Targeting environmental and genetic aspects affecting life history traits

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Explanation of the cover

The title has been depicted in the form of an individual's life span from conception (left) where all creativity is present (red circle) to the quietness of death (white horizontal line). This progression through time is affected by environmental (green circle) and genetic (blue circle) factors.

Targeting environmental and genetic aspects affecting life history traits

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Voor Frederik

Targeting environmental and genetic aspects affecting life history traits

Egon Alexander Baldal

Contents

General Introduction

General Introduction

In my thesis I start with a very broad view on the essence of life, what is it and what causes it to exist in the way it does. I will explain that, in my view, maintaining energetic states is all there is to sustaining life. This thesis revolves around the question of which strategy, for lack of a better word, an organism chooses to maintain itself for as long as possible through evolutionary time under different circumstances. Maintenance is here defined as "kept in evolutionary existence" and includes growth, reproduction, healing, etc. This has everything to do with energy and how it is allocated, how it is stored and at what rate it is used. The following chapter is abstract and philosophical, yet I think it is important to set a broad scale picture of life to portray the scientific account I have written over the past few years. I hope this will put my research in the wider context of life in general.

Life, entropy and everything

A living individual is in its essence "a part of the world with some identity that tends to become independent of the uncertainty of the rest of the world" (Wagensberg 2000). In this definition we find the marked difference between the living and non-living world[1]. The living world ends where the uncertainty of the rest of the non-living world begins. To be "certain", one needs to be in control. To be in control requires power, which in itself requires energy. Energy is what is needed to maintain order in chaos, or, to put it in other words, maintain low local entropy. "Local" means lower entropy as a body, or a somatic unit. The second law of thermodynamics states that the total entropy of a system has to become higher with every change in the system. Yet, entropy is lowered by processes that counteract chaos by putting things in order, such as growth. Following the second law of thermodynamics this would not be possible, unless when the total entropy becomes much higher. This means that for entropy lowering processes such as growth, maintenance and reproduction, the entropy of the entire system the organism lives in has to become higher. This is because lowering the local entropy without elevating the entropy of the total system would violate the second law of thermodynamics. The energy required to lower local entropy has to come from the total system and this subsequently elevates the total entropy. In order that they gain lower entropy, living organisms have to breakdown substances or redistribute energy in a way that entropy of the total system becomes higher. Seen from this point of view, life is in its essence destructive.

Lowered entropy is not an aim in itself, because it does not require life; a salt crystal is a form of locally lowered entropy. Lowered entropy is a means, but not to an end, rather to a non-end, and the same applies to the organism. There is no such thing as a purpose or target in the process of survival. There is only the continuation of an entity that has the ability to replicate itself. The form of the entity we call the

[1] T*he living world is regarded as the biotic realm, as contrasted with the non-living or abiotic world. We use the terms biotic and a-biotic or living and non-living here instead of the contrast between living and death. Dead things by definition used to live, otherwise they are not dead but lifeless. In short, death is inherent to life.*

phenotype. This entity survives when it produces a phenotype that is adapted to its environment in such a way that it can lower local entropy, which biologically means that it is able to grow, survive and replicate. To do so, the entity must efficiently transfer energy into growth, maintenance and reproduction in the environment it lives in.

The constant factor of life is that it has maintained itself through time, both through evolutionary time and during the time the organism is physically present. When an organism is not capable of reproducing, it only lives to die eventually, because of stochastic events, like for example a volcano eruption, predation or cardiac arrest. Dying without adding to procreation through time in the broad sense[2] is what I call an evolutionary dead end. This reasoning is an extrapolation of the selfish gene hypothesis (Dawkins 1976), where the only thing that matters for genes is to copy themselves into a next generation. Again, this process is not a function or a purpose in itself. It is the only way life is possible: it sustains itself or dies out. Thus, life can be approached as a self-replicating anomaly in entropy that remains present by its power to avoid uncertainty through time by the transfer of energy and the subsequent increase of total systemic entropy. The driving force behind the self-replicators is that the ones that are best adapted to their environment survive to live over evolutionary time. The fittest individuals are regarded as such always in hindsight. Because fitness can only be determined afterwards and no organism is consciously selecting itself, we should regard the evolutionary process as purposeless. The actual reproducing unit of an entity is always the essence of the entity, because the entity would be lost if it wasn't. The information on how to cope with the environment, reduce local entropy and maintain one's lineage should always be the part that is reproduced of the entity. In our world this happens because of a replication of nucleic acids, coding for the information to successfully reproduce nucleic acids into a next generation. A successful combination of nucleic acids encoding a product is called a gene. A gene encodes a product and thus a certain way of dealing with a situation. When several genes encoding several products are optimized for one or a few purposes, they may enhance the survival capacity of the total set of genes they belong to. Together these genes encode a more complex entity; the organism. The ultimate goal of the organism is to maintain the entity through time and reproduce. The exterior organism, the phenotype, is thus only a way of dealing with the environment as efficiently as possible.

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[2] *Adding to procreation can be done in a number of ways and it does not necessarily involve reproduction itself. For example, the provisioning of science, education or food, can enhance the well-being and survival capacities of youngsters of family or the community one lives in. As all humans are related to a certain extent, adding to someone else's survival is thus adding to the survival of a bit of oneself. In popular scientific literature this fact is not often acknowledged and mere redundancy of non-reproducing entities is proclaimed. I think this is rather shortsighted, because as stated this form of altruism is mere selfishness. As is everything, Nietzsche would say.*

Intermezzo

I have been writing in an extremely abstract way about biological phenomena. The rest of my thesis will be focussing on more pragmatic biological issues that have their own terminology. Because I think it is necessary for the sake of briefness to talk in jargon about science, I will shift from the philosophical terminology used in the first subparagraph to biological terms. For example, instead of using the noun "entity" I will turn to the word "organism". I will also start to use the term "evolution" more often and will then mean "the process that an organismal lineage changes over time and adapts to the environment it faces". The first paragraph discussed the most elementary principles of life. Further in this thesis, I will revert to these basic principles sometimes to illustrate how everything in biology eventually comes back to these and most of the time to show how close to the basics of life the work of this thesis comes. Also, for the sake of easy communication, I will use language that implies purpose in life, though there is no such thing.

Acquisition of energy

In the first part I explained why energy is vital to life. Here, I will explain what the importance of energy acquisition is.

In the time of physical presence of an organism, for its growth and maintenance, it needs to put energy into several mechanisms. To be able to do so, the required energy must be acquired. To do this efficiently, without having to wait for what accidentally comes along, energy acquisition systems have by definition a huge advantage in becoming more and more independent of the environment. Such systems may come into existence by chance and as a result of selective pressure then develop further to become the highly efficient energy acquisition machinery that can be observed in nature, such as catabolic enzymes, photo systems, guts etc. Arguably, systems with other features that are adaptive to new situations may come into existence in the ages that will follow. Energy acquisition via feeding, as happens in animals, requires a lot of extra structures to facilitate that the animal ends up with enough energy to be able to survive. Developing these structures and maintaining them has to cost less than it yields in terms of energy.

The environment

Acquiring energy is done in a large number of ways throughout life and has arisen as a result of positive selection pressure. The diversity varying from photosynthetic plants and chemotrophic crayfish to ants that practice agriculture indicates that there is a wide diversity of selection pressures in different systems. The environment is a very strong factor driving selective pressure. Here I explain how energy resources must be used to acquire new energy and how the organism that tries to escape environmental dependence ironically has to adapt to it.

It has been mentioned earlier in passing that the environment is everything the organism has as a reference point in terms of the "choices" that it makes[3]. Animals are forced to function in their environment as a consequence of the legacy of natural selection. The environment sets the scene in which the organism must function in order to survive and reproduce. The organism therefore has genes that are the result of the selective pressure on a viable form. Also, epigenetic regulation, such as methylation, is important in determining the phenotype. Such epigenetic factors may be seen as the way the genome can be fine-tuned to the environment without meddling with the genetics and may be involved in the basis for adaptive plasticity (Brakefield et al. 2005). Seen from this point of view, the organism is no longer the mere carrier of genes; the entropic anomaly that keeps on replicating itself by reducing uncertainty. It is a dynamic process, in which the entropic genetic entity has to adapt to external selective pressure in order to be able to compete for resources that are needed to avoid uncertainty. In other words, the environment is not only the sometimes dangerous set of conditions the organism has to escape, it also is the music to which it dances.

Allocation and trade offs

Once energy has been acquired it can be used for several purposes. The surplus energy that was gained by feeding structures gives the organism the potential to develop traits. The term 'traits' comprises many different categories, varying from developmental, physiological and behavioural to cognitive traits. In this part I will debate what underlies energy allocation to different traits.

One constant for an animal lineage is that the individuals of the lineage are eventually going to die. This implies that there can be no such thing as a Darwinian Demon[4]. It is impossible to be a Darwinian Demon because living organisms are always constrained in terms of the amount of energy that is available with which they can reduce local entropy. While it would be convenient to invest maximally in all processes[5], this is not feasible because of the limited resources one has.[6] Choices

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[3] *This is true for both the long-term ultimate evolutionary 'choices' that are shaped by natural selection, and the short-term proximate physiological mechanisms that the animal uses to respond quickly to a particular environment. In both cases the environment sets the standard the animals have to live up to.*

[4] *Darwinian Demon; i.e. an ever-living immensely reproducing unit. This Demon would need to spread fast, absorb resources and be resistant to everything that threathens it. This would, in theory, be the ideal organism towards which all selective pressure should lead (so there is a sort of end after all). Some organisms approach this situation more closely than others, the species Homo sapiens is making a good attempt to use up as many resources as possible, live long and reproduce exponentially.*

[5] *All processes an individual can invest in can be categorised as either maintenance, growth or reproduction. The most basic of these is maintenance, and the other two can be categorised in the factor maintenance as well. Maintenance should be*

have to be made; investing in growth, in reproductive output or in maintenance? These choices are not made consciously, most of the time because the organism has no such thing as a conscience, but also because consciousness, if present, has little influence on these processes[7]. Physiological and genetic constraints will make the 'choices' for you, no matter whether one's consciousness agrees or not. Because the division of energy is important and comes close to the essence of the organism, the selection process favours organisms that allocate their energy in the most efficient way. Also, because an organism has to function as a whole and not as a collection of parts (Stearns 1992), sometimes the conflict between two traits may form a constraint on the evolution of one of the two traits (for a good overview of evolutionary constraints, see Zijlstra 2002, for one on trade offs and correlated traits, see Ricklefs and Wikelski 2002). The way resources are allocated is shaped by the environment the organism lives in, because this is the major determinant of fitness. When two mechanisms are in competition for the same resource and choices have to be made, we speak of a trade off. Essential to the Darwinian Demon is that it is not constrained by a fixed amount of energy, it therefore has no trade offs. Because energy can be distributed only once and resources are limiting, there are a lot of known trade offs in organisms. The best known one will be the trade off between longevity and reproduction, which we will discuss in the next paragraph.

Life history evolution

The allocation of resources as a result of environmental selection pressure leads to favouring one trait over the other in a trade off situation. Some of these traits are very basic to life, for example development time, reproductive output and life span

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defined as "preventing yourself from becoming extinct". Growth will enable an individual to become less vulnerable, more powerful etc. which can all be seen as a way of maintenance. Reproduction can be viewed as a way of evolutionary maintenance, as is described earlier in the first chapter. Nevertheless, they are regarded as separate investments here because these are the elementary trade offs. For example, finding food should be categorised as maintenance, and once food is found it can be allocated to either of the processes categorised here.

[6] This understanding lead the famous Thomas Malthus (Essay on the principle of population, 1798) to the theory that the struggle for existence will always lead to shortage. Both Charles Darwin and Alfred Russel Wallace picked up this theory to state that favourable features must thus have an advantage over unfavourable ones and thus that natural selection must be ubiquitous. This concept forms the basis of evolutionary biology as a discipline.

[7] There can be a lot of debate about this remark, it is meant to say that animals without a central nervous system that are not complex enough to make up their own minds, have little to think about. Apart from that, conscious individuals are not capable of determining the way their physiology works as well. If that were so, then obesity would not be a problem in this society. (Arguably, people can actively choose whether to become obese or avoid it.)

(Stearns 1992). Many of these life history traits trade off or correlate with one another. When two life history traits depend on largely the same genes, selection on one will take the other along and the genetic correlation, positive or negative, becomes apparent. On the other hand, when two life history traits both require a lot of resources, they trade off and counteract one another.

Longevity, starvation resistance and reproduction.

Starvation resistance and longevity are found to be closely correlated in a number of studies (e.g. Borash and Ho 2001; Chippindale et al. 1996; Harshman et al. 1999a, 1999b; Leroi et al. 1994; Rose et al. 1992; Zwaan et al. 1991). It is often thought that longevity and starvation resistance are therefore dependent on the same genetic mechanism. However, other studies have shown that this relationship is present but hardly as straightforward as was thought earlier (Force et al. 1995; Archer et al. 2003; Phelan et al. 2003; Baldal et al. 2005). Apparently, the strong correlations found can degrade over time due to certain selection pressures, or be changed by a change in environmental conditions. This makes the supposition that both life history traits fully result from one mechanism unlikely. Thus, though starvation resistance and longevity are closely related in terms of their genetics, differences between their underlying mechanisms remain present. In Chapter 3 I present work concerning selection for increased starvation resistance. There, I also find that such selection on starvation resistance may, but does not necessarily, lead to increased longevity.

Starvation resistance and longevity are both found to trade off with reproduction in a similar way (Chippindale et al. 1993). This thus reflects the elementary trade off between maintenance and reproduction already mentioned briefly in footnote 4. The processes that underlie this trade off between maintenance and reproduction are formulated in the Disposable Soma theory (Kirkwood 1977; Kirkwood and Holliday 1979). This theory states that when the individual is in a position to procreate successfully, the individual itself becomes redundant and its offspring more important. It assumes that within the organism there is a conflict between the somatic and germ line tissues. The state of the trade off in this conflict is driven by the selection process. The trade off between starvation resistance and reproduction is very direct since they compete for precisely the same resource; fat. Depending on the evolutionary history and the environment, the allocation of fat is determined and the individual will ‘bet’ on one of the options.

Thrifty genes and phenes

That the environment is important in determining allocation models is illustrated by the fact that obesity is a serious problem in the Western society. One would expect that every lineage of organism should change towards a Darwinian Demon because of selection pressures towards infinitive life span and number of offspring. So, in theory affluent conditions in society should lead to a good allocation of the surplus energy. In practice, it is observed that our current affluence leads to a very high incidence of obesity and thus of sub-optimal usage of the energy acquired. So, I have to conclude that our allocation model is not set up for the abundant food conditions in

current society.[8] Due to adverse conditions during our evolutionary history, our genotype has adapted to poor conditions relative to present-day life in the western world. This was first observed by Neel (1962) who studied the incidence of diabetes type II in the human population. The genotypes that have adapted to adversity are very economical with their energy and are therefore called thrifty.

An illustration

The people of the island of Nauru have gone through serious bottlenecks and have become adapted to adverse food conditions. During evolution, when food conditions were more limiting, the Nauruans transition of food to reserves has been optimized. In the presence of food the Nauruan is likely to allocate energy to reserves to be able to survive more adverse times. A sudden increase in wealth gave them the opportunity to import large quantities of very fat food. This resulted in a large incidence of mortally obese people in this population where the thrifty genotype has a very high frequency (Diamond 2003)[9]. All this can be explained by the insulin signalling pathway, which is the intermediate step from food presence to phenotypic response. In this molecular pathway, the allocation is determined and insulin signalling in people adapted to adversity will lead to storage of fat. A similar trend can be observed in American Indians, non-western immigrants and Europeans. In Europeans, this problem is of a smaller magnitude because the overall food quantity in Europe has increased gradually over the ages. Apparently, a difference in evolutionary history may have lead to a difference in response to environmental challenges. These lineage specific effects are thus basically differences between genotypes. In Chapter 1, we treated three species of *Drosophila* experimentally by rearing them under different larval densities, and then examined their responses in the amount of fat, body size, longevity and starvation resistance. Different species allocate their resources in a different way because of differences in their evolutionary background. Such lineage specific effects may thus be in part responsible for differences in life history traits.

In a time of scarcity, the individual is faced with a lack of resources and will then respond in a way that has proven its worth in evolutionary history. Sometimes this

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[8] Taking into account that we, as humans, are still under the influence of natural selection, it is to be expected that in time humans in western world civilisations will adapt to the contiuous presence of large amounts of food. Evolutionary processes take time and the present situation of affluence is very young, in terms of evolutionary timescales.

[9] It could be argued that the Nauruans, with their preference for fat meals, fat-bodied partners and a sedentary life-style, are not only hindered by their genetic background but also by their culture. The frequency of the mortally obese genotype has dropped throughout recent years because of various reasons, one of them being the strongly reduced reproductive output of these obese individuals. Since the Nauruan culture increases the chance of becoming so obese, culture should be regarded an important factor shaping the genetic make-up of the human population.

may prevent the individual from incurring damage and prove to be adaptive plasticity, in other cases, this defence will not be strong enough and the individual either dies or suffers serious damage, which I would call scar. In Chapter 2 I test a theory based on the observation that in the human population individuals that have suffered from adverse conditions in the womb, had a higher incidence of metabolic syndrome as adults as found by Hales and Barker (1992; 2001). Metabolic syndrome is the common name for a group of disease types such as diabetes type II and obesity. Altered insulin signalling is hypothesised to lead to this group of diseases. The prediction that adverse pre-adult conditions lead to increased risk in adults is called the thrifty phenotype hypothesis which is also called the Barker hypothesis. I have to note here that it is important to observe that in both the thrifty genotype and thrifty phenotype hypotheses, altered insulin signalling leads to increased risk of metabolic disease. It is, thus, very easy to confound these theories.[10] Testing the Barker hypothesis could reveal an effect of nutritional conditions on life history traits.

In summary, the life history traits of an individual are a manifestation of physiological trade offs, genetic constraints and past and present environmental selection pressures. Apart from the evolutionary consequences of selection on a certain strategy, the allocation problem also applies to the individual. It has to cope with the amount of energy it can spend. The issue of how to spend energy and on what, is dependent on the environment the individual is in.

Quality and quantity of diet

Life has to a varying extent escaped the environment by becoming more and more independent from it, yet life is not possible without the environment and thus the environment is important for two things; the first one is to ensure escaping it in terms of damage, the second one as a food source. Life history characteristics have been shaped by natural selection for the successful exploitation of the environment. A change in the environment to which the lineage had no opportunity to evolve an adaptive response is an interesting test case for the processes underlying the life history configuration and potential differences among lines. In this thesis I explain how food, as an environmental condition, may affect life history traits.

Food has a very direct effect on an organisms, homeostasis and slightly different products evoke different responses, as has been found for chocolate in humans (Serafini et al. 2003). Mair et al.'s (2005) study makes it clear that in *Drosophila* it is not the number of available calories that decrease longevity. Yeast removal from the media has a substantially larger beneficial effect on longevity than does the removal of exactly the same calorific amount of sugars. Yeast, therefore, represents not only a source of energy to the fruit fly, but must also induce physiological responses

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[10] The cause of the theories is, however, fundamentally different. Where the thrifty genotype hypothesis has an evolutionary, ultimate cause, the thrifty phenotype hypothesis has a physiological, proximate cause. Clear distinguishing and thoughtful experimentation are required to obtain the proper data. We aim at this in Chapter 2.

related to the allocation of resources. This probably has to do with the induction of the reproduction process in fruit flies.

The quantity of the diet is important for inducing responses. When an animal can acquire little food, it has a smaller amount of energy available than when it can eat *ad libitum*. The amount of energy available to an animal determines its allocation, therewith inducing responses in life history traits. In addition, the amount of food taken up also poses problems to the organism that need to be solved at the same time. More food, means more build-up of resources and if an animal eats too much it may encounter negative effects of affluence. Coronary heart disease in man is, in many cases, such an effect (Anonymus 1972). In a number of animal taxa, dietary restriction leads to an increase in lifespan and thus there is a negative effect of eating *ad libitum* (Lin et al. 2002; Merry 2002; Anderson et al. 2003; Houthoofd et al. 2003; Mair et al. 2003; Fontana et al. 2004; Kaeberlein et al. 2004; Mair et al. 2004). These studies show that animals in laboratories are largely over-fed and so longevity-enhancing dietary restriction is an important process in these cases. Actually, the response curve of longevity as a function of adult diet has an intermediate maximum. Both the poor and affluent diet result in a shortened life span relative to the intermediate diet. Thus, calorie restriction leads to elevated longevity, but only up to a certain point where longevity is maximal. When calorie restriction is so harsh that the organism experiences shortage, life span will not be enhanced but rather shortened. Because dietary restriction is only used in the positive sense of the word, we use another term for the negative effect of reducing calories, namely starvation.

All chapters of this thesis are about the effect of food on the individual. In Chapters 1 and 2, I changed pre-adult environmental conditions and observe the patterns that arise. In Chapter 3, I examine the genetic effects of selection for increased survival of starvation. In Chapter 4, 5 and 6, I compare genetically different lines under different nutritional states and other environments. The next section will discuss this.

Genotype-by-environment interactions

Thus far I have discussed the effects of the environment and of genetics on life histories separately. In the very first part of this introduction I already indicated that there is a firm dependence of the individual on the environment. Because the environment can be defined as the entropic state life has to escape from and take advantage of, the organism is bound to be in contact with the environment to acknowledge changes that may affect its survival. Therefore, the individual adapts to a specific environment. Here I explain how individuals that have adapted to a certain environment respond to a different environment and how these responses may vary among different genotypes.

The organism is thus intimately dependent on the environment. With its genes as causal factors and useful tools at the same time, the organism should manage to cope with the environment in order to take advantage of the opportunities that are feasible within its genetic and physical-chemical constraints. The effects that are

observed in clonal individuals in different environments are thus always the responses of a single genotype to that environment.[11] Here, when I mention the response among different environments I speak of the reaction norm of this genotype. However, if more than one genotype is examined in these environments, one often sees the reaction norms cross. Take the example of individuals with genotype A or B that have an average longevity of 50 days in environment 1. In environment 2, A has an average longevity of 75, and B of 34. We see that the genotypes describe a different pattern across the two environments; Genotype A improves its longevity whereas B's longevity degenerates with a shift from environment 1 to 2. That is what we call genotype-by-environment interaction (see also Stearns 1992).

When species, populations, selection lines, or single gene mutants are compared in a range of different environments, or for a range of different traits, we can discover genotype-by-environment interactions. Genotype-by-environment interactions are central in my thesis.

Down to a more practical level

I have pointed out the driving forces behind life and the processes that underlie it. I have also identified which chapter is about which subject. Now I move on to a more practical level where we can implement the issues we have covered thus far. This level will be the research in *Drosophila melanogaster*, the fruit fly.

Since food is one of the major factors affecting an animal, there is likely to be plasticity in the response to certain food conditions, as has been shown by Carlson and Harshman (1999) for egg yolk mRNA. Adult fruit flies are post-mitotic in the sense that they do not grow any more after they have eclosed from the pupae (see Bhui-Kaur et al. 1998 for a more in depth study on maintenance consequences). The only thing they can do is store additional compounds as reserves. Fruit flies show plasticity in their longevity and it has been shown that reproduction and longevity trade off in fruit flies (Chippindale et al. 1993). Also, molecular signals are thought to underlie these trade offs rather than physiological resource allocation alone (Leroi 2001; Patel et al. 2002; Tu et al. 2002). Both mechanisms fit the Disposable Soma theory of ageing (Kirkwood 1977; Kirkwood and Holliday 1979).

The advantages of performing research on *Drosophila melanogaster* are numerous, but for me the following are most important: 1. *Drosophila* has a short generation time, making it easy to rear, 2. the flies are easy to handle and require conditions that are easily standardised, 3. much is known about its genetics, metabolism, physiology and life history, making it an organism with high reference potential, 4. all this gives the researcher the opportunity to do precise, in depth studies of specific well-developed fields. *Drosophila* provides us with a system where the precise effects of

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[11] I am aware of the possibility of epigenetic factors that may cause clonal individuals to produce distinctly different phenotypes (e.g. Smith and Murphy 2004). For the sake of brevity I will not go into these any further.

genetics and physiology can be examined. Combining the advantages of the laboratory with those of *Drosophila melanogaster* has proved to be a strong model system to make experimental manipulations in either genetics or environments, and examine their effects on the phenotype of the individual.

Outline of the thesis

During this project a cross fertilisation was present between the gerontologists, epidemiologists, animal ecologists, evolutionary biologists and industry, which proved to be highly productive. In this thesis Chapters 1, 2, 4 and 5 arose in close collaboration with people from various departments. Therefore, this thesis does not only invoke evolutionary paradigms but benefits from the strength of thought from many people from various backgrounds. I think this pragmatic paradigmatic plasticity added to the robustness of the thesis.

This thesis ranges from species and selection lines to single gene mutants, covering the effects of genetics, environments and genotype-by-environment interactions on life history traits. The mechanisms underlying these traits are examined by environmental manipulation, selection and state of the art expression analysis. The aim of this thesis is to identify aspects affecting life history traits at a number of different levels.

My first two Chapters deal with environmental manipulation effects on adult life history. In Chapter 3, I report on an artificial selection experiment for increased starvation resistance and an exploration of the associated correlated responses to selection in other life history and physiological traits. Chapters 4 and 5 continue with the data derived from the third Chapter. In Chapter 4, we focus on genotype-by-environment interactions among lines that are expected to show similar results on basis of earlier findings. Chapter 5 is a state of the art experiment showing the potential of micro-array studies for life history and genotype-by-environment research. In Chapter 6, the supposedly superior characters of the long-lived mutant, methuselah, are tested.

Overall, I aim to achieve an overview of how genes and environments affect life histories by experimentally altering either one or both of them. The focal point is on the effects different controlled environments have on animals with a particular genetic background. The gene-by-environment interactions of these tests yield insight in the mechanisms underlying the traits. We focus on starvation resistance and longevity and whether these traits are largely determined by the same mechanism or not. Inferring about the correlation between these traits learns about the evolution of both traits, because starvation resistance can be actively selected for, whereas longevity always will be a side-product of selection (Hoekstra 1993; Partridge and Gems 2002). I will present data on organisms with different levels of relatedness, manipulating the environment, physiology and genes to infer knowledge about the relatedness, mechanisms and sensitivities of life history traits.

Chapter 1

The effects of larval density on adult life-history traits in three species of *Drosophila*

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The effects of larval density on adult life-history traits in three species of *Drosophila*

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Abstract

There is evidence that longevity and starvation resistance are determined by a common genetic mechanism. Starvation resistance in *Drosophila* strongly correlates with both fat content and longevity, and is affected by density during rearing. In this study we examine how three species, *D. melanogaster, D. ananassae* and *D. willistoni*, respond to three larval density treatments. Starvation resistance after adult eclosion, and after 2 days of feeding, and longevity were examined in each sex. *D. willistoni* reacted differently to larval density than the other two species. This species showed an effect of density on longevity whilst *D. ananassae and D. melanogaster* showed no such effects. The results also indicate that starvation resistance is not solely determined by fat content. Resistance to starvation at two time points after eclosion differed among species. This may reflect differences in resource acquisition and allocation, and we discuss our findings in relation to how selection may operate in the different species.

Keywords

Drosophila, starvation resistance, longevity, fat, feeding, life history

Introduction

Longevity and starvation resistance are key life history traits and are studied in a wide range of organisms including, the yeast *Saccharomyces cerevisiae*, the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster* and the mouse *Mus musculus* (Longo and Fabrizio 2002; Partridge and Gems 2002). Their importance to the mechanisms of ageing in part explains the interest in these traits. Several authors working with *Drosophila melanogaster* have found that longevity and starvation resistance are correlated (e.g. Zwaan et al. 1991; Chippindale et al. 1993). Others found that selection on starvation resistance can increase longevity (Rose et al. 1992; Harshman et al. 1999b) and *vice versa* (Zwaan et al. 1995a). This indicates not only that genes for longevity affect stress resistance, but that longevity is also affected by genes involved in stress resistance.

The genetics of longevity are beginning to be unravelled and current insights reveal an important role for hormones (*e.g.* the insulin pathway, Partridge and Gems 2002; ecdysone, Tatar et al. 2003, both in *Drosophila melanogaster*). Superimposed on these mechanisms are the environmental factors that affect life span and ageing (Tu and Tatar 2003; Zwaan 2003), including larval density (Miller and Thomas 1958). In this study, we focus on the interaction between longevity and starvation resistance in relation to larval rearing conditions for three species of *Drosophila*.

Longevity, starvation resistance and fat-content all show positive responses to higher larval density (Miller and Thomas 1958; Lints and Lints 1969; Luckinbill and Clare 1986; Zwaan et al. 1991; Robinson et al. 2000; Sorensen and Loeschcke 2001). Borash and Ho (2001) confirmed that in lines of *D. melanogaster* selected for survival at high larval density, resistance to starvation increased compared to unselected controls. In addition, Mueller et al. (1993) found that *D. melanogaster* lines reared at high densities showed higher starvation resistance than the same lines when reared at low densities. These results demonstrate that larval density is an important factor in shaping life histories, and that stocks generally show an increase in starvation resistance when reared at high densities.

Selection lines for higher longevity in *D. melanogaster* showed elevated lipid content later in life (Djawdan et al. 1996), and in general starvation resistance positively correlates with fat content (Zwaan et al. 1991; Graves et al. 1992; Djawdan et al. 1998). Relative fat content is a measure for the amount of energy available per unit of body mass. It follows that more energy reserves should result in a higher starvation resistance (Chippindale et al. 1996; Djawdan et al. 1998; Zera and Harshman 2001; Marron et al. 2003). Based on the links between longevity, starvation resistance and fat content, the last of these traits is thought to be an indicator of starvation resistance.

In a study of the ecological importance of food availability for *Drosophila* dispersal, Sevenster and Van Alphen (1993) examined whether the adult food uptake during the first two days after eclosion was utilised to increase starvation resistance. In contrast to expectation, the overall analysis for several species of *Drosophila* showed that starvation resistance was decreased after 2 days of food. This interaction between food availability and starvation resistance in adults of *Drosophila* species implies that food availability triggers processes such as resource allocation or the onset of a different metabolism that reduce starvation resistance. This finding might

relate to the trade-off between reproduction and longevity (Chippindale et al. 1993). However, starvation resistance appears to be variable among species of *Drosophila* (Sevenster and Van Alphen 1993).

Thus, starvation resistance and longevity are clearly related characters, and this relationship is modulated by larval density. In this study, we examine whether three closely related species of *Drosophila* show similar responses in adult starvation resistance and longevity to rearing at three different larval densities. We perform a detailed experimental analysis of the density effects on these life history traits for each species and each sex. Fat content is also measured in relation to starvation resistance. We examine whether the responses to larval density are the same for the different species, and for males and females. The responses are recorded for starvation resistance after hatching (SR), starvation resistance after two days of food (SR2), and longevity (L). In addition, we studied fat content directly after hatching, after two days of adult feeding and two days of starvation.

Materials and methods

Stock and maintenance

The *Drosophila* species, *D. ananassae*, *D. melanogaster* and *D. willistoni*, were collected in Panama in 1998 (Krijger et al. 2001). The size of the founding population exceeded 40. The flies were maintained at population sizes of approximately 200 individuals. All maintenance and experiments took place at 25ºC and 60% RH at a 12/12 D/L cycle. Stocks were originally cultured in bottles containing 80 ml vermiculite, 40 ml water and approximately 25 gr. banana soaked in yeast suspension and enriched with 4 ml propionic acid per liter water. A foam stopper containing a small drop of honey served as additional food for the adult flies and sealed off the bottles. In 2002, the stocks were switched to bottles containing 24 ml standard medium (20 gr. agar, 9 gr. kalmus, 10 ml. nipagin, 50 gr. saccharose and 35 gr. granulated yeast per liter water). Population sizes were subsequently maintained at 800 for each species. Pieces of paper towel were added to these bottles when flies were in their third instar larval phase to allow successful pupation. Harvesting and transfer to fresh bottles was done by aspiration and shaking. Prior to the current experiment, the flies were maintained in these new conditions for 10 generations. Flies were anaesthetised briefly with CO_2 for sexing and transfer to new vials for experimental purposes.

Experimental set-up

Traits

Starvation resistance (SR) is the time between eclosion of the adult from the pupa and death of the adult under conditions without food. This was measured on 6 ml agar medium (20 gr. agar 9 gr. kalmus, 5 ml. propionic acid, 5 ml. nipagin per litre

water) to prevent the flies from dehydrating and dying of desiccation. SR2 is the time between the onset of starvation for the adult (2 days after hatching from the pupa) and death. SR2 flies were first kept on standard medium for two days after which they were transferred to agar vials. Longevity (L) is the time between eclosion of the adult from the pupa and death of the adult under standard food conditions (see above).

Larval density

Groups of approximately 100 flies were allowed to lay eggs for 24 hours on small plates containing agar medium and wet yeast. Eggs were counted the following day and groups of 10-20 (low), 50-70 (medium) and 150-170 (high) eggs were put into vials containing standard medium. These densities match values from the literature (*e.g* Graves and Mueller 1993). However, food conditions as used in the laboratory vary widely. Thus, Perez and Garcia (2002) used a more nutritional medium than Zwaan et al. (1991); the latter study used a similar medium to that used in this study. Chapman and Partridge (1996) found that the food conditions for optimal longevity in *D. melanogaster* are close to the medium used in this study. Krijger's data (2000) indicate that these species have similar development times. However, it should be noted that other features, which we do not consider here, may be differentially affected for the three species.

For all traits, observed flies were maintained in groups of 10 individuals per vial containing 6 ml standard medium, with males and females separated. Treatment effects were tested by examining 5 replicate vials containing 10 flies, for each sex and each of the 3 species, subjected to 3 densities. This resulted in a grand total of 2700 flies. Vials for both of the starvation resistance treatments were changed every two days to minimise bacterial influences due to rapid bacterial growth (Borash and Ho 2001). For each experiment dead flies were removed daily from each vial to minimise disease and feeding from the corpses. In all assays flies were checked for alive or dead status by physical stimulation. Vials for longevity were changed each week to prevent flies from sticking to the medium.

Fat content

Fat content data were obtained in a separate experiment and was measured in flies harvested within 8 hours of eclosion for the determination of relative fat content. For measuring the fat content after two days of food or starvation, flies were kept on standard medium or agar medium, respectively, and were isolated after 48 hours of treatment. Flies were kept in vials with sexes separated to measure virgin fat content. Only live flies were analysed for fat content. Three replicates of 50 individuals each were checked per treatment. For the measurement of fat content over time we examined flies each day until all flies had starved to death. Five individuals of each line and each replicate were isolated and stored at –80ºC until further analysis. Flies were dried at 60ºC for 24h and then weighed on a Sartorius® ultra microbalance to determine dry weight. Fat was extracted by adding 1 ml of diethyl ether under continuous shaking (200 rpm) for 24 hours. The flies were dried for 24h at 60ºC and then re-weighed. The fat-free dry weight value was subtracted from the dry weight value. *D. melanogaster* flies were measured in groups of 5, while *D. ananassae* and *D. willistoni* flies were measured as individuals. Relative fat content was calculated by dividing absolute fat content by dry weight (Zwaan et al. 1991).

Comparison between starvation resistance after eclosion and after two days of feeding

To make a clear comparison between the SR directly after eclosion and that after two days of feeding, we subtracted the first two days of feeding from SR2. Thus, we compared the actual time of death after the onset of starvation.

Statistics

Figure 1 illustrates the outline of the experimental set-up for the SR, SR2 and L-measurements. All data were initially analysed to detect general patterns, but specific effects of density on the three life history traits were tested in separate analyses for each species and sex. We used Tukey HSD tests to examine whether the effect of density differed between groups or within treatments (Figure 2). In addition, differences between the sexes of each species for starvation after two days of food were compared to those of starvation after eclosion.

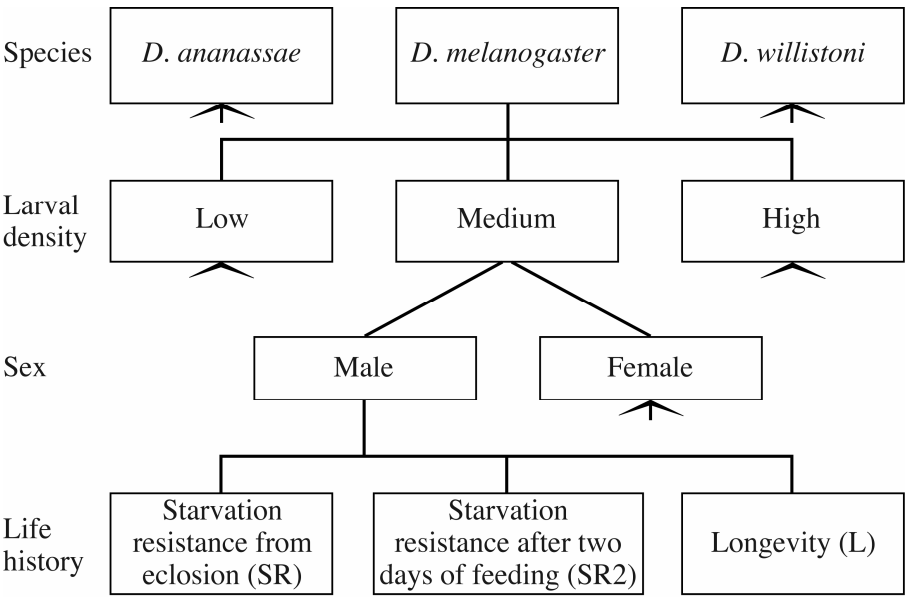

Figure 1. Set up of the experiment. The statistical analyses were applied at each horizontal level.

The data for longevity and for starvation resistance after hatching and after 2 days of food were not normally distributed (Shapiro-Wilkinson W test, data not shown). No transformation (lognormal, Weibull and Weibull with threshold) resulted in normalisation. However, tests with non-parametric models could not examine the interaction factors that are the main concern of our paper. We, therefore, determined whether the deviations from normality could bias our conclusions. There was no general pattern, such that one species, treatment or sex mainly contributed to the non-homogeneity of the variances. This suggested that using parametric models (ANOVA) would not bias these deviations from normality. So we used such models and interpreted the results with caution.

Dry weight, fat content and relative fat content data were not normally distributed, yet variances were homogeneous. As reasoned above, we still tested for differences between samples using ANOVAs. All statistical tests were performed using JMP 5.0.1.

Results

Starvation resistance

The overall analysis shows a significant negative effect of increasing density on starvation resistance. The analysis of SR showed significant effects of species ($F_{2,852}$=494.36, P<0.0001), sex ($F_{1,852}$=42.31, P<0.0001), and density ($F_{2,852}$=29.22, P<0.0001); see Figure 2. A significant species*sex interaction ($F_{2,852}$=48.73, P<0.0001) indicates that the sexes behave differently among species. This is largely explained by the differences between males and females in *D. melanogaster*.

Species-specific analysis

SR was significantly higher in female than male *D. melanogaster* ($F_{1,295}$=86.65, P<0.0001), whereas such differences were absent in *D. ananassae* and *D. willistoni* ($F_{1,269}$=0.42, P=0.53 and $F_{1,288}$=1.02, P=0.33, respectively.) SR decreased significantly with increasing larval density in *D. melanogaster* ($F_{2,295}$= 8.13, P=0.0004), *D. willistoni* ($F_{2,288}$=3.53, P=0.03) and *D. ananassae* ($F_{2,269}$=27.20, P<0.0001).

Sex-specific analysis

SR showed no significant density effects in either sex of *D. melanogaster* (females: $F_{2,149}$=0.49, P=0.62; males: $F_{2,146}$=0.24, P=0.78). Sex-specific analysis of *D. ananassae* and *D. willistoni* was not performed because no differences were found between the sexes in the species-specific analyses.

Longevity

For longevity, both species and density were significant factors ($F_{2,794}$=139.52, P=<0.0001; $F_{2,794}$=12.4, P<0.0001, respectively). However sex differences were not

found for this trait ($F_{1,794}$=1.74, P=0.19). The species*sex*density interaction showed a significant effect ($F_{4,794}$=2.48, P=0.043)

Species-specific analysis

Density was not important for *D. melanogaster* and *D. ananassae* longevity ($F_{2,272}$=0.93, P=0.40 and $F_{2,265}$=1.47, P=0.23, respectively), but was for *D. willistoni* ($F_{2,257}$=14.06, P<0.0001). The latter species also showed a significant sex*density interaction ($F_{2,257}$=5.18, P=0.0062). This is clear from Figure 2, male longevity decreased monotonically with density, whilst female longevity was lowest for intermediate densities. This largely accounts for the significant species*sex*density interaction found in the overall analysis.

Starvation resistance after two days of food

SR2 showed similar results to the SR treatment for the factors, species and sex ($F_{2,854}$=49.27, P<0.0001 and $F_{1,854}$=77.1, P<0.0001). Density effects were also significant ($F_{2,854}$=6.67, P=0.0013), and there was a significant species*sex interaction factor ($F_{2,854}$=27.82, P<0.0001).

Species-specific analysis

Density affected this trait differently in the sexes and the species in a complex fashion, and therefore no clear pattern can be distilled from the data (see also figure 2). Sex effects on SR2 were significant for both *D. melanogaster* and *D. ananassae* ($F_{1,288}$=356.95, P<0.0001 and $F_{1,286}$=29.60, P<0.0001), but not for *D. willistoni* ($F_{1,280}$=0.45, P=0.50). Larval density was important for SR2 in *D. melanogaster* ($F_{2,288}$=31.65, P<0.0001) and *D. willistoni* ($F_{2,280}$=3.4371, P=0.034), but not for *D. ananassae* ($F_{2,286}$=2.94, P=0.09). In addition, *D. melanogaster* and *D. willistoni* showed significant sex*density interactions ($F_{2,288}$=31.14, P<0.0001 and $F_{2,280}$=8.23, P=0.0003). *D. willistoni* males showed their highest starvation resistance at medium density whereas females showed their lowest starvation resistance at that density. *D. melanogaster* showed a slight decrease in SR2 with density in both sexes.

Sex-specific analysis

D. melanogaster females ($F_{2,147}$=0.66, P=0.52) and males ($F_{2,141}$=2.66, P=0.073) showed no significant effect of density on SR2. This trait in *D. ananassae* was independent of density in males ($F_{2,141}$=0.44, P=0.64), but was density-dependent in females ($F_{2,145}$=3.76, P=0.026).

The indication that density is a factor in shaping this trait comes only from the overall analysis, and from the species-specific analysis where *D. melanogaster* and *D. willistoni* showed significant effects of larval density. Higher order interactions indicate differential responses of the sexes to density.

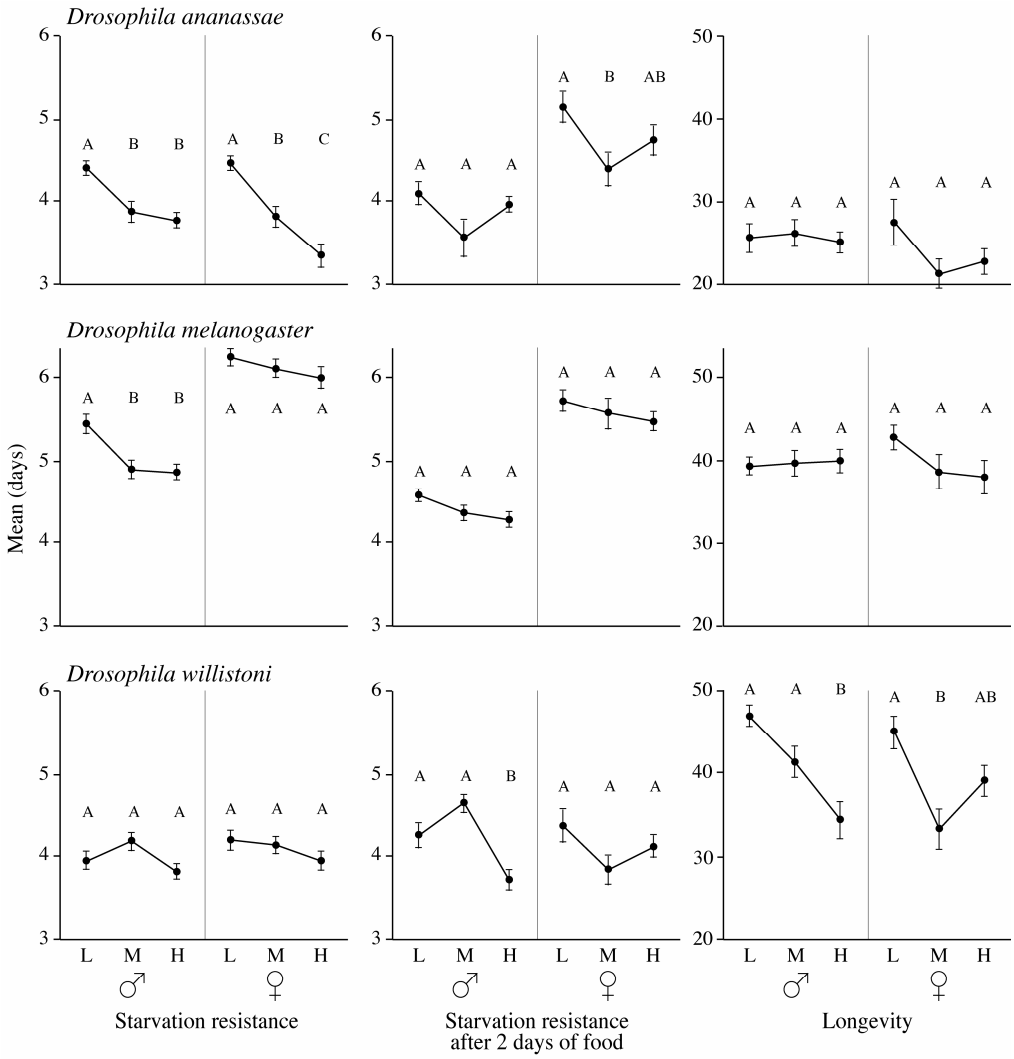

Figure 2. The average survival of males and females of three species of *Drosophila* across three larval density treatments (L, low density; M, medium density; H, high density). Life span is measured under conditions of starvation from eclosion, two days of feeding after eclosion and *ad libitum* feeding. Letters indicate significant differences between groups (after *post hoc* Tukey testing).

Dry weight and fat content

Dry weight

The size of *D. melanogaster* and *D. willistoni* females declined with increasing larval density (12% decline, $F_{2,6}$=6.19, P=0.035; 12 % decline, $F_{2,27}$=6.84, P=0.0039, respectively), whereas males showed no significant affect (5% decline, $F_{2,6}$=0.85, P=0.47; 3% decline, $F_{2,27}$=0.34, P=0.72, respectively). The density effects were not significant in *D. ananassae* (females 13% decline, $F_{2,27}$=3.17, P=0.058; males 2% decline, $F_{2,27}$=2.15, P=0.1358), though the responses for the sexes were similar to those of the other species and the decline in female size approached significance. In general, females of each species showed a stronger reduction in dry weight with increasing density than males. This effect on body size suggests that the animals were mildly stressed by higher larval density.

Fat-free dry weight

Females of all three species showed a negative effect of density on fat-free dry weight (*D. melanogaster* 15% decline, $F_{2,6}$=10.27, P=0.016; *D. willistoni* 9% decline, $F_{2,27}$=4.46, P=0.021; *D. ananassae* 15%, $F_{2,27}$=3.75, P=0.037), whilst this only occurred for males in *D. melanogaster* (15% decline, $F_{2,6}$=28.18, P=0.0015). Males of *D. ananassae* and *D. willistoni* showed non-significant declines in fat-free dry weight (6% and 5% decline, respectively) .Figure 3 illustrates the sensitivity of females with a general decline in fat-free dry weight with higher larval density.

Fat content

D. melanogaster and *D. ananassae* males showed effects of increasing larval density on adult fat content ($F_{2,6}$=5.30, P=0.047; $F_{2,27}$=5.54, P=0.0097, respectively), whereas females did not ($F_{2,6}$=2.94, P=0.13; $F_{2,27}$=0.09, P=0.91, respectively). *D. willistoni* showed a significant density effect in female fat content ($F_{2,27}$=7.32, P=0.0029) but not in males ($F_{2,27}$=1.46, P=0.25).

Relative fat content

Relative fat content in *D. melanogaster* and *D. ananassae* showed a strong effect of larval density ($F_{2,15}$=7.54, P=0.005; $F_{2,57}$=7.01, P=0.0019, respectively). Of these species males showed significant responses ($F_{2,6}$=6.33, P=0.005 and $F_{2,6}$=12.01, P=0.008, respectively) whereas females showed none ($F_{2,27}$=4.76, P=0.058 and $F_{2,27}$=1.85, P=0.18, respectively). In contrast, there was no effect in *D. willistoni* ($F_{2,56}$=1.49, P=0.23). *D. willistoni* males showed no significant effect ($F_{2,27}$=1.92, P=0.17) whilst females showed an effect ($F_{2,27}$=5.48, P=0.01). Although not all tests showed significant effects of density, the generally positive trend with increasing density is consistent with the findings of Zwaan et al. (1991).

The effect of 2 days of starvation on relative fat content

Each species showed significantly reduced relative fat content after two days of starvation as compared to immediately after adult eclosion (Table 1).

Table 1. Percentage decline of relative fat content during starvation. F- and P-values of the ANOVA test show whether relative fat content was affected when animals were first kept for 2 days without food as compared to measurement directly following eclosion. All species showed a decline in fat content. MLG, *D. melanogaster*; WLS, *D. willistoni*; ANS, *D. ananassae*.

	MLG	WLS	ANS
Overall	$F_{1,6}$=153.11; P<0.0001	$F_{1,8}$=7.88; P=0.0229	$F_{1,7}$=13.04; P=0.0086
♂	74% $F_{1,3}$=152.02; P=0.0011	16% $F_{1,4}$=0.46; P=0.5351	39% $F_{1,4}$=9.85; P=0.0349
♀	57% $F_{1,3}$=27.82; P=0.0133	60% $F_{1,4}$=13.47; P=0.0214	58% $F_{1,3}$=5.95; P=0.0926

Comparison of starvation resistance after 0 and 2 days of feeding.

The overall analysis of these two traits showed significant species, sex and larval density effects ($F_{2,1704}$=306.28; P<0.0001, $F_{1,1704}$=124.26, P<0.0001 and $F_{2,1704}$=28.10, P<0.0001, respectively). The factor treatment was not significant ($F_{1,1704}$=0.44, P=0.5081, but see Table 2) indicating that there is no overall difference between SR and SR2. All interaction factors, including the species*sex*density interaction ($F_{4,1704}$=4.62, P=0.001), were significant except for those involving the factor treatment.

Table 2. P-values of the ANOVA test of starvation resistance from eclosion (SR0) versus starvation resistance after two days of feeding (SR2) specified per species, sex and larval density; MLG, *D. melanogaster*; WLS, *D. willistoni*; ANS, *D. ananassae*; (+) means that SR0>SR2; (-) means that SR0<SR2.

	MLG ♂	MLG ♀	WLS ♂	WLS ♀	ANS ♂	ANS ♀
Low	<0.0001, +	0.0045, +	0.1143, -	0.4494, -	0.1082, +	0.0023, -
Medium	0.0002, +	0.0187, +	0.0040, -	0.1309, +	0.2762, +	0.0159, -
High	<0.0001, +	0.0021, +	0.5073, +	0.2420, -	0.1852, -	<0.0001, -

D. melanogaster males and females showed similar results for the SR-SR2 comparison to those found by Sevenster and Van Alphen (1993) at all densities. *D. melanogaster* flies generally resisted starvation better directly after hatching than after two days of food (Table 2). SR2 of *D. ananassae* males was not affected compared to SR at each density, while female SR2 was significantly higher at all densities. *D. willistoni* males were significantly different only at medium density whereas they were not at the other densities or in females (Table 2). This implies that

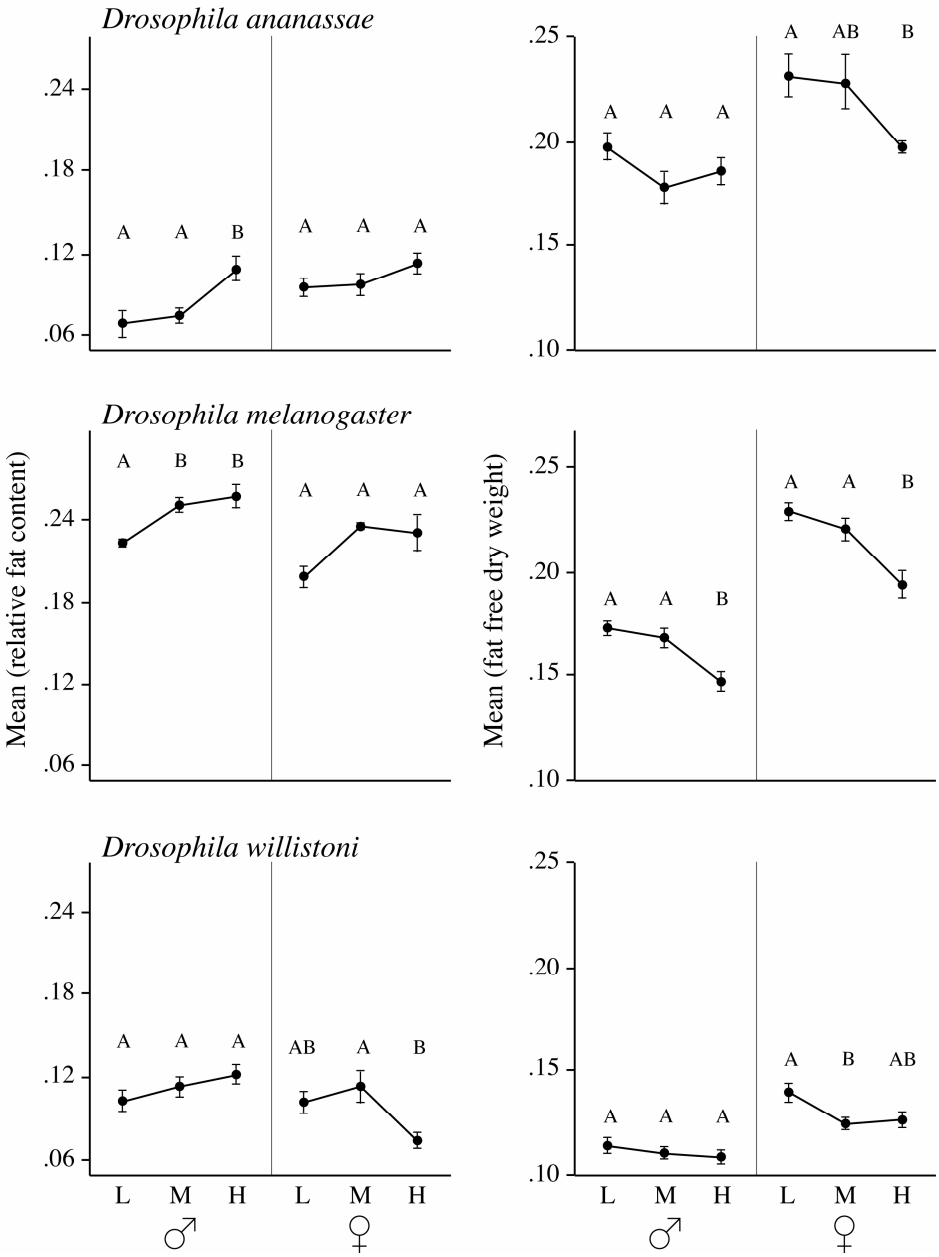

Figure 3. The average relative fat content and fat-free dry weight of males and females of three species of *Drosophila* across three larval density treatments (L, low density; M, medium density; H, high density).

for *D. willistoni*, food availability in the first two days after eclosion does not affect starvation resistance.

In general, *D. melanogaster* shows a significant decrease in starvation resistance after feeding in the adult stage. *D. willistoni* did not show differences between SR and SR2, whereas *D. ananassae* showed a significant increase in female starvation resistance. We conclude that larval density affects species and sexes in different ways thus accounting for the significant species*sex*density interaction.

Discussion

We examined effects of larval density on adult life history traits and physiology in three species of *Drosophila*. In general, the responses were similar among species, however a more detailed comparison revealed species and sex-specific responses. We will discuss whether the responses reflect phylogenetic relationships. Each of the species showed a different response of starvation resistance after adult feeding. The effect of post-eclosion feeding and the use of standard medium will also be discussed. Larval density affected dry weight and fat content in a similar way among the species. We will describe in more detail how larval density influences life history traits, and we will elaborate on the effects larval density has on fat and starvation resistance, and whether there is a causal link between these two traits. Finally we will speculate on the underlying genetics of the traits studied.

Effects of rearing conditions match phylogenetic relationships of species

D. ananassae, D. melanogaster and *D. willistoni* showed significant effects of larval density on adult starvation resistance directly after eclosion and after two days of food. We did not find effects of larval density on longevity in *D. ananassae* and *D. melanogaster*, whereas we did in *D. willistoni*. Apparently crowded larval conditions in *D. willistoni* decrease adult longevity, contrasting with the increase in longevity found in *D. melanogaster* studies (Miller and Thomas 1958; Zwaan et al. 1991). *D. melanogaster* and *D. ananassae* are phylogenetically more closely related to each other than either is to *D. willistoni* (O'Grady and Kidwell 2002). Although we used only three observations for each sex of each species, the responses of underlying physiology to larval densities seem to reflect these phylogenetic associations. Further research into how life histories reflect phylogenetic associations would be extremely valuable.

The effects of post-eclosion feeding on starvation resistance

Two days of feeding after adult eclosion in *D. melanogaster* led to a subsequent decrease in starvation survival relative to flies starved directly from eclosion. Apparently the opportunity to feed after eclosion was not used to increase energy reserves but might have triggered different metabolic processes in *D. melanogaster*, such as reproduction. Such mechanisms are known from yeast (Lin et al. 2002). Additionally, some authors have found that adding yeast to the diet of *D. melanogaster* initiated reproduction and diminished both SR and L (Chippindale et al.

1993; Simmons and Bradley 1997). This led Chippindale et al. (1993) to the finding that starvation resistance and longevity trade off against reproduction. Forced adult migration due to lack of food and oviposition site may trigger postponed reproduction and thereby influence the trade-off between longevity and reproduction. In contrast, finding food after adult eclosion may induce a reproductive response and in turn shorten starvation resistance and longevity. An eco-physiological explanation for the difference between SR and SR2 in *D. melanogaster* may be that females have a fitness advantage for early reproduction in a growing population and are capable of producing eggs within about 8 hours of eclosion. Therefore, *D. melanogaster* is likely to allocate resources to reproduction rather than to somatic maintenance under laboratory circumstances.

This scenario for *D. melanogaster* does not, however, fit our results for *D. willistoni* and *D. ananassae*. *D. ananassae* females showed an increase in SR after feeding, indicating that the food uptake prolonged, rather than shortened, SR. This is accompanied by a strong increase in relative fat content after two days of feeding in virgin *D. ananassae* (Table 3). In contrast, *D. willistoni* showed no difference in relative fat content after two days of food compared to directly after eclosion (Table 3). *D. willistoni* also showed no effect of larval density and adult feeding on starvation resistance, suggesting that there is little plasticity for starvation resistance in this species.

Table 3. F- and P-values of ANOVA on adult relative fat content at eclosion (RFC0) compared to relative fat content after 2 days of feeding (RFC2). MLG, *D. melanogaster*; WLS, *D. willistoni*; ANS, *D. ananassae*; (+) means that RFC0> RFC2; (-) means that RFC0< RFC2.

	MLG	WLS	ANS
overall	$F_{1,7}$=0.96; P=0.36, +	$F_{1,8}$=0.21; P=0.66, +	$F_{1,8}$=14.96; P=0.0048, -
♂	$F_{1,3}$=0.11; P=0.76, +	$F_{1,4}$=0.07; P=0.80, +	$F_{1,4}$=27.24; P=0.0064, -
♀	$F_{1,3}$=3.45; P=0.14, +	$F_{1,4}$=0.29; P=0.62, +	$F_{1,4}$=1.57; P=0.28, -

Feeding media

Different species of *Drosophila* could respond differently to the same larval medium, and this could introduce an artefact into our comparisons. The standard medium is widely used for *D. melanogaster* and may be less suitable for development of the other species. Non-optimal developmental conditions might then induce less optimal adult phenotypes. More natural food conditions, such as banana, are more likely to vary in nutrient concentrations and in resources thus introducing environmental variation. In *D. melanogaster* food supplements can increase longevity (Takahashi et al. 2001) and starvation resistance (Zinke et al. 2002). We reared all species on standard medium for several generations before our experiment to reduce such effects whilst retaining the ability to make direct comparisons.

Fat content and dry weight

Female dry weight and fat-free dry weight declined in response to larval density. Males had a more uniform dry weight over different larval densities. Generally, fat

content increased for all species and sexes with increasing larval density, whilst dry weight and fat-free dry weight decreased with increasing density in most species. This indicates that the higher larval densities should be regarded as a stress, as is consistent with other studies (*e.g.* Zwaan et al., 1991). Djawdan et al. (1998) showed that glycogen and lipid energy content when pooled give the best indicator of the ability to resist starvation in *D. melanogaster*. However, Graves et al. (1992) clearly showed that flies with no glycogen reserves are equally capable of resisting starvation as glycogen-containing control flies. Experiments with *D. melanogaster* lines also indicated that glycogen content was not affected by selection for starvation resistance whereas fat was (Baldal et al. unpublished results). Thus, fat appears to be the most important energy reserve underlying starvation resistance (Zwaan et al. 1991; Djawdan et al. 1998).

Density effects

Zwaan et al. (1991) found that high-density flies have the highest fat content, facilitating an increased starvation resistance. The present study does not show this; animals from high larval densities showed the shortest starvation resistance. Borash and Ho (2001) found that even though flies selected at higher larval density showed an increase in starvation resistance compared to controls when reared at normal larval density, a reduction in starvation resistance was observed when larval density increased. One explanation for these seemingly conflicting results is that Zwaan et al. (1991) measured starvation resistance 21 days after eclosion whereas in both Borash and Ho's and our own study, starvation resistance was measured directly after hatching. We found that exposure to higher larval density reduces body size. This is in line with Santos et al. (1994), who found a decreased fitness and thorax length, and thus body size, at increasing densities similar to those observed in the present study. However, compared to Zwaan et al. (1991), the reduction in body size is small. The effects of larval density in the present study tend to be small but are likely to reflect important responses to developmental conditions. Thus, we conclude that higher larval densities lead to smaller flies with a high relative fat content and reduced starvation resistance.

Fat, starvation resistance and longevity

Starvation resistance is associated with longevity, suggesting that these traits share molecular pathways. Reproduction and starvation resistance are both dependent on fat reserves and, therefore, we hypothesise a trade off between them. In the present study, relative fat content increases, and starvation resistance decreases with increasing larval density and *vice versa*. Changing density causes many physiological changes, including relative fat content. Starvation resistance is in turn affected by fat storage, as well as by at least some of the other changes. If starvation resistance does not solely depend on the amount of fat, then additional regulatory mechanisms must also play a role. Leroi (2001) argued that the insulin-signalling pathway is very important in shaping the trade off between longevity and reproduction. If this is so, then resource allocation can not be solely responsible for the trade off between longevity and reproduction, and thus neither of the traits is

dependent only on resources. This fits our finding that starvation resistance can be influenced by factors other than stored fat reserves.

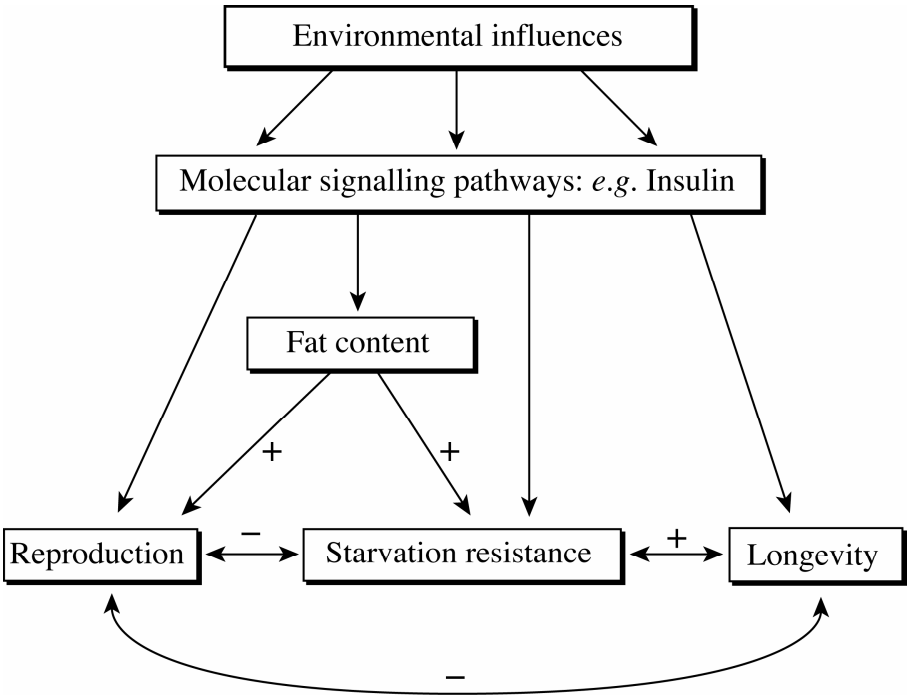

Figure 4. A model of how different factors may affect starvation resistance.A direct link is proposed between the molecular signalling pathways and starvation resistance. "+" Means that factors are positively correlated, "-" means that factors are negatively correlated.

Underlying genetic mechanisms

It is difficult to demonstrate that there is a particular molecular signalling component involved in increasing starvation resistance especially if such a pathway regulates both starvation resistance and the build up of fat. Thus fat acquisition could be an unavoidable by-product of an increase in starvation resistance and not necessarily causal for starvation resistance. Therefore, we propose that the resource allocation model for the trade off between longevity/starvation resistance and reproduction should attempt to incorporate such molecular signalling components (figure 4).

Modulating the genetic mechanism underlying any one of these life history traits would be expected to affect all the traits in a similar way if they share underlying physiological mechanisms. However, if additional pathways also affect the traits, modulation of one such pathway may not induce similar responses. Responses of SR

and longevity to larval density seem to follow a similar trend of reduced life span with increasing larval density in our experiments (Figure 2) suggesting that these life history characters share physiological mechanisms. Differences between the species lead us to believe that other regulatory mechanisms are also involved. Work of Force et al. (1995) implies the same; they found that some long-lived selection lines did not show a subsequent increase in starvation resistance. Two recent studies of *D. melanogaster* showed that initial correlations between functional traits can change when cultures are kept under continuous selection. Thus, Phelan et al. (2003) found that selection lines for longevity lost their stress resistance, whilst Archer et al. (2003) showed that selection lines for starvation resistance lost elevated longevity found earlier on after several generations. Therefore, caution is necessary before assuming that longevity and starvation resistance depend on the same genetic mechanism.

Conclusion

Different species of *Drosophila* show similar directions of response to density stress; it is the intensity of the response that differs per species. The sexes can also show different responses in different situations, probably because of differing reproductive demands across environments. High larval density acts as a stress environment and tends to lead to different resource allocation in adults with a shift from dry weight to fat content. However, a higher fat content does not lead to a higher starvation resistance or longevity. Access to food in adults also does not necessarily lead to higher starvation resistance, even though it induces higher fat content. These patterns differ in detail across species; the broad pattern is similar among species, but the fine-tuning and interdependence of the traits is not. How this is reflected on the genetic level, for example in the involvement of genetic pathways is the subject of our future research.

Chapter 2

A test of the thrifty phenotype hypothesis in *Drosophila melanogaster*

Submitted to Mechanisms of Ageing and Development

A test of the thrifty phenotype hypothesis in *Drosophila melanogaster*

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Abstract

Adverse pre-natal conditions in humans have been found to affect adult metabolism in a way that the individuals show an increased incidence of metabolic syndrome late in life. Consequently, the average life span of these individuals should be lower than that of the average population. This observation was termed the thrifty phenotype hypothesis by Hales and Barker, and is known as the Barker hypothesis. Here, we examine whether a similar process could be detected in *Drosophila melanogaster*. By rearing flies under adverse conditions, we tried to mimic adverse conditions in the human uterus. Medium with half the sugar and yeast concentrations ("half") of standard medium was used as an adverse pre-adult condition. The standard medium ("standard") was the normal rearing condition and the double medium ("double") contained twice the amounts of sugar and yeast, making it affluent. We were careful not to mix up the thrifty phenotype with the thrifty genotype hypothesis, which works in the same direction but has an ultimate rather than a proximate cause. We showed that animals reared on half medium weighed less, developed slower and had reduced egg-to-adult survival. The half medium was thus truly adverse. The animals reared at adverse medium displayed significantly shortened adult longevity, caused especially by increased mortality late in life. These data indicate that similar mechanisms underpin the Barker hypothesis and the phenomena found in our flies. Further research into this system should reveal whether the adults suffer from metabolic disease or not.

Keywords

Barker hypothesis, thrifty phenotype hypothesis, thrifty genotype hypothesis, proximate, ultimate, *Drosophila melanogaster*, scar, adaptive plasticity, longevity, metabolic syndrome

Introduction

In 1962, Neel proposed the thrifty genotype hypothesis that during human evolution, when food conditions were relatively poor, selection favoured individuals whom could endure long periods of starvation. Subsequently, in periods of food abundance, these humans would acquire as much resources as possible as a reserve against more adverse times. The adaptation to these conditions is hypothesized to be regulated by insulin metabolism which directs several key processes in response to food conditions, such as metabolism, storage and growth. Neel proposed that the increased number of diabetics observed already during his time could be attributed to the moulding of the genetic regulation of insulin metabolism in evolutionary history. As a result of this adaptation, the abundant presence of food in the Western world leads to an increased incidence of diabetes mellitus and other metabolic diseases, in turn reducing fitness and longevity (Diamond 2003).

The thrifty phenotype hypothesis (Hales and Barker 1992, 2001), also known as the Barker hypothesis, is based on the observation that pre-birth food conditions modify insulin metabolism in the human foetus and subsequently in the resulting adult. Specifically, this hypothesis was put forward to explain the observation that poor nutrition in the mother's womb would increase the likelihood of metabolic disease later in life, when food conditions are relatively better, and therewith reduce fitness and longevity. The poor nutrition can arise from adverse environmental conditions where the mother does not have access to food. Another mechanism would be the situation where the mother does not allocate sufficient food to the foetus in her womb via the placenta. Two potential mechanisms could explain the increased incidence of metabolic disease in these individuals (Brakefield et al. 2005). One mechanism is 'scar', i.e. a disruption of homeostasis. The alternative is based on the hypothesis that as the result of natural selection, developmental plasticity of insulin regulation is adaptive using the pre-natal environment as a predictor of future food conditions (Zwaan 2003). This study does not aim to distinguish between these, but mentions them for the context.

The thrifty genotype and thrifty phenotype hypotheses work in the same direction. They both predict that poor food conditions experienced early in the life history will lead to malfunctioning metabolic regulation when food conditions have become better later in the life history. Yet, the thrifty phenotype hypothesis takes place on a physiological (proximate) level, whereas the thrifty genotype hypothesis takes place on a genetic (ultimate) level. Nevertheless, the thrifty phenotype hypothesis and thrifty genotype hypothesis are easily mixed up and thus Zwaan (2003) proposed a clear separate testing of these theories.

Baldal et al. (2005) found that larval crowding in three *Drosophila* species negatively affects adult life histories. This finding could be explained by the thrifty phenotype hypothesis, as crowding involves a relative food shortage, but there were other factors involved (e.g. nitrogenous waste intoxication, competition) that prevented a firm conclusion. We sought a method that specifically tested the thrifty phenotype hypothesis without any potential interaction with the thrifty genotype hypothesis. Furthermore, the method had to exclude any other physiological responses than those involving food adversity in the pre-adult stage. To this end, here we examine whether different pre-adult food conditions significantly affect several life history and

physiological traits in the fruit fly *Drosophila melanogaster*, and whether our findings are in line with the observations in humans.

Materials and methods

Flies

One hundred and eleven females of *Drosophila melanogaster* were caught and used to initiate isofemale lines. Of these, 17 were collected in France by B. Pannebakker, 22 in Panama by K. van der Linde and C. Krijger (all from Leiden University, The Netherlands), 4 from Groningen (The Netherlands; kindly provided by R. Bijlsma and A. Boerema from the University of Groningen), and 68 were collected in the Leiden area (The Netherlands). The iso-female lines were mixed to form a Stock-population, which was maintained for over 30 generations before the onset of this experiment. Flies were allowed to lay eggs for three hours on agar medium with ample wet yeast, to induce reproduction. All experiments took place at 25ºC, 50% relative humidity and a 12/12 hour day/light regime.

Larval food conditions and crowding

In previous work, we found that high larval density reduced longevity and starvation resistance in three species of *Drosophila* (Baldal et al. 2005). In the larval environment, crowding is a stress factor that involves multiple factors such as nitrogenous waste, food competition and food depletion (Graves and Mueller 1993; Joshi et al. 1996; Borash et al. 1998). The reduced longevity and starvation resistance as an effect of high larval density in Baldal et al.'s study could thus be caused by such factors. The thrifty phenotype hypothesis revolves around malnutrition and does not take such factors into account. To test the thrifty phenotype hypothesis, we thus focused on food conditions and tested life histories of adults raised on adverse, standard and affluent conditions as larvae (figures 1 and 2).

The rationale of the experimental design

Paralleling the human womb and insect models
Extrapolating life history directly from holo-metabolous insects to man is tenuous. It would be highly contentious to simply define the larval instar stages as childhood, pupation as puberty and imago as adulthood. We regard early human development *in utero* as a phase of resource acquisition and early development that forms the infant that is supposed to eventually become a reproductive adult. We deduce that the larval stage of the fly is a developmental phase in which the organism acquires resources. After that, it metamorphoses into a stage from where it develops further, comparable to human childhood and adolescence. This reasoning is based on the traits of interest in this work: food intake, development and adult life history. Thus, we considered the larval stages as comparable to the intra-uterine environment in terms of development. Therefore, we manipulated the larval food environment to test the

thrifty phenotype hypothesis (figures 1 and 2).

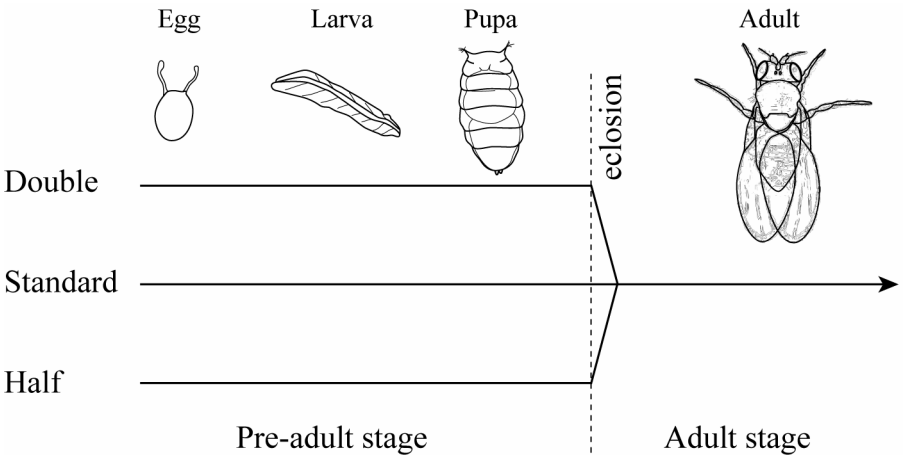

Figure 1. Overview of the experimental set-up. Eggs were put in vials containing one of three media -half, standard and double- hatched as larvae, became pupae and were from eclosion transferred to standard medium as adults. *Drosophila* stages redrawn from http://www.neosci.com/demos/10201_AP%20Lab%207/Presentation_2.html

The adult diet

The thrifty phenotype hypothesis revolves around the physiological response to adverse pre-adult conditions, independent of their evolutionary history. The effects of this process are easily confounded with the effects of a thrifty genotype, which also involve metabolic syndrome. For a clear testing of the thrifty phenotype hypothesis (i.e. examining physiological, proximate responses) we thus need to match the adult environment with the environment in the evolutionary history of the population (figure 2), being the standard medium.

Food conditions

Eggs were placed in vials containing either 6 ml of half, standard or double medium. For each of the three food conditions, 20 vials were set up containing 100 eggs each. We interpret these conditions for the larvae that hatched as adverse, standard and abundant food conditions, respectively. Pupation and subsequent eclosion took place in the same vials. At eclosion, the flies were transferred to fresh standard medium vials (figure 1). Standard medium consisted of 20 g agar, 9 g kalmus (10 parts acidum tartaricum, 4 parts ammonium sulphate, 1 part magnesium sulphate and 3 parts potassium phosphate), 10 ml nipagin (100 g 4-methyl hydroxy benzoate per liter ethanol), 50 g saccharose and 35 g granulated yeast per litre water. In half and double medium the amounts of yeast and sugar are respectively half and double of standard medium, whereas the concentrations of the other ingredients were

maintained as in standard medium. This was done in order to introduce as little variation as possible into the experiment.

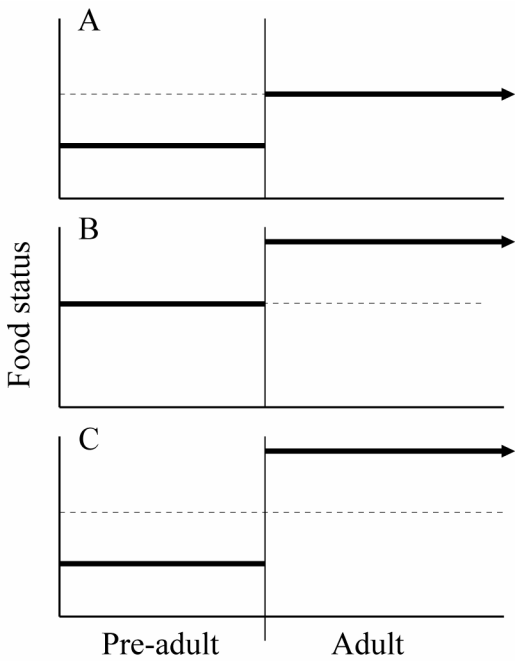

Figure 2. The difference between testing the thrifty genotype and thrifty phenotype hypotheses lies mainly in the evolutionary history of the population. When testing the thrifty phenotype hypothesis, it is important to examine the pre-adult effect on the adult phase under the food conditions to which the population has adapted by natural selection (dotted line), as is depicted in A, because then the only variable is the pre-adult environment. If an adult population is put in a situation to which it is not genetically adapted (B), we are testing the effect of the thrifty genotype hypothesis because the only variable is the condition of adult feeding. When one tries to test the Barker hypothesis by offering adverse pre-adult and affluent adult conditions (C), a confounding of proximate and ultimate mechanisms is inevitable. Both mechanisms work in the same direction, only the causality of the effect is different, resulting in similar phenotypes. Distinguishing between these causes is the aim of the present study.

Traits measured

Development time

Development time was determined by examining the number of flies that eclosed on a specific day. All flies were allowed to eclose and then counted at the same time each day. Development time was measured by taking the number of days between

egg-laying and adult eclosion. Subsequently, we calculated egg to adult survival for each of the media by dividing the number of eclosed adults by the number of eggs.

Wet weight
We measured wet weight of 5 groups of 5 females of each larval food condition directly from the peak of eclosion on a Sartorius® ultra microbalance.

Longevity
Adult longevity was measured in virgin flies as the number of days from eclosion to death. The sexes were maintained separated throughout the entire experiment. Flies were initially maintained at a density of 5 flies per vial, because this yields longevity data without the negative effects of adult density (Baldal, unpublished results).

Statistics

All data were analyzed in JMP 5.0.1 (SAS Institute). For longevity testing, the animals that died within 10 days from eclosion were censored, as they usually died by other than intrinsic causes. We used Cox Proportional hazards analysis to analyze the longevity data and used ANOVA with full factorial designs for all other data. Relative order and significant differences among the groups tested were determined by *post hoc* Tukey testing. Replicate vials were always nested in the factor sex and treated as a random factor. The age specific survival was determined by calculating the log(-log(Survival)). Age specific mortality was tested using ANCOVA. The mortality curve (fig. 4) showed two distinct phases with a linear relationship between mortality and age within each phase. Therefore, the data were analysed for 0-30 days and from 30 days onwards, separately, allowing us to obtain insight into early and late mortality patterns.

Results

The effects of larval media on development

Development time and number of eclosed adults
Females showed a significant effect of the larval media on their development time ($F_{2,2145}$=1658, P<0.0001), as did males ($F_{2,2085}$=1580, P<0.0001). Double medium yielded the fastest developing flies, followed by standard medium and then half medium. The latter medium also showed a strong increase in the variance (table 1). The differences in the number of eclosed adults from the media were striking; out of 2000 eggs put on half medium we obtained 1126 adults (56.3%), in the case of standard medium this is 1388 (69.4%) adults, and in the case of double medium 1722 (86.1%).

Wet weight
Wet weight of females was significantly lower when flies were raised on half medium ($F_{2,12}$=48, P<0.0001) compared to standard and double medium, which is also reflected in *post hoc* Tukey testing (see table 1).

Table 1. Proportion of the total number of adults of each sex that eclosed each day from egg-laying onwards on different larval diets. Peak days of eclosion are highlighted in bold. Average wet weight after eclosion in mg of 5 female flies with standard error for each of the larval media is shown in the right-hand column.

Medium	Sex	Hatching (proportion)								Wet weight (SE)
		Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15	Day 16	
Half	male	0	0.01	0.12	**0.30**	0.22	0.20	0.12	0.04	-
Half	female	0	0.03	**0.25**	0.21	0.19	0.17	0.13	0.02	3.85 (0.17)
Standard	male	0.02	**0.53**	0.33	0.07	0.03	0.01	0.01	0	-
Standard	female	0.06	**0.63**	0.23	0.05	0.01	0.01	0.01	0	6.13 (0.25)
Double	male	0.13	**0.81**	0.05	0.01	0.01	0	0	0	-
Double	female	0.48	**0.49**	0.02	0	0	0	0	0	5.87 (0.09)

Longevity

The longevity of adults was significantly affected by the larval medium (Chi^2_2=17.0, P<0.001). Both females and males showed an effect of the different larval media (Chi^2_2=7.65, P<0.05; Chi^2_2=8.9, P<0.05, respectively, see also figure 3). We found that in both sexes there was no difference between the standard and the double media (female; Chi^2_1=0.28, n.s., male; Chi^2_1=0.005, n.s.), however, flies reared on the half medium were significantly shorter-lived than those reared on standard medium (female; Chi^2_1=4.9, P<0.05, male; Chi^2_1=8.7, P<0.01) or double medium (female; Chi^2_1=7.2, P<0.01, male; Chi^2_1=4.6, P<0.05).

Analysis of the age specific mortality rate with age as a covariate reveals a pattern similar to the results of the Cox proportional hazards analysis on survivorship (see figure 4).The analysis of early and late life mortality (see materials and methods) revealed the following. Female early mortality did not differ significantly among the groups ($F_{2,20}$=0.26, P=0.77), but *post hoc* Tukey testing revealed a tendency of females reared at half medium to have slightly increased mortality. Female late mortality showed a significantly higher mortality for females reared on the half medium ($F_{2,43}$=52, P<0.0001). Male early and late mortality both showed a negative effect of the half medium ($F_{2,19}$=24, P<0.0001, $F_{2,43}$=46, P<0.0001, respectively). This indicates that there is a negative effect on longevity when larvae are reared at half medium. This effect is present throughout life, but is most significant at a later age. This confirms that life span in *Drosophila* can be affected by the pre-adult environment in a similar way as was reported in some human studies (Hales and Barker 1992, 2001) and one on butterflies (Boggs and Freeman 2005). It is noteworthy, that also in the human situation it is the late life mortality that is increased.

Discussion

General

These experiments showed that our half medium represented adverse larval conditions relative to the standard conditions. Flies from the double medium behaved in a similar manner to those from the standard medium. Adult longevity was significantly decreased by adverse larval medium. Mortality analysis showed elevated mortality, especially later in life.

The adversity of half medium and its effects on adult body size

Two physiological traits (development time and female wet weight) and the number of eclosed adults (cf. Borash et al. 1998; 2000) were negatively affected by the half medium in the larval stages. Preliminary data suggest that mass specific metabolic rates do not differ among the groups. Insects require a minimum amount of resources to initiate pupation (Ashburner et al. 1989; Davidowitz et al. 2003). Increased development time can be explained by the requirement of resources to successfully complete metamorphosis. Food intake from the critical weight onwards determines the final adult size. This explains the great reduction in adult weight of females reared on half medium; there are less resources to contribute to adult size. It may also explain the decreased egg-to-adult survival we observed in flies reared at the half medium; if it becomes more difficult to gather resources, fewer animals reach critical weight and as a result, fewer adults will eclose. Therefore, we think it appropriate to speak of adverse conditions in the case of the lowest food concentrations.

An important comparison

In the study of Tu and Tatar (2003) a significant increase in life expectancy is present in the animals that underwent juvenile dietary restriction. This holds when life expectancy is considered from eclosion onwards, not when considered from day 15 of adult life onwards. In our experiment we do not find an increase in longevity in animals that had poorer larval conditions (see figures 3 and 4). The difference between the studies implies that the condition that Tu and Tatar created, yeast deprivation in the third larval instar, is not similar to our adverse larval medium. In the third larval instar, yeast deprivation most probably commences after the critical weight (0.3 mg) has been reached for *Drosophila melanogaster*. As argued above, the critical weight is an important point in insect development. When an individual has bridged this critical point in development, disturbance results in a situation where the individual is still capable of becoming a fully functional adult. In this stage, the individual displays high plasticity and can make physiological “decisions” depending on the conditions it meets. Because in the present experiment caloric restriction commenced from hatching onwards, we judge these larval conditions as more severe than those of Tu and Tatar (2003), and also likely to affect only partly the same biological processes.

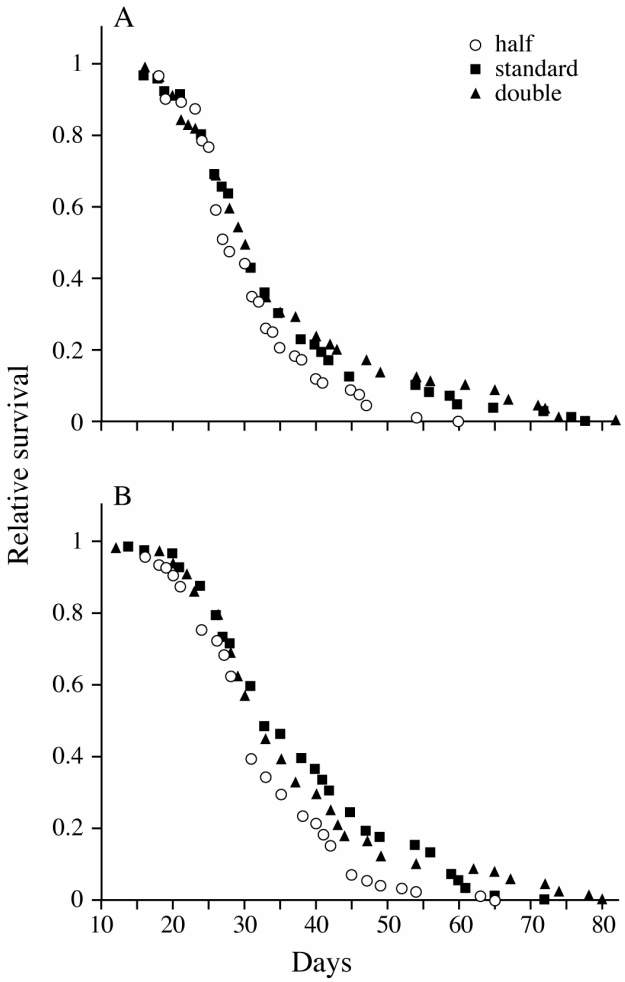

Figure 3. Survival curves of A) females and B) males on the different media. Mortality before day 10 was regarded as an effect of 'developmental hangover' and was discarded from the analyses.

Pre-adult condition dependent adult longevity

Brakefield et al. (2005) argued that environmental variation during early development determines adult features either by the mechanism of 'imprinting', which implies adaptive developmental plasticity (cf. Brakefield et al. 1998), or by the mechanism of 'scar', where homeostasis is disturbed (as proposed by Hales and Barker, 1992, 2001).

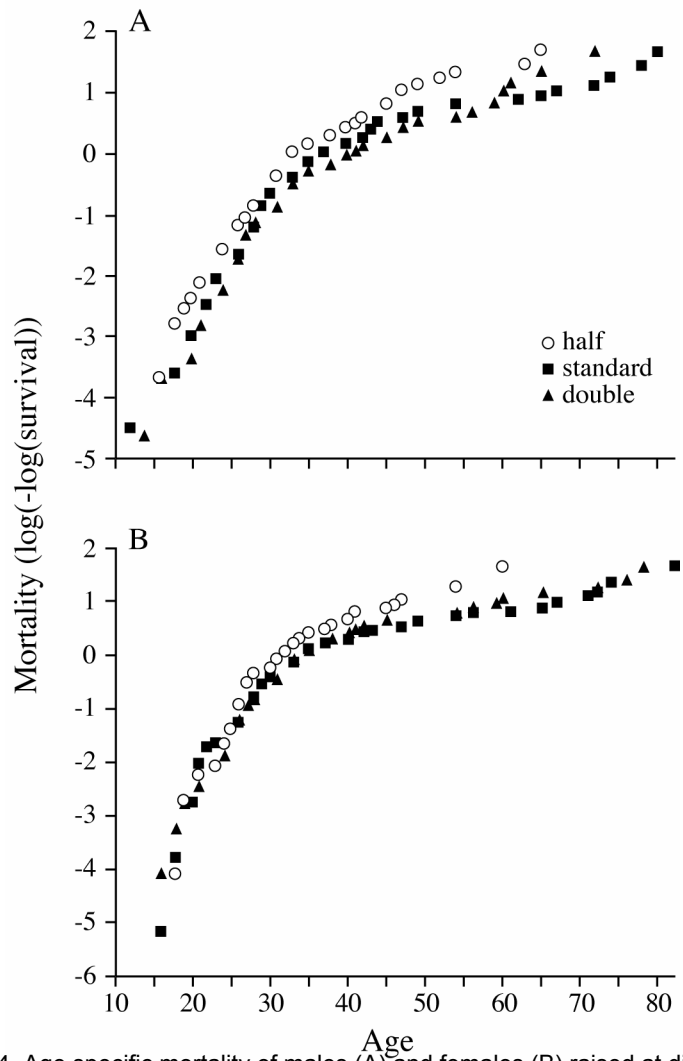

Figure 4. Age specific mortality of males (A) and females (B) raised at different larval media.

We tested the thrifty phenotype hypothesis by examining the proximate response of *Drosophila* to adverse developmental conditions. We hypothesized a response involving metabolic disease that would in turn lead to reduced longevity, the trait monitored. Mortality was elevated in animals that were reared under adverse conditions, especially late in life. This is consistent with the thrifty phenotype hypothesis, where adult metabolic disease typically appears late in life. In this experiment on *D. melanogaster*, we provide some additional credibility to the observation in humans, but the mechanism underlying this effect remains unknown. If

the reduction in longevity can be attributed to metabolic disease, then the Barker hypothesis is proven in flies.

On the basis of the literature, the thrifty phenotype is hypothesised to be a developmental scar, because it leads to a mal-adapted phenotype, relative to well-fed animals, under affluent adult conditions. Further research should focus on distinguishing between the mechanisms of ‘scar’ and ‘imprint’ by measuring lifespan, life history traits in a variety of adult conditions.

Conclusion

In summary, we tested whether the response to larval pre-adult adversity as predicted by the proximate thrifty phenotype hypothesis exists in *D. melanogaster*. Analysis of key life history traits indicates that the half medium represents an adverse larval environment. Longevity of the flies was significantly reduced when reared under adverse larval rearing conditions. This matches the observation in humans that formed the basis of the thrifty phenotype hypothesis. The next step in this line of research should be to establish whether larval adversity leads to metabolic syndrome in flies. Only then, can *D. melanogaster* become a model to investigate the mechanisms behind the thrifty phenotype hypothesis, including distinguishing between the mechanisms of ‘scar’ and ‘imprint’.

Chapter 3

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

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

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Abstract

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

Keywords

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

Introduction

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

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

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

Materials and methods

Stock population

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

Selection procedure

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

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

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

Starvation assays during selection procedure

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

Starvation assays after selection

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

Paraquat resistance

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

Longevity assays

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

Fat content and dry weight

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

Metabolic rate

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

Statistics

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

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

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

Results

Response to selection

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

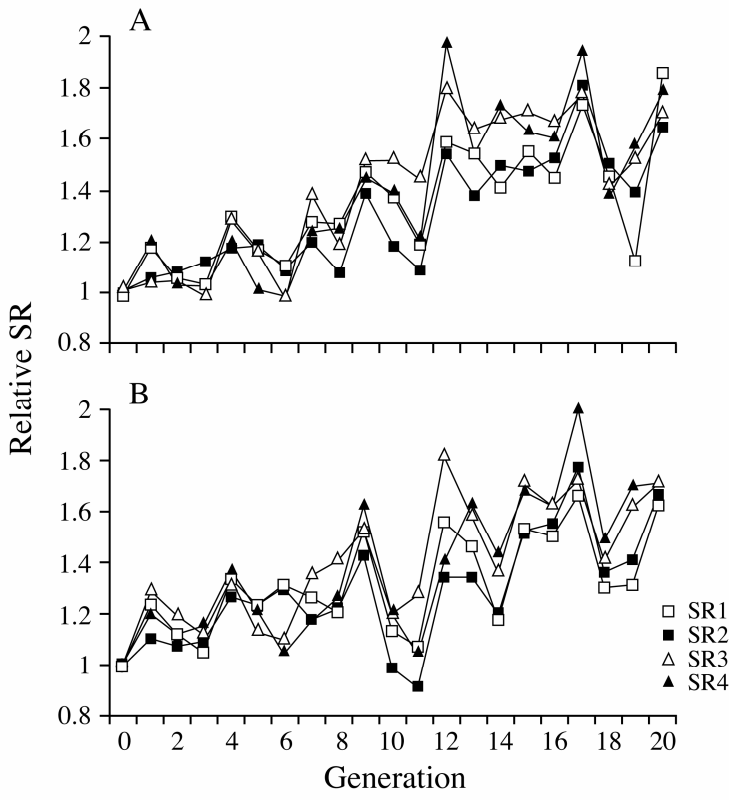

Figure 1. Changes in starvation resistance over 20 generations of selection in the 4 selected lines as scaled to the average of both control lines for males (A) and females (B) separately.

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

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

Starvation resistance after selection

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

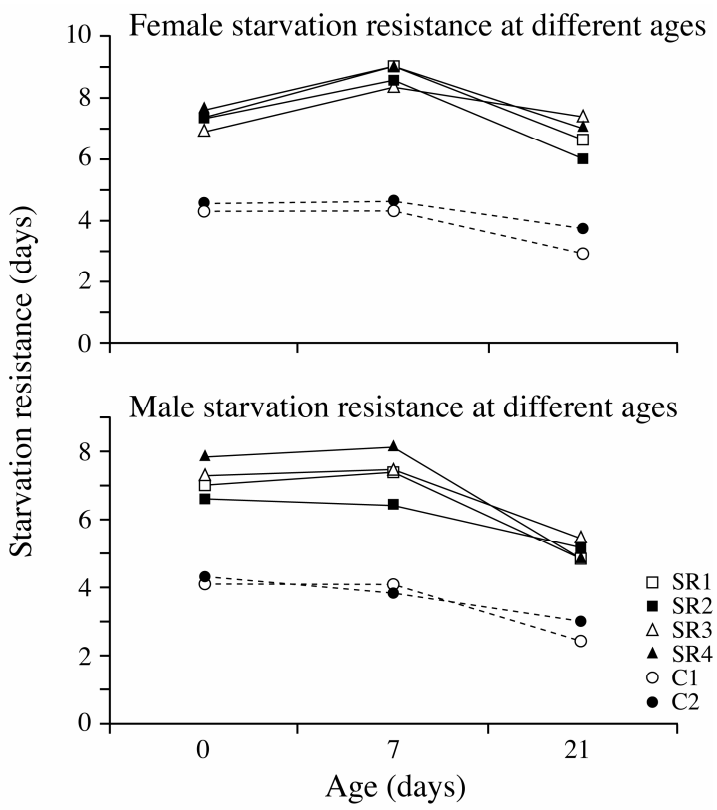

Figure 2. Average starvation resistance of males and females of each of the lines at three adult ages after 20 generations of selection. Standard error bars fell within the area of the symbols in the graph and were left out for clarity.

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

Dry weight and fat content

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

Paraquat resistance

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

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

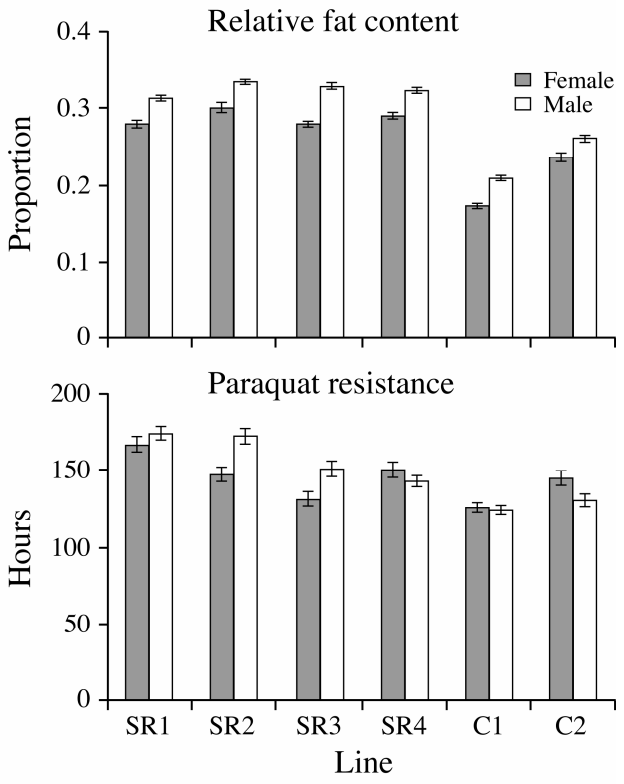

Figure 3. Relative fat content and paraquat resistance for each sex of each line (means with standard error bars).

Longevity

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

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

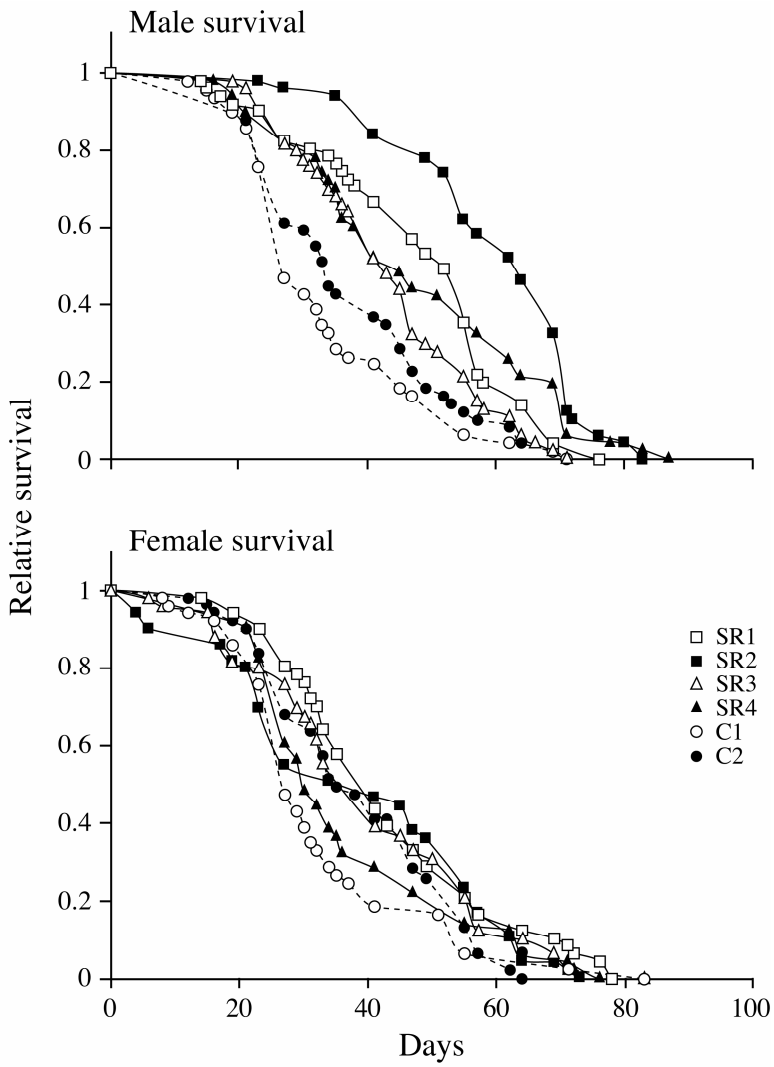

Figure 4. Mortality curves of selected and control lines.

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

Differences in metabolic rate among conditions

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

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

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

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

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

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

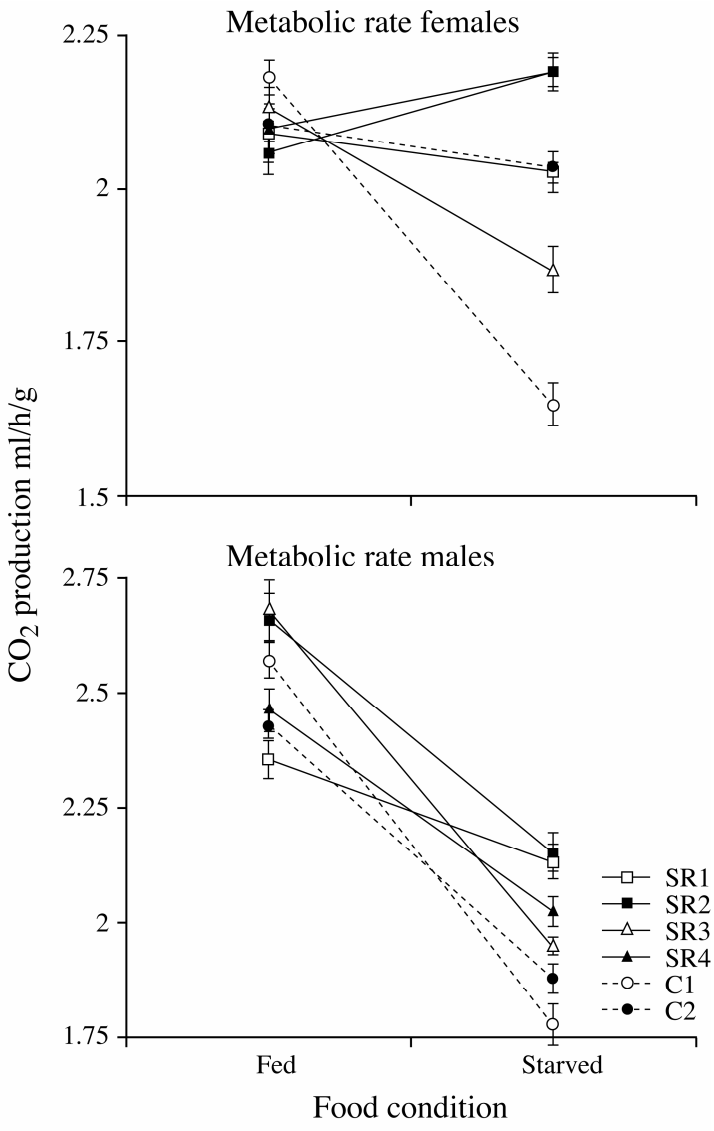

Figure 5. Average CO_2-production in ml per hour per gram of body weight of each sex of each line in both starved and fed conditions. Open symbols represent female data, dotted lines represent control lines, each line has a unique symbol-line structure.

Principal component analysis

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

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

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

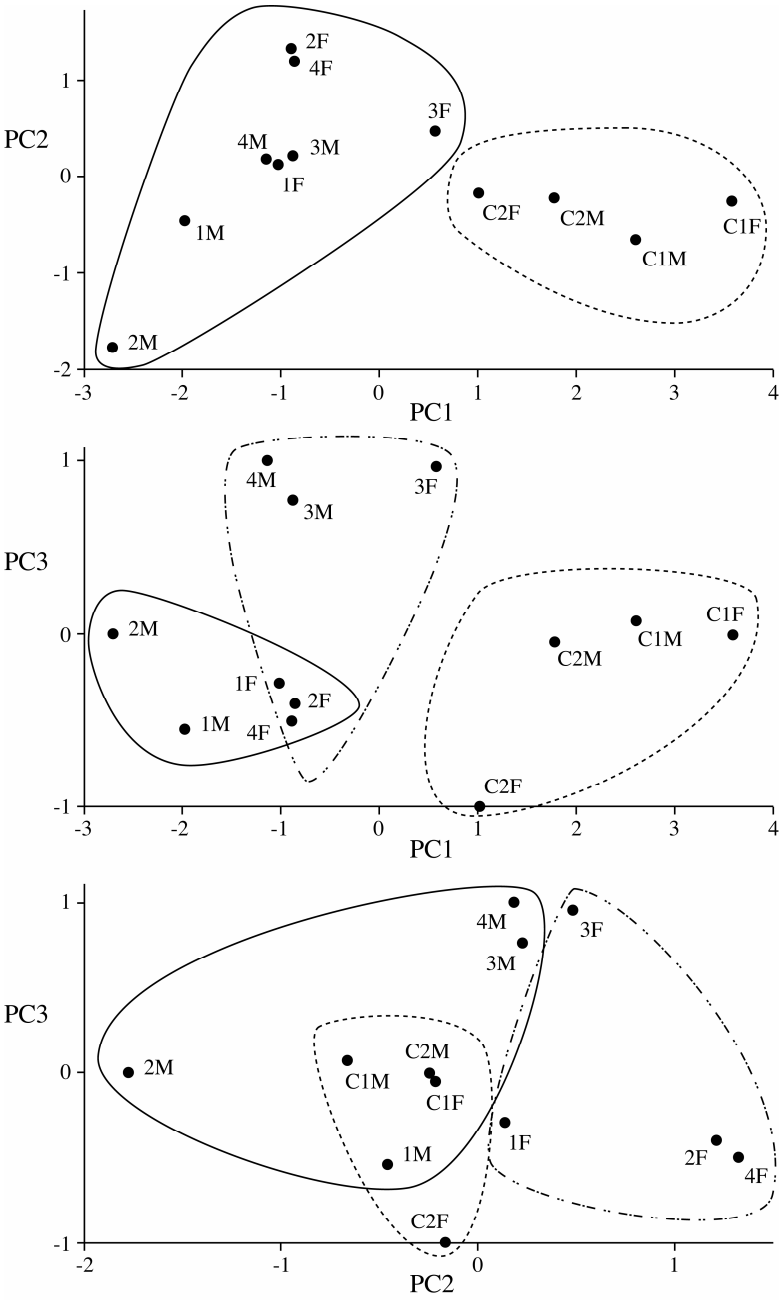

Figure 6. PCA across selection lines (1-4) and controls (C1-C2) for the variables of: starvation resistance from eclosion (SR), relative fat content (RFC), longevity (L), metabolic rate under starved conditions (MR) and paraquat resistance (PR). M = male; F = female.

Discussion

Starvation resistance

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

Fat content and body weight

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

Paraquat resistance

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

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

Longevity

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

Metabolic rate

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

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

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

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

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

Principal component analysis

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

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

Implications for evolutionary theory

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

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

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

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

Chapter 4

The interaction between food condition and life span in two sets of *D. melanogaster* lines selected for increased longevity and increased starvation resistance

The interaction between food condition and life span in two sets of *D. melanogaster* lines selected for increased longevity and increased starvation resistance

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Abstract

Lines with divergent phenotypes of interest can be acquired by experimental evolution and artificial selection. The knowledge gained from these selection lines can be substantial, especially for complex quantitative traits. Starvation resistance and longevity are such traits. They have often been found to be positively correlated in selection lines. Yet, such correlations are usually only tested in one (laboratory) environment. The universal nature of the genetic correlations that is often assumed has been questioned in earlier work. Therefore, we tested lines selected for increased starvation resistance and increased longevity over a range of environments differing in caloric food levels. The analysis showed that the lifespan profiles over the food gradient differed among lines. These interactions were consistent throughout multiple levels of analysis of the individual lines. This implies that though longevity and starvation share common mechanisms, they are also in part determined by different mechanisms.

Keywords

genotype-by-environment interactions, starvation resistance, longevity, shortened life span, food conditions, affluence, adversity

Introduction

Adaptation to a specific environment proceeds through natural selection. This process of adaptation results in a phenotype capable of surviving and reproducing in this environment. In experimental evolution designs of selection, one produces a population with the phenotype of interest through a selection environment, such as starvation. In artificial selection, a specific phenotype, such as longevity, is selected for by the experimenter and does not necessarily involve adaptation to a specific environment. Selection also produces correlated responses, either through pleiotropy and linkage, or indirect selection. Starvation resistance and longevity are often found as correlated responses (e.g. Zwaan et al. 1991; Rose et al. 1992; Zwaan et al. 1995b; Harshman et al. 1999b) . They are therefore thought to be underpinned by the same mechanism. In a number of studies, the traits were found to have become uncoupled over time (Archer et al. 2003; Phelan et al. 2003). Longevity and starvation resistance are both traits that are not only linked to lifespan, but also to specific food conditions. These traits are essential for life history and adaptation to the environment throughout distant taxa such as fungi, protostomes and deuterostomes (Longo and Fabrizio 2002; Partridge and Gems 2002; Longo and Finch 2003). Life span and starvation resistance are determined by resource acquisition and allocation, and trade off with reproduction. To elucidate part of the complexity of the relationship between longevity and starvation resistance, we examine lines artificially selected either for increased starvation resistance or for increased or decreased life span, together with their respective controls under starved, adverse and affluent conditions. There are several possible outcomes of this experiment: a. the reaction norms of the lines do not cross - both traits are determined by a shared mechanism; or b. the reaction norms of the lines cross, but the outcome is condition specific – the traits share a mechanism, but are in part independently regulated; or c. a muddled picture becomes apparent where no clear patterns can be identified – the mechanisms underlying the traits are not shared. We expect to find crossing reaction norms here and have a clear hypothesis of how these lines should interact (figure 1). In short, we hypothesise the following: Starvation resistant lines will outperform all other lines under adverse (starvation and half medium) conditions, but not under affluent (double medium) conditions. Longevity will outperform all lines under affluent conditions, but not under adverse ones. We thus think that the life span advantage of each of the selection directions (e.g. longevity) is environment specific. As has been shown in the *chico* mutant (Clancy et al. 2002), we think that the different selection lines have a relative life span optimum under different food conditions. Put in a more general context, we think that the lines selected for allocation to the soma will generally live longer than the other lines, but will interact over a food gradient because of their specific environmental adaptation.

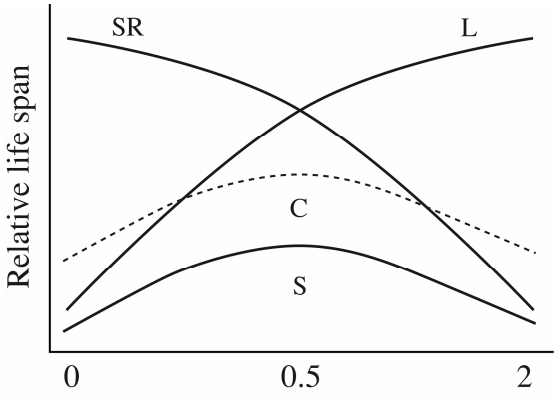

Figure 1. The hypothetical relationship between food condition and relative lifespan (cf. Chapman and Partridge 1996; Clancy et al. 2002). The horizontal axis represents the food gradient with from left to right starvation (SR), half times standard food medium (0.5) and two times standard food medium (2). On the vertical axis the relative life span among the lines is depicted. SR are the starvation resistant lines, L, the long-lived ones, S the short-lived ones and C are the control lines.

Materials and methods

Flies

We used flies from two selection experiments. Zwaan et al. (1995b) artificially selected *D. melanogaster* lines for increased and for decreased life span. After an extended period of relaxed selection the lines were re-selected in the same direction (Vermeulen 2005). This resulted in two long-lived lines (La and Lb), two short-lived lines (Sa and Sb) and two control lines (Ca and Cb). Re-selection restored most of the original life span, but failed to reveal all features found by Zwaan et al. (1995b). The most striking example of which was the decoupling of virgin and mated life span. All of these lines and their controls were disinterestedly donated by the Groningen Evolutionary Genetics laboratory for experimentation in the Leiden laboratory.

The other lines used are those where flies were selected for increased starvation resistance (SR1, SR2, SR3 and SR4) by means of experimental evolution and their controls (C1 and C2). All starvation selected lines showed a 60 to 80% increase in their starvation resistance over 20 generations of selection. After these 20 generations, the lines have been maintained under a regime of relaxed selection. Lines SR1 and SR2 showed an increase in relative fat content, longevity and paraquat resistance as correlated responses to increased starvation resistance. Lines SR3 and SR4 had only acquired an increased fat content, but showed the strongest response to selection. Metabolic rate in feeding situations was not affected in either line, whereas in starved conditions it was higher (Baldal et al. 2006). A total of 3558 flies was assayed for this experiment.

Experimental design

To prevent a bias in the comparison of the lines, we performed a 'blind' experiment. The lines were randomly coded by other members of the laboratory. The code was availed to us only after all experiments had ended. The life span selected lines from the Groningen laboratory were given three generations to adapt to the Leiden laboratory environment before the experiments began. Eggs were collected from young flies (3-5 days old) and in groups of 100 put in glass vials containing 6 ml of standard medium. Standard medium consisted of 20 g agar, 9 g kalmus (10 parts acidum tartaricum, 4 parts ammonium sulphate, 1 part magnesium sulphate and 3 parts potassium phosphate), 10 ml nipagin (100 g 4-methyl hydroxy benzoate per liter ethanol), 50 g saccharose and 35 g granulated yeast per litre water.

The resulting flies were collected within 8 hours post-eclosion to prevent the flies from mating. Adult males and females were kept separately in vials containing 5 flies throughout life, with a total of 50 flies per sex of each line for each treatment. Life span measurements were performed in glass vials containing 6 ml double medium, half medium or starvation medium. In double medium, the amounts of yeast and sugar are double that of standard medium. In half medium, the amounts of sugar and yeast are half that of standard medium. In both these media the concentrations of the other ingredients were maintained as in standard medium. Starvation medium consists of 20 gr. Agar, 9 gr. kalmus, 5 ml. propionic acid, and 5 ml. nipagin per liter water.

The vials were checked daily for living flies, dead individuals were removed immediately to prevent the living flies from feeding on corpses or body fluids and to prevent disease to spread. Immobile flies were checked for death by physical stimulation, while vials were replaced weekly. The flies were then redistributed to a density of 5 individuals per vial.

All maintenance, rearing, and experimentation took place in a 25°C cell with a 12/12hr dark/light regime and a relative humidity of 50%.

Statistics

Cox proportional hazard analysis could not be used to analyse life span because its requirement for non-crossing reaction norms was not met. The lines crossed within treatment and generally the shape of the survival curves differed. Therefore we used a full factorial ANOVA analysis. Normality was checked for each sex on each medium and did not show a significant deviation from a fitted normal distribution (Shapiro Wilkinson W test, all $P<0.0001$), while variances were equal among sexes of each medium. Because of the continuous re-distribution to 5 flies per vial, no effect of vial number could be taken into account. Redistributing the flies in this way ensures that the vial specific effects that are usually taken into account in the vial number factor are minimized and that the flies in the experiment can be regarded as a single

population. Animals that died by non-natural causes or that escaped were excluded from the analysis.

Categories of selection direction

We categorised the lines as follows; SR for the starvation resistant lines, L for the long lived lines, S for the short lived lines, CG for the Groningen control lines and CL for the Leiden control lines. When the factor line was nested in category and treated as a random factor, it was significant. The aim of this exercise was to reduce the information in the analysis and reveal a pattern that could be compared to our model. Despite the sometimes significant differences between lines within a category, we chose to treat the category of a particular line type as a single variable in category analysis.

Because the lines from Groningen and Leiden have a different genetic background for the larger part, we corrected the life span data of the selection lines by subtracting the average of the corresponding control lines for each sex and medium. In this way the distribution and variance were maintained. All tests were performed using JMP 5.0.1.

The variability of the lines was kept in mind in the interpretation of the data. We performed three analyses with increasing generalisation, so as to be able to examine whether abstracting the data changed the interpretation of the general patterns.

Results

ANOVA analysis

The overall ANOVA revealed a significant effect of medium ($F_{2,3445}$=9803, P<0.0001). On the different media, we found significant sex*line interactions (starved $F_{11,1155}$=2.7, P=0.0021; half $F_{11,1166}$=6.9, P<0.0001; double $F_{11,1124}$=6.3, P<0.0001). When analysed per sex, a significant medium*line interaction was found in both males ($F_{22,1726}$=17.8, P<0.0001) and females ($F_{22,1719}$=14.7, P<0.0001). Further analyses were performed per medium and sex, which indicated that the sex differences vary per line. Therefore, we analysed sexes separately throughout further analyses. In every further analysis the factor line was a significant factor (all P<0.0001). In appendix 1, the *post hoc* Tukey test results for the lines are listed per medium and sex. For the double medium no consistent pattern for the selection direction could be observed. However, SR2 and La have the longest life span in both sexes. The longevity of SR2 on double medium is in sharp contrast with its low ranking on half medium. There we see that the control lines ranking is scattered only in females. This can be explained by the low number of Tukey hierarchies (3) in half males. Under starvation, we observe that the lines not selected for increased starvation resistance are scattered throughout the ranking. In figure 2, we showed the average life span of the categories of different selection and control lines.

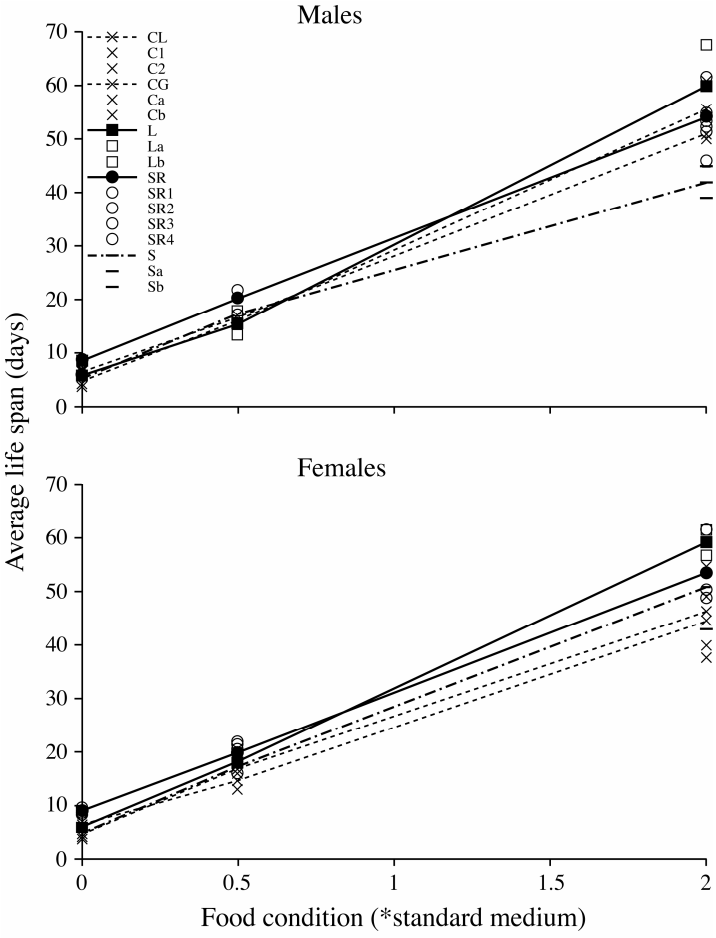

Figure 2. Average life span of individual lines for each medium and sex. Also, the grand averages are given for each selection direction. These have been connected by lines. Straight lines are starvation resistant and long-lived lines and show interactions in between the affluent and adverse media. The short lived lines average has a dash-strip-dash pattern, and control lines averages have been connected by a dotted line.

ANOVA analysis after correction for control lines

As before, for each medium significant sex*line interactions (starved $F_{7,765}$=5.0, P<0.0001; half $F_{7,781}$=12.7, P<0.0001; double $F_{7,753}$=3.8, P=0.0004) were found. Consequently, the sexes were analysed separately again. Analysis per sex revealed a significant medium*line interaction in males ($F_{14,1146}$=24.1, P<0.0001) and females

($F_{14,1153}$=13.6, P<0.0001) again. In all analyses per medium and sex the factor line was highly significant (P<0.0001). In appendix 2 we have listed the outcome of the Tukey analysis. The correction for the control line data did not result in a different hierarchical pattern in Tukey testing of the double medium data. We could see though, that in both males and females the long lived lines are among the longest lived, the short lived lines are among the shortest lived and the starvation resistant lines' life span lies between them. On half medium the starvation resistant lines generally rank highest. They are followed by the long lived lines, except for line Lb in males and eventually the short lived lines. On the starved medium, we again observe a clear pattern where the starvation resistant lines rank highest, followed by the long lived ones and then the short lived lines.

Analysis per selection direction category; two types of starvation resistance

In our earlier work (Baldal et al. 2006) we found that the starvation resistant lines could be split into two groups. We therefore designed special categories for the long lived starvation resistant lines (SR1 and SR2) and non-long lived starvation resistant lines (SR3 and SR4). In Tukey analysis (data not shown) both groups did not differ from analysis as a single group, with the exception that the SR1 and SR2 group lived significantly longer at the double medium than the SR3 and SR4 category. This is consistent with the findings of Baldal et al. (2006). However, because both groups were still neighbouring in the Tukey analysis and we wanted to examine patterns between starvation resistant and long- and short-lived lines we have excluded this factor from further analyses.

Category analysis

Because of their complexity, the data were also analysed per selection direction category, in order to obtain insight into the general patterns among the line types. Overall full factorial ANOVA analysis revealed highly significant for each factor (medium, category and sex) and their interactions. Analyses per sex revealed significant medium*line interactions for males ($F_{4,1161}$=43.1, P<0.0001) and females ($F_{4,1168}$=15.3, P<0.0001) again. Analysis per medium revealed the same for the factors sex and line and their interaction. Therefore, the analyses of category ranking were performed for each medium and sex separately. In appendix 3, the ANOVA and Tukey results are listed per medium and sex. Categories were; L for the long lived lines, S for the short lived lines, and SR for the starvation resistant lines. The double medium revealed that the long lived lines live longest, followed by the starvation resistant lines and that the short lived lines live shortest. This pattern is visible in both sexes, though in females the SR and S lines cannot be distinguished statistically. In the female data at half medium no pattern can be identified because the groups do not differ significantly. In males the long lived lines suddenly become shorter lived than the short lived lines. Life span under starvation reveals the expected ranking appears with the starvation resist lines longest lived under adversity, followed by the long lived lines and then the short lived lines.

Discussion

Here, work is presented on a set of 12 *D. melanogaster* lines, analysed for life span of both sexes under three different adult conditions. These data were analysed in three different ways: on the line level, corrected for genetic background and per selection direction. All analyses revealed similar patterns. All showed significant genotype-by-environment interactions, where lines selected for extended life span under a certain condition did not show this extension in non-selected environments. Lines selected for increased starvation resistance outperformed all other line types under adverse conditions, whereas life span of long-lived lines was highest under affluent conditions.

Control lines and the short lived lines

In general, the control lines turned out to do exactly as expected (cf. figure 1). The Groningen controls showed that they lived longer than long lived lines under adverse conditions, but not under affluent conditions. Relative to the control line, the long lived line is thus affluence-skewed in its increase in life span. The same holds for the starvation resistant lines that overlapped with the control lines on the double medium (appendix 1). There, the starvation resistant lines are adversity-skewed in their life span advantage relative to their control lines. Short lived lines turned out to be generally short-lived. If the short life span mechanism had been similar to the life span increasing mechanism then it would have been expected that under adversity they would not differ from one another and the controls. Because they differ in life span under adversity, we propose that those lines have been selected on different mechanisms than the long-lived lines.

Longevity and starvation resistance

We collected the data to test our hypothesis that the starvation resistant and long lived lines would show a genotype-by-environment interaction when examined under adverse and affluent conditions (see figure 1). There is considerable genotype-by-environment interaction for lifespan of long-lived and starvation resistant lines under affluence and adversity. This indicates that though longevity and starvation resistance are often found as correlated traits, the strength and direction of the correlation is dependent on the environment they are tested in. Starvation resistance is different from longevity because its relative peak life span is under adversity, whereas that of longevity is under affluence. This resulted in a consistent genotype-by-environment interaction in each type of analysis performed here.

Half medium, a transition between affluence and adversity

The differences among lines for life span are not clear, if present at all, at the half medium. Half medium is not as adverse as starvation, but also not as affluent as the double medium. This condition represents the transition between affluence and

adversity and neither of the lines used here is adapted to that. Here, the model that was proposed (figure 1) needs to be adjusted. The starvation resistant lines appear to be slightly longer-lived than the other lines at the half medium.

Integration

The genotype-by-environment interactions between medium and selection direction were repeatedly found in different analyses. This was irrespective of whether the life span data had been corrected for their genetic background by subtracting the average life span of corresponding control lines from the data of selected lines. In the environments where the long-lived and starvation resistant lines have been selected they outperform their control lines. The long-lived and starvation resistant lines interact and show an environment specific life span advantage. This is visible even in the intermediate condition of the half medium, where few differences could be identified. These data therefore strongly suggest that starvation resistance and longevity are not exactly two sides of the same coin (cf. Baldal et al. 2005; Baldal et al. 2006).

On the basis of the literature and of these results, we hypothesise that the phenotypes represent two mechanisms that result from changed energy/food related metabolism, that may involve insulin signalling (e.g. Partridge and Gems, 2002). As a result of selection, in both longevity and starvation resistance selection the animals will have allocation shifted to the soma instead of to the germ-line (yet, cf. Vermeulen and Bijlsma 2006), as is hinted in preliminary micro-array studies on one starvation resistant line. The starvation resistant lines will have been selected as a thrifty genotype (cf. Neel, 1962) with high storage (Baldal et al. 2006), whereas the long-lived lines will have been selected for high somatic maintenance (cf. high paraquat resistance in Vermeulen et al. 2006), which is wasteful under starvation conditions, reducing survival probability. The model we proposed in figure 1 has been proven to be largely correct. Only the low distinction that could be made at the half medium did not follow our expectations.

Appendix 1. The mean and standard error (S.E.) values of life span in days for both sexes of each line for each medium (double, half or starved). Tukey test results are given per medium and sex. Lines not represented in the same column are significantly different.

Females on double medium

	Mean	S.E.	A	B	C	D	E	F
SR2	61.7	1.68	A					
La	61.4	1.75	A					
Sa	58.7	2.1	A	B				
Lb	56.8	2.13	A	B	C			
C1	54.7	1.69	A	B	C			
SR4	53.6	2.13	A	B	C			
SR1	50.3	2.61		B	C	D		
Ca	49.4	2.09		B	C	D	E	
SR3	48.9	2.8			C	D	E	
Sb	43.1	1.51				D	E	F
Cb	40	1.99					E	F
C2	37.8	2.01						F

Males on double medium

	Mean	S.E.	A	B	C	D	E	F	G
La	67.4	2.13	A						
SR2	61.4	1.42	A	B					
C1	60.9	2.11	A	B	C				
SR4	55	2.42		B	C	D			
Ca	53.7	1.57		B	C	D	E		
SR1	53.4	1.84		B	C	D	E	F	
Lb	52.6	2.39			C	D	E	F	
C2	50.3	1.88				D	E	F	
Cb	48.6	1.15				D	E	F	
SR3	46.1	2.38					E	F	G
Sa	44.9	1.31						F	G
Sb	39	1.1							G

Females on half medium

	Mean	S.E.	A	B	C	D	E	F
SR3	21.9	0.54	A					
SR4	21.4	0.85	A	B				
SR1	20.5	0.57	A	B	C			
La	18.8	0.5		B	C	D		
Lb	18	0.48			C	D	E	
C2	17.7	0.36				D	E	
Sa	17.2	0.54				D	E	
Sb	17	0.34				D	E	
C1	16.1	0.46					E	
Cb	16	0.51					E	
SR2	16	0.81					E	
Ca	13.1	0.6						F

Males on half medium

	Mean	S.E.	A	B	C
SR4	21.7	0.52	A		
SR3	21.6	0.67	A		
SR1	20.3	0.53	A		
La	17.7	0.48		B	
Sa	17.2	0.58		B	
Sb	17.1	0.48		B	
SR2	17	0.31		B	
Ca	17	0.58		B	
C1	16.6	0.31		B	
Cb	16.3	0.52		B	
C2	15.6	0.52		B	
Lb	13.3	0.63			C

Starved females

	Mean	S.E.	A	B	C	D	E	F	G
SR1	9.36	0.16	A						
SR3	9.22	0.17	A	B					
SR4	8.58	0.19		B	C				
SR2	8.34	0.27			C				
Cb	6.76	0.14				D			
La	6.22	0.15				D	E		
Ca	6.02	0.14					E		
Lb	5.84	0.12					E		
Sa	4.94	0.12						F	
C2	4.7	0.11						F	
Sb	4.62	0.12						F	G
C1	3.92	0.11							G

Starved males

	Mean	S.E.	A	B	C	D	E	F
SR3	8.88	0.19	A					
SR1	8.5	0.15	A	B				
SR2	8.38	0.22	A	B				
SR4	8	0.16		B				
Cb	6.62	0.12			C			
La	6.14	0.12			C	D		
Ca	5.86	0.12				D		
Lb	5.72	0.1				D		
Sb	5.04	0.08					E	
Sa	5	0.13					E	
C2	4.74	0.12					E	
C1	4.06	0.12						F

Appendix 2. Tukey results for lines, for each sex and medium, corrected for controls. Lines not represented in the same column are significantly different.

Females on double medium

	A	B	C	D
La	A			
SR2	A	B		
Sa	A	B		
Lb	A	B	C	
SR4		B	C	D
SR1			C	D
SR3				D
Sb				D

Males on double medium

	A	B	C	D	E
La	A				
SR2		B			
Lb		B	C		
SR4		B	C		
SR1		B	C	D	
Sa			C	D	E
SR3				D	E
Sb					E

Females on half medium

	A	B	C
SR3	A		
SR4	A	B	
La	A	B	
SR1	A	B	
Lb	A	B	
Sa	A	B	
Sb		B	
SR2			C

Males on half medium

	A	B	C
SR4	A		
SR3	A		
SR1	A		
La		B	
SR2		B	
Sa		B	
Sb		B	
Lb			C

Starved females

	A	B	C	D	E
SR1	A				
SR3	A	B			
SR4		B	C		
SR2			C		
La				D	
Lb				D	
Sa					E
Sb					E

Starved males

	A	B	C	D
SR3	A			
SR1	A	B		
SR2	A	B		
SR4		B		
La			C	
Lb			C	
Sb				D
Sa				D

Appendix 3. Tukey results of the analysis of data per selection direction (L for long-lived; SR for starvation resistant; S for short-lived) corrected for the corresponding controls (subtraction of control average from selection line data), F and P values are also listed.

Females on double medium

$F_{2,383}$=8.4; P=0.0003

	A	B
L	A	
SR		B
S		B

Males on double medium

$F_{2,380}$=36.7; P<0.0001

	A	B	C
L	A		
SR		B	
S			C

Females on half medium

$F_{2,392}$=2.29; P=0.1029

	A
L	A
SR	A
S	A

Males on half medium

$F_{2,399}$=61; P<0.0001

	A	B	C
SR	A		
S		B	
L			C

Starved

$F_{2,393}$=1043; P<0.0001

	A	B	C
SR	A		
L		B	
S			C

Starved males

$F_{2,382}$=1046; P<0.0001

	A	B	C
SR	A		
L		B	
S			C

Chapter 5

Gene expression patterns of starvation resistant *D. melanogaster* under fed and starved conditions.

Gene expression patterns of starvation resistant *D. melanogaster* under fed and starved conditions.

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Abstract

Earlier work on *Drosophila* lines selected for increased starvation resistance gave rise to the question of which genes underlie the response to selection. Therefore, in this pilot study whole genome gene expression is surveyed using micro arrays in new lines, one selected for increased starvation resistance and one control. These lines were predicted to show the largest differences in gene expression relative to a control line under selected, i.e. starved, conditions. They were assayed for their gene expression at three days from adult eclosion on normal rearing medium, and on starvation medium. Expression patterns were analysed by PCA and full factorial ANOVA. This revealed gene-by-environment interactions in the expression of several genes. We elaborate on the importance of the gene-by-environment interactions and examine genes that are known to be involved in specific life history related processes. To our knowledge, this is the first study that implies the involvement of candidate mechanisms in underpinning standing genetic variation for starvation resistance and longevity in natural populations. The outcome is discussed in the light of the literature.

Keywords

micro-arrays, *D. melanogaster*, starvation resistance, longevity, food condition, insulin, disposable soma

Introduction

This is a preliminary study and involves the first steps into micro-array analysis for this research group. The focus of this project has been to determine the technical issues and validity of this approach, as well as a test of how to interpret the biological data.

As the world has entered the genomic era so have the evolutionary and ecological sciences. That the linking of genomic data to evolutionary and especially ecological parameters with a clear phenotype will be informative is not under debate. However, there is some scepticism about the universal applicability of gene expression data (Feder and Walser 2005). This kind of scepticism is needed to remain objective because it is all too easy to be influenced by the potential promise of a technique. On the other hand, critically considering a technique can also be overdone. Thus far, genomics has not induced a revolution in the ecological field (but see van Straalen and Roelofs, 2006). Micro-arrays can be used to identify candidate genes that need to be more thoroughly investigated requiring a high degree of specialisation. To identify candidates, one needs to have a clear phenotype to assay. Also, the phenotype must have been assayed in an environment that is relevant to the trait, the one in which the phenotype usually occurs in and has been under selection in. Only in such circumstances the investigator can be sure that at least part of the altered gene expression is relevant to the traits under study. Here, all these requirements are met.

Instead of replacing “old-fashioned” scientific labour, the application of large scale, high throughput techniques has only increased its importance. The large amount of data and the issue of their significance offer new challenges for scientists in general.

Longevity and starvation resistance are correlated, but in part distinct, complex traits that are multifactorial in their determination. Next to complex environmental dependence, these traits also seem to be influenced by a large number of genes (Geiger-Thornsberry and Mackay 2004; Harbison et al. 2004; Harbison et al. 2005). Moreover, the environment and the genotypes interact (Leips and Mackay 2000; Vieira et al. 2000), resulting in different phenotypes, depending on environment and genotype. Large scale gene expression studies, such as micro-arrays, may help to strengthen and expand hypotheses about the complex of mechanisms underlying these traits.

Here, we present work on a limited set of micro-arrays. Of interest in this study are the genetic, environmental and gene-by-environment effects that affect gene expression and life span of *D. melanogaster*. Whole genome expression patterns of flies from a control and a starvation resistant line with distinct and known phenotypes were examined. The flies had been subject as adults to either normal rearing conditions, i.e. fed, or starved conditions.

We expected that under fed conditions the gene expression patterns would differ little between the control and starvation resistant lines, whereas differences would be much more apparent under starved conditions. Under fed conditions, gene expression patterns relevant to longevity may become apparent. Though the starvation resistant line (SR2) is significantly longer lived than its control line (C1), we

expect few genes to be reminiscent of this difference at an adult age of 3 days. In the case of starvation, three days is the point where the large difference between SR2 and C1 in starvation resistance is apparent. Also, it was thought that the impact of the environmental difference between starved and fed conditions would affect a large part of the genome as a response. A difference in response to the environmental change between the lines is called a genotype-by-environment interaction. Because of the selection regime we expect to find a considerable number of genes that display genotype-by-environment interactions. Further, we expect to find genes involved in insulin signalling, lipid, sugar and protein catabolism, stress perception and neural signalling to be differentially expressed and we will thus examine these classes of genes.

In this way, it was thought that genes of interest for further, detailed research could be identified. This was done using bioinformatic tools currently available and by personal observation and interpretation. Because this is a pilot study, the data were approached with a critical view for bioinformatic or personal interpretation biases. The genes of interest that were found are considered in the light of existing literature.

Materials and methods

Flies

Previously, we selected 4 lines of *D. melanogaster* for increased starvation resistance and maintained 2 control lines (Baldal et al. 2006). The starvation resistant line examined here, SR2, is highly starvation resistant and long-lived. The control line, C1, is not starvation resistant and does not display as long a life span as line SR2 under fed conditions.

For the experiments, eggs were reared at a density of 100 eggs per vial. From both lines, female virgins were collected within 8 hours post-eclosion and subject to fed conditions on standard medium or starved conditions on agar medium. Standard medium consists of 20 gr. agar, 9 gr. kalmus [kalmus consists of 10 parts (weight) acidum tartaricum, 4 parts ammonium sulphate, 1 part magnesium sulphate and 3 parts potassium phosphate], 10 ml. nipagin [100 grams of 4-methyl hydroxy benzoate per liter ethanol], 50 gr. saccharose and 35 gr. of granulated yeast per liter water. Agar medium consists of 20 gr. agar, 9 gr. kalmus, and 5 cc. nipagin per liter water. Adult flies were maintained at a density of 5 flies per vial. After 3 days the flies were collected and flash-frozen in liquid nitrogen, before storage at -80ºC.

RNA extraction and micro-array handling

Total RNA was extracted using Macherey Nagel® Nucleospin II columns. Samples were checked for quantity and degradation using a Nanodrop® ND-1000 Spectrophotometer. Subsequently, RNA quality was analysed using Lab-on-a-Chip. Only samples were used that did not show signs of degradation (comparison of 18S and 28S rRNA peaks) and contained at least 2µg of total RNA. The samples were

then amplified and biotin-labelled using the Ambion kit and Ambion standard protocols by ServiceXS (www.servicexs.com). Samples of at least 12.5 µg were analysed using Affymetrix *Drosophila* 2.0 chips. The *Drosophila* 2.0 chip contains 18952 probe sets, analysing 18500 different transcripts. Hybridisation and readout were performed using standard protocols by the LGTC (www.lgtc.nl). In total 14 arrays were run, 3 for each line under feeding conditions and 4 for each line under starved conditions.

Bioinformatics and statistics

Quality control, normalization and ANOVA
The data in the .cel files were converted to excel sheets using the DCHIP programme (www.dchip.org). Tab-delimited files were imported into Genespring 7.2, which can link gene information to the Unigene database (http://www.ncbi.nlm.nih.gov). Measurements with an intensity of lower than 0.01 were changed to 0.01, and effectively removed from the analysis. Of the total 18952 probe sets we analysed, 14930 were labelled Marginal or Present by GeneSpring. These samples were normalized (MAS 5.0) per chip by the 50th percentile (median). They were then normalized (MAS 5.0) per gene to the median of all 14 arrays. Sample 2F1 (line 2, fed, first sample) showed signs of RNA degradation, when examined in the R-based Bioconductor (www.bioconductor.org). However, the chip was retained in the analysis to maintain a balanced design.

Samples were analysed in Genespring 7.2 by using Principal component Analysis (PCA) and full factorial ANOVA. A strict Benjamini and Hochberg False Discovery Rate (FDR) correction of 0.01 was also used to reduce the number of genes that were found but are actually not differentially expressed. Finally, a further 5247 probe sets were excluded due to lack of data for ANOVA analysis. The remaining 9683 probe sets were used for the PCA and ANOVA analysis. Differing FDRs were used in lower level analyses for arbitrary reasons and are listed in the results section. Thus, the number of genes found to be differentially expressed in the overall analysis does not match the number found in the lower level analyses.

FDR
The number of genes found to be differentially expressed is determined by the FDR. In our overall ANOVA analysis we use one FDR (0.01) and, therefore, the gene numbers between the different factors (genetic, environmental and genotype-by-environment interactions) can be compared. In our lower level analyses that focus on genotypic or environmental effects rather than an assay of the whole model, we always tried several FDRs and examined whether the trends followed expectations. Using a very low FDR will yield very few differentially expressed genes and is thus likely to miss many such genes (type I error). Conversely, a high value will display many that are actually not differentially expressed (i.e. falsely discovered, type II error). Because the FDR does not display a linear behaviour as a function of the proportion of total genes identified, a slight rise in FDR may yield a large number of extra genes. During this analysis, the point where an increase in FDR does not involve an equally large increase in the number of genes identified was used as a guide. This resulted in the difference in FDR among the lower level analyses.

Interpreting the data
Differentially expressed genes were grouped according to their Gene Ontology (GO) Biological Function level 6 category by the programme FatiGO (http://www.FatiGO.org). In Biological Function level 6 genes, are categorised in very specific processes. Approximately 10% of all *Drosophila* genes have GO annotation. Though there can be a bias in these gene lists towards processes of former scientific interest, the GO annotated samples were used to keep an overview of the processes. Only GO categories with over 10% of all the differentially expressed genes found or with a minimum of 3 genes of the annotated genes represented were used in the analysis.

From the literature, relevant categories and pathways were deduced. These include: insulin signalling, lipid, sugar and protein catabolism, stress perception and neural signalling.

Public availability

The full dataset will be made publicly available after future planned research in combination with additional data.

Results

Principal component analysis

The first principal component (PC1) reveals a large effect of nutritional status (25.7%, figure 1). PC2 indicates a genotype effect (22.3%, figure 2) with a clear separation between SR2 and C1. Combined, PC1 and PC2 show a genotype-by-environment effect (figure 2). There is a cluster of the lines SR2 and C1 on standard medium and a separate cluster for each line under starvation. Though the environmental component has a large effect, the clear distinction between the genotypes in one of the two conditions prompted a focus on the effects of genetics and genotype-by-environment interactions rather than on the environmental component. PC3 (13.5%) picked out the handling of the arrays in two batches (data not shown). The fourth PC (7.7%) explained the variation due to the sample 2F1 (data not shown). Both of these latter components indicated some technical variations in the data obtained here, and this was taken into account during the analysis.

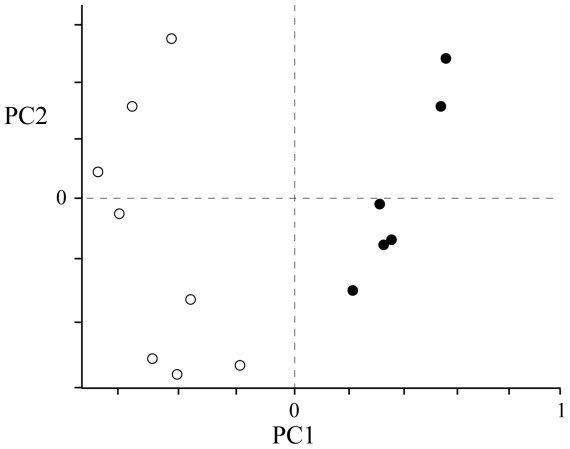

Figure 1. The first principal component (PC1, horizontal axis) on whole genome expression data, contrasting fed (filled symbols) and starved (open symbols) samples of two *D. melanogaster* lines, one control and one selected for increased starvation resistance.

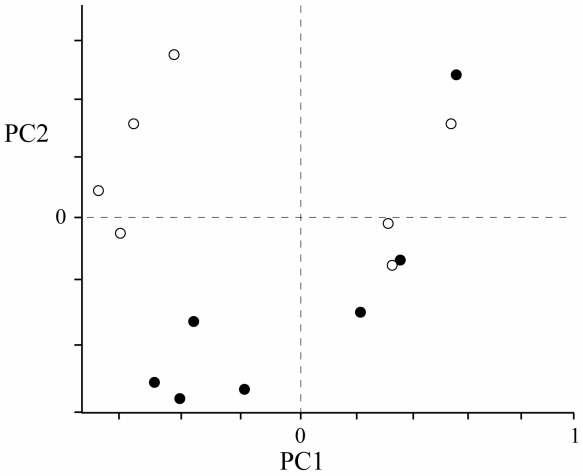

Figure 2. The second principal component (PC2, vertical axis) plotted against PC1 (horizontal axis) of whole genome expression data displaying the genotype-by-environment effect. On the right, in the fed samples as identified in the first PC (see figure 1), we observe that the starvation resistant line (SR2) and its control (C1) samples cluster together. On the left, in the starved samples, we see that the SR2 samples (open symbols) cluster separately from the C1 samples (filled symbols).

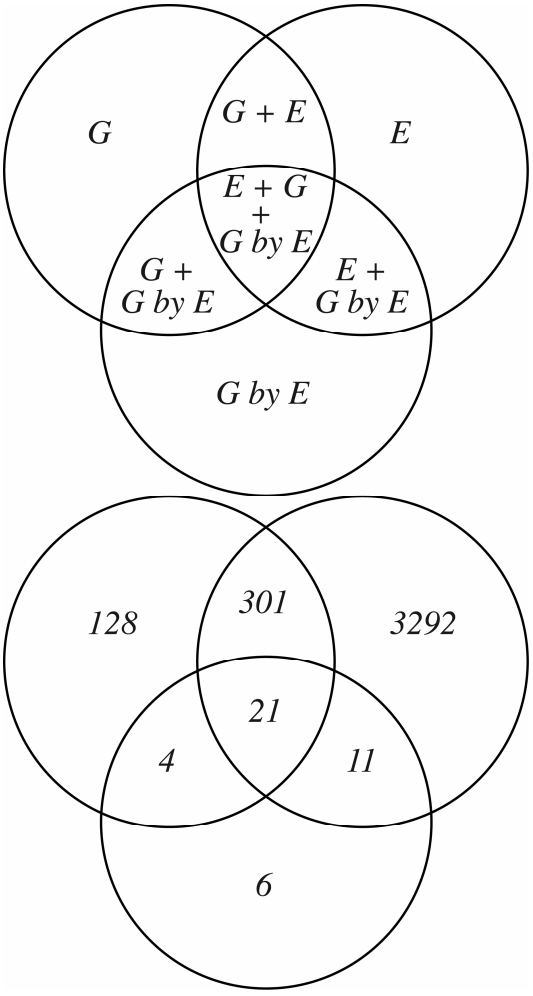

Figure 3. Outcome of the overall ANOVA. The upper Venn-diagram shows which effects the genes in a particular part of the circle are involved in. They can be involved in genetic (G), environmental (E) and gene-by-environment interactions (GbyE). Also there is overlap, where genes show, for example, genetic and environmental effects, but no gene-by-environment interaction. The lower Venn-diagram corresponds to the upper diagram. The numbers of genes that are involved in G, E effects and/or display GbyE interactions are listed in that part of the circle that represents that/those effects.

ANOVA

Figure 3 shows the numbers of genes that were detected to be differentially expressed in relation to the genotype and the environment. The full factorial ANOVA over all data showed that most genes were affected by the change in the

environment (3625 differentially expressed probe sets). This was followed by the difference between line SR2 and line C1 (454 differentially expressed probe sets). The genotype-by-environment interactions formed a minority (42 differentially expressed probe sets). A considerable number of probe sets were affected by more than one of the three factors from the full factorial design. These are shown in the overlapping regions between the circles. The low number of probe sets (138 of a total of 3763) unaffected in a consistent manner by the change in environment, but that are apparently only influenced by a genetic or genotype-by-environment cause, was striking. Of course in a genotype-by-environment interaction the environment plays an important role, but it is the genotype that responds to the environment that determines such a pattern. In this case no environment specific response could be identified from the gene expression patterns, hence the word consistent. The low number of genotype-by-environment (6 probe sets) was validated by its non-interacting counterpart, where G and E overlap (301 probe sets). In this last category, genes showed a difference in expression between the genotypes and between the environments. However, the response to the change in environment was similar in the lines.

Bioinformatics

Gene ontology analysis per food condition
The overall analysis of the genotypes (G effect) does not distinguish between the different environments, which are of considerable interest. Therefore, the G effects were subject to lower level analyses per feeding condition. Appendix 1 lists the affected biological processes from GO analysis per feeding condition. The difference between the lines when fed (G effect, FDR 0.5) comprised 62 probe sets of which 12 had a gene ontology classification. When starved, the effect of the genotype increased with 911 differentially expressed gene products (FDR 0.01), 79 of which had a GO category for biological processes at level 6.

Under fed conditions we found differential expression of GO categories involved in transcriptional regulation, nucleic acid related metabolism and neuronal signalling. Under starved conditions, GO categories were found to be involved mainly in transcription, development and catabolism.

Analysis of candidate genes

Full factorial ANOVA analysis

Genotype-by-environment interactions

In the list of genes that displayed genotype-by-environment interactions and had known functions, several functional categories were identified (Appendix 2). One set of four genes was down regulated in the control line in response to starvation, whereas there was no significant expression change in the starvation resistant line. One of these genes is the Juvenile hormone epoxide hydrolase 3 which is involved in

the catabolism of juvenile hormone. Juvenile hormone metabolism has been associated with longevity (Tatar et al. 2001).

A set of 10 genes showed down regulation in the control line and up regulation in SR2. The most striking example of this category is Turandot A, a gene involved in stress response. Gene-by-environment interactions also occur when the response of one of the lines is more extreme than that of the other line. In this category we have identified 10 genes, of which only 3 had a more extreme response in the starvation resistant line than the control line. Two of these latter genes are associated with sugar catabolism. Only one gene showed up regulation in the control line and no response in SR2, namely CG5933, an RNA methylation gene. We observe that of the genes that display genotype-by-environment interactions, 10 out of 25 have a response in the same direction only more or less extreme. Of the remaining 15 genes, 5 are not differentially expressed between environments in line SR2, which may indicate resource depletion or severe stress in line C1. The other 10 genes show a gene-by-environment effect with opposite reaction norms.

An analysis of the types of genotype-by-environment interactions that the genes displayed was performed (figure 4). In the 42 genes, 7 types of interactions were found, of a total of 10 possible (C1 up-SR2 up, C1 stronger response; C1 up-SR2 up. SR2 stronger response; C1 down- SR2 down, C1 stronger response; C1 down- SR2 down, SR2 stronger response; C1 up- SR2 down; C1 down- SR2 up; C1 stable- SR2 down; C1 stable- SR2 up; C1 up- SR2 stable; C1 down- SR2 stable). Of these, both options where C1 is stable and C1 down-SR2 down are not present. X^2 analysis showed a value of 17.4. The critical value for a two tailed test with 9 degrees of freedom was 19 at the 5% level. Thus there is no significant deviation from the expected distribution and no evidence for any forbidden categories.

Analyses per feeding condition

Fed flies

Closer examination of the genes that were differentially expressed on the basis of the GO molecular function revealed that sugar catabolising genes and chorion protein, a reproduction-related gene, were expressed at a lower level in the SR2 line. In contrast, alcohol dehydrogenase, constitutive proteins and translation initiation factors were expressed at a higher level. This suggests that SR2 flies, which have been found to have a metabolism similar to that of C1 (Baldal et al. 2006), have a different allocation pattern which involves lower expression of sugar metabolism genes and genes involved in reproduction. Instead, SR2 females could be increasing protein synthesis and detoxifying cells to help survival. The breakdown of alcohol is in line with the theory of McElwee et al. (2004) and Gems and McElwee (2005) that detoxification is a major factor involved in longevity determination. The indication of decreasing sugar metabolism matches the earlier finding that glycogen reserves increase considerably over lifetime in lines selected for increased starvation resistance, but are not elevated from eclosion (see Baldal et al. 2006, Appendix 3). High carbohydrate levels have, via desiccation resistance, often been associated with both longevity and starvation resistance (Graves et al. 1992; Force et al. 1995; Djawdan et al. 1996; Harshman et al. 1999b). Also, the decreased breakdown of

sugars may imply high hemolymph sugar concentrations, which matches the data on high glucose levels in flies that were long-lived because their insulin producing cells had been ablated (Broughton et al. 2005).

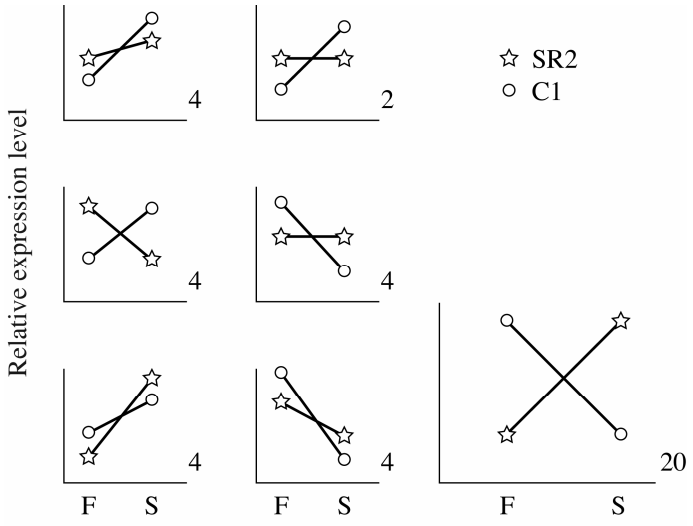

Figure 4. Gene-by-environment interactions. Graphs representing the relative gene expression level of the SR2 and C1 flies under fed (F) and starved (S) conditions. The numbers on the right side of the graph represent the number of genes out of a total of 42 we identified to be represented in this group. Depicted are only those types of genotype-by-environment interactions that were found in the data.

Starved flies: “The selection environment”

Since the gene lists are too large to give here, we have rather picked out those genes thought to be of interest. Sometimes genes occur in more than one group because they have more than one function or are involved in more than one process. We have listed the results of all genes we know to be involved in these processes.

The usual suspects
Genes that are often associated with starvation and ageing related processes are found in the gene lists. For example, *chico* is expressed at a lower level in the starvation resistant line which fits the observation that decreased *chico* expression in mutant flies shows increased life span (Clancy et al. 2001; Clancy et al. 2002; Tu et al. 2002). Juvenile hormone catabolising enzyme genes (2) are expressed at a higher level. This relates well to previous data (Tatar et al. 2001; Flatt and Kawecki 2004; but see Rauschenbach et al. 2004). The low level of heat shock protein expression in SR2 is somewhat surprising because high HSP expression levels have previously

been associated with longevity (Sorensen and Loeschcke 2001; Walker and Lithgow 2003), but may be indicative of lower levels of perceived stress in the starvation resistant line. Furthermore, the high expression level of triacyl glycerol lipase (3 genes), involved in triglyceride breakdown follows the earlier finding that the SR lines possess more lipids and have therefore more resources to burn off.

MAPKKK cascade
To survive, an organism has to recognize and deal with environmental stress as quickly as possible. In this experimental design under starvation conditions, C1 is likely to be more stressed than SR2 and, thus, should induce a higher stress response. The Jnk and MAPKKK have been found to be activated by environmental stress (Matsukawa et al. 2004; Zhuang et al. 2006). Nine genes were found of the Jnk and MAPKKK cascades that were expressed at a lower level in SR2 than in C1 (see Appendix 4a). The fact that the controls have relatively high expression levels is consistent with their higher experience of environmental stress during starvation than SR2.

Insulin signalling and sugar metabolism
Insulin signalling has often been associated with longevity (Braeckman et al. 2001; Clancy et al. 2001; Tatar et al. 2001; Dillin et al. 2002; Tu et al. 2002; Bluher et al. 2003; Holzenberger et al. 2003; McCulloch and Gems 2003; Walker and Lithgow 2003). Here we find that in SR2 flies some of the genes from the insulin signalling pathway are expressed at a lower level than in C1 (Appendix 4b).

Sugar catabolic enzymes are more highly expressed (10 genes) and the build up of glycogen is regulated at a lower level in the SR2 flies than in C1 (1 gene). We could not identify a pattern in sugar transporters (6 genes). Furthermore, glycolysis associated genes were expressed at a lower level in SR2 flies than in C1 flies (4 genes). This relates to data demonstrating that insulin signalling increases glycolysis (e.g. Beitner and Kalant 1971). Thus, there is a consistently low level of expression in the insulin pathway and its downstream targets such as glycolysis and gluconeogenesis in the SR2 flies.

Lipid metabolism
Lipid breakdown genes, such as lipases, generally have a higher expression level in the starvation selected line (9 genes, one has a lower expression level). Both cholesterol metabolism related genes found were expressed at a lower level in SR2 flies. Most genes involved in fatty acid biosynthesis (4 out of 6 genes) are also expressed at a higher level. This is consistent with higher availability of lipids of the starvation resistant flies and may be involved in fatty acid modification rather than de novo synthesis. As for the sugar metabolism related genes, the lipid transporters do not show a uniform response.

Protein metabolism
Proteolysis and peptidolysis associated genes (25 genes) were expressed at a higher level in the starvation resistant line than in the control line. Four genes with this proteolytic function were down regulated. Of these, *surfeit6* fits well with other gene expression changes because it is an ATP dependent proteolytic enzyme. Since glycolysis related genes show relatively low expression, so is the production of ATP and thus the ATP available for this enzyme to act. The remaining 10 genes, which have highly different functions, were all expressed at a lower level in line SR2. Four

of these were involved in protein modification and the other 6 were involved in diverse processes such as protein folding, transport and cell death.

Reproduction
Longevity and starvation resistance are known to be involved in a trade off with reproduction at a phenotypic (e.g. Chippindale et al. 1993) and genetic level (e.g. Zwaan et al. 1995b. We have already suggested that the insulin pathway genes were expressed at a lower level in the SR2 line compared to the control line. This pathway is thought to regulate longevity and reproduction (Dillin et al. 2002). Therefore, reproduction-associated genes can be expected to be down-regulated in starvation resistant lines relative to their control. Appendix 4c lists the 14 genes associated with the reproductive machinery, each of which was expressed at a lower level, supporting our hypothesis. Strikingly, of those genes we know to be involved in reproduction, none were up-regulated in line SR2. This consistency indicates that there is likely to be a strong effect on reproduction.

Neural tissue associated genes
A total of 26 genes associated with neural processes were found to be differentially expressed. Of these, 12 were known to be involved in development and neurogenesis, 4 of which were expressed at a higher level in SR2. The exact function of 2 genes remains unknown to us, not withstanding the fact that they are associated with neural tissue in GO annotation. A total of 12 genes were active in the transmission of neural signals. Of these, 3 were involved in neurotransmitter secretion (2 lower, 1 more expressed in SR2) and 9 were involved in nerve impulse (8 lower, 1 more expressed in SR2).Generally, we can state that neural signal transduction genes are expressed at a lower level.

Discussion

Technical issues

Many of the genes identified as differentially expressed have not been annotated yet or they do not have gene ontology categories. From this it follows that in this paper we are concentrating much more on previously investigated candidate mechanisms than on discovering new pathways and genes. This makes using current gene knowledge, bias prone. However, they supply us with valuable information about if, and to what extent candidate mechanisms are associated with standing genetic variation in natural populations for the traits they are supposed to underpin. Better annotation of the *Drosophila* genome is crucial to link the knowledge about this system to all the molecular processes responsible and not only to those that are already well-known (cf. Gems and McElwee 2005).

In the PCA analysis, 20% of all variation was explained by technical factors. On the other hand, in the ANOVA analysis, the FDR also generates errors. Micro-array data have thus not only noise because of inherent biological variation, but also because of technical and analysis variation. The question remains how worrisome this is. We have already acknowledged the necessity for caution and know that the micro-array data themselves are not enough to prove or disprove hypotheses. Therefore, we

should take the uncertainties into consideration and use them for the thing they have been designed for: revealing patterns in a large and complex dataset. In this way micro-arrays are used as a means to gain an indication of whether or not to pursue certain hypotheses.

The technical variation will not be as consistent as a biological signal. There may be a large number of genes involved in a pathway or a response mechanism. Single gene expression differences will typically be generated by technical variation. These will not yield as strong a basis for hypotheses as will pathway differences. Therefore, the technical variation need not pose an insurmountable problem upon careful consideration of the data.

General analysis

It is very important to acknowledge that micro-array experiments do not necessarily provide truth. Micro-array analysis usually does not comprise a large number of chips or high replication because of the costs. Therefore, one should be cautious in the interpretation of micro-array data and acknowledge the fact that hypotheses provided by micro-array analysis should be followed up using quantitative real time PCR. In addition, because we have 3 to 4 replicates for each treatment, the significant differences, especially in the ANOVA, are likely to be of interest. Here, we use micro-arrays within a hypothesis driven framework. The outcome of the gene expression study is not used as proof for a hypothesis, but rather to gain insight about whether testing certain aspects of the hypothesis is worthwhile. In doing so, lines were used with a distinct phenotype and known biology. We began this research with a clear hypothesis in mind; the gene expression differences of the selected line will show most differences when assayed in the environment of selection, these expression differences are likely to involve several well-known pathways, such as the insulin signalling pathway. This hypothesis drove this research, which was carried out under relevant circumstances, accordingly.

PCA analysis and ANOVA revealed that the large difference between the selected and control line is only present under starved conditions. This is not surprising since that is the condition for, and under which the starvation resistant line is explicitly selected. In our project we focus on the genes underlying starvation resistance and possibly longevity, we leave the environmentally differentially expressed genes for a more ecological genomics oriented path of research.

PCA 1 and 2 together reveal a genotype-by-environment effect; a distinct difference between the lines under starved conditions. These first two components show the power of micro-array analysis to identify processes and provide a good argument for continuing this line of research. The third and fourth components urge caution about the results. It is noteworthy that especially the third component may be reduced in strength by just normalising the expression data within batches.

Genotype-by-environment interactions

A priori we expected to find a large number of gene-by-environment interactions by comparing the transcriptome of lines C1 and SR2 under fed and starved circumstances. Line SR2 displays high starvation resistance and longevity, compared to the control (Baldal et al. in prep.; Baldal et al. 2006). By introducing a clear shift in the environment, feeding vs. starvation, we observe large differences in the gene expression patterns. The environmental change from feeding to starving induced 3625 gene products to change their expression pattern significantly. Selection on a quantitative trait, in this case starvation resistance, led to differential gene expression, but apparently in a smaller number of genes than in the case of the environmental change. Not many genes were found to show a genotype-by-environment interaction. Of the total number of 3763 differentially expressed gene products only about 1% showed a significant genotype-by-environment interaction. Genotype-by-environment interactions are established by non-parallel slopes of reaction norms; thus different genotypes show a different response to the environmental gradient (Stearns 1992).We examined one selected and one control line, SR2 and C1, respectively, for the performance of a single trait (life span) in two environments (fed and starved).

The ability to survive a period of extreme adversity such as starvation is an important feature in the life history of an organism. Selection pressure on such a trait can be very harsh in certain environments, as has been pointed out clearly for the human situation by Diamond (2003). Starvation resistance is called for by most, if not all, living organisms in adversity. The genes responsible for starvation resistance are thought to overlap with those determining resource allocation and somatic maintenance, hence the association with longevity. Life history traits such as longevity and starvation resistance are thought to rely on public mechanisms (Partridge and Gems 2002) that are shared throughout the animal kingdom and maybe more. This indicates that the mechanisms underlying these traits are evolutionarily conserved. One might argue that if such basal genes affect many important processes they will have to be more stringently regulated and are not likely to show radical changes in expression pattern. This provides a hypothesis for why in this specific example few gene-by-environment interactions are found. On the other hand, when the environment changes radically, a lot of processes may need to be changed, which is exactly the thing these key-pathways do. In the response to the environment we observe this. In a selection process, changing the expression of only a few genes, which will come on top of the normal large shift in gene expression as a result of the environment, may suffice. If the gene-by-environment interactions are reminiscent of the response to selection, then they should turn up in the loading of the factors involved in the PCA. Testing this hypothesis is one the foci of future research.

The strong genotype-by-environment effect we found in the principal components can be explained by the fact that indeed those genes that have been affected most by selection display the largest differences, add most to the variation and thus end up being reflected in the principal component. It should be noted again that PCA is a data reduction tool and should be used to gain insight from a large and difficult dataset. Close examination of the factors that attribute most to variation in combination with ANOVA analysis should reveal the extent to which those genes

contribute most to the variation are also those displaying genotype-by-environment interactions. Future work will focus on this.

Genes

The differentially expressed genes under starved conditions revealed that flies of the control line experience more stress than those of the starvation resistant line. Line SR2 shows a clearly changed pattern of gene expression and seems to burn off resources at a higher rate, down regulates insulin signalling and, consequently, glycolysis. Though burning off resources and down regulating glycolysis seems contradictory this is not necessarily the case. Glucose is only one of the resources available and its down-regulated catabolism may be a necessary consequence, which is compensated by the breakdown of other substances. Furthermore, reproduction related gene expression appears to be lowered in SR2 flies, as is neural transmission.

Resources and metabolic rate

The fact that starvation resistant lines possess more resources and can thus burn off more calories is clear from the high levels of lipid and carbohydrate down breaking gene expression levels. Earlier research in the related lines SR3 and SR4 showed that glycogen content was not higher in these lines than in control lines from eclosion onwards, but was increased considerably upon feeding. Here, we see that carbohydrate breakdown in line SR2 is higher which implies that carbohydrate levels will have been higher in this line than in C1 from eclosion onwards. Increased carbohydrate levels have been suggested to be associated with longevity through desiccation resistance (for a review, see Hoffmann and Harshman 1999). Therefore, we hypothesise that the long lived lines, SR1 and SR2, have a considerably higher desiccation resistance than lines, SR3 and SR4. Carbohydrate levels of lines SR1 and SR2 could provide additional mechanisms to increase longevity. Also, from the down-regulation of glycolysis and increased breakdown of carbohydrates it can be deduced that free glucose levels will be higher. This semi-diabetic phenotype matches that found in Broughton et al. (2005).

The burning off of excess resources explains why in Baldal et al. (2006) we found that SR2 females did not have a reduced metabolic rate after three days of starvation and had a higher metabolic rate than control lines. This suggests that in SR2 increased resources are required for increased starvation resistance. Also they explain the longer development time (unpublished result).

Proteolysis is also higher in SR2. Together with the breakdown of sugars other than glucose, it seems that SR2 flies clean themselves from excess resources to maintain homeostasis and prevent damage from secondary metabolites in the cell. Though this is speculative, it at least indicates that the flies are gaining energy from substances other than glucose. The fact that insulin signalling, glycogenesis and glycolysis related genes are expressed at a low level in SR2 indicates that the animals have down regulated these physiological processes associated with glucose metabolism. Here, we hypothesise that the high metabolic rate may be attributed by the breakdown of resources and surplus material.

Reproduction
Insulin signalling is known to regulate reproductive output in nematodes (Dillin et al. 2002) and insects (Wu and Brown 2006). SR2 is a line for which increased starvation resistance comes with an increased longevity. Here, reproduction related gene expression is suggested to be lower in the starvation resistant line. This is in line with the disposable soma theory of ageing (Kirkwood 1977; Kirkwood and Holliday 1979; Kirkwood and Rose 1991). Where reproduction is thought to trade off with somatic maintenance, therewith reducing longevity. Such a physiological trade off between starvation resistance and reproductive output has been shown to exist in *D. melanogaster* (Chippindale et al. 1993). To our knowledge this is the first study that links genome-wide expression data to the disposable soma theory of ageing.

Neural transmission
Starvation selected flies have a lower expression of genes involved in neural transmission. Earlier findings of Alcedo and Kenyon (2004) showed that ablation of specific gustatory nerve cells resulted in extended longevity in *C. elegans*. Broughton et al (2005) showed a similar increase in longevity in *D. melanogaster* flies whose insulin producing cells in the brain had been ablated. The signal that food is present (insulin like peptides) is not transmitted in the absence of these neurons in the flies. In the case of the starvation resistant lines, it may be so that neural transmission is reduced from eclosion on, as a result of selection for starvation resistance from eclosion onwards. This may result in overall lower insulin signalling, directing resource allocation to the soma instead of to reproduction and mitigating glucose metabolism, therewith extending longevity.

The high glucose levels that were found in the study of Broughton et al. (2005) are also hinted at in this study. Carbohydrate catabolic genes are expressed at a high level, whereas glycolysis specific genes are expressed at a low level. Because of this high supply, low demand situation, it can be expected that glucose levels will rise. This resembles mammalian pathological diabetes, which would, in mammals, lead to a decrease in life span. Broughton et al (2005) have acknowledged this fact. They explain that mammalian life span reduction as a result of diabetes comes from pathologies that do not occur in fruit flies, such as vascular damage.

Hypothesis

On the basis of these findings we hypothesise that as a result of selection our starvation resistant line has a lower body-wide neural transmission in the absence of food. Insulin producing cells in the *corpora cardiaca* (Kim and Rulifson 2004) produce less insulin, which results in the down regulation of the insulin pathway. This in turn results in down regulation of glycolysis and consequently, the down regulation of reproduction. The resources are then allocated to the soma side of the disposable soma theory. Because of the increased amount of resources, breakdown can continue for a longer time. Carbohydrates are effectively broken down, but the down regulated glycolysis will be a rate limiting step, through which energy will be generated over a longer time. This would have to lead to an increase in free glucose and a diabetic hyper phenotype. Figure 5 describes the pattern of changes in gene expression in broad classes of genes and pathways. More detailed analysis on these genes is needed and should be done using quantitative realtime PCR, and include

time series of gene expression. Despite the generality of the model, this study indicates that the physiological processes that have been identified in mutant studies in a variety of model organisms, are also underlying the responses to selection in lines that contain only naturally occurring variation.

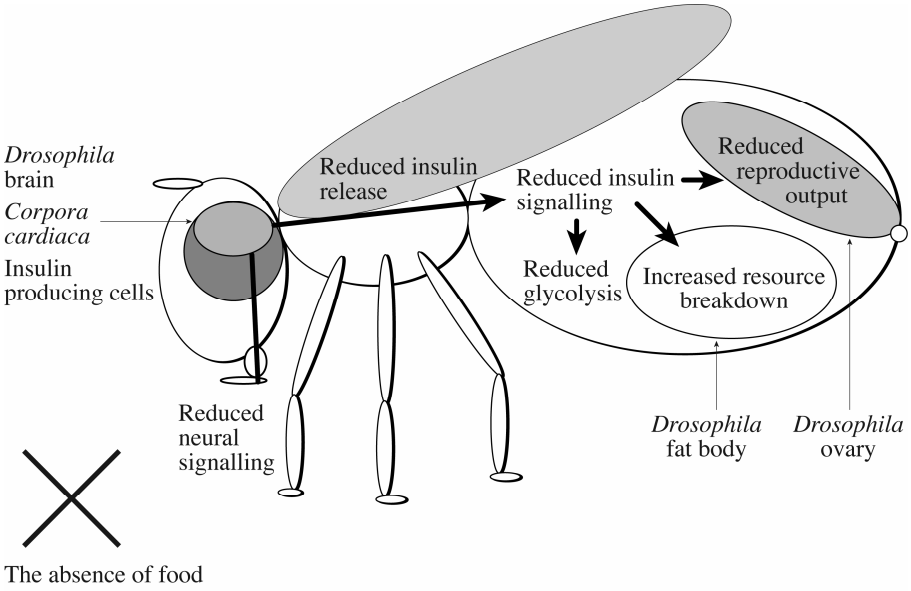

Figure 5. Schematic representation of what hypothetically happens in a female *Drosophila* in the absence of food.

Suggestions and a short outline of future research

Researchers should be aware of the technical variation that may occur in these studies. This noise can largely be overcome by synchronising experiments and controlled analysis. We, therefore, advise that in micro-array experiments all actions on samples that are to be compared take place at precisely the same time for all samples. Thus, the whole protocol for all samples should be completely synchronous. Batches of arrays that have been processed at different times should be normalized independently before analysis. In the case of time series and large sample sizes this may not be feasible. We then advise to use internal references in each separate batch.

Independent replication of the experiment (both biologically and technically) is necessary to be able to examine whether the same genes are found. Genes displaying a similar response can be regarded as being involved in the feature under study. A future project with Unilever will provide this replicate for the present pilot study.

Since pathway information is scarce for the *Drosophila* genome chips, in future analyses of this set of arrays and the replicate it would be interesting to use the human and murine orthologues and use those pathways as candidates for those involved in *Drosophila* longevity and starvation resistance. The orthologues can be found using the Ingenuity program. Also, this exercise may reveal genes of importance that have not been annotated yet.

Expression patterns of genes of interest should then be analysed in more depth by performing QRT-PCR on the remaining RNA samples. Furthermore, performing QRT-PCR on novel biological replicates will add statistical power and proof for the involvement of these genes in longevity and/or starvation resistance. Nevertheless, this pilot study illustrates the potential of applying micro-array technology to the analysis of selected lines and traits such as starvation resistance. More in depth analysis of the change in processes should be done in a time series.

Appendix 1. Gene ontology categories of genes that were differentially expressed between the genotypes under fed and starved conditions. The right hand column gives the percentage of genes involved in the GO category. Percentages add up to over 100 % because some genes are involved in several processes and thus add up to the percentage multiple times.

Fed	percentage (of 12)
transcription	67
regulation of transcription	58
regulation of nucleoside, nucleobase, nucleotide and nucleic	58
central nervous system development	42
neuroblast cell fate determination	25

Starved	percentage (of 79)
cellular protein metabolism	25
female gamete generation	23
transcription	20
imaginal disc metamorphosis	18
intracellular protein transport	18
eye morphogenesis	18
protein transport	18
cytoskeleton biogenesis and organisation	18
regulation of transcription	18
regulation of nucleoside, nucleobase, nucleotide and nucleic	18
eye-antennal disc development	15
peripheral nervous system development	13
phosphate metabolism	11
mesoderm development	11
sensory perception	11
ectoderm development	11
detection of abiotic stimulus	10
photoreceptor cell morphogenesis	10
eye photoreceptor cell differentiation	10
cell migration	10
wing morphogenesis	10
photoreceptor cell development	10
enzyme linked receptor protein signalling pathway	10
wing disc development	10
response to pest, pathogen or parasite	10
cellular macromolecule catabolism	10
M phase of mitotic cell cycle	10

Appendix 2. List of genes with known functions that showed gene-by-environment interactions. Listed are the unigene code, gene name, biological function or molecular function (indicated with an asterisk) and whether C1 and SR2 up or down regulated the expression of this gene under starved conditions relative to feeding.

Unigene	Gene name	Biological function	C1	SR2
NM_137810	CG3290	*alkaline phosphatase activity	down	equal
NM_137543	Juvenile hormone epoxide hydrolase 3	defense response; juvenile hormone catabolism	down	equal
NM_139861	CG8562	proteolysis and peptidolysis	down	equal
NM_079385	Multiple inositol polyphosphate phosphatase 1	*phosphoprotein phosphatase activity	down	equal
NM_142592	CG4462	extracellular transport	down	up
NM_080517	Turandot A	response to stress	down	up
NM_142648	CG5494	*structural constituent of cuticle	down	up
NM_143450	Odorant-binding protein 99a	*odorant binding	down	up
NM_079862	Alkaline phosphatase 4	*alkaline phosphatase activity	down	up
NM_142674	CG10827	*alkaline phosphatase activity	down	up
NM_136652	CG1809	*alkaline phosphatase activity	down	up
NM_134574	CG1304	proteolysis and peptidolysis	down	up
NM_140084	CG18180	proteolysis and peptidolysis	down	up
NM_136829	CG12350	proteolysis and peptidolysis	down	up
		stronger response in SR2		
NM_134831	CG3609	*oxidoreductase activity	up	up
NM_057277	Larval visceral protein D	glucose metabolism	up	up
NM_057280	Larval visceral protein L	glucose metabolism	up	up
		stronger response in C1		
NM_134818	CG17012	proteolysis and peptidolysis	down	down
NM_168021	Drosomycin-4	defense response	down	down
NM_141722	CG3940	*carbonate dehydratase activity	down	down
NM_079277	Astray	peripheral nervous system	up	up
NM_142777	CG7059, isoform A	glycolysis	up	up
NM_144443	CG18858	cholesterol metabolism; lipid metabolism	up	up
NM_135074	CG14031	*oxidoreductase activity	up	up
NM_142947	CG5933	RNA methylation	up	equal

Appendix 3. All the differentially expressed genes under fed conditions that have been described as genes, together with their Unigene code, gene name, molecular function or biological function (marked with an asterisk), and a column telling whether the gene was regulated at a higher or lower level in SR2 relative to C1.

Unigene code	Gene name	Molecular function	SR2 vs C1
NM_143563	CG1340	RNA binding; translation initiation factor	Higher
NM_080172	CG1856, isoform E	specific RNA polymerase II transcription factor activity; transcriptional repressor activity	Higher
NM_078990	Vismay	transcription factor activity	Higher
NM_057861	Type III alcohol dehydrogenase	alcohol dehydrogenase activity	Higher
NM_057950	Chitinase 2	chitinase activity; hydrolase activity,	Higher
NM_143456	Odorant-binding protein 99b	*Odorant binding	Higher
NM_132240	Companion of reaper	*Reaper associated	Higher
NM_057930	CG6955	structural constituent of larval cuticle	Higher
NM_057271	Larval cuticle protein	structural constituent of larval cuticle	Higher
NM_078533	Chorion protein 38	structural constituent of chorion	Lower
NM_140389	CG10154	structural constituent of peritrophic	Lower
NM_139416	CG13937, isoform A	HNK-1 sulfotransferase activity	Lower
NM_134831	CG3609	oxidoreductase activity	Lower
NM_137030	CG4812	serine-type peptidase activity; trypsin	Lower
NM_166152	CG8448, isoform D	ATPase activity, coupled	Lower
NM_057280	Larval visceral protein L	alpha-glucosidase activity; transporter activity	Lower
NM_135270	CG5177	trehalose-phosphatase activity	Lower

Appendix 4a. List of genes associated with the MAPKKK and JNK cascades. The biological function and whether the gene of interest had a higher or lower expression level in SR2 than in C1 are also listed.

Unigene code	Gene name	Biological function	SR2 vs. C1
NM_057238	Jun-related antigen	JNK cascade; MAPKKK cascade	Lower
NM_142288	CG14895, isoform A	MAPKKK cascade	Lower
NM_057532	Twins	MAPKKK cascade; response to stress	Lower
NM_140830	Mitogen-activated protein kinase phosphatase 3	MAPKKK cascade	Lower
NM_080005	Rab-related protein 4	MAPKKK cascade	Lower
NM_057750	CG8416, isoform B	JNK cascade	Lower
NM_057960	CG7693, isoform A	MAPKKK cascade	Lower
NM_079821	Protein C kinase 98E	MAPKKK cascade	Lower
NM_080050	CG12437, isoform B	regulation of JNK cascade	Lower

Appendix 4b. insulin signalling pathway associated genes.

Unigene code	Gene name	Biological function	SR2 vs. C1
NM_164899	Chico	insulin-like growth factor receptor binding	Lower
NM_080382	Protein kinase 61C	protein kinase activity	Lower
NM_057366	CG2621, isoform A	glycogen synthase kinase 3 activity	Lower

Appendix 4c. Reproduction associated genes that were differentially expressed between SR2 and C1.

Unigene code	Gene name	Biological function	SR2 vs. C1
NM_057670	Myocyte enhancing factor 2	ovarian follicle cell development	Lower
NM_167558	CG32562	oogenesis	Lower
NM_080322	New glue 1	oogenesis; reproduction	Lower
NM_080382	Protein kinase 61C	oogenesis	Lower
NM_057366	CG2621, isoform A	oogenesis	Lower
NM_057594	Cactus	oogenesis	Lower
NM_080364	Bunched	oogenesis; ovarian follicle cell development	Lower
NM_079659	Cheerio	female germ-line cyst encapsulation; ovarian ring canal formation	Lower
NM_080303	Wings apart-like	female meiosis chromosome	Lower
NM_139364	CTP	oogenesis	Lower
NM_057469	Nebbish	oogenesis	Lower
NM_078659	Bazooka	follicle cell adhesion; maintenance of oocyte identity; oocyte cell fate determination	Lower
NM_164899	Chico	egg chamber growth;	Lower
NM_080084	Spinster	courtship behavior; regulation of female receptivity	Lower

Chapter 6

Methuselah life history in a variety of conditions, implications for the use of mutants in longevity research.

Experimental Gerontology, in press

Methuselah life history in a variety of conditions, implications for the use of mutants in longevity research.

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Abstract

The laboratory has yielded many long-lived mutants of several model-organisms in the past few years. Many of the resulting claims for extended longevity have been nuanced or shown to be restricted to specific conditions, including environments and genetic backgrounds. Here, we test whether the long-lived mutant fruit fly *methuselah* (*mth*^{1}) displays its apparent superiority in longevity and stress resistance in different environments, at different ages and in correlated traits. The results demonstrate that stress resistance at different times in life is not consistently higher in the mutant relative to its progenitor strain (*w*^{1118}). Furthermore, the *mth*^{1} genotype only leads to an increase in longevity in an environment where reproduction is not stimulated. Also, virgin and mated life span were compared and showed that mating negatively affects life span, especially in the *mth*^{1} individuals. This reduced the life span enhancing effect of the mutation to zero. This apparent environment and mating dependent trade-off between longevity and reproduction supports the disposable soma theory of ageing. We conclude that these data can only provide limited information on natural variation. The data show the need to uncover the full complexity of variation in such traits in natural environments.

Keywords
Methuselah, longevity, mutant, *Drosophila*

Introduction

Several different long-lived *Drosophila melanogaster* mutants have been identified in the past years (e.g. Lin et al. 1998; Rogina et al. 2000; Clancy et al. 2001; Tatar et al. 2001). All have been created or discovered in the laboratory and do most probably not represent naturally occurring standing genetic variation. It is not known whether these mutations have fitness effects in natural environments, even if the extended longevity is the product of pleiotropy and not the sole trait affected. Longevity as a trait is situated in the selection shadow and can thus not be directly selected for in nature (Hoekstra 1993). The selection shadow comprises the age range in which natural selection cannot act because individuals are unlikely to live to these ages. This means that no selective pressure in favour of reaching this old age is present. Exception to this rule may be those species where (grand)parental care adds to survival, such as primates. There, the elderly have their use in reproductive success. Yet, also in this case there is a selection shadow, only longer after reproduction has ceased. The added scientific value of long-lived mutant strains at present lies mainly in the identification of pathways that could determine longevity. Research into such pathways should elucidate whether they are relevant to the variation in longevity in natural systems or not (cf. Flatt 2004; Geiger-Thornsberry and Mackay 2004).

The link between the laboratory and nature is important when addressing the evolution of life span using mutant analysis. One example is the dwarf *chico*^{1}-mutant, which affects insulin signalling (Clancy et al. 2001). The associated increase in longevity of this mutation was found to be highly dependent on the food environment (Clancy et al. 2002). A similar scenario applies to the long-lived *Indy* mutant (Rogina et al. 2000), a gene involved in intermediary food metabolism. Under adverse food conditions *Indy* displayed a lower fertility than controls, whereas the differences were reversed when food was present in excess (Marden et al. 2003).

Here, we focus on a mutant of the *methuselah* (*mth*) gene, which has been shown to be involved in longevity in the laboratory. The *mth*^{1} mutant line was originally derived from the *w*^{1118} strain by allowing a transposon (P-element) to randomly insert in genes (Lin et al. 1998). Strikingly, apart from the increased longevity, the *mth*^{1} mutant individuals do seem to functionally age in a similar way as the control flies (Cook-Wiens and Grotewiel 2002). This means that *mth*^{1} individuals are capable of normal behaviour for about as long as *w*^{1118} individuals, but become less active afterwards, when the control strain is already dying off. Thus, when it comes to actual life span, the *methuselah* mutant outlives *w*^{1118}. This may indicate that there is not a real advantage of living longer for the mutant *mth*^{1} individuals. *mth* encodes a G-protein coupled receptor involved in neurotransmitter release (Song et al. 2002). Natural variation in the *mth* gene has been reported to show correlations with life span and body size over a latitudinal gradient (Schmidt et al. 2000), however, no such patterns could be identified in a homolog (Duvernell et al. 2003). This indicates that *mth* is linked to life span and that its homologs do not show similar patterns. The specific selection on the *mth* gene, associated with lifespan, makes *mth* an interesting gene for further study. The fact that natural variation exists for the *mth* gene and that longevity per se is not under selection in natural *Drosophila* populations suggests that the increased life span may be the by-product of selection on other traits, such as body size and reproductive patterns, in specific environments.

In the original paper, the long-lived *mth*^{1} flies were shown to have elevated starvation, paraquat and heat resistance relative to their progenitor strain (Lin et al. 1998). In the light of the earlier comments on laboratory specific physiology, natural variation, and earlier findings in mutant strains the overall superiority of this mutant is investigated here. Lin et al. (1998) tested paraquat and starvation resistance at specific ages. These traits may develop over time and therefore, the traits are measured here at several ages.

Because the P-element interferes with *mth* expression, neural signalling will be lower. In order to function properly, we hypothesise that the mutant requires affluence, i.e. high caloric conditions (cf. Clancy et al. 2002). Notably, our food rich medium contained more yeast, inducing reproduction (Simmons and Bradley, 1997). Therefore, we hypothesised that *mth*^{1} individuals that carry the *mth*^{1} allele will suffer from a negative effect of reproduction, which is often found to negatively correlate with longevity. We tested this hypothesis by assaying lifetime reproductive success of females and examining whether differences could be found in early and late life reproductive success. Such findings would be in line with the disposable soma theory of ageing. This theory states that the body of an individual is not of interest to evolutionary processes, but that the transmission of genetic material to the next generation is. Therefore, when an individual meets conditions in which it is favourable to reproduce it does so on the cost of the body. Here, the results of the tests of both the environmental and temporal specificity hypothesis and the reproductive alteration hypothesis are presented and discussed in the light of evolutionary physiology theory.

The aim of this study was to examine the gene-by-environment interactions that modify the life history of *mth*^{1}-bearing *Drosophila melanogaster*. Our results explain why the information inferred from *mth*^{1}, and longevity mutants in general, should be treated with caution when extrapolating laboratory results to naturally occurring variation in ageing and longevity. They provide insight in the importance of the test environment on life history correlations, especially the trade off between reproduction and lifespan.

Materials and methods

Material

Mth^{1} flies were derived from the *w*^{1118} strain by Lin et al. (1998) through the creation of P-element insertion lines. *Mth*^{1} is genetically different from *w*^{1118} only because it has a P-element (P{LacW}*mth*^{1}) insert in the third intron of the gene (Lin et al. 1998). This mutant is longer lived than its control line at both 29 and 25 ºC (Lin et al. 1998).

Fly rearing

Mth^{1} and *w*^{1118} flies were kindly provided by Professor Seymour Benzer, Caltech University. They were kept in the lab for over 10 generations for acclimation to the local culture conditions. All stock maintenance and experiments took place at 25 ºC, 50% relative humidity and a 12/12 hour day/light regime, unless indicated otherwise.

Flies were maintained in half-pint bottles containing 24 ml of standard medium. Standard medium consisted of 20 g agar, 9 g kalmus (10 parts acidum tartaricum, 4 parts ammonium sulphate, 1 part magnesium sulphate and 3 parts potassium phosphate), 10 ml nipagin (100 g 4-methyl hydroxy benzoate per liter ethanol), 50 g saccharose and 35 g granulated yeast per liter water. For experiments, flies were reared at a controlled density of 100 eggs per vial of 6 ml standard medium.

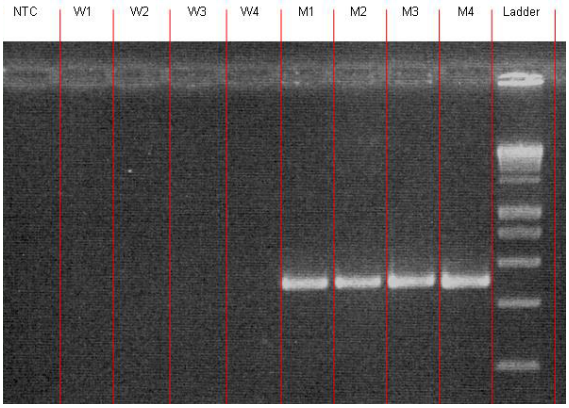

Figure 1. Gel showing the result of the PCR test on the P-element insert in methuselah and its absence in w^{1118}. NTC means No template control; W1-4 mean samples of w^{1118}, M1-4 mean samples of methuselah; Ladder means smart ladder with bands on 200, 400 500 etc. base pairs. Evidently, the no template control and w^{1118} lanes are empty, whereas the methuselah lanes show PCR product. The no template control shows that the PCR product is not produced in the absence of a template. The W-samples demonstrate that in the control strain no P-element insert is present in the *mth* gene. The M-samples demonstrate that the P-element insert is present in the *mth* gene. Therefore, the methuselah and w^{1118} strains we worked with are considered equal to the ones as used by Lin et al. (1998) and Cook-Wiens and Grotewiel (2002).

P-element check

We tested whether the P-element (P{LacW}mth^1) insert was still present in the *methuselah* gene. We isolated DNA from 4 groups of 10 female flies for both the mth^1 and w^{1118} strains using a Qiagen DNeasy minicolumn kit. We used primers that were originally used by Cook-Wiens and Grotewiel (2002);

Mth specific primer: 5'-ggtaattcctgtcaggaccaccg-3'
P-element primer: 5'-cgacgggaccaccttatgttatttcatcatg-3'

In a 25µl PCR reaction we used 2.5µl Qiagen 10* PCR Reaction buffer, 2µl 2.5µM dNTPs, 2.5 u Qiagen Taq DNA polymerase, 1µl 10µM *Mth* specific primer, 1µl 10µM P-element specific primer, 2 µl template (200ng), 16µl H_2O. We used the following PCR programme on a Biometra T-gradient thermocycler; 1. 95ºC 10 minutes, 2. 95ºC 30 seconds, 3. 72ºC 2 minutes 30 seconds, 4. 72ºC 5 minutes, 4ºC ∞, with a 30 time repeat of steps 2 and 3.

The results are shown in figure 1. The no template control shows that the PCR product is not produced in the absence of a template. The W-samples demonstrate that in the control strain no P-element insert is present in the mth^1 gene. The M-samples demonstrate that the P-element insert is present in the mth^1 gene. Therefore, the mth^1 and w^{1118} strains we worked with are considered equal to the ones as used by Lin et al. (1998) and Cook-Wiens and Grotewiel (2002).

Longevity assays

Virgin flies
Flies were sexed after eclosion and maintained as virgins. For both sexes we assayed the life span of 50 animals. In the longevity assays, flies were examined at a density of 1 individual per vial. Each standard vial contained 6 ml of either standard or double medium, the latter having the yeast and sugar concentrations doubled relative to the standard medium (table 1). These experiments were conducted at either 25 or 18 ºC. Mth^1 flies were *a priori* thought to increase their lifespan differences in the presence of more abundant food conditions (cf. Clancy et al 2002). Flies were checked daily for mortality and vials were replaced weekly for ones with fresh medium.

Table 1. Yeast and sugar quantities in grams per litre of agar medium used in relevant experimental systems with *D. melanogaster*. The column 'Extra' comprises additional nutrients, not including antibiotics and agar.

Author and medium	**Yeast**	**Sugar**	**Extra**
Baldal et al. (in prep) standard medium	35	50	-
Baldal et al. (in prep.) double medium	70	100	-
Lin et al. (1998) medium (pers. comm. G. Carvalho)	15	75	83 cornmeal
Cook-Wiens and Grotewiel (2002) medium (pers. comm.)	10	50	33 cornmeal
Mair et al. (2005) normal medium	150	150	-
Mair et al. (2005) yeast restricted medium	65	150	-

Mated female flies
Longevity of mated females of both lines was determined by daily observation of the 50 vials, each containing a single female fly together with two males of the same genotype. Vials contained standard medium and yeast granules and were changed on a daily basis during the first five days following eclosion, and from then onwards with an interval of 3 days until the death of the female. Dead males were replaced with live ones from the stocks of the appropriate genotype.

Metabolic rate, paraquat- and starvation resistance

To assess superior performance of *mth*^{1} in our laboratory, we assayed the mutant flies and their controls for metabolic rate, paraquat and starvation resistance using the standard protocols of our own laboratory. Paraquat and starvation resistance were measured in groups of 100 individuals per sex and per line, 5 flies per vial, for each time point. All flies were virgins and maintained in vials containing standard medium until being assayed on the time points of 0, 3, 5 and 10 days from eclosion. Prior to the experiments, adults were kept at an adult density between 10 and 20 per vial (except for the experiment on starvation resistance, where besides the normal slightly varying density also some flies from a controlled adult density, 10 flies per vial, were tested).

Metabolic rate was measured for 35 flies divided into 7 groups of 5 individuals for each time point and genotype, before which they were anaesthetized on ice and weighed. The flies were then assayed in a 16-channel respirometer (Li-6251 CO_2 analyzer of Li-Cor) containing a small piece of agar medium to prevent the flies from dehydrating. Two channels were left empty as independent controls in each experiment. For each channel, 5 runs of respirometry were performed. The first 2 of these runs were discarded in each case because they generally showed elevated CO_2 levels due to the experimenter's exhaling when the flies were put into the channels by aspiration. The data thus comprise 3 separate runs of 7 groups of 5 flies per sex per line per feeding condition. Data were acquired and analyzed using the program Sable. The amount of CO_2 produced in the experiments was divided by the wet weight determined just before the experiment to get an estimate of the mass specific metabolic rate.

Paraquat resistance was measured in virgin flies at a density of 5 flies per vial. Flies were given agar plugs (20gr agar per liter water) to provide moisture and 500µl of a 5% sucrose, 30mM paraquat (methyl viologen, M2254, Sigma Aldrich) solution on 5 filtration papers (1 by 1 cm, as in (Vermeulen et al. 2006)) and the flies were checked for survival twice a day. Please note that where Lin et al. (1998) used 20mM of paraquat and looked at mortality percentages after certain hours we used 30mM and used all of the mortality data available.

Starvation resistance was measured in virgin flies at an adult density of 5 flies per vial, by putting them in vials containing containing 6 ml of agar medium (20 gr. agar, 9 gr. kalmus, 5 cc. nipagin per liter water). Flies were checked daily for survival.

Reproductive output

Reproductive output was measured for each female used in the mated-longevity experiment. We examined the number of adult progeny by scoring the number of eclosed adult offspring that was produced. During the period from the second to the fifth day from eclosion we examined the total number of adult progeny for each day. From the fifth day onwards, progeny output was scored for intervals of three days. After 4 weeks, the adult progeny output was measured in alternating intervals of 3 and 4 days.

Statistics

All data were analysed using JMP 5.0.1. Longevity data were analysed using Cox Proportional hazards analysis. The starvation and paraquat resistance data as well as the metabolic rate data and those on reproductive output were analysed using full factorial ANOVA. Fixed factors always included treatment (e.g. age of testing, mated vs. unmated longevity, early vs. late reproduction) and line, and in most cases the factor sex. *Post hoc* analysis was performed using a Tukey test in order to examine which groups differed from one another in the different experiments. Replicate vials were always nested into the factor sex and treated as a random factor and did not prove to significantly contribute to the observed variation. Different time points in starvation resistance, paraquat resistance and metabolic rate were analysed as category variables.

Results

Longevity of virgins

We examined whether there was a difference between the lines for each medium and temperature. On standard medium, in general the *methuselah* mutant lived significantly longer than w^{1118} (χ^2_1=46, P<0.0001), but on double medium this was reversed (χ^2_1=11, P=0.0007, see figure 2). In the overall analysis, the effects of the different alleles was dependent on the sex of the individuals (line*sex interaction χ^2_1=5.4, P=0.02), therefore, the sexes were analysed separately. On standard medium, female mth^1 flies were longer-lived than w^{1118} flies at both 18 and 25 ºC, whereas for males there were no significant differences. On double medium, we found that w^{1118} males at both temperatures lived longer than the experimental group, whereas w^{1118} females only lived longer at 25 ºC, but the pattern at 18. Females on double medium at 18 ºC was the same (see figure 2).

Thus, under standard conditions we confirmed the longer life span of the mth^1 mutant, but we could not on double medium. Also, the sexes revealed strong differences in their life span response as a result of the different alleles.

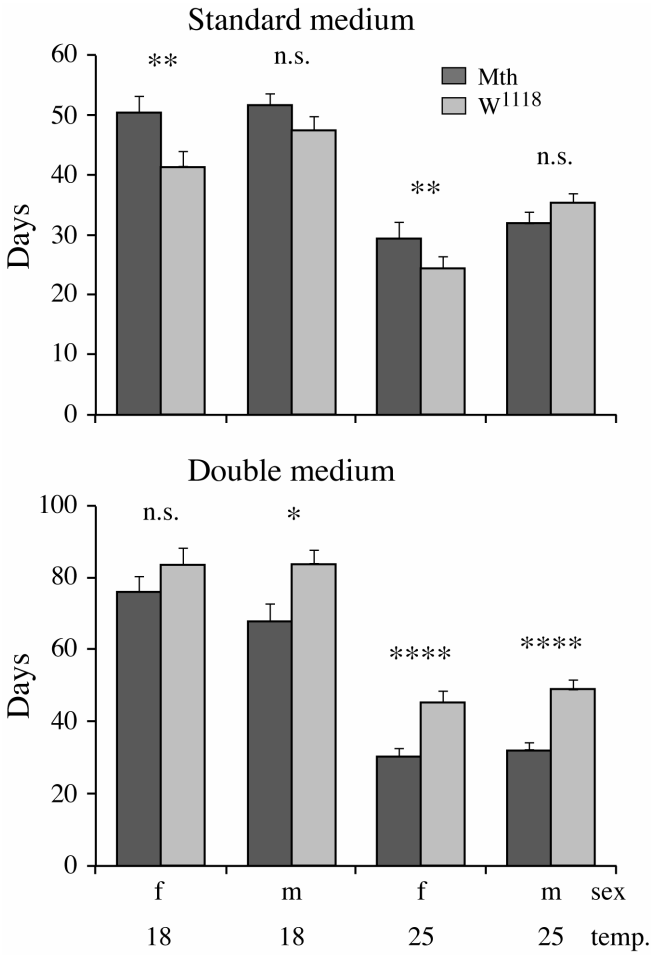

Figure 2. Longevity of both lines and both sexes with standard error bars on standard or double medium at 18 and 25ºC. n.s. = not significant, * <0.05, ** <0.01, **** <0.0001.

Longevity of mated versus virgin females at 25ºC

The overall analysis showed a significant effect of mating status (χ^2_1=5.25, P=0.022). Examining the effect of mating status per line (figure 3) reveals a significant negative effect of mating in *mth*^{1} females (χ^2_1=7.4, P=0.0064). In *w*^{1118} females a similar trend was observed, only the difference was not significant (χ^2_1=0.29, P=0.59). Overall, there was no significant effect of genotype on life span (χ^2_1=2.7, P=0.10). The apparent difference in longevity between the genotypes in virgins was not found in mated individuals. Thus, it was concluded that when mated, *mth*^{1} individuals lose their long-lived phenotype.

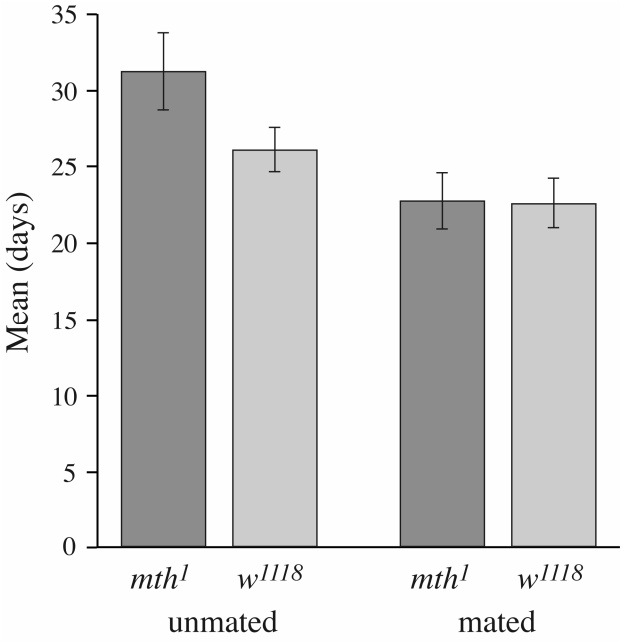

Figure 3. Average life span of unmated and mated females of lines *mth*^{1} and *w*^{1118} with standard error bars.

Metabolic rate

Mth^{1} flies of both sexes had a lower metabolic rate than *w*^{1118} flies at all ages measured (figure 4). In both sexes, at all ages, and time points, except for day 3 males, these differences were statistically significant. There was a remarkable increase in metabolic rate in *w*^{1118} males on day 5 just after the peak of female reproductive activity ($F_{3,160}$=5.0, P=0.0025, figure 5). Relative to the metabolic rate at other ages, the metabolic rate of *w*^{1118} males on day 5 proved to be significantly different from the other days when examined with *post hoc* Tukey testing. In females a similar trend was observed, but no significant differences were identified.

Paraquat resistance

There is a dramatic decrease in paraquat resistance with increasing age in both *mth*^{1} and *w*^{1118} flies (Figure 4). Males showed a higher average paraquat resistance than females ($F_{1,1583}$=18.8, P<0.0001). There is also a significantly higher paraquat resistance directly after eclosion in females of *w*^{1118} flies compared to *mth* females. Male *mth*^{1} flies remain more paraquat resistant than *w*^{1118} flies until day 10. In both sexes *mth*^{1} is significantly more paraquat resistant at days 3 and 5. The observed differences do not approach the extent of those found by Lin et al. (1998); especially the poor performance of the *w*^{1118} genotype could not be replicated.

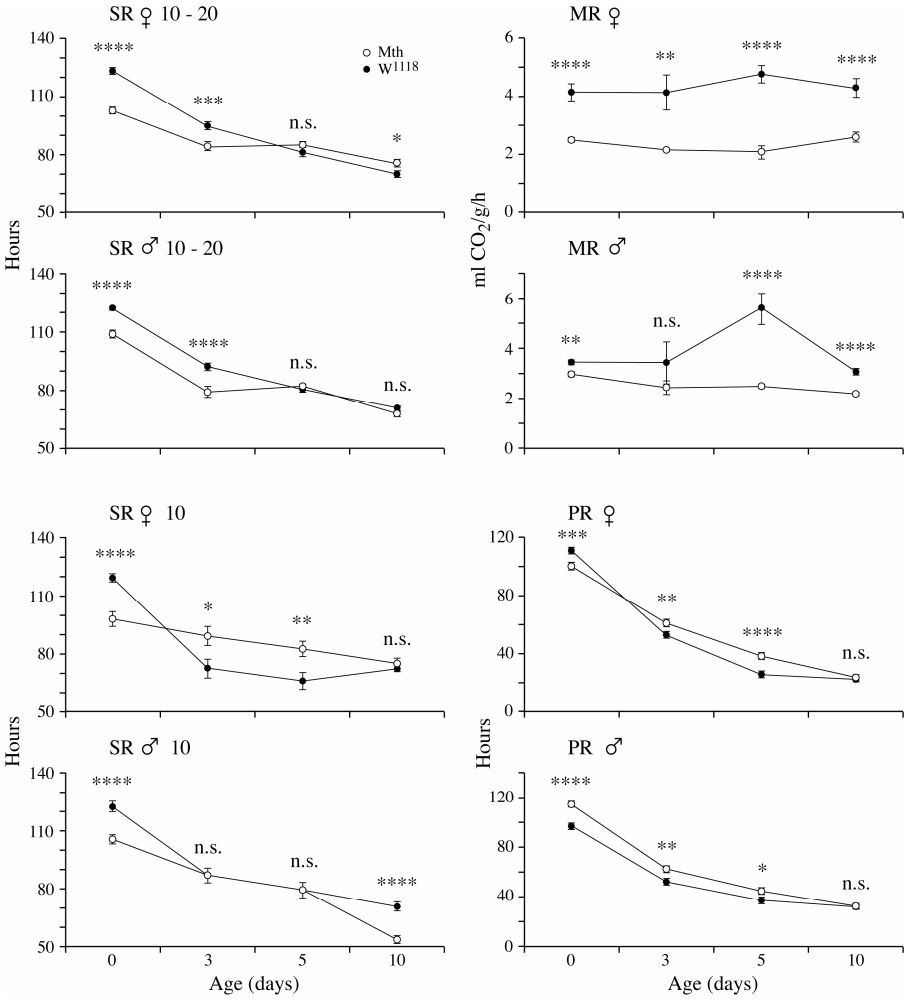

Figure 4. Patterns of traits on days 0, 3, 5 and 10 for both sexes of each line, all means ± s.e. Starvation resistance in hours (SR) at adult densities of 10 and 10-20 individuals per vial, mass specific metabolic rate in ml CO2/g/h (MR), paraquat resistance (PR) in hours. n.s. = not significant, * <0.05, ** <0.01, *** <0.001, **** <0.0001.

Starvation resistance

Starvation resistance showed a decline over lifetime as described earlier by Vermeulen et al. (2005). In adults kept at a density of 10-20 individuals, we found a noteworthy difference in starvation resistance in the first few days from eclosion when w^{1118} was highly resistant in both sexes relative to both mth^{1} and to its own resistance at older ages

(see Figure 4). From day 5 onwards, the difference became negligible and even switched to a slight but significant starvation resistance advantage for *mth*^{1} females at day 10 (Figure 4). In flies with controlled adult density, we observed a different pattern, where *mth*^{1} female flies at days 3 and 5 were significantly more resistant (Figure 4). Again, we were not able to reproduce the large differences and advantages of the P-element insert as observed by Lin et al. (1998). Our results do however suggest that at eclosion *w*^{1118} has a superior starvation resistance compared to *mth*^{1}. This result is consistent for the density groups.

Reproductive investment

The total number of live adult progeny was highly dependent on age ($F_{10,738}$=163, P<0.0001) and showed a significant age*line interaction ($F_{10,738}$=12.1, P<0.0001). Figure 5 shows that on day 2, and at a relatively old age (from day 23 on) the *methuselah* mutant flies exhibit a higher reproductive output whereas *w*^{1118} flies produce more offspring early in life (days 3,4 and days 9 to 12). On days 2 to 12 there was a significantly higher number of adult progeny output in *w*^{1118} ($F_{1,464}$=7.65, P=0.0059), whereas the reverse occurred on days 23-33 ($F_{1,92}$=8.56, P=0.0043). It is intriguing to notice that reproduction between days 0-2 is significantly lower in *w*^{1118} females compared to *mth*^{1} females. This may relate to the higher starvation resistance of the *w*^{1118} females, because starvation resistance and reproduction have been shown to phenotypically (Chippindale et al. 1993) and genetically (Zwaan et al. 1995a) trade off.

In summary, lifetime reproductive output of females of *methuselah* mutants and *w*^{1118} was equal, but *w*^{1118} displays higher early fecundity over the first 12 days, whereas *mth*^{1} had higher late life fecundity.

Discussion

General

We show here that the long-lived phenotype of the mutant *methuselah* is strongly dependent on the adult environment. We have presented evidence that implies that the earlier findings (Lin et al. 1998) on longevity and stress resistance must be treated with caution when extrapolated to other environments. Starvation- and paraquat resistance showed differences that are not consistent with earlier findings. In some cases *mth*^{1} flies outperformed their progenitor strain. However, in no case, was this increase in resistance consistently present over time. These differences may have to be attributed to differences in methodology and approach, but are reminiscent of how dependent these resistances are on test conditions. Furthermore, a novel finding is that the metabolism of *mth* mutants at 25ºC is always lower than that of *w*^{1118} individuals. We confirm the earlier findings concerning longevity of Lin et al. (1998) only in certain environments where reproductive output is low. Our data on the reproductive output suggest that the increased life span of the *methuselah* mutant may be the result of reduced early fecundity. Moreover, mated *mth*^{1} females were significantly shorter-lived than virgins, whereas the difference in *w*^{1118} females was less extreme, indicating an increased cost of reproduction in *mth*^{1} females. Maximal reproductive output at young age is probably

necessary to achieve high fitness in most environments in nature. The reduced early fecundity, together with the loss of longevity as a result of mating suggests that the particular *methuselah* mutant under study will not have superior fitness in such natural populations.

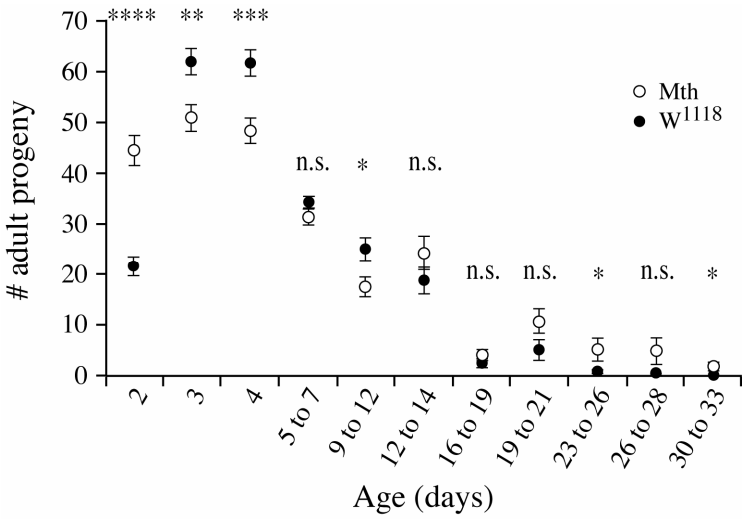

Figure 5. The number of adult progeny per day of female egg laying for each line. In the periods over more than one day, the number of individuals that eclosed were averaged to the number per day. n.s. = not significant, * <0.05, ** <0.01, *** <0.001, **** <0.0001.

Patterns over time and environments

Starvation resistance was measured at day 2 by Lin et al. (1998). Similar to their findings, in our experiments starvation resistance of animals kept at a density of 10 flies per vial showed that *mth*^{1} females have elevated starvation resistance at day 3. The flies maintained at the density of 10-20 flies per vial showed that *w*^{1118} is superior to *mth*^{1} flies in both sexes. This confirms advantages of the *mth*^{1} P-element in a certain, relatively favourable environment. In a more stressful environment, such as higher adult density, the *methuselah* mutant is apparently outperformed. Our data also indicate that the relative difference among lines for a trait depends heavily on the age at which the data is collected. That *w*^{1118} have a higher starvation resistance at eclosion than *mth*^{1} warrants further study which should involve fat allocation patterns.

Food and fecundity

The use of media other than the one used by Lin et al. may be responsible for the differences in survival. We also observed differences in life span on the double medium in the reversed direction (average longevity is generally higher in *w*^{1118}, see figure 2). The

amount of yeast the animals encountered in the double medium approximates that used by Mair et al. (2005) that facilitates life extension through caloric restriction (see table 1). The double medium yielded longevity values that approach the absolute maximum longevity of the lines on this medium. This is not the same as the maximal difference in longevity between the lines. The food condition of Lin et al (1998) is still the one under which the difference between mth^1 and w^{1118} is relatively the largest. They, like Cook-Wiens and Grotewiel (2002), used a medium where sugar concentrations (including cornmeal) were higher and yeast concentration was less than half that of our standard medium (see table 1).

Mair et al. (2005) found that reducing yeast calories had a disproportionate effect on longevity in relation to the removal of a similar amount of sugar calories from the adult media. This implies that the number of calories in yeast alone does not account for all of the longevity effects that occur with yeast removal. Yeast must therefore trigger additional processes. In *Drosophila*, the presence of yeast induces reproduction (Simmons and Bradley 1997) and is known to reduce longevity (Chippindale et al. 1993; Prowse and Partridge 1997). Here, we speculate that the relatively high proportion of yeast in our medium may reduce life span because of interactions with the reproductive system of the flies. This would be consistent with the reversal of longevity on double medium (see Figure 6). The condition dependent longevity difference between mth^1 and w^{1118} flies should therefore be explained as a classical genotype-by-environment interaction, which may involve differences in gene expression.

That reproductive biology was affected by the mutation was shown by the fact that w^{1118} females had significantly higher early life fecundity and mth^1females had significantly higher late life fecundity. Early life fecundity is often found to be associated with reduced longevity, and late life fecundity with increased longevity (Rose 1984; Leroi et al. 1994a; 1994b; Zwaan et al. 1995a, 1995b; Djawdan et al. 1996; Ackermann et al. 2001; Phelan et al. 2003). Rose's (1984, see his fig. 5) longevity associated late life fecundity seems similar to the combination of increased longevity of mth^1 under standard conditions and their late life fecundity.

Natural conditions and the relativity of superiority in the laboratory

Spencer et al. (2003) noted that there is a considerable demographic laboratory bias in the *Drosophila* populations under study. They argue that laboratory *Drosophila* are relatively short-lived. This could result in the situation where a mutant, whilst relatively 'long-lived' compared to its deficient progenitor strain, only restores a sub-optimal phenotype relative to natural populations. In such light, the superiority of the *mth* mutants is less striking. Nevertheless, the mutation causes a relative increase in life span under certain conditions and is therefore a candidate for further research into mechanisms prolonging life span. Schmidt et al (2000) found a positive correlation between latitude and both mth^1 allele frequency and longevity. They suggested that "in natural populations, selection on mth^1 may involve a pleiotropic trade-off between longevity and other fitness-related traits that are negatively affected". The fact that the *mth* locus carries imprints of past selection in natural populations (Schmidt et al. 2000; Duvernell et al. 2003) makes *mth* an even more important candidate for further study.

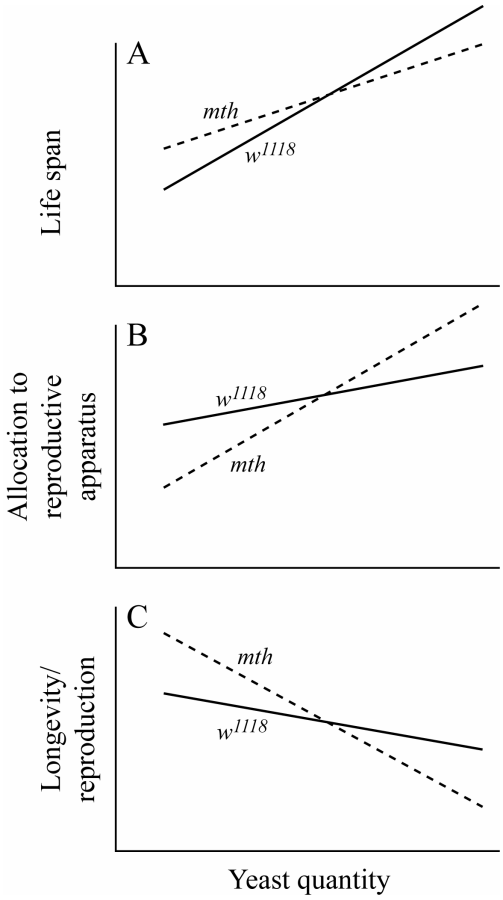

Figure 6. Hypothetical relationships of different traits with yeast quantity. In all figures the dotted line represents *mth* and the solid one *w1118*.
A. Hypothetical relationship between life span and yeast quantity in the medium. B. the hypothetical relationship between resource allocation and yeast quantity. The steeper reaction norm of the *mth* mutant indicates the larger influence of yeast on reproductive output relative to that on *w1118*. C. The relationship between longevity and reproduction as a function of yeast quantity. The steep reaction norm of longevity over reproduction in relation to yeast of *mth* results in higher longevity under poor yeast nutritional conditions.

Our results show that *methuselah* mutants underperform relative to its progenitor strain in most cases under conditions that arguably resemble a more natural situation. *mth* is likely to be under selection in nature (Schmidt et al. 2000; Duvernell et al. 2003), but our findings provide both evolutionary and mechanistic causal explanations as to why a

highly advantageous genotype similar to *mth*^{1} has not been identified in nature. One may argue that the fact that mutants like *mth*^{1} have not been found outside of the laboratory so far does not mean that they are not there. Yet, such advantageous alleles are likely to spread rapidly through the population. It would therefore be very interesting to have a functional comparison among the mutant and some long-lived individuals from natural systems with *mth* alleles with a natural selection imprint as described by Duvernell et al. (2003) and Schmidt et al.(2000). In this way, one could determine whether the selection imprint is acting in the same direction as the laboratory mutation.

Our data underline the importance of including temporal, physiological and environmental components in life history theory in general, and of trade-offs in particular. To identify these important components we will have to expand our knowledge of genetic and physiological systems at different levels. Kaitala (1987; 1991) and Leroi et al. (1994c) demonstrated the plasticity of trade-offs, which complicates investigation in natural systems considerably. Furthermore, the ubiquity of genotype-by-environment interactions makes predicting longevity on the basis of the *mth* genotype in unknown environments difficult.

Mutant analysis in life history theory

In all studies on life span enhancing genetic effects, the mutation takes place in a single gene. This gene may be pleiotropic in its nature and thus affect more than one trait. These effects are likely to induce the expression of a lot of genes. Yet, still a single gene would be responsible for the onset of increased longevity. From earlier experimentation we know that longevity is a quantitative trait (cf. Geiger-Thornsberry and Mackay 2004) and not determined by a single locus. This does not mean that the genes found in mutant research are useless and can be refuted. On the contrary, variation in these genes is likely to contribute to life span. From the wide variety of pathways and processes that are involved in life span determination, we know that even a single mechanism is unlikely to explain all of the life span variation present in nature. Variation at the *mth* locus in nature (Schmidt et al. 2000) is found to be correlated with longevity, which confirms that mth is related to longevity as found by Lin et al. (1998). Therefore, the mth locus is in part responsible for the variation in life span in natural systems, but it is not clear whether the genetic effect induced by the P-element insert is important in nature.. Because there are a lot of these kinds of mechanisms, one should be modest about the implications of finding a longevity associated gene.

When in natural populations only a smaller part of the variation can be explained by the variation on a single locus, the long lived phenotype must also be ultimately dependent on other, genetic or environmental factors. In the era of full genome expression analysis, one should be aware that the genetic background in which a single mutation operates is very important. This may or may not facilitate desirable pleiotropic effects. Even so, as proven here, the environment in which the life span enhancement is found is also of considerable importance, let alone genotype-by-environment interactions.

In the *mth*^{1} example one works with a hampered protein involved in neural transmission. The protein has been selected under natural conditions to be functional and is thus likely to be adaptive in these selective environments. If it does not, no selection imprint could

have been identified on it. A hampered protein loses part of its functionality and will thus not allow an animal to be fully functional in the environment it was selected in.

Though seemingly advantageous under certain laboratory conditions, under natural conditions large disturbances of the homeostasis are seldom beneficial. We, therefore, argue that mutation research is valuable, but only to provide us with candidate mechanisms that may form parts of the longevity puzzle, which, next to genetic components, also involves physiological and environmental aspects and the interactions among all of these. Our work thus contributes to understanding the interplay between genetics and environment in determining the ageing and longevity phenotype.

Summarising Discussion

As I am finishing up this thesis, my most important task has just begun.

Summarising Discussion

Setting the stage

In the IOP Genomics program "The genetics of longevity and disease at old age" epidemiologists, gerontologists and evolutionary biologists have integrated their knowledge and approaches to unravel the genetics underpinning longevity. The evolutionary biology projects consisted of one project on *Bicyclus anynana* and two on *Drosophila*. One of the *D. melanogaster* experiments has been conducted at the Evolutionary Genetics group from the University of Groningen and focuses on the genetics of longevity. In Leiden, I have conducted experiments concerning starvation resistance and longevity in three species of *Drosophila*, with a focus on *D. melanogaster*.

In this thesis, I have attempted to integrate knowledge from a wide variety of disciplines and levels of research. I tinkered with flies in pre-adult and adult stages, meddled with environmental and genetic factors and examined whether we could observe effects in physiology and life histories. In doing so, I compared species, selection lines, and a single gene mutant to obtain insights into the genetics underlying starvation resistance and longevity.

The focus of my thesis lies on the association between starvation resistance and longevity. Therefore, I used artificial selection, the effects of pre-adult and adult environmental manipulation on adult life history traits, genotype-by-environment interactions and micro-arrays as my tools. Throughout the thesis I attempted to unravel general patterns that may be informative about the mechanisms underlying these life history traits. Here, the accumulated knowledge is sieved, weighted, and integrated.

Starvation resistance does not necessarily mean longevity

Longevity as such is generally not a trait that is under selection in nature. This is because longevity is hidden in a so called selection shadow (Hoekstra 1993). The exceptions are mammals that provide parental and grandparental care. In these groups living longer after reproductive cessation (e.g. menopause) has an advantage in terms of provisioning offspring (e.g. Hawkes et al. 1998; Packer et al. 1998; Sherman 1998; Kirkwood 2001, 2002). This means that the chance of survival to that age is low and that those that do make it have a long-lived genotype. This may not be advantageous in the evolutionary sense, because the additional age will not result in (a lot) more offspring. Therefore, since there is no advantage, there can be no selection in favour of this trait specifically. In nature, the risk of dying early

irrespective of genotype is so large that there is no selective advantage of having a long lived genotype; selection is on optimal reproductive lifespan. Longevity is the result of a correlated response to selection on other traits that facilitate optimal reproduction. Longevity must thus hitchhike along with other traits that are under selection in nature. Since the IOP project focused on naturally occurring genetic variation in humans, I focussed on starvation resistance, which has a positive correlation with longevity. Increased resistance to adversity implies increased potential to survive adverse conditions and be prepared for better ones to invest in energy consuming processes such as reproduction and development. Starvation resistance does, however, not necessarily take longevity along proportionally (see Chapters 1, 3 and 4) and there have been reports of gradual dissociation between these traits (Archer et al. 2003; Phelan et al. 2003). Longevity is also associated with other traits, such as paraquat resistance (e.g. Vettraino et al. 2001), development time (DaCunha et al. 1995, yet see Zwaan et al. 1995a), metabolic rate (Riha and Luckinbill 1996; Braeckman et al. 2001; Lin et al. 2002, but see Hulbert et al. 2004), desiccation resistance (Graves et al. 1992; Hoffmann and Harshman 1999) and reproduction (ref Chippindale). Increased stress resistance is found in lines selected for increased longevity (Service et al. 1985; Service 1987; Leroi et al. 1994b; Harshman et al. 1999b, but not always see Force et al. 1995). More specifically, some studies showed an increase in longevity as a result of selecting on increased starvation resistance (Rose et al. 1992; Chippindale et al. 1996). In this thesis I have found that longevity and starvation resistance, though often found to be correlated, may not be as tightly linked as was previously thought.

In Chapter 1, we found that there was a general adverse effect of larval density on adult life history traits. However, we observed that in *D. melanogaster* and *D. ananassae* the effects on starvation resistance were more severe than those on longevity, whereas the reverse was observed in *D. willistoni*. Usually the life span decreased with increasing larval density, although not in all cases. This indicates that there is no strict one-on-one relationship between longevity and starvation resistance. Inducing an increase in starvation resistance by means of environmental manipulation does therefore not necessarily invoke a similar response in longevity and *vice versa*.

In our selection experiment in Chapter 3 we found that of the four selected lines with a considerable increase in starvation resistance, two showed an increase in longevity; the other two did not. Interestingly, the two lines that became long lived had also acquired an increased paraquat resistance. Apparently, by selecting on increased starvation resistance, longevity becomes selected for simultaneously in a subset of lines. Moreover, in the second principal component in Chapter 3, longevity and starvation resistance are effectively contrasted. These findings show that longevity is indeed a secondary trait that arises by selection for another trait. Again, starvation resistance and longevity were found to be not as closely related as was thought earlier.

In Chapter 4, lines selected for both traits were found to show interactions as a result of extreme environmental challenges. The long lived lines had an advantage in affluent situations, whereas the starvation resistant lines had this advantage in adverse circumstances. Relative to their controls both line types had a skew in their relative life span optimum as compared to their control lines. The differences between the lines give rise to a genotype-by-environment interaction, which indicates

that the genotypes underlying longevity and starvation resistance are to a certain extent different.

In summary, in both our genetic and environmental manipulation studies the link between starvation resistance and longevity is present, but the absence of one does not prevent the presence of the other and *vice versa*. Both traits seem to respond in a similar way to pre-adult environmental manipulation, but the associations between the traits are not tight. When examined under different adult conditions, there is a considerable genotype-by-environment interaction, which shows that the underlying genotypes are adapted to their selection environments. On the basis of the findings in Chapters 1 and 4 we state that the correlated response that is quite often found between longevity and starvation resistance is modulated by both the pre-adult and adult food conditions.

Fat content, prerequisite not cause

Fat content is often thought to be the main factor underlying genetic differences in starvation resistance (Robinson et al. 2000; Zera and Zhao 2003; Harbison et al. 2004), and the resource underpinning the phenotypic trade off between early fecundity and starvation resistance (Chippindale et al. 1993; Leroi et al. 1994a). In Chapter 1 we found that although the starvation resistance of animals is reduced with increased larval density, the relative fat content is increased simultaneously. Thus, for environmental manipulation experiments we conclude that relative fat content can not be the only resource for starvation resistance. In Chapter 3 we manipulated the genetics of starvation resistance by using artificial selection. We observed a large increase in relative fat content with a subsequent increase in starvation resistance. Metabolic rate in selection lines was comparable to controls under fed conditions and higher under starved conditions. Selection for increased starvation resistance leads to an increased build up of reserves in the pre-adult stages, so that the individuals have more resources to survive for a longer period under the absence of adult food.

We can thus state that increased relative fat content does not necessarily induce an increase in starvation resistance, but that when starvation resistance is increased, it does so through increased relative fat content. When more resources are allocated to triglyceride build up, potentially starvation resistance is higher. Yet, this is not the only factor involved; the rest of the animal's physiology also has to be set for allocation to starvation resistance. We conclude that fat is a prerequisite rather than a causal factor for increased starvation resistance.

Pre-adult conditions

The pre-adult environment is of considerable importance in determining adult longevity (see Chapters 1 and 2). Larval adversity negatively affects adult life history.

In Chapter 2, we have tested part of the Barker hypothesis. The Barker hypothesis is a medical hypothesis that states that adverse pre-adult conditions result in elevated

risk of adult metabolic syndrome, which reduces life span. We have examined what the effect was of offering little and abundant food in the pre-adult stage. The control and abundant medium-bred animals could not be distinguished in their life history traits and physiological characters. The animals reared at half medium, the adverse state, needed more time to develop, and had lower adult body weight and a reduced life span. This finding of a similar phenotype hints at the Barker hypothesis. Yet, to know this for sure, adult flies from adverse larval conditions need to be diagnosed with similar metabolic diseases as play a role in the human case. The comparability of the fruit fly model and humans on this pathological level remains to be seen, because known diabetic phenotypes have not reduced *Drosophila* lifespan, but rather enhanced it (Broughton et al 2005).

The question remains whether this effect of pre-adult stress on adult life span is a scar or adaptive plasticity. Scar is most simply explained; the individual gets damaged and consequently functions less well. Adaptive plasticity is a more complex explanation. The organism then has a “buffer” mechanism by which it is able to withstand environments other than the ideal one. During evolution these mechanisms are likely to have arisen because selective pressure never acts on a single feature. From this point of view the reduction in life span is the consequence of a programmed response in allocation. For now, we cannot answer this question, but are intrigued by the ongoing research in this field.

Reproduction, longevity and the relevance of mutant studies

Reproductive output is the ultimate measure of fitness; the traits that determine the life history of an individual are configured to maximum reproduction. The soma is the vehicle of the germ line and therefore, somatic maintenance is necessary to optimise reproductive life span. Life span is defined as the time an individual stays alive. It does not concern the condition in which the individual reaches old age. The work of Cook-Wiens and Grotewiel (2002) shows that the extended longevity of the methuselah fly does not go together with extended functional performance. This is not advantageous from a fitness point of view, especially, when this longevity comes at the cost of a reduced early fecundity, as is predicted by the antagonistic pleiotropy theory of ageing (Williams 1957). We examined this system in Chapter 6 by comparing mated and unmated individuals of the long-lived methuselah (*mth*) strain. On the whole, reproduction reduced longevity. During reproduction, the longevity advantage of the *mth* mutants is strongly reduced and their life span cannot be distinguished from that of the progenitor strain. We stimulated the reproductive output by exposing the flies to higher yeast concentrations, but made sure the flies were unable to mate. We then observed a dosage dependent decline in longevity of the long-lived mutant relative to its progenitor strain. In the most extreme case, longevity of methuselah was even lower than that of its ancestor. This shows that the validity of correlative studies is directly related to the conditions in which the correlation has been confirmed. Extrapolation of findings to nature should be done with caution in the cases where genotype-by-environment interactions have not been estimated. In addition, it shows that the stunning longevity of some mutant strains may be very condition dependent. Methuselah tends to respond more extremely to changes in the environmental reproductive cues; living longer in their absence and shorter in their presence. Their reproductive output has also shifted towards increased late-life

reproductive success and reduced early-life reproductive output. In combination with the high stochastic mortality in nature this would make fitness lower, and would lead to a lower frequency of the *mth* allele in the population after each generation and subsequently extinction. Evident from this example and literature is that mutations that seem advantageous may only be so in the laboratory. The chance that we will find a truly advantageous mutation in terms of life span and its correlated traits that is relevant to natural situations remains low, despite the great effort that is put into it. On the other hand, humans in the western society also do not live in fully natural conditions any longer, with medication, social welfare and lack of predation, making present-day society perhaps more reminiscent of the laboratory than of nature.

Correlations with paraquat resistance

Longevity is clearly not fully dependent on starvation resistance. Generally, it does show a good correlation with paraquat resistance (e.g. Arking et al. 1991; Force et al. 1995, see also Arking et al. 2000; Vettraino et al. 2001, where even different extended longevity phenotypes are found). Starvation resistance correlated in a similar way to paraquat resistance as it did to longevity in our selected lines (Chapter 3). This indicates that selection on increased starvation resistance may, but does not necessarily, induce other traits to co-evolve. We observed that paraquat resistance and longevity are increased together in lines SR1 and SR2. In the second principal component for the overall variation, we observed that paraquat resistance had a negative vector value, just as longevity, whereas all other traits show positive vector values. The paraquat resistance of the mutant line *mth* was found to be increased in the original paper by Lin et al. (1998). In Chapter 6, we found that paraquat resistance did not always differ consistently between the lines and that in most cases the progenitor line had a significantly higher paraquat resistance than the long-lived strain. Strikingly, the long lived mutant had also lost most of its longevity under the rearing condition in the Leiden laboratory.

Paraquat resistance is important because it is a measure of the resistance to oxidative damage. Protection against oxidative damage is often thought to underlie longevity. Detoxification of products that cause harm, as proposed by Gems and McElwee (2005) to be involved in longevity determination, is an important aspect of preservation and protection from oxidative damage. Starvation resistance can be the product of two types of processes: one is an increase in resources by which one can survive an adverse period, the other a reduction in expenditure, a so-called thrifty genotype (see general introduction). For both strategies, if the metabolic rate remains stable or is reduced there is no need for increased paraquat resistance, because there is no increased threat of oxidative damage. In Chapter 3, we provide evidence that metabolic rate is not increased nor decreased in the starvation resistant animals under fed conditions. Two out of four starvation resistant lines do not show an increase in paraquat resistance. The lines with increased paraquat resistance also show increased longevity, a correlation that has been shown before (Arking et al. 1991; Force et al. 1995; Arking et al. 2000; Vettraino et al. 2001). Therefore, we conclude that paraquat resistance is not causally linked to starvation resistance, but rather to longevity.

The disposable soma theory of ageing

Trade offs

The disposable soma theory of ageing has been proposed by Kirkwood (1977; Kirkwood and Holliday 1979). It states that there is a trade off between the reproductive tissues and the rest of the body. We know from the work of Chippindale et al. (1993) that such a trade off exists in *Drosophila*. The work of Patel et al. (2002) shows that germ line signalling reduces longevity and that ablation of the germ line cells increases life span in *C. elegans*. Leroi (2001) stated that such molecular signals are more likely to be the cause of the soma-germ line antagonism rather than resource allocation based trade offs. Barnes and Partridge (2003) opposed this by explaining that the findings from germ line cell ablation do not refute resource allocation based trade offs, but rather offer an additional mechanism underlying the soma-germ line contrast. Irrespective of the precise mechanism, this work underpins the antagonistic forces that separate the germ line and soma. Here, the introduction springs to mind. Life initially began as a self-reproducing unit that evolved a soma in order to convert energy more efficiently. Thus, the soma is for the germ line only a means to continue to exist over time. The fact that the germ line signals in Patel et al's work reduce longevity shows this basic interdependence.

In Chapter 3 we deduced that allocation to starvation resistance implies resources to be available for adverse times. Allocation to starvation resistance will reduce the allocation to reproduction. Therefore, reproduction and starvation resistance trade off. This was shown phenotypically by Chippindale et al. (1993). Since longevity can be correlated with starvation resistance, as was also shown in Chapter 3, longevity seems to be dependent on the allocation event (see figure 1). We have already shown that increased total fat content does not necessarily lead to an increase in starvation resistance in Chapter 1. We then concluded that there is more to starvation resistance than fat content, but it is a prerequisite for increased starvation resistance.

Mair et al. (2004) found that flies that are given less food are longer lived but do not show a reduction in reproductive rate. This may relate to the allocation of lipids. Eating a lot can be disadvantageous from a certain point onwards. A reduced access to food can therefore be relatively healthy and explain the increase in longevity. If enough resources are still available, the allocation of resources to the reproductive apparatus may not be limited by the diminished supply of food. In this way life span will be enhanced and reproductive output will remain the same. When the allocation has shifted and lipids are not allocated to reproduction but rather to the soma, the lipids will consequently be used by processes other than reproduction. One such process is starvation resistance. Here, we propose that when fat is allocated to the soma some mechanism may, but need not, simultaneously enhance potential life span. This matches the findings of Mourikis et al. (2006), who claim that lipid metabolism may play a role in life span regulation.

Because we found a large increase in fat content in the selected lines in Chapter 3, we expect that allocation in our flies is towards the soma instead of the germ line. As a result of selection under the absence of food, the animals have increased allocation to the soma at the cost of allocation to the germ line. Allocation to the germ line is not relevant in this set up, and is thus selected against.

In Chapter 5, we examined genes that were differentially expressed between a long lived starvation resistant line and its control under fed and starved conditions. Under normal, fed conditions we observed few differences between the lines, which was in sharp contrast with the large number of differentially expressed genes we found under starved conditions. We found that the starvation selected line expressed genes at a lower level that were involved in neural transmission, insulin signalling, glycolysis and reproduction. This means that the starvation selected flies have adapted to this situation by down regulating a large cascade of events that are linked to one another. Some gene categories have been discussed in more detail in Chapter 5. Here, I will focus on the low expression of reproduction related genes. Down regulation of these genes is in congruency with the hypothesis that in our selected lines reproductive output will be smaller than in the control lines.

The effect of genotype-by-environment interactions

From the above it follows that the response to starvation resistance selection has involved the trade off between soma and germ line. Leroi et al. (1994a; 1994b) showed that trade offs may be present among environments and populations and may be obscured by strong genotype-by-environment interactions. Apparently, genotype-by-environment interactions pose a caveat on the interpretation of correlative data. Hence, trade offs must always be interpreted in relation to the environment the genotypes were selected in. In Chapters 3 and 4 we found that lines selected for starvation resistance in some cases display long life span under fed conditions and in others do not. The long lived and starvation resistant lines displayed strong genotype-by-environment interactions over affluent and adverse conditions. The genotype-by-environment interactions indicated that each selected direction has its own skew in longer life span relative to the control line. Lines selected for longevity or starvation resistance should be regarded specialists that outperform in the condition they have been selected in.

Similarly, the work on the mutant methuselah revealed a strong effect of the environmental conditions on adult life histories. In Chapter 6, we showed that in conditions favouring reproduction, the longevity advantage of the mutant had faded. Also, the starvation resistance was relatively low and the longevity was not as elevated as was previously found by Lin et al. (1998). In the original study by Lin et al. (1998) little yeast was used, and methuselah lived longest. In the Leiden laboratory, we use a medium that contains a lot of yeast, which is known to enhance reproduction in fruit flies (Simmons and Bradley 1997). Under food conditions that contained even more yeast, the long-lived mutant even underperformed relative to the control. Therefore, we state that by shifting the emphasis to reproduction, longevity suffered in methuselah.

In summary, we expect that in the starvation selected lines reproductive output is reduced. This is underpinned not only by theory but also by gene expression studies. Furthermore, the trade off between starvation resistance and longevity, and reproduction is highly environment dependent, as is the relation between starvation resistance and longevity.

A new synthesis

Longevity and starvation resistance have often been found to negatively correlate with reproduction. This is in line with the disposable soma theory of ageing (Kirkwood and Holliday 1979; Kirkwood and Rose 1991). There, the allocation difference to the soma and germ line tissues, and the interaction between these determines longevity. Evidence for this hypothesis has been presented from different disciplines (Chippindale et al. 1993; Patel et al. 2002). Barnes and Partridge (2003) explained that resource allocation is at least in part responsible for life history trade offs. The initial allocation to soma or germ line causes the general observation of the link between starvation resistance and longevity. If we combine this with the skew of the life span advantage in the selected lines (Chapter 4) and the variability in the correlation found in Chapter 1, we see that longevity and starvation resistance are not intimately linked. Van Noordwijk and De Jong (1986) have shown that a positive correlation can be found among traits that are expected to trade off. Their analogy with economics provides a good insight in how these positive correlations can be found. With a certain budget one can buy a house and a car. The more one spends on the house, the less there is to spend on a car and *vice versa*. This resembles the trade off model. Yet, if the income of a family is higher, they can spend more on both housing and cars. Therefore, families having more money will probably have a large house and ditto car. This results in a positive correlation. Here, we found a similar situation. When all resources are allocated to the soma, both starvation resistance and other mechanisms can benefit. Yet, because the amount of resources is fixed there will be an allocation-fraction (cf. fraction B in Van Noordwijk and De Jong, 1986) dependent correlation between starvation resistance and longevity. On the basis of our findings and this model, I hypothesise that after allocation to the soma side there is a second allocation event that may lead to an extra increase in either starvation resistance or longevity (figure 1).

Thus far it has been found that fat and its allocation are of importance to starvation resistance (Chapters 1 and 3). Also, longevity is a by product of increased starvation resistance and is found to associate with paraquat resistance (Chapters 3 and 4). Furthermore, insulin signalling is important for the germ line-soma contrast (Chippindale et al. 1993; Patel et al. 2002; Broughton et al. 2005) and longevity is dependent on insulin signalling (Clancy et al. 2001; Tatar et al. 2001; Tatar et al. 2003; Broughton et al. 2005). Longevity and starvation resistance do not respond proportionally among species or as a result of the ablation of neurons involved in insulin signalling (Broughton et al. 2005). Thus, if present, the more subtle effects of the hypothesised secondary allocation event will depend on mechanisms other than insulin signalling. We suggest that the second allocation event between starvation resistance and longevity is dependent on lipid metabolism. Allocation to the soma side through insulin signalling makes all soma processes gain resources. The different processes on the soma side can be adjusted by selection in multiple ways. If this involves a strong increase in reserve building lipid metabolism, starvation resistance will be altered. If allocation is not targeted so specifically, other processes like somatic maintenance, will gain this energy and increase other traits, including longevity.

The fact that the high fat content in Chapter 1 did not lead to increased starvation resistance may have to do with the first allocation point. As a result of severe crowding, the surviving individuals are set to remain ahead of their competitors and

allocate their resources to reproductive output rather than to the soma. This would also explain the smaller body size at high larval density. In Chapter 3, we then observed two lines that were selected for starvation resistance on the basis of features that act before the second allocation event, taking paraquat resistance and longevity along in the selection process. The starvation resistant lines that do not show increased longevity, SR3 and SR4, will then have been selected for starvation resistance on features after the second split point. The absence of starvation resistance in the long lived lines in Chapter 4, could have arisen from selection on processes after the second split point. The single gene mutant methuselah, did not show paraquat or starvation resistance advantages in our set-up in Chapter 5, but did in the Lin et al. (1998) set-up, together with a more extreme longevity. Apparently, this mutation works on features that lie before the second split. This also explains why the extended longevity and associated features do not appear in more reproduction enhancing environments, which act on the first allocation point. A similar story may be applicable to the short lived lines from Chapter 4.

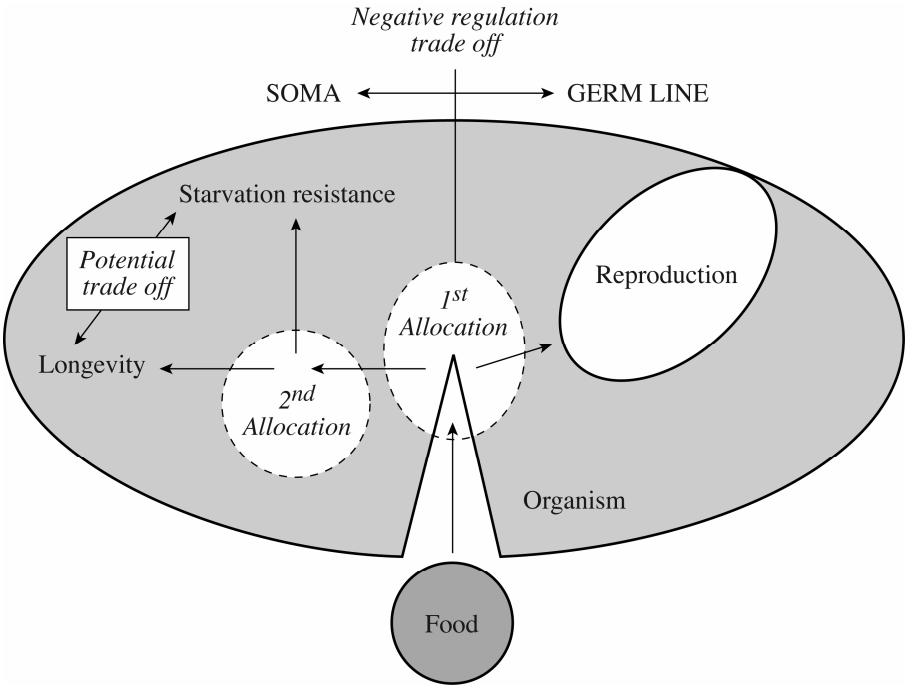

Figure 1. Hypothetical life history determination scheme. Food allocation is possibly followed by a second decision on the soma side of the organism. This process, that can be either physiological or genetic in its regulation, could lead to a trade off between starvation resistance and longevity, depending on the genotype of the organism.

In summary, I conclude that as a result of genotype and environmental cues resources are allocated to either the germ line or the soma. On the soma side at least two mechanisms act to maintain somatic stability. These traits probably trade off following an Y-model, as proposed by Van Noordwijk and De Jong (1986). One of these is starvation resistance, which acts to maintain the soma when facing adversity. The other mechanism is about maintaining the quality of the soma for a long time, which involves longevity. This last mechanism may be associated with paraquat resistance or defence from damage in general. These mechanisms trade off. The state of the trade off seems to be the result of certain genotypes, which display their phenotypic advantage only in specific environments.

Conclusion

We know that the environment is the only thing the organism, a genetic product and gene carrier, has to live up to and from which it tries to be as independent as possible. Information on the response of this genetic product to the environment provides insight in the relatedness of characters and their environmental specificity.

We have examined life history traits by environmental and genetic manipulation. These approaches sometimes yield seemingly contrasting results. Integrating the interpretation of both types of data yields novel insights. It is clear that both genetics and the environment have large impacts on life history traits. Also, conditions in the larval stages clearly affect the adult stages. Overall, the general patterns that were found earlier were also found here. Building on these, new insights into the fundamental essence of the life histories under study could be obtained. The result was that because of our findings the genetics of starvation resistance and longevity have become partly unravelled and a new and hypothetical relationship between both traits has been suggested. With this hypothesis and the data found here, I trust that the intricate genetics of longevity can be studied in more detail than before.

General statements

1. There is more than one way for a polygenic trait to adapt.

2. A prerequisite is not necessarily a causal factor. Fat is not the cause of starvation resistance. To become starvation resistant, additional processes have to be tuned for starvation resistance as well.

3. One should be very cautious in interpreting correlations among life history traits because they may represent effects of chance, environment or evolutionary history rather than common genetics.

4. The validity of correlative studies is directly related to the variation in conditions in which the correlation has been confirmed.

5. One should be very lucky to find a mutant with a truly longer life-span without a reduction in fitness.

6. Genes and genomes are ultimately dependent on environments. They have been selected to cope with them and can horribly fail if they are not adapted to a certain environment.

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Nederlandstalige Samenvatting – Summary in Dutch

Nederlandstalige Samenvatting – Summary in Dutch

Voorwoord over promoveren, het belang van de brede promotie

Promoveren komt van het Latijnse werkwoord *promovere* wat een aanzienlijk aantal betekenissen heeft, waaronder; 1. bevorderen, 2. verder komen, 3. verzetten, en 4. uitbreiden. Niet toevallig zijn de definities die ik hier geef ook degenen die dicht bij de academische bedoeling van promoveren liggen. Door te promoveren word je bevorderd tot de graad van Doctor in de wetenschap, in mijn geval in de Wiskunde en Natuurwetenschappen. Een promotie heeft ook tot doel om verder te komen, qua begrip van een bepaald wetenschapsgebied, maar ook in termen van het verrichten van onderzoek dat leidt tot nieuwe inzichten. De derde definitie, verzetten, geldt voor de hoeveelheid werk die gedaan wordt. Fysiek in sommige wetenschappen, maar met nadruk ook geestelijk worden er behoorlijke bergen werk verzet tijdens een promotie.

In tegenstelling tot wat velen denken is een promovendus in Nederland niet alleen bezig met het doen van onderzoek en het schrijven van artikelen en een proefschrift. Vandaar ook dat de vierde definitie er expliciet bij is gezet, het uitbreiden. Een promovendus breidt gedurende zijn werk een heleboel dingen uit, niet alleen de wetenschap door vondsten of de eigen expertise door het opzuigen van kennis. Een promovendus dient zich bewust te zijn van de omgeving en niet louter naar binnen gericht te zijn. Het uitbreiden dat een promovendus doet houdt ook in dat hij anderen kennis laat nemen van de zaken waar de promovendus mee bezig is, variërend van jong tot oud, van arm tot rijk, van groen tot paars. Promovendi, in de klassieke zin van het woord, zijn ook een goed deel van hun tijd bezig met een vaak onderschatte bezigheid, het geven van onderwijs. Dit komt onder andere tot uiting in het geven van colleges en practica, en het begeleiden van onderzoeksprojecten, maar zeker ook door het voorlichten van het publiek over wat wetenschappers doen, en hoe de wereld nu eigenlijk volgens hen in elkaar zit. Naast deze didactische ervaring bouwt een promovendus een behoorlijke ervaring op in projectmanagement. Het binnen de tijd afronden van een project met een gelimiteerd budget en tal van verleidingen om zijpaden in te springen vereist discipline en het vermogen om te prioriteren.

Resumerend houdt een promotie in de klassieke zin in dat iemand zich bekwaamt op een groot aantal gebieden. De term promovendus heeft in sommige maatschappelijke kringen een negatieve bijklank gekregen; oubollig, vakidioot, naar binnen gericht en communicatief gestoord. Gezien de potentieel brede en diepe ontwikkeling van een promovendus is dit dus compleet afhankelijk van de persoon en niet van de bereikte mijlpaal.

Voorwaarde voor deze brede opleiding is wel dat de verbreding wordt gewaardeerd en er tijd voor wordt vrijgemaakt. Door het stimuleren van een brede ontwikkeling en dus het breed inzetbaar laten zijn van promovendi zou de Doctor titel afgestoft en opgepoetst kunnen worden. Het korte termijn belang van zoveel mogelijk publicaties behalen zal op de lange termijn ruimschoots terugbetaald worden door vergrote efficiëntie, het integreren van meerdere vakgebieden en goed onderwezen studenten. Een betere aansluiting van de promotie op de behoeften van de

maatschappij zal een betere verbintenis tussen de academische wereld en de onderwijskundige, industriële en beleidstechnische wereld opleveren.

De wetenschappelijke samenvatting

In deze Nederlandstalige samenvatting ben ik vrijer in mijn taalgebruik en vermijd ik vakjargon waar mogelijk. Het is niet de bedoeling om dit als een zwaar wetenschappelijk stuk aan te zetten. De opzet is dat een leek die niets van dit vakgebied weet, uitgelegd krijgt wat er globaal in deze dissertatie staat. Ik wens u veel plezier met het wetenschappelijke gedeelte.

Het IOP-Genomics programma, onderdeel van SenterNovem, in opdracht van het Ministerie van Economische Zaken heeft subsidie toegekend aan het project “De genetica van veroudering en ziekte op hoge leeftijd”. Binnen dit grote project waren er deelprojecten op het gebied van de Gerontologie (de medische wetenschap van het verouderen), de Moleculaire Epidemiologie (het wetenschapsgebied dat zich bezighoudt met de verspreiding van ziekten) en de evolutionair/kwantitatieve genetica (de wetenschap die zich bezighoudt met hoe genetische eigenschappen gedurende de evolutie ontwikkelen). Binnen deze laatste categorie vonden drie onderzoeken plaats; een aan het tropische zandoogje (*Bicyclus anynana*) en twee aan fruitvliegen (*Drosophila melanogaster* en andere soorten). Er werd gekeken naar de evolutie van levensloop eigenschappen zoals hongerresistentie, levensduur en voortplanting.

Wat je eerst moet weten

Levenscyclus *Drosophila*

In hoofdstuk 2 is in figuur 1 de levenscyclus schematisch weergegeven. Fruitvliegen zijn holometabool, wat wil zeggen dat ze een complete gedaanteverwisseling of metamorfose ondergaan. Ze hebben een ontwikkelingstijd van ongeveer 10 dagen. De ontwikkelingstijd is afhankelijk van de soort, de voedselkwaliteit en -kwantiteit, en de temperatuur. Bij 25°C en voldoende voedsel is de levenscyclus van D. melanogaster als volgt. De eerste dag nadat het ei is gelegd door het fruitvliegvrouwtje ontwikkelt het ei zich tot een larve die na een dag uit het eihuidje kruipt. De volgende 5 dagen vervelt de larve twee keer (ecdysis). Dat gaat als volgt: de larve eet zoveel dat hij helemaal vol zit en gooit dan zijn oude omhulsel af. Dan probeert hij zich zo groot mogelijk te maken en maakt een nieuw omhulsel aan. Als dit nog een keer gebeurd is, komt de larve op dag 5/6 in het zogenaamde ‘crawler’ stadium. In deze fase zoekt de volgegeten larve een goede plek om te verpoppen. *D. willistoni* (een andere soort fruitvlieg) doet dit bijvoorbeeld op het voedsel, terwijl *D. melanogaster* het liever wat hogerop zoekt. Tijdens de verpopping vindt de metamorfose plaats. De metamorfose is een van de meest complexe processen die in de natuur bekend zijn. De oude larve, made of rups wordt in de pop grotendeels opgelost en omgezet in een nieuw beest, de volwassen vorm (imago) die nageslacht produceert en bij de meeste insecten kan vliegen. In het geval van de fruitvliegen is

dit de overgang van larve via pop naar vlieg. Tot zover de levenscyclus van de fruitvlieg.

Mannetjes en vrouwtjes

Fruitvliegen hebben net als mensen twee sekses, mannetjes en vrouwtjes. De sekseverhouding lijkt zelfs op die van mensen, 50-50%. Als de vliegen uit de pop gekropen zijn kunnen ze binnen 8 uur paren. In dit onderzoek werden in de meeste gevallen de volwassen beestjes kort nadat ze uit de pop kwamen van elkaar gescheiden zodat de mannetjes en vrouwtjes niet konden paren. Voortplanting heeft namelijk een nadelig effect op de levensduur. Alleen in hoofdstuk 6 hebben we proeven gedaan met vliegen die gepaard hadden.

Wat eten ze?

Fruitvliegen leven, zoals hun naam al doet vermoeden, in de natuur van fruit. In het laboratorium is fruit als een medium slecht te gebruiken omdat de ene banaan wel eens meer suikers en andere stoffen kan bevatten dan de andere. Daarom gebruiken we hier het agar medium. De proeven zijn altijd gedaan op een agar medium, waar suiker en gist in opgelost is. Soms werd de concentratie van suiker en gist verhoogd of verlaagd om rijk of arm medium te krijgen. De toevoeging van levende gist aan fruitvliegenvoer zet de fruitvlieg aan tot voortplanting. In de natuur geeft levende gist op een rottend vruchtje aan dat de larve er gemakkelijk aan zijn voedsel kan komen; suikers uit de vrucht en eiwitten uit de gist. Gist is dus een signaal voor een fruitvlieg om zich te gaan voortplanten.

P = G + E + G*E

We beginnen met een formule. Deze formule beschrijft de uiterlijke eigenschappen van een organisme. De P staat voor het fenotype (phenotype in het Engels, vandaar de P), de uiterlijke verschijningsvorm of eigenschappen van een organisme. De eigenschappen van een organisme betreffen alles van het organisme; het gedrag, de haarkleur, littekens, kleding, maar ook hoe hard hij kan rennen, hoeveel kinderen hij krijgt en hoe oud hij wordt. Het fenotype is opgebouwd uit drie componenten die bepalen wat de eigenschappen worden. De eerste is de G van genetica. De genetica bepaalt voor een deel je eigenschappen, een aanleg voor obesitas is een goed voorbeeld daarvan. De tweede component is de E, die staat voor de omgeving. Een gedeelte van je eigenschappen wordt bepaald door je omgeving, een voorbeeld is een makkelijke toegang tot veel voedsel of niet. De lastigste is de G*E interactie factor die bekend staat als het genotype bij omgeving effect. Een goede manier om dit uit te leggen is je voor te stellen dat het effect van een bepaalde genetische eigenschap in de ene omgeving wel en in de andere niet slecht voor je is. Je kunt je voorstellen dat het hebben van de genetische aanleg voor obesitas in een ontwikkelingsland een minder groot probleem vormt dan in een welvarend land met een hamburgertent op iedere hoek van de straat. Sterker nog, het zou wel eens voordelig kunnen zijn in een ontwikkelingsland, maar daar kom ik in hoofdstuk 2 op terug.

Het fenotype bestaat dus uit een genetische, een omgevings- en een genotype-maal-omgeving interactie. Het fenotype 'dik zijn' kan dus het resultaat zijn van genen (zoals het *leptine*-gen), van de omgeving (we eten nu eenmaal veel te veel in dit land), maar zeker ook van het feit dat tot obesitas leidende genotypen in deze maatschappij (omgeving!) te veel voedsel binnenkrijgen; de genotype bij omgeving interactie.

Genetica voor beginners

"Het zit in de genen" hoor je mensen die er niet toe gekwalificeerd zijn vaak zeggen. Iedereen weet tegenwoordig wel dat ieder levend wezen genetisch materiaal heeft (DNA of RNA, maar dat is een ander verhaal) waar genen op liggen. Een gen is dus niets meer dan een groot molecuul dat informatie bevat. Doordat af en toe foutjes worden gemaakt in de natuur zijn niet alle genen van alle organismen hetzelfde. De verschillende vormen van een gen worden de allelen genoemd. Zo krijg je verschillen waar de evolutie op aangrijpt. De genen evolueren onder de invloed van de natuurlijke selectie en zo ontstaan er nieuwe organismen die beter aangepast zijn aan de omgeving. Het is dus (in vrijwel alle gevallen) zo dat je niet zozeer de goede genen hebt: die heeft namelijk iedereen. Je hebt de goede allelen. Een gen is functioneel want het vormt in de meeste gevallen een overlevend fenotype. Dit doet het gen door zijn expressie, wat zoveel betekent als de activiteit van het gen. De expressie van een gen is eigenlijk vergelijkbaar met een thermostaat. Die kun je op 20 zetten, maar ook op 15. Als je de thermostaat op 20 zet gebeurt er meer (verbranding van gas) dan wanneer je hem op 15 zet. De hoogte van de genexpressie is een maat voor de activiteit van een gen en dus voor de belangrijkheid van het gen voor het fenotype van dat moment. We kunnen de expressie van alle genen van een organisme bestuderen met een zogenaamde micro-array of chip. In het geval van de *Drosophila* 2.0 chip die we hebben gebruikt gaat het om meer dan 18.000 verschillende expressievormen van genen. Fruitvliegen hebben ongeveer 13.500 genen. Sommige van die genen hebben meer dan één expressievorm. Om deze expressievarianten te bestuderen heb je meer dan de 13.500 genen nodig, vandaar dat er 18.000 varianten bekeken worden. De mens heeft ca. 20.000 genen, waarvan een groot deel vrijwel hetzelfde is in de fruitvlieg. Bedenk dit voor de volgende keer dat u met een vliegenmepper rondloopt; u vermoordt een genetische verwant.

Evolutie

Malthus was een 18^{e} eeuwse wetenschapper die zag dat er altijd meer beesten worden geboren dan waar voedsel voor is. Dit zorgt ervoor dat er dus altijd een paar beesten voedsel tekort komen en sterven. Hij snapte niet waarom er niet wat minder werd voortgeplant, zodat iedereen genoeg had. Hij observeerde echter dat dit verschijnsel overal en altijd optrad. Hij noemde dit proces 'The struggle for existence', de worsteling om het bestaan. De evolutietheorie van Darwin en Wallace bouwt hierop voort. De evolutietheorie houdt in dat ieder levend wezen probeert zich voort te planten. Om dat te kunnen moet het organisme beter aangepast zijn aan zijn omgeving dan de andere wezens die zich ook willen voortplanten. Als het organisme slechter is aangepast dan zijn concurrenten dan vergaart hij te weinig energie of bouwstoffen en kan hij zich minder succesvol voortplanten en sterft uit. Daarom stelden Darwin en Wallace dat er zoiets bestaat als 'the survival of the fittest', de best aangepaste wezens overleven.

Evolutie werkt als volgt. Er zijn alleen maar ‘slecht’ aangepaste wezens en door toeval ontstaat er een ‘goed’ aangepast wezen. De ‘goed’ aangepaste wezens doen het beter dan de ‘slecht’ aangepaste wezens. De ‘slecht’ aangepaste wezens planten zich daarom minder goed voort en sterven langzaam uit. Nu bestaan er alleen nog maar ‘goed’ aangepaste wezens. Op een gegeven dag komt er een ‘beter’ aangepast wezen. Dit ‘beter’ aangepaste wezen plant zich weer meer voort dan de ‘goed’ aangepaste wezens. Binnen een paar generaties heb je dus een hele grote groep ‘beter’ aangepaste wezens. De ‘goed’ aangepaste wezens sterven daarom op een gegeven moment uit. Zo kan het zijn dat er uit ‘slecht’ aangepaste wezens uiteindelijk ‘beter’ aangepaste wezens ontstaan. Dit proces van geleidelijke verandering van een soort over de generaties noemen we evolutie.

Trade offs

Een euro kun je maar een keer uitgeven. Het onderwerp trade offs, of wisselwerkingen, gaat daar precies over. Je kunt de energie die je vergaard hebt als organisme maar één keer gebruiken. In de natuur is het niet zo gemakkelijk als in onze maatschappij om voedsel, en dus energie, te krijgen. Dieren moeten dus zuinig doen met hun energie en goede beslissingen nemen hoe ze de energie verdelen over verschillende processen. Daarom is er in de evolutie sterk geselecteerd op genetische eigenschappen die er voor zorgt dat het genetische materiaal van het organisme doorgegeven wordt naar de volgende generatie. Als een beest zich goed kan voortplanten in een bepaalde omgeving, dan heeft dat de hoogste prioriteit. Een goed voorbeeld is de voortplanting van de fruitvlieg in de aanwezigheid van gist. Ineens draait het voortplantingsproces op volle toeren en gaat er veel energie naartoe. Dat betekent dat deze energie niet meer uitgegeven kan worden aan het bijhouden van je lichaamsonderhoud of aan de opslag van reservestoffen. Levensduur en voortplanting vormen een belangrijke trade off. Als je heel lang kunt leven, kun je minder aan voortplanting doen en *vice versa*. Dit vormt de basis voor de theorie van het wegwerplichaam, die behandeld wordt in hoofdstukken 4, 5 en 6.

Wetenschappelijke experimenten

De kracht van de experimentele wetenschap zit in het feit dat observaties altijd gerelateerd dienen te worden aan bepaalde zekerheden. We nemen bijvoorbeeld een bol als uitgangspunt. De bol is naar alle kanten toe symmetrisch en bezit de eigenschappen die bij een bol horen. Nu observeren we een totaal ander voorwerp (een kubus) en testen of dit hetzelfde is als de bol. De kubus heeft net zoals de bol een inhoud, heeft misschien dezelfde kleur en er past ook iets in. Als dat de criteria zijn waarop getest wordt, dan is een kubus hetzelfde als een bol. Als de criteria nog strenger zijn, namelijk dat er geen hoeken in een bol mogen zitten, dan valt de kubus in de categorie “niet bol”. Het mag duidelijk zijn dat de criteria waarop getoetst wordt erg belangrijk zijn om te bepalen of zaken afwijken van het uitgangspunt. Dit uitgangspunt wordt ook wel de controle genoemd. In alle wetenschappelijke experimenten die we hier presenteren is er gebruik gemaakt van een controle. Een andere belangrijke eigenschap van de wetenschap is dat alles wat wetenschappers doen falsifieerbaar moet zijn. Karl Popper heeft het begrip falsifieerbaarheid geïntroduceerd in de wetenschap. Het klassieke voorbeeld gaat over de kleur van zwanen. Een hypothese is: alle zwanen zijn wit. Dit is falsifieerbaar; ofwel alle zwanen ter wereld worden bekeken en ze blijken allemaal wit te zijn, wat de hypothese bewijst of er komt iemand aan met een zwarte, waarmee de hypothese

verworpen is. Nu is het onmogelijk om alle zwanen ter wereld te bekijken en er ook zeker van te zijn dat we ze allemaal hebben gezien[12]. Daarom gebruiken we in de biologie vaak een steekproef van de totale populatie. We moeten op basis van de gegevens van die populatie dan met behulp van statistiek berekenen hoe waarschijnlijk het is dat de hypothese wordt verworpen of aangenomen.

Hoofdstuk één: Het effect van larvale omstandigheden op de levensloop van drie soorten *Drosophila*.

In dit project heb ik me bezig gehouden met drie soorten fruitvliegen; *D. ananassae*, *D. melanogaster* en *D. willistoni*. Deze drie soorten werden onderworpen aan drie verschillende bevolkingsdichtheden als larve; laag; ~15, gemiddeld; ~80 en hoog; ~150 eieren in een buisje met standaard medium. De vliegen die uit deze larven kwamen zijn getest op hun levensduur, hongerresistentie, lichaamsgewicht en hoeveelheid vet (relatief tot het lichaamsgewicht).

We vonden dat met een verhoogde larvale dichtheid de levensduur van de fruitvliegen minder lang werd. Ook werden de beestjes dan kleiner, maar het relatieve vetpercentage ging omhoog. Kortom, door een hoge dichtheid in het larvenstadium krijg je dus kleine vette vliegjes die kort leven. We vonden ook dat in *D. willistoni* de levensduur in de aanwezigheid van voedsel sterker reageerde dan de hongerresistentie, terwijl dat in *D. ananassae* en *D. melanogaster* net andersom was.

We leren hieruit dat de levensduur en de hongerresistentie niet exact dezelfde eigenschap zijn. Dat lijkt misschien logisch, maar heel vaak vertonen deze eigenschappen dezelfde reacties op fysiologische en omgevingsveranderingen. Daarom was het feit dat we zagen dat ze niet precies hetzelfde reageren in verschillende soorten een belangrijke vondst. Wat we ook vonden was dat de relatieve hoeveelheid vet omhoog ging met een hogere larvendichtheid. Dat wil dus zeggen dat er per cel meer vet beschikbaar is. Je zou zeggen dat dit belangrijk is voor de hongerresistentie. Het lijkt logisch, hoe meer je te verbranden hebt, hoe langer je het vol kunt houden. Dat blijkt dus niet zo te zijn. Ook al hadden de beesten meer te verbranden, toch konden ze niet voor een langere tijd tegen honger. Sterker nog, ze waren zelfs minder hongerresistent. Hier concluderen we uit dat het hebben van veel reserves dus niet noodzakelijkerwijs leidt tot een grotere hongerresistentie. We weten echter wel uit ander onderzoek dat die reserves nodig zijn om hongerresistent te zijn. Dit is wat lastig: als je vet hebt ben je niet *per se* resistent, maar als je geen vet hebt ben je sowieso niet resistent. Dit betekent dat vet een voorwaarde is, maar geen oorzaak van hongerresistentie.

[12] Hoewel, soms doen we onze uiterste best en missen we er niet één. De trieste voorbeelden zijn de Moa en de Dodo die beiden zijn uitgestorven doordat de mens zo effectief te werk ging, daarbij geholpen door ratten.

Hoofdstuk twee: Het testen van de Barker hypothese.

Mijn medische collega's spraken bij het vorige stuk het vermoeden uit dat dit een goede test van de "thrifty phenotype hypothesis", ofwel de hypothese van het zuinige fenotype, zou zijn. Kortweg wordt deze theorie ook wel de Barker hypothese genoemd. Barker formuleerde zijn hypothese op basis van gegevens uit de medische wereld. Mensen die slechte omstandigheden hadden gehad in de baarmoeder bleken als volwassene een verhoogd risico te hebben op metabole ziekten. Metabole ziekten hebben te maken met het metabolisme, de stofwisseling. Voorbeelden zijn obesitas (vetzucht) en diabetes type II (ouderdomsuikerziekte). Deze ziekten verkorten het leven en resulteren dus in een gemiddeld kortere levensduur van de populatie waarin ze voorkomen. De slechte omstandigheden waar de Barker hypothese aan refereert gaan met name over een gebrek aan voedsel. In het eerste hoofdstuk waren er echter meer factoren die de levensduur konden beïnvloeden. Bij 'crowding', zoals je een hoge populatiedichtheid ook wel noemt, zijn bijvoorbeeld bij de larven van fruitvliegen competitie voor voedsel, verminderde voedselbeschikbaarheid, onderlinge agressie en vergiftiging door een verhoogde concentratie stikstofrijke excrementen (de vliegenequivalenten van ontlasting en urine) belangrijk. Omdat we voor de Barker hypothese eigenlijk alleen de voedselconcentratie wilden veranderen hebben we voor een gecontroleerde dichtheid en verminderde voedselconcentraties gekozen. Op die manier hebben we geen last van verschillen in de competitie of de excrementen, maar kijken we alleen naar de effecten van voedselverschillen. Verder liepen we tegen het probleem aan dat er naast dit mechanisme ook nog andere mechanismen zijn die hetzelfde fenotype opleveren. De Barker hypothese zoals we hem hier voorstellen is een zuiver omgeving effect. Er zijn echter ook genetische en genotype-bij-omgeving effecten die leiden tot metabole ziekten.

Een voorbeeld van genotype-bij-omgeving effecten die leiden tot ziekte komt van de natuurlijke bevolking van het Polynesische eiland Nauru. De Nauruanen hebben duizenden jaren van evolutie achter de rug waarin sterk geselecteerd werd op het zuinig omspringen met je energie. Ze leefden voornamelijk van de visvangst en hebben behoorlijke perioden van honger overleefd op hun tochten van eiland naar eiland, die vaak weken duurden. De Nauru hadden dus een zuinige motor, een 'thrifty genotype', waar ze perfect mee konden leven in de barre omstandigheden op het eilandje. Aan het begin van de vorige eeuw werd echter ontdekt dat Nauru rijk was aan fosfaat dat, ironisch genoeg gezien de gelimiteerde landbouwmogelijkheden op Nauru, goed gebruikt kon worden als bemesting. Buitenlandse investeerders stonden al snel op de stoep en maakten van Nauru een van de rijkere landen op de aardbol. Door de ineens sterk vergrote rijkdom konden de Nauruanen zich veel voedsel en inspanningbesparende materialen veroorloven. In combinatie met hun zuinige genotype leidde dit al snel tot een epidemiologische explosie van metabole ziekten.

De Nauruanen leiden aan dezelfde metabole ziekten als die bedoeld worden in de Barker hypothese (diabetes type II, obesitas *etcetera*) en ze komen beiden tot stand door een verstoring van de homeostase (metabolisch evenwicht). De oorzaak van deze ziekten is echter fundamenteel verschillend; de Barker hypothese draait om een omgevingseffect, terwijl het Nauru voorbeeld een genotype-maal-omgeving effect is dat voorkomt uit de evolutionaire historie. Omdat ze beiden hetzelfde resultaat opleveren is het belangrijk om ze te scheiden in dit onderzoek waar we maar

geïnteresseerd zijn in één van die factoren. Als we de Nauru-factor niet uit dit onderzoek weglaten kunnen we eigenlijk niets zeggen over de effecten van de slechte pre-natale (voor de geboorte, in dit geval voor de ontpopping) omstandigheden, maar weten we alleen dat fruitvliegen metabole ziekten kunnen krijgen.

Daarom lieten we larven in een normale dichtheid (80 eieren per buisje) opgroeien in standaard medium, medium met de helft aan voedingswaarde en medium met een dubbele voedingswaarde. We verzamelden de uitgekomen vliegjes en bestudeerden hoe lang het duurde tot ze ontwikkeld waren, het lichaamsgewicht en de levensduur. Om geen 'Nauru-vliegen' te krijgen hielden we de uitgekomen vliegen altijd op een medium dat hetzelfde was als het medium waar ze aan aangepast waren in het laatste gedeelte van hun evolutionaire geschiedenis.

We vonden dat er een duidelijk effect was van het medium met de halve voedingswaarde (Half), wat de Barker hypothese nabootste. De vliegen deden er langer over om te ontwikkelen, waren kleiner en er kwamen minder vliegen uit. Het Half medium was dus duidelijk een ongunstige omstandigheid. Tussen het dubbele en standaard medium was nauwelijks verschil in de eigenschappen. De vliegen op standaard en dubbel medium verschilden niet van elkaar in de levensduur als volwassene op het standaard medium. Ze leefden echter wel langer dan de beestjes die op het Half medium waren opgegroeid. Dit duidt er dus op dat de pre-natale omstandigheden wel degelijk een effect hebben op de levensduur als volwassene. Dit lijkt een indicatie te zijn voor het bestaan van de Barker hypothese in fruitvliegen. De vraag blijft of de verkorting van de levensduur ook het effect is van daadwerkelijke metabole ziekten. Het nader uitzoeken van dergelijke ziekten in de fruitvliegen onder deze omstandigheden is het onderwerp van vervolgonderzoek.

Hoofdstuk drie. De evolutie van meerdere eigenschappen door selectie op verhoogde hongerresistentie in *Drosophila melanogaster*.

In de vorige hoofdstukken heb ik het voornamelijk gehad over de effecten van de omgeving op de levensduur en hongerresistentie. In dit hoofdstuk doe ik onderzoek naar de effecten van de genetica op deze fenotypes. Levensduur en hongerresistentie zijn sterk gecorreleerde eigenschappen. Dat houdt in dat een lange levensduur ook een hogere hongerresistentie zou inhouden en *vice versa*. In hoofdstuk 1 hebben we al gezien dat deze relatie niet zo simpel is als dat hij hier voorgesteld wordt. De correlatie hield echter wel in dat de hongerresistentie een manier zou zijn om inzicht in levensduur te krijgen. Vanuit ons project gezien was het dus meer dan de moeite waard om dit te testen en wellicht lang levende fruitvliegen te krijgen.

Fruitvliegen uit Panama, Frankrijk en Nederland (Groningen en Leiden) werden verzameld en een paar generaties gemixt. Zo werd een grote genetische heterogeniteit gemengd tot een homogene populatie met een grote interne variatie. Dit betekent dat veel verschillende op zichzelf staande genetische eigenschappen van verschillende vliegenpopulaties gemengd werden tot een grote populatie waar deze eigenschappen verdeeld waren over alle vliegen. Vliegen konden dus

eigenschappen van Franse, Nederlandse en Panamese populaties hebben. Uit deze populatie werden 6 lijnen gecreëerd; 2 controle lijnen en 4 selectielijnen. De controle lijnen ondergingen exact hetzelfde behandeling als de geselecteerde beesten, behalve dat ze niet geselecteerd werden op hongerresistentie. De selectie op hongerresistentie vond als volgt plaats; de vliegen van een lijn werden als maagd na de ontpopping verzameld en per sekse op een medium gezet waar ze wel toegang hadden tot vocht, maar niet tot voedsel. Als ongeveer de helft van de fruitvliegjes was overleden als gevolg van de afwezigheid van voedsel, dan werden de overige vliegjes op een voedselmedium gezet. Daar werden ze, na een paar dagen te hebben gegeten, verenigd met de overlevenden van de andere sekse van hun lijn. De beestjes paarden en werden toegestaan om eitjes te leggen. Dan werden de eitjes in een standaard dichtheid in een buis met standaard voedsel opgekweekt. De ontpopte beestjes ondergingen weer hetzelfde regime. Dit ging dus 20 generaties zo door. Daarna werden de lijnen doorgemeten voor verschillende eigenschappen. Doordat telkens alleen de vliegen met de allelen voor hongerresistentie overbleven, selecteerden we het genetische materiaal voor hongerresistentie in onze vliegen populatie. Dit is artificiële selectie; evolutie in het laboratorium.

De selectielijnen hadden alle 4 een hongerresistentie die ongeveer 60 tot 80% hoger lag dan die van de controle lijnen. Deze lijnen kunnen dus ongeveer 3.5 à 4 dagen langer zonder voedsel dan de lijnen die er niet op geselecteerd waren. De lijnen hadden allemaal een sterk verhoogde relatieve hoeveelheid vet, zoals we al hadden voorspeld op basis van de bevindingen in hoofdstuk 1. Van deze 4 lijnen hadden er 2 (SR1 en SR2) een verlengde levensduur en de andere 2 (SR3 en SR4) niet. Ook was van de lang levende lijnen (SR1 en SR2) de resistentie tegen paraquat hoger, terwijl die van SR3 en SR4 net zo hoog was als die van de controle lijnen. Paraquat is een giftige stof die er voor zorgt dat er schade in je lichaam ontstaat door oxidatieve radicalen. Oxidatieve radicalen zijn zuurstofdeeltjes die zich binden aan het eerste het beste wat ze tegenkomen. Daarbij gaat hetgeen waar ze zich aan binden meestal direct kapot. Dat betekent dus dat door oxidatieve radicalen schade aan onder andere het DNA en eiwitten ontstaat. Meestal wordt gesuggereerd dat een betere resistentie tegen paraquat en een hogere hongerresistentie het resultaat zijn van een verlaagd metabolisme (trage verbranding). In dat geval zou er minder verbrand worden en daardoor wordt de paraquat minder snel schadelijk gemaakt, waardoor je langer mee kan en dus langer leeft. Ook wordt door minder te verbranden je reserve minder snel aangesproken en heb je dus wat achter de hand voor barre tijden. In onze analyse bleek dit echter niet zo te zijn. Als er voedsel aanwezig was bleek het ‘verbruik’ gelijk te zijn in de controle en geselecteerde lijnen. Als de beestjes uitgehongerd werden dan bleek de verbranding van de geselecteerde fruitvliegjes zelfs hoger te zijn. Een trage verbranding was dus niet de oorzaak van de hongerresistentie, en in het geval van SR1 en SR2, de verhoogde paraquat resistentie en de verlengde levensduur.

Hongerresistentie is afhankelijk van de hoeveelheid vet van een beest. Het draait allemaal om de reserves en in deze lijnen niet om een tragere stofwisseling. Ook is duidelijk dat een langere levensduur het resultaat kan zijn van selectie op een grotere hongerresistentie, zoals al eerder gevonden was. Maar het hoeft niet zo te zijn, dat blijkt uit de lijnen SR3 en SR4, die wel hongerresistent zijn maar geen langere levensduur hebben. Er zijn dus duidelijk meerdere genetische oplossingen voor het honger probleem. Ook wordt duidelijk dat een lange levensduur een bijproduct is van hongerresistentie. Met andere woorden, een langere levensduur is

niet nodig voor een goede hongerresistentie. Op een langere levensduur kan niet geselecteerd worden in de natuur. De individuen met de langste levensduur zitten in de selectieschaduw. Dat betekent dat het eigenlijk niet meer uitmaakt wat voor genetisch materiaal ze hebben voor de evolutie. Door toeval zijn veel individuen met dezelfde genen al doodgegaan en veel met andere genen zijn blijven leven. Van de lang levende individuen heeft maar een gedeelte ook echte lang leven allelen. Ook reproduceren deze beesten helemaal niet meer, waardoor er door langer te leven geen extra nakomelingen zijn en dus geen voordeel in de evolutionaire zin. De bijdrage van de langlevende allelen is zo klein dat hij in het niet valt bij de krachten die in het selectieproces werken. Lang leven moet dus altijd meeliften met een andere eigenschap. Hongerresistentie is een eigenschap waarbij dat kan.

Hoofdstuk vier. De interactie tussen voedsel en levensduur in een verzameling D. melanogaster lijnen die geselecteerd zijn voor een langere levensduur en een verhoogde hongerresistentie.

Uit de eerste drie hoofdstukken weten we dat de levensloop eigenschappen worden beïnvloed door de genetica van een organisme en door de omgeving waarin het leeft. We weten ook dat de omgeving en de genetica kunnen interacteren. Dit betekende dat de organismen met verschillende genen een andere respons laten zien over een gradiënt van omgevingen. In het vorige hoofdstuk hebben we gezien dat de lijnen die geselecteerd zijn voor verhoogde hongerresistentie niet per se langer leven. Interessant is nu om te beseffen dat hongerresistentie afhankelijk is van de afwezigheid van voedsel en een lange levensduur afhankelijk is van de aanwezigheid van voedsel. Dit lijkt simpel, maar het herbergt een wetenschappelijk interessante vraag. Ook al is er een sterke correlatie en hebben individuen die hongerresistent zijn vaak ook een verhoogde levensduur, toch worden deze eigenschappen gemeten in verschillende omgevingen. Om te weten te komen in hoeverre dezelfde mechanismen achter deze eigenschappen zitten is het belangrijk om lijnen die geselecteerd zijn voor een van beide eigenschappen door te meten in beide milieus. Hier hebben we dat gedaan. We hebben de honger resistente lijnen samen met langlevende en kortlevende lijnen uit Groningen met hun controles doorgemeten op de levensduur onder uitgehongerde, en slechte (half medium) en goede (dubbel medium) voedselomstandigheden. Door de controles van de verschillende soorten lijnen (lang levend, kort levend, hongerresistent) mee te nemen kunnen er meerdere vergelijkingen worden gemaakt. Een van die vragen is hoe de levensduur van de geselecteerde lijnen zich over de omstandigheden verhoudt ten opzichte van de controles. De andere is, hoe de levensduur van de hongerresistente en lang levende lijnen zich over de verschillende voedselomstandigheden verhoudt. Dit laatste kan geanalyseerd worden met de levensduur, maar ook met de levensduur gecorrigeerd voor de genetische achtergrond. Met de genetische achtergrond bedoelen we de effecten die alle genen waar je geen onderzoek naar doet hebben op de beesten. Zo weet je zeker dat je kijkt naar het effect van het gen van jouw interesse, omdat de effecten van de andere genen worden weggehaald uit de analyse. Als je corrigeert voor de genetische achtergrond trek je de gemiddelde levensduur van de controle af van de data van de geselecteerde lijnen. De data die overblijven, representeren het levensduurverschil dat veroorzaakt wordt door de selectie: de genetische verandering ten opzichte van de originele beesten. Als voor alle genetische achtergronden is gecorrigeerd, levert dat een dataset waarin het

effect van selectie in de verschillende selectielijnen gemakkelijk vergeleken kunnen worden.

We hebben gevonden dat als alle lijnen worden getest in de omstandigheden waar de lijnen in geselecteerd zijn, de selectielijn voor die omstandigheid altijd een superieure levensduur heeft. Op het dubbele medium lieten de honger resistente lijnen wel een verhoogde levensduur zien ten opzichte van hun controles, maar niet zo extreem als onder de honger omstandigheden. De hongerresistente lijnen lieten hier ook een levensduur zien die sowieso lager lag dan die van de langlevende lijnen. De lang levende lijnen deden het niet beter dan hun controlelijnen op het hongermedium. Dit laat zien dat de levensduur van beide lijnen verhoogd is, maar met name op het medium waarop ze geselecteerd zijn. De theorie van het wegwerplichaam gaat hierover. Die stelt dat de evolutie voornamelijk selecteert op de productie van de meeste nakomelingen. Je hebt een zogenaamde soma kant (lichamelijk onderhoud) en een reproductie kant. In het geval dat de meeste stoffen naar de soma-kant gaan is er minder reproductie en leven de dieren langer. Honger resistentie en lang leven zijn eigenschappen die dus echt bij de soma horen. Hier zien we dat in dit geval allocatie (verdeling, toewijzing) van de reserves gaat naar de soma kant in deze lijnen. Het feit dat de lijnen het langste leven in de omstandigheden waarin ze zijn geselecteerd laat dus een genotype-maal-omgevings interactie zien; in de verschillende omgevingen zijn andere genotypen superieur. Verder werd gevonden dat de voor de genetische achtergrond gecorrigeerde levensduur een vergelijkbaar patroon laat zien.

Al met al kunnen we dus zeggen dat de lang levenden en honger resistenten wel allebei een verhoogde allocatie naar de soma laten zien, maar dat het afhankelijk is van de omgeving waar hun voordeel tot uitdrukking komt. Ze doen exact het tegenovergestelde en laten zo dus zien dat als de reserves naar de soma zijn gealloceerd, er meerdere processen zijn die hun voordeel daarmee kunnen doen. Ze doen dit alleen niet noodzakelijkerwijs proportioneel. Het is duidelijk dat de genen die verantwoordelijk zijn voor de langere levensduur gedeeltelijk anders zijn dan de genen die verantwoordelijk zijn voor de honger resistentie. Anders zouden de hongerresistente en de langlevende lijnen hetzelfde reageren op de verschillende omstandigheden.Ook is het voordeel in levensduur afhankelijk van de voedselomstandigheden die ze tegen komen.

Hoofdstuk vijf. Genexpressie patronen van hongerresistente *Drosophila melanogaster* onder voedsel en honger omstandigheden.

In dit microarray experiment is gekeken naar de genexpressie van alle genen (het hele genoom) van *D. melanogaster*. De vraagstelling was welke genen er belangrijk waren voor de toegenomen hongerresistentie en misschien zelfs voor een verlengde levensduur. Daarom werd gekozen om met lijn SR2 te werken, een hongerresistente lijn met de langste levensduur. Lijn C1 werd gekozen als controle lijn voor dit experiment. Maagdelijke vrouwtjes fruitvliegen van beide lijnen werden voor drie dagen onderworpen aan ofwel voedselomstandigheden ofwel honger. Na die drie dagen werden de vliegen gedood in vloeibare stikstof om hun RNA te behouden. Dit RNA werd geïsoleerd (uit de vliegen gehaald) en middels enkele stappen op een microarray gehybridiseerd. Dit betekent dat het totale RNA van de beestjes aan

stukjes RNA waarvan we weten met welke genen ze te maken hebben op het glazen plaatje van de array worden gehecht. Daarna konden de microarrays worden bekeken (uitgelezen) en hun data worden geanalyseerd. Dit gebeurde grotendeels bij Unilever in Colworth, UK. Niet alleen was dit een experiment om te kijken hoe deze geavanceerde technologie kon worden gebruikt in onze vraagstellingen, het was ook een eerste vooronderzoek waarin we wilden kijken of we algemene patronen op konden pikken die te verklaren waren met onze theorie. De data waren uitermate interessant. In een data reducerende principale componenten analyse werd duidelijk gezien dat ongeveer een kwart van alle variatie verklaard werd door het verschil tussen gevoede en uitgehongerde dieren. Een groot deel van de verschillen wordt dus geïnduceerd door de omgeving. Iets minder dan een kwart van alle variatie in genexpressie werd verklaard door het verschil tussen de lijnen. Deze verschillen zijn dus het resultaat van verschillen in de genen. Als de eerste en de tweede principale component samen werden genomen (zie hoofdstuk 5, figuur 2) dan kwam duidelijk een genotype-maal-omgeving interactie naar voren. De controle lijnen vertoonden onder honger situaties een behoorlijk afwijkend patroon in hun genexpressie. We zagen in de derde en vierde principale componenten wel een behoorlijke technische variatie, waardoor we onze data met een behoorlijke reserve hebben geïnterpreteerd. In de genspecifieke analyse hebben we ons met name gericht op een analyse van in hoeveel genen de expressie wordt veranderd door de genetische verandering door het selectieproces (G-effect), wat er anders tot expressie kwam in uitgehongerde beesten dan in doorvoede beesten, dus een omgevingseffect (E-effect) en hoe de interactie tussen die twee was (G*E-effect). De expressie van een groot aantal genen werd veranderd door het verschil in voedselomstandigheid (3625), een kleiner deel werd beïnvloed door de genetische verandering door de selectie (454) en 42 genen vertoonden genotype-maal-omgeving interacties.

In een analyse van enkele processen die belangrijk zijn in relatie tot hongerresistentie en lang leven vonden we de bekende patronen terug. Onder andere waren alle reproductie gerelateerde genen die wij konden identificeren op een lager niveau van expressie in de hongerresistente lijnen dan in de controle lijnen. De thermostaat voor reproductie stond dus lager in de hongerresistente beesten. Dit strookt met de theorie van het wegwerplijf (soma). Ook vonden we een verminderde activiteit van zenuwsignaal doorgevende zenuwen. Stress signalering was lager in de resistente diertjes. Dit heeft er waarschijnlijk mee te maken dat de resistente beesten nog geen stress ervaren na drie dagen uithongering en de controle lijn al wel. Hetzelfde gold voor de insuline signaalroute (bekend van suikerziekte). Hierdoor wordt voedselopname minder goed doorgegeven en de beestjes dus wat zuiniger aan doen omdat ze denken dat ze minder hebben dan ze hebben. Als gevolg van verminderde insuline signalering vonden we ook dat de glycolyse, een proces in de afbraak van glucose, op een lager pitje werd gezet. De genen die betrokken waren bij de afbraak van vet, eiwitten en andere suikers dan glucose kwamen daarentegen wel veel hoger tot expressie. De dieren zullen als gevolg hiervan genoeg energie hebben om te leven. Zoals we al vonden in het derde hoofdstuk was de stofwisseling van de hongerresistente vliegen gelijk aan of zelfs hoger dan die van de controle lijnen. De glucose spiegels in het bloed (haemolymfe) zullen echter ook erg hoog zijn door de verhoogde afbraak van glycogeen (wat bestaat uit glucose moleculen die dan vrij komen) en de lage afbraak van glucose. Dit lijkt een beetje op een diabetische respons. Nu is bekend dat diabetici gemiddeld korter leven dan niet-diabetici. Hoe valt dit te rijmen met de suggestie dat onze hongerresistente, lang levende lijnen eigenlijk een soort van diabetes hebben? Diabetici hebben vaak last

van hun bloedvaten en dit blijkt ook de grootste doodsoorzaak in deze groep te zijn. Fruitvliegen hebben een compleet ander circulatiesysteem dan mensen, onder andere zonder vaten. Vandaar dat het grote hart- en vaatrisico dus uitgesloten wordt in de fruitvlieg.

Dit vooronderzoek is dus geslaagd te noemen. We weten nu dat we microarrays kunnen gebruiken voor onze proefopzet en dat er interessante patronen uit kunnen komen die eerder werk bevestigen. Wel dient de technische variatie beperkt te worden en moet er duidelijk beseft worden dat microarray werk slechts een indicatie geeft van systemen die belangrijk zijn in de eigenschappen die we bestuderen. Deze indicaties dienen verder onderzocht te worden met onder andere kwantitatieve PCR (een manier om te weten hoeveel RNA of DNA zich in een monster bevindt door terug te rekenen) om tot sluitend bewijs te komen.

Hoofdstuk zes. De levensloop van *methusalem* onder verschillende omstandigheden, implicaties voor het gebruik van mutanten in onderzoek naar levensduur.

In het verouderingsonderzoek wordt met enige regelmaat een mutant geïdentificeerd die langer leeft dan representanten van de lijn waar hij uitkomt. Een mutant is een individu met een genetische verandering, mutaties gebeuren iedere dag in ieder organisme en kunnen soms nare gevolgen opleveren zoals kanker. In veel gevallen is de vermeende superioriteit van de lijn onderuit gehaald (gefalsifieerd) doordat de mutatie bijvoorbeeld alleen het levensverlengende effect bleek te hebben in heel specifieke omstandigheden, of dat de voedselafhankelijke levensduur bleek te zijn verschoven. Zoniet bij de langlevende mutant *methusalem* (*mth*) die werd gepresenteerd als een mutant die langer leefde en beter tegen honger, hitte en paraquat kon. De evolutionaire biologie leert dat er beperkingen en grenzen (constraints) zijn waarbinnen een organisme kan evolueren. Als je goed bent in het ene, is goed zijn in het andere onmogelijk binnen dezelfde omgeving. De vraag was eigenlijk waarom we in het laboratorium een zo superieure mutant kunnen identificeren, die in de natuur helemaal niet gevonden wordt. Temeer daar we weten dat in het veld het gen onder selectie staat die geassocieerd is met levensduur. Dat wil niet zeggen dat de selectie op de levensduur plaatsvindt, maar juist op een andere eigenschap die er ook voor zorgt dat de levensduur verlengd wordt. In het onderzoek dat hier gedaan werd is gekeken naar de invloed van de omgeving waarin, en de leeftijd van de vliegen waarop eigenschappen gemeten werden. Zo bleek dat op ons medium de hongerresistentie en de paraquatresistentie lang niet zo goed waren als in de originele studie waar de *methusalem* beesten als superieur werden erkend. De levensduur van de maagdelijke mutant was wel iets langer dan die van de maagdelijke controle groep, maar niet zoveel als in de originele studie. Sterker nog, als we op het dubbele medium testten dan leefden de mth vliegen zelfs significant korter dan de controle lijn. De extrapolatie van deze gegevens en het feit dat er in het medium van de originele publicatie vrijwel geen gist zat leidde ons tot de conclusie dat als er meer gist in het medium zat, de *methusalem* minder lang leefde dan wanneer er minder in zat. Dit komt waarschijnlijk doordat meer gist de allocatie naar de reproductie vergroot en dat een negatief effect heeft op de levensduur. Of de *methusalem* mutatie inderdaad voordelen heeft is dus afhankelijk van de omgeving waar de mutant zich in bevind.

Ook hebben we proeven gedaan naar het effect van de mutatie op de reproductie. Daaruit bleek dat de mutanten en de controle vliegen dezelfde reproductiviteit hebben over hun hele leven. De levensduur van deze gepaarde mutanten was overigens identiek aan die van de controle vliegen. Het paren had dus wel degelijk een negatief effect op de verlengde levensduur van de mutant. Een nadere analyse leerde dat de mutanten een lagere reproductiviteit vroeg in het leven hadden en een hogere later in het leven. In de natuur is de reproductiviteit vroeg in het leven het belangrijkste, omdat de kans groot is dat je niet oud wordt van kans afhankelijke (stochastische) gevaren. Je kunt bijvoorbeeld gemakkelijk opgegeten worden. Dieren die later in het leven reproduceren hebben dus een behoorlijk nadeel en brengen gemiddeld minder nakomelingen in de populatie. Daardoor zullen ze langzaam uitgeselecteerd worden over de tijd. De combinatie van omgeving afhankelijke superioriteit en een verminderde reproductiviteit vroeg in het leven is dus waarschijnlijk de oorzaak van het feit dat we in de natuur geen vliegen tegenkomen die vergelijkbaar zijn met *methusalem*.

Samenvatting van de samenvatting

De samenvatting van de samenvatting dient natuurlijk kort te zijn. Om kort te gaan is het volgende uit het proefschrift gerold. Vet is in fruitvliegen een voorwaarde voor honger resistentie maar niet de oorzaak van een verhoogde honger resistentie. Daarvoor moeten andere processen ook meewerken, bijvoorbeeld de allocatie van energetische stoffen (o.a. vet, suikers) naar de soma in plaats van naar de reproductie. Ook werd duidelijk dat selectie op honger resistentie niet noodzakelijkerwijs exact dezelfde eigenschappen oplevert als selectie op een lange levensduur. Beide eigenschappen liggen aan de soma kant zoals die bedoeld is in de wegwerplichaam-theorie. Selectielijnen voor langere levensduur en hogere hongerresistentie vertonen naast een relatief verhoogde algemene levensduur echter wel een behoorlijke interactie als ze getest worden over een gradiënt van omgevingen. Daaruit werd geconcludeerd dat beide eigenschappen tot op zekere hoogte door dezelfde mechanismen worden bepaald, maar vanaf een zeker moment gescheiden zijn. De microarray analyse leverde een aardig beeld op van de processen die veranderd waren in een selectielijn. De insuline signaalroute, de glycolyse, reproductie en de katabolische (afbraak van stoffen) routes deden precies de dingen die verwacht werden van hongerresistente lijnen (zie hoofdstuk 6). In het *methusalem* onderzoek kwam duidelijk naar voren dat, net zoals in de selectielijnen, de omgeving waarin getest wordt erg belangrijk is. Ook werd daar duidelijk dat binnen een bepaald systeem de bouwstoffen maar één keer kunnen worden uitgegeven en dat niet slechts één eigenschap ten voordele kan veranderen als er verder niets verandert.

Eén studie wil ik hier dan toch graag aanhalen omdat zij zo elementair is. In 1986 publiceerden Van Noordwijk en De Jong hun Y-allocatiemodel. Ze legden hun model uit in een prachtige metafoor die ik graag mee wil geven. Als je een vast bedrag hebt kun je dat uitgeven aan een auto of aan een huis. Een grote auto betekent dat er minder geld is voor een huis en *vice versa*. Dit houdt in dat er een negatieve correlatie is tussen de grootte van je huis en van je auto. Het probleem dat het lastig

maakt om dit zonder kennis van de maatschappij te ontdekken ligt hem erin dat niet iedereen evenveel te besteden heeft. Een rijk persoon kan een heel groot huis èn een hele grote auto hebben. Hoe meer je hebt, hoe groter beiden zullen zijn. Dit leidt tot een positieve correlatie tussen de grootte van je auto en je huis. Deze negatieve en positieve correlaties heffen elkaar gedeeltelijk op en het is dus lastig om een helder beeld te krijgen als je niet weet dat er twee processen spelen. Met het systeem van lang leven en honger resistentie is het precies zo. In de selectielijnen is er meer allocatie naar de soma geweest. De soma processen hebben dus meer te besteden. Daarom wordt vaak gevonden dat zowel de honger resistentie als de levensduur is vergroot in de selectielijnen. Deze processen variëren echter zo sterk in hun onderlinge verhoudingen dat het erop lijkt dat tussen deze twee processen ook weer een grondstof-allocatie probleem ontstaat.

Al met al leidde ons dit tot de conclusie dat er na de eerste allocatie tussen soma en voortplantingssystemen een tweede bestaat tussen honger resistentie en een lange levensduur (zie de Summarising Discussion, figuur 1 op pagina 149).

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Curriculum Vitae

Curriculum Vitae

Egon was born on December 9th 1978 in Rotterdam, the Netherlands as Egon Alexander Baldal. He followed elementary school in the small village of Poortvliet at O.B.S. De Eevliet. After finishing this in 1991, he hurried to the Mollerlyceum, a Catholic high school in Bergen op Zoom, where he graduated in 8 disciplines, including the exact sciences and Dutch, English and Geography. Swiftly, he went to Groningen in 1997 to study Biology where he finished his propaedeuse in one year and graduated in 4 and half years, in March 2002, as a biologist with specializations in marine biology and population genetics. He acquired evolutionary genetic research experience on conditional lethals in *Drosophila melanogaster*. This was followed by a minor research project on nitrogen fixation in marine Cyanobacteria at the NIOO-CEME (Yerseke). On May 1st, labour day, 2002 he commenced his Ph.D. project on the genetics of starvation resistance in *Drosophila* in Leiden.

There he got involved in scientific research on a wide variety of disciplines, varying from molecular and quantitative genetics, via physiology to environmental manipulation and experimental evolution. In this project he worked with epidemiologists and gerontologists of the LUMC and industrial partners. He attended courses in Bioinformatics (EMBO, Maynooth Ireland), Life history theory (Graduate school Functional Ecology), Analysis of micro arrays (Medical Genetic Centre - Zuidwest), Patenting and intellectual property (IOP), Introduction to policy making, and Introduction to Management (PAO). He also attended a workshop on Environmental stress (Ronbjerg, Denmark). He presented his work at meetings in Leeds (2003, ESEB X, United Kingdom, poster), Texel (2003, Verweij meeting, poster), Utrecht (2003, 3rd annual fly meeting, oral presentation), Wageningen (2005, Dutch entomologists day, oral presentation) and Sandbjerg (2004, ESEB workshop on stress and evolution, Denmark, oral presentation). Furthermore, he organised and hosted a symposium on “Biology and ageing; an integrative approach” at the 2005 ESEB meeting in Krakow, Poland.

During his Ph.D. he trained four master students; Wouter Teunissen (on the effects of age on glycogen and fat content in *Drosophila melanogaster*), Jung van der Meulen (on the effects of dietary additives on longevity in two species of *Drosophila*), Wishal Baktawar (on the environmental dependence of life history advantages in the long-lived mutant *methuselah*) and Jamie Graham (on quantitative Real-Time PCR of candidate longevity genes in *Drosophila*). Also, he trained an MLO-3 technician, Hamda Mohamad Ali (various work on *methuselah*). He participated in teaching the second year evolutionary biology course at Leiden University, lecturing molecular evolution and participated in the master course on ecology and evolution, lecturing on ageing, tradeoffs and the pitfalls of genetic correlations.

His extracurricular activities consisted of membership of the IBL institution’s council for two years and being member of the financial control committee of GeNeYouS. He also participated in the Grenspost (Museon, Den Haag) and Klokhuis 750 vragen (NEMO, Amsterdam) educational projects for elementary school pupils.

Currently he is employed as a lecturer in Biology at the Roosevelt Academy in Middelburg and as an ecological advisor for the National Institute for Coastal and Marine Management (Rijkswaterstaat, RIKZ) in The Hague.

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Submitted

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