionary responses to simultaneous selection on larval diet and adult age-at-reproduction for important life history traits. We found consistent evolutionary responses for the traits developmental time and lifespan, but nor for viability and fecundity. For the former traits, our results indicate that the effects of adaptation to developmental diet and selection on age-at-reproduction are not independent of each other, but are strongly interactive. This suggests that adaptation during one life stage may be highly contingent on the selection pressures experienced in other stages, and that trade-offs may also stem from the necessity to accommodate selection pressures which select for differing adaptations. Furthermore, the evolutionary response was often highly dependent on the assay environment, indicating that phenotypic plasticity was affected as well. This was emphasised by the positive correlation between developmental time and lifespan when lines were raised in assay conditions matching their evolutionary diet, but a lack of consistent correlation in non-evolutionary conditions.

Larval survival is insensitive to our EE regime

In contrast to previous experiments which found that flies adapted to relatively poor larval diets evolved increased larval survival (Kolss et al. 2009) we found no evidence of consistent changes in larval survival in response to either selection on larval diet, or age-at-reproduction (Table 2). It is important to note that we deliberately chose our larval diets in such a way that they did not affect viability. In contrast, the diet imposed by Kolss et al., (2009) contained considerably less nutrients than our poor diet, and decreased larval survival by 20% in control lines. Since we observed little difference in larval survival of CE lines across assay diets (Supplementary Fig. 1) it is likely that there was very little direct selection for increased larval survival in our experiment. While it is still possible that changes in larval survival could have evolved as a correlated response to other aspects of selection (e.g. in a trade-off with faster larval development or longer lifespan) our results do not support this hypothesis.

Developmental time is specific to EE regimes and assay diets

We observed that the two divergent evolutionary larval diets selected for opposite adaptive responses in terms of developmental time. In the later generations of EE (P6 onwards) we observed that PE lines developed faster than CE lines when raised in the assay diet matching their evolutionary conditions (poor assay diet), while the RE lines developed more slowly than CE lines on the rich assay diet (Fig. 3). The faster development of PE lines on the poor assay diet parallels the response observed by Kolss et al., (2009) when they adapted flies to severe larval malnutritition. Kolss et al., (2009) suggested that the faster development of poor-diet adapted flies was primarily due to truncation selection imposed by the added pressure of having to reproduce at a very young age. Our study, however, shows that truncation selection is not the whole story since the PL lines also evolve faster development on the poor larval diet, though to a lesser extent than the PE lines (Fig. 3e). This suggests that even when the truncation selection pressure is relieved there is still selection for faster development on the larval diet, perhaps due to competition to access resources before they are exhausted, or to spend more time in the nutritionally replete adult conditions. The slightly increased developmental time of the RE lines relative to the CE lines on the rich assay diet suggests that there is an adaptive benefit to extending developmental time on nutrient replete larval diets. In fruit flies, some of the energy for reproduction in early life comes from the larval fat body, which is retained into early adulthood (Aguila et al. 2013; Aguila et al. 2007). Thus, the extension of developmental time in the RE lines relative to the CE lines may reflect selection to increase fat stores in the high sugar, high yeast larval environment and thereby increase early fecundity. Indeed, the RE lines do appear to have a fecundity advantage in early life (Fig. 4d) relative to the CE and RL lines, although as mentioned in the results section, the interpretation of

these differences is hampered by the inconsistency in the response of CE lines across phenotypings.

The correlation between lifespan and developmental time depends on the assay diet

For a long time, it was thought that developmental time and lifespan were genetically linked and that longer developmental time and longer lifespan were inextricably coupled (Lints & Soliman 1988). However, a series of experiments employing both experimental evolution and environmental variation showed that this relationship can be uncoupled (Zwaan et al. 1995; Zwaan et al. 1991). Our study provides further evidence for the environment specificity of this relationship: when flies were raised in the assay diets matching their evolutionary diets, lifespan and developmental time were indeed positively correlated, however, under other assay diets, the correlation was unpredictable and inconsistent (Fig. 7). That trait correlations in evolved versus novel environments may be very different is a key prediction of quantitative genetic theory (Armand et al. 1994; Fry et al. 1998; Mackay 2001; Sgro & Hoffmann 2004; Vieira et al. 2000). However, empirical evidence of such responses is still rare (Sgro & Hoffmann 2004). Here we show that this is clearly the case for the correlation between developmental time and lifespan. It then remains to be explained why selection for increased age-at-reproduction (and by extension longer lifespan) also selects for increased developmental time under matched assay diets in our experiment. One possibility is that reproduction at 14 days post-egg laying (E), the schedule to which the populations were adapted prior to commencing EE, still presents relatively strong selection for fast development. There is considerable theoretical and empirical evidence that fast growth is costly, particularly in terms of lifespan (Lee et al. 2011; Metcalfe & Monaghan 2003; Rollo 2002), thus it is plausible that by selecting for late rather than early reproduction, selection for fast growth may be removed and possibly even selected against because of its potentially negative affect on lifespan. Alternatively, selection on increased age-at-reproduction, and by extension lifespan, may actively select for longer developmental time, as increased lifespan is often coupled with increased developmental time (Khazaeli et al. 2005; Lints 1978; Promislow 1993).

Fecundity: significant but inconsistent responses

We did not anticipate the lack of a consistent response of fecundity across phenotyping sessions undertaken relatively closely in evolutionary time (Fig. 4). However, we argue that, in this particular case, the lack of a consistent response is not evidence of a lack of response, but rather, a product of our experimental design and the fickle nature of fecundity measurements/assays in general. Indeed, previous EE designs selecting on later age-at-reproduction have also found inconsistent responses of fecundity across generations (Leroi

et al. 1994), or marked sensitivity to environmental variation (Armand et al. 1994). In both phenotyping sessions we observed strongly significant effects of both age-at-reproduction and evolutionary diet (Table 3). For example, PE lines appeared to have decreased fecundity relative to PL, CE and CL lines at all ages (Fig. 4a:c). Given the large replication of our design (e.g. independent replicate populations per EE treatment) these responses are unlikely to be artefacts. Rather, we argue that the inconsistency could be merely a result of slight changes in environmental conditions coupled with the fact that we measured fecundity at three chronologically, rather than biologically determined time-points. If variables affecting the duration, onset, or magnitude of fecundity, such as temperature (Klepsatel et al. 2013) or humidity, differed slightly between the phenotyping sessions the differences observed at chronologically determined time points may not be consistent, despite the overall pattern of fecundity remaining the same. This is illustrated schematically in Fig. 5b: the relative differences between the two lines are identical across phenotypings, but in Fig. 5b.ii some hypothetical change in environmental conditions has lead to the overall compression of the fecund period relative to Fig. 5b.i, thus changing the relationship between the two curves at chronologically determined time points, despite the overall biological relationship remaining the same. Given the large size of our experimental design, phenotyping fecundity at the chronological time points at which the flies would lay eggs in the course of experimental evolution seemed a logical compromise, however, it is now a clear goal of future work to measure fecundity across a broader time frame.

The magnitude of lifespan extension in response to selection for later age-at-reproduction depends on evolutionary diet, assay conditions and sex

It was by no means a foregone conclusion that lifespan extension would still occur when flies were also forced to adapt to poor or rich larval evolutionary diets. Indeed, we hypothesised that by limiting acquisition and possibly selecting for faster development, the poor larval evolutionary diet may even prevent lifespan extension. However, we found that selection on increased age-at-reproduction was effective in extending lifespan across all evolutionary larval diets, in both sexes and across all assay diets (Fig. 6). Thus our experiment adds to the long line of experiments demonstrating that selection on age-at-reproduction can extend lifespan (Luckinbill et al. 1984; Partridge & Fowler 1992; Rose 1984). However, we show here that the extent of the response is dependent on the sex of the flies (in the case of PE & PL lines) and the assay diet (in the case of RL lines). Given that variation in diet quality is likely to be frequent in nature (Reznick et al. 2000; Tessier & Woodruff 2002) this study provides evidence that it can be an important player in life history evolution.

As we observed for developmental time, the effects of the poor and rich evolutionary diets on lifespan differed. The effect of adaptation to the poor

assay diet was sex-specific but similar across assay diets (Fig. 6a,b). In females adaptation to the poor assay diet was not at all detrimental to lifespan and even beneficial in the case of PE lines, which evolved longer lifespans than CE lines. In males, adaptation to the poor larval diet lead to less lifespan extension in PL lines than CL lines, while CE and PE lines did not differ (Fig. 6ab). A general argument can be made that selection on age-at-reproduction should affect lifespan more strongly in females than in males. For females, fitness at the later age requires being both alive and reproductively active until that age. The same is not necessarily true of males – females can utilize sperm from a single mating for several days, so males do not necessarily need to be alive at the later age to gain fitness, or indeed may gain more fitness by investing more in reproduction than survival. It is therefore possible that when nutrient availability is constrained males do not invest as much in lifespan as females, or by contrast, that gene-by-sex interactions (GSI) exist (e.g. Nuzhdin et al. 1997), such that extension of lifespan in females under low developmental nutrient availability comes at the expense of male lifespan. For the RE and RL lines, by contrast, we find no evidence of a potential trade-off between the sexes. This is consistent with the idea that trade-offs often manifest themselves only under poor conditions (Reznick et al. 2000).

The lifespan effects of adaptation to the rich assay diet were strongly dependent on the interaction between assay environment and selection on age at reproduction (Fig. 6c,d) . On the rich assay diet, RL lines showed considerably less lifespan extension than CL lines (significant in both sexes), while RE lines lived longer than CE lines (significant in females only; Fig. 6c,d). It is unclear why lines adapted to the rich assay diet should show such divergent lifespan responses when combined with selection for early and rich reproduction. It is noteworthy that the plastic effect of the rich larval diet is to slightly decrease lifespan relative to the control diet, thus the increased lifespan of RE lines relative to CE lines may be as a result of selection against this effect, or a mitigation of the detrimental effects of the rich larval diet for lifespan. Furthermore, the relatively small lifespan extension of RL lines may also reflect the inability of adaptation to mitigate completely the negative effects of the rich larval diet on lifespan. Strikingly, however, the responses of RE and RL lines with respect to CE and CL lines are completely reversed on the poor and control assay diets. Under these conditions RL and CL lines have similar lifespans and RE lines have shorter, rather than longer lifespans than CE lines. Again, this highlights the prediction from quantitative genetic theory that trait responses may be very different in evolved versus novel environments (Armand et al. 1994; Fry et al. 1998; Mackay 2001; Sgro & Hoffmann 2004; Vieira et al. 2000).

Evidence for trade-offs

For the traits we assayed, we observed no clear evidence of trade-offs. For example, lines adapted to the poor larval diet were able to both extend lifespan and speed up development at no cost to larval survival. However, it is quite likely that trade-offs exist with other traits, which we did not measure, or with fecundity, for which we obtained inconsistent results. For example, Kolss et al., (2009) found that adaptation to severe larval malnutrition decreased both adult size and early fecundity. Indeed, in Drosophila, adult body size often shows a strong positive correlation with developmental time (Hillesheim & Stearns 1992; Prasad et al. 2001; Zwaan et al. 1995), and body size, in turn, is generally positively correlated with fecundity (Honek 1993; Robertson 1957). Thus it is possible that the PE and PL lines have sped up their developmental time at the expense of adult size, and, by extension adult fecundity. In fact, there does appear to be some evidence that PE lines have decreased fecundity across the lifespan (Fig. 4a:c).

CONCLUSIONS

By utilising the combined strength of extensive replication, multiple assay environments, and assessment of evolution across multiple generations we have been able to discriminate between transient and consistent effects of adaptation to larval diet and age-at-reproduction. We show that the two life stages do not act independently but rather interact to determine phenotypes for both developmental time and lifespan, while larval survival and fecundity show no consistent evolutionary responses. Increased lifespan in response to selection on later age-at-reproduction is observed across all evolutionary diets, however, the magnitude of the response is dependent on the sex, the evolutionary diet, and the experimental assay conditions. Furthermore, across evolutionary diets we find that the adaptive response to selection on increased age-at-reproduction is increased developmental time, resulting in a positive correlation between developmental time and lifespan when flies are raised on the assay diet matching their evolutionary diet. However, on other assay diets this relationship is inconsistent, as predicted by quantitative genetic theory. Overall, we show that flies are not able to adapt to selection at two different life stages independently, but rather, must integrate both into their life history. Given that multiple selection pressures are likely the norm rather than the exception in nature, this finding argues that trade-offs should be considered not only between traits within an organism, but also between adaptive responses to differing selection pressures. Furthermore, because variation in available nutrition is so frequent in natural environments, we argue that this can play a large role in shaping the evolution and diversity of life histories in nature.

ACKNOWLEDGEMENTS

We would like to thank Kees Koops, Marijke Slakhorst, Bertha Koopmaanschap and Gabriella Bukovinszkine Kiss for help with the initial set up of the EE populations, their maintenance, and the set-up of phenotyping sessions. We would also like to thank Fons Debets, Jelle Zandveld, Florien Gorter and Claudio Valero Jimenez for helpful discussions on the experimental design and interpretation of the results. This work was supported by the European Union's FP6 Programme (Network of Excellence LifeSpan FP6/036894) and the EU's FP7 Programme (IDEAL FP7/2007-2011/259679).

REFERENCES

(2005) Development Core Team, R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0.

Aguila JR, Hoshizaki DK, Gibbs AG (2013) Contribution of larval nutrition to adult reproduction in *Drosophila melanogaster*. *The Journal of Experimental Biology* **216**, 399-406.

Aguila JR, Suszko J, Gibbs AG, Hoshizaki DK (2007) The role of larval fat cells in adult *Drosophila melanogaster*. *Journal of Experimental Biology* **210**, 956-963.

Armand ML, Chippindale AK, Rose MR (1994) Long-term laboratory evolution of a genetic life-history trade-off in *Drosophila melanogaster*. 1. The role of genotype-by-environment interaction. *Evolution* **48**, 1244-1257.

Baldal EA, Brakefield PM, Zwaan BJ (2006) Multitrait evolution in lines of *Drosophila melanogaster* selected for increased starvation resistance: the role of metabolic rate and implications for the evolution of longevity.*Evolution* **60**, 1435-1444.

Bochdanovits Z, Jong Gd (2003) Experimental evolution in *Drosophila melanogaster*: Interaction of temperature and food quality selection regimes. *Evolution* **57**, 1829-1836.

Bubliy OA, Loeschcke V (2005) Correlated responses to selection for stress resistance and longevity in a laboratory population of *Drosophila melanogaster*. *Journal of Evolutionary Biology* **18**, 789-803.

Chippindale AK, Chu TJ, Rose MR (1996) Complex trade-offs and the evolution of starvation resistance in *Drosophila melanogaster*. *Evolution*, 753-766.

de Jong G, van Noordwijk AJ (1992) Acquisition and allocation of resources: genetic (co) variances, selection, and life histories. *The American naturalist* **139**, 749-770.

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

Fry JD, Nuzhdin SV, Pasyukova EG, Mackay TFC (1998) QTL mapping of genotype–environment interaction for fitness in *Drosophila melanogaster*. *Genetics Research* **71**, 133-141.

Hillesheim E, Stearns SC (1992) Correlated responses in life-history traits to artificial selection for body weight in *Drosophila melanogaster*. *Evolution* **46**, 745-752.

Hoffmann AA, Hallas R, Anderson AR, Telonis-Scott M (2005) Evidence for a robust sex-specific trade-off between cold resistance and starvation resistance in *Drosophila melanogaster*. *Journal of Evolutionary Biology* **18**, 804-810.

Honek A (1993) Intraspecific variation in body size and fecundity in insects: a general relationship. *Oikos* **66**, 483-492.

Kawecki TJ, Lenski RE, Ebert D, *et al.* (2012) Experimental evolution. *Trends in Ecology & Evolution* **27**, 547-560.

Khazaeli AA, Van Voorhies W, Curtsinger JW (2005) The relationship between life span and adult body size is highly strain-specific in *Drosophila melanogaster*. *Experimental Gerontology* **40**, 377-385.

Kirkwood TB (1977) Evolution of ageing. *Nature* **270**, 301-304.

Kirkwood TBL, Holliday R (1979) The Evolution of Ageing and Longevity. *Proceedings of the Royal Society of London B: Biological Sciences* **205**, 531-546.

Kirkwood TBL, Shanley DP (2005) Food restriction, evolution and ageing. *Mechanisms of Ageing and Development* **126**, 1011-1016.

Klepsatel P, Gáliková M, De Maio N, *et al.* (2013) Variation in thermal performance and reaction norms among populations of *Drosophila melanogaster*. *Evolution* **67**, 3573-3587.

Kolss M, Vijendravarma RK, Schwaller G, Kawecki TJ (2009) Life-history consequences of adaptation to larval nutritional stress in drosophila. *Evolution* **63**, 2389-2401.

Lee W-S, Metcalfe NB, Monaghan P, *et al.* (2011) A Comparison of dynamic-state-dependent models of the trade-off between growth, damage, and reproduction. *The American naturalist* **178**, 774-786.

Leroi AM, Chen WR, Rose MR (1994) Long-term laboratory evolution of a genetic life-history trade-off in *Drosophila melanogaster*. 2. Stability of Genetic Correlations. *Evolution* **48**, 1258-1268.

Lints FA (1978) Genetics and ageing S. Karger.

Lints FA, Soliman MH (1988) *Drosophila* as a model organism for ageing studies Springer.

Luckinbill LS, Arking R, Clare MJ, Cirocco WC, Buck SA (1984) Selection for delayed senescence in *Drosophila melanogaster*. *Evolution* **38**, 996-1003.

Mackay TFC (2001) Quantitative trait loci in *Drosophila*. *Nat Rev Genet* **2**, 11-20.

Mair W, Goymer P, Pletcher SD, Partridge L (2003) Demography of dietary restriction and death in *Drosophila*. *Science* **301**, 1731-1733.

Medawar PB (1952) An unsolved problem of biology College.

Metcalfe NB, Monaghan P (2003) Growth versus lifespan: perspectives from evolutionary ecology. *Experimental Gerontology* **38**, 935-940.

Nakagawa S, Lagisz M, Hector KL, Spencer HG (2012) Comparative and meta-analytic insights into

REFERENCES

life extension via dietary restriction. *Aging Cell* **11**, 401-409.

Nuzhdin SV, Pasyukova EG, Dilda CL, Zeng Z-B, Mackay TFC (1997) Sex-specific quantitative trait loci affecting longevity in *Drosophila* melanogaster. *Proceedings of the National Academy of Sciences* **94**, 9734-9739.

Partridge L, Fowler K (1992) Direct and correlated responses to selection on age at reproduction in *Drosophila melanogaster*. *Evolution* **46**, 76-91.

Prasad N, Shakara M, Anitha D, Rajamani M, Joshi A (2001) Correlated responses to selection for faster development and early reproduction in *Drosophila*: the evolution of larval traits. *Evolution* **55**, 1363-1372.

Promislow DE (1993) On size and survival: progress and pitfalls in the allometry of life span. *Journal of Gerontology* **48**, B115-B123.

Reznick D, Nunney L, Tessier A (2000) Big houses, big cars, superfleas and the costs of reproduction. *Trends in Ecology & Evolution* **15**, 421-425.

Robertson FW (1957) Studies in quantitative inheritance XI. Genetic and environmental correlation between body size and egg production in*Drosophila* Melanogaster. *Journal of Genetics* **55**, 428-443.

Roff DA (2001) *Life History Evolution* Sinauer Associates, Sunderland.

Rollo CD (2002) Growth negatively impacts the life span of mammals. *Evolution & Development* **4**, 55-61.

Rose MR (1984) Laboratory evolution of postponed senescence in *Drosophila melanogaster*. *Evolution* **38**, 1004-1010.

Schlichting C, Pigliucci M (1998) Phenotypic evolution: A reaction norm perspective Sinauer.

Sgro CM, Hoffmann AA (2004) Genetic correlations, tradeoffs and environmental variation. *Heredity* **93**, 241-248.

Stearns SC (1992) *The Evolution of Life Histories* Oxford University Press, Oxford.

Tessier AJ, Woodruff P (2002) Trading off the ability to exploit rich versus poor food quality. *Ecology Letters* **5**, 685-692.

Van Noordwijk AJ, de Jong G (1986) Acquisition and allocation of resources: their influence on variation in life history tactics. *American Naturalist*, 137-142.

Vieira C, Pasyukova EG, Zeng Z-B, *et al.* (2000) Genotype-environment interaction for quantitative trait loci affecting life span in *Drosophila melanogaster*. *Genetics* **154**, 213-227.

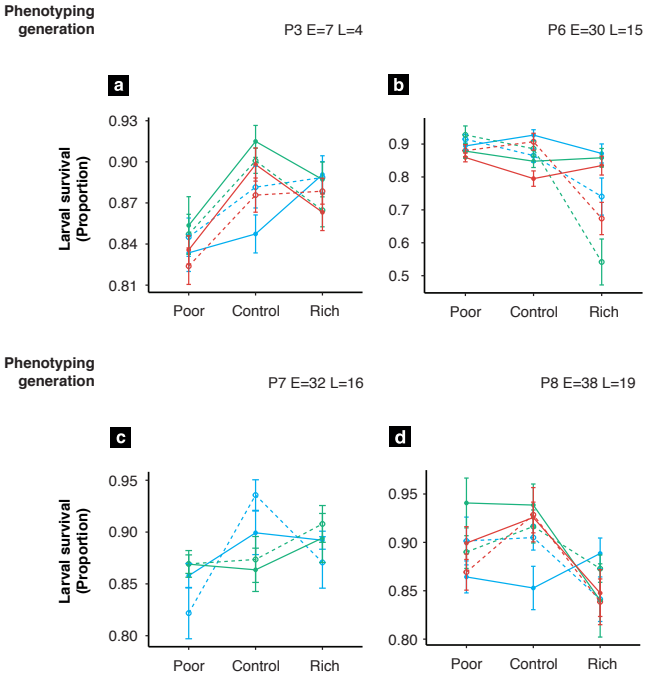
Vijendravarma RK, Narasimha S, Chakrabarti S, *et al.* (2015) Gut physiology mediates a trade-off between adaptation to malnutrition and susceptibility to food-borne pathogens. *Ecology Letters* **18**, 1078-1086.

Vijendravarma RK, Narasimha S, Kawecki TJ (2013) Predatory cannibalism in *Drosophila melanogaster* larvae. *Nat Commun* **4**, 1789.

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

Zwaan BJ (1999) The evolutionary genetics of ageing and longevity. *Heredity* **82**, 589-597.

Zwaan BJ, Bijlsma R, Hoekstra RF (1991) On the developmental theory of ageing. I. Starvation resistance and longevity in *Drosophila melanogaster* in relation to pre-adult breeding conditions. *Heredity* **66**, 29-39.



APPENDICES

Appendix 1. Larval survival from egg to adult (y-axis) across assay diets (x-axis) and phenotyping sessions for each combination of evolutionary diet (color) and age-at-reproduction (line type). Blue, green and red represent the poor (P), control (C) and rich (R) EE larval diets respectively. Solid and dashed lines represent early (E) and late (L) reproducing lines respectively. All error bars are standard errors of the mean across replicate lines. For simplicity, only phenotyping sessions carried out using all three assay diets are included: P3 (a), P6 (b), P7 (c) and P8 (d). In general, larval survival remains high across phenotyping sessions and assay diets and shows no clear or consistent effect of EE.

6

General Discussion

GENERAL DISCUSSION

The aim of this thesis was to increase our understanding of how developmental diet influences life histories in the fruit fly. Within this broad mandate and as outlined in the introduction, I addressed three main questions: first, to what extent does the phenotypic effect of developmental diet depend on the adult environment, second, does variation in developmental diet have consequences for gene expression across the lifespan, and finally, how do life histories evolve in response to variation in developmental diet? The first two questions were addressed in the first three experimental chapters of the thesis (**Chapters 2 through 4**) and lead to two main findings. First, developmental diet does affect both adult phenotypes and gene expression in the fruit fly across the lifespan, but except for at the very beginning of adult life, this effect is considerably smaller than that of the adult environment, suggesting that fruit flies retain extensive plasticity into adulthood. Second, in general, the developmental and adult environments exert independent effects on both life history traits and the transcriptome, suggesting that the effect of development on late life health in flies is congruent with the "silver-spoon" hypothesis. The last experimental chapter (**Chapter 5**) addressed the third question: how do life histories evolve in response to variation in developmental diet, and how does selection for increased age-at-reproduction during adulthood depend on this (developmental) diet. The main finding here was that, in contrast to the plastic response, individuals are not free to independently respond to variation in developmental and adult conditions, but rather over evolutionary time the adaptive responses to developmental diet are contingent on the selection pressures experienced during adulthood and vice versa.

In this discussion, I will synthesise the findings across the experimental chapters, and address how they relate to each other. Furthermore, I will discuss some more general points that emerged, such as the potential importance of allometry or tissue specific effects in mediating effects of larval diet, and the difficulty of linking life history phenotypes to variation in gene expression. Finally, I will address future directions suggested by this thesis, as well as the potential insights that can be gained from this work in the context of theories linking developmental conditions to late life health in humans.

Under and over-feeding during development: similarities and differences

In **Chapter 2**, I showed that both the poor and rich larval diets lead to increased developmental time and decreased adult size relative to the control diet. Both of these responses are classically considered indicative of a sub-optimal environment in flies and suggest that over and under-feeding during development may share some similarities, a finding that has also been observed in mammalian studies (reviewed in Ford & Long 2011). Indeed, this was also

supported by patterns of variation in gene expression in early life (**Chapter 3**) as the transcriptomes of poor-raised flies were more similar to rich than control-raised flies in both sexes. However, the two diets differed in whether they also showed "sub-optimality" of lifespan and fecundity relative to the control diet. Surprisingly, flies raised on the poor larval diet lived longer (males and females) and reproduced more at certain ages and under certain reproductive conditions (females), while the rich-raised flies showed the opposite response. This effect on lifespan and fecundity was also identified when these traits were measured across adult diets (**Chapter 4**) indicating that these effects are robust.

Given the generally positive correlation between body size and fecundity in insects (Honek 1993), the increased fecundity of the flies raised on the poor larval diet, which are smaller, is surprising. Furthermore, their increased fecundity does not appear to come at any cost to lifespan, giving the outward appearance that that they have circumvented the general trade-off between lifespan and fecundity (Roff 2001; Stearns 1992). However, acquisition-allocation theory predicts that the appearance of an absence of such trade-offs can occur when individuals differ in resource acquisition levels, as they do in our experiment (de Jong & van Noordwijk 1992; Van Noordwijk & de Jong 1986). In general this theory relates to the apparent alleviation of the lifespan-fecundity trade-off in individuals who acquire more resources, allowing them to both reproduce more and live longer, but it is also allows for the possibility of differing allocation strategies with a given amount of resources. Thus the poor-raised flies may have allocated more of their limited resources to lifespan and fecundity, at the expense of another trait or traits.

The role of the immune system

One possibility is that poor-raised flies may have invested less in their immune system, allowing greater investment in reproduction, but at little cost in the relatively clean (e.g. microbe-free) laboratory environment. The immune system is costly, and competes with repair mechanisms (longevity assurance) and fecundity for resources (Lochmiller & Deerenberg 2000; Moret & Schmid-Hempel 2000; Norris & Evans 2000; Schwenke et al. 2015). Indeed, one of the only other studies to address the consequences of developmental conditions for gene expression found that the expression of candidate immune genes in young adults is positively correlated with the protein content of the larval diet and concluded that larval diet lead to a plastic reallocation of resources to immunity (Fellous & Lazzaro 2010). In our study of the transcriptome in late life (**Chapter 4**), immunity did not emerge as a major functional group affected by larval diet across the lifespan. It should be noted however, that this may be due to our emphasis on groups of co-expressed genes, rather than the individual genes with the largest difference in expression across the treatments. In fact, when I focused on this aspect of the transcriptome, immunity genes

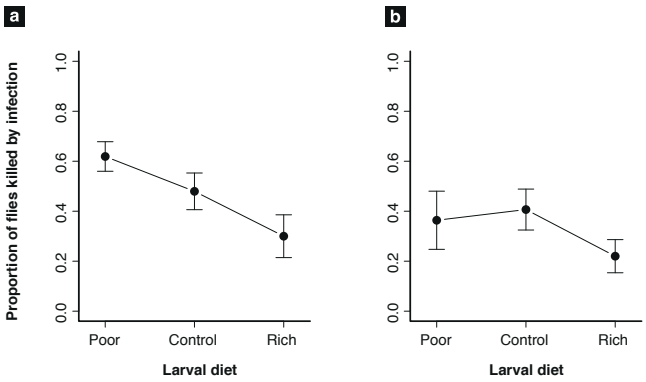


Figure 1: Proportion of flies killed by infection with the gram positive bacteria Enterococcus faecalis in female (a) and male (b) flies raised on either poor, control, or rich larval diets (along x-axis). Error bars represent 95% confidence intervals.

were clearly overrepresented and their expression was lower in the poor relative to the control and rich-raised flies (analyses not shown). This prompted me to perform several extensive pilot experiments in which I septically infected adult flies raised on the different larval diets with the gram-positive bacteria Enterococcus faecalis. I found that the poor larval diet decreases the ability to survive infection, while the rich larval diet increases it, significantly so in females (Fig. 1a, GLM: $F(2,12) = 4.60$, $p=0.003$), but not in males (Fig. 1b). These results are consistent with differential allocation of developmentally acquired resources to immune function on the one, and lifespan and fecundity on the other hand. In addition, the fact that this effect is much more visible in the susceptibility to infection of female than male flies suggests that fecundity is a much more likely "beneficiary" of this resource re-allocation than lifespan as the reproductive investment in females in much larger than in males. Thus these experimental results show that (i) the poor larval diet is not unequivocally "good" relative to the rich larval diet, and, (ii) that although lifespan and reproduction are the "textbook" fitness traits to place on the opposite sides of a trade-off, there are many other physiological and life history traits that may play a role. The latter message too often goes unheeded in studies of ageing and lifespan, perhaps because of the siren-song of cost-free lifespan extension. There are certainly many examples of lifespan extension with no clear cost to fecundity and vice versa (reviewed in Flatt 2011), however to my mind, in order to establish the existence of truly "cost-free" lifespan extension, we must look more carefully at the full life-history phenotype, beyond lifespan and fecundity, across a range of different resource acquisition levels, and in environments more similar to the environments in which the species evolved.

The role of the microbiome

Changes in immune function are also known to be related to the microbiome in flies (Broderick et al. 2014), and the microbiome is known to be dependent on diet (Chandler et al. 2011; Gilbert 2005), thus I hypothesised that one potential reason for the change in immune function may be due to flies acquiring

different microbiomes during development, and that this may also relate to the observed lifespan differences. I assessed whether "curing" adult flies raised on the different larval diets of their microbiome by antibiotic treatment had any effect on the lifespan differences observed in Chapters 2 and 4. I reasoned that if the effects disappeared, then they may have been due to changes in the microbiome. I found no effect of the curing on the lifespan differences we observed (unpublished data) however, a role for the microbiome cannot be completely excluded as flies may have re-established similar microbiomes after antibiotic treatment, or the effects may already have been established during development or in early adulthood, prior to curing. Thus addressing how developmental diet affects the microbiome requires further work.

The role of ribosomes, transcription and translation

Another clue about the potential mechanism underlying the lifespan extension in poor-raised flies and the lifespan decrease in rich raised flies comes from the finding in **Chapter 4** that larval diet affects the expression of a cluster of genes related to ribosomes, transcription and translation in both sexes, across adult diets and in middle and old-age. The expression of this cluster is negatively associated with the lifespan effects of larval diet, in a manner consistent with the known effects of knock-down of ribosome and translation related genes in model organisms - i.e. knockdown of the expression of ribosomal subunits or transcription and translation machinery usually increases lifespan (Chen et al. 2007; Chiocchetti et al. 2007; Curran & Ruvkun 2007; Hansen et al. 2007; e.g. Kaeberlein et al. 2005; Larson et al. 2012; McCormick et al. 2015; Pan et al. 2007; Steffen et al. 2008). Given that the generation of ribosomes and transcription and translation are fundamental cellular processes, it seems plausible that changes in their expression may affect lifespan and fecundity. For example in growing yeast cells, ribosomal RNA makes up approximately 80% of total cellular nucleic acid and nearly 50% of all RNAP II transcription initiation events occur on ribosomal protein genes (Lempiäinen & Shore 2009; Warner 1999).

The question then becomes: why or how would larval diet affect expression of these genes at middle and old-age. Because I used whole flies I cannot exclude that these changes reflect larval-diet induced changes in the allometry of certain tissues, however, given the ubiquitous and essential nature of ribosomes and translation, no obvious candidate tissue presents itself. An alternate hypothesis is that larval diet somehow affects the activity of ribosomes. Ribosome expression level is known to be modulated in two ways: for short-term regulation in response to dietary changes or stress, transcription rate at already "active" rDNA is altered, while for more stable changes (such as tissue-specific levels of expression) expression of ribosome protein genes is turned either on or off through epigenetic changes that lead to an altered

chromatin state (reviewed in Grummt & Ladurner 2008). For example, even in rapidly growing yeast cells, about 50% of the rDNA copies are silenced through epigenetic modification (Lempiäinen & Shore 2009). Intriguingly, a recent study in Drosophila found that increasing levels of dietary yeast during development lead to increased expression of ribosomal RNA genes during development and that this in turn is correlated with increased rDNA instability and loss of rDNA copies in adults (Aldrich & Maggert 2015). Furthermore, the loss of rDNA copies is known to be associated with the loss of heterochromatic induced silencing (Paredes & Maggert 2009) and the loss of heterochromatin in turn, is known to de-repress RNA synthesis and decrease lifespan in Drosophila (Larson et al. 2012). This has lead to the hypothesis that epigenetic preservation of genome stability, especially at the rDNA locus, and repression of unnecessary rRNA synthesis, might be an evolutionarily conserved mechanism for prolonging lifespan (Larson et al. 2012). Thus one plausible hypothesis is that as yeast concentration in our larval diet increased (poor -> rich) de-repression of RNA synthesis was decreased during development, leading to loss or rDNA copies in adults, resulting in the loss of heterochromatic induced silencing and de-repression of rRNA synthesis in adulthood. At the moment this is very clearly only a hypothesis, but measuring rDNA copy number in adult flies raised on the different larval diets would give a relatively quick insight into whether this mechanism may be important. If such differences exist it may also prove insightful to look for such changes in the experimental evolution lines (**Chapter 5**) to determine whether such changes will persist and become fixed in the genome.

Given the crucial nature of ribosomes, transcription and translation for cellular growth and reproduction, it is difficult to reconcile the increased fecundity of poor-raised flies with their down-regulation of these processes. One possibility is that the increased lifespan of the poor-raised flies may be mediated via the ribosomes and the increased fecundity via the immune response "savings" discussed above - i.e. the lifespan and fecundity effects may be underpinned by different processes.

Young adulthood as a unique epoch in the life of a fly

In **Chapter 3**, I showed that most of the variation in gene expression in young adult flies is attributable to larval and not adult diet, while, in **Chapter 4**, I found that this pattern is reversed at both middle- and old-age. This suggests that the beginning of adult life is a unique phase in which gene expression is still largely dependent on developmental conditions. Furthermore, comparison with the middle- and old-aged time points with PCA analysis shows that the first day of adult life is unique not only in the relative magnitude of the effects of larval and adult diet, but also in its overall expression (Males: Fig. 1a; Females: Fig. 1b). Indeed, this large distinction (PC1 explains 66% of all variation in males and

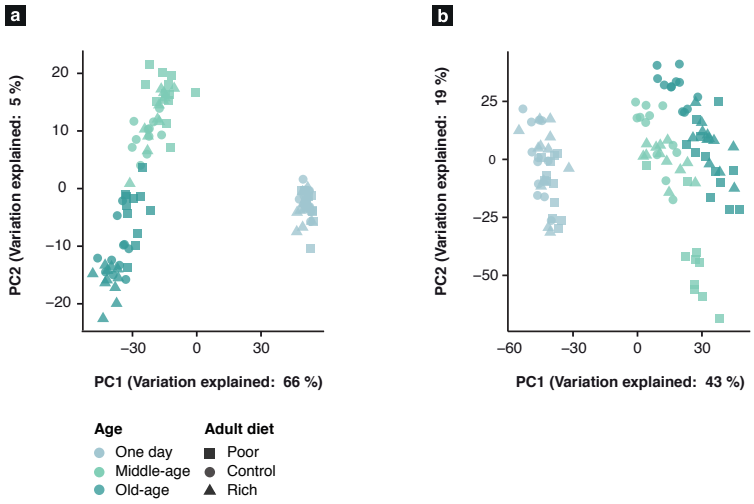


Figure 2: Plots of PC1 versus PC2 in males (a) and females (b) when samples taken at one day of age, middle-age, and old-age are normalised together. In both sexes PC1 explains most of the variation in expression and clearly divides samples taken at one-day old from the rest.

43% in females) formed the rationale behind analyzing the early and later life transcriptomes separately.

I hypothesised that the large effect of larval diet in early life may be due to the carry over of larval tissues into adulthood, thus affecting the whole body transcriptome either through changes in regulation of the tissues, or through changes in their overall size induced by larval diet. Such changes are clearly plausible, as for example, Zwaan et al., (1991) showed that overall fat content (arguably related to the size of the fat body) later in life is influenced by both developmental density and diet. In females I hypothesised that the effects of the developmental diet on the young adult may be mediated by either changes in ovariole number due to larval diet (thus changes to the adult tissue, not carry-over of the larval tissue) or due to the carry-over of the larval fat body from development into adulthood. In males tissue-specific expression profiles suggested a role for the malpighian tubules, the gut and the larval fat body, all of which are known to survive pupation and persist into adulthood (Aguila et al. 2013; Aguila et al. 2007; Klapper 2000; Nelliot et al. 2006; Riddiford 1993). The relative absence of such effects later in life suggests that this is purely a transient phenomenon. In the case of the larval fat body, the transience of the effect is expected, as it disappears within one week of adulthood (Aguila et

al. 2007). However, there is no evidence that the gut and malpighian tubules are later lost, thus if the tissues are being carried over, one might expect the expression changes to persist, especially if they are due to changes in allometry. Intriguingly though, in males there is evidence that alongside the effect of larval diet on probes with high expression in these tissues, there is also a concomitant and independent effect of adult diet. Thus one hypothesis is that while flies carry these larval tissues over into adulthood, they adapt them to prevailing adult conditions upon emergence. Indeed, a recent study by van den Heuvel et al., (2014) found that when maintaining flies on a so-called "yo-yo" diet, in which they switch between different diets every week of adult life, the first diet experienced after eclosion determines both the manner and extent to which life-history traits vary. The authors suggested that it might be beneficial for a short-lived organism to be able to alter life-history decisions in response to early adult experience. Thus one possibility is that flies "adjust" tissues carried over into adulthood to the prevailing adult environment, which is especially interesting in the case of the gut, as it has been shown that regional expression patterns are not set until approximately three days after eclosion (Buchon et al. 2013). To test this hypothesis it would be useful to employ a similar yo-yo design to that employed by van den Heuvel et al., (2014) and address whether certain tissue-specific aspects of the transcriptome are "set" during early life.

Despite the relative transience of the effect of larval diet on the adult transcriptome, it may still have important consequences for our understanding of life history evolution. For example, in my experimental evolution regime (**Chapter 5**) flies selected for early reproduction have only four days of adult life prior to laying eggs for the next generation, thus these differences in expression in early life might be important constraints or determinants of the potential response. Furthermore, a more practical point is that (uncontrolled) differences in larval diet may explain inconsistencies observed between studies of adult traits, especially when they pertain to early life phenotypes and gene expression. In the fly literature there is no defined developmental diet, thus slight differences in the developmental diet used may have profound effects, at least for early life responses. Thus the fly community may also benefit from a "defined" developmental diet, as has been proposed for studies addressing the effects of diet in adults (Bass et al. 2007; Piper et al. 2014).

Adapting to selection in both development and adulthood

In **Chapter 5**, I addressed how life history traits evolved in response to adaptation to different levels of larval acquisition combined with classical selection for early and late-life reproduction. Most Experimental Evolution (EE) studies address only a single environmental manipulation, however, individuals likely need to cope with several different selection pressures at the same time, and, furthermore, must balance the costs and benefits of adaptations in one

stage such as development, with their consequences in another stage, such as adulthood. I showed that the response to selection depended on both the developmental and adult selection regime, however, the interactions were generally of "magnitude" rather than of "sign". This is surprising given previous studies which suggest that there may be a conflict between the two selection pressures: low developmental acquisition levels tend to select for faster development (Bochdanovits & de Jong 2003; Kolss et al. 2009) while faster development is generally associated with shorter lifespan (Lints 1978) (but see Zwaan et al. 1991)). Despite this potential conflict, lifespan extension was achieved across all acquisition levels and flies adapted to the poor larval diet and late reproduction were able to both extend lifespan and decrease developmental time simultaneously, suggesting that such perceived conflicts are not set in stone, and are highly dependent on the actual experimental evolution regime employed.

These lines continue to evolve in the laboratory and intermittent phenotypings (unpublished data) show that the life history responses identified in this thesis persist. One intriguing question for further work is whether fecundity has or has not evolved - I found no consistent evidence of this, but argued that it was due to the experimental design employed. It seems unlikely that no changes in fecundity have evolved, and the transition from poor larval food conditions to the control diet in combination with early reproduction requires particular further attention as the most likely place in which trade-offs may be involved. Furthermore, as mentioned in the introduction, evolve and resequence (E&R) approaches allow the identification of the pathways and processes accessible to evolution in natural populations (Kawecki et al. 2012; Long et al. 2015). An exciting future direction that is ongoing is to apply E&R to these populations in order to identify the underlying genomic changes involved in mediating the phenotypic changes. Because of the full-factorial design employed here these lines offer especially attractive candidates for E&R, especially with respect to understanding the evolution of increased lifespan, as both shared and unshared genomic changes correlated to lifespan extension can be expected depending on the specific combination of acquisition and selection on age-at-reproduction involved. Thus the analysis of the EE lines as described in this thesis represents an essential and solid basis with respect to what we can learn from them, and they will hopefully provide a resource for understanding the nature of the relationship between acquisition levels and lifespan for many years to come. This is a badly needed and essential addition to the "knock-in/knock-out" mutagenesis approaches in model organisms that have provided crucial insights into public and private pathways and processes that are involved in determining lifespan (Kuningas et al. 2008; Partridge & Gems 2002), but for which it is unclear and under-researched whether they explain the variation in lifespan and other life history traits in natural populations, including those in humans.

The difficulty of matching gene expression to phenotype in life history traits

In general, transcriptomic studies aim to link expression variation with phenotypic variation. While not one of the main goals of this thesis, an important conclusion is that this is far from a straightforward exercise, especially when addressing life history traits, which likely are a reflection of the overall physiological status of an individual. In our studies we used whole bodies of flies for gene expression analysis, both because there was no a priori hypothesis about which tissues or mechanisms might play a role, and because this could yield an idea of the systemic response. Of course the potential shortcoming of this approach is the inability to discriminate between expression changes due to regulation or allometry (Harrison et al. 2015), however, the opposite end of the coin is that it can point at tissues that may play a role at a systemic level. Two alternatives to our approach are the use of single tissues (e.g. Robinson et al. 2013) or, as has recently become possible, single cells (e.g. Xue et al. 2013). The tissue-specific approach is powerful when there is a good a priori reason to suspect the involvement of a certain tissue, however, tissues are also generally composed of a mix of different cell types, thus the potential still exists to measure overall differences in cell type populations, rather than identifying true "effector" genes (Harrison et al. 2015). The single-cell approach avoids problems of allometry within the sample itself and gives exciting insight into cellular physiology, but contains little information about the physiological status of the whole individual. Ideally, one would apply a combination of all three approaches to gain a more nuanced understanding of the link between gene expression and phenotype. Furthermore, to gain true understanding, it appears we may have to take a step backwards from exciting new technologies and look more deeply at phenotypes - or at least to consider phenotypes and gene expression together. For example, by using three diets instead of two as in our study, we show that most variation in gene expression is not linearly related to the observed phenotypic differences between diets: i.e. in male fruit flies we see considerable changes in testes-related genes across the lifespan whose expression shows no linear correlation with the observed lifespan differences. Thus studying phenotypes and expression together, with more than two treatments, is essential for studies that aim to make such links.

PARs, silver spoons, and where to look next for long-term effects on phenotypes on late life health

This thesis was embarked upon in the context of theories linking the observed effects of poor developmental conditions in humans to increased risk of metabolic disease in adulthood, primarily the "Silver Spoon" and "Predictive Adaptive Response" hypotheses (reviewed in Monaghan 2008). Briefly, the predictive adaptive response hypothesis predicts that in utero, humans use cues about the future environment to develop a phenotype that will be well-suited to the predicted conditions, but with fitness consequences in the case of

an incorrect prediction (Bateson et al. 2014; Gluckman & Hanson 2004), while the silver spoon hypothesis predicts that individuals that develop under good quality conditions will have an advantage across adult conditions and vice versa (Grafen 1988). Flies are clearly not humans, and have evolved in a very different ecological context, thus whether or not flies exhibit PARs (it appears they do not; this thesis) does not tell us anything about whether or not we can expect the existence of PARs in humans (though there is a relatively convincing body of literature stating that they are unlikely (Hayward & Lummaa 2013; Hayward et al. 2013; e.g. Rickard & Lummaa 2007; Wells 2012)). However, as research on flies has helped to identify dietary restriction, insulin signalling (IIS) and TOR pathways as important modulators of mammalian and possibly human lifespan (Fontana et al. 2010; Partridge et al. 2011), it can also serve as a tool to identify potential future directions for understanding the link between development and late life health in humans. One might expect that the mechanisms involved may be the same, even if the way in which they are used differs: e.g. to respond to variation in diet, individuals use conserved nutrient sensing mechanisms, but depending on their evolutionary history, differ in how they use them (e.g. van den Heuvel et al., 2016, submitted theoretical model). In any case, the work in my thesis is one of the first, if not the first, to partition the variation in gene expression to developmental and adult diets across the lifespan. Moreover, in general the effects of "developmental exposures" on the epigenome and gene expression are exclusively measured in late-life and lack (for understandable reasons) a longitudinal component. I show that long-lasting effects on gene-expression can be found, but that linking them "as is" to observed phenotypes is likely to lead to spurious conclusions. Thus the possibility that many of the observed differences in molecular (epi) genetic and even physiological measures may merely reflect the "shadow of development past" should serve as both a source of caution and a thoughtfully considered competing hypothesis for researchers attempting to link long-term epigenetic changes to late life health.

In addition to these more general points on the value and possible impact of my work, I think there are two more specific insights to be gleaned from my thesis. First, I show that the expression of ribosomes, transcription and translation are affected across the lifespan by larval diet in both sexes and are correlated with lifespan. Given the conserved functional role of the transcriptional and translational machinery across eukaryotes, coupled with the recent discoveries that similar to mutations in TOR and IIS, knock-outs of ribosomes and translational machinery increase lifespan (Chen et al. 2007; Chiocchetti et al. 2007; Curran & Ruvkun 2007; Hansen et al. 2007; e.g. Kaeberlein et al. 2005; Larson et al. 2012; McCormick et al. 2015; Pan et al. 2007; Steffen et al. 2008), this may be an intriguing area to investigate in "higher" organisms such as mice or rats. Second, our use of whole flies suggested that some of the long-term change

in gene expression were related to certain tissues (i.e. the testes in males). While this response was not correlated to variation in lifespan, it does suggest that developmental diet might disproportionately affects certain tissues. Indeed, a tissue-specific explanation was one of the first proposed, when Barker et al., (1989) discovered the link between low birth weight and cardiovascular disease many years ago. It was suggested that individuals in a poor in utero environment prioritise the brain at the expense of other tissues, and that the "compromised" tissues may form the source or cause of late-life disease (Hales & Barker 1992; Osmond & Barker 2000). This angle has since been relegated to the back-burner but might deserve re-examination. This would require extensive phenotypic analysis of the relative size and composition of different tissues in response to variation in the developmental environment. It is noteworthy in this respect that in one of the best studied tissues in humans, blood, differences in cell type and counts are treated as confounding rather than explanatory variables.

CONCLUSIONS AND FINAL REMARKS

In this thesis I have shown that developmental diet affects both adult phenotypes and gene expression in fruit flies, whether experienced within a single generation, or across multiple generations. For plastic responses, the developmental and adult environments exert largely independent effects on phenotypes and gene expression suggesting that flies retain extensive plasticity into adulthood. Furthermore, the effect of adult diet is generally much larger than that of developmental diet, suggesting that while effects of developmental diet do exist, they are dwarfed by adult plasticity. Furthermore, most long-term effects on gene expression show no linear correlation with phenotypes, however I do identify a long-term effect of developmental diet on the expression of ribosomes and transcription and translation related genes that is correlated with the lifespan effects of larval diet, suggesting that these genes may play a role in modulating the long-term effect of diet, and that they deserve further consideration. Most excitingly, in the last experimental chapter I begin to categorise responses to developmental diet over evolutionary time, showing that the extent of lifespan extension in response to selection on increased age-at-reproduction is dependent on evolutionary larval acquisition levels. This analysis is only the tip of the iceberg and offers great potential to understand how organisms cope with variation in acquisition over the long-term, but also which genes and pathways underlie this process. It is hoped that this thesis has provided a thorough baseline for the types and magnitude of responses that can be expected in response to developmental diet. Future work should address the candidate mechanisms identified in this thesis further, as well as make full use of the extraordinary potential of the experimental evolution lines, for example by identifying the genomic changes underlying the observed evolutionary phenotypic responses.

REFERENCES

Aguila JR, Hoshizaki DK, Gibbs AG (2013) Contribution of larval nutrition to adult reproduction in *Drosophila melanogaster*. *The Journal of Experimental Biology* **216**, 399-406.

Aguila JR, Suszko J, Gibbs AG, Hoshizaki DK (2007) The role of larval fat cells in adult *Drosophila melanogaster*. *Journal of Experimental Biology* **210**, 956-963.

Aldrich JC, Maggert KA (2015) Transgenerational inheritance of diet-induced genome rearrangements in *Drosophila*. *PLoS Genet* **11**, e1005148.

Barker D, Winter P, Osmond C, Margetts B, Simmonds S (1989) Weight in infancy and death from ischaemic heart disease. *Lancet* **2**, 577-580.

Bass TM, Grandison RC, Wong R, et al. (2007) Optimization of dietary restriction protocols in *Drosophila*. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences **62**, 1071-1081.

Bateson P, Gluckman P, Hanson M (2014) The biology of developmental plasticity and the Predictive Adaptive Response hypothesis. *The Journal of Physiology* **592**, 2357-2368.

Bochdanovits Z, de Jong G (2003) Temperature dependent larval resource allocation shaping adult body size in *Drosophila melanogaster*. *Journal of Evolutionary Biology* **16**, 1159-1167.

Broderick NA, Buchon N, Lemaitre B (2014) Microbiota-induced changes in *Drosophila melanogaster* host gene expression and gut morphology. In: *mBio*, pp. e01117-01114.

Buchon N, Osman D, David Fabrice PA, *et al.* (2013) Morphological and molecular characterization of adult midgut compartmentalization in *Drosophila*. *Cell Reports* **3**, 1725-1738.

Chandler JA, Morgan Lang J, Bhatnagar S, Eisen JA, Kopp A (2011) Bacterial communities of diverse*Drosophila*species: ecological context of a host–microbe model system. *PLoS Genet* **7**, e1002272.

Chen D, Pan KZ, Palter JE, Kapahi P (2007) Longevity determined by developmental arrest genes in *Caenorhabditis elegans*. *Aging Cell* **6**, 525-533.

Chiocchetti A, Zhou J, Zhu H, *et al.* (2007) Ribosomal proteins Rpl10 and Rps6 are potent regulators of yeast replicative life span. *Experimental Gerontology* **42**, 275-286.

Curran SP, Ruvkun G (2007) Lifespan regulation by evolutionarily conserved genes essential for viability. *PLoS Genet* **3**, e56.

de Jong G, van Noordwijk AJ (1992) Acquisition and allocation of resources: genetic (co) variances, selection, and life histories. *The American naturalist* **139**, 749-770.

Fellous S, Lazzaro BP (2010) Larval food quality affects adult (but not larval) immune gene expression independent of effects on general condition. *Mol Ecol* **19**.

Flatt T (2011) Survival costs of reproduction in *Drosophila*. *Experimental Gerontology* **46**, 369-375.

Fontana L, Partridge L, Longo VD (2010) Extending healthy life span-from yeast to humans. *Science* **328**, 321-326.

Ford SP, Long NM (2011) Evidence for similar changes in offspring phenotype following either maternal undernutrition or overnutrition: potential impact on fetal epigenetic mechanisms. *Reproduction, Fertility and Development* **24**, 105-111.

Gilbert S (2005) Mechanisms for the environmental regulation of gene expression: Ecological aspects of animal development. *Journal of Biosciences* **30**, 65-74.

Gluckman PD, Hanson MA (2004) The developmental origins of the metabolic syndrome. *Trends in Endocrinology and Metabolism* **15**, 183-187.

Grafen A (1988) On the uses of data on lifetime reproductive success. In: *Reproductive success* (ed. Clutton-Brock TH), pp. 454-471. University of Chicago Press.

Grummt I, Ladurner AG (2008) A metabolic throttle regulates the epigenetic state of rDNA. *Cell* **133**, 577-580.

Hales C, Barker D (1992) Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. *Diabetologia* **35**, 595-601.

Hansen M, Taubert S, Crawford D, *et al.* (2007) Lifespan extension by conditions that inhibit translation in *Caenorhabditis elegans*. *Aging Cell* **6**, 95-110.

Harrison PW, Wright AE, Zimmer F, *et al.* (2015) Sexual selection drives evolution and rapid turnover of male gene expression. *Proceedings of the National Academy of Sciences* **112**, 4393-4398.

Hayward AD, Lummaa V (2013) Testing the evolutionary basis of the predictive adaptive response hypothesis in a preindustrial human population. *Evolution, Medicine, and Public Health* **2013**, 106-117.

Hayward AD, Rickard IJ, Lummaa V (2013) Influence of early-life nutrition on mortality and reproductive success during a subsequent famine in a preindustrial population. *Proceedings of the National Academy of Sciences* **110**, 13886-13891.

Honek A (1993) Intraspecific variation in body size and fecundity in insects: a general relationship. *Oikos* **66**, 483-492.

Kaeberlein M, Powers RW, Steffen KK, *et al.* (2005) Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients. *Science* **310**, 1193-1196.

Kawecki TJ, Lenski RE, Ebert D, *et al.* (2012) Experimental evolution. *Trends in Ecology & Evolution* **27**, 547-560.

Klapper R (2000) The longitudinal visceral musculature of *Drosophila melanogaster* persists through metamorphosis. *Mechanisms of Development* **95**, 47-54.

Kolss M, Vijendravarma RK, Schwaller G, Kawecki TJ (2009) Life-history consequences of adaptation to larval nutritional stress in drosophila. *Evolution* **63**, 2389-2401.

Kuningas M, Mooijaart SP, Van Heemst D, *et al.* (2008) Genes encoding longevity: from model organisms to humans. *Aging Cell* **7**, 270-280.

Larson K, Yan S-J, Tsurumi A, *et al.* (2012) Heterochromatin formation promotes longevity and represses ribosomal RNA synthesis. *PLoS Genet* **8**, e1002473.

Lempiäinen H, Shore D (2009) Growth control and ribosome biogenesis. *Current Opinion in Cell Biology* **21**, 855-863.

Lints FA (1978) Genetics and ageing S. Karger.

Lochmiller RL, Deerenberg C (2000) Trade-offs in evolutionary immunology: just what is the cost of immunity? *Oikos* **88**, 87-98.

Long A, Liti G, Luptak A, Tenaillon O (2015) Elucidating the molecular architecture of adaptation via evolve and resequence experiments. *Nat Rev Genet* **16**, 567-582.

McCormick Mark A, Delaney Joe R, Tsuchiya M, *et al.* (2015) A comprehensive analysis of replicative lifespan in 4,698 single-gene deletion strains uncovers conserved mechanisms of aging. *Cell Metabolism* **22**, 895-906.

Monaghan P (2008) Early growth conditions, phenotypic development and environmental change. *Philosophical Transactions of the Royal Society B: Biological Sciences* **363**, 1635-1645.

Moret Y, Schmid-Hempel P (2000) Survival for immunity: the price of immune system activation for bumblebee workers. *Science* **290**, 1166-1168.

Nelliot A, Bond N, Hoshizaki DK (2006) Fat-body remodeling in *Drosophila melanogaster*. *genesis* **44**, 396-400.

Norris K, Evans MR (2000) Ecological immunology: life history trade-offs and immune defense in birds. *Behavioral Ecology* **11**, 19-26.

Osmond C, Barker D (2000) Fetal, infant, and childhood growth are predictors of coronary heart disease, diabetes, and hypertension in adult men and women. *Environmental Health Perspectives* **108**, 545.

Pan KZ, Palter JE, Rogers AN, *et al.* (2007) Inhibition of mRNA translation extends lifespan in Caenorhabditis elegans. *Aging Cell* **6**, 111-119.

Paredes S, Maggert KA (2009) Ribosomal DNA contributes to global chromatin regulation. *Proceedings of the National Academy of Sciences* **106**, 17829-17834.

Partridge L, Alic N, Bjedov I, Piper MDW (2011) Ageing in *Drosophila*: The role of the insulin/Igf and TOR signalling network. *Experimental Gerontology* **46**, 376-381.

Partridge L, Gems D (2002) Mechanisms of aging: public or private? *Nature Reviews Genetics* **3**, 165-175.

Piper MD, Blanc E, Leitão-Gonçalves R, *et al.* (2014) A holidic medium for *Drosophila melanogaster*. *Nature methods* **11**, 100-105.

Rickard IJ, Lummaa V (2007) The predictive adaptive response and metabolic syndrome: challenges for the hypothesis. *Trends in Endocrinology Metabolism* **18**, 94-99.

Riddiford LM (1993) Hormones and *Drosophila* development. *The development of Drosophila melanogaster* **2**, 899-939.

Robinson SW, Herzyk P, Dow JAT, Leader DP (2013) FlyAtlas: database of gene expression in the tissues of *Drosophila melanogaster*. *Nucleic Acids Research* **41**, D744-D750.

Roff DA (2001) *Life History Evolution* Sinauer Associates, Sunderland.

Schwenke RA, Lazzaro BP, Wolfner MF (2015) Reproduction-immunity trade-offs in insects. *Annual Review of Entomology*.

Stearns SC (1992) *The Evolution of Life Histories* Oxford University Press, Oxford.

Steffen KK, MacKay VL, Kerr EO, *et al.* (2008) Yeast life span extension by depletion of 60s ribosomal subunits is mediated by Gcn4. *Cell* **133**, 292-302.

van den Heuvel J, Zandveld J, Mulder M, *et al.* (2014) The plastic fly: the effect of sustained fluctuations in adult food supply on life-history traits. *Journal of Evolutionary Biology* **27**, 2322-2333.

Van Noordwijk AJ, de Jong G (1986) Acquisition and allocation of resources: their influence on variation in life history tactics. *American Naturalist*, 137-142.

Warner JR (1999) The economics of ribosome biosynthesis in yeast. *Trends in biochemical sciences* **24**, 437-440.

Wells JCK (2012) A critical appraisal of the predictive adaptive response hypothesis. *International Journal of Epidemiology* **41**, 229-235.

Xue Z, Huang K, Cai C, *et al.* (2013) Genetic programs in human and mouse early embryos revealed by single-cell RNAsequencing. *Nature* **500**, 593-597.

REFERENCES

Zwaan BJ, Bijlsma R, Hoekstra RF (1991) On the developmental theory of ageing. I. Starvation resistance and longevity in *Drosophila melanogaster* in relation to pre-adult breeding conditions. *Heredity* **66**, 29-39.

SUMMARY

Long-term effects of developmental diet on adult life history traits and health have been identified across a range of organisms, including humans. However, there has been considerable debate about the adaptive significance of such effects and the mechanisms responsible. In particular, we still lack insight into whether and how much such effects depend on the adult environment (as has been hypothesised to be the case in humans), and if and how such effects manifest as long term effects on gene expression, a proposed mechanism for the long-term effects.

The aim of this thesis was to use the fruit fly, Drosophila melanogaster, as a model to gain insight into the effects of developmental diet on adult phenotypes and gene expression, and if and how this effect is also contingent on the actual adult environment experienced. I addressed this question from two angles by assessing the effects of developmental diet both within a single generation (plasticity) and when experienced across many generations (adaptation). **Chapters 2, 3 and 4** address the plastic effects of developmental diet on phenotypes and gene expression across adult diets and across the lifespan, while **Chapter 5** uses experimental evolution to address how flies adapt to differing larval diets over many generations, and how this interacts with classical selection for increased age-at-reproduction. Throughout the thesis I used the same three diets (poor, control, and rich) which differ 10 fold in the amount of sugar and yeast they contain and thus represent drastically different nutritional environments.

Chapter 2 begins by determining the general effects of the three diets used throughout this thesis on both developmental and adult traits. It addresses the fundamental question of whether variation in developmental diet affects developmental and adult life history traits, with a particular emphasis on whether the effect of developmental diet depends on the reproductive potential of the adult environment. I found that both the rich and especially the poor developmental diet lead to what would classically be considered negative effects on developmental traits relative to the control - both slowing development and decreasing adult size in young adulthood, however, their effects on adult traits were distinct: the poor larval diet lead to increased virgin lifespan and increased female fecundity at certain ages and in certain adult reproductive environments relative to the control, while the rich larval diet had the opposite effect. This suggests that while over- and under-feeding share certain similarities with respect to their effects on traits in early life, their long-term effects differ. It also indicates differing nutritional optima between the developmental and adult stage in flies, as the poor diet is known to drastically decrease both lifespan and fecundity when experienced during adulthood, but is shown here to be largely beneficial for these traits when experienced in development. In addition, we found that the adult reproductive

environment was considerably more important for determining traits than the developmental environment, thus, while long-term effects of developmental diet do exist, they are marginal when related to the plastic response effected in adulthood - a recurring theme in this thesis.

In **Chapter 3** the focus shifts from the effect of developmental diet on life history traits to its effect on the adult transcriptome. It has frequently been proposed that long-term effects of developmental diet on adult phenotypes are mediated by changes in gene expression. Thus, in this chapter I addressed the scope for such effects in fruit flies, by determining the relative effect of developmental versus adult diet on gene expression in very young adult flies (one-day old) in both sexes. I used a full factorial design combining three larval and three adult diets (9 treatments total). I found that the largest contributor to transcriptional variation in one day old flies is larval, not adult diet, especially in females. Furthermore, the global effect of increasing caloric content of the larval diet on gene expression was not linear, but rather followed the same pattern as that observed for developmental phenotypes in **Chapter 2** (i.e. rich-raised flies were intermediate between poor and control) suggesting that calories per se do not drive global patterns of gene expression variation. Next, using Weighted Gene Correlation Networks Analysis (WGCNA) I identified modules of co-expressed genes whose expression was affected by larval or adult dietary conditions. In females, larval diet modulated the relative expression levels of reproduction versus non-reproduction related genes, while in males a large portion of the transcriptome was unaffected by dietary conditions, suggesting a lesser role for both larval and adult diet in affecting gene expression. The modules affected by diet in males related primarily to nutrient sensing and metabolism and showed no evidence of the reproduction and cell-cycle related processes identified in females, however, their expression in external tissue specific data sets suggested a role for the gut and fat body in mediating the effects of diet in males, potentially through the carry over of the larval versions of these tissues into adulthood. Overall, these results suggested that there is scope for long-term effects of developmental diet on gene expression, which is necessary for all hypothesised mechanisms that link developmental conditions to late-life health and disease.

Chapter 4 combines phenotypic and transcriptomic approaches to look at the longer term effects of developmental diet in adulthood. Using the same full factorial approach applied in **Chapter 3**, I assessed virgin and mated lifespan and fecundity as well as gene expression at middle and old age. I found that for the most part larval and adult diet exerted independent effects on the phenotype and on gene expression, and thus there was no evidence for Predictive Adaptive Responses (described in **Chapter 1**) operating in Drosophila melanogaster.

Rather, the responses followed the silver spoon hypothesis which predicts that the effect of developmental conditions will be similar across adult conditions. Furthermore, in contrast to the beginning of life (**Chapter 3**), adult diet explained considerably more variation in gene expression and phenotypes than larval diet, showing that flies retain extensive plasticity into adulthood, and suggesting that the long-term effects of developmental diet likely reflect the inability or lack of incentive to erase such effects, rather than an adaptive response. I did identify some genes that retain a legacy of developmental diet in their expression into middle and old-age. Many of these genes show no linear correlation with the observed phenotypic responses, however, in both sexes, I identified a cluster of genes whose expression was negatively correlated with the observed lifespan differences and that were enriched with terms related to transcription and translation, particularly with respect to ribosomes. Given several recent studies which show that the down-regulation of ribosomes and other aspects of transcriptional and translational machinery increases lifespan these genes provide promising candidates for mediating the long-term effects of larval diet on lifespan. As these processes are highly conserved across the tree of life our results may be relevant for other species as well, including for humans.

In **Chapter 5**, I address the evolutionary, rather than the plastic response to developmental diet by evolving flies on the three different larval diets in combination with selection for early or late age-at-reproduction in a full factorial design. This approach addresses how life histories evolve under different levels of larval acquisition, as well as how individuals cope with potentially competing selection pressures experienced at two different life stages. I found that the two life stages do not act independently but rather interact to determine both developmental time and lifespan. Across all evolutionary developmental diets, selection for later age-at-reproduction increased lifespan, however, the magnitude of the response was dependent on the sex, the evolutionary diet, and the experimental assay conditions. Developmental time from egg-to-adult also showed a similar dependency on both evolutionary diet levels and selection on age-at-reproduction. Given that multiple selection pressures are likely the norm rather than the exception in nature, this finding argues that trade-offs should be considered not only between traits within an organism, but also between adaptive responses to differing selection pressures. Furthermore, because variation in available nutrition is so frequent in natural environments, we argue that this can play a large role in shaping the evolution and diversity of life histories in nature.

In the discussion in **Chapter 6** I synthesise the findings across the experimental chapters, and address how they relate to each other. I also discuss some more general points that emerged, such as the potential importance of allometry

or tissue specific effects in mediating effects of larval diet, and the difficulty of linking life history phenotypes to variation in gene expression. Finally, I point out future directions suggested by this thesis, as well as the potential insights that can be gained from this work in the context of theories linking developmental conditions to late life health in humans.

ACKNOWLEDGEMENTS

PhD's are certainly not done in a vacuum, and over the last five years I have benefitted from the expertise, support, and good company of many people. Here I would like to take the opportunity to thank each of you for making this experience an enjoyable and instructive one. While writing this thesis I spent a fair amount of time trying (and usually failing) to be concise. Here I abandon all attempts at brevity in favour of a more effusive recounting of the many thanks that are owed. Be warned!

To begin with, my supervisor Bas. You played several roles simultaneously during the course of my PhD - boss, promotor, and daily supervisor. In all of these capacities I have learned a lot from you. As a boss, your fair, open and enthusiastic treatment of all members of the department results in an inspiring and productive workplace. Furthermore, you bring together all of the disparate lines of research in the genetics department under one umbrella, making each part (and person) feel integral to the whole. As a promotor, you are always able to see the bigger picture, while also remembering more details about my past experiments or results than even I do. To come to your office is to leave enthusiastic about pursuing an exciting new direction, or less puzzled about a strange result, or wondering whether years of healthy lifespan might more easily be gained by investing in community and social infrastructure than ageing research?! As a daily supervisor, you always made time for discussing fly lab phenomena, and were even known to lend a hand in the lab. I truly appreciated your patience in reading (and re-reading, and reading again) draft manuscript versions, despite all of the other demands on your time. It has been a pleasure to work in the atmosphere of supported independence that you foster, and I look forward to continuing to work together to get these papers into the world.

Fons, you were originally meant to be a second promotor/daily supervisor for myself. Indeed in the first two years there were many interesting meetings with yourself, Jelle, Bas and I. You are always able to think of many, many different ways to solve a problem. The frequency of our meetings has decreased in the last years as I shifted from experiments towards analysis. However, you were always ready to answer questions (usually about bacteria or fungi), and it is always a pleasure to see your smiling face in the department.

Agnieszka, your influence as my Masters thesis supervisor prompted me to start a PhD. From you I first learned about the promise of using model systems to understand ageing, and about many other things besides. Your patience and ability to explain difficult concepts are truly extraordinary. Through the years it has always been a pleasure meeting up to discuss old data or new career paths, and I hope we have not seen the last of such discussions.

While my supervisors laid the groundwork for my scientific endeavours, the other members of the Genetics department provided an essential forum for discussing ideas and opinions, and a friendly and social environment in which to do so. Thank you to Aina, Andy, Anneloes, Alex, Ana Carolina, Arjan, Bart N., Bart P., Bertha, Charles, Claudio, Corrie, Cris, Duur, Diana, Eric B., Erik W., Eveline, Felipe, Florien, Frank, Gabriella, Jelle, Jianhua, Jimmy, Johanna, Jose, Joost, Justin, Julio, Katja, Kim, Kristiina, Klaas, Lennart, Laurens, Lidia, Margo, Marcin, Marijke, Marjon, Magda, Mark A., Mark Z., Merijn, Martijn, Mina, Mohamed, Natalia, Nihal, Padraic, Paola, Ping Ping, Ramon, Rik, Robert, Ross, Roxanne, Sijmen, Sabine, Tania, Thierry, Valeria, Vivian, Wytske, Xianwen, Ya-Fen, Yanli, and Zeshan.

On top of contributing to the happy atmosphere in the Genetics department, Bertha, Corrie, Frank, Gabriella, Marijke and Wytske also ensure that the department runs smoothly. Thanks especially to Bertha, Gabriella, Frank and Marijke for help with the fly rearing. I know it was most decidedly no ones favourite job, but I appreciate the freedom you afforded me to (slowly) finish writing my thesis. Gabriella, thank you for being the fly lab fairy god-mother, secretly drying the stoppers and doing other jobs without being asked! Bertha, thank you for having (or finding) the answers to any lab-related questions and for your always calm and kind presence. Wytske, you are the organisational hub and chief carer of the genetics department. Thanks for always telling me what I need to do and when, and for keeping me on my toes with Dutch!

A great deal of time was spent behind my desk, and here I was fortunate to be surrounded by some very clever and friendly people: Claudio, Jianhua, Florien, Jelle, Magda, Alex, and Erik. Thanks very much for the discussions about PhD life and PhD data (always at an appropriate volume for an open work space). Florien, it was great fun to organise the “unofficial” Friday genetics drinks with you, they were a success while they lasted! Jelle - it was a pleasure to have another “fly” person to discuss things with. It is also great to see you bring your creativity to bear on communicating evolution. Claudio, thank you for the many talks about PhDs, possibilities and almost finishing Chapter 4 of the Python book. I’m glad at least one of us can now claim to have “Basic Python Skills”. Jianhua, it was a pleasure to talk to you over a cup of hot water or a room temperature beer and to listen in shock and awe as you describe your latest bicycle accident. Alex, seeing your friendly face first thing in the morning always gets the day off to a good start. When you are not at your desk it feels like all is not well in the realm of genetics. Erik, it was comforting to see the top of your head across from me! I’m glad that you will be returning from the chilly north to resume caring for your sap-dripping rescue plant. And thank you also

to Robert, the time spent getting to and from these desk mates was made much enjoyable with your friendly train conversations.

I also had the opportunity to supervise several Masters and Bachelors students. While this was initially terrifying, I learned a lot from each of you, and really enjoyed working together. Roel, I almost feel that you could have supervised me in many respects. Since you were so well organised and good at solving problems supervising you was like having training wheels on a bike. Thijs, it was inspiring to witness your creativity. Aina Maria and Berdien, you were both very diligent and clever, and I enjoyed our time working together.

To the members of the IDEAL consortium, it was a great to learn more about the genetics of ageing from you, and also the location of the best Irish bars in several cities across Europe! Erik, it was a pleasure to work together and analyse data over Skype. I think we've done something quite cool. Thank you for your patience in teaching me to work with network analyses. Seeing your organised R-scripts has been a revelation for me.

I also had the opportunity to be a part of the PE&RC PhD committee (PPC). Thank you Claudius, Lennart and all of the other members of the committee. It was great fun gaining better insight into the inner-workings of the university with you.

While a large part of the last 5 years was spent in Wageningen, I also had the opportunity to meet many other fantastic people in the outside world! Utrechtenaars from around the globe - Alice, Gordon, MG, Gareth B. & Gareth W., Fiona, Marleen, Carl, Hannes, Vief, Carol-Ann, Ricky, Helen, (Hesham, Richard, Tom and Leontien (Honorary Utrechtenaars)) - thank you for making the many Kings nights and regular nights in Utrecht so enjoyable. It feels like the end of an era, and I will miss you all very much. Thank you Alice, for taking on the role of paranymph, ensuring that we can have at least one more reunion on Dutch soil! I hope there will be many more to come. And thank you MG, for making this beauty, for getting me to bootcamp, and for introducing me to your lovely friends (Marleen ik mis je nu al)!

Friends from B.P. (before PhD): old friends and new partners, Yvonne, Julia, Danielle, Ashley, Martha, Chrissie, Joe, Lauren, Jackie, Ryan, and Laura. Thank you for being on board with my hands-off intermittent brand of long-distance friendship. I can't wait to pick up where we left off 7 years ago, older, wiser, and with a slightly more discerning taste in wine!

Schoonouders (Antoinette, Frans) en schoonfamilie (Oma, Cynthia). Bedankt voor het warme welkom in jullie familie. Door jullie voelde ik me nooit alleen in Nederland. Bedankt voor de leuke weekendjes in Duitsland, de gezellige ontbijten en wandeltochten in de Hoek, en dat je mijn slechte gedrag nog steeds niet hebt verraden aan mijn vader, Frans. Ik zal jullie heel erg missen maar ik kijk uit naar onze vakanties.

Sisters (Micha, Doris), brothers (Peter, John), brothers- and sister-in-law (Randy, Mark, Morgan), nieces (Maria, Rebecca, Hannah, Jessica, Julia, Claire, Isabelle, Kaylee, Sarah) and nephews (Chris, Alex, Noah, Nicholas, Aaron, Mikey, Lucas): thank you for tolerating my absence, and for having a brood of tech-savvy kids who can entertain me on Skype. I am looking forward to making up for lost baby-sitting time. And thank you Micha for getting all gussied up (did you?) and sitting up front with me.

Mama and Papa, to say thank you seems so inadequate it is bordering on silly. Without your support and encouragement through every step of my education (and life) I would not have written this book. Thank you Papa, for passing on your love of nature to me, and thank you Mama, for making me see what a privilege it is to be able to continue to learn. I am grateful.

Marf - you probably won't believe that this was a challenge for me to write. But there are too many things to thank you for, and too few words to do so. To be honest, I don't know how people finish a PhD without your support. Thank you for everything: the fly collecting, the enthusiasm, the voice of reason, the support and the fun! Together we made a home in Utrecht and I will miss all the good times we had in our little house. I feel very lucky and gratefull to have you in my life, and I can't wait to start the next stage of our life in Canada!

Despite all my wordiness, I am sure I've managed to forget someone. I will happily make it up to you over a beer.

BIOGRAPHY

Christina Maria May was born in 1986, in the suburbs of Vancouver, Canada. After completing her high school degree at Little Flower Academy in Vancouver in 2004, she went on to obtain her Bachelors Degree in Zoology at the University of British Columbia with honours in 2009. During this time she spent a year on exchange at Uppsala University in Sweden (2006) and acquired a taste for the European education system. This lead her to the Netherlands to complete her MSc in Evolution, Biodiversity and Conservation at Leiden University (cum laude; 2010). In the course of her masters she researched two topics: in the Behavioural Biology Group she studied how anthropogenic noise influences the courtship behaviour of cichlid fishes, while in the Evolutionary Biology Group she began to study the link between developmental diet and longevity in fruit flies. For her Masters research she won the Unilever Research Prize in 2011.

In 2011, Christina began her PhD at Wageningen University under the supervision of Bas Zwaan and Fons Debets. Her project (described in this thesis) formed a small part of a larger EU funded project to understand the mechanisms linking variation in developmental conditions to late-life health (IDEAL). During her PhD, Christina was an active member of the PE&RC PhD Committee, and helped to organise the PE&RC day in 2010. After spending more than seven years in the Netherlands, and acquiring a taste for bitterballen, hopjes vla and oliebollen, Christina plans to return to Canada.

LIST OF PUBLICATIONS

Baker DW, May CM, Brauner CJ. 2009. A validation of intracellular pH measurements in fish exposed to hypercarbia: the effect of duration of tissue storage and efficacy of the metabolic inhibitor tissue homogenate method. Journal of Fish Biology 75:268-275.

May CM, Doroszuk A, Zwaan BJ. 2015. The effect of developmental nutrition on lifespan and fecundity depends on the adult reproductive environment in *Drosophila melanogaster*.
Ecology and Evolution 5:1156-1168.

SUBMITTED:
May CM and Zwaan, BJ. submitted. Relating past and present diet to phenotypic and transcriptomic variation in the fruit fly

IN PREPARATION
May CM, van den Heuvel, J., Doroszuk, A and Zwaan, B.J. submitted. Balancing fitness: adaptation to developmental diet influences the response to selection on age at reproduction in the fruit fly

May CM, van den Akker, EB and Zwaan, BJ. Growing up and getting old: Life history traits reflect the interaction between developmental and adult selection regimes

May CM, Valero Jimenez, CA & Zwaan, BJ. Adaptation to developmental diet and food quality selection regimes affects tolerance to fungal infection in the fruit fly


The C.T. De Wit
Graduate School
PE&RC
PRODUCTION
ECOLOGY
& RESOURCE
CONSERVATION

PE&RC Training and Education Statement
With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks
of activities)

Review of literature (5.6 ECTS)
The development environment and aging: an integrative perspective from genotype to phenotype

Writing of project proposal (3 ECTS)
The development environment and aging: an integrative perspective from genotype to phenotype

Post-graduate courses (14.4 ECTS)
Vitality and aging summer school course; Leyden Academy, Leiden, the Netherlands (2011)
Summer school on genome biology and evolution; Instituto Gulbenkian de Ciencia & Volkswagen, Oeiras, Portugal (2011)
Evolutionary biology workshop in the Alps; University of Lausanne, Arolla, Switzerland (2012
Microarray analysis; University of Tromso, Tromso, Norway (2012)
Survival analysis; PE&RC, Wageningen, the Netherlands (2013)
Weighted gene correlation networks analysis; LUMC (2014)
Epigenesis and epigenetics; Wageningen University, the Netherlands (2014)
Bayesian statistics; PE&RC, Wageningen, the Netherlands (2014)

Laboratory training and working visits (0.3 ECTS)
Discussing transcriptional responses to diet in flies and viewing fly-rearing facilities and tools; University College, London (2013)

Invited review of (unpublished) journal manuscript (2 ECTS)
Ecological Entomology: effects of diet on life history traits in Drosophila (2014)
BMC Genomics: transcriptomic responses to infection in Drosophila (2015)

Competence Strengthening / skills courses (3 ECTS)
PhD Competence assessment; PE&RC (2012)
Techniques for writing and presenting a scientific paper; PE&RC (2012)
Reviewing a scientific paper; PE&RC (2013)
Career orientation; PE&RC (2013)

PE&RC Annual meetings, seminars and the PE&RC weekend (2.1 ECTS)
PE&RC first year PhD weekend (2011)
PE&RC Day (2011-2014)

Discussion groups / local seminars / other scientific meetings (6 ECTS)
Experimental Evolution Discussion Group (EEDG) (2011-2014)
Wageningen Evolution and Ecology Seminars (WEES) (2011-2014)

International symposia, workshops and conferences (6.6 ECTS)
European Meeting of PhD Students in Evolutionary Biology 18 (EMPSEB); Virrat, Finland (2012)
IDEAL Annual meeting (2011-2015)
NERN Meeting; Lunteren, the Netherlands (2013)
Biology of Aging Gordon Research Conference (2015)

Lecturing / supervision of practicals / Tutorials (5.4 ECTS)
Insect ecology (2012-2015)
Concepts and theories of healthy aging (2012-2015)
Evolution and systematics (2012-2015)

Supervision of MSc students
Experimental evolution of Drosophila melanogaster on larval food levels: the role of IIS/TOR signalling
Testing the relationship between developmental diet, the microbiome, immune function and lifespan in Drosophila

COLOFON
The research in this thesis was carried out at the Laboratory of Genetics at Wageningen University, Wageningen, the Netherlands and was financially supported by grants from the EU's 6th (Network of Excellence LifeSpan FP6/036894) and 7th (IDEAL FP7/2007-2011/259679) Framework Programmes.

COLOPHON
Cover design by: Margriet Hogenbirk
Thesis Layout by: Christina May
and Margriet Hogenbirk
Printed by: Drukkerij Peters, Amsterdam