

**The evolution of lifespan
in the butterfly
*Bicyclus anynana***

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The evolution of lifespan in the butterfly *Bicyclus anynana*

PhD Thesis, Faculty of Science, Leiden University, the Netherlands

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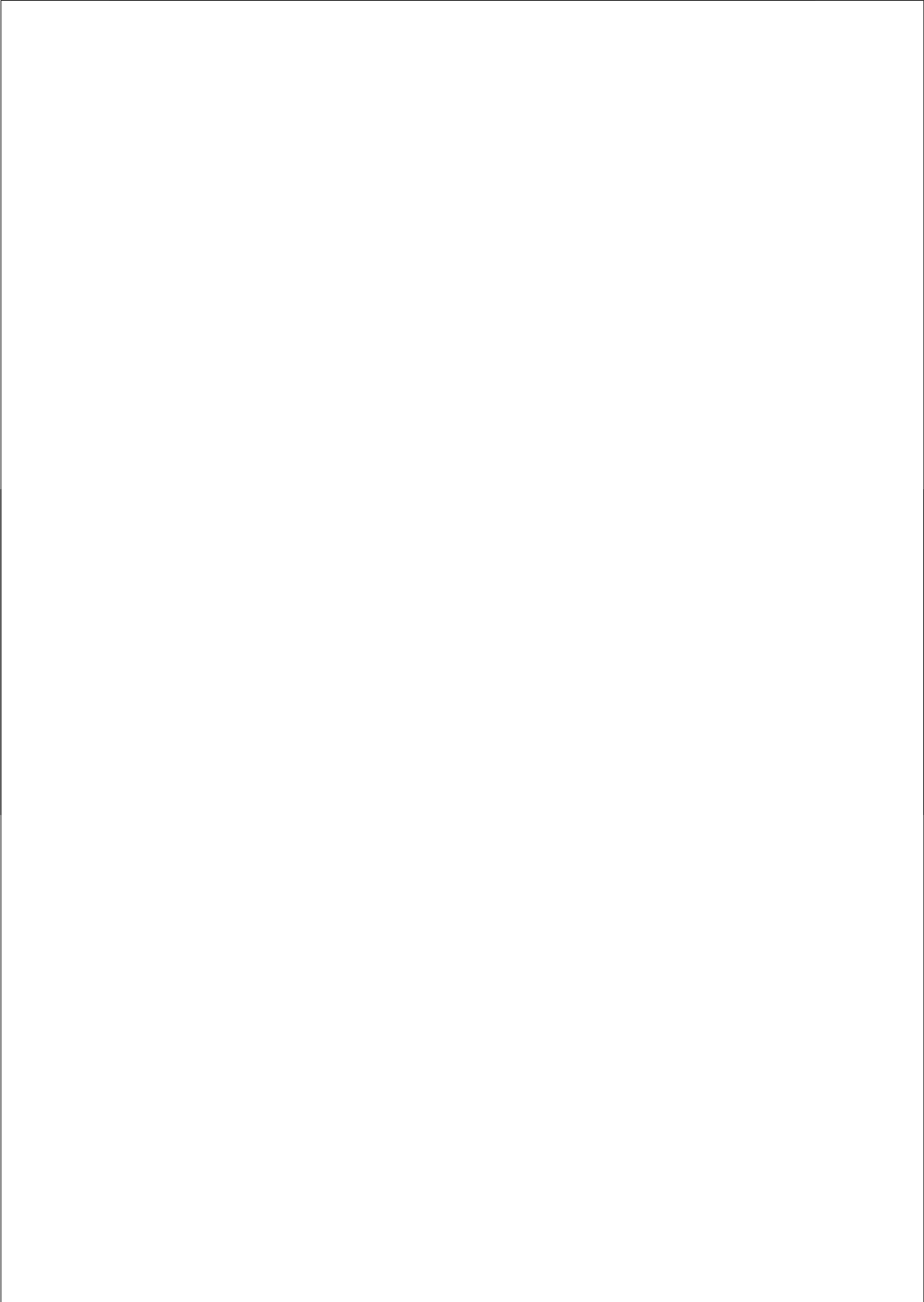
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Chapter
1

Introduction

Overview

In the introduction of this thesis, I aim to provide the reader with three things: (1) the main rationale for my studies, (2) a short review of the main issues in present-day research on ageing, and (3) the necessary background for a broad audience to understand in general terms the work described in this thesis. More detailed background on the issues addressed in the chapters 2-7 is described in the chapter introductions and the references therein.

I have divided this introduction into three sections, each starting with a question. To answer the first question, “what is ageing?”, I briefly discuss the main framework in which all the work in this thesis was performed: life history evolution and the evolutionary theories of ageing. In the section “Why study ageing?”, I introduce the multi-faceted and interdisciplinary project ‘The genetic determination of longevity and disease at old age’ to which my work contributes. In “How to study ageing?”, I describe the approaches and the methodology that I have used to address the relevant questions in biological ageing research. Here, I also explain the rationale for using a tropical butterfly species for the study of lifespan and ageing-related traits, namely the extraordinary life history of *Bicyclus anynana*. Finally, I outline the content of the chapters of this thesis.

What is ageing?

People (including scientists) seem to have a concept of what ageing is, but as soon as one contemplates ageing in detail it is evidently very difficult to define. Ageing is a process that affects all of us; although it has positive aspects, ageing is generally viewed as a negative process that is closely related to death. This life-opposing process is also referred to as senescence. Here, I use the term ageing for exactly this process. From a scientific point of view, ageing is in fact one of the great mysteries in modern biology. How it comes about, and how it works is not exactly known, and hence hotly debated. Consequentially, many mechanisms for ageing have been proposed, and the relationships and overlap among them have not been well established. This thesis does not aim to answer the important question ‘What is ageing?’ posed above. Rather, its aim is to discover what causes variation in lifespan between individuals. However, as I hope will become evident from this introduction,

it is necessary to describe what ageing is and to describe the dominant evolutionary views on ageing that provide the framework for this thesis.

So what is ageing? Ageing is purely an intrinsic process, but what is this process? The answers are manifold. It is the gradual build-up of errors in our cellular machinery. It is the increased deviation from homeostasis, the 'normal' physiological functioning of the body, with age. It is the decline of the ability to cope with stress. It is also the increased susceptibility to harmful external factors. It is important to note that ageing does not cause death; it merely enhances the likelihood of death. To summarise these points, a workable definition of ageing would be:

'Ageing is the total effect of intrinsic changes accumulating in the course of life that negatively affect the vitality of the organism, and that makes it more susceptible to the factors that can cause death.'

Lifespan and ageing

In nature, most organisms die at a relatively young age. Relatively, because most organisms have the capacity to live much longer than they, on average, do in their original, natural environments. We know this from two observations. First, humans in the recent history of the developed world have been able to reach much higher ages than recorded for humans in pre-industrial times mainly because of improved sanitation and other insights from modern medical science that have led to changes in the patterns of survival (Fig. 1).

Second, the animals and plants that humans have in captivity, such as domesticated

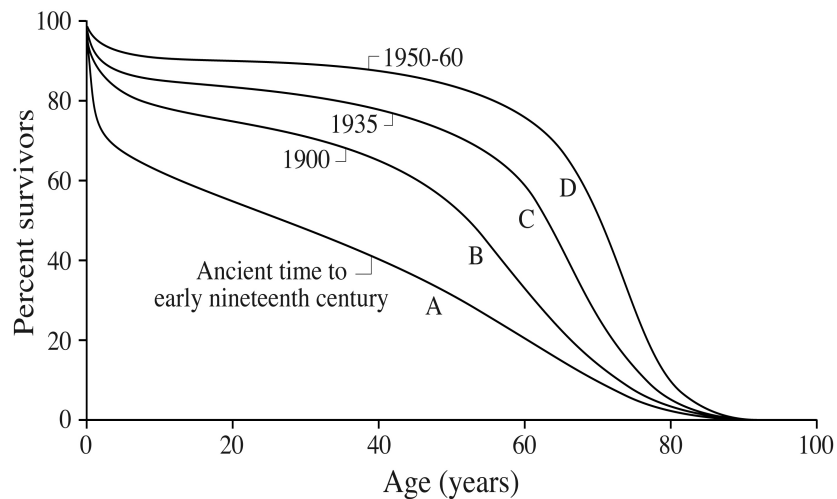


Figure 1 The rapid change of the survival curve in humans from ancient to modern industrial populations. Redrawn with permission from Kandel et al 2000.

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species and species in zoos and botanical gardens, typically reach much higher ages than they would do in the wild (Carey and Judge 2000). The cause of the increase in lifespan in both cases is essentially the same: a reduction of external mortality. External mortality is the cause of death for most individuals, and it remains a dominant factor in human populations. Examples of external mortality are: predation, starvation, infectious disease, and accident. These sources of external mortality do not contribute directly to ageing, but they clearly contribute to variation in lifespan. As these external mortality sources are minimised, as is attempted in modern societies by for example vaccination and traffic regulation, then ageing becomes increasingly apparent.

Ageing is under genetic control.

Under more or less optimal environmental conditions, maximal lifespan is relatively uniform within species. For instance, the average maximum lifespan of humans has not increased much in the last 200 years, although survival at any given age has increased dramatically (Fig 1). However, among species the maximal lifespan differs widely. Even within relatively uniform groups of species, such as mammals or insects, the difference in lifespan can be significant. For example, bats generally live much longer (3.5 times longer maximum lifespan, on average) than mice or other non-flying mammals of comparable size. Some bat species have a maximum lifespan of 25 years. Such differences clearly indicate a strong genetic basis of species-specific lifespan. Thus, the different evolutionary histories of these species should best explain the differences in lifespan between species. However, within a species lifespan can also dramatically differ. The most extreme example is that of ant queens, which can reach ages of over 20 years, up to 50 times longer than workers of the same colony. These observations raise some important questions that are highly relevant for an understanding of ageing. How do these differences come about? How important is the genetic component? Do the genetic factors that influence the differences between species also contribute to variation in lifespan between populations of the same species, and between individuals of the same population?

A short history of the evolutionary theories of ageing.

The first recorded modern scientific thinking on ageing comes from August Weismann (1881). Amongst other ideas, he postulated a sharp distinction between an immortal germ line and a mortal soma, at least in multicellular organisms. This idea was recently found to also explain ageing in relatively simple, single cell organisms such as bacteria (Ackermann et al. 2003; Stewart et al. 2005). Thus, ageing is somehow a consequence of reproduction through cell division. Because reproduction is a hallmark of life itself, such findings suggest that ageing is a fundamental part of all life forms. In other words, this idea can be considered a

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'public' mechanism of ageing, shared by most lineages of species, in contrast to 'private' mechanisms that work only in some of these lineages and, thus, can not be extrapolated to other lineages (Martin et al. 1996; Partridge and Gems 2002).

The first modern evolutionary explanation for the occurrence of ageing was formulated by J.B.S. Haldane in the early 1940's. He realised that some lethal dominant genetic diseases (he used the example of Huntington's disease) continue to occur in human populations because they can not be removed by natural selection. The reason is that such diseases typically manifest themselves only after the age of 35, when most of the reproduction has taken place. Moreover, this age is beyond the expected lifespan in most of human evolutionary history. This post-reproductive period has later become known as the 'selection shadow' (Hoekstra 1993).

With the discovery of particular molecules as the carriers of heritable information ('genes'), thinking about the origins of ageing changed accordingly. Peter Medawar took Haldane's idea further and developed his 'mutation accumulation' theory (Medawar 1952). This states that the accumulation of late-acting, harmful mutations (single changes in the genetic code) within an individual and over the generations causes ageing. In this light, one could say that ageing itself is a genetic disease. Importantly, these mutations can arise at any time during evolution, and are thus more likely to be private mutations belonging to a single lineage, like a species or even a population.

A few years later, Medawar's theory inspired George C. Williams to formulate an alternative but related theory: the antagonistic pleiotropy theory of ageing (Williams 1957). This theory also assumes that mutations in genes are causing ageing, but the reason they persist and spread in populations is not the selection shadow but a beneficial effect of the mutation in early life. This theory assumes that such a gene is pleiotropic: it has multiple effects, or it affects more than one trait or process. It also assumes that these multiple effects have an opposing (antagonistic) influence (beneficial versus detrimental) in different stages of life. This theory appears to apply to specific genes. However, there are many genes that have pleiotropic effects on traits closely related to fitness, and thus selection acts strongly to maintain them. Therefore, such mutations are more likely to be evolutionarily ancient and will contribute to public mechanisms of ageing.

The idea of opposing selection forces in the antagonistic pleiotropy theory and Weismann's distinction between germ line and soma come together in the 'disposable soma' theory (Kirkwood and Holliday 1979). Here, the central notion is that ageing is a consequence of the choice of allocating limited resources to reproduction (germ line) or longevity (soma). As resources (in the physiological meaning, *i.e.* food or reserves) are always limited, all individuals are and have been forced to make decisions about their priorities: Put all the available energy in current reproduction, or invest in a durable body to wait for better conditions or to spread the risk for the offspring? Such decisions, and how they depend on past, present and future environmental conditions are the key elements of theories

formulated by another school of biologists and are known collectively as 'life history theory' (Stearns 1992).

A life history perspective on ageing

Ageing is a complex process because it is multifactorial: It involves many, if not all aspects of adult organismal biology. The lifespan and rate of ageing of an individual organism are the outcome of a history of interactions between genetics and environment, both in the lifetime of the individual and that of its ancestors. In other words, they evolve. However, they are not necessarily adaptive, but can be merely a by-product of other processes (development, reproduction) earlier in life that have their own evolutionary dynamics and are typically under strong natural selection. Life history evolution is a well-suited approach to study such a complex trait, as it can be regarded as a 'theory of all' in biology. The life history approach involves studying 'life history traits': traits that directly contribute to the central concepts in biology, reproductive success and fitness. Typically, relations between traits are measured, often finding trade-offs. A trade-off is a negative relationship between two traits; when a change in one trait potentially has a positive effect on fitness, another trait changes in such a way as to have a negative impact on fitness. The net effect on fitness is dependent on the outcome of this balance, but also on third traits and trade-offs with third traits.

How trade-offs are regulated is mostly unknown. Sometimes, mechanisms of physiology and/or endocrinology that are potentially involved in the relation between traits are investigated, but often these mechanisms are considered a 'black box'. However, with the increasing availability of genomic information, scientists have begun to incorporate molecular genetic approaches in life history evolutionary research. This synthesis of different research areas is comparable to a 'systems biology' approach. Theoretically, systems biology adopts a holistic, integrative view (Institute of Systems Biology website: www.systemsbiology.org). In practice, however, it is usually large-scale reductionism: it takes a bottom-up approach, using information from complex interactions among lower level (molecular) processes to explain the functioning of cells and organisms. This is crucially different from life history evolution, which uses a top-down approach, starting at the level where selection acts (typically the individual organism), and moving down the organisational hierarchy to eventually find a mechanistic explanation. Both approaches have their advantages and disadvantages and should be used in a complementary manner. An important and frequently ignored advantage of life history evolutionary studies for medical science is that the mechanistic knowledge they generate is more likely to have a medical application, because it is relevant in populations of real organisms that live (and die) in real environments. In other words, knowledge of the functioning of regulatory mechanisms, and how they vary between individuals, is gathered in natural populations across environments, including the natural environment.

Why study ageing?

From the previous section, it becomes clear that although we know a lot about several individual mechanisms of ageing, we lack a full and unitary understanding of the whole ageing process. Whilst the quest for this fundamental knowledge continues, an equally important reason to study ageing is because it is relevant to us humans. With the linear rise in life-expectancy in the past century (see Fig. 1), the relative and absolute amounts of old-aged people have increased substantially in modern industrialised countries. The direct and indirect social and economic consequences of this demographic shift have long been foreseen but have only recently prompted significant action in politics and science. In addition, it is evidently in the interest of everyone, young and old, to reduce the burdens of old age. Medical science can significantly contribute to such a process, but gerontologists are only beginning to investigate how this can be done most effectively. A basic requirement to promote a healthy old age is to minimise invasive treatments. This is envisaged to be possible with person-based intervention schedules: individually tailored care programmes that include medication, diet and lifestyle. This goal critically depends on the full understanding of the biomolecular pathways that regulate the processes of life and death.

'Lang leven' IOP-Genomics study

A better understanding of ageing and the treatment of the diseases of old age are the ultimate goals of an integrative research project that started in 2002: the 'Lang Leven' study¹. This project, funded by the Innovative Research Programmes (*Innovatieve Onderzoeks Programmas, IOP*) of the Dutch Ministry of Economic Affairs, is one of the first of its kind worldwide. It brought together gerontologists and evolutionary biologists, and combined the study of experimental models with analysis of human cohorts.

The focus is on the genes that have evolved for somatic maintenance, repair mechanisms, and longevity assurance, in order to achieve long active and rewarding lifespans whilst minimizing the potential for disease-causing genetic problems. The model organisms are two insect species: the fruit fly *Drosophila melanogaster* (and closely related species) and the tropical butterfly *Bicyclus anynana*. In these species, evolutionary functional genomics was used to identify novel pathways and to

¹ Participants: A. Ayriñac, E.A. Baldal, M. Beekman, G.J. Blauw, D.I. Boomsma, P.M. Brakefield, B.W. Brandt, R. Bijlsma, D. van Heemst, B.T. Heijmans, J. van Houwelingen, D.L. Knook, I. Meulenbelt, P.H.E.M. de Meijer, S.P. Mooijaart, J. Pijpe, P.E. Slagboom, R.G.J. Westendorp, L.P.W.G.M. van de Zande, B.J. Zwaan.

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examine how associated genes are expressed across environments. The human studies comprised extensive surveys of the very old in the present Dutch *Homo sapiens* population at large, together with various smaller-scale surveys of the elderly in Leiden. The success of the Lang Leven study depended on the interactions of both sets of researchers and their study subjects: the processes that determine lifespan must be directly comparable between model organisms and humans, and the researchers must have insight into the possibilities and limitations of each others' approaches. While the latter remained to be demonstrated at the start of the project, there was ample *a priori* evidence that the former was possible. Developmental biologists have long discovered the similarities across animals, including humans, in their development. This is true for the ontogeny of morphology as well as for the underlying genetic program. This homology is one of the hallmarks of the evidence for a common origin of all animals (Carroll 2005). The availability of whole genome information on, for example *D. melanogaster* and *H. sapiens*, has revealed that the majority of human disease genes have orthologues in distantly related animal species (Rubin et al. 2000). Recently, it has become evident that there are central regulatory pathways for the ageing process that are conserved between yeast, worms, flies and mice. These pathways typically underpin the central hormone systems of the body that contribute to the regulation of growth and reproduction, that is the life history traits most closely related to fitness. It is very likely that these pathways are also important for human ageing. In addition, one of the key life history trade-offs influencing lifespan in animals, that between reproduction and longevity, has been shown to account for some of the variation in lifespan in humans (e.g. Westendorp and Kirkwood 1998). In other words, there is ample evidence that the extrapolation of evolutionary theory and experimental data from insects models to the human situation, and vice versa, is valid.

How to study ageing?

In a functional evolutionary approach to the causes and consequences of ageing, it is important to identify several prerequisites. First, one needs to have a suitable study or model organism in which the traits of interest are readily investigated and their function well understood. Second, some powerful experimental tools are needed to be able to make qualitative and quantitative measurements of the traits of interest. Third, genetic tools need to be available or readily implementable in order to get to the mechanisms underpinning the traits. Each of the three prerequisites receives extensive attention in this thesis because the power and success of the functional evolutionary approach is in their combination and integration.

Model organisms in the study of ageing

The very use of model organisms in modern science and medicine is founded and spurred on by the idea of homology at most levels of biological organization. In biology, model organisms are chosen to best fit the question at hand, while in medicine the model is mostly chosen based on the disease phenotype. As in most other areas of research, research on ageing has benefited from the study of a wide range of species. Fish, rats, fruit flies, nematode worms, and more recently mice, yeast and *E. coli* have been used to investigate ageing, but not necessarily as a model for human ageing. A limitation to the use of such models is a lack of knowledge on functionality of the findings in 'standard' model species. Functionality of a finding would mean: knowing what the trait, pathway or molecule under investigation does in the organism when it is living and functioning in its 'normal' environment, the environment it evolved in. With the advance of the reductionist program, the focus of medical science is more and more on the molecular (genetic) level, and the presence of such information became the most important criterion of choice for animal models. Currently, the vast majority of biomedical research uses only a handful of species. The increasing use of these models has delivered many scientific breakthroughs and it will continue to do so; some of these having major impact on human medicine. However, until now there has been very little attention in all the 'standard' model animals for what the findings on the molecular (genetic) level will mean for the every day life of patients around the world, *i.e.* their functionality. The information in such studies has been obtained in particular environments (animal housing in laboratories), using particular populations (often traditionally used, often inbred lab strains) in which the evolutionary and ecological context is almost completely missing. This would not be such a problem if this information were present, and extrapolation of the original to the new condition (and *vice-versa*) were possible. However, the ecological context, present or past, is typically more or less unknown in the 'standard' model species.

Another issue when using model organisms in the light of medical application is that it will only be useful when investigating public mechanisms. Research in model organisms will only find mechanisms that are conserved among animals and humans. Private mechanisms in humans that could prove the key to a healthy long life will not be found using model organisms. On the other hand, studying only one or a few model organisms involves the risk of finding mechanisms private to those species with no relevance to human ageing. So, first we need to know whether certain interesting mechanisms identified in model organisms are public or private.

Bicyclus anynana: a new model organism for the study of ageing

From the previous section, it is clear that the field of ageing research would benefit greatly from the use of a model species with known ecology. The tropical butterfly *Bicyclus anynana* is an excellent candidate. *B. anynana* (Butler 1879), the squinting

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Figure 2. Wet season *Bicyclus anynana* mating pair resting on a young maize leaf. Left is the male, right is the female. (Photo by W.H. Piel, 2005 ©)

bush brown, is a small butterfly species of the Nymphalidae family and occurs in tropical and subtropical East Africa (Figure 2).

The ecological habitat of this species is primarily savannah grassland and open forest, where the adult butterflies feed on (fallen) fruit with occasional mud and dung puddling. It has an overall brown colour with distinctive pattern elements on the wings, most notably a band of eye-like spots close to the distal wing margin. The biology of the species is characterised by seasonal polyphenism in the adult butterflies. There are 3 or 4 generations a year and the butterflies that fly in alternative seasons have dissimilar phenotypes. Like in most tropical areas, in Malawi, from where our stocks originate, there are two seasons: a wet season and a dry season. Adult *B. anynana* appear in two modes, or forms, which are induced in the pre-adult stage by environmental conditions that prevail in either the wet season or the dry season (Brakefield and Reitsma 1991). The wet season has, on average, high daily temperatures (above 23°C), and a high humidity and rainfall that enables a lush growth of grasses, some of which are larval host plants of *B. anynana*. This is the reproductive season when butterflies are actively flying around, in search of mates or food. The butterflies reproduce quickly and tend to die young. The eyespot pattern on the wings contributes to mate choice and is important in deflecting predator attacks away from the body towards the wing edge, which can readily tear away. The dry season has a lower daily temperatures (on average, below 21°C) and because of a lack of rain, the plant life, in particular the grasses,

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progressively die back. The availability of fruits also diminishes with time, and thus this season requires strong survival characteristics. The dry season form adults that emerge at the end of the rains are well suited for survival and avoid predation by their highly cryptic appearance when resting on dead leaves. They probably also save energy by their relative inactivity (Brakefield and Frankino 2007).

It is well established that the seasonal polyphenism is mediated by phenotypic plasticity. This means that the adult phenotype is induced by environmental conditions during development. The changing environmental conditions are used as a predictor for the future season by the larva in its final stage of development, and around this stage the adult phenotype is determined. The phenotypic plasticity can explain much of the variation observed in many adult traits, from morphology to physiology to behaviour. Crucially, the life history configuration is also determined in part by the plasticity, in addition to acclimation. Mechanisms underlying the phenotypic plasticity have been traced back to the endocrinological (hormone) level (Koch et al. 1996) and the gene-expression level (Brakefield et al. 1996).

Environmental tools: temperature and food

An important aim of this thesis is to investigate whether lifespan and ageing-related traits are an integral part of the seasonal polyphenism, and if so whether this is regulated by the same mechanisms. One can investigate genetic and physiological relations among traits by placing individuals under variable environments and measuring how they respond. In my thesis, I have varied two important components of the environment that the butterflies encounter in the wild: temperature and food.

Temperature

Temperature is highly relevant for any poikilothermic organism (when body temperature is mainly determined by external temperature, in contrast to endothermy) because it determines the rate of cellular physiology, and hence contributes to the rate of ageing: above some threshold, lower temperatures will allow insects to live longer than higher temperatures. Poikilothermic organisms can acclimatise to changing temperatures by altering their physiology, from changing respiration to heat or cold tolerance. They can also acclimatise by behaviour, such as basking or burrowing, or more extremely, by migrating. Additionally, in *B. anynana*, the temperature during development is important as a major operator of phenotypic plasticity that determines the seasonal dimorphism in the adult. These are two separate processes involving temperature, although they are likely to be regulated by similar mechanisms. Thus, both pre-adult and adult temperatures have a crucial impact on the expression of the phenotype, whether it concerns morphological, physiological, life history, or behavioural traits. Thus manipulating temperature in pre-adult and adult life in the laboratory to a large extent mimics wet and dry season field conditions, and enables the disentanglement of the

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contributions of genetic and environmental factors, and their interactions, to the phenotype.

Starvation stress

In addition to temperature, some of the butterflies experienced another, particular stringent artificial environment: absence of food. There are several reasons why inducing starvation stress will help to elucidate the mechanisms of longevity. Firstly, the ability to resist starvation is closely related to survival in many species. It is highly relevant in *B. anynana*, when occasional feeding is necessary to survive the dry season when resources are limiting; the alternative wet season is, in contrast, a period of plentiful resources. There are indications that famine has had a major impact on human evolution, the more recent of which can be backed-up by historical data (Prentice 2005). It is likely that such processes have shaped human genetic variation. Secondly, there is evidence that organisms use the same mechanisms to deal with a variety of stresses, including starvation stress. The general ability to deal with stress is an important component of longevity: the longer living individuals in a population are generally those that have the best stress resistance, whether they experience stress or not. An important aspect of general stress resistance is the extent of stress during development. Both adverse and beneficial effects of stress early in life on lifespan are observed; the outcome depends strongly on the degree of stress later in life. Thirdly, there is evidence for a strong genetic link between the regulation of both food stress resistance and lifespan. In nature, starvation resistance is under strong selection, whereas ageing is not. However, in artificial selection experiments (explained in the next section) using *Drosophila*, a positive genetic correlation between starvation resistance and longevity is observed, suggesting that the same set of genes is underpinning both. Molecular genetic studies have shown that some of these genes may well be members of the Insulin pathway, the hormonal system that regulates the energy availability for cells and that has an evolutionarily conserved function in all animals. Thus, imposing starvation will yield information on physiological and genetic mechanisms that are also (in part) regulating lifespan under better food conditions.

Genetic tools: artificial selection

In addition to procedures that use environmental manipulation to investigate underlying mechanisms, one can address the genetic architecture of a trait directly, by (artificially) selecting on it. Typically, artificial selection is the procedure of unidirectional selection in an artificial evolution setting. The individuals are allowed to grow, move or behave, and only those individuals that meet the selection criterion are allowed successful reproduction. It is essentially a breeding program, much like those used in an agricultural context. In evolutionary biology, the aim of artificial selection can be fourfold: (i) to assess whether genetic variation exists in a trait, (ii) to assess the proportion of phenotypic variation in a population that is

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attributable to genetic variation among individuals for a trait: the heritability, (iii) to look for constraints on, and the potential for the evolution of a trait, or (iv) to give insights in to the physiological or genetic regulation of a trait. Genetic variation for lifespan and other ageing-related traits has been shown in *Drosophila melanogaster*. Here they tend to have a lower heritability than is found for morphological traits, as is typical for life history traits. Based on these findings, and on our knowledge of field biology, a similar situation is assumed in *B. anynana*. The focus in this thesis is on aims iii and iv.

The strength of artificial selection is that it targets standing genetic variation, that is, the natural genetic variation that is present in a given population. Thus, in contrast to mutational analyses and transgenic animals, artificial selection is able to identify the mechanisms responsible for the variation in ageing in natural populations, and for the evolution of lifespan. This is because the whole organism is considered in all its complexity, rather than putting emphasis on the manipulation of a single gene. Moreover, the common use of specific strains of model organisms that clearly express the gene-alteration may give information not relevant in natural populations and environments of the same species.

There is much evidence that genetic background is very influential in the expression of genes or phenotypes (Hartman et al. 2001). The reason is that many genes are linked, either physically on the genome (linkage) or by an interactive influence on the phenotype (epistasis). These effects are considered unwanted in mutant studies, even though they are common in organisms and characteristic of the complexity and pleiotropy of gene networks. Nonetheless, artificial selection provides the best method to probe the standing (natural) genetic variation for any given trait, if one also takes into account other important correlated responses. Moreover, a thorough analysis of (potentially) correlated traits will yield more information about the genetic mechanisms underlying phenotypic changes that resulted from artificial selection. The additional advantage of using *B. anynana* is that findings from artificial selection experiments can be readily fitted within a substantial biological framework that has been built in part by previous artificial selection experiments.

Aim & outline of this thesis:

The general aim of the work described in this thesis is to help explain the variation in ageing by using the life history framework of *B. anynana*. Each chapter focuses on a different aspect of the life history, together giving a complete picture of the origins of variation in ageing in this species. A central theme to every chapter is the relative influence of genes, the environment, and how they related to plasticity.

Chapters 2 and 3 focus on the role of development in variation in adult lifespan. Chapter 2 focuses on phenotypic plasticity in response to pre-adult temperature and its influence on adult physiology and survival. Chapter 3 investigates the influence of life history traits that are rooted in development in a unique experimental set-up

that enables a distinction to be made between the effects of genetics and environment.

Chapters 4 and 5 describe populations artificially selected for increased lifespan. The butterflies from these populations in the course of generations have accumulated particular combinations of alleles at different genes that together contribute to a longer life under either starvation conditions or optimal lab food conditions; they are ideal to investigate the genetic and physiological mechanisms underlying differences in survival. Chapter 4 describes the response and correlated responses in physiological, developmental and reproductive traits to artificial selection for longevity in males. Chapter 5 describes similar experiments with artificial selection for starvation resistance in both sexes.

Chapters 6 and 7 further investigate potential mechanisms for the increased starvation resistance and longevity in the butterflies described in chapter 5. Chapter 6 investigates differences in male mating behaviour. Chapter 7 compares variation in candidate gene expression of genes involved in cellular maintenance processes in both selected and unselected butterflies.

Chapter 8 contains a summarising discussion of all chapters, and a perspective on how the ideas in this thesis can contribute to ageing research in the future.

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Chapter
2

**Phenotypic plasticity of
starvation resistance in the
butterfly *Bicyclus anynana*.[†]**

Abstract

Starvation resistance is an important trait related to survival in many species and often involves dramatic changes in physiology and homeostasis. The tropical African butterfly *Bicyclus anynana* lives in two seasonal environments and has evolved phenotypic plasticity. The contrasting demands of the favourable, wet season and the harsh, dry season have shaped a remarkable life history, which makes this species particularly interesting for investigating the relationship between starvation resistance, metabolism, and its environmental modulation.

This study reports on two laboratory experiments to investigate the effects of pre-adult and adult temperatures that mimic the seasonal environments, on starvation resistance and resting metabolic rate (RMR) in adult *B. anynana*. In addition, we investigate starvation resistance in wet and dry seasonal form genotypes; artificial selection on eyespot size has yielded lines that only produce one or the other of the seasonal forms across all rearing environments.

As expected, the results show a large effect of adult temperature. More relevant, we show here that both pre-adult temperature and genetic background also influence adult Starvation resistance, showing that phenotypic plasticity in this species includes starvation resistance. The dry season form genotype has a higher starvation resistance when developed at dry season temperatures, indicating a genetic modulation of starvation resistance in relation to temperature. Paradoxically, dry season pre-adult temperatures reduce starvation resistance and raise RMR. The high overall association of RMR and starvation resistance in our experiments suggests that energy expenditure and survival are linked, but that they may counteract each other in their influence on fitness in the dry season. We hypothesize that metabolism is moderating a trade-off between pre-adult (larval) survival and adult survival in the dry season.

[†] Pijpe, J., Brakefield, P.M., Zwaan, B.J. (2007) *Evolutionary Ecology* 21(5): 589-600

Introduction

Many organisms encounter periods of starvation in their life, and most organisms have evolved some form of adaptive physiology to cope with them. In times of food abundance, the excess of energy is typically stored as lipids that can then be used as resources in less affluent periods. Starvation resistance that enables survival under prolonged conditions of no food is an extreme form of adaptive physiology that occurs in some species. It has often been suggested that the mechanisms that induce survival under starvation also underlie the regulation of longevity. In a range of well studied animal species, long-lived individuals appear to be more resistant to multiple stresses, often including starvation (Service et al. 1985; Zwaan et al. 1991; Chippindale et al. 1993; Zwaan et al. 1995; Djawdan et al. 1998; Harshman et al. 1999b; Johnson et al. 2001; Longo and Fabrizio 2002).

The presence of a clear correlation between starvation resistance and longevity strengthens the proposal that regulation of metabolism, homeostasis and energy resources is crucial in determining lifespan (Pearl 1928; Van Voorhies and Ward 1999; Speakman 2005). However, the relation between metabolic rate and lifespan is more complex. There is much variability in this relation across species (Austad and Fischer 1991; Speakman 2005). Within species, metabolic rate does not always influence the rate of ageing directly (Liu and Walford 1975; Dillin et al. 2002; Van Voorhies et al. 2004). Nonetheless, alteration of metabolism is a potent physiological response for organisms to rapidly cope with environmental change, such as an absence of food, thereby enhancing survival. It has been shown that metabolic rates respond quickly to starvation in *Caenorhabditis elegans* (Van Voorhies 2002) and *Drosophila melanogaster* (Djawdan et al. 1997).

In poikilothermic organisms, including insects (Loeb and Northrop 1917), nematodes (Klass 1977) and fish (Liu and Walford 1975), ambient temperature has a marked influence on standard or resting metabolic rates and on lifespan, but little is known about the role of natural selection in shaping these relationships. The life history of the tropical African butterfly, *Bicyclus anynana* (Butler), makes it particularly interesting for investigating the relationship between starvation resistance, metabolism and the environmental temperature. The evolution of adaptive phenotypic plasticity in these butterflies has led to dramatically contrasting life histories in alternative seasonal environments. Depending on external temperature conditions close to pupation, *B. anynana* can develop into a physiologically well-adapted adult phenotype in each of the two annual seasons that occur in the field. The 'wet season' phenotype has a rapid reproduction, an active lifestyle, and a lifespan of a few weeks. The 'dry season' phenotype appears to have the physiological and metabolic properties of a thrifty lifestyle and thus will be able to survive stressful conditions that may include long periods of starvation without losing the capacity to reproduce at an advanced age of six to eight months (Brakefield and Reitsma 1991; Brakefield and Frankino 2006).

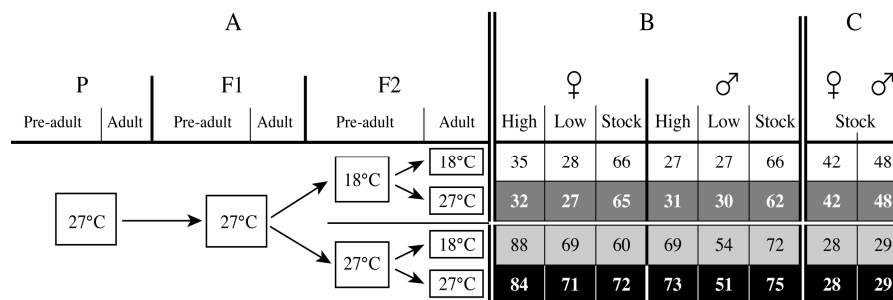
The aim of our study is to investigate whether starvation resistance could have an adaptive role in the dry season for *B. anynana* in the wild, and whether metabolic rate provides a physiological basis for such a role. In *B. anynana*, key life history traits are endocrinologically coupled to the dry or wet season temperature in the sensitive phase prior to pupation (Koch et al. 1996; Fischer et al. 2003b; Zijlstra et al. 2004). However, adult life history traits are also influenced by ambient temperature directly, and these two temperature effects need to be distinguished. We report on two experiments that investigate the effect of pre-adult and adult temperature on starvation resistance and resting metabolic rate in adult *B. anynana*. The first experiment explores the ability to resist starvation under differential wet and dry season temperatures during development and in adulthood. In addition to an unselected stock population, we used two populations of butterflies from lines artificially selected for eyespot size either to produce a 'wet season' phenotype or a 'dry season' phenotype for the wing pattern elements across all rearing environments (Brakefield et al. 1996). By including these lines, the hypothesis of a coupling of wing phenotype with starvation resistance can be tested on a genetic level. We thus equate the selection lines to dry and wet season form genotypes, expecting to find a higher starvation resistance in the 'dry season' lines and possibly a lower starvation resistance in the 'wet season' lines. In the second experiment, the same temperature regime was used for measuring adult resting metabolic rate, recorded as CO₂ production, in unselected stock butterflies. In both experiments, two temperatures (18°C and 27°C) were used to simulate dry and wet seasonal conditions, respectively. The results show the importance of considering pre-adult and genetic factors for adult starvation resistance and its relationship with fitness.

Material & Methods:

Experimental populations

The lines of *Bicyclus anynana* used in this experiment were all established from a stock population originating from over eighty gravid females collected in Malawi in 1989 and maintained at sufficiently high adult population size to maintain high levels of heterozygosity (van't Hof et al. 2005). The Stock population used in the starvation assay and the resting metabolic rate measurements were derived directly from this population. In addition, populations selected for a 'wet season' phenotype (High) or a 'dry season' phenotype (Low), based on the size of ventral wing eyespots were used in the starvation resistance assay. The High and Low lines were established by selecting the parents that were most similar to wet-season or dry-season season form, respectively, at intermediate temperature based on relative eyespot diameter (Brakefield et al. 1996). Initial intense selection for over 20 generations was followed by selection by eye, ensuring the phenotypes are clearly distinct until the present day. Virgin butterflies were used in both experiments. Humidity was 75±5% at 27±1°C and 65±5% at 18±1°C, with a 12-12 hour light-dark

Figure 1. Schematic overview of the experimental set-up that explores the influence of genetics (line), pre-adult and adult temperature on starvation resistance and metabolic rate. (A): temperature scheme indicating the relation of the 4 treatment groups. (B): number of butterflies used by line (High, Low, Stock) in the starvation resistance experiment (C): number of butterflies used in the resting metabolic rate experiment. The treatment groups are grey-scale coded which relate to the colour coding in figures 2, 4 and 5.



cycle at both temperatures. Larvae were reared on maize in population cages. Apart from the starvation assay, butterflies were fed slices of moist banana.

Starvation resistance experiment:

Stock, High and Low populations were all reared at the same conditions at 27°C for two generations to minimise uncontrolled environmental effects on development. The F₁ adults of each group were then randomly divided into two temperature groups (18°C and 27°C) to produce the F₂ generation that was used in the assay. F₁ mating for both temperature groups took place at 27°C. Since temperature at oviposition has an effect on several life-history traits (Fischer et al. 2003a), the butterflies were held in their designated environment for three days before egg collection. The F₂ eggs in the 18°C group were allowed to hatch at 20°C before transfer to 18°C for further development and assays because hatching success can be low at 18°C. F₂ larvae were reared in sleeve cages as described in Zijlstra et al. (2003) The F₂ adults were allowed to eclose, separated according to sex, and again randomly divided over the two temperature groups (for a schematic overview, see figure 1). They were then subjected to a starvation assay performed in small cylindrical hanging cages (25 Ø x 60 cm) with *ad-lib* presence of water-saturated cotton wool to prevent desiccation effects. Starvation commenced immediately after eclosion so adults had no access to food, only water. Deaths were scored every day at 18°C and twice a day at 27°C, always at the same time relative to the photoperiod.

Resting metabolic rate experiment

In a separate experiment with an identical set-up (figure 1), the resting metabolic rate (RMR) was measured for Stock butterflies reared at 27°C or 18°C and subsequently kept at 18°C or 27°C as adults. With this set-up that mimics seasonal average temperatures (Brakefield and Mazzotta 1995), results can be compared directly with the starvation resistance data for the Stock population. The butterflies were 2 days old when measured at their adult temperature. CO₂ levels (ml CO₂/h) as measured at temperature conditions similar to those used for adult maintenance, were used as an index of RMR. A Li-Cor LI-6251 CO₂ analyzer in a respirometer set-up (Sable Systems) with a push-through flow of 100 ml/min was used to measure respiration from individual butterflies in small cylindrical containers (4Ø x 9 cm). Measurements were performed in the dark part of the life cycle in a temperature controlled climate cabinet rendering the butterflies inactive (Zijlstra *et al*, unpublished data). Individual adult mass was then measured directly and used as a measure for body size. CO₂ data from two consecutive replicate measurements ($r > 0.95$) were analysed using Datacan 5.4 (Sable Systems) and averaged.

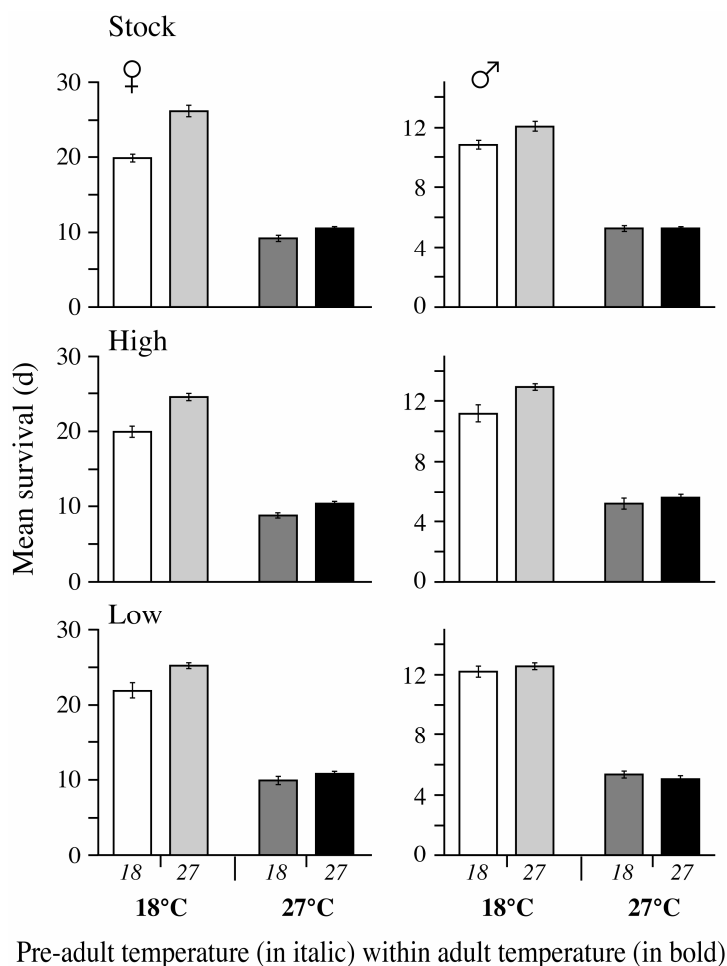
Statistics

Survival in the starvation assay was analysed by a Cox Proportional Hazard model, a non-parametric, conservative model that uses both likelihood-ratio test statistics and risk ratios \pm 95% confidence intervals to quantify factor effects. If the 95% confidence interval falls completely below or above 1.00, the risk of dying is significantly lower or higher, respectively. Initially, we build a full factorial model with the pre-adult temperature, adult temperature, sex, line, and the interactions between them. The final model presented below excluded non-significant factors while explaining most variation in SR. To quantify differences in survival between selection lines, a pair-wise comparison with the Stock line as reference group using risk ratios from a Cox Proportional Hazard model is most appropriate. Such analyses can only be performed pair-wise, so by sex and within pre-adult and adult temperatures. For the metabolic rate data, a minimal adequate General Linear Model was used, with individual adult dry mass as a covariant. Survival and metabolic rate data were standardised by treatment and sex before the correlation estimate over all lines was calculated as a Pearson's correlation coefficient. All tests were done with JMP 5.01 statistical software from SAS Institute Inc.

Results:

The factors sex and adult temperature together explain the majority (95 %) of the variation in starvation resistance (see table 1). Figure 2 shows that for Stock butterflies, females are, on average, nearly twice as starvation resistant as males (risk ratio = 0.27 [0.25 – 0.29]), and both sexes are more starvation resistant at the

Figure 2. Mean adult survival of Stock line (top), High (middle) and Low (bottom) line butterflies under starvation in the four temperature treatments. Results for females are indicated on the left, males on the right. Standard errors are indicated on top of bars. For statistical analyses, see text. The grey-scale coding of the treatment groups relate to figure 1.



Phenotypic plasticity of starvation resistance

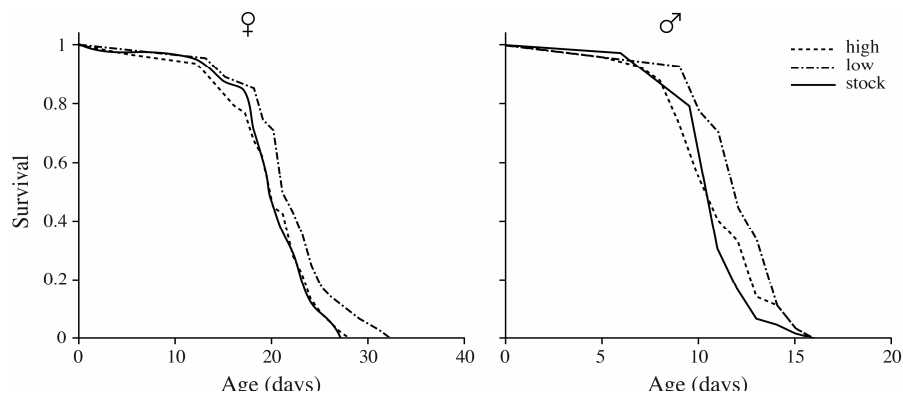
dry season adult temperature (risk ratio = 0.18 [0.16 – 0.20]). All interactions with sex are significant as well, indicating the importance of sex specific physiology in starvation resistance.

More surprising is the effect of pre-adult temperature (see table 1): butterflies that had developed at 18°C have a lower starvation resistance (risk ratio = 1.24 [1.17 – 1.31]), resulting in an opposite effect of ambient temperature in the two life stages on starvation resistance. Overall, the line effect in the Cox Proportional Hazard model is not significant, but the line x developmental temperature interaction is. This warrants a detailed analysis of line-specific survival. Analyses that compare lines by sex and within pre-adult and adult temperatures revealed that Low line females and males that developed at 18°C show a significantly higher starvation resistance at 18°C (risk ratio = 0.79 [0.61 – 0.99] and 0.76 [0.60 – 0.96], respectively; see figure 3).

Table 1. Minimum adequate Cox Proportional Hazards Fit for survival under starvation. df: degrees of freedom, L-R ChiSquare: likelihood ratio chi-square test value, p: probability.

Factor	df	L-R ChiSquare	p
Pre-adult temperature	1	46.6	<0.001
Adult temperature	1	1504.3	<0.001
Line	2	4.4	0.11
Line * Pre-adult temperature	2	11.0	<0.01
Sex	1	977.7	<0.001
Line * Sex	2	18.2	<0.001
Sex * Pre-adult temperature	1	32.9	<0.001
Sex * Adult temperature	1	16.8	<0.001

Figure 3. Adult survival curves under starvation of the selection lines High and Low and of unselected Stock at 18°C that had developed at 18°C. The Low line in both females and males is significantly more starvation resistant compared to Stock and High lines. Note the different age scales. For statistical analyses, see text.

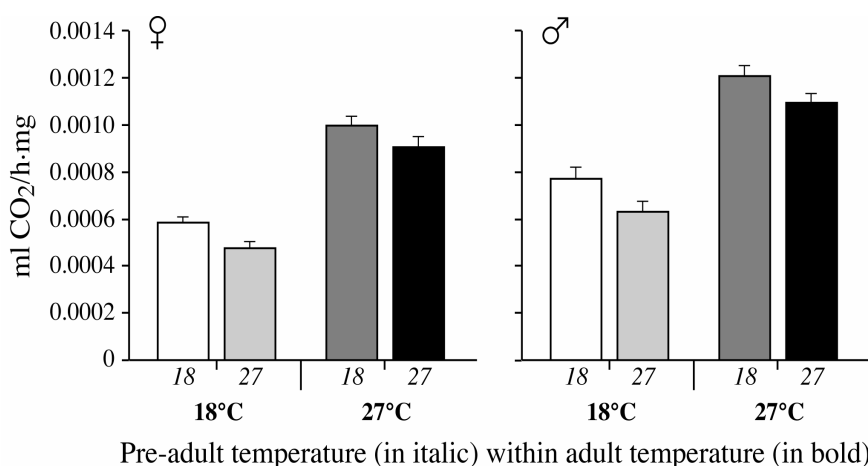


Chapter 2

Table 2. Minimum adequate model of an ANOVA of the Resting Metabolic Rate (CO₂/mg). Df: degrees of freedom. p: probability.

Factor	df	Sum of Squares	F Ratio	p
Pre-adult temperature	1	3.2·e ⁻³	21.5	<0.0001
Adult temperature	1	40.1·e ⁻³	269.2	<0.0001
Sex	1	0.47·e ⁻³	3.1	0.0772
Dry mass	1	6.1·e ⁻³	41.3	<0.0001
Adult temperature * Dry mass	1	1.9·e ⁻³	12.5	<0.001

Figure 4. Average CO₂ production per hour, corrected for individual dry mass, for Stock males and females in the 4 temperature treatments. Standard errors are indicated on top of bars. The grey-scale coding of the treatment groups relate to figure 1. See table 2 for ANOVA statistics.



In addition, Low line females that developed at 18°C show a marginally higher starvation resistance at 27°C (risk ratio = 0.82 [0.65 – 1.03]) (data not shown). Starvation resistance of males or females that developed at 27°C is similar for all lines, as there were no other significant differences between lines (data not shown). Females have a lower resting metabolic rate (RMR) compared to males (see figure 4 and table 2). Adult CO₂ production was significantly affected by ambient temperature in both pre-adult and adult stages (table 2). Consistent with the results for starvation resistance, the effect of temperature for adult RMR is opposite in the two life stages. Low adult temperature leads to lower CO₂ production while low pre-adult temperature results in a higher adult RMR (see figure 4). The correlation between standardised survival and standardised resting metabolic rate over all lines and sexes is significantly negative (Pearson correlation = 0.80, p<0.02, see figure 5).

Discussion & Conclusions

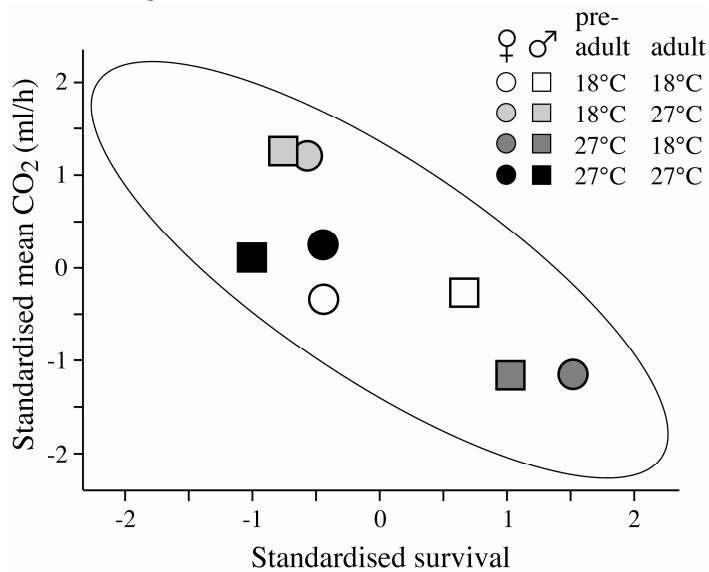
Developmentally induced polyphenism versus adult plasticity

The results show that survival under starvation conditions is highly dependent on both pre-adult and adult ambient temperature in *B. anynana*. The negative effect of temperature (within normal physiological ranges) on poikilotherm lifespan under ad lib food conditions is well described as a consequence of metabolic rate (Loeb and Northrop 1917; Liu and Walford 1975; Klass 1977). Here, we show that survival under starvation is similarly dependant on temperature, and that this is at least partly mediated by metabolic rate. An intriguing novel finding is that the pre-adult temperature has a substantial effect on survival under starvation such that butterflies reared at the low temperature are *less* starvation resistant. Whilst the effect is not as large as that of adult temperature, at first sight it is at odds with what is interpreted as being beneficial in the field. A possible explanation for this phenomenon is discussed below (also see figure 6). The pattern of survival over the temperature treatments is, however, in agreement with a higher resting metabolic rate in butterflies reared at a lower pre-adult temperature as is indicated by the high overall negative correlation of RMR and starvation resistance in our experiments (figure 5). Probably, a higher metabolism leads to a faster depletion of energy reserves, which is highly relevant under starvation conditions (Djawdan et al. 1998; Marron et al. 2003). Our study did not include the investigation of body size and fat content, which are both positively correlated with starvation resistance (Chippindale et al. 1996; Harshman et al. 1999a). In particular, body size could be important in mediating the effect of pre-adult temperature on starvation resistance. However, this is very unlikely because the effect of temperature on starvation resistance and body size is opposite. In general, insects growing at lower temperature become larger (Davidowitz and Nijhout 2004), and we find that such butterflies are less starvation resistance. Whether survival under non-starving conditions is equally dependent on metabolic rate remains to be tested in *B. anynana*.

Sex differences in starvation resistance

The higher starvation resistance in females is expected to follow from the higher body weight (Brakefield and Kesbeke 1997) that reflects a higher absolute fat content (Zwaan et al. 2001; Fischer et al. 2003b) and possibly a higher carbohydrate content in females (Simmons and Bradley 1997; Djawdan et al. 1998). This higher starvation resistance could partly be an artefact because we used virgin butterflies. Females, compared to males, could have used more of their resources for survival, that after mating are usually allocated to reproduction in the wild. Although this could be true in the wet season, in the dry season there is very little if any reproduction-related activity, as reproduction is postponed until the start of the

Figure 5. Pearson's correlation between standardised mean RMR and standardised mean survival per sex, pre-adult and adult temperature, averaged over all lines. Ellipse indicates the 95% confidence interval, with an overall correlation of -0.80 , $p < 0.02$. The grey-scale coding of the treatment groups relate to figure 1.



next wet season (Brakefield and Reitsma 1991). In addition, sex differences in survival under starvation can at least partly be explained by a lower resting metabolic rate of females that enables them to cope better with periods of starvation.

Genetic coupling between seasonal wing pattern phenotype and starvation resistance.

We included the High and Low lines in the starvation experiment because we expected the genetic difference in wing patterning to be reflected in the physiology corresponding to the seasonal environment. Both the developmental plasticity of wing patterns (Koch et al. 1996; Brakefield et al. 1998) and life history traits (Fischer et al. 2003b; Zijlstra et al. 2004) are regulated by ecdysteroids. From the starvation resistance results it can be inferred that there is genetic variation underpinning a coupling of starvation resistance and wing pattern phenotype in *B.anynana*. In several cases the Low line is significantly more starvation resistant following development at 18°C. The effect is remarkable because it occurs in lines selected purely on wing pattern phenotype. The fact that enhanced starvation resistance is only present at the dry season developing temperature suggests that gene by environment interactions are important in the expression of aspects of the seasonal

phenotypes. Physiological candidate mechanisms for increased starvation resistance in the Low line include lower metabolic rate and enlarged reserves. It would be useful to couple further studies on such mechanisms with energy allocation to the reproductive system.

Adaptive plasticity in starvation resistance

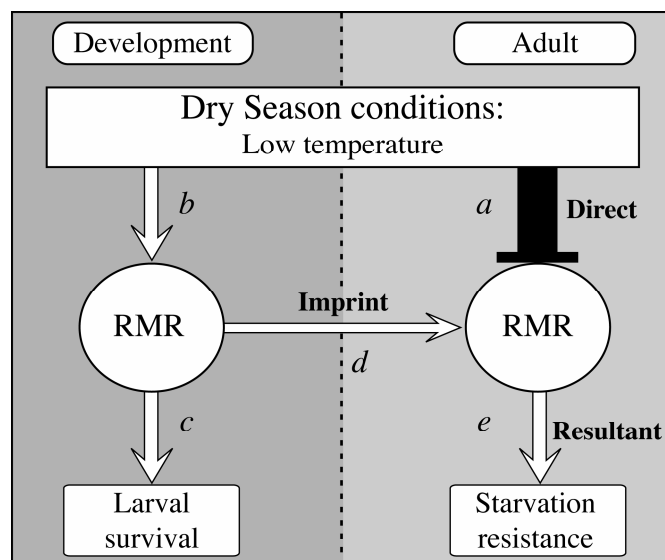
Although our results clearly indicate that *B. anynana* has phenotypic plasticity in starvation resistance, it is not clear whether this is due to direct adaptation to periodical starvation conditions in the dry season. The data lead to a paradox. Thus whilst dry season form selected butterflies (Low line) are a little more starvation resistant than Stock or wet season form selected (High line) butterflies, low pre-adult temperatures, as occur in the early part of the dry season, have a negative effect on adult survival under starvation conditions in the laboratory. The strong association between RMR and starvation resistance may offer an explanation for the negative effect of low pre-adult temperature on adult survival. This hypothesis is visualized in figure 6.

Similarly to adult metabolism (a), larval metabolism depends strongly on ambient temperature. However, the larvae cannot afford to stop feeding for too long because their food plants of grasses will dry out in the early dry season (Brakefield and Reitsma 1991). They may then need to raise their metabolism (b) to cope with the low developmental temperature in the dry season and successfully metamorphose to the adult stage (c). Our results suggest that their relatively elevated level of resting metabolism is apparently imprinted on adults (d), meaning that the level of larval metabolism is partly fixed when expressed in adults. This results in a decrease in adult survival under starvation conditions (Resultant, e) compared to butterflies that were not constrained to maintain a higher resting metabolism as larvae. Thus, the potential negative effect of pre-adult temperature on adult survival may be the result of selection for increased RMR with low, dry season temperature. This suggests that metabolism is moderating a trade-off between pre-adult (larval) survival and adult survival, at least in the dry season. However, the net result of these effects is the increased resistance to starvation in the dry season, the environment in which this matters.

The imprinting of metabolic rate could be regulated by Ecdysteroid signaling. Interestingly, Thyroid Hormone, the mammal orthologue of Ecdysone, is central in the regulation of homeostasis and metabolism and is implicated in genomic imprinting (Tsai et al. 2002). Thyroid Hormone signaling is also tentatively involved in ageing (Tatar et al. 2003). Combining hormone and metabolic rate measurements in larvae at various temperatures and stages should test this hypothesis.

Figure 6. Conceptual figure depicting the hypothetical relations between temperature and resting metabolic rate (RMR) that matter for survival in the dry season in nature. Pointed arrowheads indicates positive influence, blunt arrowhead indicates negative influence. The thickness of the arrows indicates the magnitude of their effect.

In the dry season, there is a balance between the effects of temperature at the pre-adult stage and adult stage. At the adult stage, the low temperature directly lowers adult RMR (a) and hence increases starvation resistance (Fig. 4). At the pre-adult stage, a low temperature raises RMR (b), perhaps to increase larval survival (c). The imprint of the raised pre-adult RMR on adults (d) is in conflict with the adult RMR. Thus, the balance between the direct temperature influence and the imprint will determine adult survival under starvation conditions (e). The result predicts a trade-off between larval and adult survival.



Conclusions

Adult starvation resistance differs in the two seasonal forms of *B. anynana*. Butterflies with a dry season genetic background have a higher adult starvation resistance. However, a dry season pre-adult temperature results in a lower starvation resistance. We hypothesise that this is the consequence of a trade-off favouring pre-adult survival. We conclude that starvation resistance is one component of the complex adaptive physiology required to meet the conditions that prevail in the dry season. The striking correlation of RMR and starvation resistance in our experiments suggests that metabolic rate is an important factor in this adaptive physiology.

Acknowledgements

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Phenotypic plasticity of starvation resistance

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Chapter
3

Consequences of artificial selection on pre-adult development for adult lifespan under benign conditions in the butterfly *Bicyclus anynana*[†]

Abstract

The genetic architecture underlying the regulation of lifespan is shaped by evolutionary history, thus including selection in past environments. In particular, the developmental environment is important, because selection pressure for survival is highest during development. From this life-history point of view, the ageing phenotype is the outcome of these factors, and links between the developmental and adult life stage are expected. In this study, we specifically address whether genetic variation in pre-adult traits affects adult lifespan. We use lines artificially selected for divergence in development time, pupal mass or egg size, thus exploiting the standing genetic variation in pre-adult traits present in natural populations of *B. anynana*. We then reared individuals from each line and the unselected base population in a common environment, and recorded each selected trait and adult longevity. In general, differences in adult lifespan across selection lines were small. This is not surprising given the benign conditions used here. The minor differences in adult survival were only partially the result of environmental influences, as indicated by low phenotypic correlations. However, significant genetic correlations point to possible intrinsic mechanisms involved in lifespan regulation. Genetic variation in egg mass or pupal mass did not contribute to variation in lifespan. However, we found a negative genetic correlation between developmental time and lifespan, suggesting a genetic coupling of faster development with a longer adult lifespan in this species. A follow-up study with an identical set-up that introduces stress during development should give a more detailed insight into the role of development in the regulation of lifespan.

[†] Pijpe, J., Fischer, K., Brakefield, P.M., Zwaan, B.J. (2006) Mechanisms of Ageing and Development 127: 802-807

Introduction

Arguably, two main life stages can be differentiated in successfully reproducing organisms: development and adulthood. Classically, in sexual multicellular organisms development initiates with conception, although pre-zygote factors are recognised to be influential (Mousseau and Fox 1998). Development ends with sexual maturity and the start of the reproductive period, while adulthood ends with death.

The transition from development to adulthood can be fairly gradual in mammals whilst in holometabolous insects these two stages are highly distinctive. Development essentially ends in metamorphosis and the eclosion of an imago or adult. The major transition of metamorphosis occurs in the pupa, when imaginal discs developed from epidermal cells in the embryo divide and grow to form the adult (Wolpert, 1998). The role of development in shaping the adult phenotype is well established. In insects, many fitness-related traits, such as body size, fat content, colour pattern and the timing of eclosion are determined during development (Brakefield et al. 2003). Thus, the environment during development may leave its footprint in adulthood, influencing adult life history, including ageing (Zwaan 2003; Gluckman et al. 2004; Brakefield & Frankino 2006).

Developmental variation and adult lifespan

When investigating the role of development on lifespan, it is crucial to discriminate between variation in development that results from environmental or genetic sources, or by their interaction (Zwaan 2003). Much research on environmentally induced variation in adult lifespan has been performed in the past using *Drosophila* (Miller & Thomas 1958; Lints & Soliman 1977; Zwaan et al. 1991), mostly in relation to the developmental theory of ageing (Lints & Lints, 1971). It has proven to be difficult to show a clear relation of developmental life history traits with lifespan. This has two main reasons. First, the environmental manipulation that was used in such experiments introduces additional variation. Secondly, the context of the natural environment was missing. As a consequence, the attention of ageing science has diverted away from studying development. An exception is the influence of stress during development on adult lifespan (Zwaan et al. 1991; Sorensen & Loeschke 2001; Brakefield & Frankino 2006). This has led to the stress theory of ageing, which states that fitness traits in development and adulthood are linked through stress resistance, which is omnipresent in all life stages (Parsons 2003). Investigating developmental variation caused by genetic factors typically involves mutants of large-effect (Clancy et al. 2001) or transgenic manipulation (Aigaki et al. 2002) that allow successful screening for genes involved in longevity.

It is unclear to what extent standing genetic variation in developmental traits contributes to variation in adult lifespan. This information is important because

variation in lifespan can only be fully understood when taking into account the whole life-history, including development. Natural selection should result in pre-adult growth traits that maximise fitness in the adult stage. All developmental traits are fitness related and have been under strong selection. Lifespan may evolve, but never independently of development and the genetic architecture underpinning development. This is recognised by a recent initiative that highlights the role of development in ageing (see Westendorp & Wimmer 2005). By focusing on the standing genetic variation present in natural populations it is possible to identify the genes that matter in the evolution of lifespan, which may eventually help to the implementation of treatments for age-related diseases in humans (Kirkwood and Austad 2000; Zwaan 2003).

An excellent organism to study natural variation in life-history traits in relation to development is the tropical butterfly *Bicyclus anynana*. This African species occurs in an environment with wet and dry seasons and has evolved adaptive phenotypic plasticity in the form of seasonal polyphenism (Brakefield & Frankino 2006). Many traits of the adult phenotype are determined late in development through endocrinological responses to temperature (Brakefield et al. 1996; Koch et al. 1996; Zijlstra et al. 2004). We have used artificial selection to establish separate pairs of upward and downward lines with divergent phenotypes for development time, pupal mass and egg size, thus exploiting the standing genetic variation in life history traits present in natural populations of *B. anynana*. These are all key developmental traits and closely related to fitness (Brakefield et al. 2003). We use these to tease apart the environmental and genetic effects on variation in adult lifespan.

The present study aims at uncovering the role of genetic variation in several pre-adult life-history traits on adult lifespan. Our experiment is unique in that we have used the same base population representing an outcrossed stock to set up independent pairs of selected lines for each of the key life history traits in pre-adult development and then used a common garden experiment to examine the consequences for adult lifespan. Since the experiment was performed in a single benign environment, we can investigate the influence of genetic variation in pre-adult traits on variation in adult lifespan directly, minimising the introduction of additional variation (e.g. caused by different environments).

Materials & Methods

Experimental populations

All butterflies were derived from a single stock population that has been reared in the laboratory for over 100 generations since the parental generation was collected in Malawi in 1988. High levels of heterozygosity have been maintained (Van 't Hof et al. 2005). Butterflies from seven selection lines established from this stock were used in the present experiment. Six lines were artificially selected for increases or decreases in the three major life-history traits: egg size, pupal mass and

Table 1. Overview of the lines used in this experiment and their abbreviation (Code) used throughout this paper.

Life-history trait		Egg size	Pupal size	Development time	Unselected
Line name (Code)	Selection increased trait value	↑ Large Egg Size (LES)	Large Pupae (LP)	Long Time (LT)	Stock (Stock)
	Selection decreased trait value	↓ Small Egg Size (SES)	Small Pupae (SP)	Short Time (ST)	

development time (egg to adult). In addition, the Stock population was used as unselected control. Please refer to table 1 for an overview and their names.

The unique designs of the present experiment posed practical limitations and forced us to use only a single replicate of each selection line. However, all lines were replicated with significant realised heritabilities and replicates generally showing comparable results ((egg size selection: Fischer et al. 2006); (pupal mass selection: Frankino et al. 2005); (multiple selection lines, including development time: Brakefield & Kesbeke 1997 and Zijlstra et al. 2003)). In addition, all past selection was performed at 27°C in environments similar to the one used in the present experiment. Consequently, the extensive phenotypic divergence of the lines in the targeted traits has a clear genetic basis, and on the basis of our previous work (Fischer et al. 2006), are typical representatives of the full set of selection lines.

Experimental design

A total of 700 individuals, 100 per line, were followed individually from egg to death. Temperature was 27°C ($\pm 1^\circ\text{C}$), humidity 70% ($\pm 5\%$) and photoperiod 12:12h light:dark throughout the experiment. The parental generation of all lines was also reared under these conditions to eliminate potential cross-generational effects.

Eggs were laid on maize cuttings over a 12 h light period, then weighed to the nearest $1 \cdot 10^{-4}$ mg on a micro-balance and transferred to 1.5 ml Eppendorf tubes. Hatchlings were individually transferred to *Oplismenus africanus* plants, a natural food plant of *B. anynana*. The plants were propagated vegetatively, to standardize environmental conditions as far as possible. Larvae were reared in a common garden environment (one level of a single climate room).

One larva from each line was placed individually in a plastic pot (10 \varnothing x 15 cm) on a separate stolon of a single plant (*i.e.* seven larvae per plant, each in a separate

container, with 100 plants in total). The resulting pupae were weighed to the nearest $1 \cdot 10^{-2}$ mg one day after pupation and transferred to a small plastic pot (5 \varnothing x 7 cm) until eclosion. Egg development time, larval development time and pupal development time were also recorded.

Following eclosion, adults were individually numbered on their left hind wing, separated by sex and randomly divided among four large population cages (60 \varnothing x 100 cm; two per sex) with a maximum of 140 butterflies per cage. Thus, all butterflies were virgin. Food was present *ad libitum* as ripe banana with moistened cotton wool on top and at the bottom of the cage. Deaths were recorded on Mondays, Wednesdays and Fridays throughout the experiment. For further details on pre-adult experimental conditions and measurements see Fischer *et al.* (unpublished manuscript).

Statistical analysis

Pre-adult mortality was analysed using a nominal logistic regression (LR) of all lines pooled. Odds ratios (with their 95% confidence intervals) are used to identify the relative deviation from the unselected Stock line.

Analyses of factors influencing lifespan used two statistical approaches. First, Cox Proportional Hazard (PH) models using a stepwise procedure constructing a minimal adequate model was used. Likelihood Ratio test statistics are used to quantify factor effects. The initial model contained the factors egg size, development time, pupal mass, replicate cage, line and sex. Only single factors and two-way interactions were considered, with line and sex treated as fixed factors. Survival among replicate cages did not differ significantly, so they were pooled to increase statistical power. Lifespan differences were quantified by pair-wise comparisons between individual selection lines and the Stock line, using risk ratios and their confidence intervals from a Cox PH model by sex with line as the single factor.

Secondly, phenotypic and genetic correlations were calculated using Pearson's (product-moment) correlations (r) between lifespan and all other measured life-history traits. In addition, partial phenotypic correlations (pr) between two variables while controlling for the effects of a third variable, were calculated. We here focus on correlations between adult lifespan and pre-adult traits. Correlations among the pre-adult traits can be found in Fischer *et al.* (unpublished manuscript).

Phenotypic (partial) correlations (r_p / pr_p) were calculated by pooling data across all lines, yielding a single population with extreme variation in pupal mass, development time and egg size. To analyse only correlations resulting from non-genetic effects, the data were corrected for genetic variation caused by selection lines and sex differences by using the residuals of a two-way ANOVA with factors line and sex. The analyses were performed for each sex, and the sexes combined.

Genetic (partial) correlations (r_G / pr_G) were calculated from line means for each trait and by sex (7 lines and 2 sexes). Exploiting the genetic divergence between the selection lines, r_G 's are used as a proxy for genetic correlations among traits. Thus,

the variation in the genetic correlation data will be caused by the factors line and sex only, which we consider to be genetic factors.

In order to separate physiological lifespan under benign conditions from deaths caused by impairment of food intake, the small cohort of early dying animals was excluded from the analyses. As a cut-off point we used 9 days for females (11 cases) and 5 days for males (7 cases), which relate to the upper 95% cases of the survival under starvation in Stock butterflies at the same conditions as found in another study (Pijpe *et al.*, unpublished). In addition, butterflies that eclosed with deformed or missing appendages were excluded from the analyses. In total 434 individuals were incorporated in the lifespan analyses. All analyses were done using JMP 5.01 and SPSS 12.0.1.

Results

Pre-adult mortality

Pre-adult mortality is typically around 20%. There is a significant line effect on pre-adult mortality (LR: $\chi^2 = 14.13$, df= 6, $p < 0.05$). This seems to be caused mainly by the LT line (40%; Odds ratio = 0.39 [0.20 – 0.74]).

Variation in adult lifespan across sexes and selection lines

Results from the Cox PH model suggest that sex is the main factor affecting lifespan in *B. anynana* (table 2): median lifespan in males is over 20% longer than in females. This sex difference is consistent across all lines except the SP line, where lifespan does not differ between sexes. Pupal mass had a small but significant influence, as had the interactions between egg mass and pupal mass and between development time and line (table 2). The latter may indicate that selection lines may sometimes

Table 2. Factors in the minimal adequate Cox Proportional Hazard model for lifespan in *Bicyclus anynana*. Deaths that occurred in the first 5 days for males and 9 days for females were excluded. Their inclusion would not change any of the results qualitatively. The total number of included individuals was 434. df: degrees of freedom, L-R Chi-Square: Value for the Likelihood Ratio test statistic, p: probability.

Factor	df	L-R Chi-Square	p
Line	6	9.10	0.17
Sex	1	52.38	<0.0001
Egg mass	1	0.95	0.33
Pupal mass	1	6.56	0.015
Pupal mass * Egg mass	1	4.86	0.027
Development time	1	0.36	0.55
Development time *Line	6	14.70	0.023

Consequences of artificial selection for pre-adult development

Figure 1. Survival patterns over time for males and females from six selection lines and an unselected stock population of *Bicyclus anynana*.

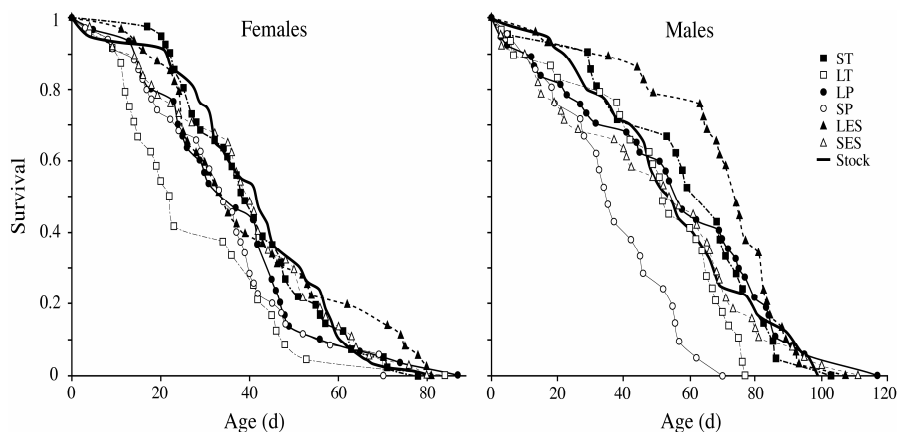


Table 3 Risk ratios for pair wise comparisons between individual selection lines and the Stock line. Significant deviations from the Stock are indicated in bold. Cases in italic are not significant but show a trend deviating from the Stock line. F: females, M: males. CI: 95% confidence interval. n: sample size

Sex	Line	Risk ratio	CI	n
Females	ST	1.07	[0.85 - 1.34]	31
	LT	1.35	[1.02 - 1.76]	22
	LP	1.12	[0.87 - 1.44]	29
	SP	1.18	[0.93 - 1.51]	32
	LES	1.01	[0.80 - 1.29]	35
	SES	1.02	[0.81 - 1.30]	34
	Stock	1	- -	36
Males	ST	0.90	[0.67 - 1.17]	20
	LT	1.19	[0.92 - 1.54]	28
	LP	0.91	[0.72 - 1.15]	33
	SP	1.57	[1.16 - 2.10]	20
	LES	0.83	[0.65 - 1.06]	29
	SES	0.97	[0.77 - 1.23]	35
	Stock	1	- -	40

have different relations between these traits and lifespan. Separate models for each sex (data not shown) revealed that much power is lost and that the only factors that remain significant are a line effect in males (L-R: $\chi^2 = 26.73$, $p < 0.001$), and a pupal mass effect in females (L-R: $\chi^2 = 5.18$, $p < 0.05$).

Regarding differences within lines (figure 1), SP males and LT females lived significantly shorter than Stock males and females, respectively. Further, there were (non-significant) tendencies towards a longer lifespan in LES males and a shorter one in LT males compared to Stock males, and once again a shorter lifespan in SP females compared to Stock females (table 3).

Phenotypic correlations

Correlations between adult lifespan and the pre-adult traits measured were all weak and in most cases non-significant (table 4). Only pupal mass was significantly related to lifespan, with larger individuals tending to live longer in females and, when data were pooled, across sexes. Partial correlations were similar to Pearson's correlations throughout, indicating that the latter are not strongly affected by variation in the third trait.

Table 4. Phenotypic correlations (r) and partial correlations (pr) of the residuals of the three pre-adult variables after a two-way ANOVA with factors line and sex. Significant correlations ($p < 0.05$) are depicted in bold. n: sample size, df: degrees of freedom, p: probability.

A Sexes combined						
Variable	r	n	p	pr	df	p
Egg mass	-0.02	434	0.71	-0.036	430	0.45
Development time	-0.07	434	0.17	-0.024	430	0.62
Pupal mass	0.11	434	0.017	0.10	430	0.034
B Females						
Variable	r	n	p	pr	df	p
Egg mass	-0.026	229	0.69	-0.044	225	0.51
Development time	-0.12	229	0.062	-0.074	225	0.27
Pupal mass	0.14	229	0.034	0.11	225	0.11
C Males						
Variable	r	n	p	pr	df	p
Egg mass	-0.0107	205	0.88	-0.0314	201	0.66
Development time	-0.0178	205	0.80	0.0200	201	0.78
Pupal mass	0.0970	205	0.17	0.1015	201	0.15

Genetic correlations

Genetic correlations between lifespan and pre-adult traits were low and not significant for egg mass or pupal mass. However, the correlation is significantly negative for development time (table 5, figure 2). Partial correlations indicate that the latter is not strongly affected by variation in the third trait. To further assess whether drawing conclusions on the genetic correlations are not based on a single line, we analysed the genetic correlations while excluding single or multiple lines. Although excluding the LT females or both sexes from the analysis reduces the correlation, it remains relatively high (r around 0.5; data not shown). Removal of LP females has a similar effect on the correlation coefficient, but they do not differ in survival from the unselected Stock line. In addition, removal of LT males (which also have a slightly reduced lifespan) or SP males (highly reduced lifespan) has a negligible influence on the correlation coefficient, indicating that the correlation is robust. Reduction of the significance of the observed correlation is thus a result of the relatively low number of lines and not of a strong influence of any one particular line.

Figure 2 Genetic correlation of line mean adult lifespan and line mean development time using combined sexes. Large symbols indicate females line means, small symbols males line means. The solid line is the linear regression fit. The dashed line is the orthogonal fit indicating the correlation. See table 5 for details.

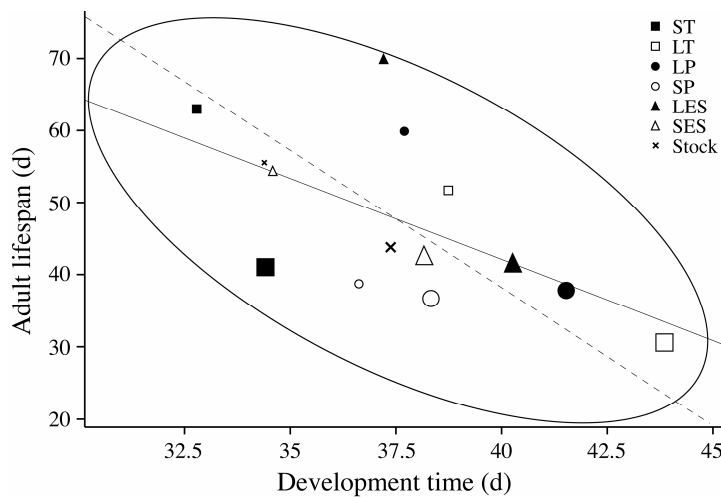


Table 5. Genetic correlations (r) and partial correlations (pr) of line means lifespan with line mean of egg mass, pupal mass and development time. Significant correlations are depicted in bold. N: number of cases, df: degrees of freedom, p: probability.

Variable	r	n	p	pr	df	p
Egg mass	0.28	14	0.33	0.55	10	0.064
Development time	-0.59	14	0.026	-0.63	10	0.027
Pupal mass	-0.22	14	0.44	-0.11	10	0.74

Discussion

Variation in adult lifespan across sexes and selection lines

The higher lifespan of males is highly consistent across lines and most of the other relations between lifespan and other life-history traits have to be evaluated in this light (Kirkwood 2001; Wiklund et al. 2003). The significant individual line effects are reflected in the general model. If this is largely underpinned by the traits targeted by artificial selection in the lines, trait values up and down should show opposite effects on lifespan when different alleles of the same genes underlie the selection response. However, this was not found, indicating that “private” mechanisms coupled to the trait selected may have influenced adult lifespan differently in some lines, i.e. different genes are involved in the response of the traits in the alternative directions. Disregarding interactions, pupal mass is the only consistent significant continuous factor affecting lifespan; butterflies from larger pupa tend to show longer lifespan. How the significance of the factors sex and line relate to the influence of the pre-adult traits on the variation in lifespan is difficult to uncover in a general model. Such complex relationships are more appropriately investigated by multivariate correlation analyses.

Variation in adult lifespan within selection lines

The LT line females clearly had a reduced lifespan and males show a similar trend. These lines do not show reduced levels of genetic polymorphism at a set of enzyme loci nor do they show hybrid vigour (Brakefield and de Vos, unpublished data), making inbreeding depression an unlikely explanation. The pupal mortality data suggest that development for this line is especially stressful. This may provide a more general explanation. Perhaps the prolonged pre-adult development makes the individuals more prone to random environmental influences (including infection)

that form a “developmental scar”, which may also negatively affect adult life history (c.f. Brakefield et al. 2005).

SP males and to a lesser extent also SP females are at a higher risk of dying. This seems to be due to developmental processes specific to the SP line. One possible clue about the mechanism comes from values for the weight-corrected resting metabolic rate that are higher in SP line butterflies (Zwaan, Kesbeke, Zijlstra, unpublished data).

LES line males have an increased lifespan, and although this is not significant, it is consistent with other experiments using these lines (Pijpe and Bot, unpublished data). The possible mechanisms for this effect include beneficial effects of early growth (Fischer et al. 2003) and altered endocrine regulation. Studies on endocrine function of reproduction in *B. anynana* are ongoing and may provide more insight into correlated responses in such lines.

Phenotypic and genetic correlations

The results generally show weak phenotypic correlations, indicating that lifespan depends only loosely on environmental influences in our experiment. Although important environmental conditions such as food availability, density, temperature and humidity were the same for all individuals, there is phenotypic variation in developmental traits, independent of the line mean. This is not surprising and can have two causes. First, the micro-environmental differences that we cannot control may still exist between some individuals. Secondly, it can be caused by genotype-by-environment interactions during development. Apparently, pupal mass is particularly sensitive for such effects. The phenotypic correlation with pupal mass is significantly positive but explains less than 2% of the variation in adult lifespan.

The results from the genetic correlation analyses indicate that a faster development is correlated with a longer lifespan, while egg mass and pupal mass seem less important. Because line means are used to calculate correlations, individual lines may disproportionately influence the observed correlations. The low survival in the LT line could explain most of the correlation. However, although removing the LT females or both sexes reduces the correlation, it remains relatively high, indicating the negative correlation of development time with lifespan is independent of a single line. It also indicates that using a single set of replicate selection lines does not hinder the generalisation of our results. Thus, we conclude that the robust pattern we find despite the relatively small data set indicates a strong genetic relationship between faster development and longer life.

Perspective

In this study, we address the link between pre-adult developmental traits and adult lifespan under benign conditions. We concentrate on the question of whether past

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selection has left a footprint in the genetic architecture by determining the genetic correlations of egg size, development time, pupal size with lifespan.

The results from individual selection lines do not point to a general developmental mechanism that underlies differences in lifespan. The observed differences in survival are only partially the result of environmental influences, as indicated by the low phenotypic correlations. However, our experimental set-up allowed us to detect some interesting genetic correlations that point to possible intrinsic mechanisms involved in lifespan regulation. There is a clear negative genetic correlation between lifespan and development time: faster developing individuals live longer. However, we find that genetic variation in pupal mass - the final mass attained after larval growth - is not related to variation in adult lifespan. How can we explain this, and what is the impact of this finding?

A trivial explanation is that in our analyses we missed a third trait that is mediating the correlation between development time and lifespan (Pease & Bull 1988). This is a possibility, although it was our explicit goal to take into account such confounding factors. The exceptionally wide scope created by using selection lines of the three most studied key developmental traits, based on the same population and in the same environment, is unique and provides an answer to the problems of interpreting correlated responses. Because of our experimental set-up, we are able to conclude that body mass is not such a third trait. This is remarkable because development time and final mass have been considered to be highly positively correlated, although there is much discussion on whether this reflects reality in nature (Klingenberg and Spence 1997). It seems that the relationship of development time and pupal mass is partly uncoupled when it comes to genetic correlations with lifespan and that any costs involved in accelerated growth do not apply with regard to lifespan.

A second explanation is provided by the stress hypothesis of ageing, which states that because stress is the rule in natural populations, survival to old age will strongly depend on genes that confer stress resistance (Parsons 2003). A positive relation between fast development and adult lifespan is predicted by this hypothesis, also under benign conditions. A possible mechanism is that the genetically most healthy individuals, through enhanced developmental stability and homeostasis, would be able to develop fast as well as have a reduced mortality rate. Whether these processes can promote longevity is unknown. A complementary study of correlations of developmental traits with lifespan under stress conditions is needed to confirm whether the correlation found is fundamental. Under the stress hypothesis, we expect the genetic correlation between development time and adult lifespan to be magnified.

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Chapter
4

Artificial selection for male longevity in a butterfly

Abstract

Ageing and species-specific lifespans have been successfully explained within the framework of evolutionary theory. Various specific mechanisms have been proposed and discovered that are important in the regulation of lifespan. In particular, the trade-off between reproduction and lifespan has received much attention. However, whether the evolution of reproductive strategy actually underpins the evolution of lifespan in nature is rarely addressed. Here we attempt this for the tropical butterfly, *Bicyclus anynana*. This species has evolved phenotypic plasticity to adapt as adults to the alternating wet and dry seasons it encounters. This has resulted in two alternative life histories: in the wet season, butterflies are short-lived and reproduce rapidly; in the dry season, butterflies are long-lived and postpone reproduction many months until the next wet season. We used artificial selection to investigate genetic correlations between life-history traits relevant to the regulation of lifespan. In an environment resembling the wet season, we selected two lines for increased male longevity for five generations. We then measured the response to selection, as well as correlated responses in development time, body size, fat content and metabolic rate and, for females, in early and late reproduction. The selection was successful with an increase of about 25% in median longevity in males. In addition, females of one line showed a similar increase in lifespan. Correlated responses in female reproduction indicate a reduced fecundity in favour of a larger egg size. The fact that we find a change in female reproduction in response to selection on male longevity suggests that the genetic trade-off between reproduction and survival is fundamental to the regulation of lifespan. Our results indicate that we selected for a phenotype that resembles the long-lived dry season form that is induced by environmental temperature (in the lab and) in the field. We conclude that the genetic variation for lifespan in this population is involved in co-ordinating the whole life history of the species including the adaptation to alternative seasonal environments, and supporting interactions between phenotypic plasticity and the regulation of lifespan.

Introduction

Life-history evolution (Stearns 1992) and the evolutionary theories of ageing (Zwaan 1999) have greatly enhanced our understanding of the causal factors that influence ageing and longevity in animals. The evolutionary genetic theories of mutation accumulation (Medawar 1952) and antagonistic pleiotropy (Williams 1957) were introduced to explain the evolutionary origin of ageing. Antagonistic pleiotropy is a central theme in ageing (Leroi et al. 2005), and the genetic argument was further developed to a more physiological level in the disposable soma theory (Kirkwood 1977). Essentially a life-history theory, it tackles the problem of ageing from a checks-and-balance point of view, arguing that during their evolution, organisms have continuously been forced to shape their life histories in terms of investment of a limited amount of energy in somatic maintenance or in high fecundity. The outcome then is slower or faster ageing, respectively (Kirkwood and Austad 2000).

The use of artificial selection has proven to be particularly successful in testing theories of ageing. Selection was frequently aimed at uncovering the fundamental life-history trade-off between reproduction and survival (Maynard Smith 1958; Luckinbill et al. 1984; Rose 1984; Partridge and Fowler 1992; Zwaan et al. 1995). Remarkably, such experimental approaches have been performed almost exclusively in *Drosophila melanogaster*, probably because selection for such a trait requires a well established and easily manipulatable laboratory animal with a lifespan that allows multiple generations per year. Exceptions include other *Drosophila* species (Wattiaux 1968), the bean weevil *Acanthoscelides obtectus* (Tucic et al. 1996), and the flour beetle *Tribolium spp.* (Mertz 1975). As most selection is on delayed reproduction or on the time of reproduction, it is not surprising that a trade-off of longevity and reproduction is often found. Selection in *D. melanogaster* for increased and decreased lifespan independent of time of reproduction generally confirmed a trade-off with reproduction (Zwaan et al. 1995). However, the details of the trade-off differ between the various selection experiments, especially in terms of whether early fecundity or overall fecundity traded off with longevity. How the evolution of reproductive strategy affects lifespan in nature is rarely addressed, perhaps because a detailed understanding of the ecology is lacking. We attempt to address this question explicitly by investigating the evolution of lifespan in an ecological context, using artificial selection in the tropical butterfly, *Bicyclus anynana*.

B. anynana has evolved phenotypic plasticity as an adaptation to the alternating wet and dry seasons it encounters in East Africa. The species expresses two alternative seasonal forms with strikingly different life histories. Butterflies with rapid adult reproduction fly in the wet season whilst butterflies with long lifespan and delayed reproduction are found in the dry season (Brakefield and Frankino 2006). Artificial selection applied to increase longevity is expected to lead to correlated responses in those traits associated with the long-lived form and especially to fewer and larger

eggs (Fischer et al. 2003). In this paper we combine the power of applying artificial selection in this system, with our extensive knowledge of the life-history and ecology of the species in the field to investigate the fundamental mechanisms underpinning the evolution of ageing and longevity.

Materials and methods

Animals

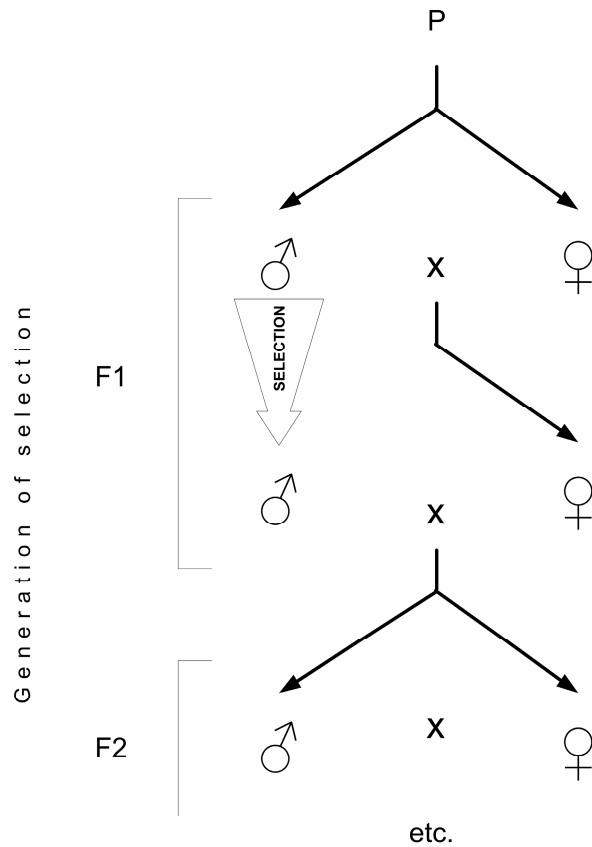
The selection lines and unselected control populations used were derived from a Stock population founded by over 80 gravid females caught at a single location in Malawi in 1988. It has been maintained in outbreeding conditions without loss of heterozygosity (van't Hof et al. 2005). During selection and in the assays, all butterflies were reared and maintained at wet season conditions of 27°C, 70% relative humidity (RH) and LD 12:12 h in a climate controlled room, unless indicated otherwise. Larvae were reared ad lib on maize in large population cages. Adults had ad lib access to moist banana refreshed 3 times a week.

Selection Procedure

Two replicate lines, LS1 and LS2, founded from the Stock population in May 2003 were selected for increased male longevity, while maintaining the ability to reproduce at an advanced age. This was done by using a procedure that involves rearing an intermediate generation for crossing with the selected males (see Fig. 1). The F1 sexes were separated at eclosion. When females were 3-7 days old, they were allowed to mate to the (unselected) males to produce an intermediate F2 generation. Only the mated males entered the selection population, others were discarded. The mated F1 females were allowed to oviposit within a week. The F2 were reared at 20°C and 60% RH (and if needed partly at 27°C) to regulate development time to synchronise the timing of eclosion with the selected, long-lived F1 males. Females from this intermediate generation were used for mating to the selected F1 males.

The F1 males were kept at 27°C in large cylindrical hanging cages (60 Ø x 100 cm), which were frequently moved randomly through the climate room to minimise local environment effects. A sufficiently large number of males (>200) was used in each generation of the selection in each line to increase selection intensity and minimise inbreeding effects. Deaths were scored every Monday, Wednesday and Friday. When mortality in the selection population had reached 60%, 1-2 week old intermediate generation females were introduced into the cage to allow mating. Males that had finished copulation were re-introduced to obtain more matings. The criterion of 60% was established from pilot studies and ensured a sufficient number of matings (>35).

Figure 1. Schematic overview of the selection procedure that shows how young F1 males are first crossed to F1 females. Then, F1 males that are relatively long lived (selection) are crossed to young unselected females reared from the whole parental population to provide the next generation for selection (F2). P: parental generation.



Longevity Assay

After five generations of selection and one generation without selection, adult lifespan at 27°C was measured in a standardised manner for the selection lines and the unselected Stock population they were derived from. At eclosion, sexes were separated and virgin butterflies of the same line were transferred to small cylindrical hanging cages (25 Ø x 60 cm) with a maximum of 10 per cage. Deaths were scored every Monday, Wednesday and Friday. Cases of mortality in the first 10-day period of adult life were excluded from analysis in order to separate physiological lifespan under benign conditions from deaths caused by feeding

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disability or other causes unrelated to ageing. This comprised 41 cases, evenly distributed over the sexes and lines

Correlated Responses

To investigate the trade-off between reproduction and longevity, we measured correlated responses in female reproductive traits three generations after selection ceased. In separate experiments, we measured development time, body mass, fat content and metabolic rate.

Female reproduction

To measure female reproductive traits of egg number, egg size and fertility, individually numbered females were mated to males of the same line when 3 to 4 days old and immediately allowed to oviposit for 72 (\pm 2) hours (day 1-3 after mating) on cuttings of a natural host plant, *Oplismenus africanus*, in a plastic pot (5 \varnothing x 7 cm) to measure early reproductive traits. This was repeated after 3 weeks (day 22-24 after mating) to measure late reproductive traits. All eggs were counted directly after the oviposition period, and from a small subset, the size was measured using digital image analysis (Fischer et al. 2002). The remaining eggs were used to score the fertility of each pairing as the presence or absence of developing embryos or hatched larvae. Only the fertile pairings were considered for analysis of egg size and egg number. Although we did not measure any reproductive traits between days 4-21, females had access to egg laying plants for oviposition. After the experiment on day 24, the females were killed and frozen, and subsequently their dry mass was measured to 0.01 mg after drying at 50°C for 48 hours. These individual body mass data were used as a covariate in the statistical analyses.

Development time, body mass, fat content, and metabolic rate

Development time at 27°C was measured in a separate experiment as the number of days between the date of oviposition and the date of emergence from the pupa. Body mass, fat content and metabolic rate were measured in the same individuals in a single experiment carried out from January to February 2006. First, individual Resting Metabolic Rate (RMR) of 2 to 6 day old butterflies was measured in the dark part of the circadian cycle in a temperature controlled climate cabinet at 27°C. Values for CO₂ ($\mu\text{l}\cdot\text{h}^{-1}$) respired from individual butterflies in small cylindrical glass containers (4 \varnothing x 9 cm) were used as an index of RMR. These data were obtained with a Li-Cor LI-6251 CO₂ analyzer in a respirometer set-up (Sable Systems) with a push-through flow of 100 ml/min. CO₂ data from two consecutive replicate measurements ($r > 0.95$) were analysed using Datacan 5.4 (Sable Systems) and averaged. The butterflies were then killed and frozen, and individual body mass was

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measured as dry mass to 0.01 mg after removing wings, legs and antennae and drying at 40°C for 48h. Total fat mass (triglyceride and fatty acid) is measured as the difference of the previously measured dry mass and the dry mass after fat extraction. Fat extraction begins by incubating the butterfly body in a shaking (100 rpm) solution containing dichloride-methane and methanol in a 2:1 ratio at room temperature for 48 h. This extraction protocol is then repeated with fresh solution, after which the cadaver is weighed after 24h drying at 40°C (Zwaan et al. 2001). Fat content is the percentage of fat mass relative to body mass. For both RMR and fat content the uncorrected data were used in the statistical analyses with body mass as a covariate. However, the presented means and standard deviations of relative RMR and fat content are corrected for body mass.

Statistical Analyses

Differences in lifespan between lines were estimated pair-wise by comparing risk ratios from Cox Proportional Hazard models with line as the single factor, for each sex separately. Such models account for mortality throughout life and better describe survival than median survival or maximum lifespan. Development time and body mass were analysed using one-way ANOVAs. RMR data were ln transformed to satisfy the assumption of equal variances. RMR and fat mass were analysed with body mass as a covariate in ANCOVAs. Egg number and size were analysed for early and late reproduction separately by ANCOVAs with line, body mass from the same individuals as factors, and their interaction. Following AN(C)OVAs, significant differences between lines were estimated with post-hoc Tukey-Kramer HSD tests (T-K-tests) with $\alpha = 0.05$. Differences between early and late reproduction were analysed by repeated measures MANOVA. Fertility of both selection lines was analysed by G-tests using the Stock probability of fertile eggs as expected values. All statistical analyses were performed with JMP 5.01 (SAS Institute).

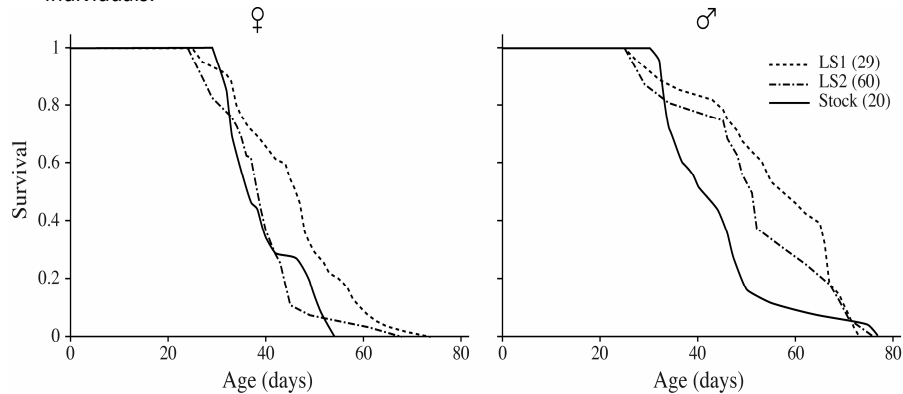
Results

Longevity

The response to selection for male longevity after five generations is shown in Figure 2. Lifespan increased significantly by 20-30% compared to the unselected Stock in the LS2 line females (RR = 0.72 [0.55-0.97], $p=0.033$) and males (RR = 0.72

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Figure 2. Survival of females (top) and males (bottom) of LS1, LS2 and Stock lines at 27°C. For statistics on differences between lines, see text. 'n'= number of individuals.



[0.55-0.97], $p=0.040$). Such an increase is also seen in LS1 males, although not significantly (RR = 0.76 [0.54-1.05], $p=0.098$) due to the relatively small cohort size. LS1 females do not show a response to selection (RR = 1.01 [0.75-1.38], $p=0.95$), resulting in a significant difference in lifespan for the two selected lines (LS1 vs. LS2: RR = 1.36 [1.07-1.71], $p=0.011$).

Correlated responses

Female reproduction

Figure 3B shows that egg number at early reproduction (week 1) differs between lines ($F_2 = 5.65$, $p<0.01$) and is significantly lower in both selection lines compared to Stock. Egg size (figure 3A) also differed between lines ($F_2 = 4.03$, $p<0.05$) and was significantly larger in the selection lines. At late reproduction (week 3), no significant differences between lines are found for egg number ($F_2 = 1.24$, $p=0.30$) or size ($F_2 = 0.18$, $p=0.67$), although the ranking of lines for egg size is identical to that in early reproduction. Interestingly, there is a difference in the influence of body mass on the reproductive traits over time, as analysed in ANCOVA models. Early reproductive traits are not dependent on body mass, whereas in the third week egg number (dry mass: $F_1 = 9.17$, $p<0.005$; line x dry mass: $F_2 = 10.72$, $p<0.001$) and egg size (line x dry mass: $F_2 = 4.12$, $p<0.05$) appear to be dependent on body mass.

The MANOVA analyses indicate that both egg size ('time', $F_1 = 4.87$, $p<0.001$) and number ('time', $F_1 = 1.44$, $p<0.001$) are reduced in week 3 compared to week 1, whilst the pattern of egg size across lines is constant over time ('time x line', ns). For egg number, a time x line interaction is marginally significant ($F_2 = 0.12$, $p<0.053$). Fertility was not significantly lower in LS1 ($G1 = 2.77$, $p=0.096$) or LS2 ($G1 = 1.51$, $p=0.22$) compared to Stock.

Figure 3. Mean egg number and size (\pm standard error) in week 1 and 3 for LS1, LS2 and Stock females. Sample sizes, n , are given. For statistics on differences between lines, see text.

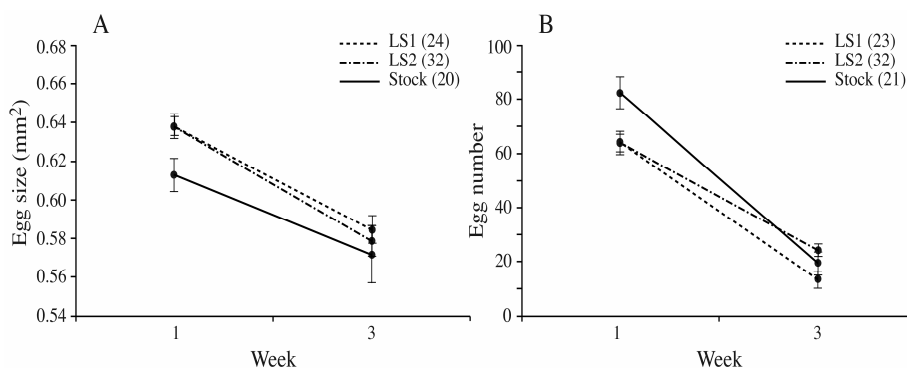


Table 1. Means values with standard deviations (in parentheses) for development time, body mass, fat content and resting metabolic rate (Relative RMR) for each sex of butterflies in the lines (sample sizes, n , are given).

	Line	Development time (days)	Body mass (mg)	Fat content (%)	Relative RMR ($\mu\text{l}\cdot\text{h}^{-1}\cdot\text{mg}^{-1}$)
Females	LS1	30.5 (0.24)	23.3 (0.94)	20.7 (1.4)	5.8 (0.47)
	n	87	35	35	35
	LS2	30.7 (0.20)	21.3 (0.96)	21.0 (1.5)	5.2 (0.43)
	n	116	33	33	33
	Stock	27.9 (0.19)	18.0 (1.01)	19.3 (1.6)	4.3 (0.51)
	n	86	30	30	30
Males	LS1	28.8 (0.21)	11.9 (0.61)	26.9 (1.6)	7.9 (0.90)
	n	129	23	23	23
	LS2	28.7 (0.24)	11.4 (0.54)	24.7 (1.4)	6.9 (0.81)
	n	124	29	29	29
	Stock	26.5 (0.13)	11.2 (0.62)	25.5 (1.7)	8.5 (0.92)
	n	63	22	22	22

Development time, body mass, fat content and metabolic rate

Development is significantly longer (+10%) in the LS lines of both sexes compared to the Stock line (line effect: $F_2 = 54.6$, $p < 0.0010$; T-K-test for both sexes: $LS1=LS2 > Stock$) (Table 1). Body mass is significantly higher (+15%) in selection line females (line effect: $F_2 = 7.62$, $p < 0.001$; T-K-test: $LS1=LS2 > Stock$), but not in males (line effect: $F_2 = 0.31$, $p = 0.73$). Fat content (line effect for females: $F_{2, 94} = 0.41$, $p = 0.66$; males: $F_{2, 70} = 0.56$, $p = 0.57$) and resting metabolic rate (RMR) (line effect females: F_2 ,

$t_{94} = 1.69$, $p=0.19$; males: $F_{2, 70} = 2.03$, $p=0.14$) did not differ between the lines in either sex.

Discussion

Responses to Selection:

The selection has produced longer-lived butterflies in a relatively short time. This is especially noteworthy considering the two factors in the selection design that lower selection intensity: the intermediate generation females, and the use of selection on males only. This may indicate that only a few genes are contributing to the response, as suggested by Zwaan et al. (1995) in *D. melanogaster*. In other words, a limited number of genes may have segregating alleles for longevity in our selected populations. Recent investigation into the selection lines created by Zwaan et al. (1995) reveals constraints on the responses to long term selection, further suggesting a fixation of relatively few genes or alleles that underlie variation in lifespan (Vermeulen and Bijlsma 2006). Continuing selection in combination with genomic analyses is needed to establish what changes occurred at the genomic level in our selection lines. We predict that the lack of a significant effect on LS1 butterflies is the result of the relatively low selection intensity and limited amount of generations.

Correlated Responses to Selection

The major correlated response in our experiments is the change in female reproductive traits. Our results from female reproduction indicate the general importance of reproduction in lifespan regulation as predicted by life history theory. At early reproduction, selection line eggs are larger and fewer in number compared to the Stock line. At late reproduction, there are no significant differences between the lines. This suggests that early reproduction is not traded-off against a higher late reproduction, as is often found in *D. melanogaster* lines selected for increased lifespan (Luckinbill et al. 1984; Rose 1984; Gasser et al. 2000), but that selection produces a shift within the reproductive program from quantity (many but small) to quality (few but large). Such a trade-off is important in many species (Fox and Czesak 2000; Fischer et al. 2006) and our result is the first that reveals such a trade-off in context of a genetic relationship with lifespan.

The longer development time suggests that selection has indirectly targeted developmental processes. These could be linked to resource acquisition, and the results for body mass suggest that this may have happened in females, but not in males. However, the possibility of increased resource assimilation in the selection lines is not likely to involve fat content, which is not different between lines. Alternatively, a slower metabolism could explain a correlated response in

development time. The role of metabolic rate in lifespan extension has always received much attention, although the nature of the relation is much debated (Khazaeli et al. 2005). However, we do not expect the selection response to include a general lowering of metabolism because selection also acts on male mating ability which requires highly active behaviour. Indeed, our results on RMR show that an increase in lifespan is not accompanied by a lowered metabolism in both sexes. It is very likely that the selection has favoured behaviour that is associated with successful mating, especially at an advanced age. We did not observe any change in the number of successful matings during selection. However, we noted that several old males mated 2-3 times in a few hours, a behaviour that is infrequent even in young males (Brakefield et al. 2001). It would be interesting to collect data on the reproductive success of these males.

The Trade-Off Between Reproduction and Lifespan in *B. anynana*.

An issue in evolutionary ageing research has been whether artificial selection experiments devised to demonstrate a trade-off could discriminate between selection directly on lifespan and indirectly on reproduction (Zwaan et al. 1995). Evidence for the important role of reproduction in lifespan regulation has come typically from studying female reproduction (Partridge et al. 2005). It is important to note that our selection was on the ability of males to live a long time combined with an ability to mate successfully at an advanced age. Thus, we selected on longevity directly, but not independently of reproduction. This may bias our interpretations in favour of the importance of reproduction in the evolution of ageing. However, even though selection was on males only, female reproduction has been altered towards lower early egg production independently of the selection procedure. Clearly, the genetic correlations of traits involved in reproduction and lifespan are high between the sexes, and we predict that a shift in reproductive investment in males has occurred, comparable to that seen in females. We confirm that reproduction has traded-off with lifespan in the selection lines, as has been shown previously (Maynard Smith 1958; Luckinbill et al. 1984; Rose 1984; Partridge and Fowler 1992; Zwaan et al. 1995). However, the nature of the trade-off we find has not been implicated before in the regulation of lifespan and can adequately be explained in the light of the life history in the field.

Life History and the Regulation of Lifespan: Clues from *B. anynana*

We performed laboratory artificial selection on a population for which we have good knowledge on ecology and evolution. Literature on mortality in wild populations of various butterfly species in the tropics (F. Molleman, B.J. Zwaan, P.M. Brakefield, J. R. Carey, unpublished) show that mortality rates change in concert with other life history traits, suggesting the importance of studying various aspects of the life history simultaneously. Our selection changed the relation between

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reproduction and lifespan to produce longer-lived butterflies. More specifically, investment in reproduction is diverted from quantity (number of eggs) to quality (larger egg size) of offspring. This is consistent with a shift towards the dry season form (Fischer et al. 2003) that lives longer and is adapted to survive up to eight months until the end of the long dry season when it reproduces. Given the evolution of different life histories in *B. anynana* in the alternative seasonal environments, including divergent natural lifespans, we can assume that lifespan itself is under selection. During evolution, the physiological and genetic mechanisms governing lifespan must have been embedded in the regulation of the life history. Because of the central place of phenotypic plasticity in the regulation of life history in *B. anynana* (Brakefield et al. 2003), we can speculate that our selection for longevity has at least partly targeted the pathways that regulate the phenotypic plasticity. Our future research aims at uncovering these pathways.

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Chapter
5

**Increased lifespan in a
polyphenic butterfly artificially
selected for starvation
resistance[†]**

Abstract

Starvation resistance (SR) is closely associated with fitness in natural populations of many organisms. It often covaries with longevity, and is a relevant target for understanding the evolution of ageing. We selected for increased starvation resistance in the seasonally polyphenic butterfly, *Bicyclus anynana*, in a warm, wet-seasonal environment over 17 generations. We measured the response to selection for two selected lines compared to an unselected stock. Results show an increase in survival under adult starvation of 50 to 100%. In addition, selection lines showed an increase in lifespan under normal adult feeding of 30 to 50%. Female reproduction was changed towards laying fewer, but larger eggs. The results indicate a sex-specific response to selection: females re-allocated resources towards a more durable body, whereas males appear to increase starvation resistance through changed metabolic rate. The phenotype produced by artificial selection resembles the form that occurs in the cool, dry-season environment, which suggests that selection has targeted the regulatory mechanisms for survival that are also involved in the suite of traits (including starvation resistance) central to the adaptive plastic response of this butterfly to seasonal conditions. In general, these results implicate that the regulation of lifespan involves mechanisms of phenotypic plasticity.

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Introduction

Deprivation of nutrition is a prevalent threat to survival and successful reproduction for organisms. The ability to deal with this stress is called starvation resistance (SR) and is often closely associated with fitness. It has often been suggested that the mechanisms that induce prolonged survival under starvation also underlie the regulation of longevity (Parsons 1995). In a range of well studied animal species, long-lived individuals appear to be more resistant to multiple stresses, including starvation. This has been shown for long-lived single gene mutants in several species (Johnson et al. 2001; Longo and Fabrizio 2002), and for fruit flies that experience different environments (Zwaan et al. 1991; Chippindale et al. 1993). It has also been shown in some long-lived *Drosophila melanogaster* populations produced by artificial selection (Service et al. 1985; Zwaan et al. 1995; Bubli and Loeschcke 2005), although not in other such long-lived populations (Force et al. 1995). In addition, some experiments that have increased starvation resistance through direct artificial selection show enhanced longevity (Rose et al. 1992), but others do not (Harshman et al. 1999b; Bubli and Loeschcke 2005). Although a clear correlation between starvation resistance and longevity is not present in all studies, probably due to the non-linearity of such relationships (Archer et al. 2003; Vermeulen and Bijlsma 2006), the presence of such a link strengthens the notion that regulation of metabolism, homeostasis and energy resources are crucial in determining longevity.

Experiments investigating the correlation between starvation resistance and longevity have been conducted almost exclusively with *D. melanogaster*; additional data from other organisms will aid in making more general conclusions (Hoffmann and Harshman 1999). However, the major problem with interpreting the mixed results for the correlation between starvation resistance and longevity in the various populations of *D. melanogaster* may result from the lack of an appropriate ecological context. The ecology of the African butterfly *Bicyclus anynana* (Butler 1879), the Squinting Bush Brown, and its implications for the life history of this species are well known. *B. anynana* exhibits seasonal polyphenism as an adaptive response to living in alternating dry and wet seasons throughout East-Africa (Brakefield and Reitsma 1991). The advantage of starvation resistance occurs mainly in the dry season, when individual butterflies must survive a long dry season before reproducing at the start of the next wet season. Their main survival strategy is to remain mostly inactive and cryptic on the carpet of dead leaves on the (open) forest and savannah floors. Although energy demands are lower in the dry season because of lower environmental temperature and reproductive dormancy (Brakefield and Frankino 2007), these fruit-feeding butterflies need to locate fallen fruit scattered spatially and temporally to ensure survival. Periods of intermittent starvation are thus likely to occur regularly during each dry season. It has previously been shown that butterflies selected for a dry season wing pattern exhibited a small but significant increase in starvation resistance when developed in dry season

conditions (Pijpe et al. 2007). Thus, both starvation resistance and longevity are part of the suite of life history traits that differ between the two seasonal forms; they are involved in the adaptive phenotypic plasticity of this species, and influenced by natural selection. Consequently, *B. anynana* offers a unique opportunity to study the relationships between starvation resistance, longevity, and other life-history traits (Pijpe et al. 2006), and to shed more light on the role of stress resistance in the evolution of lifespan.

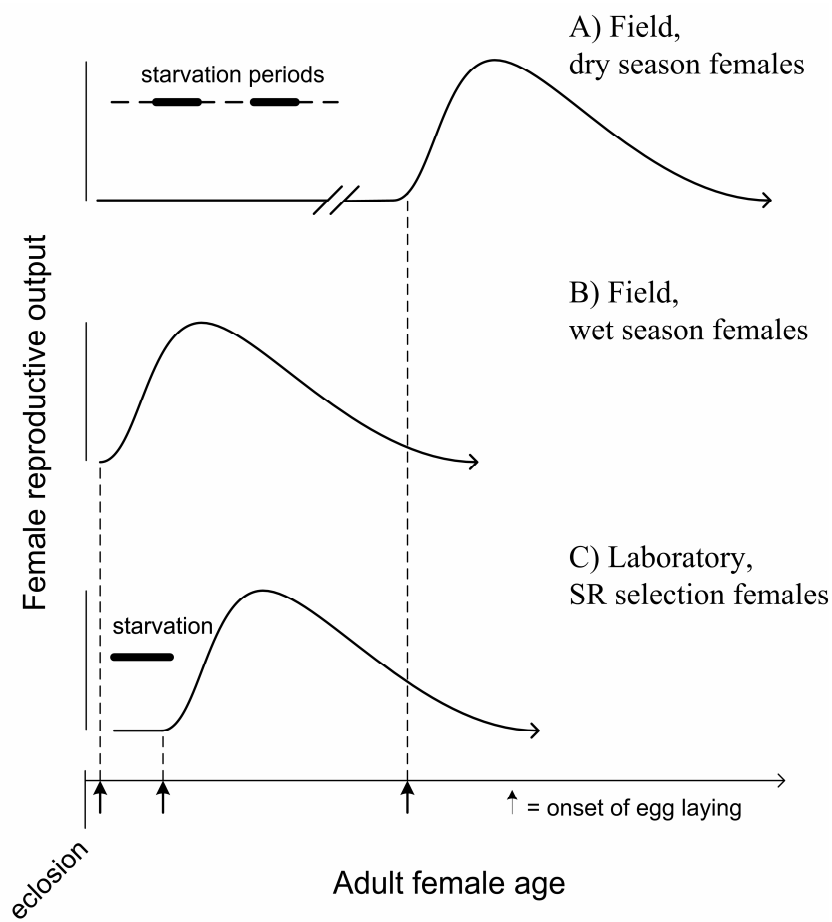
We applied artificial selection to explore the standing genetic variation for starvation resistance in such a way that is relevant for the field biology of this species in its seasonal environments (figure 1A, B). We successfully selected for increased starvation resistance in *B. anynana* in both sexes over 17 generations. We then measured correlated responses in longevity, reproduction, development time, and several physiological traits to obtain information about the genetic correlations of starvation resistance with other life-history traits. This will indicate possible physiological and genetic mechanisms shared by starvation resistance and longevity and how they evolved; knowledge that is highly relevant for understanding the biology of ageing. Because of the central role of adaptive phenotypic plasticity, and the significance of starvation resistance and longevity for the *B. anynana* in the dry season, we expected our artificial selection in wet season conditions to produce a phenotype that more resembles the dry season form as induced through environmental plasticity.

Materials & Methods

Animals

The butterflies of the selection lines (SR1 and SR2) and unselected Stock (US) were derived from the same population, which was founded from over 80 gravid females in 1988 and has subsequently been reared in the laboratory in outbreeding conditions with several hundred individuals per generation without loss of heterozygosity (van't Hof et al. 2005). The rearing schedule is such that slow and fast developing animals have broadly equal chances of contributing to the next generation. Larvae are fed young maize (*Zea mays*) plants, and adults are fed moist slices of banana (*Musa acuminata*). Mating takes place in a single, large cage, and egg laying typically takes place when females are 1-3 weeks old, thereby minimizing selection towards a peaked early reproduction. In this study, all assays, rearing and selection were performed at wet season environmental conditions in climate controlled chambers. The temperature was 27°C, relative humidity 70% and photoperiod 12:12 L:D.

Figure 1. Schematic comparison of the female reproductive schedules in the dry season (A), and the wet season (B) environment in the field, and in the artificial selection procedure (C) in the laboratory that incorporates a temperature and humidity regime similar to that of the wet season environment.. The duration of the egg laying period is similar for all three situations, but the timing of the onset differs. Artificial selection has delayed the onset of egg laying compared to the wet season environment and the Unselected Stock (US) control line. However, this delay is small relative to the total period of egg laying and the total length of life (not shown).



Selection Procedure

Two replicate lines were selected from March 2002 for the ability to resist death from adult starvation. Daily eclosing butterflies were separated by sex and transferred to a cylindrical hanging cage (25 \varnothing x 60 cm). Over the full period of eclosion, new hanging cages were used daily to keep butterflies by line and sex. In each cage, butterflies had continuous access to water (moistened cotton wool) and were deprived of food until about 50% had died. In this way we ensured that selection was on starvation resistance, rather than on desiccation resistance. A minimum of 40 males and 40 females per replicate remained alive for selection in each generation to minimize inbreeding. Under laboratory conditions effective population sizes are about 60% of adult census number (Brakefield et al. 2001). Thus, any consequences of random genetic drift and founder effects are expected to be small (Saccheri et al. 1999). Male deaths were scored twice a day, and female deaths once a day. The surviving individuals were rescued by feeding them moist banana, reducing mortality to less than 5% by the next day, and they were pooled by line and sex. Selected butterflies were then transferred to 20°C until the final females had been selected. After selection, males and females were put together to mate and reproduce by allowing females to oviposit 3 days later. This procedure was continued in each of 17 generations. No selection was performed thereafter, but at generation 25 (March 2006), the selected lines still exhibited their strongly increased starvation resistance (Pijpe et al., unpublished data).

Response to Selection

We compared the selection response to an Unselected Stock (US) line at generation 7 and 14 for both sexes, and for males again in generation 21. Due to practical limitations, we did not use an 'internal control': the US lines were taken from the Stock population one generation before the response measurements to synchronize the life stages, and subsequently reared in the same climate chamber as the selection lines. The mean starvation resistance of the Stock line did not change when measured repeatedly over a 4 year period (data in this study, and Pijpe et al., unpublished data). The set-up was similar to the selection procedure, except for the exclusion of the selection itself: starvation continued until all butterflies had died. The US differed from the selection lines in the selection treatment and the age of egg collection. For the US this was on average 14 days, and for the selection lines on average 14 days for early generations up to 21 days for later generations, and all lines had the same age ranges. In other words, there may have been inadvertent selection for increased lifespan and/or delayed reproduction in the selected lines. However, any such effect will be minimal because the difference in age at reproduction is small compared to the maximum lifespan, and the eggs were collected at the same time relative to the onset of egg laying (Figure 1). Thus, our

comparisons for the correlated response are unlikely to be confounded by the small difference in treatment other than selection for starvation resistance.

Correlated Responses

Longevity

In generation 20, after 3 generations without selection, lifespan under standard ad libitum food conditions was measured in the SR1, SR2, and US populations. At eclosion, sexes were separated and virgin butterflies were transferred to cylindrical hanging cages (25 Ø x 60 cm) with a maximum of 10 per cage. All butterflies were fed ad libitum on moist banana, refreshed three times a week. Deaths were scored every Monday, Wednesday and Friday. We excluded butterflies from the longevity analysis that eclosed with deformed wings or missing legs. In addition, butterflies that died in the first 10-day period were excluded to separate physiological lifespan under benign conditions from deaths caused by feeding disability. In total, 28 cases ($\approx 10\%$) evenly distributed over the sexes and lines were excluded. Individuals that escaped or became damaged by handling during the experiment (17 cases) were right-censored, thereby still partly contributing to the analysis.

Female reproduction

In generation 22, females of the SR1, 2, and US populations were set up to measure egg size and egg number in a common, wet season environment. Eclosing females were individually numbered on their wings and mated to males of the same line when 3-4 days old. Female reproductive traits were measured twice during their egg laying period: early (week 1), corresponding to the peak of reproductive output (Brakefield et al. 2001) and late (week 3). For early reproduction, females were individually set-up directly after mating (day 1-3 after mating) to lay eggs for 72 (± 2) hours on cuttings of *Oplismenus africanus*, a natural host plant, in small plastic pots (5 Ø x 7 cm). Moist banana was provided and refreshed as required. This was repeated after 3 weeks (day 22-24 after mating) to measure reproductive traits in the late part of the egg-laying period. In the intermediate period (days 4-21), females had continuous access to egg laying plants for oviposition in cylindrical hanging cages (25 Ø x 60 cm), with a maximum of 5 butterflies per cage. From a small subset of eggs collected to evaluate early and late reproduction, the size was measured using digital image analysis (Fischer et al. 2002). The remaining eggs developed further and after 4-7 days were scored for fertility by eye as the presence or absence of fully developed larvae. Directly after the experiment, individual females were weighed to 0.01 mg on a microbalance following drying at 50°C for 48 hours. Adult dry body mass was then used as a covariate in the statistical analyses. Data obtained from females that died during a three-day measuring period were excluded from the analysis. Data for females that died before late reproduction

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were retained for the early reproduction analyses. Only data of fertile females were considered.

Development time, body mass, metabolic rate and fat content

Development time at 27°C of both females and males was measured in the female reproduction experiment as the number of days between the date of oviposition and the date of emergence from the pupa. In a separate experiment in generation 23, populations of SR1, SR2 and US butterflies were reared to measure resting metabolic rate (RMR), body mass and total fat content. Butterflies were 2 to 6 days old when metabolism was measured in the dark part of the diurnal cycle and in a temperature controlled climate cabinet at 27°C (the diurnal cycles of climate room and cabinet were synchronized). CO₂ levels ($\mu\text{l}\cdot\text{h}^{-1}$) respired from individual butterflies in small cylindrical glass containers (4 \varnothing x 9 cm) are used as an index of RMR. CO₂ production is measured with a Li-Cor LI-6251 CO₂ analyzer in a respirometer set-up (Sable Systems; www.sablesys.com) with a push-through flow of 100 ml/min. CO₂ data from two consecutive replicate measurements ($r > 0.95$) were analyzed using Datacan 5.4 (Sable Systems) and averaged. Then, body mass was measured as dry weight to the nearest 0.01 mg following removal of wings, legs and antennae and drying at 40°C for 48h. Fat weight is calculated as the difference of dry weight and fat-free dry weight (FFDW), as obtained after extraction of the total fat (triglyceride and fatty acid) in individual butterflies (Zwaan et al. 2001). Fat extraction was done by incubating the butterfly body in a shaking (100 rpm) solution containing dichloride-methane and methanol in a 2:1 ratio at room temperature for 48 h. The extraction protocol was repeated with fresh solution, followed by drying at 40°C for 24h and weighing to estimate FFDW. Fat content is the percentage of fat weight relative to FFDW.

Statistical Analyses

In the analyses for development time, body size and physiological traits, both replicate selection lines (SR1 and 2) are contrasted to a single control line (US). To account for a potential lack of statistical independence, replicates were nested within line in the models, thereby contrasting SR to US. However, because of the low number of replicate lines, statistical power is limited when replicate is treated as a random effect. Therefore, we based our interpretation of the analyses on a three step approach. First (I), a significant effect of line in the random model is interpreted as a positive effect. Secondly (II), in the case of a non-significant (NS) line effect in the random model, the analysis was repeated using a nested model with replicate as a non random factor (thereby testing effects against residual error). A repeated non-significant effect is interpreted as a negative result, while a significant effect is interpreted as an indication of significant differences. Thirdly (III), confirmation of an indication from step II is sought through a post-hoc test when both SR1 and SR2 are significantly different from the US. This approach

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balances the issue of independence with the severe reduction in power in the random nested ANOVA.

Data for development time, body size and physiological traits were analysed using minimum adequate ANOVA models. Initial models included sex as a factor and it had a significant influence on all traits investigated, and complicated the model building. Therefore, the final analyses were performed separately in each sex. Development time, dry weight and FFDW were analyzed with one-way ANOVA. Data for RMR and fat weight were analyzed with fat-free dry weight as a covariate in ANCOVAs, thus calculating relative RMR and fat content, respectively. The RMR data were natural log (ln) transformed prior to analysis to satisfy the assumption of equal variances. The means for relative RMR and fat content presented in Table 1 are calculated as the fraction to FFDW. The female reproductive traits of egg number and size were analyzed for early and late reproduction separately by ANCOVAs with line and body mass from the same individuals as factors, and their interaction. Following AN(C)OVAs, significant differences between lines were estimated with post-hoc Tukey-Kramer HSD tests (T-K-tests) with $\alpha = 0.05$. Differences between early and late reproduction were analyzed by repeated measures MANCOVA. In the AN(C)OVA results, F test statistics are presented with respectively numerator and denominator degrees of freedom (df) in subscript. For the random nested analysis, the df for the denominator are calculated according to Satterthwaite (1946) as implemented in the software package. For each trait, the result of step I is given. If step I is non-significant, steps II and III are given consecutively. In all cases, a significant effect in step I is accompanied by a significant effect in steps II and III. Therefore, in such cases steps II and III are not shown. Fertility (yes or no) was analyzed in a nominal logistic regression with line as the single factor.

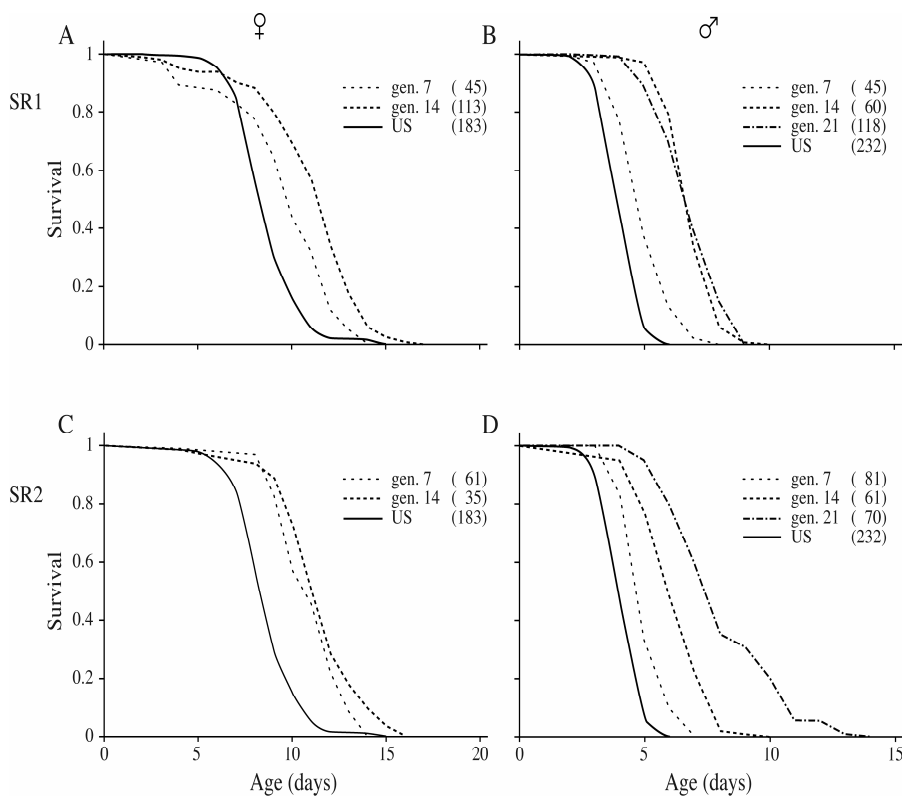
Analyses of variation in survival in the starvation resistance assays and the longevity assays were done with non-parametric Cox Proportional Hazard (PH) models. These conservative models are most appropriate for these data because they do not make a priori assumptions about the shape of the underlying mortality distribution and because censored data can be included. A disadvantage of such models is that random effects can not be included. Therefore, a significant effect of line is confirmed by pair-wise comparisons of a replicate selection line to the US line using Risk Ratios (RR) from Cox PH models with replicate line as the single factor, for each sex separately (comparable to step III in the ANOVA results). Kaplan-Maier survival curves are used only to visualize qualitative differences in survival. All statistical analyses were performed with JMP 5.01 (SAS Institute; www.jmp.com).

Results

Selection Response

Unselected Stock (US) females are more resistant to starvation than males: their median survival times are about 8 and 4 days, respectively. This gender difference was also found in the SR lines.

Figure 2. Survival curves of male and female *B. anynana* butterflies, after 7 and 14 (females) and 7, 14, and 21 (males) generations of selection for increased starvation resistance. Panels A and B represent females and males of line SR1, respectively; panels C and D represent females and males of line SR2, respectively. The survival curves for the unselected Stock (US) are derived from the pooled data from generation 7 and 14 for females and 7, 14 and 21 for males, and identical in panels A and C, and in B and D. US survival was not significantly different between generations in either sex. Sample sizes are indicated in parentheses in the figure legend.



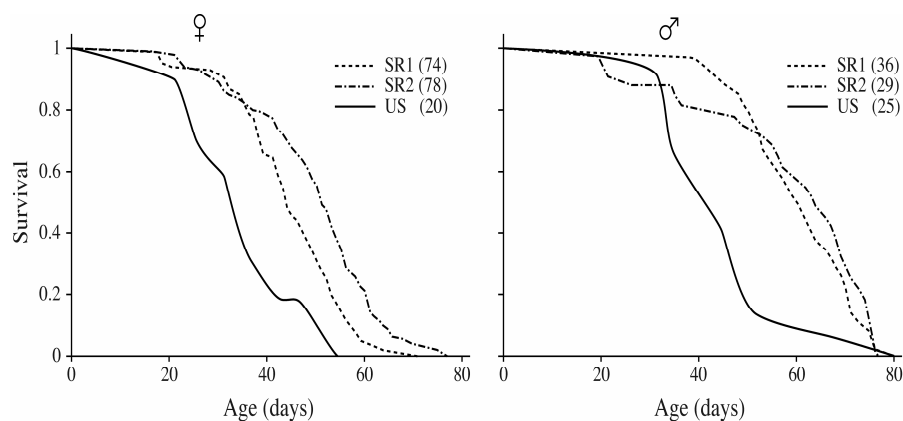
Selection over 7 generations increased starvation resistance by about 2 days (+25%) in SR1 (Risk Ratio (RR)= 0.78 [0.65-0.92]) and SR2 females (RR= 0.66 [0.54-0.79]). By 14 generations, it had increased by a further 2 days in both SR1 (RR= 0.38 [0.31-0.47]) and SR2 (RR= 0.40 [0.31-0.51]), amounting to about 50% longer median survival under starvation. In SR1 males, starvation resistance increased significantly by 1 day or 25% in generation 7 (RR= 0.70 [0.57-0.84]), by 3 days (+67%) in generation 14 (RR= 0.51 [0.43-0.60]), and showed a limited further increase in generation 21 (RR= 0.30 [0.21-0.42]). In SR2 males, starvation resistance increased by 1 day (+25%) in generation 7 (RR= 0.74 [0.57-0.95]), 2 days (+50%) in generation 14 (RR= 0.62 [0.51-0.74]) and 4 days (+100%) in generation 21 (RR= 0.27 [0.14-0.34]) (see figure 2 for all survival curves). This is comparable to the female responses, and indicates an average increase in starvation resistance of 0.2 days per generation, amounting to a near doubling of survival under starvation by the end of selection.

Correlated Responses

Longevity

Both sexes in each selection line also showed a significant increase in lifespan under normal feeding conditions after 21 generations of selection as compared to the US (figure 3). In males, both SR1 (RR= 0.61 [0.47-0.81], $p < 0.05$) and SR2 (RR= 0.58 [0.44-0.78], $p < 0.001$) had an increased median lifespan of 50% or 20 days, compared to the US. In females, the SR1 line had an increased median lifespan of 20% or 10 days (RR= 0.69 [0.53-0.92], $p < 0.02$), and the SR2 line had an increased median lifespan of

Figure 3. Survival curves under optimal food conditions of SR1 and SR2 lines of *B. anynana* after 17 generations of selection for starvation resistance, compared to the unselected Stock (US). Lifespan of virgin butterflies was measured at 27°C for females (left panel) and males (right panel). Sample sizes are indicated in parentheses in the figure legend.



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30% or 15 days (RR= 0.50 [0.38-0.68], $p < 0.0001$), compared to the US. SR1 females are significantly shorter lived than SR2 females (RR= 1.36 [1.07-1.71], $p < 0.05$).

Development time, body mass, fat content and metabolic rate.

Mean values and standard deviations for developmental time, body mass, fat content, and metabolic rate are given in table 1. The SR lines take significantly more time (about 10%) to reach adulthood in both females (line effect: I (random model): $F_{1, 0.98} = 11.13$, $p = 0.19$; II (non-random model): $F_{1, 276} = 45.30$, $p < 0.001$; III (Tukey-Kramer-HSD test): SR1=SR2> US) and males (I: $F_{1, 1.02} = 10.09$, $p = 0.19$; II: $F_{1, 203} = 39.96$, $p < 0.001$; III: SR1=SR2> US). Adult body mass, measured as fat-free dry weight (FFDW), is significantly higher by 20% in selection line females (line effect: I: $F_{1, 2.45} = 98.01$, $p < 0.005$). In males, fat-free dry mass is not significantly different between lines (line effect: I: $F_{1, 4.96} = 4.20$, $p = 0.10$; II: $F_{1, 79} = 1.40$, $p = 0.24$; III: NS. For dry weight, a similar result was found (statistics not shown). In females, there is a trend towards higher absolute fat mass as a response to selection (line effect: I: $F_{1, 1.11} = 2.40$, $p = 0.35$; II: $F_{1, 99} = 5.32$, $p = 0.023$; III: SR1<US, SR2=US, SR1=SR2). However, relative fat content in the selection lines does not differ from the US in females (I: $F_{1, 1.50} > 0.01$, $p = 0.98$, males: $F_{1, 1.67} = 0.59$, $p = 0.54$; II: $F_{2, 98} = 1.07$, $p = 0.35$; III: SR1=SR2=US) or males (I: $F_{1, 1.50} > 0.01$, $p = 0.98$, males: $F_{1, 1.67} = 0.59$, $p = 0.54$; II: $F_{2, 78} = 0.61$, $p = 0.54$; III: SR1=SR2=US). Resting metabolic rate (RMR) is significantly lower in males (line effect: I: $F_{1, 10.02} = 48.16$, $p < 0.001$) but not in females ($F_{1, 8.69} = 0.28$, $p = 0.61$; II: $F_{3, 98} = 0.87$, $p = 0.42$; III: SR1=SR2=US). In addition, for males a line x FFDW interaction was significant (I: $F_{1, 77} = 11.43$, $p < 0.05$), resulting from a greater slope of the regression of RMR on FFDW for the US compared to SR lines (data not shown).

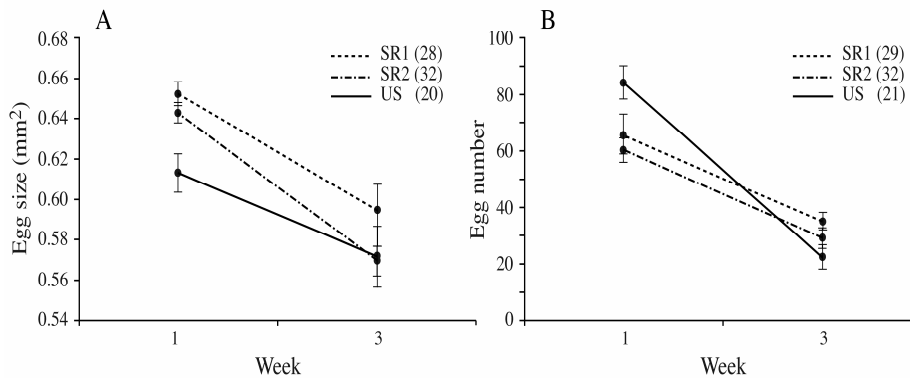
Female reproduction

Early-life egg size is significantly larger in both selection lines compared to the US (Fig. 4A; line effect: I: $F_{1, 1.69} = 10.44$, $p = 0.10$; II: $F_{1, 85} = 8.23$, $p < 0.01$; III: SR1=SR2> US). For early egg production there is a trend towards laying fewer eggs in the SR line females (figure 4B; line effect: I: $F_{1, 1.49} = 5.15$, $p = 0.22$; II: $F_{1, 83} = 4.98$, $p < 0.05$; III: SR2< US, SR1= US, SR1=SR2). At late reproduction, egg production tended to be higher in the selection lines (I: $F_{1, 5.12} = 4.40$, $p = 0.09$; II: $F_{1, 65} = 4.42$, $p < 0.05$; III: SR2< US, SR1= US, SR1=SR2). Late-life egg size was not significantly different between lines ($F_{1, 1.48} = 0.01$, $p = 0.95$; also NS in II and III). The reduction of both egg number and size over time was significant (MANOVA, time effect: $F_{1, 60} = 29.9$, $p < 0.001$ and $F_{1, 60} = 47.2$, $p < 0.001$, respectively). In addition, the time x line interaction in the MANOVA analysis for egg number approached significance ($F_{2, 60} = 2.8$, $p = 0.07$). Female fertility did not differ between lines (Wald $X^2 = 0.26$, $df = 2$, $p = 0.87$).

Table 1. Means values with standard errors (in parentheses) for development time, body mass (as dry weight and fat-free dry weight (FFDW)), fat content, relative fat content (as percentage of FFDW), resting metabolic rate (RMR), and relative RMR (per unit of FFDW) for each sex of butterflies in the lines. Data for development time are from a different series of individuals. Sample sizes, n, are given. Note that the data for relative fat content and relative RMR presented here are different from those used in the ANCOVA statistical analyses.

Line	n	Development time (days)	n	Dry weight (mg)	FFDW (mg)	Fat content (mg)	Relative fat content (% FFDW)	RMR (ul/h)	Relative RMR (ul/h*mg)
Females									
SR1	58	29.3 (0.23)	38	24.34 (0.93)	19.30 (0.69)	5.04 (0.35)	26.55 (1.73)	88.57 (8.92)	4.67 (0.44)
SR2	135	29.9 (0.18)	34	23.12 (0.77)	18.86 (0.60)	4.26 (0.37)	22.89 (1.79)	94.80 (11.16)	5.04 (0.55)
US	86	27.9 (0.18)	30	17.98 (0.89)	14.40 (0.67)	3.58 (0.39)	25.44 (2.89)	75.80 (6.30)	5.24 (0.35)
Males									
SR1	54	28.3 (0.35)	34	12.06 (0.35)	8.73 (0.32)	3.33 (0.21)	39.93 (2.95)	71.06 (8.19)	8.08 (0.86)
SR2	89	29.1 (0.30)	26	12.04 (0.50)	8.93 (0.28)	3.10 (0.25)	34.23 (2.76)	76.32 (6.75)	8.47 (0.69)
US	63	26.4 (0.13)	22	11.22 (0.63)	8.31 (0.43)	2.92 (0.27)	34.67 (1.76)	101.51 (14.55)	11.45 (1.17)

Figure 4. Mean and standard error of size (A) and number (B) of eggs laid during a three day period in the early egg-laying period (week 1) and the late egg-laying period (week 3) at 27°C for SR1, SR2 and the unselected Stock (US). The data for week 1 represent peak reproduction. Sample sizes are given in parentheses in the figure legend.



Discussion

The rationale for our study is twofold. Firstly, to explore the genetics and physiology of starvation resistance in an insect species that allows the linking of the mechanisms to the ecology and relevant selection pressures in natural populations. In other words, we have selected for starvation resistance in a wet season environment and in a manner relevant to the field biology (Fig. 1). Secondly, our results will further a conceptual unification of the mechanisms of starvation resistance and ageing that were hitherto based almost exclusively on one major study species, *D. melanogaster*.

A strong genetic link between starvation resistance and longevity

We successfully targeted the available standing genetic variation in the original Stock population for alleles, and their combinations, that promote starvation resistance. The results are similar to previous findings in *Drosophila melanogaster* (Rose et al. 1992; Harshman and Schmid 1998; Baldal et al. 2006). The survival curves (Fig. 2) show that the increase in median and maximum starvation resistance is due to a later onset of mortality. This suggests that the selection line butterflies are able to maintain homeostasis longer than unselected butterflies, but once this fails, the pattern in mortality, and possibly the underlying causes, appear to be similar. Longevity also increased significantly, and to a remarkable extent. For example, the percentage increase in starvation resistance and longevity relative to the US are similar, suggesting a high value for the genetic correlation. Such an

increase is unlikely to result from small differences in reproductive schedules. Previous findings in *Drosophila* have shown that starvation resistance and longevity can be genetically linked (Zwaan et al. 1991; Rose et al. 1992; Chippindale et al. 1996) but this was not always found (Harshman et al. 1999b; Baldal et al. 2006).

Mechanisms of increased starvation resistance

Both the response to selection and the correlated responses indicate that at least part of the response in survival under starvation is the result of selection for thriftiness. In addition, selection has targeted developmental traits. This can be explained by two non-exclusive mechanisms; enhanced resource acquisition and lowered metabolism (i.e. Chippindale et al. 1996; Djawdan et al. 1998; Harshman et al. 1999a; Baldal et al. 2006). Interestingly, we find evidence in *B. anynana* for both mechanisms, the first in females, the second in males. Females seem to have invested in a more durable body, as evidenced by the increase in body mass. However, relative fat content was not significantly different between the lines, indicating that the body composition did not change, in contrast to findings from *Drosophila melanogaster* (Chippindale et al. 1996; Djawdan et al. 1998; Harshman et al. 1999a; Baldal et al. 2006). Apparently, the female larvae increase body mass by increasing fat as well as other resources, such as carbohydrates and/or proteins (Djawdan et al. 1998). A previous finding in females of populations of *D. melanogaster* artificially selected for increased starvation resistance report highly increased lipid and carbohydrate content, but no change in metabolic rate (Djawdan et al. 1997). In contrast, our results for males suggest that selection has targeted genes that have lowered metabolism, which then may have caused growth to slow down. We have measured metabolic rate at rest, which indicates the base level of energy consumption. Other experiments with these lines show that a lowered RMR affects activity and active behaviour, such as is involved in mating, as investigated in male-male competition trials for females in large flight cages (Pijpe et al., unpublished results). The possibility of different mechanisms in the sexes in the evolution of starvation resistance or longevity has been reported (Nuzhdin et al. 1997; Hoffmann et al. 2005; Tower 2006), and our results indicate that sex differences should be taken into account in relation to lifespan. The mechanisms highlighted by our results may well be involved in the general pattern of female-male lifespan differences in the animal kingdom (Smith 1989).

The relationship between lifespan and reproduction has always received much attention in life history evolution and ageing research alike. The selection experiments performed in *Drosophila* (Service et al. 1985; Rose et al. 1992; Harshman et al. 1999b; Bubli and Loeschcke 2005) have identified this relationship as a trade-off; lifespan increases are realized at the expense of reproductive output. We show that females are more likely to change their reproduction to produce eggs of higher quality at the expense of quantity when confronted with repeated generations of adult starvation. In life-history theory (Stearns 1992), this is a

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textbook example of a trade-off that is highly relevant in many species (Fox and Czesak 2000), including in *B. anynana* (Fischer et al. 2006). Moreover, the change in the female reproductive program is very similar to what is observed at the end of the dry season form as a plastic response to low temperatures during development and in adulthood (Fischer et al. 2004). Thus, our results provide valuable details of the trade-off between reproduction and lifespan, and allow interpretation of these details relevant for the ecology of this species.

Perspective: ageing and the mechanisms of life history plasticity

Increased development time, larger adult size, the shift in female reproduction and the increased longevity are all hallmarks of the dry season phenotype of this species (Fig 1a, Brakefield and Frankino 2007). The dry season phenotype is a result of phenotypic plasticity and readily induced by dry season environmental conditions prior to pupation (Brakefield and Reitsma 1991; Brakefield 1997). The similarities of the selection phenotype to the dry season form in nature suggest that the genes we have targeted by artificial selection for starvation resistance comprise at least some that are involved in the plasticity to induce a dry season life history. A more detailed genetic analysis of the selection lines is required to test this intriguing parallel. The adult dry season phenotype shows characteristics of reproductive dormancy. Our result supports the conclusion that reproductive dormancy or diapause is central to the regulation of lifespan in many organisms (Tatar and Yin 2001). We postulate that the mechanisms of ageing are to a large extent plastic, and may underlie adaptive phenotypic plasticity to variable environmental conditions, within an individual's lifespan, as well as over generations. The notions that the processes that determine lifespan can be environmentally malleable within the course of a life, and that the interaction between genetic variation and the environment may have shaped species-specific lifespan and ageing rates, are important for understanding observed patterns of ageing in natural populations, including in humans (c.f. Bateson et al. 2004).

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Chapter
6

**Male longevity at the cost of
mating success**

Abstract

Evolutionary theories of ageing predict a cost of increased lifespan. In invertebrates, a cost to reproduction has often been shown at a physiological level, but mainly in females. Here, we show that for male butterflies, an increased lifespan also occurs at a cost to reproductive success under conditions of plentiful food: long lived males mate with fewer females when competing against males of normal lifespan. However, in conditions of mild adult starvation resembling natural environments long-lived males mate at least as successfully as males of normal lifespan. Our results highlight the need to consider behaviour together with natural environmental when seeking to explain variation in ageing

Chapter 6

The increasing size of the ageing human population has substantial social, medical and economic consequences. Hence, it is important to unravel the mechanisms of the ageing process to promote a healthy old age. Research has greatly benefited from an evolutionary analysis demonstrating that ageing is the by-product of selection for an optimal reproductive lifespan (Kirkwood and Austad 2000).

Here we focus on male reproduction, and especially on mating related behaviour in long-lived populations of the butterfly, *Bicyclus anynana*, that have been selected for increased starvation resistance in adults (Pijpe et al. 2007). Starvation resistance (SR) of two replicate lines increased under artificial selection over 17 generations by 100%. Their longevity also increased by 50% relative to the unselected Stock from which they were derived. Similarly to other populations of insects known to be comparatively long lived, we found that in females an increased body size and altered reproductive output contributed to the increase in lifespan. In males we found only a slightly reduced resting metabolic rate which is unlikely to account for the observed increase in lifespan (Pijpe et al. 2006). In a similar way to females, a trade-off between reproductive investment and lifespan might explain the enhanced lifespan in males but this has seldomly been investigated. We hypothesized that in males such a trade-off would involve behaviour and activity rather than the physiological mechanisms of resource allocation.

Initially, we competed 3-7 day old males of SR and unselected Stock lines for a limited number of receptive females in a large cage that allows natural mating behaviour (Frankino et al. 2005). Food conditions were then changed from ad lib to 2 or 3 days of adult starvation for the males; this treatment causes no additional mortality. This was then repeated for different cohorts of male butterflies.

Males of the long-lived populations have low mating success when in competition with Stock males (Fig. 1). Interestingly, this pattern is reversed after 2-3 days of adult starvation prior to the competition test with starvation resistance selected males now outcompeting Stock males for mating (see figure 1 caption for details).

The results clearly demonstrate that male reproduction is affected by selection for increased survival under starvation. Moreover our results highlight the importance of studying gene-by-environment interactions in the evolution of lifespan. The impaired mating ability is condition dependent. In the selection environment, the SR males perform equally or better compared to control males.

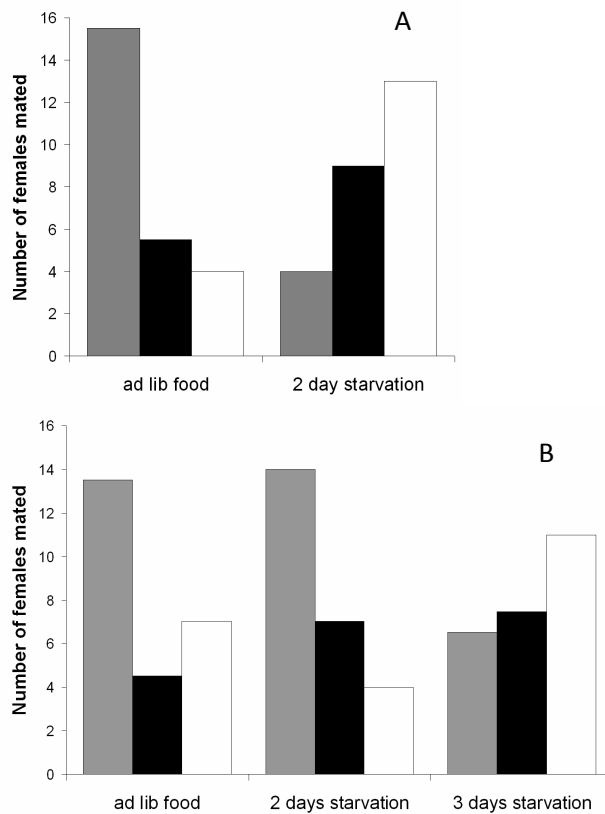
Fitness-associated behaviour, such as results in the observed reduction of mating capacity, presents a significant factor in the evolution of stress resistance and longevity. In natural populations of this species, SR and longevity are especially important in the dry season when the adult butterflies must survive up to eight months before reproducing at the start of the wet season (Brakefield and Frankino 2007). We hypothesize that the SR selection lines display a permanent mild reproductive dormancy, which is crucial to survival in the dry season in the wild.

Trade-offs between traits are typically considered a consequence of physiological constraints that incur some cost in another (Stearns 1992). This study reveals a direct cost of increased lifespan in males that involves behaviour. Moreover, we show that increased lifespan can come at a direct fitness cost in under natural,

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restrictive conditions. Our results indicate a need to take behaviour together with more natural environments into account when seeking to explain variation in ageing.

Figure 1. The male mating trials (analyzed with G-tests) show that in competition under optimal food conditions SR line males (black bars) mate significantly with fewer females than Stock males (grey bars) (Trial A: $G = 4.71$, $P = 0.030$; trial B: $G = 4.96$, $P = 0.026$). Starving the males for 2 days (trial A: $G = 10.50$, $P = 0.001$) or 3 days (trial B: $G = 5.20$, $P = 0.023$) reduces Stock male mating frequency, and increases SR male mating frequency, compared to ad lib food conditions. White bars represent females that did not mate within 24 hours. In all trials, 25 males of each line were competing for 25 Stock females.



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Chapter
7

Candidate ageing mechanisms underpin standing genetic variation for lifespan in the butterfly *Bicyclus anynana*.

Abstract

Clear candidate genes and mechanisms for the regulation of ageing and lifespan have emerged from studies that use mutants and genetically manipulated model organisms under laboratory conditions. However, it is rarely addressed whether these genes contribute to standing genetic variation in natural populations of these species. Here, we explore the natural variation in candidate ageing genes in *Bicyclus anynana*, a butterfly species with well understood ecology. We investigated whether the expression of candidate genes related to metabolism (*Indy*) and oxidative defense biology (*sod2*, *catalase*), is altered in *B. anynana* populations artificially selected for increased adult starvation resistance. The adult lifespan of these butterflies is not only considerably increased under starvation, but also under *ad libitum*, optimal food conditions, thus providing evidence for a genetic correlation between starvation resistance and longevity. We measured gene expression in butterflies of various ages, both under optimal conditions and under mild and prolonged starvation stress. *Indy* and *catalase* are up-regulated in response to stress; *sod2* expression is very high under mild stress, but drops to normal levels under prolonged stress. Under optimal conditions, *Indy* is down-regulated at a later age. More importantly, we find expression differences between selected and control populations. Under stress conditions, *catalase* and *sod2* have a higher expression in selected butterflies compared to unselected butterflies. *Sod2* expression is also higher in the selected populations under optimal conditions. We conclude that *sod2* and *catalase*, but not *Indy*, are involved in the response to artificial selection for increased starvation resistance. In addition, *sod2* appears to contribute to the genetic correlation between starvation resistance and longevity.

Introduction

Since the discovery of single gene mutants with a long-lived phenotype in laboratory stocks of species such as the nematode *Caenorhabditis elegans* (Friedman and Johnson 1988; Kenyon et al. 1993; Lakowski and Hekimi 1996), the fruitfly *Drosophila melanogaster* (Clancy et al. 2001; Lin et al. 1998; Rogina et al. 2000) and the yeast *Saccharomyces cerevisiae* (Kennedy et al. 1995), a major focus of ageing research has been to identify genes and/or pathways that regulate the rate of ageing. Since then, mutational mapping has greatly expanded our knowledge of the regulation of ageing, and this is likely in turn to contribute to a healthier long life in our own species (Partridge and Gems 2006). To make this connection, we need to know whether these genes are contributing to variation in human lifespan. The first step in this analysis is to study the contribution of the identified candidate genes contribute to variation in ageing in natural populations of the model organisms. A few studies on *Drosophila* have now taken this direction. Recently, Mackay and co-workers (Geiger-Thornsberry and Mackay 2004; Harbison et al. 2004; Leips et al. 2006) have begun to combine quantitative trait loci mapping and complementation tests to find small genomic regions that contribute to variation in many phenotypes, including ageing related traits (Flatt 2004). Their approach includes the use of long term laboratory strains of *D. melanogaster*, including inbred lines. More relevant, geographic clines for genetic variation in the candidate ageing gene *Methuselah* have been described in natural populations of *D. melanogaster*, suggesting positive selection on this gene (Duvernell et al. 2003; Schmidt et al. 2000). However, the connection with variation in lifespan is unclear. In addition, research on the expression of anti-oxidant genes in long-lived artificial selection lines can give important indications but do not always give consistent results; *catalase* expression can be higher (Foley and Luckinbill 2001), but also lower (Mockett et al. 2001). Another study showed that increased lifespan is correlated with higher *sod2* expression in one replicate line, but with higher *catalase* expression in another (Arking et al. 2000a). Thus, the potential role of genes for anti-oxidant enzymes in the evolution of longevity and stress resistance remains unresolved in the laboratory, and unknown for natural populations. Here, for the first time, we study in detail how the gene expression of candidate ageing genes relates to variation in starvation resistance and lifespan in selected populations of *Bicyclus anynana*.

From variation in candidate gene expression to variation in lifespan

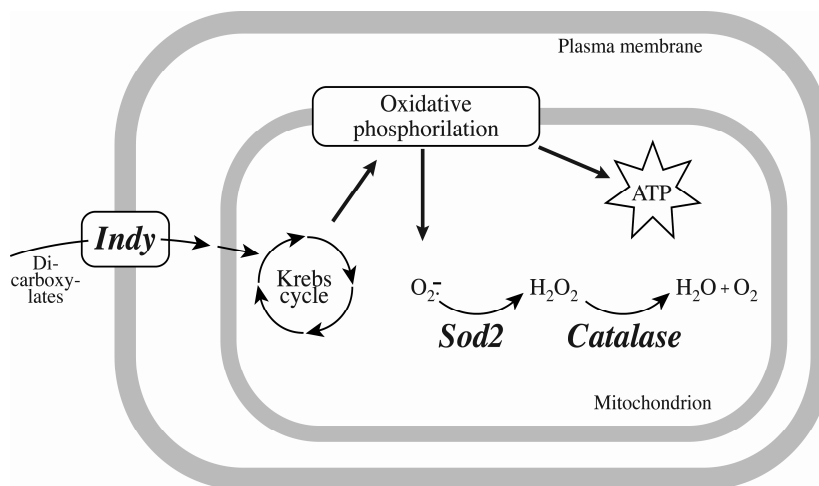
Both long adult lifespans and the necessity of high starvation resistance occur naturally in the life history of the butterfly *Bicyclus anynana* (Butler 1879), an emerging model species for the study of lifespan and ageing in an ecological perspective (Pijpe et al. 2006; Zwaan 2003). *B. anynana* is a tropical, multi-voltine,

Candidate ageing mechanisms underpin standing genetic variation for lifespan

fruit-feeding species that lives in a highly seasonal environment in East Africa (Brakefield and Reitsma 1991). The evolution of phenotypic plasticity in this species has led to two distinct seasonal forms. Butterflies of the dry season form butterflies are long-lived; they must survive the dry season (5-7 months) to be able to reproduce at the start of the next wet season. In contrast, individuals of the two generations of the wet season form show rapid reproduction and short lifespans (Brakefield and Frankino 2007). In both seasons, but especially in much of the dry season, the availability of fruit in tropical forest typically varies in space and time. Therefore, mild starvation occurs regularly in the life of this butterfly and starvation resistance may be especially important in the dry season form (Pijpe et al. 2006). To investigate the genetics, physiology and adaptive role of starvation resistance in *B. anynana*, two lines were artificially selected for starvation resistance (see chapter 5). These lines were founded from a natural population and showed increased median lifespan under starvation stress (60%) and under optimal food conditions (30%). This then is a positive genetic correlation between starvation resistance and adult lifespan.

On the basis of availability in the *Bicyclus anynana* EST database (Beldade et al. 2006), we chose three genes that have been shown to be important in other organisms in both starvation resistance and ageing: *Indy* (Rogina et al. 2000), *sod2*

Figure 1 Pathway relationship between the three candidate genes investigated in this study. In cells of the gut or intermediary metabolic tissue, *Indy* is responsible for the transportation of Krebs (citric acid) cycle intermediates for across the plasma membrane. In the mitochondrion, the Krebs cycle reduces oxygen to phosphorylate ADP to ATP, mainly through the oxidative phosphorylation chain at the inner membrane. In this process, reactive oxygen species (ROS) are formed. The oxygen anion (O_2^-) is scavenged by SOD2 and reduced to another ROS, hydrogen peroxide (H_2O_2). This is reduced by Catalase to water and oxygen.



(e.g. Honda and Honda 1999; e.g. Tyler et al. 1993) and *catalase* (e.g. Arking et al. 2000a; Mockett et al. 2001). These genes are part of pathways that regulate metabolism and are important for homeostasis (see figure 1).

This allows us to study the interactions between the genes at the level of gene expression, the first and foremost step from the genotype to the phenotype. We used quantitative real-time PCR (qPCR) to measure candidate gene expression in adult males sampled at various conditions and at various ages: (i) at eclosion (emergence from pupa); (ii) at 2 and 5 days after eclosion under starvation stress conditions; and (iii) at 14 and 35 days after eclosion under *ad libitum*, optimal food conditions. Importantly, our set-up allows us to distinguish between environmental factors and genetic factors influencing gene expression, and their interactions.

Material and Method

Butterflies and their survival

The laboratory stock of *Bicyclus anynana* was established in 1988 from around 80 gravid females caught at a single location in Malawi. This stock is kept in outbred conditions (Van't Hof et al. 2005) in climate controlled chambers. The larvae are fed on young maize plants and adults on moist banana for both stock and experimental rearing, unless indicated otherwise. In 2002, two starvation resistance lines (SR1 and SR2) were started from the stock population and selected for increased starvation resistance. After 14 generations of selection, each line showed an increase in SR and in longevity of over 50% (chapter 5).

For our experiment we used adult males of three lines: an unselected Stock line, and the two selected lines, SR1 and SR2. Animals were kept in a climate controlled chamber at 27°C, 70% relative humidity and 12:12 light to dark regime. A minimum of 100 butterflies were reared to eclosion, and separated by sex. Male butterflies were collected in a cylindrical hanging cage (25 Ø x 60 cm) on each day of eclosion, with a maximum of 15 per cage to minimise density stress. Butterflies in the *ad libitum* food treatment were supplied with banana slices on water saturated cotton wool, whereas those in the food stress treatment were supplied with water saturated cotton wool only (to prevent desiccation). Both were refreshed three times a week. Due to practical limitations we focused on one sex, males, to increase exploratory power. Survival was measured by scoring individual deaths daily. The butterflies not sampled for RNA extraction were followed to measure survival under starvation. We gave priority to an adequate measurement of survival under starvation and therefore had to divide the butterflies over the treatment groups in such a way that numbers of butterflies were insufficient to properly assess survival in the *ad lib* food treatment. However, we have shown previously that lifespan under *ad lib* food conditions is 30% higher in the selection lines, compared to the unselected Stock (see chapter 5).

Candidate genes

Indy (I'm not dead yet) codes for a sodium dicarboxylate co-transporter. These membrane proteins are responsible for the uptake or re-uptake of Krebs cycle intermediates (Knauf et al. 2002). In *D. melanogaster*, hypomorphic mutant flies with reduced expression live up to 80% longer under laboratory conditions (Rogina et al. 2000). Researchers suggested that mutations in the *Indy* gene may reduce the availability of these key metabolic intermediates to the cell, thus reducing the generation of metabolic energy. Because this resembles the lifespan-increasing effect of caloric restriction that has been documented in various species, it is thought that *Indy* may provide a genetic mechanism for caloric restriction (Rogina et al. 2000). In adult *D. melanogaster*, *Indy* is expressed in the fat body, midgut and oenocytes (Knauf et al. 2002). These organs are thought to be the primary sites of intermediary metabolism, absorption and metabolic storage in adult insects. In *B. anynana*, we hypothesise a reduced *Indy* expression in the lines selected for increased starvation resistance compared to the unselected Stock. However, under food stress we expect an up-regulation of *Indy* that limits gene expression differences between lines, because nutrients mobilised from reserves need to be available for survival. Age effects are difficult to predict; the expected reduction in metabolic efficiency with age could give a lower (causal) or higher (compensatory) expression.

There is a large body of literature that suggests that genetically increased stress resistance and lifespan are accompanied by up-regulation of anti-oxidant enzymes (e.g. Beckman and Ames 1998; Hekimi and Guarente 2003; Landis et al. 2004). *Catalase* and *sod2* (Manganese superoxide dismutase 2) are central to oxidative stress resistance; their products neutralise reactive oxygen species (ROS), and form a main line of anti-oxidative defense enzymes in eukaryotes. The mitochondrial respiratory chain that reduces oxygen to water is the metabolic engine of high-energy aerobic metabolism. ROS such as superoxide radicals are a by-product of oxidative phosphorylation in all aerobic organisms, and are thought to be a major cause of intra cellular damage that increases the rate of ageing (Beckman and Ames 1998).

The Superoxide Dismutases (SODs) are a family of antioxidant enzymes present in most eukaryotic cells; SOD2 is located in the mitochondrial matrix to detoxify superoxide radicals released during respiration and convert these to hydrogen peroxide (Landis and Tower 2005). Loss of *sod2* activity in *D. melanogaster* causes a net increase in superoxide radical load in mitochondria resulting in mitochondrial membrane damage, which in turn leads to lethal cell death in heart, brain and muscle (Duttaroy et al. 2003). Transgenic studies that over-express *sod* genes typically find a higher stress resistance and often a higher lifespan in *D. melanogaster* and *Saccharomyces cerevisiae* (reviewed in Landis and Tower 2005), but this depends critically on the method used (Orr and Sohal 2003), and on genetic background and sex (Spencer et al. 2003). The hydrogen peroxide formed in the reduction by SOD2 is further reduced by *catalase* to water and oxygen. *Catalase* is

typically present in excess, and thus artificial over-expression does not seem to influence lifespan (Landis et al. 2004).

In *D. melanogaster*, the expression of *catalase* in adults is restricted to intermediary metabolic, digestive and adipose tissues, and oenocytes, highly comparable to the location of *Indy* expression (Klichko et al. 2004). In *B. anynana*, we expect a higher expression of both *catalase* and *Sod2* genes in the starvation resistance selected lines compared to the unselected line. We expect such a difference to become more evident with age in the optimal food treatment. Under starvation stress, we expect expression to be higher in all lines and the difference between lines to be decreased.

Gene sequence information

All sequence information was obtained from the *Bicyclus anynana* EST database (Beldade et al. 2006). The sequenced contigs were BLASTed against relevant gene collections for annotation. Additional Gene Ontology information (Ashburner et al. 2000) was obtained by direct or indirect BLAST to *D. melanogaster* gene collections. GenBank accession numbers and primer names and sequences are given in table 2. GenBank EST sequence information does not include annotation information at this point.

Table 1. GenBank accession numbers and primer sequence information for the genes used in this study.

Gene	GenBank accession number	Primer name	Dir.	Sequence (5'-3')
<i>Indy</i>	DY763534	Indy_2_F	F	AGCATTCCAAGCTGCCTAAA
		Indy_2_R	R	TTGGAGACCCACATGGAGAT
<i>Sod2</i>	DY768811	Sod2_1_F	F	CAACTTGCAACTGCGTCTGT
		Sod2_1_R	R	GGTTCTGGCAAGTGGTGATT
<i>Catalase</i>	DY766654	Catbov_F	F	TGTTGCAAGGACGACTGTTC
		Catbo_R	R	CCTGGTTATTGATGGCTTGG
<i>FK506</i>	DY768120	FK506_2_F	F	AAACTAACCTGCAGCCCTGA
		FK506_F_R	R	CAAGACGGAGAAGTTCCACA
<i>EF-α</i>	DY766179	Ba_EF_F	F	GCCTGGAGACAATGTTGGTT
		Ba_EF_R	R	ATTTGACCAGGGTGGTTGAG
<i>VhaSFD</i>	DY767355	VhaSFD_2_F	F	GAGGGGCAAACACATCATAGA
		VhaSFD_2_R	R	TTCCCAGTTGTGAACCATGA

Sample selection

To adequately investigate the effects of starvation stress and ageing on gene expression, we chose 5 time-points at the three lines were sampled at random. They are labelled as follows. 'Ecdysis' is on the first day of adult life, within 10 hours of eclosion. 'Early' is at the age of 2 days under starvation or at 14 days under optimal conditions. 'Late' is at the age of 4 (for the Stock line) or 5 (for both SR lines) days under starvation, and 35 days under optimal food conditions. Under starvation, the Stock mortality was in exponential phase at the age of 4 days, but this had not begun in the SR lines. For the sake of proper comparison we wanted to capture the effect of starvation, thus we chose the age of 5 days for the selection lines. The samples were taken as an optimal balance between the comparisons we wanted to focus on, and the experimental constraints of cost and time. We sampled five male butterflies per time point, treatment and line, thus obtaining a total of 75 samples. The chosen live butterflies were frozen instantly in N₂(l), put in micro tubes after removal of the wings and stored at -80°C until subsequent RNA isolation. We always sampled at the same hour (\pm 30 min) during light in their circadian cycle.

RNA isolation and cDNA synthesis

Total RNA from individual abdomens was extracted using the Nucleospin® RNA II kit and additional DNase treatment (Machery-Nagel) according to the kit protocol. The initial and final steps were deviate from the protocol as follows: before and again after disruption of the abdomen, 175 μ l of a mixture of RA1 buffer (Nucleospin®) and β -mercaptoethanol was added to the micro tube; RNA was eluted in 50 μ l RNase free water and stored at -20°C until cDNA synthesis. RNA concentration and purity were measured using a ND1000 spectrophotometer (NanoDrop Technologies). Samples of low quantity (<100 ng/ μ l) and/or quality (A260/A280 ratio outside the range of 1.9 to 2.1) were not used. cDNA was synthesised in 20.0 μ l reactions using 1.00 μ g total extracted mRNA in the First Transcription System (Promega).

Real time qPCR

To measure the gene expression we used quantitative real-time PCR (qPCR) with the cDNA as a template. The amount of amplified product during the qPCR reaction was detected using SYBRGreen® (Eurogentec), a fluorescent a-specific DNA binding dye. Per gene, a fluorescence threshold was set, at which the number of reaction cycles (C_t) was scored. qPCR reactions were performed on a MJ Research PTC 200 thermal cycler with a mounted Chromo4 Real-Time Detector (BioRad). Control and data acquisition was done with the accompanying Opticon Monitor 3.1 software (BioRad). We checked the raw (C_t) data for outliers using one-way-ANOVA, and values that deviated more than 2 standard deviation from the mean (per line and

treatment) were excluded from further analysis. The RT control samples (sample reactions that were not reverse transcribed and thus did not contain cDNA) indicated that not all DNA was removed during RNA isolation. A major advantage of real time qPCR is that this contamination can be controlled for: in all cases the RT control C_t values were >10 C_t higher than cDNA sample C_t values, indicating the very low (2^{10} fold lower) fluorescent signals from DNA contamination do not influence the cDNA amplification (and hence quantification) until well after the threshold cycle.

Primer sequences

Primer design was done using Primer3 software (Rozen and Skaletsky 2000). Adjustments to the default conditions were as follows: product size range: 100-150 base pairs, $T_m = 60.0^\circ\text{C}$ minimum, GC content at 50% maximum, and maximum T_m difference for primers = 1.0°C . All primer pairs were tested for specificity and dimer formation by visual inspection of ethidium bromide stained agarose gels after regular PCR. The primer sequences are shown in table 2.

Calculation of relative expression

The calculation of relative expression is performed with GeneX software (Bio-Rad). It essentially uses the method of Pfaffl (2001). In short, the raw C_t data are normalised to a single randomly chosen sample, and consecutively normalised to the geometric mean of the expression of three reference genes. GeneX allows correcting for PCR efficiency (E) by giving more weight to the relative expression values with higher E before averaging. E is calculated from the slope of a standard curve using the following formula:

$$E(\%) = ((10^{(-1/\text{slope})} - 1) * 100).$$

The standard curve was determined per gene from 5 reactions from a dilution series, using cDNA from a pool of all samples as a template.

Rereference genes

Reference genes must possess two main properties. First, they should be ubiquitously expressed in all tissues, for example because they are essential for the maintenance of cellular function and viability ('housekeeping gene'). Secondly, their transcription should be minimally affected by the experimental context, which in our case includes a wide range of environments. We initially chose 5 genes based on their supposed role as 'housekeeping gene'. We then tested whether they met the second condition by using geNorm (Vandesompele et al. 2002), which allows ranking of the variation in expression of genes based on a calculated stability measure. C_t values were scaled relative to the lowest C_t value (highest absolute expression) among the samples. The three most stable expressed genes that were

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used as reference genes were *EF- α* (Elongation factor 1 alpha), *FK506* (FK506 binding protein 2) and *VhaSDF* (Vacuolar ATP synthase subunit H). The genes *β tubuline* [GenBank:DY767109], *α tubuline* [GenBank:DY772209], and *developmental embryonic B* [GenBank:DY766034] exhibited a too wide range across all samples.

Statistical analysis

Relative expression values were log-transformed to satisfy the requirement of equal variances before analysis of variance (ANOVA). Two separate analyses were performed. First, an overall model was built with factors line and treatment, and their interaction. The treatment factor differentiates stress and optimal food conditions, with the eclosion category included in both treatments. Secondly, models by treatment were built, with time (0, 2 and 4/5 days for starvation, and 0, 14 and 35 days for optimal), line and their interaction to investigate stress intensity and age effects, respectively. Significant differences between factors were qualified by post-hoc Tukey HSD tests ($\alpha = 0.05$), or with a Student's t-test when appropriate. All three lines (Stock, SR1, SR2) were treated as independent lines.

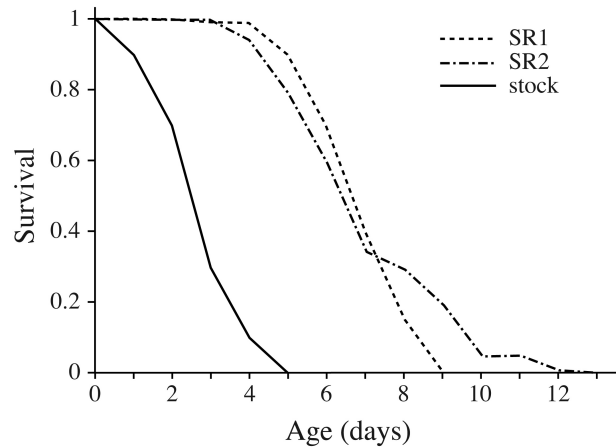
The effect of line on survival under starvation was analysed with a Cox Proportional Hazard model. Lines were compared pair-wise to quantify survival differences with risk ratios. All analyses were performed using JMP 5.0.1.2 (SAS Institute).

Results and discussion

Survival

Males of both selection lines show a doubling of survival under starvation compared to the unselected Stock (figure 2) (chapter 5). The cohorts of SR males show a later onset of the exponential mortality phase, suggesting that SR selection males suffer less from starvation stress. In addition, we have previously shown that males of both selection lines are long-lived; their mean lifespan has increase by 50% (chapter 5). The aim of this study is to investigate whether these genetic differences in mortality can be traced back to differences in candidate gene expression.

Figure 1 Survival under starvation of the males that were not sampled for gene expression analyses. As measured previously (chapter 5), the starvation resistant selection lines live ca. twice as long on average under these stress conditions.



Gene expression

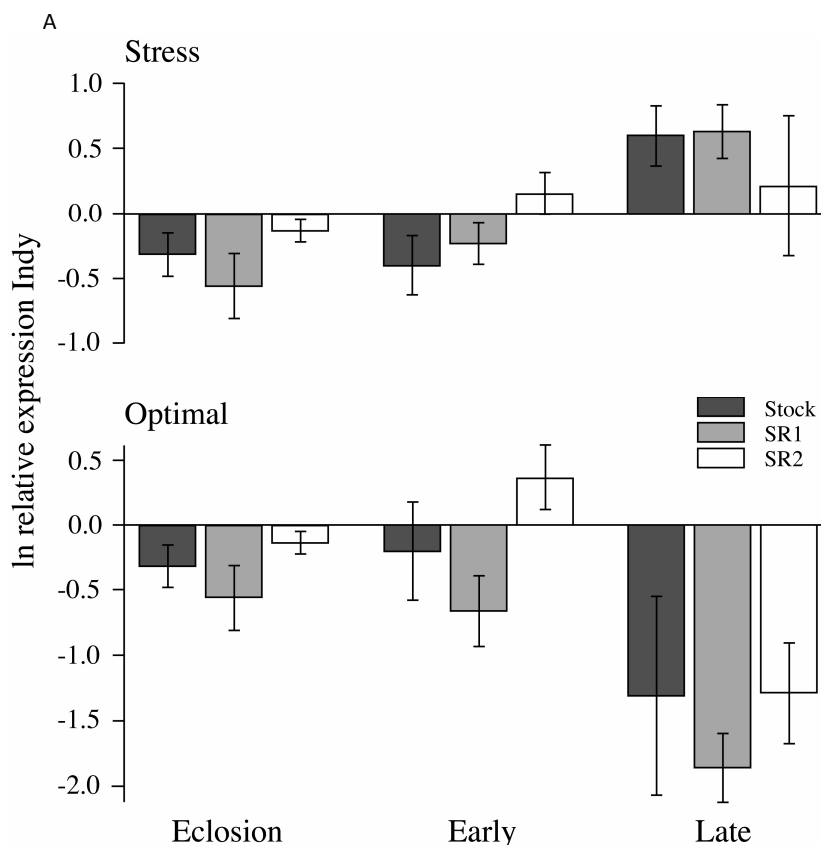
Indy

No line effect is evident in the overall analysis that includes line and treatment. Treatment is highly significant, with a higher expression under stress conditions (Figure 3A, Table 1). Two patterns are observed in an analysis by treatment. First, *Indy* expression is down-regulated at later ages under optimal food conditions (Table 1; Tukey: Eclosion: A, Early: A, Late: B), suggesting that *Indy* expression is associated with age. Secondly, *Indy* is up-regulated upon prolonged starvation, compared to mild starvation (Table 1; Tukey: Eclosion: A, Early: A, Late: B). An absence of differences between lines indicates that *Indy* expression was not involved in the response to selection for starvation resistance, or in the correlated response of increased longevity. However, in general, there is high variation in *Indy* expression that makes it difficult to find more subtle expression differences, such as those expected between lines.

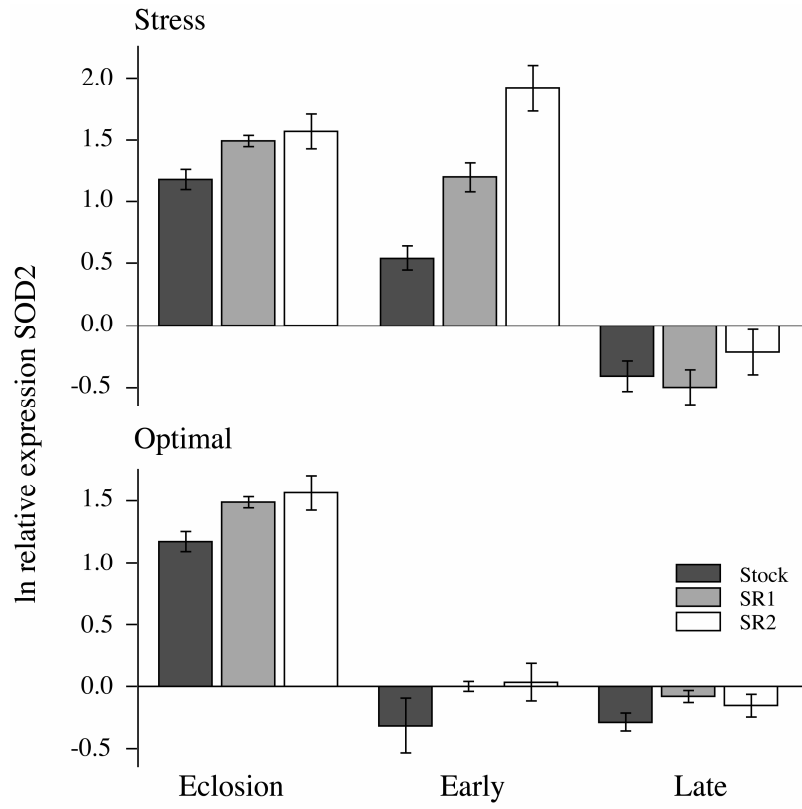
At present we conclude that altered *Indy* expression is not causally related to variation in lifespan in our study. Our findings represent a significant advance in understanding the role of *Indy* in organismal biology. We conclude that *Indy* is involved in the environmentally induced response to food stress. Up-regulating *Indy* increases the transport of metabolites (Knauf et al. 2002), which probably results from butterflies accessing energy reserves to provide the necessary energy in absence of nutrition. Expression levels under mild starvation are similar to those on day 0 or day 14, indicating that mild starvation is apparently not a sufficient stimulus

to trigger the access to energy reserves. This suggests that a starvation period of two days lies within the normal physiological range of the population. In addition, we show that *Indy* expression is lowered at higher ages. This may be explained by an ageing effect: either by a reduced demand for citric acid cycle intermediates with age, or by a reduced efficiency of citric acid metabolism or of the upstream pathway necessary for normal *Indy* expression.

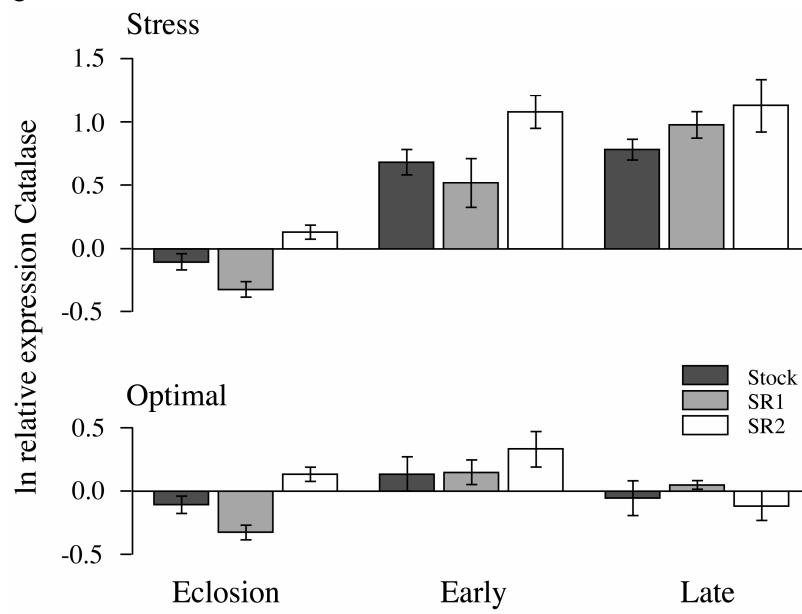
Figure 6 (this and next page) Gene expression of A: *Indy*, B: *sod2* and C: *catalase* in two starvation resistance selection lines and the unselected line under 2 food treatments: stress (top) an optimal or *ad libitum* (bottom) at three ages: at eclosion (0 days), early (2 days for stress, and 14 days for optimal) and late (4/5 days for stress, and 35 days for optimal). The data are natural log (ln) transformed for the sake of analysis. Thus, a difference in expression of 1.0 ln is a 2.72 fold difference. An expression of +2,0 compared to -0,5 in the graphs is a difference of 2.5 ln, and thus a 12.8 fold difference. Error bars indicate the standard error of the mean. All data from a gene are normalised to the same sample, making expression levels under stress and optimal conditions directly comparable.



B



C



sod2

The overall analysis of *sod2* reveals no significant differences in expression (Table 1) between lines nor between treatments. This seems in part due to the remarkably high expression at eclosion (day 0) and at mild stress (2 days of starvation) (figure 3B). The expression pattern could be explained by an early age effect, with high expression in the first days after eclosion followed by low expression from day 5 or day 14 onwards. Possibly, the dissimilarity of expression on days 0, 14 and 35 could indicate that the period following eclosion is an exceptional moment in the lives of the butterflies. Metamorphosis is a particularly physiologically dynamic time, characterised by a multitude of processes, including a high metabolism and a high oxidative stress (Hilliker et al. 1992; White et al. 1999). In addition, some tissues may not have fully matured immediately after eclosion. However, the expression of the other genes and the standard errors do not seem to be exceptional. For these reasons, we have performed analyses with and without the eclosion group.

Line and especially age are highly significant factors affecting expression in the stress treatment (Table 1). Post hoc analysis of the line effect shows that SR2 has a significantly higher expression than SR1 and Stock over all ages (Tukey: SR1: A, SR2: B, Stock: A). Analysis of only the early stress treatment reveals that both SR lines express *sod2* more strongly than the Stock, and that SR2 has a higher expression than SR1 (Tukey: SR1: A, SR2: B, Stock: C).

The expression levels show a remarkable change with age. Whether or not the data at eclosion are excluded, it is evident that *sod2* expression is much lower under prolonged stress compared to mild stress. A possible explanation is that *sod2* is involved in a general stress response and is up-regulated under the influence of upstream pathways, such as the Insulin(-like) signalling pathway (Fabrizio et al. 2001; Honda and Honda 1999; Kops et al. 2002), as soon as individuals experience stress. In the face of death from prolonged starvation, all energy resources are shifted towards survival, and away from reactive oxygen scavenging. Experiments that sample at a wider variety of ages, including during development, are needed to fully explain the observed *sod2* expression pattern.

There is a significant age effect in the optimal treatment (Table 1). At day 0, *sod2* expression is significantly higher than at 14 and 35 days, which do not significantly differ from each other (Tukey: day 0: A, day 14: B, day 35: B). Lines differ significantly in expression when all ages are taken together (Table 1). Both SR lines have a higher expression than Stock (Tukey: SR1: A, SR2: A, Stock: B). Excluding day 0 with exceptionally high expression renders the line effect non-significant ($F_{2, 18} = 3.14$, $p = 0.068$). However, a less conservative Student's t post hoc test shows that expression of both selection lines is significantly different from Stock (Student's t: SR1: A, SR2: A, Stock: B), indicating that the trend towards higher expression in selection line males is evident in all cases.

The patterns of expression over time demonstrate a clear difference between lines. Compared to Stock, SR selected butterflies have up-regulated *sod2*, independent of treatment or age. In some cases, SR2 butterflies seem to have an even higher *sod2*

expression compared to SR1. Remarkably, the starvation survival analysis suggests that SR2 butterflies live even longer than SR1. This could be explained by a different distribution of active *sod2* alleles over the replicate selection lines (Tyler et al. 1993). Thus, in line with our a priori expectations and with some previous findings (Arking et al. 2000a; Arking et al. 2000b; Dudas and Arking 1995; Mockett et al. 2001) we have found that SR line butterflies have an intrinsically higher *sod2* expression that may be fundamental to their ability to better resist starvation and to live longer under normal food conditions.

catalase

The analysis over all treatments shows a significant effect of treatment and of line on the expression of *Catalase* (Table 1). This gene is significantly up-regulated under stress compared to optimal conditions (figure 3C). Over all treatments, SR2 has a significantly higher expression compared to both SR1 and Stock (Tukey: SR1: A, SR2: B, Stock: A).

The analysis of the optimal treatment shows no effect of line. Age has a significant effect on gene-expression (Table 1). Thus, at day 14, expression is significantly higher than at days 0 and 35, which have a similar expression (Tukey: Eclosion: A, Early: B, day35: A).

Analysis within the stress treatment shows that both line and age significantly affect expression (Table 1). Expression under mild or prolonged stress is significantly higher compared to day 0, and also compared to optimal conditions (figure 3C). Thus, *catalase* expression seems to be positively correlated with stress. Moreover, there is a trend towards a linear relationship between duration of stress and the level of *catalase* expression. This pattern is comparable with the results for Indy, suggesting that *catalase* expression is also coupled to the level of energy generation through citric acid cycle metabolism. A post hoc analysis of the age effect shows that SR2 has a higher expression than SR1 and Stock (Tukey: SR1: A, SR2: B, Stock: B).

Our hypothesis on the role of the oxidative defense gene *catalase* in maintenance and the evolution of stress resistance in *B. anynana* is partly supported. We show up-regulation of expression under stress conditions, confirming a role for *Catalase* in stress resistance (Foley and Luckinbill 2001). Elevated *catalase* expression appears to be required for the increased starvation resistance as a result of selection, at least for the SR2 line. *Catalase* does not play a role in the variation of survival under optimal conditions.

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Table 1. Results of the gene-expression analysis with General Linear Models.

Gene	Model	N	Effect	df	Sum of Squares	p
<i>Indy</i>	Overall	65	Model	5	9.983	
			Line	2	0.625	
			Treatment	1	8.724	**
			Line x treatment	2	0.617	
	Optimal	37	Model	8	14.206	
			Line	2	2.672	
			Age	2	10.840	**
			Age x line	4	0.652	
	Stress	41	Model	8	7.038	
			Line	2	0.140	
			Age	2	4.984	**
			Age x line	4	1.467	
<i>sod2</i>	Overall	65	model	5	2.920	
			Line	2	1.794	
			treatment	1	<0.000	
			Line x treatment	2	1.573	
	Optimal	38	Model	8	21.626	
			Line	2	0.657	*
			Age	2	20.470	***
			Age x line	4	0.083	
	Stress	41	Model	8	30.250	
			Line	2	2.757	***
			Age	2	25.668	***
			Age x line	4	1.879	**
<i>catalase</i>	Overall	65	Model	5	12.336	
			Line	2	0.973	*
			Treatment	1	11.768	***
			Line x treatment	2	0.136	
	Optimal	38	Model	8	1.539	
			Line	2	0.185	
			Age	2	0.687	*
			Age x line	4	0.401	
	Stress	41	model	8	10.668	
			Line	2	1.147	**
			Age	2	8.945	***
			Age x line	4	0.310	

N: number of individuals, df: degrees of freedom, p: probability; *:p<0.05, **:p<0.001, ***:p<0.000

Genetic mechanisms of starvation resistance and longevity.

The goal of our study is to investigate whether variation in candidate gene expression is associated with starvation resistance and longevity. The key to our approach is the combination of artificial selection lines that probe the standing genetic variation for these traits in the context of knowledge about their roles in the life history and ecology of the species in natural, seasonal environments. Thus, the selection lines are expected to represent populations enriched in alleles that contribute to increased survival in relevant environments encountered in nature.

We have measured the expression of three candidate ageing genes in different lines, under various conditions and at various ages. On a functional level, the analysis of gene expression is more detailed than in most previous studies on this topic. The variation in expression levels is very different for each gene but we can identify some important patterns. The environment has a more profound effect on gene expression than variation in genotype (c.f. Li et al. 2006). All genes show differential expression with (increasing) starvation. However, line differences are found in some analyses, and these findings lead to important conclusions about genetic mechanisms of ageing. First, both the anti-oxidant genes *catalase* and *sod2* are associated with increased starvation resistance: in the populations selected for increased starvation resistance, they have a higher expression under starvation conditions. Secondly, *sod2* is constitutively up-regulated in selection lines, independent of environmental conditions. This indicates that variation in the expression of *sod2* is closely associated with the selection response. Most importantly, our results suggest that *sod2* contributes directly to the genetic correlation between starvation resistance and longevity observed in our selection experiments (chapter 5). The significance of this correlation is debated in genetic studies that use *D. melanogaster* (Baldal et al. 2006). However, in the light of our knowledge of the species' ecology (Brakefield and Frankino 2007; Brakefield and Reitsma 1991), such a correlation is expected to be substantial.

Our study indicates that some, but not all, genes identified through mutant screens may underpin standing genetic variation in stocks of butterflies established from natural populations. Thus, this approach narrows down the list of candidates to be tested in human populations. Moreover, the success of this approach is critically dependent on the inclusion of multiple ages and multiple environments, including the selected environment.

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Chapter
8

**The evolution of lifespan in
Bicyclus anynana, a synthesis**

The goal of this thesis is to investigate the evolutionary mechanisms of ageing from an ecological perspective. By using a model organism with a well known ecology, life history, and, in particular, alternative seasonal forms, we can try to understand variation in ageing and ageing related traits in a more fundamental manner than is possible in most other model organisms, or in humans. The main question in this chapter is: did we succeed in painting a clearer picture of the proximate and ultimate mechanisms that lead to variation in ageing? To answer this question, I first identify general patterns among the various chapters and then discuss their broader implications.

Development and ageing

Survival to adulthood is the first and foremost prerequisite for successful reproduction as an adult. Therefore, development is under stringent selection whereas many adult traits are less so, and increasingly less so with age, when the selection shadow becomes longer. Moreover, the antagonistic pleiotropy theory of ageing postulates that the same genetic mechanisms that assure survival through development have a negative influence on adult lifespan. A different class of genes, the longevity assurance genes, is very likely to have the same consequence for survival early in life. Two recent papers acknowledge the importance of pre-adult development in the search of genes that regulate adult lifespan (Curran & Ruvkun 2007; Chen et al. 2007). The range of pathways and cellular processes they find suggest that the mechanistic relationship between pre-adult development and adulthood is as complex and intricate as development itself. Do our results give more insight into possible mechanisms?

In chapter 2, we find that pre-adult temperature has an important influence on adult survival under starvation conditions. This influence is independent from, and opposite to the effect of adult ambient temperature. We find a high positive correlation of starvation resistance with resting metabolic rate (RMR), suggesting that a direct effect of ambient temperature is mediated by RMR. The mechanism by which pre-adult temperature influences survival is different. We speculate that this is caused by an imprint of a lower RMR on adult RMR as a consequence of the sub-optimally low pre-adult temperature. Such a developmental imprint can be considered a form of phenotypic plasticity (Brakefield et al. 2005).

Further evidence for a role of developmental processes in the variation in adult lifespan comes from the artificial selection experiments (chapters 4 and 5): in both

experiments, development time is increased in both sexes. What causes this increase is unknown, and thus it could be that different mechanisms are involved in the two selection experiments. However, it is likely that metabolic processes have been altered, but can we be more precise? Adult resting metabolic rate is lower in SR line males, but not in females or in LS butterflies. Thus, this could play a minor role in the delay of developmental growth, if it is assumed that resting metabolic rate is relatively constant throughout life. However, as also suggested by the results of chapter 2, adult RMR is likely to be a consequence of other events during development. Another potential mechanism is the increase in larval fat content. *A priori*, this is expected to be especially relevant in the SR lines, since their selection regime did not favour any accumulation of reserves as adults. However, there is no increase observed in relative fat content. In absolute amounts, fat did increase in SR females, as did the total body mass.

For a full understanding of the role of metabolic processes in determining adult lifespan, it is crucial to establish what these reserves are, and how and when they are built up. This encompasses studying growth and storage, rather complex processes in themselves. An important target of study is the fat body, the tissue that is the site of storage and intermediary metabolism, but recent research shows it is important in many adult traits in insects, including those of the reproductive system (e.g. Lazareva et al. 2007) and starvation resistance (Aguila et al. 2007).

The genetic relationships between key pre-adult traits and adult lifespan are investigated in chapter 3. The experiment used a wide range of artificially selected genotypes in a common garden set-up with near-optimal lab conditions. Most of the observed variation in adult lifespan was line-specific. Although mechanisms are proposed in each case, these do not hold when analyzing all genotypes. The only consistent result is that, on average, faster developing butterflies live longer as adults. This directly contradicts the results for the artificial selection for increased longevity and starvation resistance. In the selection for starvation resistance, different mechanisms may be targeted, as the selection environment was very different. Responses to artificial selection are highly conditional. For the longevity selection, there is no obvious explanation, although it is relevant to mention that a genetic correlation between populations (in the case of selection for survival) is not the same as a genetic correlation within populations (the data for selection on pre-adult traits were corrected for line differences). Unfortunately, there are no data available on individual developmental speed and survival time in the longevity and starvation resistance selected lines. Clearly, the complexity of genetic relationships between (life history) traits warrants care when interpreting the results of artificial selection (Fischer et al 2007). Whatever the details, these experiments provide strong evidence for ample standing genetic variation for adult lifespan and adult starvation resistance.

Phenotypic plasticity of lifespan

The past environments have shaped the life history of a species or a group of species. This sets the limit to maximal lifespan. Within this limit, there is ample room for variation. This variation is largely dependent on the present environment, and the life history decisions the organism makes to be successful within the environment. A parallel with development is appropriate (Partridge and Gems 2006); in fact, ageing can be seen as a continuum of development into adult life (Zwaan 2003). Although this mechanistic framework is powerful and can yield new insights, for *B. anynana* there is an alternative, more expanded framework to explain variation in lifespan that also includes development: phenotypic plasticity. In *Bicyclus*, this plasticity comes in two forms: the irreversible developmental plasticity that determines the seasonal polyphenism as an adaptation to the wet and dry seasons, and the facultative plastic responses in the adult that govern stress resistance and reproductive patterns.

We investigated the role of the two forms of temperature plasticity in starvation resistance in chapter 2. The results suggest that developmental plasticity and adult plasticity operate via different mechanisms to influence adult starvation resistance. However, we find that starvation resistance is highest in the conditions that most resemble the dry season. Further evidence that starvation resistance is well embedded in the seasonal polyphenism comes from the finding that the butterflies that were selected to express a dry season wing phenotype are more starvation resistant under dry season-like conditions.

Artificial selection on adult longevity was performed in a laboratory environment with warm, favourable conditions comparable to the wet season environment in the field. There were several correlated responses consistent with a shift in this wet season environment towards a dry season-like phenotype, involving other life history and reproductive traits: pre-adult developmental time was extended and females at early reproduction laid larger but fewer eggs. The complexity of the response to selection is probably because although we targeted longevity directly, it was not independently of reproduction; we screened for males that were both long-lived and mated in later life as in the life history of dry season form adults in the field (although much of these males' extended lifespan occurs in a cooler environment). In other words, our selection for longevity has apparently, at least in part, targeted the same pathways regulating the developmental plasticity that have evolved under a history of natural selection involving responses to the seasonal cycle in favourable and stressful environments.

The results from the longevity selection are echoed by those from the selection for increased starvation resistance in the absence of adult food in chapter 5. Most striking was the accompanying changes in adult lifespan under conditions of normal feeding, with longevity up to 50% higher than in unselected controls. Females appear to have responded to selection principally through higher energy reserves and a thrifty phenotype in terms of allocation of resources to a durable body. In males there appears to be a more prominent role for a changed metabolism

resulting in more conservation of resources. This is also reflected in the mating success of these males. In competition with unselected stock males, this is reduced under optimal food conditions but more prolonged under starvation conditions (Chapter 6). It remains to be tested whether this phenomenon is caused by a reduced activity (a metabolic origin) or reduced sensitivity or motivation (a sensory origin). The finding that artificial selection for starvation resistance yields a phenotype that is both more starvation resistant and longer-lived suggests a substantial genetic correlation between these traits. Given this correlation, the longevity selection line butterflies are expected to show increased starvation resistance. This hypothesis remains to be tested. Thus, the genetic variation for lifespan and stress resistance in these populations appears to be involved in coordinating the whole life history of the species including the adaptation to alternative seasonal environments. This strongly supports interactions between phenotypic plasticity and the regulation of stress resistance and lifespan.

Molecular mechanisms of ageing

The selection lines represent novel genotypes that can provide excellent material to study the genetic and physiological bases of variation in lifespan and stress resistance, as well as the role of quiescence and phenotypic plasticity in the evolution of these traits. In chapter 7, we have begun to use qPCR for the first time to explore the genetic basis of the response to artificial selection for starvation resistance. We expected selection line butterflies to have increased the expression of genes that promote survival through enhanced resistance against cellular oxidative stress. The results indicate that the candidate genes, *SOD2* and *Catalase*, indeed are involved in the response to selection for increased starvation resistance. Furthermore, the results suggest that *SOD2* is involved in the genetic correlation between starvation resistance and longevity.

These genes are likely to be in pathways that are under control of the upstream pathways that are also underpinning plasticity. The main candidate mechanisms are the pleiotropic, evolutionary conserved hormone pathways that link tissue level internal processes and external perception. This involves the nervous system (sensory tissues, brain), the hormone system, and the receptors on cell membranes with transcriptional modulation functions, such as nuclear hormone receptors. It will prove fascinating to determine the extent to which this phenomenon involves the same underlying gene networks and pathways as those involved in the irreversible developmental plasticity induced by the alternative larval environments in this butterfly.

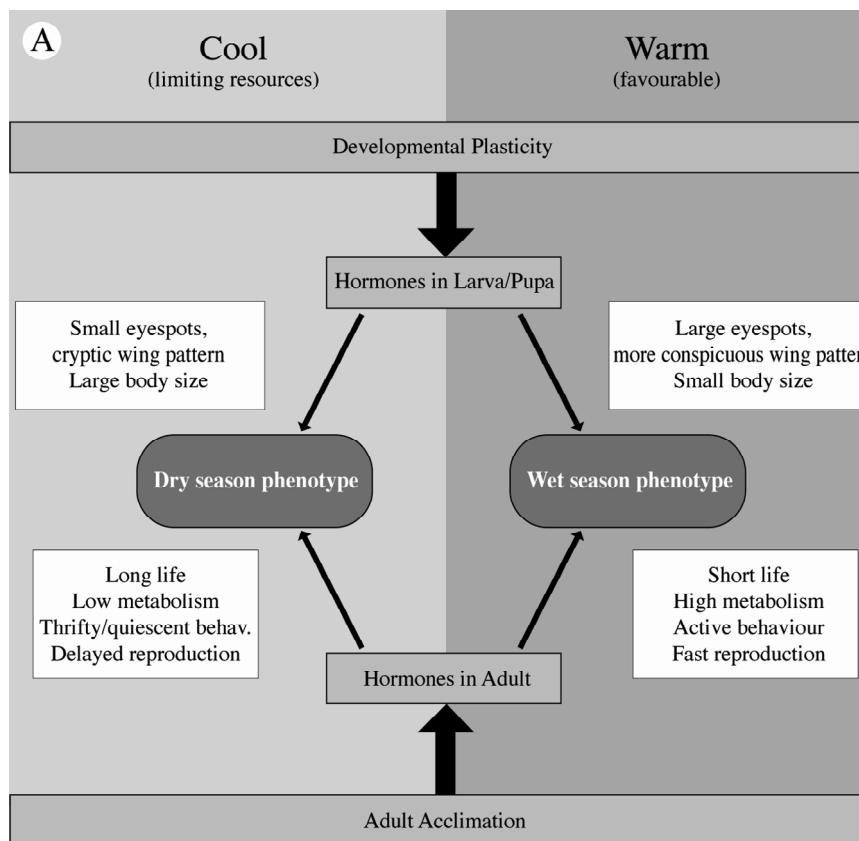
A butterfly model of ageing

The outcome of the artificial selection for enhanced male longevity and for starvation resistance is similar. However, more intriguing is that in both cases our

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findings suggest that we selected for a quiescent phenotype that resembles the long-lived dry season form as induced by cool larval temperatures whether in the laboratory or the field. This also includes behavioural changes, in the form of a reduced activity or motivation. Reproductive dormancy, a mild form of diapause, has been suggested to contribute to the survival of dry season form butterflies in the wild. In the dry season, comparatively few butterflies are caught in fruit traps, and they are rarely seen flying. Manipulating temperature during pre-adult development is a very direct way of inducing the seasonal phenotypes. This has facilitated evolutionary and genetic analyses of morphological, behavioural and life history traits (Brakefield and Frankino 2007). Lifespan in the lab is probably not as long as in comparable conditions of average temperature in the field (Molleman et al. 2007). Additional triggers, such as larval food stress related to food quality, may also be involved in the induction of reproductive dormancy.

Figure 1 A framework of adaptive responses in *Bicyclus* butterflies. See text on next page for explanation



Adult plasticity centres around making decisions that have consequences for reproduction. The relationship between reproduction and lifespan has received much attention in evolutionary physiology and in ageing research, because it can provide a mechanistic explanation for the notion that longevity can only be accomplished through investment in maintenance processes (Partridge, Gems, Withers 2005). The results in this thesis do suggest that this relationship is important. However, the details are best understood in the light of the ecology of the species. The most striking result is that there are apparently sex specific mechanisms. This, then, should be presumably be accounted for by different selection pressures on the sexes. To address this issue, functional studies on sexual selection that integrate physiological experiments on pheromones with behavioural assays have been initiated

We conclude that systems of resource allocation and energy conservation are components of those regulatory mechanisms shared by starvation resistance and longevity in adult *Bicyclus* butterflies. The responses of each of these traits to artificial selection involve changes in many of the same suite of traits that contribute to the developmental plasticity of the seasonal polyphenism (also see Zijlstra et al 2004). The ability of *B. anynana* to persist through periods of both plentiful and limited resources involves a combination of irreversible developmental plasticity and of facultative plastic responses in periods of acclimation in the adult. The figure illustrates a framework for thinking about how both the processes of plasticity cued by different environments in pre-adult development and those involved in acclimation to changes in adult environments are crucial components of the adaptive responses of *Bicyclus* butterflies. Both sets of processes contribute to phenotypic differences found between the generations of adults flying in alternating wet-dry seasons. There is an overlap in the sets of adult traits involved in these responses that probably reflects some shared components in the mechanisms of their regulation. In addition, whilst the major players in the hormonal regulation may differ across stages in the life cycle, for example from ecdysone around the pupal moult to juvenile hormone and insulin signaling in the adult stage, downstream pathways probably have more in common. Development of tools of functional and ecological genomics for *B. anynana* provides a rich promise for the further exploration of such issues (Beldade et al 2006, 2007).

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Summary

Chapter 2 investigates whether there is phenotypic plasticity in adult starvation resistance in *B. anynana* in response to wet and dry season temperatures during development and in adulthood, and whether this related to resting metabolic rate (RMR). Two lines of evidence, with antagonistic effects, support the existence of plasticity in starvation resistance. First, pre-adult temperature contributes to variation in SR, independent of adult ambient temperature. Developing at a wet seasonal temperature results in a higher SR. Secondly, when controlling for pre-adult and adult temperature, the genetics underlying phenotypic plasticity contribute to variation in SR. A population that has been artificially selected to resemble a dry season wing phenotype under all seasonal conditions has a higher SR, when developed at dry seasonal temperatures. A high correlation of SR with RMR, as measured under normal food conditions, was found over all temperature conditions. Thus, the mechanisms that regulate the metabolic state of individuals in response to temperature are largely overlapping with those needed for SR. This suggests that low RMR is an important manner to achieve a high adult SR. We hypothesise that a low RMR during development (like is occurring in the dry season) has a negative effect on adult performance, which may then explain the opposing effect that pre-adult temperature has on adult SR.

Chapter 3 describes an experiment that was designed to distinguish between phenotypic and genetic correlations of key pre-adult traits with adult lifespan, and to maximise discovery of genetic correlations. Butterflies of artificial selection lines for the three pre-adult traits egg size, development time, and pupal body size were followed individually from egg to death in a common garden design experiment. We did not find general developmental mechanisms that underlie differences in lifespan. We found low phenotypic correlations between pre-adults traits and adult lifespan. In addition, there was no genetic correlation between egg size or body size with adult lifespan. However, there was a strong negative genetic correlation between development time and adult lifespan. Although some selection lines showed a significant change in adult lifespan, the genetic correlation found did not depend on the effect of one particular line. Thus, we find a genetic coupling of faster development with a longer adult lifespan in *B. anynana*. Such a relationship is predicted by the stress hypothesis of ageing, which states that longevity strongly depends on genes or alleles that confer stress resistance. Such a relationship is most likely to be found under stress conditions. We show that it also exists under conditions close to optimal.

Summary

Chapter 4 is on the first of two sets of artificial selection lines in this thesis: selection for increased adult male longevity. We selected on the ability of males to live long and still be able to mate at an advanced age. This ability is the target of selection in the dry season. Despite the low number of 5 generations of selection, the selection was successful with an increase of about 25% in median virgin lifespan in males. In addition, females of one line showed a similar increase in lifespan. Correlated responses in female reproduction suggest a reduced early fecundity in favour of a larger egg size. This corresponds to a dry season mode of reproduction. The fact that we find a change in female reproduction in response to selection on male longevity indicates that the genetic trade-off between reproduction and survival is fundamental to the regulation of lifespan. Our results suggest that we have selected for a dry season phenotype. Continued selection is needed to gain a stable further increase in lifespan before genetic analyses can be performed to investigate what genes or alleles underpin the response.

Chapter 5 describes the selection for adult starvation resistance in males and females, its response and correlated responses. Adult starvation resistance is closely associated with fitness in *B. anynana*, and it often covaries with longevity in other species. After 17 generations of selection, median and maximum lifespan under starvation doubled compared to the unselected stock the line were derived from. In addition, artificially selected butterflies are 30-50% longer lived under ad lib conditions. Results on physiological traits indicate a sex-specific response to selection. Males have lower metabolic rates, possibly to conserve resources. In contrast, females have an elevated body mass, suggesting a re-allocation of resources towards a more durable body. In addition, females, reproduction was altered towards fewer but larger eggs. Our results suggest that we selected for a phenotype that resembles the long-lived dry season form that is induced by dry season environmental temperatures (in the lab and) in the field. We conclude that the genetic variation for lifespan in this population is involved in coordinating the whole life history of the species including the adaptation to alternative seasonal environments, and supporting interactions between phenotypic plasticity and the regulation of lifespan. The newly created genotypes provide an excellent basis to further study the genetic and physiologic basis of variation in lifespan, and the role of quiescence and phenotypic plasticity in the evolution of ageing. Chapter 6 and 7 describe the first of such studies.

Chapter 6 continues on other aspects of correlated responses to selection for adult starvation resistance. We describe experiments that measure the mating performance of males in a large flight cage. This environment allows the full expression of behaviour needed to court females, similar to natural environments. We placed SR selection males in competition with unselected control males for a limited number of females under various food conditions, ranging from ad lib to 3 days of starvation. We show that unselected males outperform selection males under 'optimal' ad lib conditions, indicating a cost to selection for increased

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lifespan. However, when in competition under mild starvation conditions, comparable to the selection environment, SR males perform as well or even better. Under such conditions, most unselected males fail to mate with a female, while selection males mate as many females as under optimal food conditions. Thus, males perform best in the environment in which they are selected. We conclude that selection for increased survival has affected the motivation to mate. We hypothesize this to be an important aspect of the state of quiescence that enables the survival of butterflies that occur in the dry season in Africa.

Chapter 7 investigates aspects of the molecular genetic mechanisms that underlie differences in survival in the butterflies selected for starvation resistance. We expected selection line butterflies to have increased the expression of genes that promote survival through enhanced maintenance. We investigated the expression of three genes involved in maintenance processes in males at various ages under 'normal' ad lib food conditions and under starvation. Expression of *Indy*, which codes for a transport molecule crucial for intermediary metabolism, is higher under prolonged starvation, but is not different between the selection lines, suggesting that prolonged stress induces active catabolism of reserves. In addition, *Indy* expression is lower at late age under normal food conditions, perhaps indicating a decreased metabolic efficiency with ageing. We also measured expression of two genes involved in anti-oxidant defence. The protein coded by *SOD2* is crucial in reducing the damaging super oxide load in mitochondria. Its expression is up-regulated upon starvation, and it is significantly higher expressed in the selection lines compared to unselected butterflies under both starvation and normal food conditions. *Catalase* through its enzyme reduces the toxic hydrogen peroxide, the product of the reaction catalysed by *SOD2*, to oxygen and water. Under starvation, expression is higher, and at least one of two selection lines has a higher mean expression compared to unselected butterflies. Under normal food conditions, there are no line differences, but expression is lower at later age. Together, this suggest that *SOD2* and *Catalase* are involved in the response to selection for increased SR. In addition, *SOD2* appears to be underpinning in the genetic correlation between starvation resistance and longevity.

Nederlandse Samenvatting

De wetenschap die veroudering bestudeert heeft de afgelopen jaren vele nieuwe inzichten opgeleverd over individuele verouderingsmechanismen. Een volledig en diepgaand inzicht in het verouderingsproces hebben wij echter nog niet. Intussen zijn in moderne industrielanden door de gestage stijging van de levensverwachting in de afgelopen eeuw de relatieve en absolute hoeveelheden oude mensen wezenlijk gestegen. De directe en indirecte sociale en economische gevolgen van deze demografische verschuiving zijn ingrijpend. Bovendien is het in het belang van iedereen, jong en oud, om de lasten van de oude dag te verminderen. Een beter inzicht krijgen in het verouderen en de mogelijke behandeling van de ziekten in ouderen zijn de doelstellingen van een onderzoekproject dat in 2002 begon: de 'Lang Leven' studie. Dit project, dat door de Innovatieve Onderzoek Programma's (IOP) werd gefinancierd door het Nederlandse Ministerie van Economische Zaken, is één van eerste in zijn soort. Het bracht gerontologen en evolutiebiologen samen en combineerde de studie in dieren met analyse van menselijke cohorten.

Door hun gezamenlijk evolutionaire oorsprong zijn er veel gelijkenissen tussen dieren, met inbegrip van de mens. Dit geldt voor de ontwikkeling, morfologie, fysiologie en genetica. Sinds er informatie beschikbaar is over het gehele genoom van, bijvoorbeeld, de fruitvlieg *Drosophila melanogaster* en dat van de mens is het bekend dat de meerderheid van menselijke ziektegenen in relatief onveranderde vorm aanwezig zijn in (ver) verwante diersoorten. Recent is ook duidelijk geworden dat de centrale genetische signaalroutes die het verouderingsproces reguleren gelijk zijn in gist, wormen, vliegen en muizen. Deze signaalroutes vormen de basis van de centrale hormoonsystemen van het lichaam die bijdragen aan de regulatie van groei en reproductie; kortom die kenmerken en eigenschappen van organismen die de biologische fitness bepalen. Het is zeer waarschijnlijk dat deze signaalroutes ook belangrijk zijn voor menselijke veroudering. Een ander proces belangrijk voor veroudering in dieren is de balans tussen reproduceren en lang leven. In meer fysiologische zin wordt wel gesproken van een uitruil of 'trade-off' van energie(voorraden) tussen deze processen. Ook deze uitruil is aangetoond in mensen. Kortom, deze vertaling van theorieën en van experimentele bevindingen over veroudering van zogenaamde modelorganismen (zoals insecten) naar mensen en omgekeerd is een geldige en zeer nuttige wetenschappelijke aanpak.

In het 'Lang Leven' onderzoek ligt de nadruk op het vinden van genen die zijn geëvolueerd ter bevordering van lichamelijk onderhoud en cellulaire reparatiemechanismen. Deze genen zijn waarschijnlijk belangrijk om gezond een hoge leeftijd te bereiken en voor het minimaliseren van potentiële

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ziekteveroorzakende genetische problemen. De modelorganismen zijn twee insectsoorten: de fruitvlieg *Drosophila melanogaster* en de tropische Afrikaanse vlinder *Bicyclus anynana*. In deze soorten werd een evolutionair functionele aanpak gebruikt om nieuwe signaalroutes te identificeren en te onderzoeken hoe de bijbehorende genen in verschillende omgevingen tot expressie komen. De menselijke studies bestonden uit uitgebreide onderzoeken van zeer oude mensen in de huidige Nederlandse bevolking en ook van ouderen in Leiden.

De biologie van *B. anynana* wordt gekenmerkt door een seizoensgebonden polyfenisme in de volwassen vlinders. Dit betekent dat vlinders die in alternatieve seizoenen leven verschillende fenotypes hebben. Zoals in de meeste tropische gebieden zijn er in Malawi, waar onze populaties vandaan komen, twee seizoenen: een nat seizoen en een droog seizoen. Volwassen *B. anynana* komen dus voor in twee vormen. Welke dat is wordt bepaald door de omgevingsfactoren van het natte of het droge seizoen tijdens ontwikkeling (rups stadium). In het bijzonder de temperatuur heeft een grote invloed. Het natte seizoen heeft gemiddeld hoge temperaturen (boven 23°C) en ook een hoge vochtigheid en regenval die een weelderige groei toelaat van de grassen waarvan de rupsen eten. Dit is het reproductieve seizoen waarin de vlinders actief zijn en rondvliegen op zoek naar partners en voedsel. De vlinders reproducen snel en sterven jong. Het droge seizoen heeft lagere temperaturen (gemiddeld onder 21°C) en door de afwezigheid van regen sterven veel planten af, in het bijzonder de grassen, waardoor reproductie niet succesvol is en niet plaatsvindt. De vlinders in dit seizoen wachten tot het volgende regenseizoen begint, iets wat langer dan 6 maanden kan duren. Daarnaast vermindert met de tijd in het droge seizoen de beschikbaarheid van vruchten, het voedsel van de volwassen vlinders. Dit seizoen vereist dus goede overlevingseigenschappen, waarbij vooral hongerresistentie belangrijk is. Het is al lang bekend dat het seizoengebonden polyfenisme door fenotypische plasticiteit wordt veroorzaakt. Hierdoor kan de rups variatie in omgevingsfactoren gebruiken als voorspeller voor het toekomstige seizoen. De fenotypische plasticiteit kan veel van de variatie in volwassen eigenschappen verklaren, van de morfologie tot fysiologie en gedrag. De mechanismen die ten grondslag liggen aan de fenotypische plasticiteit zijn deels bekend op het niveau van de hormonale fysiologie en de genexpressie. Het algemene doel van het werk beschreven in dit proefschrift is het verklaren van de variatie in levensduur, en het onderzoeken van de rol van fenotypische plasticiteit daarbij. De unieke levensgeschiedenis van *B. anynana* is hiervoor bij uitstek geschikt.

In Hoofdstuk 2 onderzoek ik of er fenotypische plasticiteit is voor hongerresistentie in volwassen *B. anynana* in reactie op natte - en - droog seizoenstemperaturen tijdens ontwikkeling en in volwassenheid. Daarnaast onderzoek ik of dit te maken heeft met de snelheid van het rustmetabolisme. Twee resultaten, met tegenstrijdige effecten, ondersteunen het bestaan van plasticiteit in hongerresistentie. Ten eerste draagt de ontwikkelingstemperatuur bij tot variatie in hongerresistentie, onafhankelijk van volwassen omgevingstemperatuur. Ontwikkeling bij een natte seizoenstemperatuur resulteert in een hogere

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hongerresistentie. Ten tweede draagt de genetica die ten grondslag ligt aan fenotypische plasticiteit bij aan variatie in hongeresistentie. Een populatie die kunstmatig is geselecteerd om onder alle seizoensomstandigheden een vleugelpatroon te hebben dat lijkt op dat van het droge seizoen fenotype, heeft een hogere hongeresistentie wanneer het ontwikkelt bij droge seizoenstemperaturen. Een hoge correlatie van hongeresistentie met snelheid van rustmetabolisme, zoals die bij normale voedselomstandigheden wordt gemeten, werd gevonden over alle temperaturen. Ik concludeer dat de mechanismen die het metabolisme reguleren in reactie op temperatuur grotendeels overlappen met de mechanismen die nodig zijn voor hongeresistentie. Dit maakt het aannemelijk dat het laag houden van de snelheid van het rustmetabolisme een belangrijke manier is om een hoge adulte hongeresistentie te bereiken. Om deze resultaten te verklaren stel ik de volgende hypothese voor: een lage snelheid van rustmetabolisme tijdens ontwikkeling (zoals voorkomt in het droge seizoen) heeft een negatief effect op volwassen prestaties, wat dan het tegengestelde effect van de ontwikkelingstemperatuur op volwassen hongeresistentie verklaart.

Hoofdstuk 3 beschrijft een experiment dat onderscheid maakt tussen fenotypische en genetische correlaties van belangrijke ontwikkelingskenmerken met levensduur, en om de ontdekking van (nieuwe) genetische correlaties te maximaliseren. De vlinders van kunstmatige selectie lijnen voor de drie ontwikkelingseigenschappen eigrootte, ontwikkelingstijd, en popgrootte zijn individueel gevolgd, van het ei tot de dood in een experiment waarbij alle individuen in een gelijke omgeving leven. Ik vond geen algemene relatie tussen variatie in ontwikkeling en levensduur. Ik vond lage fenotypische correlaties tussen alle onderzochte eigenschappen en adulte levensduur. Bovendien was er geen genetische correlatie tussen eigrootte of popgrootte met levensduur. Echter, er was een sterke negatieve genetische correlatie tussen ontwikkelingstijd en adulte levensduur. Hoewel sommige selectielijnen een significante verandering in volwassen levensduur lieten zien, hing de gevonden genetische correlatie niet af van het effect van één lijn in het bijzonder. Ik concludeer dat er een genetische koppeling is tussen snellere ontwikkeling en langere adulte levensduur in *B. anynana*. Een dergelijk relatie wordt voorspeld door de zogenaamde 'stress hypothese' van veroudering, die stelt dat de levensduur sterk afhangt van genen die zorgen voor stressresistentie. Deze verhouding wordt meestal gevonden in stressvolle omstandigheden. Echter ik toon aan dat deze relatie ook onder optimale omstandigheden kan bestaan.

Hoofdstuk 4 gaat over het eerste van twee kunstmatige selectie experimenten in dit proefschrift: selectie voor verhoogde adulte mannelijke levensduur in *B. anynana*. Door kunstmatige selectie evolueert een populatie in een bepaalde opgelegde richting. Deze populatie verandert genetisch omdat vooral dat deel van de variatie van genen (allelen) zal overblijven dat nodig is om de opgelegde richting van selectie in te gaan. Ik selecteerde op de capaciteit van mannen om lang te leven en dan op gevorderde leeftijd te kunnen paren. Deze capaciteit hangt waarschijnlijk sterk samen met natuurlijke selectie in het droge seizoen. Ondanks het lage aantal

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van 5 generaties van selectie was de selectie succesvol met een verhoging van ongeveer 25% van de gemiddelde levensduur van maagdelijke mannen. Bovendien vertoonden de vrouwen van één lijn een vergelijkbare verhoging van de levensduur. Daarnaast was voor de vrouwelijke reproductie te zien dat een verminderde eiproduktie vroeg in het leven samen gaat met een verhoogde eigrooite. Dit is een soortgelijk patroon als waargenomen in het droge seizoen. Het feit dat er een verandering in vrouwelijke reproductie is als respons op selectie voor een toename in mannelijke levensduur wijst erop dat de genetische uitruil tussen reproductie en overleving voor de veroudering van levensduur fundamenteel is. Mijn resultaten maken het aannemelijk dat ik voor een 'droog seizoensfenotype' heb geselecteerd. Verdere selectie is nodig om een grotere, stabiele verhoging van levensduur te bereiken alvorens de genetische analyses kunnen worden uitgevoerd om te onderzoeken welke genen en allelen de respons op selectie mogelijk hebben gemaakt.

Hoofdstuk 5 beschrijft de selectie voor hongerresistentie in volwassen mannetjes en vrouwtjes, en de respons en gecorreleerde respons hierop. In *B. anynana* wordt adulte hongerresistentie sterk geassocieerd met fitness in het droge seizoen en het is vaak gerelateerd aan levensduur in andere soorten. Na 17 generaties van selectie waren de gemiddelde en maximale levensduur onder condities van verhongering verdubbeld in vergelijking met de ongeselecteerde populatie waar de selectielijn uit voortgekomen was. Bovendien leefden de kunstmatig geselecteerde vlinders 30-50% langer onder optimale voedselomstandigheden. De resultaten van metingen aan fysiologische kenmerken wijzen op een verschil in de geslachten in respons op selectie. De mannetjes hebben lagere metabolische snelheden, wellicht om langer met de beschikbare energievoorraden te kunnen overleven. Vrouwtjes daarentegen hebben een hogere lichaamsmassa, wat kan wijzen op een herverdeling van voorraden naar een duurzamer lichaam. Daarnaast was de vrouwelijke reproductie veranderd naar minder maar grotere eitjes. De resultaten wijzen erop dat ik voor een fenotype selecteerde dat lijkt op de langlevende droge seizoensvorm die (in het laboratorium en) in het veld door een droge seizoensomgeving wordt geïnduceerd. Ik concludeer dat de genetische variatie voor levensduur in deze populatie betrokken is bij het coördineren van de gehele levensgeschiedenis met inbegrip van de aanpassing aan alternatieve seizoensgebonden milieus. Het onderbouwt de interactie tussen fenotypische plasticiteit en de regulatie van levensduur. De nieuw gecreëerde genotypen vormen een uitstekende basis voor verdere studie van de genetische en fysiologische basis van variatie in levensduur, en de rol van diapauze en fenotypische plasticiteit in de evolutie van het verouderen. In hoofdstukken 6 en 7 zijn de eerste van dergelijke studies beschreven.

Hoofdstuk 6 gaat verder in op de aspecten van gecorreleerde responsen op selectie voor hongerresistentie. Ik beschrijf experimenten die de paringsprestaties van mannetjes meten in een grote kooi. Deze kooi is vergelijkbaar met een natuurlijke omgeving als het gaat om het balts- en paringsgedrag. Ik liet mannetjes geselecteerd voor hongerresistentie concurreren met ongeselecteerde (controle)

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mannetjes voor een beperkt aantal vrouwtjes onder diverse voedselomstandigheden, van optimaal tot 3 dagen hongering. Ik toon aan dat onder optimale omstandigheden ongeselecteerde mannetjes in staat zijn meer paringen te krijgen dan de selectiemannetjes. Dit is een aanwijzing dat er kosten zijn verbonden aan een langere levensduur. Echter, onder milde honger, vergelijkbaar met de situatie tijdens selectie, presteren de selectiemannetjes even goed of zelfs beter. In dergelijke omstandigheden slagen veel ongeselecteerde mannetjes er niet in om te paren, terwijl de selectiemannetjes met meer vrouwtjes paren dan onder optimale voedselomstandigheden. Kortom, de mannetjes presteren het best in het milieu waarin zij zijn geselecteerd. Ik concludeer dat de selectie voor verhoogde overleving de motivatie om te paren heeft beïnvloed. Ik stel dat dit een belangrijk aspect is van de overlevingsstrategie van de vlinders in het droge seizoen in Afrika.

Hoofdstuk 7 beschrijft het onderzoek naar aspecten van de moleculaire genetische mechanismen die ten grondslag liggen aan verschillen in overleving in de vlinders die voor hongerresistentie zijn geselecteerd. Ik verwachtte dat de vlinders van de selectielijn een verhoogde expressie ('activiteit') zouden hebben van genen die overleving bevorderen, zoals via onderhoudmechanismen van het lichaam. Ik onderzocht in mannetjes de expressie van drie genen betrokken bij onderhoudsprocessen op diverse leeftijden onder normale (optimale) voedselomstandigheden en onder hongersomstandigheden. Expressie van het gen *Indy*, dat codeert voor een molecuul dat essentieel is voor het vervoer van metabolieten in de cel, was hoger onder langdurige hongering, maar de expressie is niet verschillend tussen de selectielijnen. Misschien wijst dit op het actief cataboliseren van reserves onder langdurige voedsel stress. Daarnaast is *Indy*-expressie lager op latere leeftijd onder normale voedsel omstandigheden, wat kan wijzen op een verminderde metabolische efficiëntie en/of behoefte door veroudering. Ik mat ook expressie van twee genen betrokken bij de verdediging tegen oxidanten. Het eiwit dat wordt gecodeerd door *SOD2* is essentieel voor het verminderen van de schadelijke superoxide concentratie in mitochondriën. *SOD2* expressie is hoger onder hongering, en het is beduidend hoger in de selectielijnen in vergelijking met ongeselecteerde vlinders onder zowel verhongering als de normale voedselomstandigheden. Het door *Catalase* gecodeerde enzym zet het giftige waterstofperoxide, het product van de reactie die door *SOD2* wordt gekatalyseerd, om tot onschadelijk zuurstof en water. Onder hongering is de expressie van *Catalase* hoger, en minstens één van twee selectielijnen heeft een hogere gemiddelde expressie in vergelijking met ongeselecteerde vlinders. Onder normale voedselomstandigheden zijn er geen verschillen tussen lijnen, maar de expressie is lager op hogere leeftijd. Ik concludeer dat *SOD2* en *Catalase* betrokken zijn bij de reactie op selectie voor verhoogde hongerresistentie. Bovendien vond ik dat *SOD2* ten grondslag ligt aan de genetische correlatie tussen hongerresistentie en levensduur.

De algemene conclusie van het onderzoek is dat de regulatiemechanismen van hongerresistentie en levensduur in adulte *Bicyclus* vlinders gedeeltelijk overlappen, en dat allocatie en behoud van energie(reserves) hier belangrijke componenten van

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zijn. Eigenschappen zoals reproductie, fysiologie en gedrag zijn door de respons op kunstmatige selectie voor hongerresistentie en levensduur zodanig veranderd dat ze vergelijkbaar zijn met die eigenschappen zoals die voorkomen in de droge seizoensvorm. De kunstmatige selectie lijkt dus te hebben aangegrepen op de ontwikkelingsplasticiteit van het seizoengebonden polyfenisme. De capaciteit van *B. anynana* om periodes van overvloed en schaarste te overleven wordt mogelijk gemaakt door een combinatie van onomkeerbare ontwikkelingsplasticiteit en facultatieve plastische reacties in de volwassen vlinder. De overlap van deze twee processen maakt het waarschijnlijk dat de onderliggende regulatiemechanismen voor een deel hetzelfde zijn.

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I have spent a significant part of my adult life at the Evolutionary Biology group, first for my undergraduate studies and later for my PhD research. It has always felt like home.

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Curriculum vitae

Jeroen Pijpe was born on Friday April 8th 1977 in the former Hofpoort hospital in Woerden, the Netherlands. He lived in Bodegraven, Saint-Nom-la-Bretèche (France), Sint-Genesius-Rode (Belgium), and Bilthoven where he became a 'worker' at Kees Boeke's high school 'Werkplaats Kindergemeenschap' in 1989. He doubled the pre-final year, exchanged history for biology and received his Athenaeum diploma in 1996. In the same year he moved to Wageningen to start Biology at the local (Agricultural) University. Inspired by the lectures of prof. dr. Jan Osse, he specialised in animal biology. The taxonomy course in the Pyrenees invoked his fascination for insects. In 2000, he started his first research project on left-right asymmetry in early developing pig embryos, under the supervision of Serge van de Pavert, dr. Henri Strobant and prof. dr. Johan van Leeuwen. In search for a more evolutionary topic, he stumbled upon the work on butterflies in Leiden. He started a 7 month project on protandry in *Bicyclus anynana* supervised by Wilte Zijlstra, dr. Bas Zwaan and prof. dr. Paul Brakefield. For a third project he went to Riverside and the breathtaking Mojave desert in California, USA together with fellow students Maja Roodbergen, Bas van de Meulengraaf and Martijn Schenk, where he studied the evolutionary ecology of *Trichogramma* parasitic wasps and their *Wolbachia* sex-ratio distorters under the supervision of Ties Huigens, prof. dr. Bob Luck and prof. dr. Richard Stouthamer.

Back in the Netherlands, he received his M.Sc. diploma in 2001 and was offered a PhD position on the IOP Genomics funded project 'The genetic determination of longevity and disease at old age' (IGE014), which he happily accepted and started in 2002. The same year, he went to Princeton, New Jersey, USA for 3 months trying to obtain DNA sequences for important candidate genes. He presented his work at various national and international meetings, including various IOP Genomics meetings, the IBL symposia (2004 and 2005), the Functional Ecology - Verweij dagen (2002, 2003), the Benelux Congress of Zoology (2003), the NEV Entomologendag (2003) and the ESEB meetings in Leeds (2001), Krakow (2005), and Uppsala (2007) where he also co-organised a symposium. He also attended inspiring workshops of the ENS in Saint Pierre-les-Nemours (Paris; 2003), FE/Ludens in Groningen (2004) and EMBO in Oeiras (Lisbon; 2006).

Currently, Jeroen is working as a post doc researcher on human evolution and population genetics at the Human Genetics department of the Leiden University Medical Center, in the group of prof. dr. Peter de Knijff.

List of publications

- Pijpe J**, Brakefield PM & Zwaan BJ Increased lifespan in a polyphenic butterfly artificially selected for starvation resistance. *American Naturalist* *In press* (chapter 5 of this thesis)
- Pijpe J.**, Brakefield P.M. & Zwaan B.J. Phenotypic plasticity of starvation resistance in *Bicyclus anynana*. *Evolutionary Ecology* 2007, 21 (5): 589-600 (chapter 2 of this thesis)
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