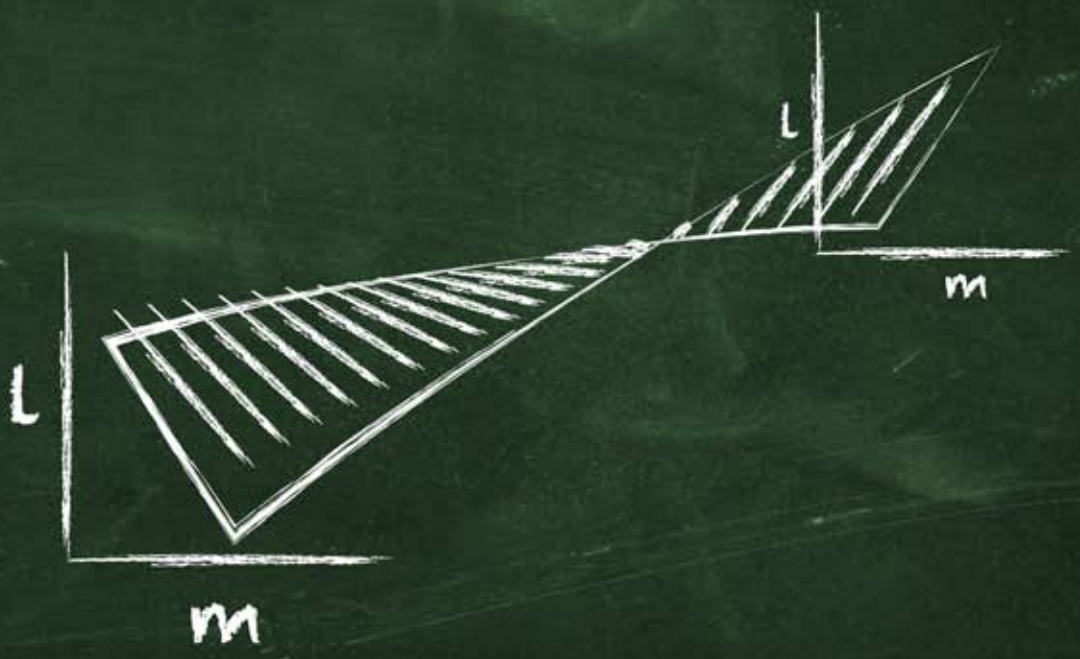
The evolution and plasticity of life histories upon variation in nutrition

an ageing focused integrative approach



Joost van den Heuvel

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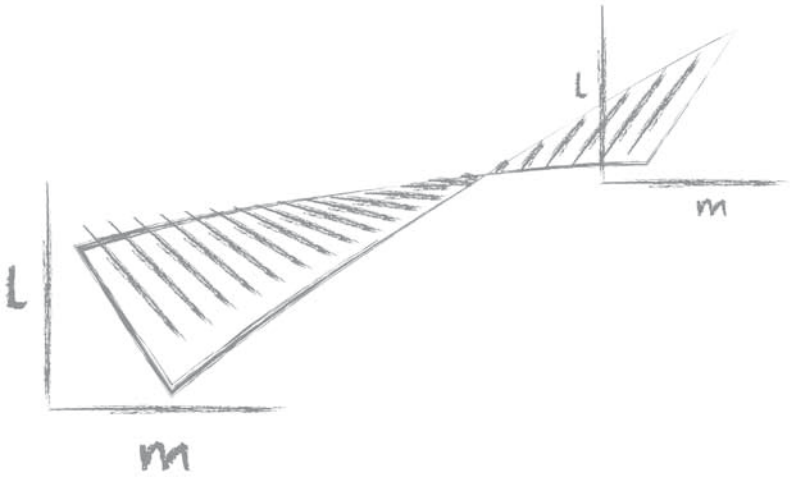
by Joost van de Heuvel



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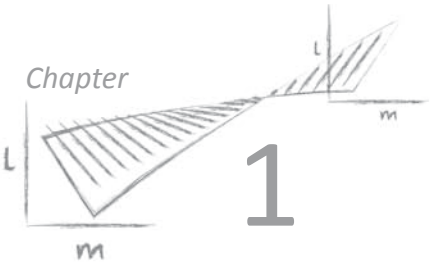

Introduction

Joost van den Heuvel

"Longevity is generally related to the standard of each species in the scale of organization, as well as to the amount of the expenditure in reproduction and in general activity. And these conditions have, it is probable, been largely determined through natural selection."

Charles Darwin

Darwin, demography, life history

The life history of an organism is the description of events that affect Darwinian fitness. The traits we call life history traits affect fitness directly. Examples of these traits are (in chronological order for an individual) size at birth, growth rate, age and size at maturity, size and number of offspring and lifespan. Darwin (1859) expressed the success of a species or variety as the geometrical rate of increase. The species or variety which has the tendency to produce the most offspring successfully, becomes the most abundant type in an environment and is therefore, best adapted to this environment. Euler (1760) had already described how this rate of increase depends on the different life history traits, rediscovered by Lotka (1907), now known as the Euler-Lotka equation. A simple formulation is provided by considering the expected number of offspring born at a specific time, which is

$$N_{t,0} = \sum_{X=a}^{X=\omega} N_{t-X,0} l_X m_X \qquad (1)$$

(taken from Stearns 1992, p. 24). Here $N_{t.0}$ is the total number of offspring born at time *t*, which is the sum of all individuals alive at age *X*, which is $N_{t\text{-}X,0}$ multiplied by the chance that they are still alive l_x at age *X,* and the number of offspring they each produce m_x. Only individuals that are mature, and have passed the age of maturity (α) and individuals that are young enough to reproduce (ω) are considered. When the number of offspring produced (m_x) increases, or the chance to survive to a specific age (l_x) increases, the total number of individuals born at a specific time will increase. Furthermore, eq. (1) suggests that when individuals would start reproduction earlier, when age at maturity α would be lower, then we could expect the number of newborns to increase. If the total number of individuals alive increases with time we consider the rate of increase of a population to be larger than 0.

As an illustration, considering the Darwinian fitness for an example of phenotypic variation, where a white mouse would have a higher chance to survive (i.e. l_x is larger) than a brown mouse on a white background. In this case, all else being equal, the white mouse would have a higher fitness, because the rate of increase of the white mice population would be larger than the brown mice. If two populations of mice would have equal survival probabilities but different reproductive capacities, (i.e. m_x differs) the mice that produce more offspring will have a higher fitness. In life history biology we refer to these differences between individuals, populations, varieties or species as 'strategies'. The ones with the most successful strategy will have the highest fitness. In reality, especially in this thesis, we do not consider two distinct populations separated in space, but genotypes expressing phenotypes that represent strategies within one population. If a strategy is (partly) genetically determined, as an analogy to two populations with different survival or reproductive modes, within a population individuals with the (partly) genetic determined higher fitness will have more offspring in the next generation(everything else being equal) and therefore this genotype will take over the population. Therefore, within one population, if coat color is genetically determined, white mice will produce more offspring than brown ones and therefore after a couple of generations the populations will consist of more white mice. Charlesworth (1980) has shown, using mathematical models, that comparing the demography of populations adopting different strategies is similar to comparing different genotypes within one population dependent on their genetically determined strategies. The examples outlined above show how in principle the evolution in life history traits can take place. In reality it needs to be proven that variation in

phenotypes, partial genetic determination of the phenotypes and a difference in fitness exists. If this is done, we can conclude the evolution of life history traits by natural selection explains variation in the studied traits.

Genetic determination of life history traits

Gregor Mendel discovered how traits are inherited by performing experiments with the common pea (*Pisum sativum*) where he used phenotypes that can be put in two classes. For instance, he used plants which produced wrinkled or smooth seeds, or peas that were green or yellow. When he crossed a parent wrinkled seed plant with a smooth seed plant, the first generation offspring were all smooth seed plants. From these first generation offspring a second generation with 25% wrinkled seed plants were produced. He concluded that there are two copies of every gene in an organism, and sometimes one copy overrides the effect of another (called dominance, as in the first generation of the common pea example).

In general, phenotypes are determined by a genotype and the environment. To what degree genotype influences the phenotype can be studied in many ways, but the four most often adopted are 'common garden' experiments, study of mutants, parent-offspring regressions and artificial selection experiments. In a common garden experiment genetically diverse populations of individuals are reared under similar conditions. Potential differences in phenotypes expressed are genetically determined since the environment is similar. Studies of mutants, completely lacking for instance a functional form of a gene, have been very informative in the analysis of many traits. Mutants are often discovered coincidently, mostly in inbred populations under laboratory conditions. If traits are partly heritable, then parents which on average have a higher trait value will also produce offspring with on average a higher trait value (Falconer and Mackay 1996). Therefore, if a trait is partly heritable, the phenotypes of parents and offspring will be related to each other, which can be quantified by performing a regression of the phenotypes of the offspring on those of the parents. This might be exploited by breeding only with the individuals with a specific trait value (most often the highest or lowest). In the next generation the average of the distribution of phenotypes will be different if a trait is heritable. Such artificial selection experiments have been used to analyze the genetic component of traits.

Some common garden experiments

Longitudinal clines have been used in common garden experiments to test for the genetic determination of traits. Populations that differ in the proximity of the equator are expected to have evolved under different means and variations of temperatures and therefore, all kinds of abiotic and biotic factors correlated between these environments. *Drosophila* clines along several continents have been studied. Individuals from populations at higher longitudes tend to be larger (Alpatov 1930; Watada *et al.* 1986; Coyne and Beecham 1987; James and Partridge 1995; Gilchrist *et al.* 2001; Hallas *et al.* 2002; Calboli *et al.* 2003), have more ovarioles (Watada *et al.* 1986; Schmidt *et al.* 2005) but develop faster (James and Partridge 1995), are more starvation resistant (Karan and Parkash 1998; but see Robinson *et al.* 2000), are less desiccation resistant (Karan and Parkash 1998), have shorter chill coma recovery (Hallas *et al.* 2002), live longer (Hallas *et al.* 2002; Schmidt *et al.* 2005) and higher diapause incidence (Schmidt *et al.* 2005). Body size clines are also related latitude in other organisms (Arendt 2007). In other species common garden experiments have shown differences in age at maturity and growth rate (Dudycha

and Tessier 1999; Tracy 1999; Merila *et al.* 2000; Johnson 2001; Aday *et al.* 2004), senescence (Dudycha and Tessier 1999; Reznick *et al.* 2004), fecundity (Reznick *et al.* 2004) (Dudycha and Tessier 1999; Kinnison *et al.* 2001) body size (Tracy 1999; Amarillo-Suarez and Fox 2006), development time (Skelly 2004). This small selection from the literature concerning genetic variation between populations in life history traits shows that the differences among life history traits between natural populations in many different species are partly genetically determined.

Mutant studies

Under laboratory conditions, once in a while, individuals are born that show dramatically different phenotypes which can be inherited. Examples of these are for instance the Snell (Snell 1929) and Ames dwarf mice (Brown-Borg *et al.* 1996), which show a diminished growth hormone signaling and are smaller than wildtype (individuals that carry the normal variant of a gene) mice. The dwarf mice, besides their smaller size, also show a slower ageing process and therefore live much longer. Similar type of mutants have been found in the worm *Caenorhabditis elegans* (Kenyon *et al.* 1993) and in flies (Tatar *et al.* 2001), where lifespan and or growth is affected. Oocyte production mutants have been found in *Xenopus* (de Vantery *et al.* 1994), mice (Perez-Sanz *et al.* 2013) and *Drosophila* (Bohrmann *et al.* 1992). In *Bombyx mori* a large effect mutants has differ in the number of larval instars, reducing the larval stage and adult body size (Daimon *et al.* 2012) compared to wild type moths. In *Drosophila* mutants were discovered with changed cell growth rates which ultimately led to size and development time differences (Oldham *et al.* 2000). Again, similar to the common garden experiments, genetic regulation of several life history traits has been found, including studies in which multiple traits are affected by individual mutations.

Parent-offspring regressions

Male-father and female-mother regressions of body weight, clutch size and several morphological characters significantly different from zero in a population of pied flycatchers, indicating that parents resembled their offspring (Gustafsson 1986). Heritability of dispersal rate, body mass, age at first reproduction, egg mass, clutch size and lifetime reproductive success was measured using parent-offspring regression under field conditions in the Glanville fritillary butterfly (Saastamoinen 2008). Heritability of lifespan in different populations has been shown in mice (Klebanov *et al.* 2000) and worms (Johnson and Wood 1982). In red deer, traits that were associated with longevity had a lower similarity between parents and offspring, compared to a trait such as average annual reproductive success, because of the large effect of environmental variation on longevity (Kruuk *et al.* 2000). Many traits show similarities between parents and offspring, in several types of organisms, measured in the laboratory as well as under natural conditions.

Artificial selection experiments

The genetics of life history traits have been studied using artificial selection in many organisms for many life history traits. What is very noticeable is that often correlated responses to selection are found. Mice have been selected on body weight, and became fatter with higher blood glucose levels (Buenger *et al.* 2003). Body weight has been altered in chicken by selection which has caused correlated responses in age at first egg production, egg quality traits and egg fertility (Dunnington and Siegel 1985). Guppies have been successfully selected for divergent

body size and time at maturation (Van Wijk *et al.* 2013). Insects have been selected for divergent developmental time, with correlated responses in body weight, viability, pupal mass, fat content, starvation resistance and longevity (Zwaan *et al.* 1995b; Pijpe *et al.* 2006; Cortese *et al.* 2007), although the correlated responses varied per study. Selection on starvation resistance has resulted in correlated changes in longevity, activity, dry weight, fat content, and age-specific egg size and number (Rose *et al.* 1992; Baldal *et al.* 2006; Pijpe *et al.* 2008; Schwasinger-Schmidt *et al.* 2012). Selection on lifespan has resulted in populations that differ in body weight, developmental time, oxidative stress resistance, starvation resistance and viability (Zwaan *et al.* 1995a; Arking *et al.* 2000). Selection for age-specific reproduction results in differences in lifespan, while early and late fecundity are variably affected (Luckinbill *et al.* 1984; Rose 1984a; Partridge and Fowler 1992; Nagai and Sabour 1995). Selection on male longevity has also resulted in increased lifespan in both sexes and changes in age-specific egg size and number in the butterfly *Bicyclus anynana* (Pijpe 2007), while female fecundity was not affected in the cricket *Teleogryllus commodus* (Hunt *et al.* 2006).

Many of the studied traits show a significant heritability. More interestingly, few of the traits show an evolutionary constraint, indicating that many traits can still be selected in any direction. One might expect traits such as developmental time to be optimized so that offspring would grow as fast as they can or adults would be selected to reproduce as much as they can. The general reason for the lack of minimization or maximization of single life history traits is that these traits are related to each other, and therefore the increase of one trait alters another. In many of the above mentioned examples, whether we consider common garden experiments, mutants or artificial selected populations a change in one trait results in a change in another.

Some traits are negatively related to each other. These relationships are called trade-offs. Traits can be negatively related because of genetic or physiological trade-offs. The first is true when populations of organisms show heritable variation in two traits, where one trait is higher in one population, while the other is higher in the second populations. For instance we saw in the *Drosophila* clines that body size and fecundity increase with latitude, while lifespan decreased. The physiological trade-off is caused by the general need for time and resources to develop traits. Therefore a reduced time spent on feeding during a larval stage (which represents a reduced developmental period) may impose a cost of body size (since the larva has grown less because of lower amount of acquired resource). The general framework describing how physiological variation in environments causes variation in strategies of time and resource is referred to as ecological evolutionary physiology. This framework can explain how that what underlies genetic correlations between life history traits are physiological relationships and also point to the type of genes that might vary between populations and species that vary in life history traits.

Ecological evolutionary physiology

The ecological evolutionary physiological framework (ecoevophrame) has been described by Calow, Sibly and Townsend (Calow and Townsend 1981; Calow 1987; Sibly and Calow 1987). The principle assumption of the ecoevophrame is that time, space and resources are limited. Organisms have to make strategic choices about how to invest time, when and where to go and how to use acquired resources. A second assumption is that natural selection will act in such a way upon genetic variation (that ultimately translates in variation in phenotypes which we refer to as the strategies), that time, movement and resources are allocated so that fitness is maximized

of an individual in an environment. If the correct assumptions within a framework concerning a specific trait are made, an optimization on the level of phenotypes can be performed, without considering possible genetic limitations. For instance, it makes sense to assume that a fish cannot swim around the entire world in a day while ingesting all the resources in its proximity and mating all the time, producing endless numbers of eggs. The framework needs to consider certain physical allometric rules, such as for instance described by Kooijman (2010). When this is done properly, optimal decisions can be calculated using tools to determine maximum fitness.

An example based on physiological rules will show how the ecoevophrame can be used to look at genetic correlations between populations under different selection regimes. When an organism starts its life it needs to develop and grow. Let's assume constant ingestion of resources, and therefore, a negative relationship between development time and size at maturation. Everything else being equal, it would be adaptive to develop faster when mortality is higher during this phase of life. Therefore, in populations for which juvenile mortality is higher, it is expected that development time will be smaller and adults will be smaller. In general, smaller organisms have a lower fecundity (because of size limitations). Therefore, the allometry of size dependent fecundity determines to some degree the relationship between juvenile mortality and developmental time. In this example, over a range of environments in which juvenile mortality increases, development time would decrease with body size and fecundity. Thus, it is expected that the genetics of development time, body size and fecundity partially overlap. Indeed we have seen this in artificial selection experiments.

Now we will quantify this example. If we assume that at every time step an adult is alive it can produce a number of offspring which is proportional to its weight, then eq. (1) will become the expected number of offspring an adult will produces. We replace $N_{t\text{-}X,0}$ by 1, since we consider 1 individual, and m_x by a size dependent measure namely, W^b. Juveniles increase 1 mg of weight per day and we can therefore express size and development time conveniently as d, which also then represents size. If we assume a mortality for juveniles of μ(J) per time step, the expected proportion of juveniles surviving d numbers of time steps of development equals $e^{-d\mu(J)}$. We want to maximize the number of offspring produced, and have to quantify at which level of d, the expected number of offspring d^b multiplied by the chance that juveniles survive to the age at maturity, $e^{-d\mu(J)}$. The result of this simple quantification is shown in figure 1 for a juvenile mortality of 0.05 and 0.1, where b=2 and adult mortality is 0.1. In an environment where juvenile mortality is 0.05, the optimal size at maturity (and development time) is 40, which leads to a total expected number of offspring of over 2000. In an environment with a juvenile mortality of 0.1, the optimal size a maturity is 20, with an expected number of lifetime fecundity of 500. Therefore, we have produced a physiologically based model that predicts that development time and lifetime fecundity would be genetically negatively related, if resource would be similar between populations of organisms living under various juvenile mortality regimes. Moreover, since the traits are physiologically linked, a (genetic) change in one trait changes the others as well. Solving this problem was quite simple, but the problem might become more complicated when we consider an adult which can choose to allocate its resources to survival and reproduction. Then we have to consider a decision for every time step the adult lives. If the maximum number of time steps is 10 and the number of choices per time step is 2, we would have to consider 2^{10} choices during adulthood. In reality we would like to quantify the amount of allocation to survival, hence, we would have to use a number of possible choices per time step, instead of 2. To perform such a calculation, a dynamic programming algorithm can be used (Mangel and Clark 1988; Houston and Mcnamara 1999).

The dynamic programming algorithm

Dynamic programming is a generally applied technique that uses reverse iteration to reduce what would be a very large problem into a series of smaller tractable problems. Essentially, at each time step the algorithm computes the fitness for each state that an individual may be in. This in turn depends on the state that the individual will be in at the next time step, the fitness of this new state and the probability that the new state will be reached. When organisms experience mortality, it is a general rule that decisions made in the far future, have a small effect on total fitness. With respect to optimal life histories, in a dynamic programming algorithm this knowledge is used by first determining the optimal life history decisions at some hypothetical time horizon. Beyond this horizon, we do not know the effect of the decision and there are no quantifiable fitness consequences. We simply assume that all fitness values in the time immediately following the time horizon are equal for all decisions that an individual may make.

The decisions available to an individual depend on the stage of the life history e.g. juvenile or adult. Typically, for a juvenile, we would evaluate the effect of growing in one additional time step, taking into account that in the next time step a juvenile that grows will be 1 mg larger for example. Or a juvenile can mature, and will be an adult at the next time step. We let the maximum accumulated reproductive success of an adult that ultimately develops from an juvenile with development time *d* at time *t* be $F_J(d,t)$. Then the future fitness of a growing juvenile is,

$$V_{GROW}(d,t) = e^{-\mu(J)} F_J(d+1,t+1) \tag{2}$$

In this example development time and size was updated by 1, hence, size and development time in the future is *d+1* at time *t+1* and mortality is μ(J*).* Mortality generates a probability that the future state is achieved. The future fitness of a juvenile that matures is dependent on the maximum accumulated reproductive success of an adult, which ultimately survives from an adult with size *d* at time *t*, represented by $F_A(d,t)$. We can write the fitness of a juvenile that matures at time *t* as,

$$V_{MAT}(d,t) = e^{-\mu(J)} F_A(d,t+1) \tag{3}$$

Note that the juvenile is not allowed to grow further, hence, the size of the adult at time *t+1* when it was an juvenile at time *t* is *d*. Now we can calculate the optimal decision of a juvenile at time *t* as,

$$F_J(d,t) = \max[V_{GROW}(d,t), V_{MAT}(d,t)] \tag{4}$$

We consider the two options available to the juvenile of growth or maturation, for all possible values of *d* at time step *t*. The number of offspring an adult produces in this example is equal to d^b and since we now computed the fitness value for juveniles at all stages of development we can assign the fitness value for a newly produced offspring as $F_J(1,t+1)$. Therefore the maximum accumulated reproductive success of an adult with size *d* at time *t* is,

$$F_A(d,t) = e^{-\mu(A)}[d^b F_J(1,t+1) + F_A(d,t+1)] \tag{5}$$

This to some degree resembles again eq.(1), where we evaluate the fitness of an individual as its chance to survive is ($e^{-\mu(A)}$) between till *t+1*, the added value of reproduction and the future

maximum accumulated reproductive success of an adult with size *d* at time *t+1*. In the dynamic program we solve this problem by assuming that all fitness values at the last time step, the time horizon (*T*), for juvenile and adults is 1 (i.e. $F_J(d,T)$=1 and $F_A(d,T)$=1) for all possible values of *d*. We then fill in these values in equations (2-5) and determine the optimal decisions made in equation (4). Then we have the fitness values for all values of all $F_J(d,T-1)$ and $F_A(d,T-1)$ for all values of *d*. We continue this process until all the fitness values converge to specific levels and the optimal decision has thereby stabilized for all juvenile sizes and adult states. We can use the optimal decision now to infer, as done in figure 1, what the development time and size at maturity is depending on the parameter values. Also we can start a population consisting of juveniles with *d=1* at a specific time and simulate forward to get an impression of the traits and relationship between traits amongst individuals. This can again yield information about the expected total number of offspring produced. In general, dynamic programming saves enormously on computing time: we calculate N decisions in T time steps, giving a total of NxT calculations. If we simply computing the N decisions forward in time, we would have to calculate N^T decisions to consider the same state space.

We have seen that the ecoevophrame can be used to quantify the optimal allocation of resources dependent on the state of the organism (*d* in the previous example) and the environment (mortality). Two important assumptions of the model could interfere (when not correct) with how well the model predicts the outcome of differences between populations that have evolved in the different conditions. The first is that we expect organisms to have equal acquisition rates of resources. An increased acquisition rate would lead to organisms that might develop faster and become larger, but also produce more offspring. Hence, additional environmental variation can obscure genetic variation of allocation (Calow and Townsend 1981; Calow 1987; Sibly and Calow 1987). In reality there will be always small stochastic differences between individuals that lead to more positive estimates of negative genetic relationships when organisms are taken into the laboratory. The acquisition of resources itself is also a trait which evolves and therefore, the acquisition between populations that differ in mortality might also differ genetically in acquisition rates (Jorgensen and Fiksen 2010). A quantitative genetic model (Y-model) which considers genetic variation in acquisition and allocation also predicts this complication, where more variation in acquisition leads to more positive relationships between life history traits (Van Noordwijk and De Jong 1986; De Jong and Van Noordwijk 1992). Therefore, when testing the outcomes of these type of models, one is more likely to find genetic correlations when variation between populations is considered, rather than individuals within populations, because it is expected that the genetic variation in allocation is larger between populations. A second complication is the effect of allocating resources in a specific trait. For instance, above we assumed that once an adult has reached a size of *W* it will produce W^b number of offspring every time step it lives. Although we did not study the effect of different values of *b*, it is important to consider the correct allometric relationships between traits and allocation of resources. The most intriguing one is the allocation to survival and the increase of survival, at the cost of other traits such as growth and reproduction. For this we need to consider the biology and evolution of ageing.

Ageing from cell to ecosystem

Ageing is the accumulation of intrinsically caused physical changes over time, that lead to a decrease of vitality, fecundity and an increase of the chance of morbidity and mortality. Over the life of an individual changes in the ability to move, to reproduce (but not in semelparous organisms, which reproduce only once) or to learn can be measured longitudinally, but the chance to become diseased and die can only be measured in a population of individuals. At the basal level of the organism and population dynamic traits, are stochastic cellular change causes damage to DNA, shortened telomeres and dysfunctional mitochondria. When cells are derived from a single founding cell, they show a large variation in the capability to multiply (Smith and Whitney 1980). When only DNA or mitochondrial mutation or telomere loss is modeled separately, this cannot explain fully this large variation in the ability of cells to replicate. Modelling these three features of cellular ageing in interaction with each other, Sozou and Kirkwood (2001) showed that these interactions are needed to explain this variation. The interactions between DNA damage, telomere shortening signaling and mitochondrial dysfunctioning signaling all relate to the retrograde response pathway, and ultimately these lead to the replicative senescence of the cell (Passos *et al.* 2010). The retrograde response is a signal to the nucleus from mitochondria, when they become dysfunctional, often leading to altered functioning of the whole cell, including cellular senescence (Butow and Avadhani 2004). A proposed molecular pathway, (DNA damage -> p53 -> CDKN1A -> GADD45 -> MAPK14 -> TGFβ) leading to cellular senescence was verified. Considerable work has been done on the genes of this pathway. For instance, increases in sublethal oxidative stress causes an increase in CDKN1A expression, and later replicative senescence in human fibroblasts (Dumont *et al.* 2000).

Moreover, replicative senescence is not the only pathway a cell can go adopt when damaged. Cellular damage may be repaired. If this does not succeed, some cells continue to replicate while others (depending on cell type) go into apoptosis. When cells continue to replicate, the damage in the cell propagates, which can lead to the development of cancer cells (Kirkwood 2011). Indeed genetic manipulation of CDKN1A also affects cellular senescence and possibly tumor cell proliferation (Garcia-Fernandez *et al.* 2014). While organisms are ageing, their tissues contain more senescent cells, and when for instance healthy donor hearts are compared to failing hearts, the failing contain much more senescent stem cells, which have an upregulated levels of for instance CDKN1A (Cesselli *et al.* 2011). These examples show that cellular ageing regulated via DNA damage or mitochondrial dysfunction can be related to tissue level senescence which via cancer or heart failure can lead higher chances of organismal morbidity and mortality.

Ageing at the level of the organism reduces the chance to survive and therefore fitness (see eq. [1]). Therefore it seems counterintuitive that ageing has evolved. Many hypothesis of why ageing has evolved have been proposed (Medvedev 1990). Evolutionary theories of ageing are based on the fact that the presence of continued extrinsic mortality (by predation, disease, hunger) will lead to a exponentially decreasing chance of survival at older ages (as in the above mentioned example, survival till time t is $e^{-t\mu}$, where μ is mortality rate). Therefore, a gene with negative effect will have less bearing on fitness when it is expressed late in life, compared to early in life. Medawar (Medawar 1952) suggested that alleles of genes that negatively affect survival would be able to increase in populations because selection against these genetic variants is

weak. Taking this further, when an allele has a positive effect early in life, but negative late in life, it can easily spread in a population because selection is stronger during earlier life stages. These theories of antagonistic pleiotropy (Williams 1957) and mutation accumulation both use age classes in their assumption. Although one could separate individuals by chronological age, in a mixed age population age classes only exist when physiological ageing occurs. Therefore, without ageing, there would not be any biologically relevant age classes. Therefore, the arguments of mutation accumulation and antagonistic pleiotropy are circular, as they explain the evolution of ageing assuming it already exists. The realization that ageing occurs because of stochastically caused damage opened up a new possibility of the evolution of ageing, by which organisms need to allocate resource to maintenance of the soma to repair this damage. According to the disposable soma theory (Kirkwood 1977) this allocation to repair has an optimal value which is below maximum repair since organisms die anyway because of extrinsic mortality. Hence, organisms that would allocate all their resource to maintenance would produce no offspring, but die anyway, while organisms that would allocate resource to reproduction at the cost of maintenance would reproduce. They would age (faster) because of the mechanistic increase of damaged DNA molecules in cells that cause ageing. The prediction from this theory is that extrinsic mortality is related to the increase of intrinsic mortality caused by ageing which is verified for instance by selection experiment mentioned above, where selection of age specific reproduction affects lifespan (Luckinbill *et al.* 1984; Rose 1984b; Partridge and Fowler 1992). Furthermore, this theory of ageing puts ageing and survival in the broader framework of life histories (Roff 1992; Stearns 1992) and the ecoevophrame (Calow 1987; Sibly and Calow 1987). Lastly, this theory of ageing is based on the mechanistic causes of ageing, which include DNA damage, telomere shortening and mitochondrial dysfunction. Also it predicts that what modulates ageing are pathways that respond to resources (nutrient sensing) and deal with these resources in alternating the allocation of resources into specific functions, which was the basis of the ecoevophrame. Indeed nutrient sensing pathways such as the growth hormone and nutrient sensing pathway generally affect ageing processes in multiple species (Kenyon *et al.* 1993; Brown-Borg *et al.* 1996; Clancy *et al.* 2001; Tatar *et al.* 2001; Tu *et al.* 2002; Barbieri *et al.* 2003; Holzenberger *et al.* 2003; Bartke and Brown-Borg 2004; Hwangbo *et al.* 2004; Selman *et al.* 2009; Selman *et al.* 2011). Cells use information of both the outside (via this signaling pathway) as well as inside, via p53 and AMPK to make decisions ultimately by regulating the action of transcription factors (Zoncu *et al.* 2011). The outcome of a model based on the ecoevophrame would be that variation in nutrients would relate to variation in function, which can be modulated by these pathways, since they regulate both stress resistance and survival regulating transcription factors as well as transctiption factors that regulate growth and storage via lipid synthesis. Therefore, the discovery of such pathways regulating these functions fits well with the general framework that describes the evolution of life histories.

In field situations, the selection pressures that shape life histories come in the form of other organisms and abiotic variables. For instance, mortality can be related to the number and type of predators around. Variation between environments in food could be regarded as differences in production of the lower trophic level organisms than the focal organism studied. When variation in predation pressure between populations causes differences in densities of the focal organism, this in turn can cause differences in competition for food. From life history theory it would be

expected that increased extrinsic mortality will lead to more allocation to reproduction at the cost of somatic maintenance. Additional acquisition variation because of density differences will lead to other relationships between genetic variation in allocation patterns. A well-studied example is the evolution of high and low predation guppies. In 1976 guppies were transplanted from populations where predation occurred on selectively at on large, mature-sized class of guppies to populations where only smaller predators occurred, which predate on smaller, juvenile-sized class of guppies (Seghers 1973; Liley and Seghers 1975). Guppies mature earlier, increase reproductive effort and produce more and smaller offspring per brood in the control, adult predation sites (Reznick *et al.* 1990), which was replicated at several sites (Reznick and Endler 1982), and has a genetic basis (Reznick 1982). The overall predation level is higher at the adult predation sites (high predation, HP), compared to the juvenile predated sites (low predation, LP), and densities and total biomass of guppies are higher at LP sites (Reznick and Endler 1982; Reznick *et al.* 1996; Rodd and Reznick 1997; Reznick *et al.* 2002). Therefore, it is likely that the HP populations have evolved at a higher per capita resource environment (Bassar *et al.* 2013) and they have different feeding habits (Bassar *et al.* 2012). The fact that HP guppies live longer in a common garden experiment (Reznick *et al.* 2004), might be because of possible interactions between acquisition and allocation. In the study of the evolution of ageing, it is therefore important to note that differences in one parameter of the environment can be related to other parameters.

Plasticity

We have now seen that life history traits are phenotypically variable, heritable and there exist clear predictions on how selection varies between environments. Therefore life history traits evolve. The variation that different populations experience in space can also be experienced in time within populations. Differences in seasons can cause organisms to hibernate in winter, and be active during the warmer seasons. When the environment shows heterogeneity, organisms (although similar in genotype) tend to produce different phenotypes in the different conditions that they experience, which is referred to as phenotypic plasticity. For instance, upon the experience of predator kairomones *Daphnia* change their age specific reproductive traits and survival, but also grow longer spines on the head (Black 1993). Tadpoles develop in carnivorous or even cannibalistic types under high shrimp or high conspecies densities (Pfennig 1990). The tropical butterfly *Bicyclus anyana* produces conspicuous wing patterns, develops and reproduces faster and lives shorter during the wet season, compared to the dry season during which the offspring cannot feed (Brakefield and Zwaan 2011). Horned beetles develop horns under high feeding conditions (Moczek 1998), larvae of butterflies grow faster or slower dependent on photoperiod (Gotthard 2008), and different invertebrate species develop into forms that upregulate somatic maintenance by partly the same mechanism in periods in which reproduction is unfavored (Flatt *et al.* 2013). When the cue for developing into a certain form is very reliable, sometimes plasticity can develop over generations. In such a case, the female produces offspring of a certain kind. For instance, aphids that develop from overwintering eggs will produce female offspring clonally, and feed on host plants. At some point, the quality of the host drops, by destruction of the aphids, and the female produces winged offspring, which can disperse and colonize new plants. Towards the end of the season, temperature and photoperiod change and females will produce sexual male and female offspring, which will mate to produce a new generation of overwintering eggs (Miura *et al.* 2003; Shingleton *et al.* 2003; Braendle *et al.* 2006). This example shows that depending on

the cues from the environment, for instance quality of the plant or temperature and photoperiod, the females produce offspring that match the requirements of the future environment. Aphids would starve if they could not disperse from a plant that dies and would freeze if they would not produce overwintering eggs. Similarly, locusts avoid nourishment declines under high densities, by producing dispersal adapted forms (Pener and Yerushalmi 1998).

Phenotypic plasticity does not have to be adaptive, especially when the environment experienced is atypical for a population or species (Ghalambor *et al.* 2007). Furthermore, it can be seen from the aphid example that the cues that lead to the production specific offspring were very reliable. In theoretical work, the reliability of the cue is of major importance for the evolution of plasticity, as is the frequency over on type of environment encountered over the other, as well as whether variation in spatial or temporal (Moran 1992). Moreover, there might be costs associated with phenotypic plasticity. Obviously there are mismatch costs, when a form is developed according to the wrong cue in the wrong environment. These costs are already accounted for by the proportion of present microenvironments and the reliability of the cue (Relyea 2002). There could be mechanistic costs though, where the adaptation to two environments by one genotype, because of antagonistic actions on genes, leads to an average lower adaptation compared to the fitness of to two genotypes adapted to the two environments. Still, if in time or space both type of environments would be experienced, and the plastic genotype would have on average a higher fitness, the costs do not outweigh the benefits (Relyea 2002). Using a method with a large number of independent variation between genotypes in phenotypic variation and the degree of plasticity, costs of plasticity has been shown (DeWitt 1998), but costs in general are very rare (DeWitt *et al.* 1998).

Dietary restriction

A well-known intervention that affects various life history traits is mild dietary restriction (DR). It extends lifespan in many organisms such as yeast (Muller *et al.* 1980), worms (Klass 1977), flies (Chippindale *et al.* 1993) and rodents (McCay *et al.* 1935; Weindruch *et al.* 1986) and some positive health effects have been shown in primates (Mattison *et al.* 2012). Although it has been discussed that this response upon DR might be widely evolutionary conserved, many organisms do not show a lifespan extension upon DR (Kirk 2001; Carey *et al.* 2002; Cooper *et al.* 2004; Beck 2007; Weithoff 2007; Sutphin and Kaeberlein 2008; Kasumovic *et al.* 2009; Swindell 2012) and it has been suggested that organisms that are showing lifespan extension upon DR are simply better adapted to laboratory conditions (Nakagawa *et al.* 2012). An evolutionary explanation for the variation in organisms is that lifespan extension upon DR is adaptive, to outlive periods where food is scarce, and offspring are unlikely to survive. A theoretical model, based on the disposable soma theory, parameterized for mice has shown this is possible when resource level is related to offspring survival and when a reproductive overhead needs to be paid before the unset of reproduction (Shanley and Kirkwood 2000). Therefore the modeling predicts an upregulation of somatic maintenance upon DR in experiments. This indeed seems to be the case (Holehan and Merry 1986; Yu 1994). There are differences even within groups of organisms that increase lifespan upon DR. For instance, although both mice and rats generally have increased lifespan upon DR, besides some variation between strains, there are differences in how they respond to this intervention, most clearly how body temperature is regulated (Rikke

et al. 2003). Lastly, organisms generally respond much more differently to DR then appreciated in literature (Shanley and Kirkwood 2006), and simple differences between species such as the ability of flies to maintain reproduction while reproduction is absent in mice upon DR may shed more light on how organisms respond increase lifespan upon DR than looking at the similarities.

Studies on nutritional geometry, where nutrients are combined in specific ratios rather than all nutrients are changed in one ratio (Raubenheimer and Simpson 1997), has led to a more mechanistic view DR and life history traits (Lee *et al.* 2008; Skorupa *et al.* 2008; Grandison *et al.* 2009; Fanson *et al.* 2012). It is unclear where this line of research is going and whether it can use theory based hypotheses to predict the outcome of experiments done with specific ratios of for instance proteins and carbohydrates (Lee *et al.* 2008; Skorupa *et al.* 2008). Evolutionary theory would probably predict that organisms would optimize their nutrition intake to maximize fitness and evolve to cope with a protein carbohydrate ratio from the environment which it lives in. Another prediction would be that the carbohydrate protein ratio and total amounts which maximizes fitness will be different from the ones that maximize reproduction or lifespan.

Gene expression upon dietary restriction

Another way of mechanistically studying the response to dietary restriction is by gene expression measurements. The function of many genes in the genome are known, and summarized in gene ontology terms, such as the biological functions *egg production*, *determinants of lifespan* and *neuropeptide signaling*. Gene expression profiles of the whole genome can therefore indicate what kinds of processes are related to the DR intervention. Processes that are generally affected by DR in different organisms are metabolism (carbohydrate, amino acid, protein or lipid), stress and immune responses, energy metabolism and regulation of transcription, cellular growth and apoptosis, xenobiotic metabolism and signal transduction (Han and Hickey 2005). These groups of genes that are affected by dietary restriction can be related to processes associated to somatic maintenance. Examples are protein turnover, DNA repair activity, cytosolic antioxidants and heat shock protein expression, which are often seen in rodents upon dietary restriction (Kirkwood and Shanley 2006). The insulin signaling pathway is regarded as one of the most important pathways regulating both lifespan increases by genetic manipulations, as well as, by differences in nutrition (Mair and Dillin 2008). Problematically, the insulin pathway itself is regulated by post translational status rather than by transcription levels of the genes (see for models Smith and Shanley 2010; Dalle Pezze *et al.* 2012). Since many genes become differently expressed in organisms experiencing DR, at some point regulation must be different on the transcription level. Indeed, the signaling pathway changes the activity of transcription factors, such as Foxo, SREPB and PPARγ directly regulate transcription (Zoncu *et al.* 2011) and indeed, Foxo in fruit flies regulates partly the type of processes via transcription that are differently expressed between DR and fully fed flies (Alic *et al.* 2011). Gene expression studies can to some extent indicate the type of processes that are regulated by nutrition manipulation.

Unrestricted diet

The opposite of restriction of diet is overfeeding. In modern human populations many age related diseases are prominent that have a relationship to the excess intake of food. For instance, the use of fast food is associated with higher BMI (Boutelle *et al.* 2006) and a higher BMI

(>30) is associated with higher risk for all causes of death (Flegal *et al.* 2013). In the thrifty gene hypothesis it is proposed that modern humans carry alleles that have high frequencies because of past selection in a feast and famine environment (Neel 1962). In the past when highly nutritious food items were scarce, these alleles provided an advantage by storing food as body fat to be able to survive scarce periods. In modern societies this leads to overweight and other metabolic syndrome related diseases such as diabetes mellitus and coronary heart disease because of too much high fat and carbohydrate food. The ApoE gene poses an interesting possible example since it increases the risk for age and BMI related disease and where between alleles fecundity and survival are negatively related (Corbo *et al.* 2008).

The observation that experiences in utero seems to 'program' offspring to develop into thrifty individuals, by developmental plasticity, has led to the thrifty phenotype hypothesis (Hales and Barker 1992). Under low nutrition, individuals that develop the thrifty phenotype, can complete development successfully, but will also develop higher incidences of diabetes mellitus. In the Dutch hunger winter cohort, there is indeed an association between the lowered rations of food, higher incidence of metabolic syndrome in affected humans and a methylated gene, known to be important for development and associates with lifespan (Heijmans *et al.* 2008). A related cohort of war-time famine in Leningrad showed a different relationship between famine in utero and the development of diseases that make up the metabolic syndrome (Stanner *et al.* 1997). A possible reason for this is the lack of an increase in rations after birth for this cohort. Therefore, there is a match between in utero and after birth nutrition levels in the Leningrad cohort, which was absent in the Dutch hunger winter cohort. This has led to the development of a mismatch theory of the development of metabolic syndrome, the predictive adaptive response (Gluckman and Hanson 2004). The predictive adaptive response is an adaptive plastic response of the mother, directing the development of the offspring towards a phenotype, better fit in the environment that it will experience later in life (adult life time). Whether this response is adaptive cannot be tested now, but must be tested in ancient, or at least pre-industrial societies (Hayward *et al.* 2013). In general, modelling approaches show that it is quite unlikely that this kind of response can evolve in humans (Nettle *et al.* 2013), but since information on the reliability of a cue in ancient human populations is lacking, it is difficult to test the evolutionary basis of the model.

Aims and chapters

In this thesis I aim to address unsolved problems in life history research with a focus on ageing. These topics specifically interest me, but have implications for the field in general. Besides using various types of measurements in experiments, I also use theoretical models and have studied several organisms. In general, the theoretical models can be used to verify the validity of claims made in the literature. Secondly, the theoretical models can link experimental data to proposed field situations, to better understand what is found in the laboratory. In general, findings from experiments and models have been discussed side by side in this introduction, and ideally should always be used side by side. Models can be used to zoom in on specific cases, where they have explanatory power, but can also be used to extrapolate, where they have predictive power. This shows that they can link different levels of biological organization, such as the individual physiological and the evolutionary ecological level, which is difficult to do experimentally. The experiments performed also have an integrative approach, where we do not want to uncover the relationships between evolution and ecology with mechanisms of life histories. Below each chapter is briefly introduced.

Theoretical models

In **chapter 2** the applicability of the predictive adaptive response for a seasonal tropical butterfly, *Bicyclus anynana* is tested. This butterfly lives in an environment that very predictably alternates in dry and wet seasons in which reproduction only takes place in the wet season (Windig *et al.* 1994). Experimental data manipulating the larval and adult conditions of the butterfly suggests that larvae that are stressed cope better with adult stress (Saastamoinen *et al.* 2010). Using a state-dependent energy allocation model we test whether a response found in the experiment would be adaptive in the natural environment of *B. anynana*. This chapter will address not only the predictive adaptive response hypothesis specifically, but will also reveal how well ecological knowledge about the species can tell us something about specific experiment done in the laboratory.

Dietary restriction is the most widely used manipulation that affects organismal ageing and seems to increase lifespan for many very different organisms. Although commonly referred to as evolutionarily conserved, there are many exceptions. This pattern is not explained yet by evolutionary theory. In **chapter 3** we will use a modelling approach to test in a wide variety of theoretical environments whether an increase in allocation to maintenance and repair at the cost of reproduction can explain the divergent patterns in lifespan increase upon DR in the wide variety of taxa. The model is based on a theoretical approach specifically developed for mice (Shanley and Kirkwood 2000). It provides an evolutionary view on a topic which can use an ecological evolutionary approach since the validity of the claim that lifespan extension upon DR is conserved has been reviewed for data from a wide variety of taxa, but this review does not consider an ecological evolutionary approach (Nakagawa *et al.* 2012).

Plasticity of Drosophila life history upon variation in nutrition

Organisms live in a heterogeneous world. In laboratories, in general, the effects of nutrition are tested using constant levels of food. It is known that flies can respond very quickly to nutrition changes with an effect on survival (Mair *et al.* 2003). In **chapter 4** we will study the effect of continued variation in nutrition on weight, egg production and survival of the fruit fly, *Drosophila melanogaster.*

Gene expression measurements can indicate how changes in phenotypes are regulated. In model organisms such as *Drosophila* it is relatively easy to use genome wide transcription arrays to study the effect of manipulations on gene expression. In **chapter 5** we will look at the plasticity of the transcriptome of the flies that changed in nutrition compared to constant flies. First we will study to what degree the transcriptome of the flies on variable food resembles that of the controls. Secondly, since we will look at longitudinal data as described in chapter 4 we can relate gene expression to phenotypes to study how life history variation in *Drosophila* is regulated.

In **chapter 6** we develop an individual based model to look at individual life histories obtained from the study from chapter 4. In this chapter we will describe how well state dependent allocation theory works on differences within populations of flies, while in general, it is much easier to look at differences between allocation strategies between genetically divergent populations. Therefore, chapter 6 deals with the applicability of allocation theory upon individual differences rather than populations.

Linking genetic with phenotypic variation in natural populations

In **chapter 7** we will look at the genetic variation in candidate genes that are known to

affect life history traits, in natural populations of the Least Killifish, *Heterandria formosa.* The ecological differences between populations of this species have been studied very thoroughly. Relationships between predation pressure, density of the populations and life history traits have been discovered (Schrader and Travis 2012). Similar relationships exist in another livebearing fish, the Trinidadian guppy, where guppies from high predation environments mature earlier, grow larger and produce more but smaller offspring (Reznick *et al.* 2004). Also in *H. formosa* these traits are heritable (Henrich and Travis 1988; Leips *et al.* 2000; Schrader and Travis 2008; Schrader and Travis 2009; Leips *et al.* 2013). The species is found throughout the southeastern United States and population structure and biogeography are known (Baer 1998). Density estimates relate well to heterozygosity measurements (Soucy and Travis 2003; Schrader *et al.* 2011). In this chapter, we will describe the sequencing of candidate genes and test whether these relate better to phenotype than control genes. We will use allele frequencies and average phenotypes from eight populations. This will reveal whether continuous variation in phenotypes from field populations relate to candidate genes often discussed in life history research.

Discussion

Finally, in **chapter 8** the findings from the previous chapters will be summarized and discussed. Then I will focus on how the different parts of the thesis are related to each other. The contribution of this work to life history research and ageing will be discussed, and I will give an opinion on where this field should go. In the end I will discuss how the use of theoretical models and experiments on various organisms can be used to compile a wholesome picture of the biology of life histories and ageing.

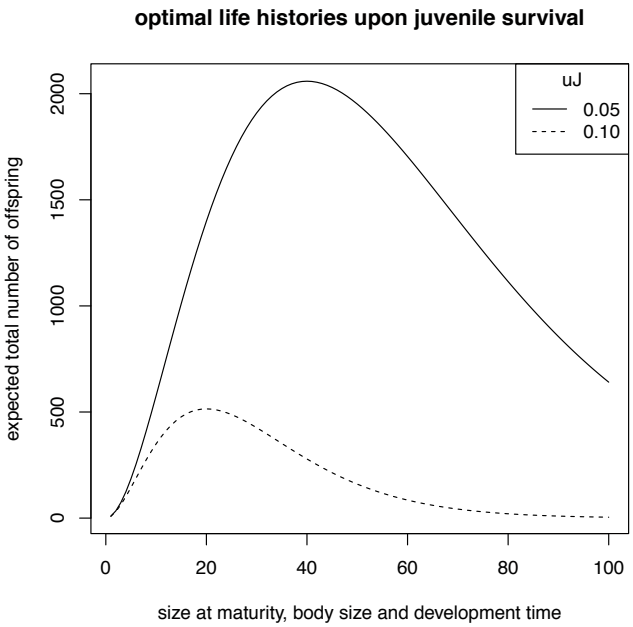

Figure 1. Optimal size at maturity and developmental time for different values of juvenile mortality. In this example b=2 and adult mortality is 0.1.

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The predictive adaptive response: Modeling the life history evolution of the butterfly, *Bicyclus anynana*, in seasonal environments.

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"You are a splendid butterfly
It is your wings that make you beautiful
And I could make you fly away
But I could never make you stay"

Stephin Merritt

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Abstract

A predictive adaptive response (PAR) is a type of developmental plasticity where the response to an environmental cue is not immediately advantageous but instead later in life. The PAR is a way for organisms to maximize fitness in varying environments. Insects living in seasonal environments are valuable model systems for testing the existence and form of PAR. Previous manipulations of the larval and the adult environments of the butterfly *Bicyclus anynana* have shown that individuals that were food restricted during the larval stage coped better with forced flight during the adult stage compared to those with optimal conditions in the larval stage. Here, we describe a state-dependent energy allocation model, which we use to test whether such a response to food restriction could be adaptive in nature where this butterfly exhibits seasonal cycles. The results from the model confirm the responses obtained in our previous experimental work and show how such an outcome was facilitated by resource allocation patterns to the thorax during the pupal stage. We conclude that for *B. anynana*, early-stage cues can direct development towards a better adapted phenotype later in life and, therefore, that a PAR has evolved in this species.

Keywords: predictive adaptive response, developmental plasticity, energy allocation model, life history, stochastic dynamic programming

Introduction

Developmental plasticity is the ability of a genotype to direct development in response to changes in its environment (Stearns 1982; Piersma and Drent 2003; West-Eberhard 2003). Plasticity is adaptive when the resultant change in the phenotype causes a fitness benefit in the new environment (Ghalambor *et al.* 2007). The predictive adaptive response (PAR) is a specific type of developmental plasticity where the response to a cue is advantageous at a later life stage, rather than immediately (Gluckman and Hanson 2004a; Gluckman and Hanson 2004b; Gluckman *et al.* 2005). The concept of PAR was first introduced in the context of human development. It is suggested that the human fetus evolved an ability to interpret cues from the early-life environment which results in development of a phenotype better matched to the environment predicted to occur later in life (Gluckman and Hanson 2004a; Gluckman and Hanson 2004b; Gluckman *et al.* 2005). Some empirical data support the idea of PAR in humans (e.g., Jasienska *et al.* 2006), but its general validity remains uncertain. Concerns involve factors such as honesty of cue, mother offspring conflict, and environmental variance (Wells 2006; Rickard and Lummaa 2007; Wells 2007; but see Gluckman *et al.* 2005; Gluckman *et al.* 2008). Theoretical work suggests that plasticity is less likely to evolve when the relationship between cue and late-life environment weakens (Reed *et al.* 2010; but see Moran 1992; Sultan and Spencer 2002).

One of the best candidates for PAR is the development of meadow vole (*Microtus pennsylvanicus*) fur, which is thicker in autumn-born voles than in those born in the spring (Lee and Zucker 1988). There is no immediate advantage of the differential fur thickness, as the nest temperatures are similar in autumn and spring. However, when the voles mature and leave the nest, the external temperatures are very different. Other possible examples of PAR include the dispersal response in the desert locust *Locusta migratoria* (Pener and Yerushalmi 1998; Simpson *et al.* 1999; Simpson *et al.* 2001) and in the great tit *Parus major* (Tschirren *et al.* 2007), alteration of morphology of water flea *Daphnia cucullata* (Agrawal *et al.* 1999), and growth rate and developmental time in the guppy *Poecilia reticulate* (Gosline and Rodd 2008). Even though such examples demonstrate that organisms respond to environmental cues by changes in physiology and/or life history traits, what is often lacking is a definitive test of the adaptive value of the responses. This may be partially explained by the fact that measuring fitness in the field can be logistically difficult and that interpretations of such data may be confounded by environmental and genetic factors (Monaghan 2008).

Ideal opportunities to test the presence of evolved PARs exist in organisms experiencing seasonal environments, particularly when generation times are relatively short compared to the seasonal variation. Moreover, in testing PAR it is important that the environment can be easily manipulated and that extensive knowledge exists about the ecology to which the adaptive suitability of the observed response can be related (Rickard and Lummaa 2007). One example of such an organism is the tropical butterfly *Bicyclus anynana* (Brakefield and Zwaan 2011). This species exhibits seasonal polyphenism, appearing in two distinct phenotypes (wet and dry season morphs), which differ in a number of morphological and life-history traits (Brakefield and Larsen 1984; Brakefield and Frankino 2009). The dry season form is, on average, larger and longer-lived, with delayed reproduction, higher metabolic rate and fat content, and it is better camouflaged on its resting background (Brakefield *et al.* 2007). Seasonal variation is highly predictable, with warm, wet seasons of abundant food alternating with cool, dry seasons when there is no food for larvae.

Recently, Saastamoinen *et al.* (2010) conducted an experiment on *B. anynana* to assess whether poor nutritional conditions during development shaped the adult phenotype so that it could better deal with stressful conditions later in life. Results from this experiment showed that females experiencing food restriction during larval development did not increase their tolerance for adult food limitation but did alter their body allocation via an increased thorax ratio (i.e. thorax mass/body mass) which led to an enhanced flight performance (Saastamoinen *et al.* 2010). These results reveal a substantial effect of plasticity in response to larval nutrition on variation in adult flight performance. They also suggest that such plasticity may be adaptive, as food-stressed individuals could disperse more effectively to higher quality habitats when this is favoured.

Here we describe a state-dependent energy allocation model of the life history of *B. anynana*, in which organisms can evolve strategic decisions based on their physiological state and the state of the environment (McNamara and Houston 1996). Individuals respond dynamically to condition from the past and present, providing a basis to predict and adapt to future environments. We address whether the life history traits, as observed for *B. anynana* in nature, are predicted to evolve and whether the responses observed in the experiment of Saastamoinen *et al.* (2010) are likely to be adaptive.

Methods

Overview of the model

In the model, we characterize an individual in one of a set of states that describes variables such as weight, developmental time and biological age, which are known to affect the biological outcomes of interest. Transitions between states and options such as feeding and egg production are described by mathematical relationships using energy as a common currency. Environmental factors, such as temperature, predator pressure, and resource abundance, are also included in the model. We use a daily time step, and each day the individual makes decisions concerning, for example, larval development time, pupal allocation pattern, and adult behavior, which will affect its future state. The optimal decisions in a given environment are determined using stochastic dynamic programming (SDP, Mangel and Clark 1988; Houston and McNamara 1999; Clark and Mangel 2000). We assume that natural selection acts to optimize the life history of an individual by maximizing the number of progeny that the individual produces. Once the optimal decisions have been found, the traits of the individuals that define its state in the different environments can be modeled by forward simulation. As nutritional status at the larval stage influences allocation strategies in the pupal and adult stages, all these stages of the life cycle are modeled. Figure 1 presents an overview of the model. We first explain how environmental variation is addressed and then briefly describe each stage of the life cycle. Further mathematical details are provided in the appendix.

Environmental variation

Generations of *B. anynana* live in two alternating seasons, the dry and the wet seasons. A year in the model consists of 366 time steps, each representing 1 day. During most of the dry season, which lasts 6 months, there are no host plants for larvae to feed on. At the start of the wet season, ambient temperature increases, followed by an increase in rainfall (Windig *et al.* 1994)

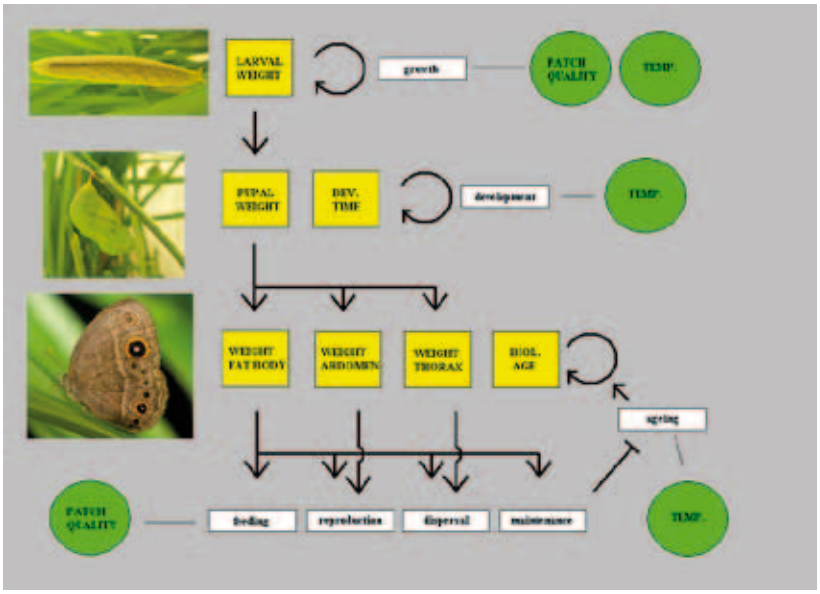

Figure 1. Overview of the model. The different stages of the butterfly's life history (larva, pupa, adult) are shown from top to bottom. Individuals at each stage are characterized by the traits shown as yellow squares (biol. age = biological age). The processes in the model are shown as white rectangles. The environmental factors are shown in green circles. Photos by Oskar Brattström.

and an increase in host plant density. Hence, during the wet season at every time step a proportion of the patches changes in quality. As the wet season progresses, temperature, rainfall and the number of host plants decrease. For each time step in the model, we assigned a temperature (table S1, figure S1) and a proportion of good patches based on climate information available for Malawi, from where the laboratory population of *B. anynana* was derived (WINDIG *et al.* 1994). A good patch contains host plants where larvae can feed: see table S1 and figure S1. Every patch becomes good and bad once a year, with the percentage of good patches fixed for a given day. A good patch remains a good patch unless the proportion of good patches decreases. We let

$\lambda_{gb}(t)$ = Pr{good patch at time t is followed by bad patch at time t+1}. (1)

This probability is

$$\lambda_{gb}(t) = \frac{\varphi_g(t) - \varphi_g(t+1)}{\varphi_g(t)} \quad (2)$$

where ϕ_g(t)is the proportion of good patches at time t (table S1). The chance that a good patch remains a good patch is $1 - \lambda_{gb}(t)$. Equivalent relationships hold for the probability of a patch being bad.

The probability that a butterfly reaches a good patch by dispersal is equal to the relative frequency of good patches present in the next time step and is therefore equal to ϕ_g(t+1), if the butterfly disperses at time *t*. This is independent of the patch quality from the point of dispersal, and therefore there is no spatial correlation. The chance of reaching a bad patch is 1- ϕ_g(t+1). The seasonal variation is predictable in the sense that when a good patch turns into a bad patch, this patch does not become a good patch again until the next wet season. Hence, the only chance of

reaching a good patch is by dispersal. At the very end of the wet season there are so many bad patches that the chance of reaching a good patch is negligible. From year to year, the time at which a specific patch becomes good or bad is variable.

In our model, we separate data by generation, which is possible since we can trace each individual from birth. Individuals born from parents surviving the dry season are called, "first generation, wet season morphs". The offspring they produce are called "second generation, wet season morphs". These produce the individuals that survive the dry season, and these individuals are therefore called the "third generation, dry season morphs". Butterflies sometimes survive longer than one generation, but lose the ability to reproduce, so in reality no overlap in generations exists.

Larval stage

During the larval stage, the state of an individual is described by its body weight. Larvae are located in either a good or a bad patch, with food being available only in a good patch. The growth of a larva depends on its current weight, the availability of food, and temperature. At every time step (day) a larva consumes an amount of food that is partitioned between daily costs and growth. This is modeled as

$$w_L(t+1) = w_L(t) + E_L(p)[i_1 w_L(t)^{i_2+i_3 T(t)} - c_1 w_L(t)^{c_2+c_3 T(t)}] \quad (3)$$

where $w_L(t)$ is the weight of the larva with an initial value 1 mg, $E_L(p)$ is the effect of patch quality in the larval stage taking the value of 0 in a bad patch and 1 in a good patch. The constants i_1, i_2 and i_3 relate larval weight and temperature (T) to daily intake and c_1, c_2 and c_3 to daily costs. We select the values of these constants so that the model behavior corresponds to larval growth in Lepidoptera. Larval growth follows an S-shaped curve (Parker and Johnston 2006) with an exponential initial phase (Mackey 1978). At lower temperatures growth rate decreases, but final size at pupation increases due to longer development time (Atkinson 1994; Davidowitz and Nijhout 2004, see also figure A1), and this is also true for *B. anynana* (Oostra *et al.* 2010). Therefore, we model growth as being faster at higher temperatures, but with a lower asymptote of final weight. Pupation can be initiated when a larva reaches a weight larger than the critical weight for pupation, which is assumed to be independent of temperature, but see (Davidowitz *et al.* 2004; Nijhout *et al.* 2006). Alternatively, the larva can keep growing and pupate later. These two possibilities generate two different relationships for fitness which are compared in the SDP.

We let $F_L(t,p,w_L)$ denote the maximum accumulated reproductive success of an adult that ultimately emerges from a larva with weight w_L, in patch p at time t . For a larva opting to grow at time t, future fitness is

$$V_{grow}(t,p,w_L) = \sum_{p'=g}^{b} \lambda_{pp'}(t+1) F_L(t+1,p',w_L) S\{\mu_L, p(t), p(t-1)\} \quad (4)$$

We calculate the fitness of this individual for the cases that the patch will be a good patch or a bad patch at time t+1, and sum over these two options using the probabilities ($\lambda_{pp'}$) from equation (2), where p' indicates the patch quality at time t+1. Survival (S) depends on daily mortality (μ_L) which is constant, and thus independent of weight, and patch quality at this time step and the former

time step (t-1). Starvation is known to increase mortality rates for *Bicyclus* larvae (Bauerfeind and Fischer 2009) which is modelled by a severe reduction in survival if a bad patch is experienced for more than 1 day; they also tend to increase for insect larvae at higher temperatures (Oloumi-Sadeghi and Levine 1989; Padmanabha *et al.* 2011) - see appendix for further details.

We let $F_p(t,p,w_p,d)$ denote the maximum accumulated reproductive success of the adult that ultimately emerges, given that the pupa is in a patch of kind p, with pupal mass w_p and developmental state d at time t . Thus, for a larva that pupates, future fitness is

$$V_{pupate}(t,p,w_L) = \sum_{p'=g}^{b} \lambda_{pp'}(t+1) F_P(t+1,p',w_P,d) S\{\mu_L, p(t), p(t-1)\} \quad (5)$$

The developmental state of the pupa is initially zero (*d=0*), and the pupa will start development in the next time step. Survival is equal to that in equation (4). Again the fitness is summed over both kinds of patches with probabilities (λ) that the individuals will be in a good or bad patch as in equation (4).

The optimal decision (to grow or pupate) is then determined by the larger of the fitness values in equations (4) and (5):

$$F_L(t,p,w_L) = \max\{V_{grow}(t,p,w_L), V_{pupate}(t,p,w_L)\} \quad (6)$$

Pupal stage

During the pupal stage, an individual's state is described by weight and pupal developmental state. The development state is temperature- and time-dependent as a fixed number of degree-days are required before the adult can eclose and at higher temperatures it takes fewer days to complete pupal development (Koch *et al.* 1996; Oostra *et al.* 2010). For simplicity, pupal mortality (μ_p) is assumed to be constant as there are no data available for *Bicyclus,* and data concerning the relationship between pupal size and survival other Lepidoptera are inconsistent (Tammaru *et al.* 2002). Using the definition for $F_p(t,p,w_p,d)$ provided in equation (5), future fitness for a pupa that does not eclose and continues to develop is

$$V_{develop}(t,p,w_P,d) = \sum_{p'=g}^{b} \lambda_{pp'} F_P(t+1,p',w_P,d') \exp(-\mu_P) \quad (7)$$

where the developmental state increases from *d* to d' in both a good and bad patch, given by probability (λ), and survival is given by $exp(-\mu_p)$.

At the end of the pupal stage, the pupal weight is partitioned into thorax, abdomen, and fat, which represents the second strategic choice in the model. During the pupal stage, the costs for development, growth and maintenance differ among tissues (Kooijman 2009), which is implemented in the model as follows: for each 10 mg of pupal weight, 10 eggs of 0.1 mg each can develop. This yields a weight ratio from the pupal to the adult stage of 10:1 for abdominal tissue; for muscle tissue the corresponding ratio is 2:1 and for fat 1:1. Although no direct comparison can be made to data, a 90% decrease in muscle tissue in de-alated crickets only led to a doubling of the number of eggs produced (Tanaka 1991). Thus egg production is most costly, as eggs not only consist of proteins, but also need to be maintained in a damage-free condition (Kooijman 2010). The actual tissue-specific costs are unknown, but since exploration of a range of costs shows no qualitative change in the results, we continue with the ones described above, which

we consider to be reasonable estimates.

Fitness at the end of the pupal stage depends on the amount of resource allocated to fat, abdomen and thorax, and biological age *X* [see eq. (11) below] of the adult. Thus, if $F_A(t+1, p', w_f, w_a, w_t, x)$ represents the maximum accumulated reproductive success of an adult that is in patch of kind *p* with fat, abdominal and thorax masses w_f, w_a, w_t and biological age *x* at time *t* the fitness value of eclosing is

$$V_{eclose}(t, p, w_P, d) = \sum_{p'=g}^{b} \lambda_{pp'} F_A(t+1, p', w_f, w_a, w_t, 0) \exp(-\mu_P) \tag{8}$$

The conversion of pupal mass (w_p) to fat, abdominal and thorax masses depends on the allocation pattern as described above.

If the pupae are in a developmental state lower than the boundary (d_{max}) at which they will eclose, the fitness during this development (eq. [9a]) is equal to that described in equation (7). When the developmental state is equal to or larger than this boundary, the fitness value (eq. [9b]), at eclosion, is determined by the optimization of the allocation to the different adult tissues as described in equation (8). Therefore,

$$F_P(t, p, w_P, d \mid d < d_{\max}) = V_{develop}(t, p, w_P, d) \tag{9a}$$

$$F_P(t, p, w_P, d \mid d \geq d_{\max}) = \max_{allocation} \{V_{eclose}(t, p, w_P, d)\} \tag{9b}$$

where *allocation* refers to all possible ways the energy acquired during the larval stage can be allocated to the adult tissue fat (w_f), abdomen (w_a) and thorax (w_t) as described in equation (8).

Adult stage

During the adult stage, the state of a butterfly is described by the weight of its tissues (fat, abdomen and thorax) and its biological age. At every time step, an adult can undertake one of three behaviors, B: to feed, disperse or reproduce. When feeding, the weight of fat increases. This increase in fat decreases with biological age so as to reflect lower feeding rates (Wong *et al.* 2009). If the butterfly disperses, fat decreases by an amount dependent on total weight, weight of the thorax and temperature. Reproduction decreases the number of eggs present in the abdomen, and additionally the development of these eggs costs 1 mg of fat per egg. The effect of the different strategic choices on the daily changes in fat is summarized as feeding:

$$w_f(t+1) = (1-q)w_f(t) + I_A - C_A \tag{10a}$$

dispersing:

$$w_f(t+1) = (1-q)w_f(t) - C_A - T(t) f\{w_f(t), w_t(t), w_a(t)\} \tag{10b}$$

and reproducing:

$$w_f(t+1) = (1-q)w_f(t) - C_A - N_{eggs} \tag{10c}$$

where $w_f(t)$ is the weight of fat at time *t*, I_A is daily intake for feeding individuals, C_A are the daily costs, *T* is temperature and N_{eggs} represents the cost for laying eggs. The function which relates

the weight of fat, abdomen and thorax to cost of dispersal ($f\{w_f(t), w_t(t), w_a(t)\}$) increases with weight of fat and abdomen, but decreases with thorax. Thus a relatively larger thorax ensures that dispersal is less costly. The variable *q* represents the proportion of fat used for maintenance and repair, which affects the rate of increase in biological age, *X(t)*. The actual value of *q* is a strategic choice for adults and can change every time step. Biological age is irreversible and increases at every time step by an amount determined by the energetic investment in maintenance and repair processes, total weight, and temperature,

$$X(t+1) = X(t) + \frac{a_1 w_{total}(t) + a_2 (T(t) - a_3)}{rqw_f(t)} \quad (11)$$

The parameter *r* is a conversion constant relating an amount of fat ($w_f(t)$) to an amount of damage repair. Damage increases faster for individuals with more tissue and at higher temperatures. Further constants a_1, a_2 and a_3 are also introduced.

Mortality during the adult stage is dependent on predator pressure, biological age and weight of the adult. Predator pressure is lower in the bad patches compared to good patches corresponding to field observations (Lyytinen *et al.* 2004; Brakefield and Frankino 2009; Joiris *et al.* 2010). We model total mortality rate as

$$\mu_{total} = \mu_X(X(t)) + \mu_{pred}(p) + \mu_w(w_{total}) \quad (12)$$

where intrinsic mortality (μ_X) depends on biological age (*X(t)*), predation-related mortality ($\mu_{pred}(p)$) depends on patch quality, and weight-related mortality ($\mu_w(w_{total})$) depends on total weight. The latter sets a lower boundary for the weight at which butterflies can still survive. Mortality due to biological age is

$$\mu_X(X(t)) = a_4 + a_5 \exp(a_6 X(t)) \quad (13)$$

where a_4, a_5 and a_6 are constants.

The future fitness for an adult following behavior *B* (see eq. [10]) is

$$V_{B,q}(t, p, w_f, w_a, w_t, X) =$$

$$[\sum_{p'=g}^{b} \lambda(B) F_A\{t+1, p', w_f', w_a', w_t', X'\} + N_{eggs}(B) F_L(t, p, 1)] \Delta(B) \exp(-\mu_{total}) \quad (14)$$

where *B* represents the decision for behavior (see eq. [10]). The chance of reaching a certain type of patch in the next time step (λ) is dependent on behavior since in the adult stage the adult can either feed or reproduce, and λ will then be calculated using equation (1), or disperse, when λ will be calculated using equation (2). When individuals lay eggs, $N_{eggs}(B)$ is 10; otherwise it is zero. Each egg, equivalent to a larva of weight 1 mg, provides a fitness benefit of $F_L(t,p,1)$, which is dependent on patch quality (*p*) and (*t*) and equivalent to the fitness of a larva with a weight of 1 mg from equation (3). The reason for modeling $F_L(t,p,1)$ as dependent on patch quality and time is that larvae cannot survive in the dry season. Therefore, eggs laid by a butterfly in the dry season would be expected to result in a lower fitness benefit than those laid in the wet season. Survival ($exp(-\mu_{total})$) is multiplied by a term (Δ(B)) which is required because survival is affected by dispersal and takes a value of 0.75 for individuals that disperse, otherwise it is 1. The change in biological age from *X* to *X'* depends on *q*, the amount of energy allocated to maintenance and repair.

The optimal decisions for *B* and *q* give the maximum accumulated reproductive success of an adult, $F_A(t, p, w_f, w_a, w_{t,}, x)$:

$$F_A(t, p, w_f, w_a, w_t, x) = \max_{B,q} V_{B,q}(t, p, w_f, w_a, w_t, x) \quad (15)$$

Backwards iteration

We determine the optimal strategies by finding the maximum value for fitness from equations (6) for the larval stage: (9) for pupae and (15) for adults. The SDP algorithm, which works backwards in time, is initiated at a nominal time horizon by assigning the fitness for all larvae with a weight of 1 mg (newly born) a value of 1, i.e. $F_L(t,p,1) = 1$ in equation (4). The fitness of all other states are 0. As the environmental parameters between years for a given time step are the same, the fitness values on a specific day for each combination of states converge to a single value. Following convergence, the fitness values and optimal strategies for each state are stored to be used for forward simulations.

Forward simulation

In the forward simulation we model two scenarios. Firstly, individuals are simulated by the introduction of 100 larvae of a size of 1 mg at the start of the wet season (time step 175). The temperature and proportion of good patches used for these simulations are the same as those used in the backwards iterations, see table S1. The output is the numbers of individuals, their life history strategies, and their traits. Secondly, we simulate the experimental setup of Saastamoinen *et al.* (2010). In our model, there are good and bad patches and during the backward optimization the patches vary seasonally. In the laboratory experiment one group was fed optimal food as larvae throughout development, while another was starved in the last stage of larval development. For the model all patches are assumed to be good at the start of a forward simulation, but all turn into bad patches when food manipulation during the larval stage is needed. In the optimization, the adults can choose to feed, fly or reproduce. During the forward simulation, adults are forced to perform a series of flight bouts by fixing their strategy to flight rather than following their optimal strategy in the first few time steps but can then behave optimally according to when in the seasonal cycle they live (first, second or third generation).

States and strategic decision possibilities

We discretize state variables as detailed in the appendix (table S2). For the strategies, in every time step, larvae can choose to start pupation. Pupae can allocate from 5% to 90% to every tissue in steps of 5% with the total constrained to 100%. Adults can choose one behavior from feeding, dispersal, reproduction in each time step. Also they can reallocate energy to maintenance and repair for 0 to 8% of the fat tissue every day in 10 steps between 0 and 8%. In initial runs of the model, no individual ever spent more than 8% on maintenance and repair; therefore, we reduce the choice to this number.

Typically in a state dependent model a robustness test is performed. This is carried out by altering the parameters systematically and checking whether the optimal strategic decisions and overall results show dramatic changes. If such sensitivity is displayed, then the generality of the results is limited; however, if the results are stable, the generality is increased. In the appendix, a detailed description is given of the robustness tests we perform. Most of the results are qualitatively stable, except for the number of generations, which can be altered by varying larval growth rates (i.e. by changing parameter $E_L(p)$ in a good patch, eq. [3]).

Results

Population and the seasonal life history strategies based on the model

The numbers of larvae, pupae and adults in good and bad patches in the model are shown in table 1 and figure 2. As in the wild, the dry season morph survives throughout the dry season without reproducing, and reproduction is initiated at the start of the next wet season. The first wet season generation of butterflies will produce the second wet season generation, which in turn produces offspring that survive over the next dry season.

Most larvae (98.0%) in the first wet season generation pupate in good patches, while this number is reduced (87.9%) in the second generation. If during larval growth a patch changes from good to bad, larvae initiate pupation. In the dry season generation, most larvae are in a patch that changes from good to bad, and therefore, they start to pupate in a bad patch (99.5%). Based on our model, the average pupation size is larger in the first than in the second wet season generation (table 1 & fig. S2). Individuals in the third generation (dry season) are on average the largest, (table 1 & fig. S2). Those larvae of the second wet season generation that pupate later, do so at a smaller size even in good patches, which explains the variation in size. Within the dry season, higher variance is explained by habitat heterogeneity, and consequently by the fact that some individuals are able to prolong their growth and increase their size, whereas others run out of food and remain small.

Life history strategies (summarized in table 1) are determined by the resource allocation patterns of individuals, as those with larger abdomens can lay more eggs, while those that allocate resources into thorax require less energy for a single dispersal event between patches. Fat is used as storage to support activities such as egg production, dispersal, and damage repair. During the pupal stage, individuals of the first wet season generations allocate more to fat than abdomen when they are in good patches, while this is reversed in the second wet season generation (table 1 & fig. S3). Individuals of the two wet season generations in bad patches allocate more resources to fat than to abdomen, while dry season individuals allocate equal to fat and abdomen in good and bad patches (table 1 & fig. S3). Individuals of the wet season generations allocate much more to thorax in bad, compared to good patches, indicating investment in dispersal (table 1 & fig. S3).

As adults, individuals have a choice of feeding (acquiring more resources), reproducing or dispersing, and these behaviors are highly dependent on season and patch quality. Since only bad patches are available during the dry season, all individuals feed in order to survive the dry season (fig. 3*A*). Reproduction is not beneficial in terms of fitness, as the larvae would not survive. Reproduction only takes place in good patches (table 1 & fig.3*B*). Individuals in the wet season that are still able to reproduce but are in a patch that changes from good to bad, disperse (table 1 & fig. 3*C*). In every generation some individuals also disperse from good to bad patches (table 1 & fig 3C), but these individuals have lost the capacity to reproduce as they have insufficient fat storage (not shown), and hence their behavior does not influence their fitness or the number of larvae in the population. Young individuals without fat reserves can take up energy and regain the capacity to reproduce, whereas old individuals that survived the dry season cannot.

In the wet season, adults use fat mostly for reproduction (fig. 3*D*), whereas in the dry season, individuals do not reproduce or disperse, and rather use fat to reallocate resource to maintenance and repair (fig. 3*G*). Therefore, butterflies age more rapidly in the wet season compared to the dry season (fig. 3*F*).

Table 1: Life history traits and strategies separated by generation and patch quality. Average values (sd) of indicated strategies and traits for the different stages (larval , pupal and adult) of the three generations in good and bad patches. Larval and pupal traits are measured once while those of the adults were measured every day in the life of the individual. N indicates the number of individuals in a single simulation

	Wet season form 1		Wet season form 2		Dry season form	
	Good	Bad	Good	Bad	Good	Bad
Larval and pupal strategies:	N=19759	n=407	n=83486	n=11484	N=863	n=200274
Size at pupation	138.7 (17.9)	151.2 (21.9)	145.8 (22.9)	141.8 (18.8)	239.5 (1.2)	179.9 (32.5)
Allocation fat	0.48 (0.02)	0.45 (0.01)	0.46 (0.022)	0.46 (0.04)	0.45 (0.00)	0.47 (0.02)
Allocation abdomen	0.47 (0.02)	0.41 (0.02)	0.49 (0.023)	0.44 (0.02)	0.45 (0.00)	0.47 (0.02)
Allocation thorax	0.05 (0.00)	0.14 (0.03)	0.05 (0.01)	0.10 (0.05)	0.10 (0.00)	0.06 (0.02)
Adult optimal behavior:	N=60782	n=42396	n=55912	n=67411	N=127158	N=526666
Proportion feeding	0.78 (0.002)	1.00 (0.000)	0.67 (0.001)	0.99 (0.000)	1.00 (0.00)	1 (0)
Proportion reproducing	0.21 (0.002)	0 (0)	0.29 (0.002)	0 (0)	0.00 (0.00)	0 (0)
Proportion dispersing	0.01 (0.000)	0.00 (0.000)	0.00 (0.001)	0.01 (0.000)	0.00 (0.00)	0 (0)
Body composition adults:	N=60782	n=42396	n=55912	n=67411	N=127158	n=526666
Proportion fat	0.37 (0.002)	0.40 (0.002)	0.39 (0.002)	0.31 (0.002)	0.03 (0.000)	0.28 (0.001)
Proportion abdomen	0.48 (0.002)	0.43 (0.002)	0.50 (0.002)	0.61 (0.002)	0.84 (0.001)	0.63 (0.001)
Proportion thorax	0.15 (0.001)	0.17 (0.002)	0.11 (0.001)	0.08 (0.001)	0.13 (0.001)	0.09 (0.001)
Adult traits:	N=60782	n=42396	n=55912	n=67411	N=127158	n=526666
Fecundity	44.6 (16.9)	40.6 (3.2)	23.3 (27.1)	3.9 (12.8)	1.1 (3.1)	0.0 (0.2)
Age	16.9 (14.8)	57.5 (26.5)	16.9 (38.6)	79.6 (63.4)	230.3 (32.6)	88.7 (61.0)
Biological age	16.6 (15.9)	51.0 (20.2)	9.7 (19.3)	30.1 (30.1)	105.4 (38.6)	23.0 (25.2)
Fat used per day for repair	0.24 (0.13)	0.26 (0.06)	0.66 (0.55)	0.63 (0.33)	0.20 (0.09)	0.87 (0.49)

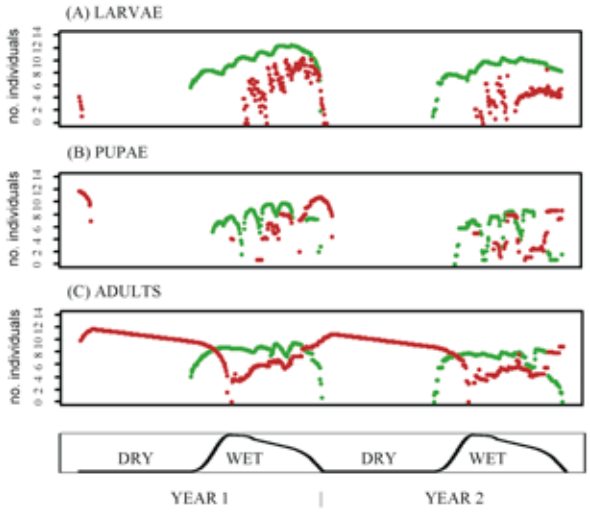

Figure 2. Number (log transformed) of individuals ((A) larvae, (B) pupae, & (C) adults) across the seasons in good (green) and bad (brown) patches. Each point indicates the number of individuals on a specific time step (day) during the simulation. Two years are simulated, consisting of two dry and two wet seasons, as indicated below the graph. In the lowest panel, the line represents the proportion of good patches in each time step.

Finally, the average lifespan differs among the three generations, and is highest in the dry season morph when butterflies can only reproduce at an advanced age as the next wet season begins (table 1 & fig. 2). Due to lower survival and reproduction at an older age, the average fecundity in the dry season generation is much lower than in the two wet season generations (table 1 & fig. 2). Within the wet season, individuals of the first generation have a lower lifespan but higher fecundity compared to the second generation (table 1). In good patches, fat used for maintenance and repair per day is highest in the second wet season generation, while in bad patches, it is highest in dry season individuals (table 1).

Predictive adaptive response

We test whether food shortage during the larval stage or an increase in dispersal events during the adult stage influenced lifespan and fecundity. Figure 4 shows the effect of larval food manipulation on thorax ratio and early fecundity compared to the previous laboratory experiment. Both in the experiment and in the model, the thorax ratio is higher for individuals that were restricted during late larval development (fig. 4*A* & *B*). In the experiment, early fecundity was lower for individuals that were food-restricted (fig. 4*C*). In the model, the abdomen weight (proxy for fecundity) is also lower for restricted individuals (fig. 4*D*). The percentage of thorax in the experimental butterflies was 24.1 and 25.7% of the total body weight for *ad libitum* and restricted individuals, respectively (Saastamoinen *et al.* 2010), while in the model these values are lower, 5.0 and 10.0%. The standard deviation of the thorax ratio is larger in the bad patch individuals in the model prediction. This is because the timing of encountering a bad patch varies widely among individuals, leading to higher variation in their weight compared to those in good patches.

Figure 5 shows the effect of larval and adult manipulation on lifespan and fecundity. These two traits are less affected by the adult treatment (forced flight events) when larvae had

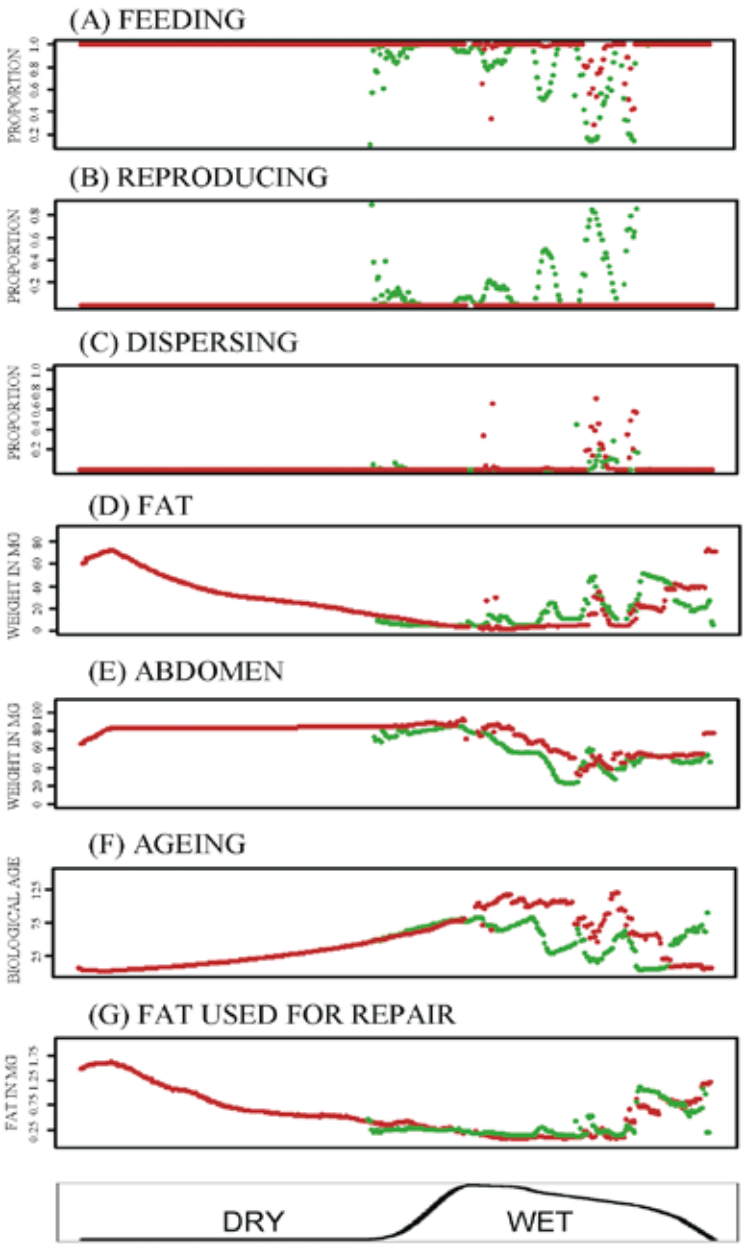

Figure 3. A-C show the proportion of individuals feeding, reproducing, and dispersing (in respective order). D and E represent the average weight of fat tissue and abdomen, and F and G show variation in aging and the amount of fat used for repair. The data is represented for individuals in good (green) and bad (brown) patches dependent on the seasonal variation, which is indicated by the bar below the graph (where the line represents the proportion of good patches in each time step).

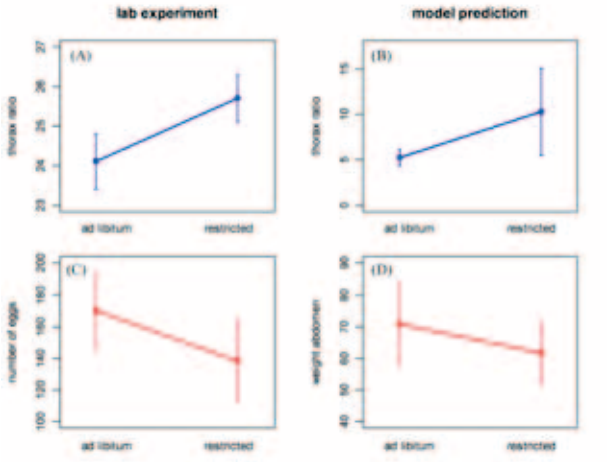

Figure 4. Comparison between the experimental data and the model predictions. A & B show the average thorax ratio (±sd) in the experiment and in the model, respectively, from one simulation. The lower panels show the mean number of eggs (±sd) during the first two weeks in the experiment (C) and the weight of the abdomen in the model (D).

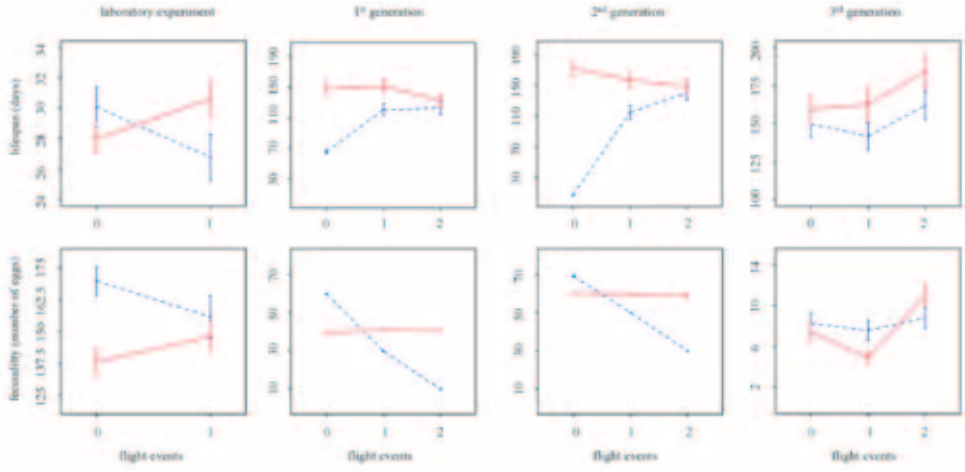

Figure 5. Comparison of the data acquired in the laboratory experiment and the first, second and third generation of butterflies in the model. Top and lower panels present lifespan and fecundity, respectively. Note that the y-axis might differ per subgraph. Individuals which were food restricted as larvae are shown in red (solid lines) and individuals from the optimal larval treatment are shown in blue (dashed lines). Error bars indicate standard errors for mean values.

experienced food limitation, compared to individuals with an optimal larval period in the wet season (fig. 5). This is because in both wet season generations, allocation to the thorax is higher for individuals deprived of food in the late larval stage (data not shown). In the wet season, lifespan is positively related to flight events while fecundity is negatively related to flight events for individuals with an optimal larval period (fig. 5). For individuals that experience food shortage during the larval stage, lifespan and fecundity are both largely unaffected by the adult treatment. Larval treatment groups do not respond differently to adult treatment for dry season individuals (fig. 5). When comparing the results between model and experiment, the effects of flight on fecundity are more similar than those on lifespan (fig. 5), as the optimally-reared individuals have decreased fecundity when forced to fly, both in the model and in the experiment. Fecundity and lifespan within a larval treatment group are negatively related in the model, while in the experiment they were positively related.

Discussion

In this study we describe a state-dependent energy allocation model of the life history of *Bicyclus anynana*, which we use to test whether a specific plastic response observed under laboratory conditions could potentially be adaptive in nature. Food-restricted larvae in both the experiment and the model allocated more energy to dispersal ability, which in the model made them more likely to reach a good-quality patch and hence reproduce successfully. We thus conclude that a predictive adaptive response evolved in *B. anynana*. However, this specific predictive adaptive response is likely to be only adaptive in one of the seasons, the wet season, as in the dry season no extra allocation to dispersal ability occurred in response to larval food restriction. This is expected as the benefit of increased dispersal is lacking in this season due to lack of good quality habitat patches.

Life history and seasonal variation

Seasonal change in our model consists of variation in temperature and the percentage of good patches, both of which are lowest in the dry season and peak in the wet season. This pattern is comparable to measurements taken from the site where *B. anynana* has been studied in Malawi (Brakefield and Reitsma 1991; Windig *et al.* 1994). Similarly, predicted seasonal population dynamics emerge from the model as the dry season form adults delay their first reproduction and initiate it only early in the wet season to yield the first generation of the wet season form. This generation then produces a second wet season generation, which is followed by a new dry season generation.

An important question for any state-dependent energy-allocation model is to consider the relationship between the assumed physiological rules and the inferred optimal behavior. In our model, larval growth is influenced by temperature, so that at higher temperatures growth rate is higher but maximum possible size is lower, as is common for insects (Atkinson 1994; Davidowitz and Nijhout 2004). In the dry season, individuals are, on average, larger and temperature is lower. Therefore, we expect the optimal life history decision to be to prolong larval growth. This is partly facilitated by the assumed physiology in the model, since larvae can reach a higher plateau of size at lower temperatures. In a version of the model in which temperature does not influence larval

growth, the size differences between the different generations of butterflies remain, suggesting that another factor additional to the assumed physiology contributed to the size variation. A possible explanation is that optimal size varies between seasons, and that larger body size is favored in the dry season.

In addition to variation in size, we also find variation in allocation patterns, which vary with the generation and the quality of the patches in which the larvae are present. We find a large number of larvae in the second wet season generation in patches that change from good to bad in quality. Our model shows that when this occurs, individuals allocate more resources to their thorax during the pupal stage, which is comparable to the findings of earlier laboratory studies (Saastamoinen *et al.* 2010). An increased thorax ratio allows individuals to disperse more easily to a new, good-quality patch, where they can successfully reproduce. In our model, we presume that individuals which allocate more to thorax during the pupal stage use less energy per flight event based on the observation that individuals with a higher thorax ratio are better fliers (Marden 2000). Thus the cue of food deprivation during the larval stage directs the allocation pattern in the pupal stage towards a more optimal phenotype (i.e. higher thorax ratio). The pupae of the dry season generation do not show this allocation pattern since the allocation to thorax is equal in bad and good patch individuals. The model's prediction is thus that in the field, pupae from dry patches with low quality plants will allocate more to the thorax during the middle of the wet season when some good patches remain. Later in the wet season, this allocation to the thorax becomes less favorable because the likelihood of reaching a good patch decreases as the number of bad quality patches increases.

Predictive adaptive response

The second aim of the model is to test whether the environment of *B. anynana* is expected to favor evolution of a predictive adaptive response. Experiments have previously shown that individuals reared as the wet season form had an increased resistance to adult manipulation (flight stress) when food-restricted during the final larval stage (Saastamoinen *et al.* 2010). Interestingly, in our model, individuals are also less affected by this adult treatment if they are food-restricted during the final larval stage. The increased ability to cope with forced flight events is facilitated by an increase allocation to thorax in the model, consistent with what was observed in the experiment (Saastamoinen *et al.* 2010). The relationship between food limitation, increased thorax ratio, and resistance to increased flight events does not appear in the model for the dry season generation.

The patterns of relative response to food manipulation in the experiment and in the model with respect to allocation to thorax are thus very similar. However, the thorax ratio in the model is, on average, lower than in the experiment (fig. 4*A* & *B*). This difference can be explained by a lack of detailed realism concerning the function of the thorax in the daily routine movement of a butterfly. In the model, the thorax is not necessary in the search for food or mating partners, but only for dispersal. However, the argument that increasing thorax ratio in restricted conditions is adaptive is supported by the model, which suggests that these adaptations can be studied in this type of model. In addition, (Oostra *et al.* 2010) showed that the relative thorax weight is higher for individuals reared on good quality plants as dry season morphs compared to wet season morphs which is true in our model for the individuals in good patches (but not in bad patches, fig S3).

There are also differences between the model and experiment in the results concerning the relationship between fecundity and lifespan, a positive correlation between these traits

being found only in the experiment. This difference may be partially due to any allocation to fecundity leading to a larger increase in intrinsic mortality rate in the model. In reality a number of other traits are also likely to co-vary with fecundity and lifespan (e.g. immunological responses, metabolic rate, activity, stress resistance; (Boggs 2009) but such relationships are not included here. Since the larvae in both the model and experiment are treated equally, the inconsistency is unlikely to be caused by a difference in acquisition during the larval stage, which may potentially also lead to positive relationships between life history traits (De Jong and Van Noordwijk 1992).

We have modeled an organism which lives for a short time compared to the length of the seasonal period (i.e. shorter than a year). Besides sampling the environment directly (Krebs *et al.* 1978; Mangel and Roitberg 1989), information can additionally be passed on via maternal effects. Whether it is adaptive to pass on information from one generation to the next is very much dependent on the lifespan compared to the length of a seasonal period (Lachmann and Jablonka 1996). The type of maternal effect that evolves is likely to be highly dependent on parameters such as life-expectancy, environmental fluctuations and predictability, presence of parent-offspring conflict and constraint and costs of producing offspring with specific phenotypes (Marshall and Uller 2007). These will be quite different between long-lived organisms and short-lived insects, and, therefore it is unlikely that any maternal effects in the modeled organism could be translated to the cases of long-lived organisms. A model similar to the one described here can be developed for long-lived organisms, when agreement is reached about the relevant ecological parameters. Such an approach is likely to benefit the discussion around PAR.

In conclusion, the evolution of developmental plasticity resulting in the two distinct adult morphs emerged as a robust prediction of the model. In addition, we show that a short-lived organism, such as *B. anynana*, living in a seasonal environment can evolve a PAR. The model is based on the considerable biological and ecological knowledge we have on this species, accumulated through extensive field and laboratory studies. However, even without this extensive knowledge state-dependent modeling is a valuable tool as it allows for freedom of parameters. The combination of modeling and experiments promises to be a constructive way to test the adaptive value of plasticity in this species and potentially in others, as it facilitates the evolutionary and ecological interpretation of laboratory experiments.

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Table A2. List of traits with their minima, maxima, and number of steps for the larval, pupal and adult stage.

Larval stage			
Trait	Minimum	Maximum	Number of steps
Weight	1	290	500
Pupal stage			
Trait	Minimum	Maximum	Number of steps
Weight	65	300	42
Development time	0	540	55
Adult stage			
Traits	Minimum	Maximum	Number of steps
Weight fat storage	0	147.5	60
Weight abdomen	0	110	12
Weight thorax	0	12	3
Ageing	0	197.5	80

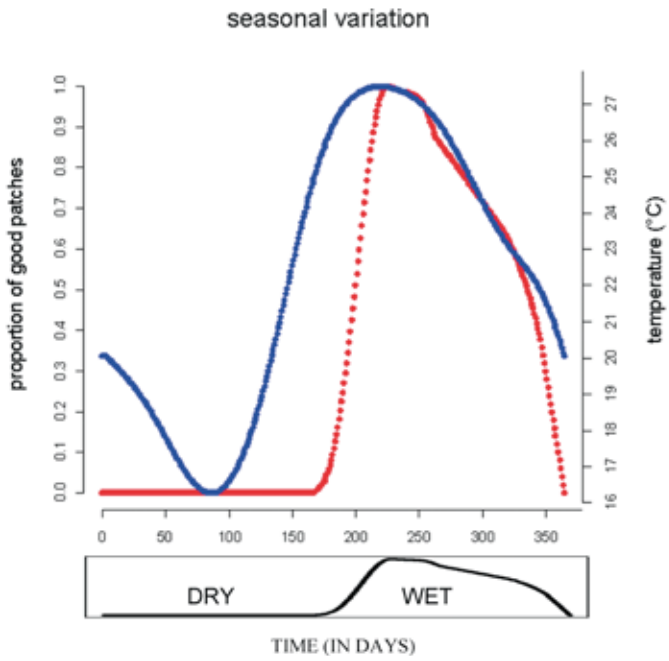

Figure S1. Seasonal variation in proportion of good patches (red) and in temperature (blue). The panel below indicates the season, and the line represents the proportion of good patches in each time step (numbers in x-axes).

Table S1. Environmental variation. For every day the temperature and proportion of good patches are shown. The proportion of bad patches is equal to one minus the proportion of good patches.

Day	Temperature	Proportion good patches	Day	Temperature	Proportion good patches	Day	Temperature	Proportion good patches
1	20.0448	0	123	18.4462	0	245	27.2211	0.979902
2	20.0682	0	124	18.5678	0	246	27.1987	0.978828
3	20.0605	0	125	18.6921	0	247	27.1753	0.977754
4	20.0398	0	126	18.8192	0	248	27.1509	0.976682
5	20.0125	0	127	18.9488	0	249	27.1253	0.97454
6	19.9815	0	128	19.081	0	250	27.0987	0.972402
7	19.9485	0	129	19.2156	0	251	27.0709	0.970268
8	19.9142	0	130	19.3525	0	252	27.042	0.967073
9	19.8791	0	131	19.4916	0	253	27.0119	0.963889
10	19.8434	0	132	19.6328	0	254	26.9807	0.959655
11	19.8073	0	133	19.7761	0	255	26.9482	0.954384
12	19.7709	0	134	19.9212	0	256	26.9146	0.948091
13	19.7342	0	135	20.068	0	257	26.8798	0.940792
14	19.6972	0	136	20.2165	0	258	26.8437	0.93251
15	19.6599	0	137	20.3665	0	259	26.8064	0.923267
16	19.6223	0	138	20.5178	0	260	26.7678	0.913089
17	19.5843	0	139	20.6704	0	261	26.728	0.90402
18	19.5458	0	140	20.824	0	262	26.6869	0.896032
19	19.5069	0	141	20.9785	0	263	26.6446	0.884688
20	19.4674	0	142	21.1339	0	264	26.6011	0.875328
21	19.4272	0	143	21.2898	0	265	26.5562	0.871054
22	19.3865	0	144	21.4463	0	266	26.5102	0.866779
23	19.345	0	145	21.603	0	267	26.4628	0.862505
24	19.3027	0	146	21.7599	0	268	26.4143	0.858231
25	19.2597	0	147	21.9169	0	269	26.3645	0.853957
26	19.2157	0	148	22.0736	0	270	26.3135	0.849683
27	19.1709	0	149	22.2301	0	271	26.2613	0.845409
28	19.1251	0	150	22.386	0	272	26.2079	0.841135
29	19.0783	0	151	22.5413	0	273	26.1534	0.836861
30	19.0305	0	152	22.6958	0	274	26.0978	0.832587
31	18.9817	0	153	22.8494	0	275	26.041	0.828313
32	18.9319	0	154	23.0018	0	276	25.9832	0.824039
33	18.881	0	155	23.1529	0	277	25.9243	0.819765
34	18.829	0	156	23.3026	0	278	25.8644	0.815491
35	18.776	0	157	23.4508	0	279	25.8036	0.811217
36	18.7219	0	158	23.5972	0	280	25.7418	0.806943
37	18.6668	0	159	23.7418	0	281	25.6791	0.802669
38	18.6107	0	160	23.8843	0	282	25.6155	0.798395
39	18.5536	0	161	24.0248	0	283	25.5512	0.794121
40	18.4956	0	162	24.163	0	284	25.4862	0.789847
41	18.4367	0	163	24.2988	0	285	25.4204	0.785573
42	18.3769	0	164	24.4321	0	286	25.354	0.781299
43	18.3163	0	165	24.5629	0	287	25.287	0.777025
44	18.255	0	166	24.6909	0	288	25.2195	0.772751
45	18.193	0	167	24.8162	0.000945	289	25.1516	0.768477
46	18.1304	0	168	24.9386	0.002282	290	25.0832	0.764203
47	18.0673	0	169	25.0581	0.004024	291	25.0145	0.759929
48	18.0037	0	170	25.1746	0.006192	292	24.9456	0.755655
49	17.9398	0	171	25.288	0.00881	293	24.8765	0.75138
50	17.8756	0	172	25.3983	0.01191	294	24.8072	0.747106
51	17.8113	0	173	25.5054	0.015529	295	24.7379	0.742832
52	17.7469	0	174	25.6093	0.019711	296	24.6687	0.738558
53	17.6825	0	175	25.71	0.024508	297	24.5995	0.734284
54	17.6182	0	176	25.8074	0.029983	298	24.5305	0.73001
55	17.5542	0	177	25.9016	0.036209	299	24.4618	0.725736
56	17.4905	0	178	25.9925	0.043268	300	24.3933	0.721462
57	17.4273	0	179	26.0801	0.051259	301	24.3253	0.717188
58	17.3647	0	180	26.1645	0.060297	302	24.2577	0.712914
59	17.3028	0	181	26.2456	0.070515	303	24.1907	0.70864
60	17.2416	0	182	26.3236	0.082068	304	24.1242	0.704366
61	17.1814	0	183	26.3983	0.095135	305	24.0584	0.700092
62	17.1222	0	184	26.4699	0.109928	306	23.9934	0.695818

63	17.0641	0	185	26.5383	0.126692	307	23.9292	0.691544
64	17.0072	0	186	26.6037	0.144389	308	23.8658	0.68727
65	16.9517	0	187	26.6661	0.163024	309	23.8033	0.682996
66	16.8977	0	188	26.7255	0.182597	310	23.7418	0.678722
67	16.8453	0	189	26.7821	0.203104	311	23.6812	0.674448
68	16.7945	0	190	26.8357	0.224533	312	23.6218	0.670174
69	16.7455	0	191	26.8867	0.246866	313	23.5634	0.6659
70	16.6984	0	192	26.9349	0.27008	314	23.506	0.661626
71	16.6532	0	193	26.9804	0.294144	315	23.4499	0.657352
72	16.6102	0	194	27.0235	0.319018	316	23.3948	0.653078
73	16.5693	0	195	27.064	0.344657	317	23.3409	0.648592
74	16.5306	0	196	27.1021	0.371008	318	23.2881	0.643762
75	16.4943	0	197	27.1379	0.398008	319	23.2364	0.638594
76	16.4604	0	198	27.1715	0.42559	320	23.1858	0.633096
77	16.429	0	199	27.2029	0.453676	321	23.1363	0.627275
78	16.4002	0	200	27.2321	0.482182	322	23.0878	0.621139
79	16.3741	0	201	27.2594	0.511017	323	23.0402	0.614698
80	16.3507	0	202	27.2847	0.540082	324	22.9936	0.607961
81	16.3301	0	203	27.3081	0.569274	325	22.9477	0.600936
82	16.3123	0	204	27.3297	0.598481	326	22.9026	0.593634
83	16.2975	0	205	27.3496	0.627587	327	22.8581	0.586064
84	16.2856	0	206	27.3679	0.656471	328	22.8141	0.578238
85	16.2769	0	207	27.3845	0.685008	329	22.7705	0.570164
86	16.2711	0	208	27.3997	0.713071	330	22.7271	0.561856
87	16.2686	0	209	27.4133	0.740529	331	22.6839	0.553323
88	16.2692	0	210	27.4256	0.767251	332	22.6406	0.544577
89	16.2731	0	211	27.4365	0.793104	333	22.597	0.53563
90	16.2802	0	212	27.4461	0.817957	334	22.5531	0.526493
91	16.2906	0	213	27.4545	0.841682	335	22.5086	0.517178
92	16.3044	0	214	27.4617	0.864151	336	22.4633	0.507696
93	16.3215	0	215	27.4677	0.885241	337	22.4171	0.498061
94	16.3421	0	216	27.4727	0.904836	338	22.3697	0.488283
95	16.3661	0	217	27.4766	0.922825	339	22.3209	0.47721
96	16.3935	0	218	27.4794	0.939102	340	22.2706	0.465776
97	16.4245	0	219	27.4813	0.953573	341	22.2185	0.454009
98	16.4589	0	220	27.4822	0.96615	342	22.1645	0.441942
99	16.4968	0	221	27.4821	0.976757	343	22.1083	0.429603
100	16.5382	0	222	27.4811	0.985327	344	22.0498	0.417024
101	16.5832	0	223	27.4793	0.991806	345	21.9887	0.404236
102	16.6317	0	224	27.4766	0.996151	346	21.925	0.391268
103	16.6838	0	225	27.473	0.998333	347	21.8585	0.378152
104	16.7393	0	226	27.4685	0.998333	348	21.7891	0.358303
105	16.7985	0	227	27.4632	0.998333	349	21.7167	0.338454
106	16.8611	0	228	27.4571	0.998333	350	21.6411	0.318606
107	16.9273	0	229	27.4501	0.99724	351	21.5624	0.298757
108	16.997	0	230	27.4423	0.996148	352	21.4804	0.278908
109	17.0702	0	231	27.4337	0.995057	353	21.3953	0.259059
110	17.1469	0	232	27.4243	0.993968	354	21.3069	0.239211
111	17.227	0	233	27.414	0.992879	355	21.2155	0.219362
112	17.3106	0	234	27.4029	0.991792	356	21.1209	0.199513
113	17.3976	0	235	27.3909	0.990706	357	21.0234	0.179664
114	17.4879	0	236	27.378	0.98962	358	20.923	0.159815
115	17.5816	0	237	27.3643	0.988536	359	20.82	0.139967
116	17.6787	0	238	27.3497	0.987453	360	20.7145	0.120118
117	17.7789	0	239	27.3341	0.986371	361	20.6067	0.100269
118	17.8824	0	240	27.3177	0.98529	362	20.4969	0.08042
119	17.9891	0	241	27.3003	0.984211	363	20.3853	0.060572
120	18.0989	0	242	27.282	0.983132	364	20.2722	0.040723
121	18.2117	0	243	27.2627	0.982054	365	20.1579	0.020874
122	18.3275	0	244	27.2424	0.980978	366	20.0428	0.001025

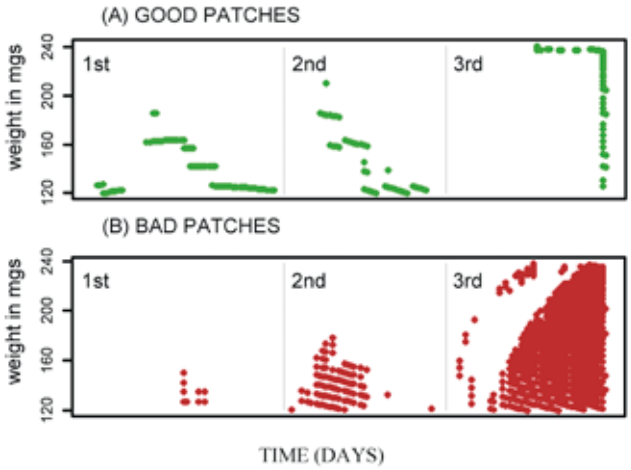

Figure S2. Size at the start of pupation in good (A) and bad (B) habitat patches. The x-axis indicates time steps in days in the wet (1st & 2nd generation) and dry (3rd generation) seasons.

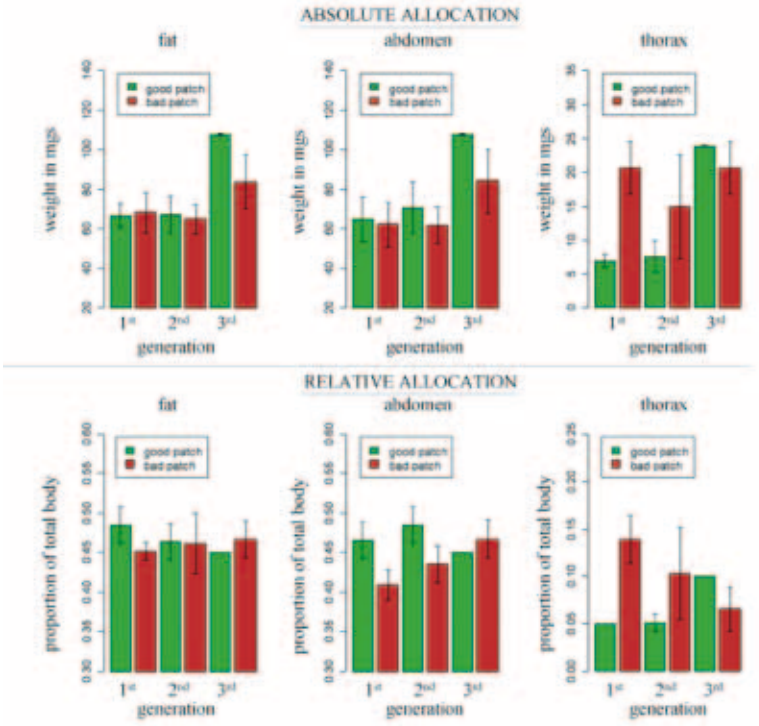

Figure S3. Mean (±sd, over one simulation) allocation to fat, abdomen and thorax in absolute (above) and relative terms (below). In every panel the 1st, 2nd, and 3rd generations are shown. Green and brown bars represent individuals from good and bad patches, respectively.

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Appendix A: Mathematical Equations

In this appendix, mathematical descriptions of metabolism and the effect of strategic decisions on the traits are given. Energy uptake and use for specific processes depend on the environment and state of the organism. Strategic choices can influence how energy is invested. The different strategic choices represent either genetic variation or plastic responses. The model calculates which strategy is the best in which environment. The equations of which the model consists will be described for each stage (larva, pupa, adult) of the life history. We then describe how the environment is modeled with respect to patch quality and its changing nature. All constants and variables that take two levels (patch quality, predation pressure) are listed in table A1.

Table A1. Model constants and their values

Constant	Value
a_1 a_2 a_3 a_4 a_5 a_6 a_7	2.5 0.1 15 0.0025 0.0005 0.025 0.001
c_1 c_2 c_3 c_4 c_5	0.045 0.8 0.021 0.025 1
d_1 d_{max}	1.5 540
E_L(p=good) E_L(p=bad) E_L(p=good) E_L(p=bad)	1 0 3 2.5
f_1 f_2 f_3	0.4 20 2.5
i_1 i_2 i_3 i_4 i_5	0.27 0.5 0.02 0.022 0.9
P_L P_P P_A(p=good) P_A(p=bad)	0.015 0.0075 0.02 0.01
s_1 s_2	1.125 0.03125
t_1 T_{min}	0.05 15
R	12.5
w_1 w_2 w_3	0.01 -0.12 0.7
Δ (feeding) Δ (reproducing) Δ (dispersing)	1 1 0.75

Larval stage

The most important feature of larvae is that they feed. There are also daily costs which are directly paid from intake. Growth is modeled as income minus the daily costs. In a bad patch, no food for larvae is available.

In this model every time step (day) a larva consumes an amount of food that is partitioned between daily costs and growth. This is modeled as,

$$w_L(t+1) = w_L(t) + E_L(p)[i_1 w_L(t)^{i_2+i_3 T(t)} - c_1 w_L(t)^{c_2+c_3 T(t)}] \quad \text{(A1)}$$

Where w_L is the weight of the larvae, $E_L(p)$ is the effect of patch quality that varies between 0 and 1 bad and good patches. The constants i_1, i_2 and i_3 relate weight and temperature with intake and constants c_1, c_2 and c_3 relate weight and temperature to maintenance costs. These constants are chosen in such a way that larvae grow faster at higher temperatures but can reach a larger size at lower temperatures (PARKER and JOHNSTON 2006).

Figure A1 shows the effect of temperature on larval growth. Because the value of *i3* is lower than *c3*, the plateau reached in the late stage of growth is higher at lower temperatures. The manipulation of the constants i_1 and c_1 together lead to a change in relative growth rates between temperatures but not in the plateaus the larvae can reach. In the power functions, the effect of temperature is multiplied by i_3 and c_3 and then added to i_2 and c_2 because otherwise the effect of an increase in temperature would lead to a very large decrease in the final growth plateau, which is not biologically realistic.

Intake is dependent on surface area of the organism and daily costs depend on volume (KOOIJMAN 2010). In the model this is realized by a larger value of $c_2+c_3T(t)$ than $i_2+i_3T(t)$. The growth curves at different temperatures have an S-shape (fig. A1). Larvae grow slower at lower temperatures but can potentially reach a higher weight because of a higher growth plateau at lower temperatures, as has also been shown for larvae of the phantom midge (*Chaoborus flavicans*) (HANAZATO and YASUNO 1989; ATKINSON 1994).

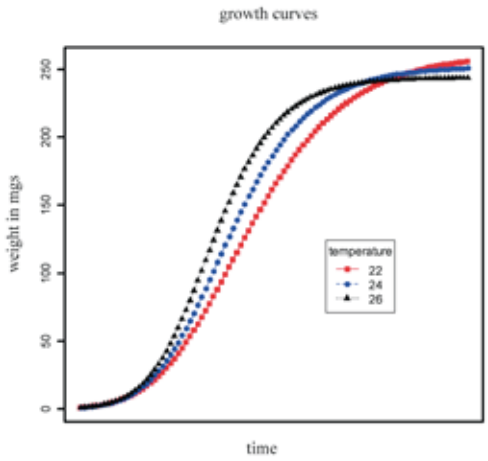


Figure A1. The effect of temperature on growth of the larvae. Different colors, shapes and lines indicate growth at different constant temperatures given in degrees Celsius.

To test robustness, the parameters $E_L(p)$, i_2, i_3, c_2 and c_3 are varied. When $E_L(p)$ increases, overall growth rate also increases. This changes the number of generations from three to four, but does not increase the size of each generation. The size difference between the first and second wet-season generations remains similar. Also, the average size of the dry season form is larger than any other generation of butterflies. Another test is whether the size differences between generations are influenced by temperature dependent growth. Constants i_3 and c_3 are reduced to zero, and i_2 and c_2 increased to maintain the same average growth rate. Absolute size differences between generations of butterflies are altered, but relative differences remain the same.

Larval mortality is dependent on predation risk, food availability and temperature, when larvae are deprived of food. Predation risk is independent of size. Although tests have been done which relate predation risk to weight in butterfly larvae, this risk may vary between species and is dependent on the type of predator and background (Mand *et al.* 2007; Remmel and Tammaru 2009). Here we model predation risk as,

$$\mu_L = P_L \tag{A2}$$

where P_L is predation risk of larvae. Survival of larvae in a good patch is equal to survival in a bad patch if it is the first day for the larvae in a bad patch.

$$S = \exp(-\mu_L) \tag{A3}$$

When the larvae continue to be in the bad patch for more than one day then survival is further reduced. This is modeled as

$$S = \{s_1 + s_2 T(t)\} \exp(-\mu_L) \tag{A4}$$

The constants s_1 and s_2 relate the survival decrease in a bad patch to $T(t)$, which is temperature. This extra reduction in survival is necessary since larvae cannot live very long without food. The constants we use are based on an experiment where larvae of *B. anyana* were starved for different numbers of days (Bauerfeind and Fischer 2009). Increase in temperature negatively affects survival under starvation (Oloumi-Sadeghi and Levine 1989; Padmanabha *et al.* 2011) therefore in our model constant s_2 is negative.

At a point called the 'critical weight', the larvae have reached a size at which they can start pupation. The critical weight is associated with a decline in growth rate and in this model is assumed to be independent of environmental factors, but see (Davidowitz *et al.* 2004; Nijhout *et al.* 2006). In the model the extra weight increase after the critical weight is not fixed. As a strategic choice, the model admits the possibility of further growth. Individuals that choose to grow larger are less likely to reach the pupal stage, because it takes more days to grow and thus the cumulative mortality is higher. During the larval stage individuals thus have a choice along the axis of trade-off between the size at pupation (and thus adult size) and the chance to survive until pupation and adulthood.

Pupal stage

During the pupal stage the individuals develop the organs specifically needed for adult

life. The amount of time needed is dependent on temperature. A fixed number of degrees of temperature multiplied by number of days, is necessary to complete this developmental stage. This is modeled as

$$D(t+1) = D(t) + d_1 T(t) \quad \text{(A5)}$$

where D represents the developmental state during pupal stage in units degrees x days. At higher temperatures, pupal development is shorter (Koch *et al.* 1996; Oostra *et al.* 2010). Weight is positively related to survival in the laboratory and negatively related to survival in a test with predators for *Orygia* species (Tammaru *et al.* 2002), but the field situation is not known for *Bicyclus.* Pupal survival is modeled as a fixed predatory pressure with

$$\mu_P = P_P \quad \text{(A6)}$$

and survival is modeled as for the larval stage, i.e.

$$S = \exp(-\mu_P) \quad \text{(A7)}$$

Because at lower temperatures development takes longer, the cumulative survival of pupae at lower temperatures is reduced.

As pupae become fully developed, they must divide their energy between tissue (which becomes fat as storage, abdomen which contains eggs and thorax for dispersal ability). These tissues are not uniformly costly. To produce one egg, 1mg of pupal energy must be paid. During the adult stage to produce an egg this egg has to be matured which also costs 1mg. The weight of an egg when it is not matured is 0.1 mg, which makes egg production ten times more costly than mere weight. 1 mg of pupal weight can be developed into 0.5 mg of thorax tissue. Fat can be transferred from the pupal to adult stage as a 1:1 ratio. These ratios of tissue conversion mean that pupae will generally lose weight and adults become lighter in weight than pupae.

Because measurements of the costs of specific tissues are not yet available, a robustness test is done with equal costs for fat, abdomen and thorax. This leads to an increase in fat in all generations of butterflies, but does not alter the allocation differences and behavior between good and bad patch individuals.

Adult stage

Adults can feed, disperse or reproduce. Each activity has different effects on the amount of fat storage. Energy can also be put into reducing ageing and thus the intrinsic mortality rate.

Adult weight is divided in three parts

$$w_{total} = w_f + w_a + w_t \quad \text{(A8)}$$

where w_f is the weight of the fat, w_a is the weight of the abdomen which contains the eggs and w_t is the weight of the thorax which enables dispersal.

During each time step, adults must pay daily costs. This is modeled as

$$C_A = f(T) c_4 w_{total}^{\ c_5} \quad \text{(A9)}$$

where C_A are the daily costs, constants c_4 and c_5 relate weight to the costs, and $f(T)$ is a function of temperature

$$f(T) = 1 + t_1\{T(t) - T_{\min}\} \quad \text{(A10)}$$

where t_1 and T_{min} are constants. Although the actual costs for specific processes within and between organisms might differ, costs decrease overall with a decrease in temperature (Kooijman 2010). In the model, temperature is also positively related to intake (eq. [A17]) and daily costs (eq. [A9]) at each time step through $f(T)$ (eq. [A10]).

Intrinsic mortality rate is modeled as an indicator of biological age. Biological age, X, increases with each time step, which depends on repair, temperature and weight. This is modeled as

$$X(t+1) = X(t) + \frac{a_1 w_{total}(t) + a_2(T(t) - a_3)}{rqw_f(t)} \quad \text{(A11)}$$

where a_1, a_2 and a_3 are constants. The constant r relates the amount of repair to an amount of energy allocation from fat. A part of the fat, q, is used to repair damage and thus to decrease the rate of ageing. As in other models, the increase in biological age increases partly as a function of volume, which is here represented by weight (Mangel 2008). Intrinsic mortality rates in *Drosophila melanogaster* were previously shown to be positively related to temperature at a population level (Miquel *et al.* 1976) as well as the molecular level (Jacobson *et al.* 2010).

Adult mortality has three different causes. The first is intrinsic mortality which increases exponentially with biological age as

$$\mu_X(X(t)) = a_4 + a_5 \exp(a_6 X(t)) \quad \text{(A12)}$$

where a_4, a_5 and a_6 are constants.

The second mortality cause is predation which is a constant that depends on the patch quality, since the butterflies have a conspicuous coloring in a good (green) background, but a cryptic one in a bad (brown) background (Brakefield and Frankino 2009).

$$\mu_{pred}(p) = P_A(p) \quad \text{(A13)}$$

where P_A(p) is a function of patch quality. Predation pressure takes values 0.02 and 0.01 in a good and bad patch, respectively.

The third cause of mortality is associated with weight. There is a lower boundary for weight, below which butterflies cannot survive. Weight dependent mortality is modeled as,

$$\mu_W(w_{total}) = \frac{w_1 \exp(w_2 w_{total})}{w_3 + \exp(w_2 w_{total})} \quad \text{(A14)}$$

where w_1, w_2 and w_3 are constants.

Total adult mortality per time step is thus

$$\mu_{total} = \mu_X(X(t)) + \mu_{pred}(p) + \mu_w(w_{total}) \quad \text{(A15)}$$

and survival is modeled as

$$S = \exp(-\mu_{total}) \tag{A16}$$

Adults can exhibit a range of behaviors, each associated with some additional considerations, as follows.

Feeding

Feeding adults gain an intake-based income dependent on weight, biological age and temperature. Income increases with temperature and decreases with biological age and is modeled as

$$I_A = E_A(p) i_4 W^{i_5} f(T) f(X) \tag{A17}$$

where $E_A(p)$ is the effect of patch quality in the adult stage, i_4 and i_5 are constants which relate weight to intake (Molleman *et al.* 2005), $f(T)$ is a function of temperature according to equation (A10) and $f(X)$ is a function of biological age according to

$$f(X) = 1 - a_7 X \tag{A18}$$

The rates of several processes linked with intake and food digestion are positively associated with temperature in a variable number of taxa (Angilletta 2009). Biological age is negatively related to intake because it is expected to relate to all functions including feeding. In fruitflies, feeding has been shown to decrease with age (Wong *et al.* 2009).

Daily costs are subtracted from the amount of fat, while income is added to the fat, which is modeled as,

$$w_f(t+1) = (1-q) w_f(t) + I_A - C_A \tag{A19}$$

where I_A is income and C_A represents daily costs. The weight of the thorax and abdomen does not change while feeding. The fraction of the fat which remains after allocation to repair is (1-q).

Flying

For flying individuals, fat is reduced further, according to

$$w_f(t+1) = (1-q) w_f(t) - C_A - T(t) f_1 \frac{f_2 + w_f(t) + w_a(t)}{w_t(t)^{f_3}} \tag{A20}$$

where f_1, f_2 and f_3 are constants, w_a is the weight of the abdomen and w_t is the weight of the thorax. When the weights of the fat and abdomen are high, flying is more costly. With a larger thorax, flying is less costly. Total weight has been shown to influence the costs of moving (Bejan and Marden 2006) and butterflies with higher thorax ratios have a better flight performance (Berwaerts *et al.* 2008). Flight is considered to be a cost for storage since it can influence how fast energy can be allocated to other functions such as reproduction, which has also been shown to need energy from the fat body in the butterfly *Pararge aegeria* (Gibbs *et al.* 2010).

Reproducing

When an individual reproduces, the number of eggs present in the abdomen decreases and fat decreases by an amount of 1 mg per egg. Fat is further depleted by reallocation of energy to repair and maintenance and daily living costs.

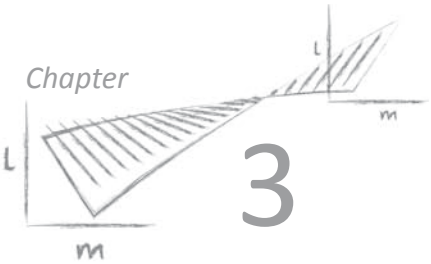
$$w_f(t+1) = (1-q)w_f(t) - C_A - N_{eggs} \quad \text{(A21)}$$

where N_{eggs} number of eggs produced by a female.

The abdomen contains the eggs. When an individual reproduces the abdomen is decreased in a similar fashion,

$$w_a(t+1) = w_a(t) - 0.1N_{eggs} \quad \text{(A22)}$$

Because one egg weighs 0.1 mg, abdomen is reduced 0.1 mg per egg.



A theoretical model of dietary restriction responses in temporally and spatially varying environments

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Bas J. Zwaan, Daryl P. Shanley

"The future is uncertain, and the end is always near."

Jim Morrison

Manuscript

Abstract

It is well known that dietary restriction (DR) enhances lifespan in many organisms. It is often emphasized that the DR response is conserved between all animal taxa, but there are many exceptions and a comprehensive explanatory theoretical model has so far only been produced for mice. Here we describe a resource acquisition and allocation model based on the classical principles of the disposable soma theory and the Y-model. In this model the environment varies temporally and spatially in terms of food availability and in the level of predation in which organisms are selected to optimize/maximize their reproduction and fitness. We then examine whether this range of environments aimed at representing the conditions in which animals have evolved across the evolutionary tree, favors a plastic response that resembles the empirically described DR response. Results indicate that the DR response only evolves in a temporally varying environment when variation in food availability correlates with juvenile survival and when extrinsic mortality is low enough to enable organisms to experience different 'seasons'. In a spatially varying model very short lived organisms also evolve a DR response, but the food abundance where the combination of acquisition and allocation maximizes lifespan differs among environments, and thus between the hypothetical species. When temporal variation in patch quality is present, all organisms show a DR response, in which the magnitude of the DR effect is dependent on the relative abundance of good and bad food "patches" in the environment. An extended period of reproductive investment for long-lived organisms (e.g. where the age at sexual maturity is higher) removes the DR response dependent on spatial variation. We conclude that temporal and spatial heterogeneity differ in their effects on the chance that the DR response will evolve in a lineage, and that it is not expected to be conserved over the whole evolutionary tree. Most notably, our theoretical results indicate that DR is unlikely to evolve for organisms in which food availability at the time of reproduction does not influence juvenile survival.

Keywords: dietary restriction, energy allocation, stochastic dynamic programming, life history, lifespan, fecundity

Introduction

Many species show a specific phenotypic plasticity for which there are reasonable adaptive explanations. For example, seasonal plasticity occurs in the tropical butterfly *Bicyclus anynana*, where the wet season morph has a more conspicuous wing coloring, develops faster, reproduces faster and lives shorter than the dry season morph (Brakefield and Zwaan 2011). Locusts show phenotypic plasticity in response to density of conspecifics with higher density inducing dispersal phenotypes (Pener and Yerushalmi 1998), which is an indirect response to expected future resource availability. A more direct response is shown in the spade-foot toad where tadpoles develop into large carnivores when a certain amount of shrimp are ingested (Pfennig 1990) and in the horned beetle which develop horns when well fed which are absent in undernourished individuals (Moczek 1998). These examples show that variation in nutrition can lead to extremely variable plastic responses in different organisms.

Lifespan extension, with a reduction in fecundity upon dietary restriction of the adult has been cited as an evolutionary conserved response to food in a wide variety of species. For example, upon mild dietary restriction (DR) lifespan is increased in yeast (Muller *et al.* 1980), flies (Chippindale *et al.* 1993), worms (Klass 1977), and rodents (McCay *et al.* 1935; Weindruch *et al.* 1986). Although this response seems widely conserved, the effect of dietary restriction is variable between closely related species such as rats and mice but also between genotypes within these species (Swindell 2012). Similar variation between genotypes is seen in the nematode *C. remanei* (Sutphin and Kaeberlein 2008) and between species of rotifers (Kirk 2001; Weithoff 2007). Lifespan of the fruit fly *Drosophila melanogaster* increases when both the yeast and / or sugar components of food is decreased (Lee *et al.* 2008; Skorupa *et al.* 2008), although this is also dependent on feeding method and severity of food deprivation and probably interacts with sex (LeBourg and Minois 1996). A decrease of food resource of Mediterranean fruit fly *Ceratitis capitata* decreases lifespan though (Carey *et al.* 2002) and an increase in lifespan upon DR is only seen when protein is reduced in houseflies, while when the sugar component is reduced, lifespan decreases (Cooper *et al.* 2004). Furthermore in butterflies (Beck 2007), the spider *L. hasselti* (Kasumovic *et al.* 2009) and in rhesus monkeys (Mattison *et al.* 2012) DR does not prolong live. The reason for variation in lifespan extension upon dietary restriction has been suggested to be due to some organisms being better adapted to laboratory conditions (Nakagawa *et al.* 2012). On the other hand, the variation in responses might also have been caused by the differences in the evolutionary past of the studied organisms.

The increase of lifespan upon DR is often related to a decrease in reproduction. Because these two traits, survival and reproduction are part of the energy budget (Kooijman 2010), they can be modeled using a framework of acquisition and allocation of resources (Van Noordwijk and De Jong 1986; Boggs 2009). Based on the disposable soma theory (Kirkwood 1977; Kirkwood and Holliday 1979) it is expected that organisms allocate energy to maintenance and repair, at a cost of reproduction, in a way that maximizes fitness in the environment in which they evolved. An increase of lifespan, a reduction of reproduction with DR, informed by the disposable soma theory, leads to the hypothesis that allocation to maintenance and repair at the cost of reproduction is promoted in environments with lower food levels. This has been theoretically tested specifically for mice (Shanley and Kirkwood 2000). At intermediate food levels selection was shown to favor an increased allocation to maintenance and repair. This plastic response is

only favored when juvenile mice have a lower survival chance in an environment in which female mice have lower food intake and when an amount of resource needs to be allocated to initiate reproduction (Shanley and Kirkwood 2000). Although it is specified that food is decreased for a certain amount with a certain chance, it was not specified what the nature is of the heterogeneity in food availability is in this model.

Here we present a more ecological model of DR in which we test how different organisms should respond to variation in food conditions. We thereby also address the question of how conserved this response between different organisms is expected to be. In a natural environment food availability can vary in many different ways. One of the most visible is seasonal variation, where a food item can be present in one season while absent in another. The availability of food may vary spatially with items at different local concentrations. In our model food will first be varied in a seasonal manner, while at later simulations the food is varied in a spatial manner. A third way is to combine the two approaches, where spatial variation varies temporarily.

In addition to variation in food, we model differences in environments in other dimensions. First the organisms differ in the parameter extrinsic mortality. Fruit flies for instance are known to live for a very short period in natural conditions (Dobzhansky and Wright 1947; Crumpacker and Williams 1973), which reflects a high extrinsic mortality rate, while mice can live for more than a year with an extrinsic mortality rate of 0.15 per month (Shanley and Kirkwood 2000). Many organisms are known that can live for half a century, such as the mates of Lonesome George which might have had a lifespan of more than 50 years before they were faced with modern humans (Swingland 1977; Hamann 1993). The variation in extrinsic mortality rates therefore reflects the differences in ecological lifespans of organisms.

Furthermore we model a juvenile stage, which can represent the larval stage of a fruit fly or that of a juvenile period for a mouse. We do not model any traits during this stage, but the survival rate of this stage might be related to food quality in some environments, while not in others. This is modeled in this way because juveniles of mammals greatly depend on the mother, and therefore on present nutrients. A bad quality patch for the adult therefore reduces the fitness of the juvenile as well. This can be modeled as a reduction in survival, while developmental time is always similar. This is therefore a way to model variation reduced fitness costs for individuals reproducing in a bad food quality environment.

In our model organisms may move between patches and we draw on the rich history of studies concerning optimal movement and feeding (Macarthur and Pianka 1966; Rapport 1971; Schoener 1971; Krebs *et al.* 1974; Pulliam 1974; Charnov 1976; Parker and Stuart 1976; Sibly and Mcfarland 1976; Mangel and Clark 1986; Mcnamara and Houston 1986) but we will not specifically consider free choice of patch, rather we focus on optimal allocation to reproduction and maintenance and repair. We thereby align our focus with the majority of the dietary restriction experiments where organisms are not free to choose their food resource, but rather remain in a constant environment throughout life (but see Lee *et al.* 2008). Also mortality of the adults is similar between patches, although this might influence optimal decisions (Houston and Mcnamara 1986). Our study more resembles the aims of optimal oviposition and clutch sizes dependent on the state of the organisms (Mangel 1987) and resembles a model in which the adult can move after completing development (Mangel and Roitberg 1993). In our model the state of the patch is not influenced by number of ovipositions although this does influence the adaptive behavior (Van alphen and Drijver 1982; Driessen and Bernstein 1999). Lastly, our

model considers only number of offspring, while size and number varies with age and state of the organism (KINDSVATER *et al.* 2010; JORGENSEN *et al.* 2011).

The major difference between the above mentioned models and the model presented here is the nature of the variation of resource. In our model we specifically test whether a lifespan increase is favored by selection in period or patches with lower resource abundances. Furthermore we test whether theoretical species that differ in life histories due to the differences in environments, show a consistent or conserved response. The results from this model will indicate whether a lifespan increase upon DR is expected in different types of organisms and can therefore clarify why in some organisms lifespan is increased while in others not.

Method

Patch quality, temporal and spatial variation

The environment consists of patches that vary in quality which affects survival in the juvenile stage and food intake in adult stage. We consider twelve patch qualities where Q_1 has the lowest quality and Q_{12} the highest. The frequency of patch Q_n with quality n at time t is given by,

$$\varphi(Q_n, t) = a / b^{n-1} \quad (1)$$

Where the frequency of patch Q_1 is a, and the relative frequencies of the other patches are related through the patch quality parameter b. Parameter b is always larger than 0. If b is smaller than 1, patches have a higher frequency when they increase in quality. When b is larger than 1, patches have a lower frequency when the quality increases. The value of a is chosen so that the sum of the frequencies of all patch with different qualities is 1. Patch quality may also vary spatially. In the general case of combined spatial and temporal variation we introduce a time dependence for parameter (b). The chances that a patch will change in quality are then dependent on the frequencies of patches in the next time interval.

$$b(p,t) = b_{ampl} \sin(\frac{2\pi t}{365}) + b_{mean} \quad (2)$$

Transition probabilities in patch quality

We consider adults as the mobile stage, with random transfer between patches at each time step. The juveniles remain in the patch of their birth, which only change in quality if patches are also subject to temporal variation.
We let

$\lambda_{p,p'}(t)$ = Pr{individual situated in a patch with quality p at time t, is in a patch with quality p' at time $t+1$} (3)

Adults move around randomly in space and $\lambda_{p,p'}(t)$ is simply,

$$\lambda_{p,p',A}(t) = \varphi(Q_{p'}, t) \quad (4)$$

Juveniles are sedentary, and the patch can only change in quality if the frequency of patches changes in time. We assume that patch quality can only change by a single increment. When a juvenile is in the lowest quality patch and overall the patch quality increases, then the chance that the patch remains a patch with lowest quality is,

$$\lambda_{1,1,J}(t) = \frac{\varphi(Q_1,t) - \varphi(Q_1,t+1)}{\varphi(Q_1,t)} \tag{5}$$

The probability that it changes into a patch with quality two is therefore,

$$\lambda_{1,2,J}(t) = 1 - \lambda_{1,1,J}(t) \tag{6}$$

For a patch with quality 2, these equations are,

$$\lambda_{2,2,J}(t) = \frac{\varphi(Q_2,t) - \varphi(Q_2,t+1) + \lambda_{1,2,J}(t)\varphi(Q_1,t)}{\varphi(Q_2,t)}, \text{ and } \lambda_{2,3,J}(t) = 1 - \lambda_{2,2,J}(t) \tag{7}$$

Where the term $\lambda_{1,2,J}(t)\varphi(Q_1,t)$ is required to take into account patches of quality 2 in time *t+1* that were quality 1 at time *t*. Similar equations apply for patches with quality 3 to 11. The probability that a patch of highest quality will remain of highest quality is 1. When the overall quality of patches decreases, the probabilities are,

$$\lambda_{12,12,J}(t) = \frac{\varphi(Q_{12},t) - \varphi(Q_{12},t+1)}{\varphi(Q_{12},t)} \tag{8}$$

For the highest quality patch remaining the same, and

$$\lambda_{12,11,J}(t) = 1 - \lambda_{12,12,J}(t) \tag{9}$$

for a decrement in quality. For patch quality 11 the transition probabilities are,

$$\lambda_{11,11,J}(t) = \frac{\varphi(Q_{11},t) - \varphi(Q_{11},t+1) + \lambda_{12,11,J}(t)\varphi(Q_{12},t)}{\varphi(Q_{11},t)}, \text{ and } \lambda_{11,12,J}(t) = 1 - \lambda_{11,11,J}(t) \tag{10}$$

With similar equations for patches of quality 10 to 3 and finally for lowest quality patch,

$$\lambda_{1,1,J}(t) = 1 \tag{11}$$

Organism

Our model organism has a juvenile and an adult stage. The juvenile takes 10 time intervals to develop to an adult. The chance to survive a time interval depends on patch quality which is the only means that a patch influences the life history in the juvenile stage. Development is

modeled as,

$$D(t+1) = D(t) + 1 \tag{12}$$

And survival is modeled as,

$$S(t) = \exp\{-M(p,t)\} \tag{13}$$

Mortality *M(p,t)* can vary seasonally described by a sinusoid with an amplitude of M_{ampl}. We assume the same seasonal variation every year and mortality varies with time according to,

$$M(p,t) = M_{ampl} \sin(\frac{2\pi t}{365}) + (1 - l_{s0} - p(t)l_{si}) \tag{14}$$

Where l_{s0} and l_{si} are patch dependent juvenile mortality parameters. We consider fitness *F(t,D,p)* as the maximum accumulated reproductive success of an adult that ultimately develops from a juvenile with developmental stage *D*, in a patch of type *p*, at time *t*. A juvenile that has a developmental state lower than 10 time intervals will remain a juvenile in the next time interval and *F(t,D,p)* is given by,

$$F(t,D(t)<10,p) = \sum_{p'=1}^{12} \lambda_{p,p'}(t) F\{t+1, D(t+1), p'\} S(t) \tag{15}$$

Where fitness at time *t* is the total future fitness obtained from the sum of the fitness calculated in each patch of quality *p'* at time *t*+1 multiplied by the probability of reaching this patch. On completing development (*D(t)*=10) the juvenile becomes an adult of age 0. For an adult with biological age *x* in a patch of type *p*, we consider *F(t,x,p)* to be the maximum accumulated reproductive success at time *t*. The future fitness of such a juvenile is therefore,

$$F(t,D(t)=10,p) = \sum_{p'=1}^{12} \lambda_{p,p'}(t) F\{t+1, 0, p'\} S(t) \tag{16}$$

Adults acquire resource at every time step. The amount of acquired resource is dependent on biological age and patch quality. We consider *I(x,p,t)* as the acquired resource of an adult that has biological age *x*, in a patch of type *p*, at time *t*.

$$I(X(t),p,t) = \frac{i(p,t)}{\exp\{cX(t)\}} \tag{17}$$

As biological age increases, the intake of food decreases, hence, *c* is a negative constant. *i(p,t)* is a function which relates resource level to the quality of a patch and time in the season. For seasonal variation,

$$i(p,t) = i_{ampl} \sin(\frac{2\pi t}{365}) + i_{mean} \tag{18}$$

Where i_{ampl} and i_{mean} are the amplitude and mean of the sinusoid curves. During the adult stage, intake is divided between reproduction and maintenance and repair. This allocation of energy is the only decision made in the model, and depends on state of the individual, the patch quality and time of the year. Investment in maintenance in repair slows ageing which is modeled as,

$$X(t+1) = X(t) + d(1 - \frac{qI(X(t),p)^2}{qI(X(t),p)^2 + e^2}) \quad (19)$$

Here *q* is the proportion of energy allocated to maintenance and repair which corresponds to efficiency of repair (Mangel and Munch 2005). With no allocation to maintenance and repair biological age increases by *d*. When *qI(X(t),p)* equals *e* half of the damage is repaired and repair is more costly as *e* increases.

Adult survival is influenced by biological age and predation level, with mortality modeled as,

$$M(t) = P + m_1 \exp(m_2 X(t+1)) \quad (20)$$

Where *P* is the level of extrinsic mortality rate and constants m_1 and m_2 link biological age to mortality. Survival is given by,

$$S(t) = \exp(-M(t)) \quad (21)$$

The maximum accumulated reproductive success of an adult at time *t*, of biological age *X(t)* in a patch with quality *p* is described as,

$$F(t,X(t),p) = \max_{q=0}^{1}[(1-q)I(X(t),p)F(t+1,D(t+1)=0,p) + \sum_{n=1}^{12}\lambda_{p,p'}(t)F(t+1,X(t+1),p')S(t)] \quad (22)$$

where *q* is the proportion of total acquired resource allocated to maintenance and repair. The value *(1-q)* is the portion of acquired resource that is allocated to reproduction, which is multiplied by the fitness of a larva with development 0 born in the patch as calculated by equation (4). The future fitness is the total calculated for all patches in which the adult could be at the next time step. The probability λ is calculated as in equations 4-11 and *F(t+1, X(t+1),p')* is the fitness value of an adult in the next time step, with a biological age of *X* (eq. [19]) in patch *p'*, given it survives (eq. [20]). The optimal value of *q* that maximizes *F(t,x,p)* is stored at each time step. This is indicated by *max*, which is taken over a set of possible values of *q*, which ranges between 0 and 1.

An extension on the model considers the case in which an adult needs to invest in reproduction for two time steps instead of one. We introduce an additional parameter *v* for developmental time that can take values of 1 or 2 for first and second day of investment. For the first day of investment in reproduction, the maximum accumulated reproductive success *F(t,x,p,r,v)* of an adult at time *t*, with biological age *X(t)*, in a patch with quality *p*, with zero reproductive investment *r* is,

$$F(t,X(t),p,0,1) = \max_{q=0}^{1}[\sum_{n=1}^{12}\lambda_{p,p'}(t)F(t+1,X(t+1),p',R_1,2)S(t)] \quad (23)$$

where R_1 is the investment in reproduction when for day 1 and given by,

$$R_1 = (1-q)I(X(t),p) \tag{24}$$

Equation (23) is computed for all *q* and the optimal value of *q* which maximizes *F(t,x,p,r,v)* is stored. For day 2, the investment in reproduction is described in a similar fashion to equation (24),

$$R_2 = (1-q)I(X(t+1),p) \tag{25}$$

Since developmental time has increased by one, we denote time as *t+1* to make equation (25) logically comparable to equation (24). Now the maximum accumulated reproductive success can be calculated as,

$$F(t,X(t),p,R_1,2) = \max_{q=0}^{1}[2\min[R_1,R_2]F(t+1,D(t+1)=0,p) + \sum_{n=1}^{12}\lambda_{p,p'}(t)F(t+1,X(t+1),p',0,1)S(t)] \tag{26}$$

We take the minimum of R_1 and R_2 as the second investment in reproduction has to at least match that on day one. Note that $min[R_1,R_2]$ replaces *(1-q)I(X(t+1),p)* from equation (22) and therefore replaces the term for investment in reproduction. We multiply this minimum by 2, so that the two days of investment equals the normal reproduction in two days. Equation (26) is again calculated for all *q* and the value of *q* that maximizes the left hand side of equation (26) is saved as the optimal strategy.

Backward iteration

We use a dynamic programming algorithm (Mangel and Clark 1988; Houston and Mcnamara 1999; Clark and Mangel 2000) to solve equations (15), (16), (22), (23), and (26) by initiating all fitness values at the time horizon (*t=T*) to 1 and working backwards from that time point. Juvenile development and biological age are discretized using steps of 1, with a minimum of 0 and maxima of 10 for development and 499 for biological age. As absolute fitness can potentially increase or decrease rapidly, we monitor relative fitness which is calculated by dividing all absolute fitness values by the maximum in every time interval, such that the highest fitness value is 1. This does not affect the outcome of the model and the decisions of which strategies are optimal. The algorithm is stopped when all optimal strategies in all state combinations remain constant. From that point optimal strategies for every state combination are saved for forward simulation. The values of the parameters are given in the appendix tables A1 and A2.

Theoretical DR experiments by forward simulation

To perform theoretical DR experiments, we simulate 100 adults of biological age 0, and initiate these populations at different time points in the theoretical year. In laboratory experiments, lighting and temperature conditions are usually kept constant, and it could be argued that organisms perceive time as constant. Depending on temperature lightning organisms are known to alter growth which is likely to be adaptive (i.e. a strategic decision) in the field (Gotthard 2008; Lee *et al.* 2010). Therefore we simulate the individuals as if every time step is the same day

in the year, i.e. keeping *t* constant. Furthermore, because we mimic laboratory experiments, the extrinsic mortality parameter (*P*) is set to 0 as, apart from possible infections, organisms do not normally die from extrinsic hazards in DR experiments.

Results

Seasonal variation

First we considered an environment that varied in food quality in time (within a year) with and without an effect on juvenile survival. We used the dynamic programming algorithm to determine the optimal allocation strategy for individuals at each age at every day of the year. We then simulated 100 adult individuals fixed at a specific day in the year and allowed them to follow their optimal strategies. We started the simulation with individuals that had a biological age of 0, which corresponded biologically to those that they were just matured. We monitored their survival and reproduction. Since the food availability and juvenile survival varied sinusoidally, these features first increased during the year and then decreased with maxima and minima at a fourth and three fourths of the year.

The upper two rows of Figure 1 show the results of a simulation where only food availability is varied in time, with different amplitudes of variation indicated by different lines. In the column to the left extrinsic mortality is low and juvenile survival does not vary. If food varied sinusoidally, lifespan (first row, figure 1) and early fecundity (second row, figure 1) followed a similar pattern. This indicates that resource was allocated to each of these traits, but the relative allocation did not vary at different times of the year. The amplitude of food availability determined the variation in the traits. As extrinsic mortality rates increased (from left to right in columns in figure 1) the overall allocation to maintenance and repair decreased. Because allocation to maintenance and repair decreased to zero at a very high level of extrinsic mortality rate lifespan was similar for environments in which the food availability varied with different amplitudes (see upper right panel figure 1). Early fecundity in these environments still varied similarly to food availability. Since in general lifespan and early fecundity increased with acquisition whereas juvenile survival was not related to environmental quality, the variation in life history traits were positively correlated and lifespan was not predicted to increase upon dietary restriction.

When juvenile survival increased with food availability (lower half of figure 1) allocation to reproduction was increased in the first half of the year (left side) at very low extrinsic mortality rates. This was costly for lifespan, as it decreased in the first half of the year, while lifespan increased for individuals that were simulated to live in the second half of the year. With a large variation in food availability, the acquisition was very high early in the year while low late in the year. Because this effect was opposite to the allocation to maintenance and repair, there were environments in which juvenile survival was related to food availability and yet lifespan was not extended upon dietary restriction (see figure 1). Early fecundity was always highest in the first half of the year. When extrinsic mortality rates were again very high, the variation in allocation pattern between the different times of the year disappeared, and lifespan was not extended upon dietary restriction.

In summary, in an environment where food availability varied seasonally, individuals only extended their lifespan at the cost of reproduction upon dietary restriction when juvenile

survival was lower when food availability decreased. This pattern was only present if variation in acquisition was not sufficiently large that it overrode the effect of increased allocation to maintenance and repair and when organisms lived long enough to experience variation in time (i.e. when extrinsic mortality is low). In an earlier model, the effect of dietary restriction on mice was simulated (Shanley and Kirkwood 2000). The allocation to maintenance and repair increased when acquisition of resources decrease from fully fed to and intermediate dietary restriction food level. A further reduction led to a decrease in total resource allocated and therefore a reduction in lifespan, which in reality was caused by starvation. The green line in the third row in the left in figure 1 represents such a pattern in which the lifespan in not maximized at the point in which resource acquisition in maximum, but is maximized at earlier and later times in the year. The kind of data simulated here is comparable to studies done with hamsters. Individual hamsters invested differently in testis weight when different latitudes were simulated by lighting conditions. Also individuals that were kept at constant longer days invested differently in testis weight (Gorman and Zucker 1995).

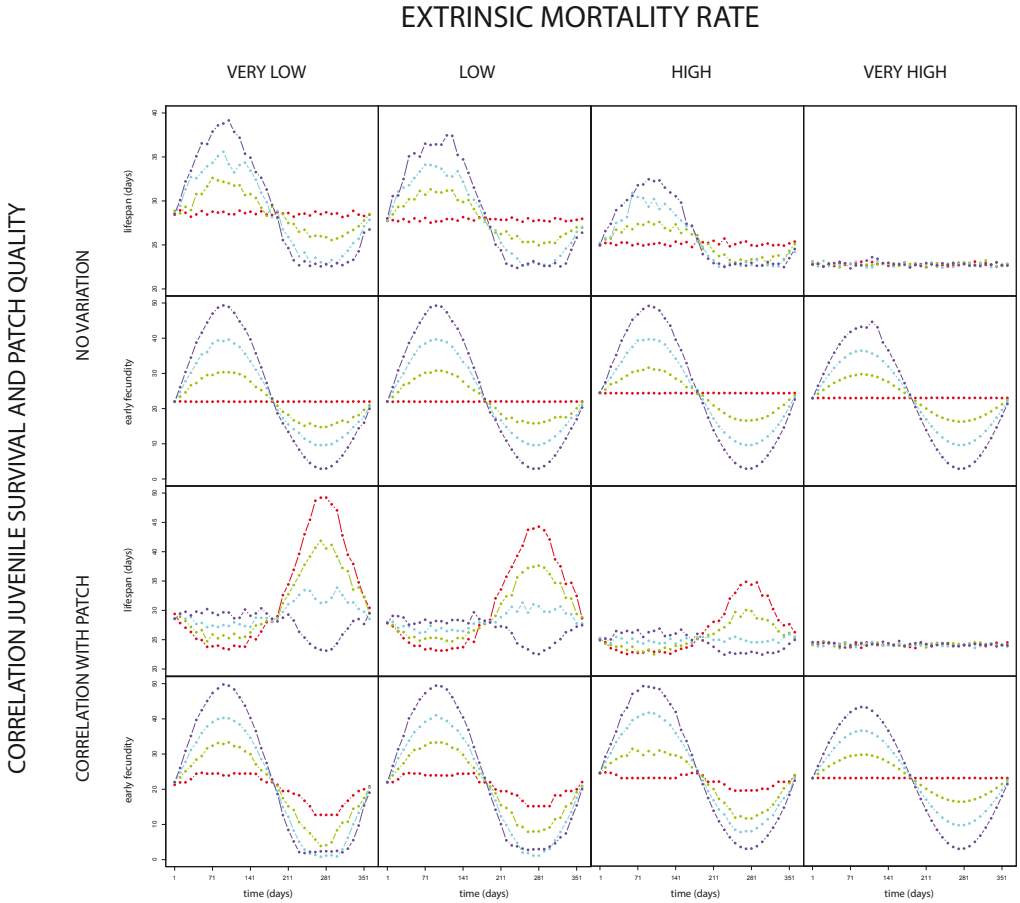

Figure 1. Effects of seasonal variation in food availability and juvenile survival combined with extrinsic mortality rates on lifespan and early fecundity. In every subgraph the average trait value of 100 simulated individuals are given by a point, where the different lines indicate differences in amplitudes of food availability (from low to high; red = 0, green = 1.75, blue = 3.5 and purple = 4.25) and the x axes indicate time within a year. In columns the different values for extrinsic mortality are indicated increasing from left to right. In the upper half of the figure juvenile survival is not related to quality of the environment, while in the lower half it is.

Spatial variation

In addition to a temporal variation in food availability and juvenile survival organisms might also experience spatial variations. We have modeled an organism that has to move every time step between 'patches' that may vary in food availability and / or juvenile survival in a random (Brownian motion) fashion. Although the reasons that individuals might disperse can be various and the can be influenced by the size of the patches (Bowler and Benton 2005) at least for *Drosophila* this random type of dispersion seems justified (Dobzhansky and Wright 1947; Crumpacker and Williams 1973). We consider 12 different types of patches, which can vary in food availability and / or larval survival, again combined with different levels of extrinsic mortality. Because we modeled distinct patches, we also simulated the response of the organisms in environments that differ in the patch frequencies (see figure 2). Like the seasonal model, the organisms evolved in an environment in which individuals experienced variable conditions, but we then simulated how individuals would respond to fixed food levels. Then we expressed lifespan as dependent on these 12 different food levels.

If only food availability varied with patch quality, again lifespan increased with higher quality patches (first row, figure 2), especially when extrinsic mortality rates were low (left column figure 2). In an environment where juvenile survival was low in low quality patches, lifespan was highest when there was no variation in food (second row, red lines, figure 2). This was because selection for reproduction was lower when juvenile survival decreased, and therefore allocation to maintenance and repair was favored. When the decrease of this allocation to maintenance and repair over increased patch quality was combined with food availability variation then an optimum of lifespan was reached at an intermediate patch quality, which was similar to experiments done with *Drosophila melanogaster* (Clancy *et al.* 2001). Lifespan extension upon dietary restriction can evolve in an environment in which food availability varies spatially and juvenile survival is related positively to adult food acquisition. Although lifespan was maximized at a intermediate food level, it has been shown for fruit flies that fecundity was maximized at high levels of yeast (Skorupa *et al.* 2008). As in the simulation with seasonal variation, fecundity in these simulations is maximized at the highest quality patches (see figure 7 in appendix C).

When different levels of extrinsic mortality were considered, average lifespan decreased with increasing mortality rates (from left to right in figure 2), but the patterns did not change. This means that in contrast to the seasonal variation model, in environment with spatial variation lifespan extension upon dietary restriction can also evolve for very short lived organisms, although the effect of dietary restriction on lifespan was smaller. In environments in which the frequency of patches decreased with the quality of patches, lifespan in general was lower. Furthermore, the patch quality in which lifespan was maximized was different and decreased with decreasing frequencies of higher quality patches. This indicates that how lifespan varies with food availability depends not only on how patches vary, but also on the frequencies of specific patches.

To illustrate how variable responses were between individuals within a specific environment (stochastic differences) we calculated the standard deviation of lifespan for all individuals simulated with low extrinsic mortality, in an environment where juvenile survival relates to patch quality and in which patch frequency increased with quality (see appendix B, figure 1). The standard error indicated by the error bars are calculated using 10 individuals as a replicate number. The 95% interval of the average of for instance the lifespan of the individuals simulated in the lowest quality patch in the environment in which food does not vary (red line) did not overlap with the individuals

simulated at the patch that was two steps higher in quality. Also the survival curves were very distinct, when plotted for all the simulated individuals when all the 12 groups of individuals were considered in the 12 patches within this environment were considered (see figure B1, right side).

In summary, again lifespan was increased upon dietary restriction when juvenile survival was positively related to adult food availability. Also, when variation in food acquisition was too high or too low, the effect of allocation was not visible in the traits. At intermediate variation levels in acquisition lifespan was also extended for organisms that were very short lived, which was different from the seasonal variation model.

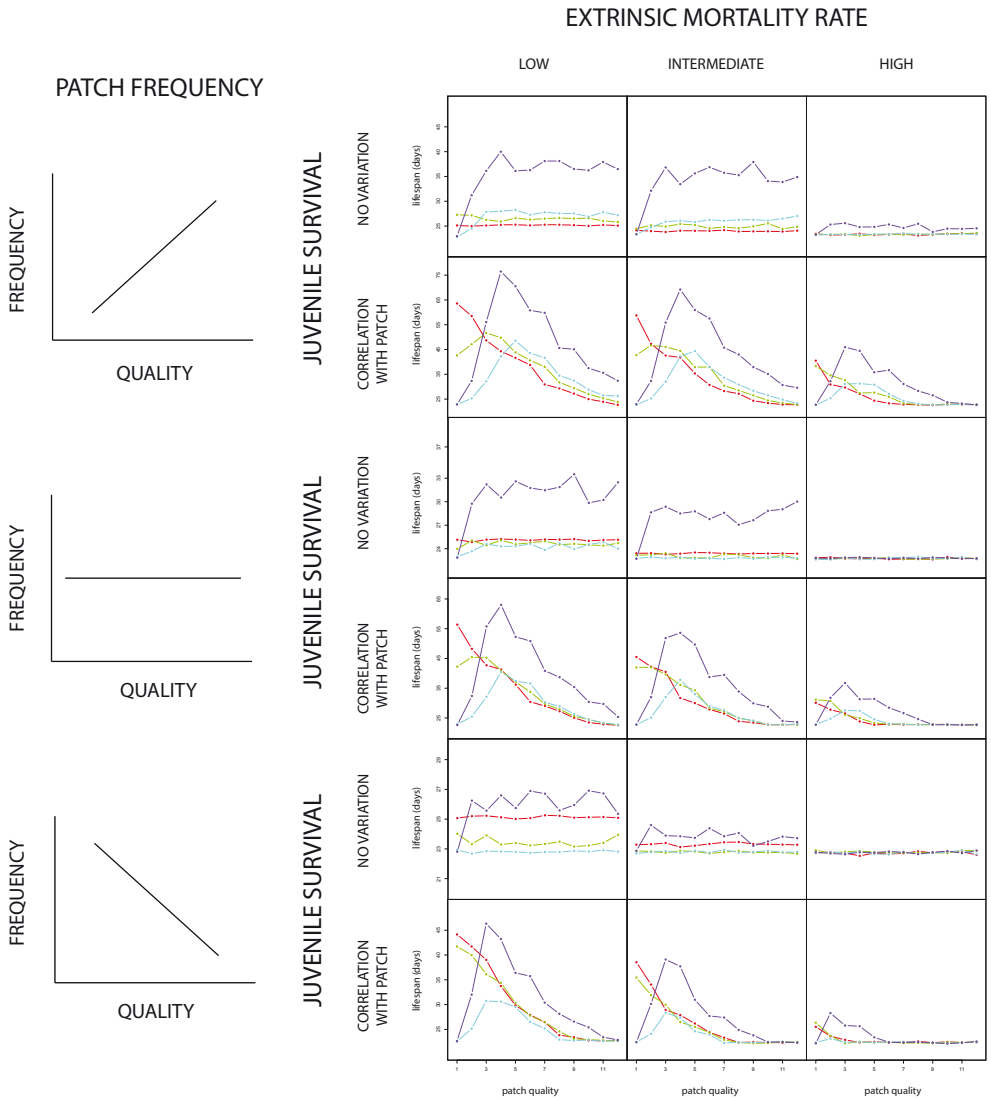

Figure 2. The effect of patch quality (x axis every panel) variation in food availability (different lines, red= no variation, green = low increase with patch quality, blue = intermediate increase with patch quality, purple = high increase with patch quality) and juvenile survival (rows 1, 3 and 5 no variation in juvenile survival, rows 2, 4 and 6 positive relationship patch quality and juvenile survival) in combination with extrinsic mortality rate (different per column) and patch frequency (upper six panels = increasing frequency with quality, middle six panels = equal frequencies, lower six panels = decreasing frequency with patch quality) on lifespan. (Note that the y axes differ per row).

Seasonal changes in spatial heterogeneity

The effects of temporal and spatial variation can be combined when we consider seasonal changes in patch frequencies. We modeled this by varying parameter *b* from equation 2 (methods section) according to a sinusoid, instead of food acquisition or larval survival. We then considered different environments in which the patch qualities were related to food acquisition and / or juvenile survival in combination with extrinsic mortality. When parameter *b* increased early in the year, this meant that there were less high quality patches. In figure 3 the joint effect of extrinsic mortality rate, juvenile survival variation, food variation and seasonal patch frequency variation on lifespan is shown.

First, if we considered an environment in which there was no variation in juvenile survival, and food and patch frequencies remain constant, we expected no variation in lifespan between individuals simulated to live in different patches. This was indeed the case (see fig. 3 row 1 left and right) where it can also be seen that the lifespan was reduced at the higher extrinsic mortality rate environment (right column compared to left column, figure 3). When juvenile survival was different between patches, comparably to the spatial model, allocation to maintenance and repair was increased in patches where juvenile survival was lowest. In figure 3 the patches vary from blue to red which represent patches that vary from high to low juvenile survival respectively (see second row, figure 3). Therefore the red line patches have the highest lifespan. In the third and fourth row of figure 3 the patches vary in resource availability. Indeed, when juvenile survival was not variable, individuals from high quality patches had the highest lifespan. When juvenile survival was combined with food acquisition variance, individuals in intermediate quality patches had the highest lifespan, comparably to the spatial variation model.

The results shown in the upper half of figure 3 are comparable to the results of the spatial model. In the fifth row of figure 3, the patch frequencies vary, but the patches do not vary in juvenile survival and resource availability. Therefore, the lifespan between patches was similar to that of the first row. When there was variation in patch frequency though, lifespan was lower in the first half of the year, while higher in the second, but only for low quality patches (see figure 3, sixth row). Again this was similar to what we have seen in the spatial model, where in high quality patches lifespan in general was higher (see figure 2, second row compared to sixth row). When patches varied in food availability individuals in high quality patches had a higher lifespan (see row 7 figure 3).

When juvenile survival and food availability were combined with variation in patch quality, individuals from intermediate quality patches had the highest lifespan, indicating that in this environment lifespan was extended upon dietary restriction. Similarly to the spatial model, it was dependent on the frequency of patches at which patch quality (i.e. when in the year) lifespan was maximized.

Summarizing, the model in which patch frequency varied in time, by combining the seasonal model with the spatial model, led to similar responses as the spatial model. To test whether there was an effect of time or whether it was only an effect of space, we simulated individuals that evolved in an environment in which the patch frequencies varied in time. The results are shown on the left side of figure 4. The data is clearly similar to that of figure 3, bottom left. Then for a specific day in the year we simulated individuals that evolved in a constant environment, but with different patch frequencies of day 1, day 2, et cetera, and compared the results of these individuals with those of the seasonally variable environment (see figure 4, right side). The variation of the

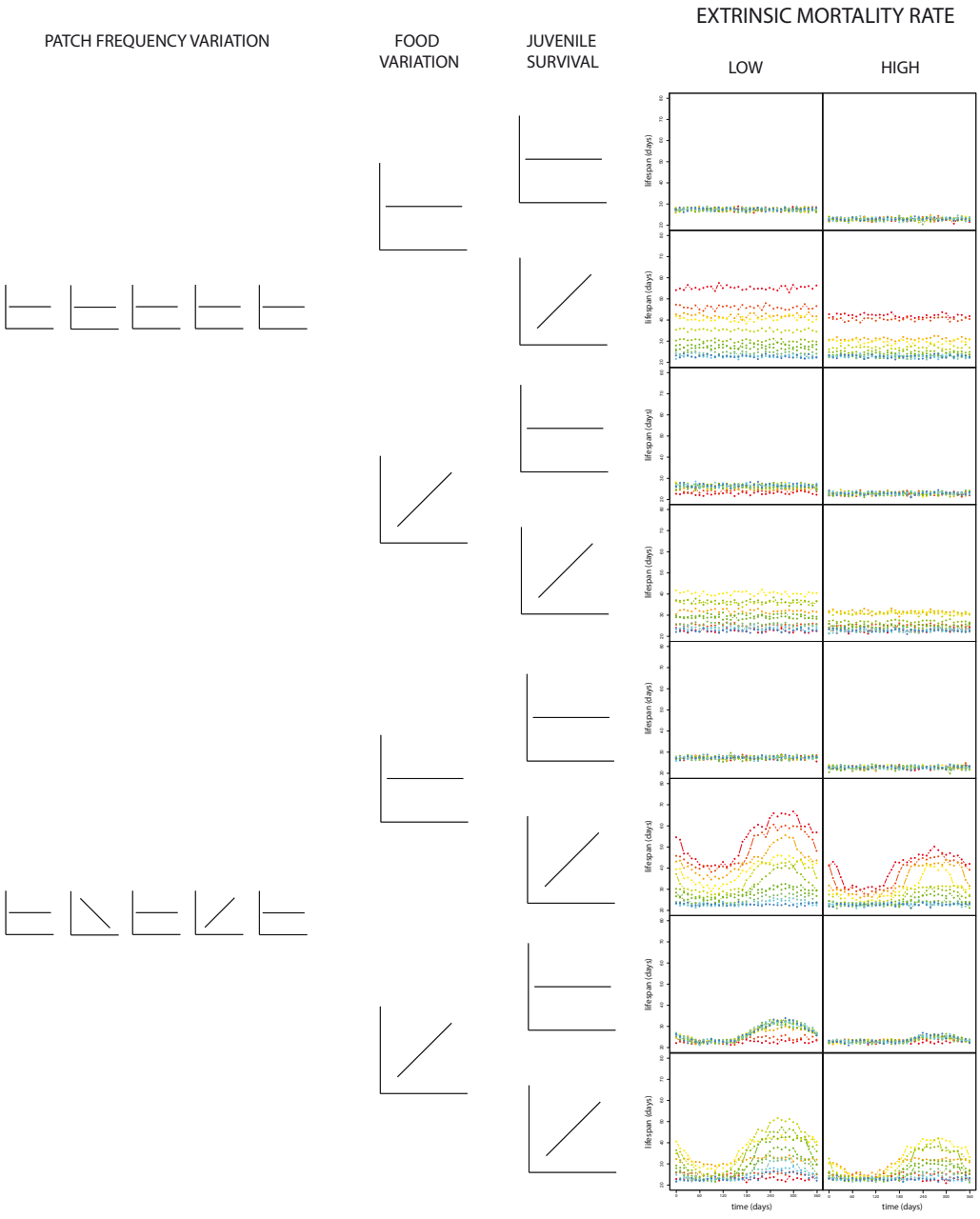

Figure 3. The effect of variation in patch frequencies in time on average lifespan within a population of 100 simulated individuals per point. On the x axis of every subgraph time is indicated, the different lines indicate patch quality where colors vary from red to blue representing patches from bad to good. In rows 1, 3, 5 and 7 juvenile survival is equal between patches, where in the other rows juvenile survival is lower in lower quality patches. In rows 1,2, 5 and 6 food acquisition is equal between patches, where in the other acquisition increases with patch quality. In the upper half the patch frequencies do not change with time, while at the lower half of the figure low quality patches increase in frequency early in the year, while they decrease to a lowest point ¾ of the year. In the left column the extrinsic mortality in the environments was high, while low in the right column.

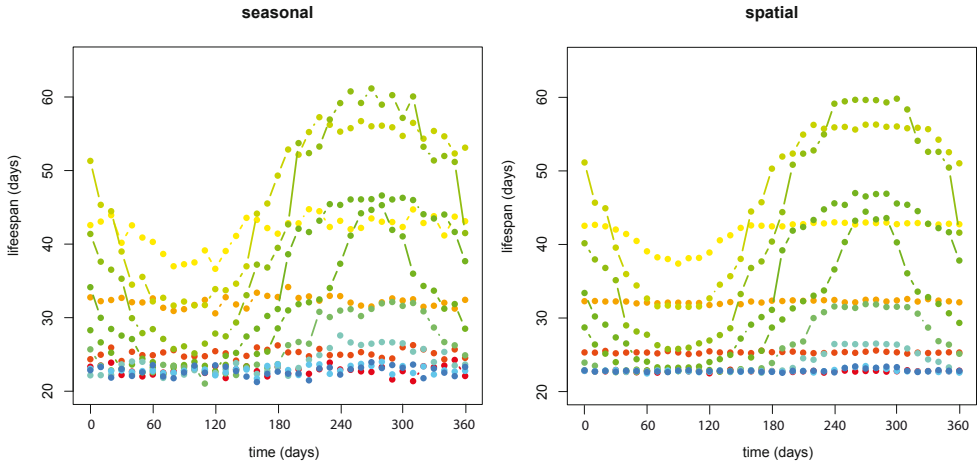

Figure 4. Comparison of lifespans resulting from optimal decisions with seasonal variation in patch frequency (left) with the equivalent values for patch frequencies along the x axis, but where all the time points are different runs in the case of spatial variation.

non-seasonal individuals (right side, figure 4) was similar to that of the seasonal individuals (left side, figure 4). This means that although it seems that the individual vary seasonally in how they respond to patch frequency, because they change their response according to time, they actually respond similarly to individuals that do not live in a seasonal environment, but always experience a similar patch frequency.

One important feature of this model, in which seasonal and spatial variation in combined, is that organisms in general live longer in a part of the year that contain more high quality patches. Fitness was apparently maximized in the second part of the year by not investing in more offspring within one patch, but by trying to live longer to be able to visit more high quality patches. Even for very short-lived organisms this was the outcome, but only when juvenile survival was very low at lower quality patches. For flies it has been shown that longer light regimes led to higher fecundity, but lower lifespan (Sheeba *et al.* 2000). Therefore, in the case of *Drosophila,* if the second part of the year in reality is related to the season where flies experience longer days, and find higher quality patches more often, then the model would predict the opposite of the outcome of the experiment. When the relationship between ageing dependent mortality and efficiency of repair are altered, this pattern was different though. Therefore, this model could be used to quantify these efficiencies by comparison with experiments.

A more realistic model of reproduction for long lived organisms

So far, we have modeled different types of organisms in a very general way. This was done by altering food availability, juvenile survival, extrinsic mortality rate and patch frequency. Although responses did vary when these parameter were altered, the response in figure 4 shows that the seasonal changes in spatial variation led to immediate responses, which is even true in longer lived organisms (where P is lower). This would suggest that even mice would respond very directly and immediately to spatial variation in the environment. This outcome might be not very

realistic, as mice need to invest a longer period in reproduction (to fully developed offspring). Fruit flies on the other hand can up- or down-regulate the output of eggs within days. To test whether longer investment might influence the response to variation in food on a seasonal and spatial scale, we simulated organisms that need to invest in reproduction beyond 1 day to get a payoff. For reasons of computational feasibility we only consider a 2 day investment but consider that this will provide an indication of the effect of longer period. Again we separate these in a seasonal and a spatial model.

Figure 5 (left panel) shows the lifespan resulting from an optimal life history for seasonal variation of a 1 day reproductive investment organism compared to a 2 day investment organism. This is shown for simulations in which the amplitude in food is 1.75 and 4.25. All other parameters are similar. In a seasonal environment, lifespan was still maximized in the part of the year where juvenile survival was minimal when food amplitude was 1.75. The response though was much weaker compared to the one day investing organism. When the amplitude was larger, the lifespan was minimized at this point, and maximized at the point where food was maximized.

When organisms need to invest more days in reproduction and are faced with spatial variation (figure 5, right side), the response to food variation was very different. Because they needed to invest equally in reproduction for two days to get the payoff, they invested less in reproduction when they were in a good patch, because this investment could not be matched the next day when they were likely to be in a patch with lower quality. This was why lifespan actually increased again at higher food levels. This effect was indeed smaller when more good patches are present and when predation was higher.

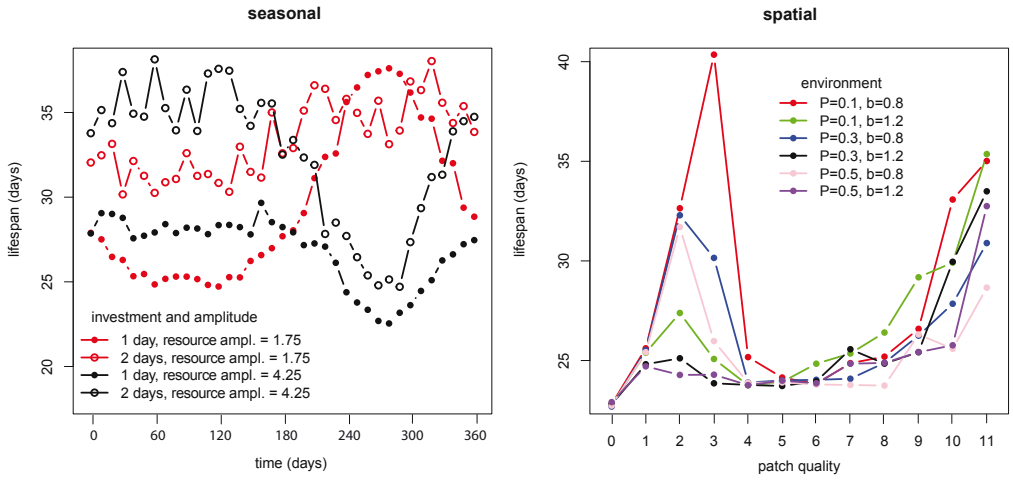

Figure 5. Left; comparison of how the number of days individuals have to invest in reproduction affects lifespan in a seasonal environment in which predation level is 0.03, larval survival amplitude is 0.2 and food amplitude is 1.75 (in red) or 4.25 (in black). Right; the effect of spatial variation in patch quality when an organism needs to invest an equal amount of resource into reproduction for two days to get a payoff. Response is dependent on predation level (P) and patch frequency parameter (Bp).

Discussion

Our model describes the optimal allocation pattern between maintenance and repair on the one hand and reproduction on the other hand, for organisms in temporally and spatially varying environments. With this model we address the question of whether an increase in lifespan upon dietary restriction (DR) is expected to be conserved. Temporal (or seasonal) variation leads to a DR response when organisms are not too short-lived. In environments with spatial variation, short-lived organisms also evolved a DR response, but only when the investment in reproduction pays off directly. None of these responses evolved when food low quality patches did not relate to lower juvenile survival. Hence, in organisms for which the relationship between juvenile survival and current food availability does not exist, it is not expected that a typical DR response would evolve. We conclude that DR response is not expected to be conserved. If an increase in lifespan upon DR is conserved between very different organisms, for instance short- and long-lived organisms, the response is expected to be adaptive in environments differing from spatially to seasonally varying environments.

Although we made a strict distinction between temporal and spatial variation in our model, the difference in reality is much more quantitative than qualitative. When in a spatially varying environment reproduction can be easily up or down regulated within the time food availability varies, allocation patterns are expected to change very quickly. Only when organisms can experience dramatically different patches within a period of investment in reproduction, do the results qualitatively differ. This shows therefore that the time it takes to develop eggs or offspring is a major component of the plastic response to food variability, and possibly not lifespan itself.

The reason why in our model the organisms do show a DR response in the seasonal model when they have to allocate more time steps into reproduction is because seasonal variation is very predictable, while in the spatial variation model this is not the case. When the spatial variation would be more predictable, and the organisms had a choice to move or not, they would remain in high quality patches, while disperse from low quality patches (van den Heuvel *et al.* 2013; chapter 2). Also we modeled a 'fine-grained' environment, which might differ from optimal strategies compared to an environment with larger 'grain-sizes' (Macarthur and Pianka 1966; Mathias *et al.* 2001). Furthermore we modeled organisms that lived longer due to a decreased extrinsic mortality rate. We did not take body size into account although interspecific size does covary with mortality regimes (Reznick and Endler 1982; Reznick *et al.* 1990) and an increase in intraspecific lifespan relates positively to body size and resting metabolic rates (Speakman 2005; Kooijman 2010). This will alter the way organisms move around in spatially varying environments (Schoener 1969) and therefore also how they perceive the environment.

We expect longer lived organisms to be able to respond to season, varying allocation patterns dependent on time. In a field situation this would mean that organisms might adjust their allocation pattern in response to temperature and / or daylight. Therefore we would expect in that relatively longer lived organisms are more like to show different allocation patterns depending on seasonal cues such as temperature and lightning. The satyrine butterfly *Lasiommata petropolitana* and the three-spined stickleback (Gotthard 2008; Lee *et al.* 2010) are species that clearly show responses to seasonal cues. For very short lived organisms, which are expected to respond to spatial variation more than to temporal variation, we do not expect these results. The latter organisms would allocate equally to reproduction independent of when they live, and therefore

would not respond to changes in lighting and temperature. On the other hand, when the frequency of patches is different at different times of a year, then this would alter the relative lifespan between high and low quality patches. These specific hypotheses can be tested in the laboratory.

In some organisms the effect of restriction in calories is highly dependent on what type of resource is restricted. In *Drosophila melanogaster*, an increase in protein can decrease lifespan, while fecundity increases, whereas an increase in sugar can affect both traits negatively (Lee *et al.* 2008; Skorupa *et al.* 2008). This response seems to be caused by the addition of specific amino acids decreasing lifespan (Grandison *et al.* 2009). This means that the lack of a response in other organisms might be caused by the fact that the resource added, leads to an overall decrease in performance of the organism, such as in butterflies (Beck 2007). Our model suggests though that for these organisms (i.e. butterflies), where the larvae and adult eat completely different things, it is unlikely that a lifespan extension upon DR will evolve, since the relationship between adult food availability and larval survival is weak.

Although it seems that the mechanisms by which DR leads to an increase of lifespan might be conserved between yeast, worms, flies and mice (Alic and Partridge 2011) our model indicates that these widely varying organisms should actually respond in various ways to different types of environmental heterogeneity. Therefore to completely understand the evolution of the life histories regulatory mechanisms should be incorporated into theory (Flatt and Heyland 2011). Future models should include multiple resources that have various mechanistic effects on life history parameters. Furthermore, studies into the mechanisms of DR should not only include description of the mechanisms that seem to be conserved or similar. If one wants to understand the evolutionary pressures that shape different organisms, one must also look at the differences in responses in relation to the organisms that are under study. For instance, genes which are commonly up- or down-regulated in microarray studies are involved in the nature of the DR response (Han and Hickey 2005) represent a relatively small number of genes of the whole genome of these organisms. Therefore, it might be more informative to think about how organisms differ and how this might be represented in gene expression rather than to look for consistencies between species that are expected have a different response to DR. Since our model indicates that short- lived organisms respond more to spatial variation while long- lived organisms should respond more to temporal variation, we should also expect that nutrient sensing pathways (i.e. insulin and TOR pathways) of these organisms cross communicate with different types of pathway in for instance flies and mice. How organisms perceive the environment has been shown to influence the response to DR in both mammals (Nelson 1988; Froy 2007) and insects (Libert *et al.* 2007) and the common up- regulation of abiotic stimulus genes (Han and Hickey 2005) might indicate the perception of time and space in organisms under DR regimes.

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Appendix A: tables of parameter values

Table A1A. Variable parameters per figure of results section.

Figure	b_{ampl}	b_{mean}	M_{ampl}	l_{s0}	l_{si}	i_{ampl}	i_{mean}	P
1 upper row, outer left	NA	NA	0	0.8	0	0, 1.75, 3.5, 4.25	6	0.0075
1 upper row, middle left	NA	NA	0	0.8	0	0, 1.75, 3.5, 4.25	6	0.03
1 upper row, middle right	NA	NA	0	0.8	0	0, 1.75, 3.5, 4.25	6	0.12
1 upper row, outer right	NA	NA	0	0.8	0	0, 1.75, 3.5, 4.25	6	0.48
1 lower row, outer left	NA	NA	0.2	0.8	0	0, 1.75, 3.5, 4.25	6	0.0075
1 lower row, middle left	NA	NA	0.2	0.8	0	0, 1.75, 3.5, 4.25	6	0.03
1 lower row, middle right	NA	NA	0.2	0.8	0	0, 1.75, 3.5, 4.25	6	0.12
1 lower row, outer right	NA	NA	0.2	0.8	0	0, 1.75, 3.5, 4.25	6	0.48
2, left column, row 1	NA	0.8	NA	0.9	0	NA	Variable[1]	0.03
2, middle column, row 1	NA	0.8	NA	0.9	0	NA	Variable[1]	0.12
2, right column, row 1	NA	0.8	NA	0.9	0	NA	Variable[1]	0.48
2, left column, row 2	NA	0.8	NA	0.72	0	NA	Variable[1]	0.03
2, middle column, row 2	NA	0.8	NA	0.72	0.02	NA	Variable[1]	0.12
2, right column, row 2	NA	0.8	NA	0.72	0.02	NA	Variable[1]	0.48
2, left column, row 3	NA	1	NA	0.9	0	NA	Variable[1]	0.03
2, middle column, row 3	NA	1	NA	0.9	0	NA	Variable[1]	0.12
2, right column, row 3	NA	1	NA	0.9	0	NA	Variable[1]	0.48
2, left column, row 4	NA	1	NA	0.72	0	NA	Variable[1]	0.03
2, middle column, row 4	NA	1	NA	0.72	0.02	NA	Variable[1]	0.12
2, right column, row 4	NA	1	NA	0.72	0.02	NA	Variable[1]	0.48
2, left column, row 5	NA	1.2	NA	0.9	0	NA	Variable[1]	0.03
2, middle column, row 5	NA	1.2	NA	0.9	0	NA	Variable[1]	0.12
2, right column, row 5	NA	1.2	NA	0.9	0	NA	Variable[1]	0.48
2, left column, row 6	NA	1.2	NA	0.72	0	NA	Variable[1]	0.03
2, middle column, row 6	NA	1.2	NA	0.72	0.02	NA	Variable[1]	0.12
2, right column, row 6	NA	1.2	NA	0.72	0.02	NA	Variable[1]	0.48
3, left column, row 1	0	1	NA	0.8	0	NA	6	0.06
3, right column, row 1	0	1	NA	0.8	0	NA	0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11	0.24
3, left column, row 2	0	1	NA	0.7	0.02	NA	6	0.06
3, right column, row 2	0	1	NA	0.7	0.02	NA	0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11	0.24
3, left column, row 3	0	1	NA	0.8	0	NA	6	0.06
3, right column, row 3	0	1	NA	0.8	0	NA	0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11	0.24
3, left column, row 4	0	1	NA	0.7	0.02	NA	6	0.06
3, right column, row 4	0	1	NA	0.7	0.02	NA	0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11	0.24
3, left column, row 5	0.3	1	NA	0.8	0	NA	6	0.06
3, right column, row 5	0.3	1	NA	0.8	0	NA	0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11	0.24
3, left column, row 6	0.3	1	NA	0.7	0.02	NA	6	0.06
3, right column, row 6	0.3	1	NA	0.7	0.02	NA	0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11	0.24
3, left column, row 7	0.3	1	NA	0.8	0	NA	6	0.06
3, right column, row 7	0.3	1	NA	0.8	0	NA	0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11	0.24

3, left column, row 8	0.3	1	NA	0.7	0.02	NA	6	0.06
3, right column, row 8	0.3	1	NA	0.7	0.02	NA	0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11	0.24
4, left side	See text	See text	NA	0.6	0.04	NA	0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11	0.12
4, right side	0.3	1	NA	0.6	0.04	NA	0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11	0.12
5 left side	NA	NA	0.2	0.8	0	1.75, 4.25	6	0.03
5 right side	NA	0.8, 1.2	NA	0.74	0.02	NA	0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11	0.1,0.3,0.5

[1]Variable food changes per line color per patch. Values are given in table A1B (below).

Table A1B, variable food levels as indicated above in footnote per line color.

	0	1	2	3	4	5	6	7	8	9	10	11
Red	6	6	6	6	6	6	6	6	6	6	6	6
Green	3	3.5	4	4.5	5	5.5	6	6.5	7	7.5	8	8.5
Blue	0	1	2	3	4	5	6	7	8	9	10	11
Purple	0	2	4	6	8	10	12	14	16	18	20	22

Table A2. Constant parameters and their values.

Parameter	Value
c	0.01
d	20
e	1
m_1	0.001
m_2	0.01

Appendix B: Error of measurement on lifespan

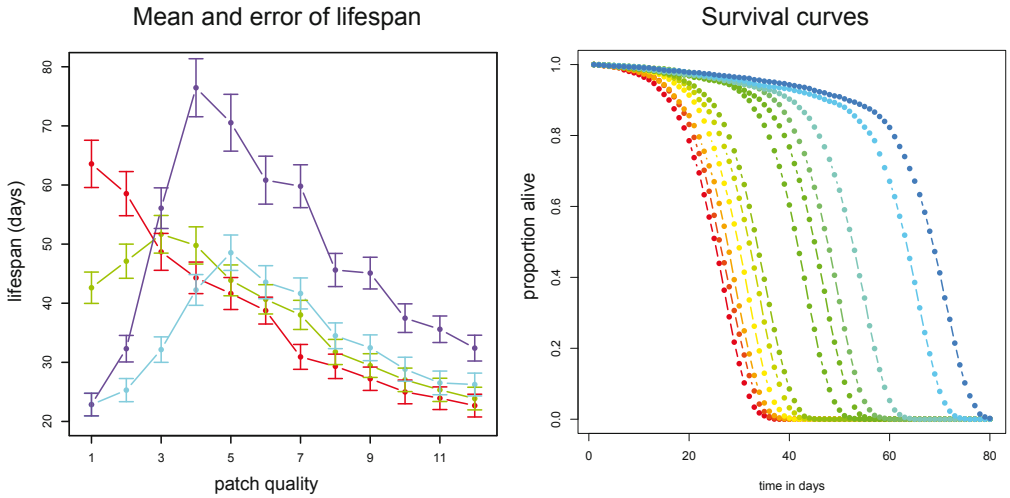

Figure B1. Individual variation expressed as standard error bars for the panel expanded from the second row left in figure 2 on the left together with the corresponding survival curves from of the twelve food levels of the red line from the left figure (on the right panel). In the left panel, standard deviations used to calculate the standard error came from 3700 simulated individuals per point, but the standard error was calculated using an N of 10. On the right panel the highest food quality curve is indicated by red while the lowest food quality curve is indicated in dark blue. For these survival curves again data from 3700 individuals were used. The condition used was chosen because the average lifespan from low quality to high quality goes steadily down.

The plastic fly: the effect of sustained fluctuations in adult food supply on life history traits

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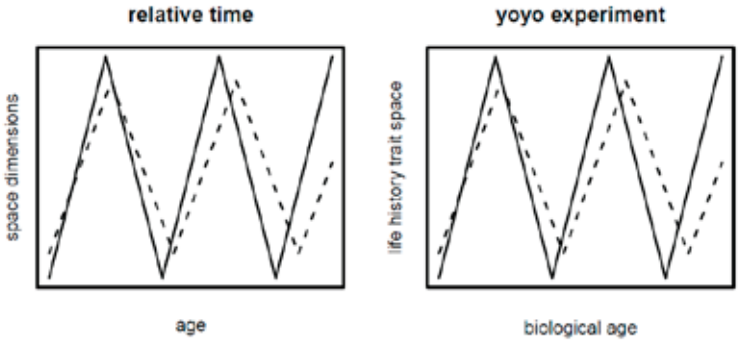

According to the theory of relativity an individual ages slower if, everything else being equal, if moves faster through space (y axis, left panel). Similarly, we ask, if an organism moves faster in life history trait space (y axis right panel, number eggs, weight) will it then age slower?

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Abstract

Many adult traits in *Drosophila melanogaster* show phenotypic plasticity and the effects of diet on traits such as lifespan and reproduction are well explored. Although plasticity in response to food is still present in older flies, it is unknown how sustained environmental variation affects life history traits. Here we explore how such life-long fluctuations of food supply affect weight and survival in groups of flies and affect weight, survival and reproduction in individual flies. In both experiments, we kept adults on constant high or low food, and compared these to flies that experienced fluctuations of food either once or twice a week. For these 'yoyo' groups, the initial food level and the duration of the dietary variation differed during adulthood, creating four 'yoyo' fly groups. In groups of flies, survival and weight were affected by adult food. However, for individuals, survival and reproduction, but not weight, were affected by adult food, indicating that single and group housing of female flies affects life history trajectories. Remarkably, both the manner and extent to which life history traits varied in relation to food depended on whether flies initially experienced high or low food after eclosion. We therefore conclude that the expression of life history traits in adult life is affected not only by adult plasticity, but also by early adult life experiences. This is an important but often overlooked factor in studies of life history evolution and may explain variation in life history experiments.

Keywords: Phenotypic plasticity, lifespan, reproduction, weight, diet, life history, ageing, resource allocation

Introduction

Phenotypic plasticity is the ability of a genotype to express different phenotypes in response to environmental variation. Some plastic traits such as wing coloration in butterflies or horn length in beetles are fixed at a specific developmental stage and cannot be changed once the phenotypes have been expressed. Such developmental plasticity may be maladaptive if the environment changes in an unexpected way after a phenotype is fixed (Ghalambor *et al.* 2007; Reed *et al.* 2010). Other traits such as metabolism and metabolic rate remain phenotypically plastic, for instance in response to food availability (Karowe and Martin 1989; Compher *et al.* 2006; Jobling 2006).

Many adult traits of the fruit fly *Drosophila melanogaster* are plastic in response to different adult environments. Lifespan has been shown in many studies to vary with food availability and temperature (Miquel *et al.* 1976; Chippindale *et al.* 1993; Partridge *et al.* 1995; Pletcher *et al.* 2002; Mair *et al.* 2003; Doroszuk *et al.* 2012). Amounts of protein and fatty acids and other traits such as reproduction co-vary with lifespan between different types of food (Lee *et al.* 2008; Skorupa *et al.* 2008). When flies are transferred once between different types of food in later stages of adult life, lifespan and reproduction can still be affected (Carey *et al.* 1998; Mair *et al.* 2003). Nevertheless, it remains unknown to what age and to what extent fruit flies can respond plastically when environments change multiple times in a lifetime, and how variation in early life traits relates to variation in traits later in life.

In this study we manipulate the environment of adult fruit flies using the nutritional level of food as the main treatment. We compare flies living in constant environments with flies that received fluctuations of food throughout adult life. Four 'yoyo' treatment groups were designed along two variables in a full-factorial design. The first variable relates to the frequency of the nutritional fluctuations; flies were transferred either once or twice a week between high and low food. The second variable constitutes the early life experience; at eclosion, flies either initially received high or low food. In a first experiment, we measured survival and weight of female flies that lived in vials at a density of five individuals (Exp#1). To enable us to follow the response in life history traits on an individual based level, we repeated the experiment with individually housed flies (Exp#2) and also monitored egg production at every transfer. These experiments were designed to reveal whether sustained fluctuations of food would have an effect on survival and to quantify the degree of plasticity in weight and reproduction in response to food. This study aims to enhance the understanding of how life histories are shaped in a variable environment.

Methods

Food

Three food levels were used in this experiment, indicated by 1x (low), 2x (intermediate) and 5x (high) medium. These food levels vary in amounts of sugar (50, 100 and 250 gram per liter in 1x, 2x and 5x medium, respectively) and yeast (35, 70, 175 gram per liter in 1x, 2x and 5x medium, respectively). The food contains agar (20 gram per liter), nipagine (15 ml of 100 g 4-methyl hydroxy benzoate per liter alcohol), and propionic acid (3 ml per liter).

Flies

Flies (*Drosophila melanogaster*) were wild-caught from six different populations along a transect between Vienna and Athens in the summer of 2008. Once established in the laboratory, they were crossed in a scheme that ensures a balanced contribution of each source population to the newly established outbred population. This latter population was reared in half-pint bottles for 50 generations with at least 300 individuals per generation on 1x medium before the experiments were started. These populations were originally established for the purpose of starting experimental evolution lines and the choice of keeping them on 1x medium was made earlier and unconnected to the present study. Rather, we used these flies because they were genetically diverse and therefore the results are expected to be relatively 'public' and more widely relevant. The experimental media were 1x and 5x, and therefore, in addition, to avoid trans-generational effects on adults, flies were reared for at least three generations on 2x medium prior to the experiment. This means that the flies are possibly adapted to one of the food types (1x) and that the data might be affected by this. Because we did not rear flies under 5x medium, we cannot control for this. If adults clearly perform better for all traits on 1x medium, this might be an effect of the short prior period of evolution in the laboratory to this medium. The larvae were reared in vials with 6 ml of intermediate food, with a density of 50 eggs per vial. After eclosion, the sex of the flies was determined, and unmated female flies were distributed over experimental vials in experiment 1 (Exp#1) using ice as anesthesia, while in experiment 2 (Exp#2) we randomly put flies in either a low food vial (6 ml of food throughout the experiment) or a high food vial (6 ml of food throughout the experiment) without using anesthesia.

The singly-housed flies were all checked for mating and possible fertilized eggs in the first three days, and fertilized females were removed from the experiments. All reported results in this study come thus from *virgin female* flies. We used virgins because fecundity in once-mated flies is strongly affected by sperm depletion during the first weeks of life. Life history of females (lifespan and fecundity) is affected by mating frequency and this additional component of variation is also avoided in our study by using virgin females.

Adult food treatment

In both Exp#1 and Exp#2, six food treatments were used. We compared flies living in constant environments of high (CH) and low food (CL) with flies that received fluctuations of food throughout adult life ('yoyo' treatment). These latter flies also received different treatments with groups that were transferred either once a week (slow yoyo) or twice a week (fast yoyo) between high and low food. Furthermore, we controlled for the first adult food vial experienced by separating both the slow and fast yoyo cohorts between flies that were initially on high food or low food. This resulted in four different yoyo fly groups: slow yoyo, high start (SYH); slow yoyo, low start (SYL); fast yoyo, high start (FYH); and fast yoyo, low start (FYL).

All flies from different treatments were transferred on the same day, even if nutrient levels did not change. Furthermore, the vial transfers were performed in such a way that, in total, the flies of the slow and fast yoyo groups fed for similar number of days on low or high food (namely always 7 days on low and 7 days on high medium per two weeks). In Exp#1, flies were kept in densities of five flies per vial. Flies were redistributed between vials when flies had died so that the density remained five for most vials. In Exp#2, flies were kept individually. In Exp#1, we started with 25 vials of flies that we weighed (125 individuals per food treatment), and a similar number

of flies that were not weighed. In Exp#2 we started with 65 individuals per food treatment.

Trait Measurements

In both experiments, flies were weighed before transfer. Weight was measured to the nearest 0.01 mg (Sartorius). Survival was checked daily, and escaped or accidentally crushed flies on vial transfer were right-censored in the analyses. In Exp#1, a control group of flies was not weighed to examine the effect of anesthesia on survival. In Exp#2 we counted the number of eggs in every vial after flies were transferred.

Statistics

The program R was used for all statistics (R Development core Team 2011). We used Chi-square-tests to determine heterogeneous survival within the first 4 days of Exp#1. For other survival analyses we fitted a Cox proportional hazard test (Cox 1972). For weight measurements in Exp#1, we fitted an ANOVA model with age (as a polynomial covariate), food level (high or low), yoyo treatment (constant, slow, fast), initial food (high or low), and possible interactions. For Exp#2, we included individual as a random effect, therefore fitting a repeated measures ANOVA with a similar model to Exp#1. In both experiments residuals fitted well with a normal distribution and variances were not unequal, and we thus fitted the data using a Gaussian error distribution. For weight data we simplified the inference by performing type II Wald test implemented in the car package (Fox and Weisberg 2011). With egg production we started with a generalized linear model (GLM) with similar factor as with weight, but with a Poisson error distribution. Because egg production showed a complex relationship with age, we fitted several GLM models, differing in the exponent used for the polynomial relationship between age and egg production, using AIC to identify the best model. The analysis was continued including individual as a random effect (GLMM), but this still lead to a polynomial with high exponent number, and, therefore, many terms. We then fitted a generalized additive model (GAM) that uses smoothing functions (Zuur *et al.* 2009). Because the fit and residual variation (mean and variances) were not equal, a negative binomial error distribution fitted the data better than a Poisson distribution. We used the mgcv package in R that automatically fits a smoothing function without a user biased degree of smoothing. It does so by penalized regression splines which maximize the explained variance taking into account the smoothness, and where a penalty of a narrower window is applied to less smoothing. The advantage is that users do not choose a specific degree of smoothness, but the smoothness is determined by an objective algorithm, and given that data are similar, fits should be similar for different users (Wood 2006). For pairwise testing of differences in weight and number of eggs between short- and long-lived cohorts of flies, t-tests were used. The relationship between weight and egg number was performed using an ANOVA and GLM with age and food as factors using a Gaussian and Poisson error distribution respectively.

Results

Experiment 1: 5 flies per vial

Survival

A higher proportion of flies that were weighed died in the first four days of the experiment, while this did not happen for the group of flies that were not weighed (256 of the 609, 42.0% of the weighed flies, 61 of the 638, 9.6% of the un-weighed flies, $\chi^2_{d.f.=1} = 173.32$, $p<0.001$, see Fig S1, table S1). We tested whether the number of deaths was distributed heterogeneously over the food treatment groups. This was not the case ($\chi^2_{d.f.=5} = 2.42$, $p = 0.79$ for un-weighed flies, $\chi^2_{d.f.=5} = 7.25$, $p = 0.20$ for weighed flies), and, therefore, the analysis was conducted by removing the data from the first four days to improve the fit of the Cox proportional hazard tests. The survival analysis using food treatment and weighing treatment as explanatory variables indicated that the two-way interaction between food and weighing, and weighing as a main effect were not significant ($Z=0.956$, $p=0.34$, for the latter). The survival curves (Fig. 1) and hazard ratios per term (Table 1) indicate that the survival of the CL flies is significantly lower than all flies in all other treatments. Although the slow yoyo flies that started high did not have a higher survival compared to the constant high flies, they did have an improved survival compared to all the other groups (Table 1). All other groups of flies, besides the CL flies, were not significantly different in survival compared to the CH flies. Therefore, flies that received sustained fluctuations had an intermediate survival, but significantly higher than the constant low flies.

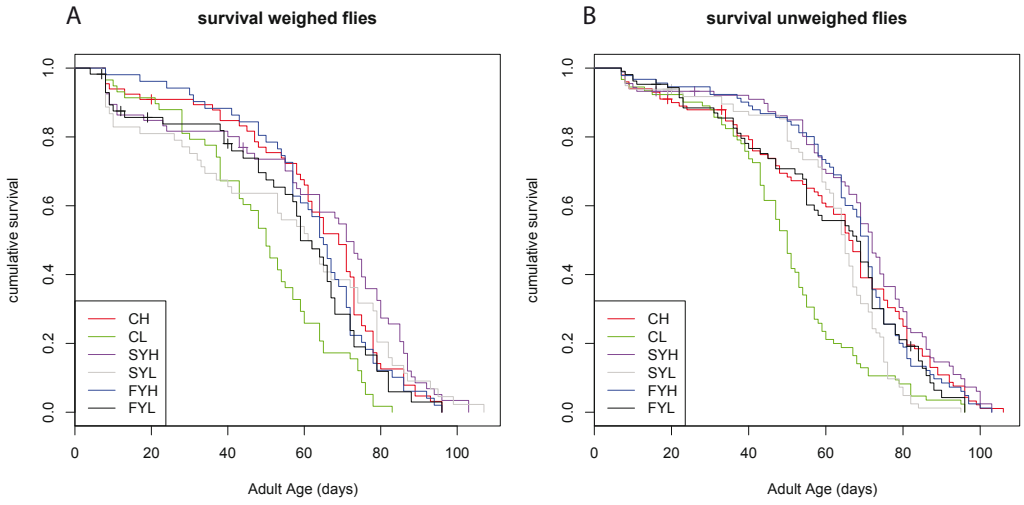

Figure. 1 (A) Survival of flies weighed with a lifespan longer than 4 days and (B) survival of flies not weighed with a lifespan longer than 4 days. Food treatments are indicated by lines with different colors.

Table 1. Statstics of the survival analysis of experiment 1.

Comparison	Hazard ratio	Con. Int. h. r.*	Z test statistic	P value
CL vs CH	2.155	1.715-2.708	6.59	<0.001
SYH vs CH	0.800	0.637-1.004	-1.93	0.0539
SYL vs CH	1.227	0.974-1.547	1.74	0.0822
FYH vs CH	1.025	0.815-1.288	0.21	0.8349
FYL vs CH	1.187	0.930-1.516	1.38	0.1683
Weighing	1.105	0.961-1.271	1.40	0.1680

*95% confidence interval hazard ratio

Weight

In this experiment we weighed all individuals in groups of flies from one vial before they were transferred to a new vial. Because we redistributed the flies to maintain the number of flies per vial as close as possible to 5, we could not perform a statistical analysis with individual or vial number as a random variable (e.g. repeated measures ANOVA). However, there remained a considerable number of measurements taken for flies in a vial with 1, 2, 3, 4 or 6 individuals, which allowed us to include number of flies in a vial in the statistical model. We only tested for treatment effects on weight until measurement 23 (84 days), because the number of replicate vials then fell below 5 for some treatments. A polynomial linear model was fitted because the effect of age was not linear with respect to weight. In the model the effect of yoyo mode (constant, slow yoyo, fast yoyo) was separated from the nutritional value of the food in the first vial after eclosion. These two are fitted as a crossed design, together with food level, time (polynomial), and number of flies in a vial.

The food effect on weight of flies in the different food treatments is shown in figure 2. Food level ($F_{1,1652}$=228.03.14, p<0.0001) was highly significant, while the effect of yoyo treatment less so ($F_{2,1652}$=4.17, p=0.016); flies were heavier when they were on high food. Interestingly, flies that began adult life on high food were on average heavier ($F_{1,1652}$=101.07, p<0.0001), but also maintained higher weights throughout life ($F_{1,1652}$=46.21, p<0.0001). Age of the flies had a large effect on weight ($F_{1,1652}$=381.28, p<0.0001, $F_{1,1652}$=196.23, p<0.0001, for terms with exponent of 1 and 2 respectively). The interaction of age and food level in the initial vial significantly affected weight ($F_{1,1652}$=46.21, p<0.0001), but also the three-way interactions with yoyo treatment ($F_{1,1652}$=9.41, p<0.0001), and to a smaller degree the number of flies ($F_{1,1652}$=2.054, p=0.0252). Unexpectedly, the number of flies in a vial as a main effect was also significant ($F_{5,1652}$=2.497, p=0.029). Lastly, the interaction between age and yoyo treatment was significant ($F_{2,1652}$=6.020, p=0.0025).

The effect of initial vial could be largely dependent on the effect of the constant lines, where the initial vial is similar to the food level throughout life. Therefore, a similar analysis was performed but only for the slow and fast yoyo lines. Both these models confirm that age, food, initial vial, and the interaction between age and initial food vial are significantly affecting weight. Therefore, the effect of initial vial was not due to the effect of the constant lines and also present when only data was taken from either the slow or either the fast yoyo lines.

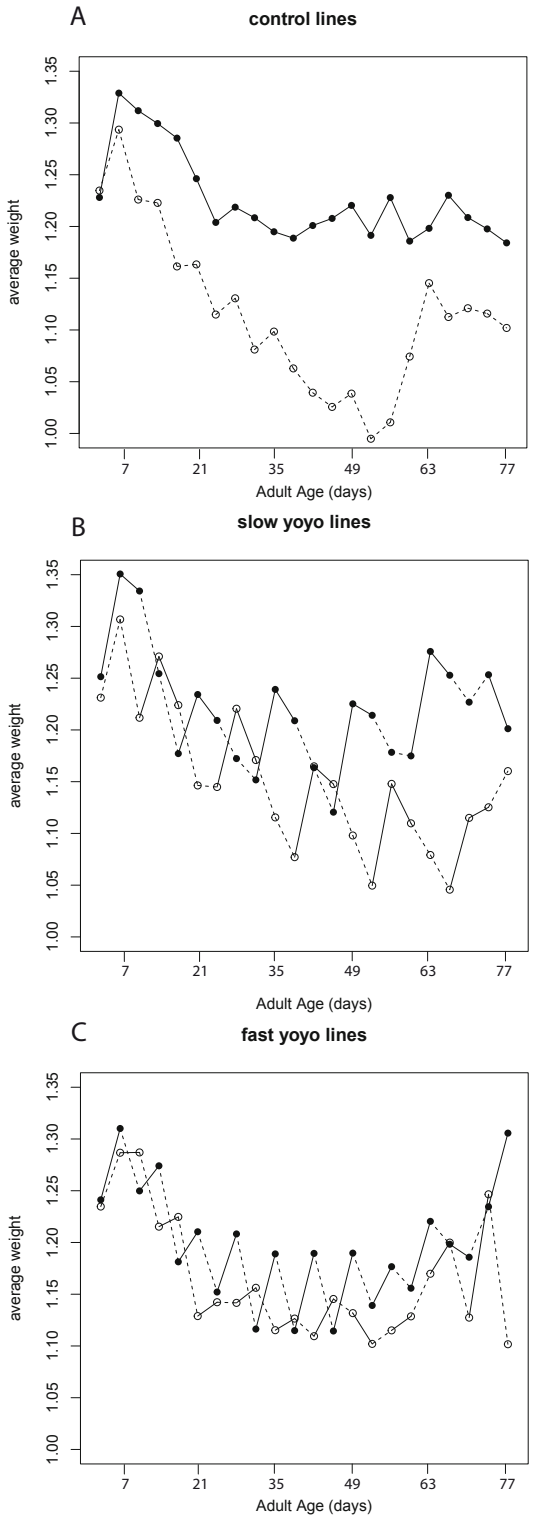

Figure 2 suggests that the effect of food for the slow yoyo lines differs depending on whether flies are moved from low to high food or from a high to low food vial. To study this further, we assigned the weight on the first high food vial as period H1, the second as period H2, the first on low food as period L1, and the second as period L2. The effect of this can then be tested for both the high and low slow yoyo lines, although they are never on the same food at the same time. Figure 3 shows, and table S2 lists, the average and standard errors per line, per period for the first 16 measurements. The flies from the SYH treatment lost weight between the high and low food vial ($t_{99.69}$=3.84, p<0.001), but then gained weight again between the low and high food vial ($t_{122.37}$=-6.32, p<0.001, Fig. 3). In contrast, SYL treatment flies lost weight during the low food period ($t_{109.66}$=2.88, p<0.005), between the first and second low food vial, and then gained weight between the low and high food vials ($t_{94.68}$=-2.75, <0.005). Remarkably, the difference in how food affects weight between SYH and SYL flies is only caused by the food level in the first week of adult life.

Figure 2 Average weight of groups of flies for constant treatments (A), slow yoyo treatment (B), and fast yoyo treatment flies (C). Filled points indicate the flies that started high, open points those that started low. Dashed lines connect two consecutive data points with low food, solid lines with high food.

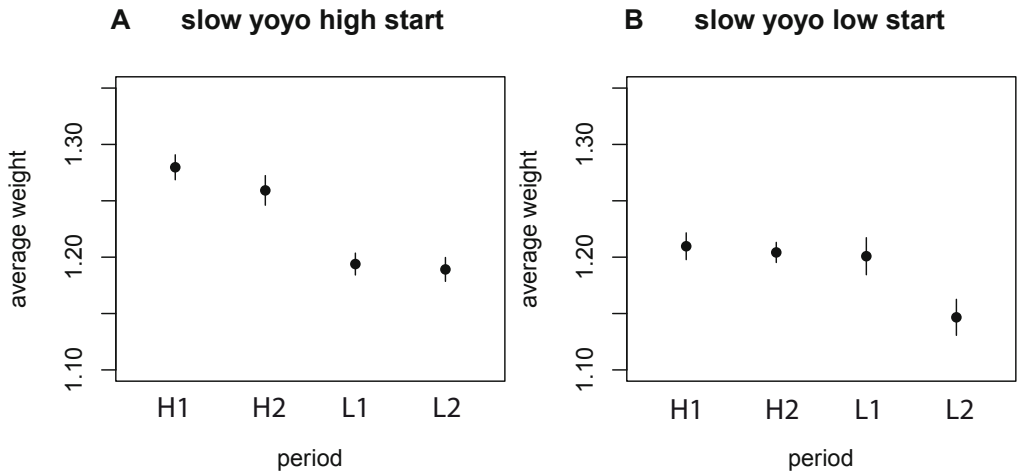

Fig. 3 Average weight per period, as explained in the text, for the slow yoyo line started on high food (A) and on low food (B). Error bars indicates 95% confidence intervals of the mean. The x axis gives the period where H1 and H2 are the first and second high food vial, and L1 and L2 are the first and second low food vial. Please note that since these are the slow yoyo lines, the SYH lines first experienced two periods high food (H1 & H2) and then two periods low food (L1 & L2), while the SYL first experienced two low food periods (L1 & L2) and thereafter two high food periods (H1 & H2).

Experiment 2: one fly per vial

In Exp#2, we monitored the dynamics of adult weight using single virgin female flies, in addition to counting the number of eggs laid. Because there was a large effect of the weighing treatment in Exp#1 (probably due to the use of anesthesia during sexing of the flies), we distributed flies in vials without sedating them in Exp#2.

Survival

The hazard ratio for mortality was the highest for the CL flies, while it was the lowest for the CH flies (Table 2, Fig. 4; $Z=5.62$, $p<0.001$). The fast yoyo treatment flies tended to have a lower hazard ratio compared to the slow yoyo treatment, which was significant when the FYL flies were compared to the SYL flies (Table 2, $Z=-2.55$, $p<0.05$). The FYH ($Z=2.15$, $p<0.05$) and SYH flies ($Z=2.678$, $p<0.01$) had significantly lower survival rates compared to the CH, but significantly higher than the CL. Thus, these flies had a significant and intermediate survival compared to the controls, while those started on low food were only significantly different compared to one of the controls (Table 2). These results are in line with the intermediate survival rates for 'yoyo' flies in Exp#1, including the higher resemblance to the CH flies.

Table 2. Statstics of the survival analysis of experiment 2.

Comparison	Hazard ratio	Con. Int. h. r.*	Z test statistic	P value
CL vs CH	2.9543	2.024 – 4.311	5.617	<0.0001
SYH vs CH	1.6525	1.143- 2.390	2.668	<0.01
SYL vs CH	2.0975	1.435 – 3.065	3.828	<0.001
FYH vs CH	1.4956	1.037 – 2.158	2.151	<0.05
FYL vs CH	1.3053	0.905 – 1.882	1.427	0.15

*95% confidence interval hazard ratio

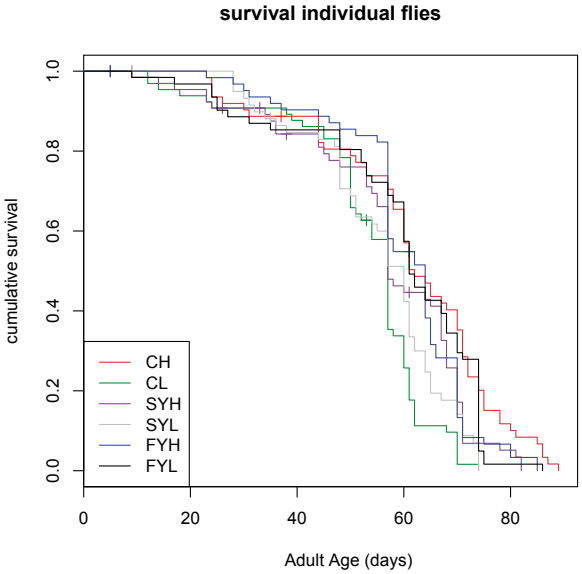

Figure. 4 Survival curves for individual flies for the six food treatments, indicated by lines with different colors.

Weight

In contrast to Exp#1, the weight of the individuals was not affected by food ($\chi^2_{d.f.=1}$ = 0.24, p=0.62) although flies with initial high food were lighter ($\chi^2_{d.f.=1}$ = 7.04, p<0.01), and lost weight faster ($\chi^2_{d.f.=1}$ = 9.52, p<0.005). In general, flies lost weight with age ($\chi^2_{d.f.=1}$ = 773.21, p<0.0001). Lastly, the interaction between food level and initial food was significant ($\chi^2_{d.f.=1}$ = 6.53, p<0.05). Flies that began life on low food were heavier on low food, while flies that began life on high food were heavier on high food. To test whether the large effect of initial food level was due to the constant food level treatments, we inspected similar statistical models per yoyo treatment. In the separate data sets, age was significant in all three yoyo treatments, and only the interaction between age and food in the constant food treatment and the interaction between age and initial food in the slow yoyo treatment were significant. Therefore, the weight of flies was affected by age and initial food level, which reaches very high significant levels when all the data are pooled.

In Exp#1, the effect of food on weight was dependent both on the type of food and on how long a fly remained on the food. In Exp#2, weight is similar between the first and second time on high food for both the SYH and SYL flies (Fig. 5, Table S3). The SYH flies lost weight after transfer to the first low food vial and then gained weight again. The SYL flies have higher weights than the SYH flies in period 1, but lost weight in the second low vial. This difference in the first and second low food vial features is paralleled by the virgin (unfertilized) egg production data, although on average the number of eggs is higher on low food for both types of slow yoyo treatment flies (Fig. 5). Again, as in Exp#1, the variation of weight (and now also the number of eggs) is both dependent on current food, the time flies spent on a specific food, and on whether they began adult life on high or low food. In contrast, the actual effect of food and time on weight differs between Exp#1 and #2 (compare Fig. 3 with Fig. 5).

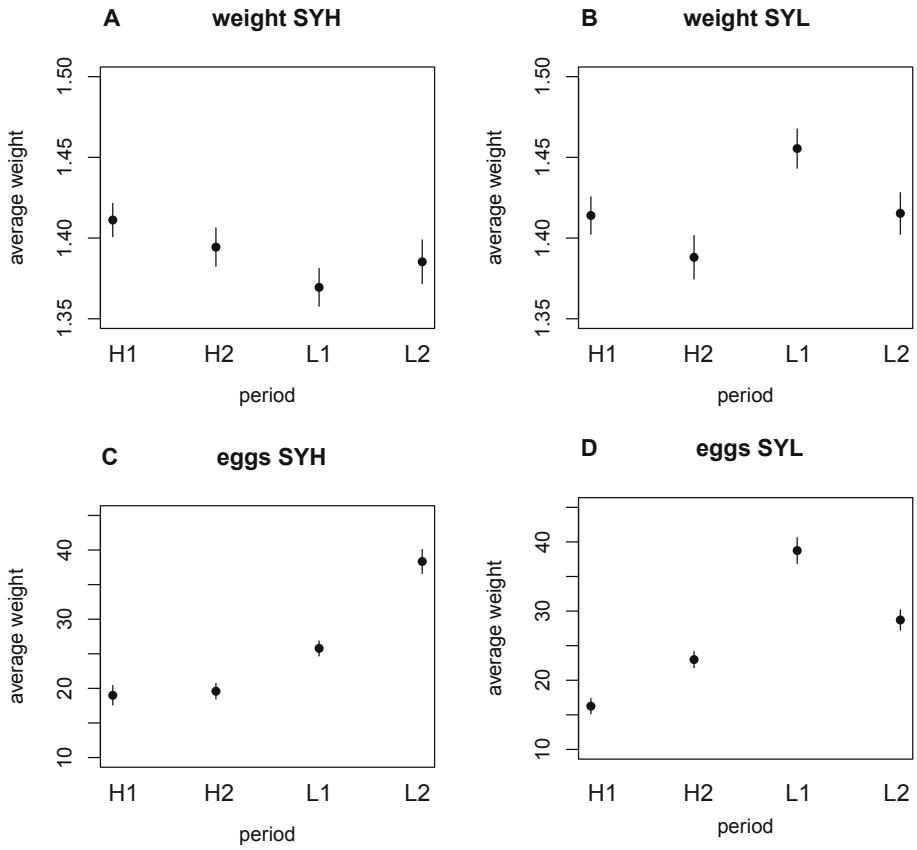

Fig. 5 Average weight (A,B) and number of eggs (C, D) for two food treatments (SYH, SYL). Error bars indicate 95% confidence interval from a normal distribution with the average trait value as mean. Please note that since these are the slow yoyo lines, the SYH lines first experienced two periods high food (H1 & H2) and then two periods low food (L1 & L2), while the SYL first experienced two low food periods (L1 & L2) and thereafter two high food periods (H1 & H2).

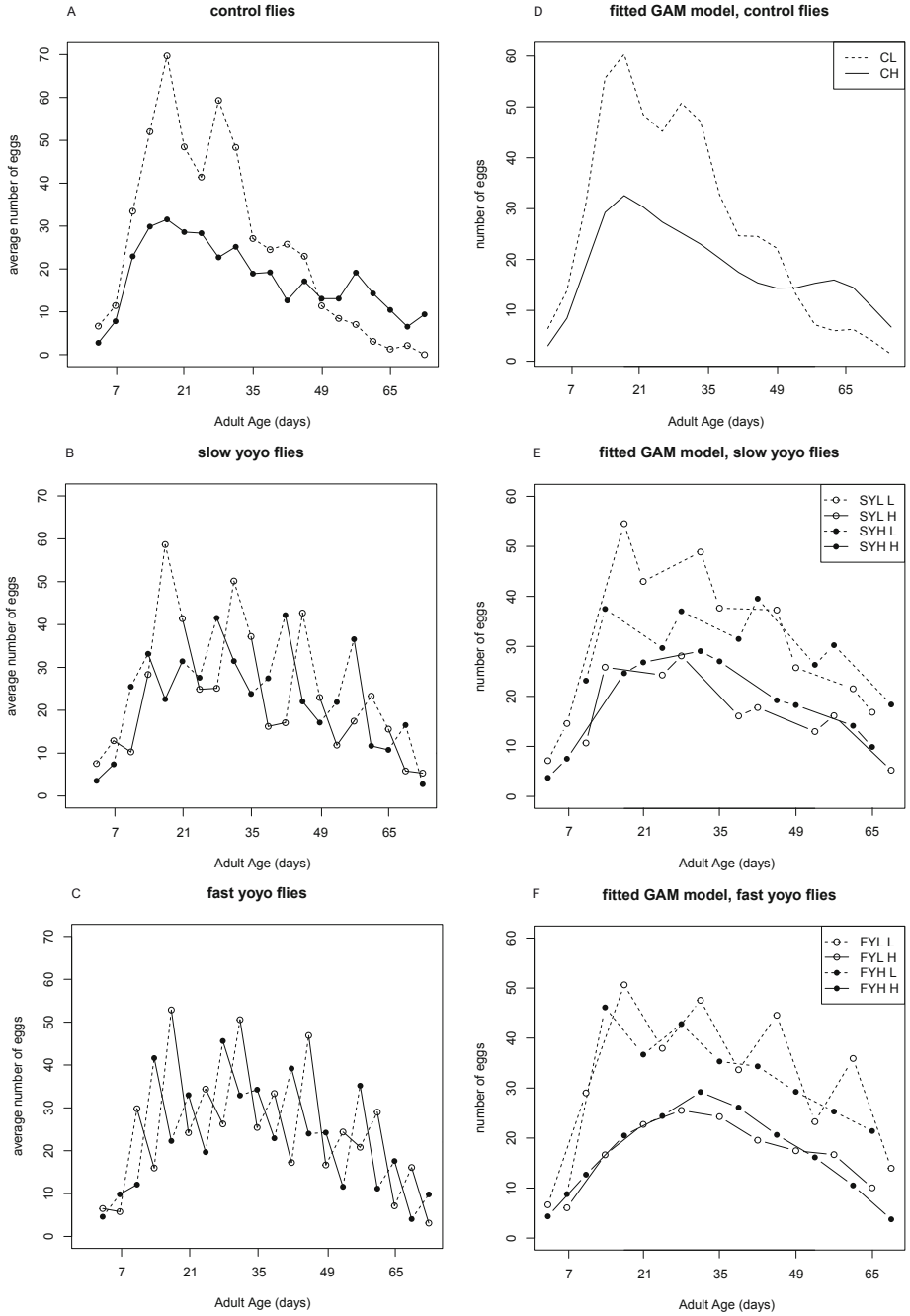

Figure. 6 The average number of eggs per food treatment shown for the two control fly cohorts (A), the slow yoyo flies (B) and the fast yoyo flies (C). In the left column (A, B, C) filled points indicate the flies that began life on high food, open points the flies that began on low. Dashed lines connect two consecutive data points with low food, solid lines indicate with high food. In the right column the fitted statistical model is given for the control flies (D), the slow yoyo flies (E) and the fast yoyo flies (F). Here solid lines indicate fitted smoothers on high food, while the dashed lines indicate the fitted smoothers on low food. For the yoyo fly panels (E and F) fitted smoothers are indicated for flies that started on low food by open circles, while closed circles indicate flies started on high food.

Egg production

In Exp#2 we also measured the egg production for each female at every transfer. A visual inspection of the data clearly indicates that the relationship between age and number of eggs is not linear (Fig. 6). Therefore we first tested what the best fit was for the data using a polynomial model with Poisson errors. This was first done with a GLM (therefore without individual as a random factor). Using AIC as test for improvement of the model, a polynomial model with terms with an exponent of 15 was the best fit, including all (and significant) two-way interactions between age, food, yoyo and start treatments. A GLMM (therefore including individual as random effect) verified that a polynomial model of age with high exponent number was the most significant, while the AIC was already lower for a linear model with individuals as random effect compared to the polynomial with exponent 15 without individual as random effect. Further verification of the interaction was done by fitting a GAM (generalized additive model), which uses smoothing functions over a covariate rather than terms for polynomial functions. The best model was one with specific smoothers for every separate food level in every food treatment for the yoyo groups and start treatment for the constant groups, indicating that flies respond differently to food dependent on yoyo treatment and initial vial food level (Table S4). This is the outcome of three separate different statistical models, and therefore is perceived to be a robust outcome of the analysis. Therefore, egg number was affected by food level, yoyo treatment, initial adult food level treatment, and age. In addition, how flies responded to food was dependent on age, yoyo treatment and initial food treatment (i.e. their interactions). For instance, although on low food the yoyo flies always produced more eggs on average, the difference between egg number on low and high food on consecutive time points is larger in slow yoyo flies compared to fast yoyo flies, and larger for flies that started on low food (for SYL; 27.19, SYH; 25.78, FYL; 16.41, FYH; 13.69 eggs more on low food). Furthermore, as flies get older, they first increase and decrease in plasticity (Fig. 6). Lastly, the improvement of explanatory variation from a GLM to a GLMM indicates that there is substantial variation among individuals. The average number of eggs per individual on both the high and the low food varies between individuals, resulting in more eggs on low food for most, but not all individuals (Fig. S2).

How do the different life history traits relate?

Weight loss per time step is significantly related to number of eggs ($F_{1,3293}$ = 243.42, p<0.001, Fig. S3) they produced in the same time period. This indicates that when flies laid more eggs per time step, they also lost more weight. This effect is much stronger when flies are on high food ($F_{1,3293}$ = 57.00, p<0.001; comparable results when tested per food treatment). When a fly gained 0.1 mg per time step, it would on average produce three eggs less, while on low food this would be four eggs. This is in addition to the overall negative effect of high food on egg number. In the models we also took into account age itself as this significantly affected the number of eggs ($F_{9,3293}$ = 84.64, p<0.001). This was true for both a linear model with a normal error distribution, as well as for a generalized linear model, with a Poisson error distribution (Fig. S3).

We further investigated the relationship between both the number of eggs, weight, and lifespan by separating the flies into short- and long-lived individuals using median lifespan (Fig. S4 & S5). For the two constant food treatment flies, egg production is higher for relatively short-lived individuals early in life, while egg production is lower later in life. This was significant when tested pairwise at several ages, but also in general the interaction between time and cohort was

significant in a full model. The relationship between time-specific egg production and lifespan was less clear for the yoyo treatment flies. Weight was significantly lower for flies that were short-lived, especially in both the slow yoyo and the high fast yoyo flies. In the pairwise tests, few points were significant due to extensive variation in weight. In a full mixed model with age, food treatment, and lifespan cohort, correcting for multiple testing within individuals, cohort had a significant effect on weight (fast yoyo: $\chi^2_{d.f.=1} = 4.18$, $p<0.05$, slow yoyo: $\chi^2_{d.f.=1} = 9.09$, $p<0.005$).

Pooled survival

Finally, we pooled the survival data from the two experiments (Fig. 7). We tested for food treatment effect (6 levels) and experiment effect (3 levels) where the levels were five individuals un-weighed, five individuals weighed and one individual (Exp#2, all weighed). The interaction between these two factors was also examined. The interaction was significant ($\chi^2_{d.f.=10} = 19.495$, $p<0.05$), but only marginally so compared to the effect of treatment ($\chi^2_{d.f.=5} = 88.790$, $p<0.001$) and experiment ($\chi^2_{d.f.=2} = 44.100$, $p<0.001$, see also Fig. 7). The interaction was due to the SYH treatment flies having a higher survival in the experiment with individual flies. The large effect of experiment was caused by a considerably lower survival of the individual flies compared to that of the SYH flies when kept in groups. The effect of treatment was mainly the effect of the CL treatment flies with a much lower survival and that of the SYL treatment flies with a marginally lower survival. In this analysis the flies that died in the first week were excluded.

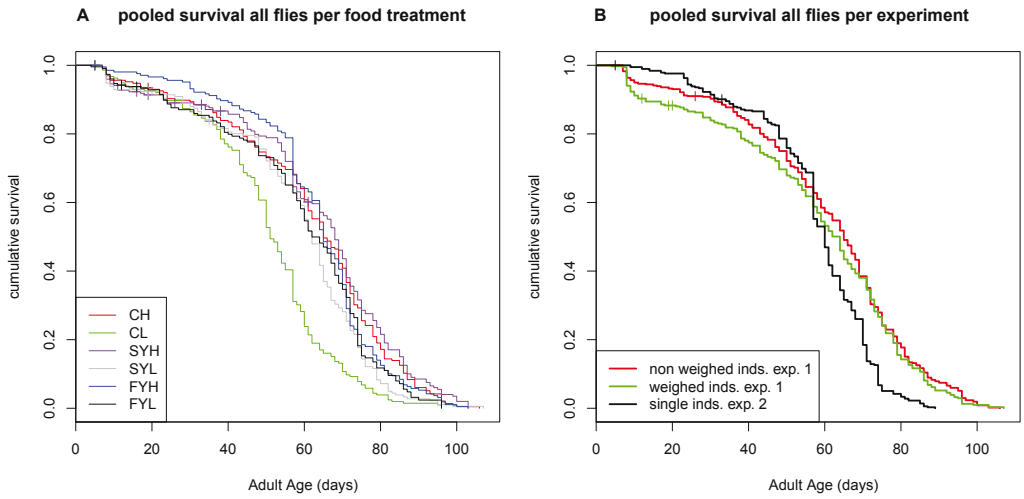

Fig. 7 Survival curves for all pooled flies of Exp#1 and #2 in A) the effect of food in all the experiments; and in B) the survival curve per experiment, separated for weighed and unweighed flies in experiment 1. The individuals that died in the first week were omitted.

Discussion

Integrating of results: some general observations

We examined whether adult flies kept on food that varied over time differed in life history traits from those maintained on constant food. Figure 8 gives an overview of the effects found of variation in food level on the measured traits. Survival of flies on sustained varying food was not lower than that of controls. The former showed an intermediate survival, and the control flies on low food had a decreased survival compared to those on several other food treatments. This suggests that there is little, if any, cost in being variable in weight (Exp#1) or in the number of eggs produced (Exp#2). Strikingly, the lifespan was very similar across experiments when food treatments were compared. Most interestingly, in addition to evidence of adult plasticity, there was also a large effect on life history traits throughout life of the initial food level experienced by a fly after eclosion. A similar effect of early adult experience was shown by (Pearl *et al.* 1927) where flies were kept in bottles with various densities which affected lifespan. For instance, when a fly was transferred from a bottle in which the density was 35 flies to one of 200 at the 16th day of age, they lived longer than flies that lived under a density of 200 throughout life (Pearl *et al.* 1927). Our study on nutrition and (Pearl *et al.* 1927)'s study of the effect of density, demonstrate the importance of early adult life experience.

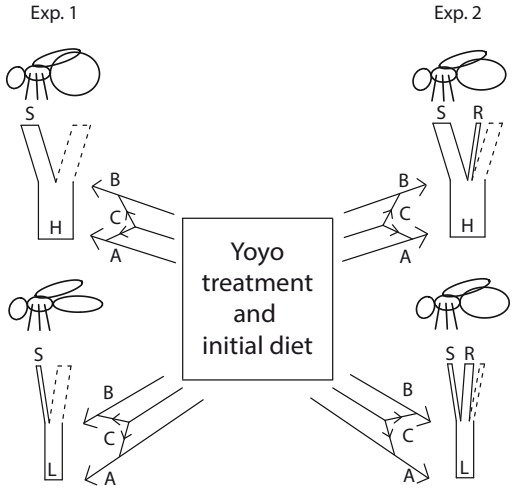

Fig. 8. A schematic overview for the outcome of the two experiments. High and low food treatments are indicated by the H and L at the stem of the 'Y'. We quantified the life history traits survival (S) and virgin egg production (R) which are indications of how the acquired resources are allocated (by the width of the stem). In experiment 1 and 2, survival is higher at high food represented here by a broader branch towards survival. In Exp#1 no other target for allocation of resource was quantified; we represent any other resource allocation by the dashed branch. The other trait measured, weight, was higher at higher food, indicated by the 'fat' fly at high food and the 'slender' fly at low food. In Exp#2 we also quantified the number of eggs: at high food allocation to survival was high but to egg production low, while the reverse was true for low food. In Exp#2 a smaller amount of the acquired resource has an unknown allocation (dashed branch). Weight was equal between high and low food in Exp#2 shown by the equal flies. In both experiments the general scenario of differential allocation holds, but the detailed relationships between acquisition and allocation of resource varies with yoyo treatment and especially initial food level experienced in the early adult life of a fruit fly. Yoyo treatment and initial food level could have affected the details of the outcome in three ways, namely, (A) by variation in acquisition, (B) by variation in allocation, and (C) by a combination of acquisition and allocation. Lastly, there are differences mainly in weight between experiments.

Weight was affected differently by food in the two experiments. Flies on high food had a higher weight in Exp#1. This was true when control flies were compared, but also when the flies on variable food were transferred from low food to high food. This was not, however, repeated in Exp#2. Rather, weight was higher, on average, for CL compared to CH flies. Weight was also higher for all yoyo treatment flies when on low food (except for the SYH). Although the food effect was not significant in Exp#2, the trend was in the opposite direction to Exp#1, indicating that food had a different effect on weight in the two experiments.

Egg production was much higher on low food in Exp#2, while flies typically produce more eggs on high food (Lee *et al.* 2008; Skorupa *et al.* 2008), although these were mated. Furthermore, gene expression studies of flies kept on high food indicate higher reproductive rates (Pletcher *et al.* 2002; Doroszuk *et al.* 2012). Other studies show that weight and reproduction are correlated and higher on high food levels (Morris *et al.* 2012). In our Exp#2 weight and reproduction are also correlated between food levels, but increased at low food. Furthermore, our FYH and FYL flies tend to be heavier, produce more eggs and have also been shown to up-regulate genes associated with reproduction, e.g. gene associated with female gamete production and chorion structure genes (chapter 5). We therefore suggest that it is likely that the flies on high food in Exp#1 also produced more eggs. This would mean that not only weight, but also reproduction is affected in a different way by food in Exp#1 and #2. In general reproduction can be differentially regulated by the environment, which is matched by the expression of reproduction-related genes. .

Methodological reasons for differences between Exp#1 and #2.

Our two experiments differed in how flies were treated. In Exp#1, a large proportion of the flies died in the group that was weighed. Therefore, in Exp#2, we did not sedate them during the distribution of flies to vials. Furthermore, we did not measure egg production in Exp#1 which was increased on low food in Exp#2. Although we repeated Exp#2 and similar differences between food levels were found in egg number, we did not repeat Exp#1, using five flies per vial to count the eggs. Our conclusion that flies are very plastic in response to food and that these responses are in a largely determined by yoyo treatment and initial food level remain, with or without the addition of eggs number in Exp#1, since proof of the involvement of these factors have been found in both experiments.

The effects of living in a group

Although the differences between the experimental outcomes might have been caused by variation in treatments, there could be other, more biological explanations, such as an increased feeding rate of flies when kept at higher densities (Wong *et al.* 2009). It is known that an increase of sugar and yeast has interactive effects on life history outcome (Grandison *et al.* 2009). In other species of fruit flies it has been shown that the effect of feeding rate on reproduction and lifespan interacts with level of carbohydrate and protein content of food (Fanson *et al.* 2009; Fanson *et al.* 2012). In our experiment, a difference in feeding rate between Exp#1 and Exp#2 might have led to a change in the relationship between high and low food and the measured life history traits (see also Fig. 8). In Exp#2, flies on high food produced fewer eggs and tended to have lower weights. If we had only considered these two traits, we might have concluded that more acquisition (i.e. high food) leads to lower resource output (egg and weight), which is opposite of that expected from the difference in nutritional value of the food. According to the Y model, relationships

between traits are the composite effect of both variation in acquisition and allocation of resource (Van Noordwijk and De Jong 1986). Because survival was higher in the flies on high food (when control flies are considered), the Y model is sufficient to explain the variation in life history traits in Exp#2, where flies on high food might have allocated more resource to maintenance and repair, and therefore have the potential to live longer. Hypothetically, they could then have allocated less resource to weight gain and egg production, and therefore, flies on high food are both lighter and lay fewer eggs, while increasing survival. Although the Y model can be extended to contain more loci underlying the variation in traits (De Jong and Van Noordwijk 1992) it is also important to consider the physiology of more than two traits (Calow and Townsend 1981; Sibly and Calow 1987; Boggs 2009).

Furthermore, a particular prediction of the Y model hypothesis is that individuals that have a higher acquisition of resource might show less negative relationships between life history traits compared to those acquiring fewer resources. A more negative relationship between weight gain and egg production for individuals on high food was found in Exp#2. Similar patterns have been found in *Daphnia*, where on higher food levels, relationships between survival and egg rate have been found to be more negative (Olijnyk and Nelson 2013). Because it is not clear how much resource any particular trait costs to develop, it is uncertain how relationships between multiple traits play out, even more so when acquisition is varied. In our experiment, the more negative relationship between weight gain and egg production on high food can be explained by the Y model if the increase in egg production were more costly because of higher allocation to survival on high food. However, it remains unclear how costly these specific functions are and how the costs of these functions relate to each other, and also whether these costs are similar on different food types. These costs must be incorporated into the Y model to completely model the actual relationships between traits on different food types (*cf* (Olijnyk and Nelson 2013).

Adult plasticity and early adult experience

In this study we set out to examine the influence of adult acclimation on life history traits. Survival, weight, and egg production were affected by adult plasticity. Interestingly, strong and persistent effects were found of the initial food condition of the adult flies. For instance, the influence of food on fly weight differed between SYH and SYL flies in Exp#1. Furthermore, the FYH and FYL differed widely in how they responded to high food (see Figure 3, lower panel). Similarly to Exp#1, the effect of initial food experience on weight in the SYH and SYL differed in Exp#2, as well as that on egg production.

We conclude that while many studies have considered the influences of developmental plasticity on adult life histories in numerous organisms, the influence of the earliest adult experience, at least in *Drosophila*, is also of great importance. (Pearl *et al.* 1927) showed in early work that density in young flies can have a long lasting effect on their life histories (Pearl *et al.* 1927). In our study we have demonstrated long-lasting effects of nutrition in early life on late life history. Because fruit flies cannot perceive changes in environmental nutrition during the pupal stage and rely on information from the larval stage, it might be beneficial for a short-lived organism to be able to alter the life history decisions immediately dependent on (very) early adult experience. Although these changes are persistent, their adaptive value is likely to be on a short time scale in the field as fruit flies are thought to experience high mortality rates (Dobzhansky

and Wright 1947; Crumpacker and Williams 1973). Following the main evolutionary theories of ageing (Medawar 1952; Williams 1957; Kirkwood 1977), selection is considered to act primarily on adults early in life which will have affected the life history including the nature and extent of plasticity. Thus, in the ecological context, fixation of life history traits in very young adult flies is more likely to be adaptive in the early adult life history rather than through any long-lasting effects or predictive abilities of future conditions to be experienced in later life. Nevertheless, we consider that the type of substantial consequences revealed in our experiments of the dietary conditions experienced immediately after eclosion will repay further investigation in other organisms. This may be particularly important in those invertebrates in which some adults in the wild can have extended reproductive lifespans. Such effects could then play a role alongside developmental plasticity in pre-adults in forming predictive responses regarding environments to be experienced later in adult life (Brakefield and Zwaan 2011; Flatt *et al.* 2013).

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Appendix Tables

Table S1. Individuals alive and dead after 4 days per line and weighing treatment.

	CH	CL	SYH	SYL	FYH	FYL
Alive non weighed	100	91	89	98	92	107
Alive weighed	66	58	67	53	52	57
Deaths non weighed	9	13	8	8	12	11
Deaths weighed	39	44	39	45	45	44

Table S2. Descriptive statistics and t tests for weight of the slow yoyo lines.

	Mean SYH	S.e. mean SYH	Mean SYL	S.e. mean SYL
Period L1	1.2797	0.0111	1.2097	0.0119
Period L2	1.2592	0.0131	1.2042	0.0089
Period H1	1.1939	0.0097	1.2008	0.0164
Period H2	1.1891	0.0106	1.1466	0.0160
T tests	T value SYH	P val SYH	Mean SYL	P val SYL
Per L1 – L2	1.342	0.098	0.276	0.609
Period L2 – H1	3.843	<0.001	0.241	0.595
Period H1 – H2	0.300	0.382	2.878	<0.005
Period H2 – L1	-6.319	<0.001	-2.754	<0.005

Table S3. Descriptive statistics and t tests for weight and egg number of the slow yoyo lines

Weight	Mean SYH	S.e. mean SYH	Mean SYL	S.e. mean SYL
Weight Per. L1	1.411	0.010	1.414	0.012
Weight Per. L2	1.394	0.012	1.388	0.013
Weight Per. H1	1.369	0.012	1.455	0.012
Weight Per. H2	1.385	0.014	1.415	0.013
T tests	T value SYH	P val SYH	T value SYL	P val SYL
Per L1 – L2	1.062	0.144	1.457	0.073
Period L2 –H1	1.492	0.078	-3.711	<0.001
Period H1 – H2	-0.883	0.189	2.266	0.012
Period H2 – L1	-1.518	0.064	0.0748	0.560
Eggs	Mean SYH	S.e. mean SYH	Mean SYL	S.e. mean SYL
Eggs Per. L1	19.00	1.405	16.26	1.101
Eggs Per. L2	19.59	1.146	22.99	1.202
Eggs Per. L3	25.78	1.091	38.75	1.887
Eggs Per. L4	38.35	1.730	28.72	1.452
T tests	T value SYH	P val SYH	T value SYL	P val SYL
Per L1 – L2	-0.323	0.373	-4.130	<0.001
Period L2 – H1	-3.915	<0.001	-7.067	<0.001
Period H1 – H2	-6.144	<0.001	4.227	<0.001
Period H2 – L1	8.681	<0.001	6.838	<0.001

Table S4. AIC values of the GLM, GLMM and GAM models of egg number. A description of the model is given, where exponent indicates to what term age is modelled including all the interactions between all the terms. A GLM with age polynomial exp. 2 means that a model is fitted with a parameter for age as a covariate as well as age to the power of 2, therefore a second degree polynomial functions. All two way interactions are fitted as well, between initial food level, yoyo treatment, food level and age terms. In a GLMM also individual as random factor is taken into account. In a GAM, the variation of egg number is modelled using a smoothing function for the relationship between age and egg number. The GAM models vary in the number of smoothing functions. A GAM can consist of one smoothing function for the average effect of time over all food treatments, added with factors for yoyo treatment, food level and initial food level. This can then be compared with a model that contains a smoothing function for every yoyo treatment added with additional factors for food and initial food such as GAM1. The model with most smoothing functions is the analogous model in GLMs which have the most factors present, but then using additive modelling.

Model	AIC	Description model
GLM1	136956.9	GLM age polynomial exp. 1
GLM2	117055.1	GLM age polynomial exp. 2
GLM3	111601.4	GLM age polynomial exp. 3
GLM4	109344.6	GLM age polynomial exp. 4
GLM5	109070.3	GLM age polynomial exp. 5
GLM15	104575.3	GLM with age polynomial exp 15
GLMM1	103798.1	Glmm with age polynomial exp 1
GLMM2	84261.5	Glmm with age polynomial exp 2
GLMM3	79043.4	Glmm with age polynomial exp 3
GLMM4	76771.4	Glmm with age polynomial exp 4
GLMM5	76482.4	Glmm with age polynomial exp 5
GAM1	105688.4	GAM with separate food smoothing functions per yoyo treatment, with poisson error distribution
GAM2	103513	GAM with separate food smoothing functions per yoyo treatment and start treatment within yoyo treatment, with poisson error distribution
GAM3	45792.1	GAM with separate food smoothing fucntions per yoyo treatment, with negative binomial error distribution
GAM4	45754.9	GAM with separate smoothing functions per yoyo treatment, food level per start treatment within yoyo treatment, with negative binomial distribution

Appendix Figures

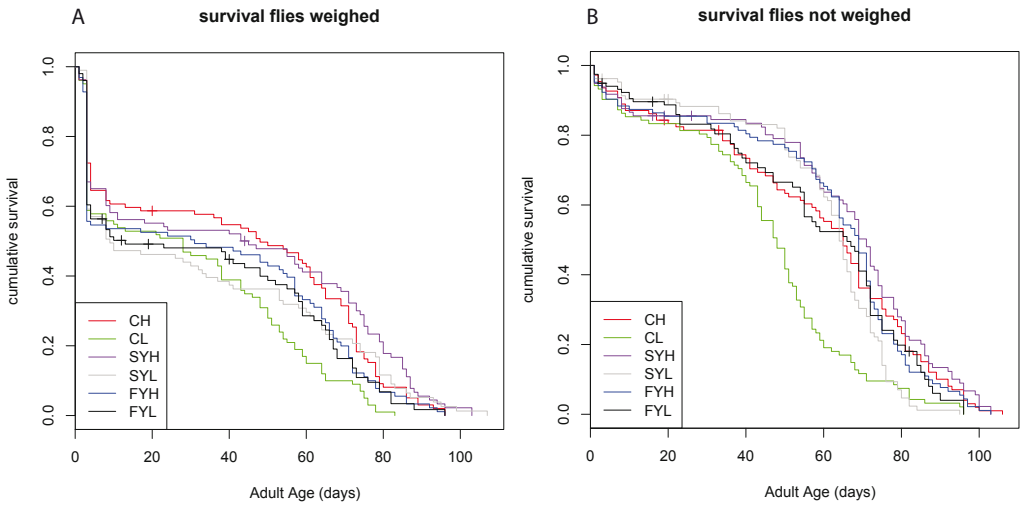

Figure S1. A: cumulative survival of the flies that were weighed (left), and B: cumulative survival of the flies that were not weighed (right) of all flies, including the ones that died in the first four days. The different lines indicate the difference in food treatment.

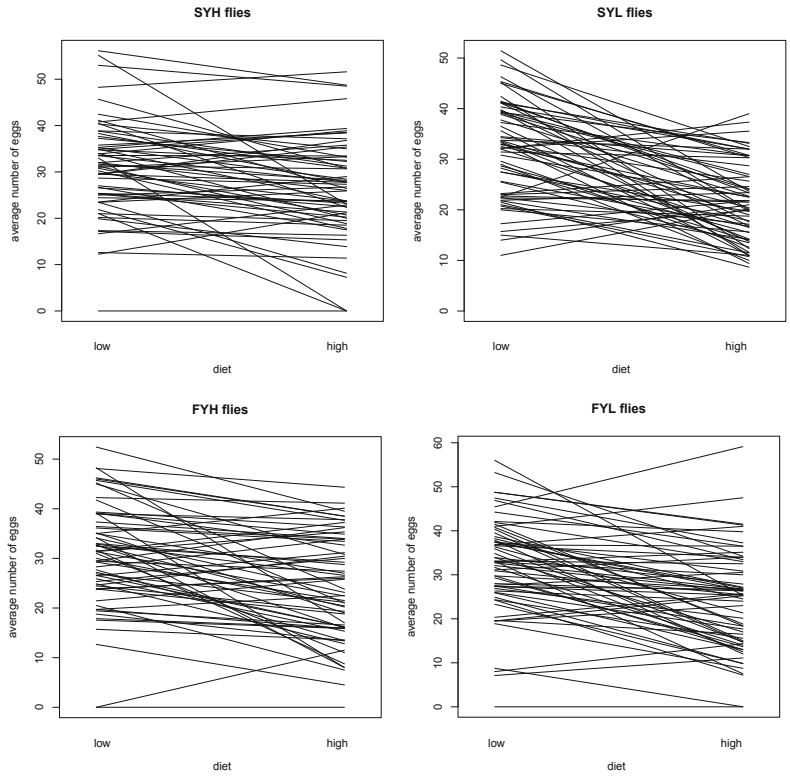

Figure S2. Individual plasticity in the average number of eggs on low and high food. Every point indicates the average number of eggs per individual with on the left, the average on low food and the right the average number of eggs on high food.

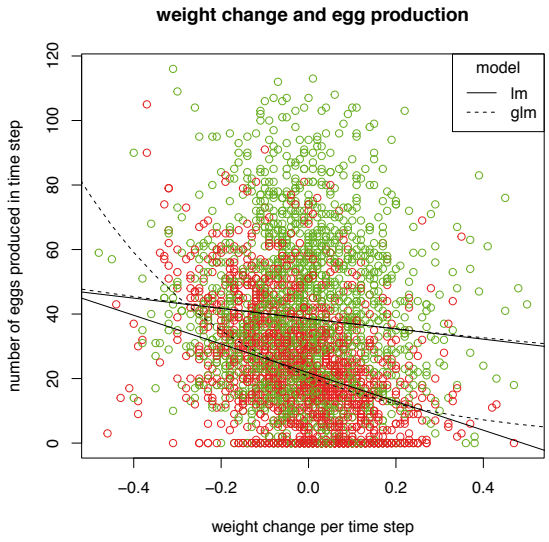

Figure S3. Weight loss and number of egg produced per time step for low food (green) and high food (red). Lines indicate models where the average fit (intercept) is higher for low food, but also the effect of weight loss on number of eggs is more negative. Solid lines indicate fit from a linear model with normal error distribution, dashed lines indicate the fit of a model fit a Poisson error distribution.

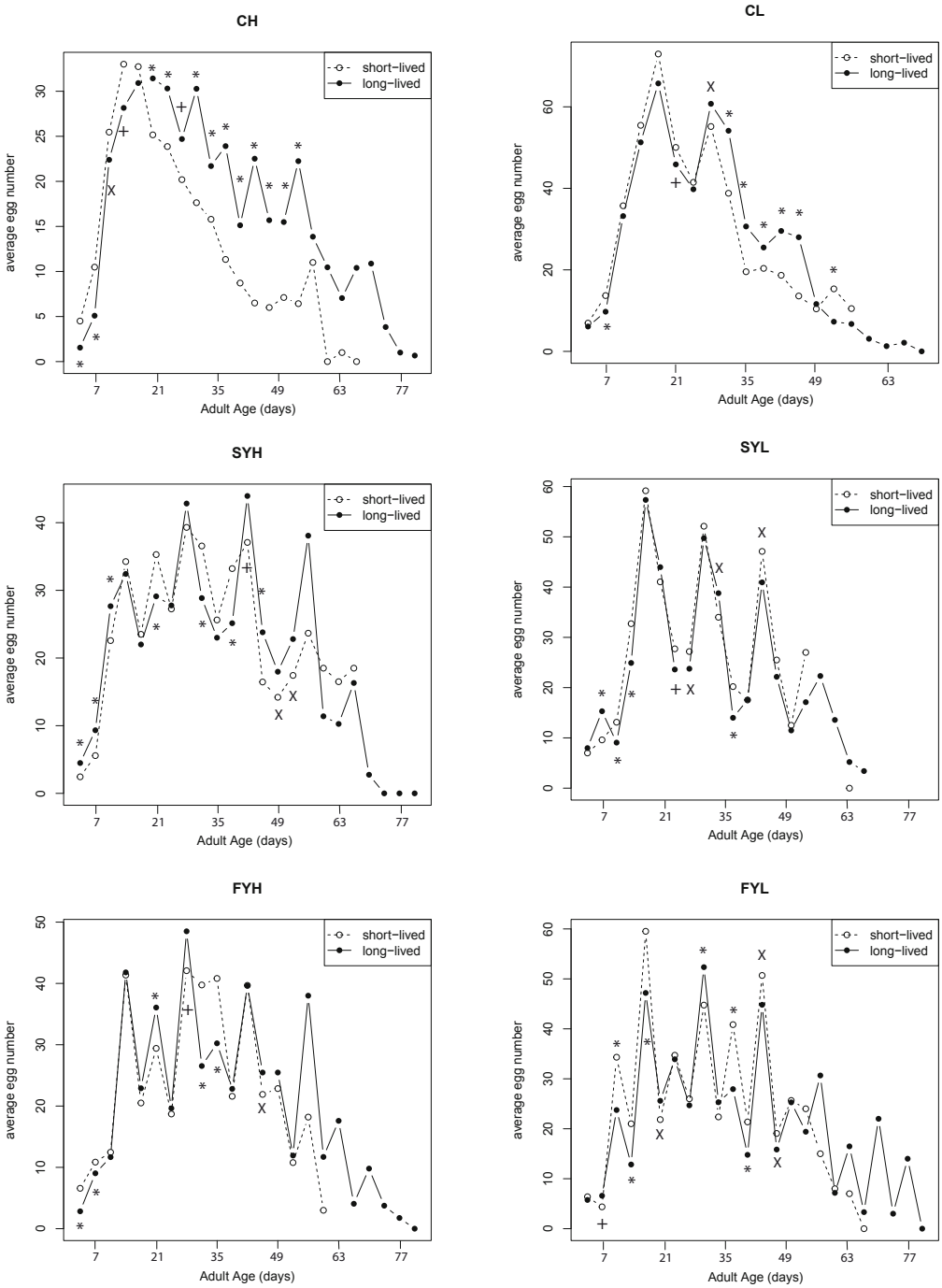

Figure S4. Egg production for flies that are short- and long-lived within every treatment. Symbols indicate pairwise means that differ significantly at the level: p<0.000275 (*), p<0.0025 (+), or p<0.025 (X). Only the first 15 measurements were pairwise tested.

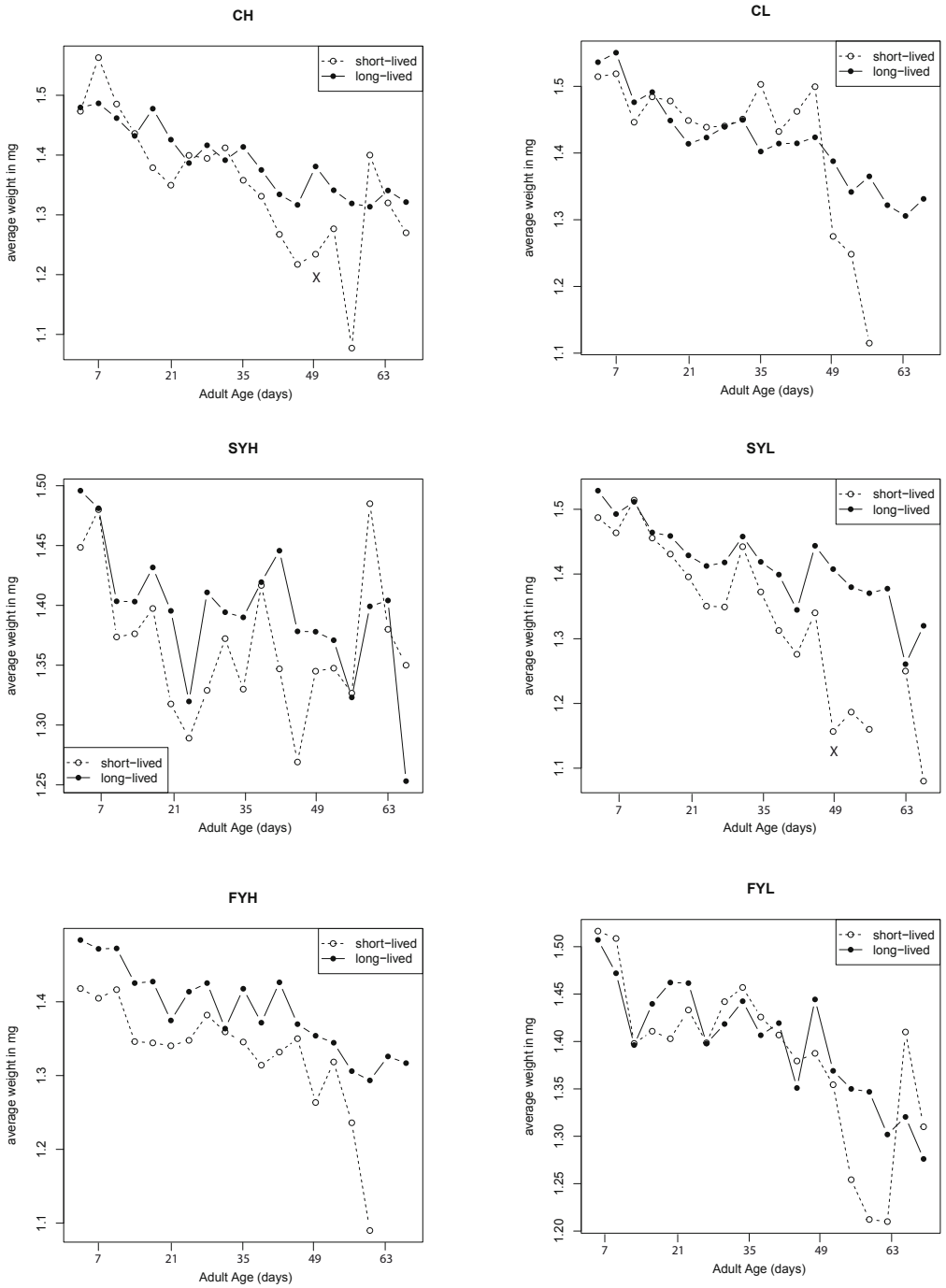

Figure S5. Egg production for flies that are short- and long-lived within every treatment. Symbols indicate pairwise means that differ significantly at the level: p<0.000275 (*), p<0.0025 (+), or p<0.025 (X). Only the first 15 measurements were pairwise tested. Only significant difference at a level of p<0.025 were found.

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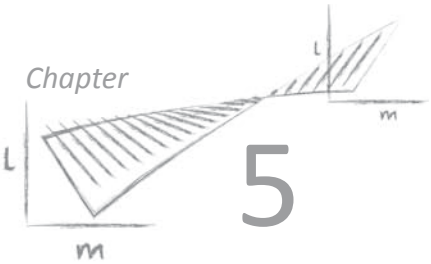

Linking life history trait and transcriptome variation using cyclical nutritional environments in *Drosophila melanogaster*

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"I'm the yo-yo man, always up and down,
so take me to the end of, your tether"

McCulloch, Sergeant, Pattinson, de Freitas

Manuscript

Abstract

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

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

Introduction

Phenotypic plasticity is the ability of a genotype to produce alternative phenotypes in different environments (Piersma and Drent 2003; West-Eberhard 2003) and is a very important concept in the study of life history evolution (Roff 1992; Stearns 1992; Flatt and Heyland 2011). Although phenotypic plasticity is often viewed as adaptive (Stearns and Koella 1986), whether this is true is rarely tested (van den Heuvel *et al.* 2013; chapter 2, but see for individual traits under laboratory conditions Partridge *et al.* 1994; Fischer *et al.* 2003). If fact, phenotypic plasticity can be maladaptive if the phenotype does not match the requirements of the changed environment and the likelihood of this occurring critically depends on how predictable the environment is (Reed *et al.* 2010). Both the evolution (Roff 1992; Stearns 1992) and plasticity of life history traits are greatly influenced by resource availability. For instance, organisms might develop into distinct phenotypes (Maret and Collins 1997; Frankino and Pfennig 2001; Bento *et al.* 2010) depending on resource availability, which in turn leads to variable food utilization (Pfennig and McGee 2010). Such plastic responses may be subtle and continuous in nature. This may be reflected in results from laboratory experiments. For instance, in some species lifespan increases trough mild dietary restriction (McCay *et al.* 1935; Klass 1977; Muller *et al.* 1980; Weindruch *et al.* 1986; Chippindale *et al.* 1993), while other species respond differently (Carey *et al.* 2002; Cooper *et al.* 2004; Beck 2007; Kasumovic *et al.* 2009). This variation between species and populations within species may reflect the degree of laboratory adaptation (Nakagawa *et al.* 2012), but, alternatively, evolutionary explanations might be able to account for the species and population-specific responses to dietary restriction.

Individuals acquire resources from their environment that they allocate to the many diverse functions such as growth, development, egg production and maintenance and repair, and past selection has resulted in an allocation pattern that maximizes fitness (Calow and Townsend 1981; Calow 1987; Sibly and Calow 1987). This framework of acquisition and allocation has been central for the development of the theory of life history evolution (Roff 1992; Stearns 1992; Flatt and Heyland 2011) and can for instance explain variation in lifespan between species (Kirkwood 1977; Kirkwood and Holliday 1979; Kirkwood and Austad 2000). Importantly, combining genetic variation in acquisition and allocation can lead to trait correlations that vary between positive and negative (Van Noordwijk and De jong 1986).

The resource acquisition and allocation framework (Calow and Townsend 1981; Van Noordwijk and De jong 1986; Calow 1987; Sibly and Calow 1987) has been very helpful to understand the relationship between food content and various life history traits (Boggs 2009). Using this framework, for instance, it can be explained why it is adaptive for mice to extend lifespan upon dietary restriction by increasing maintenance and repair at the cost of reproduction at intermediate food levels (Shanley and Kirkwood 2000). Similarly, in flies it has been shown that relatively more energy is invested in protein turnover compared to reproduction in dietary restriction regimes compared to fully fed individuals. In contrast, total energy spent on protein turnover, which can serve as an indicator for maintenance, was higher in fully fed flies (Tatar 2011). Animals with a reduced energy intake may also produce less damaging molecular species because of a reduction in metabolic rate (Mccarter and Mcgee 1989), and therefore, total maintenance and repair of DNA and protein turnover must be seen in a relative amount, not only to total intake, but also compared to how much cellular damage is inflicted. Furthermore, additional acquisition of specific amino acids may increase or reproduction (Grandison *et al.* 2009) without affecting lifespan, which may indicate that organisms do not always fully use all

resource in maximizing these two traits. These results indicate that the evolutionary framework of acquisition and allocation can be used to explain why relationships between life history traits vary between food levels, but mechanistic extensions to this framework are needed to explain specific outcomes such as those found in specific cases such as the fruit flies.

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

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

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

Methods

Diet

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

Flies

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

Adult diet treatment

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

Trait Measurements

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

Microarray experiment

For the microarray experiment a new cohort of flies was set up. The flies received he same treatment as those for life history assessment, with the exception that after being anesthetized,

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

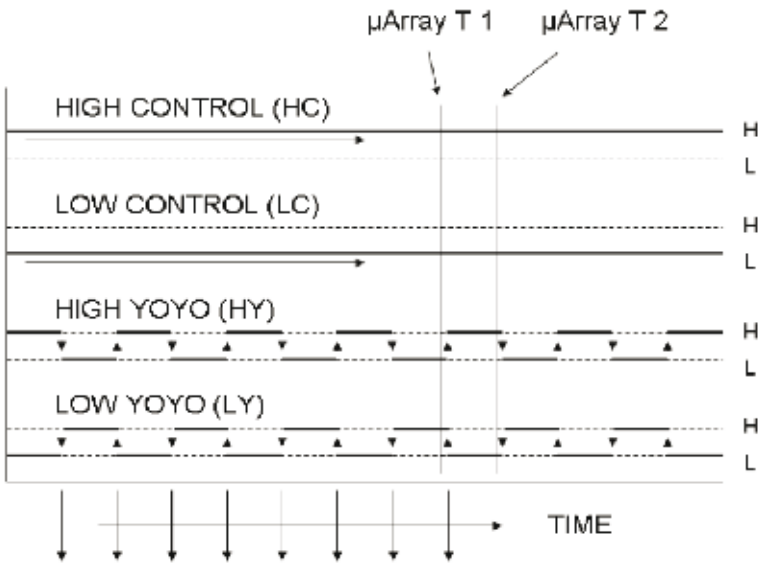

Figure 1. Schematic overview of the setup of the experiment. The four food treatments vary in when they get high (H) or low (L) food (as indicated by the horizontal solid line on the dashed lines). The high control (HC) treatment flies are on high food throughout the experiment. The low control (LC)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

Statistics

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

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

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

GO terms (see Appendices C1, C2, D1, D2, E1, E2, F1, F2 for GO term enrichment results, G1 for results of this comparison, G2 for lists of probes). These enriched GO terms were represented by probe sets in our study. The average expression of the probe sets per GO term was calculated per sample for the four other studies to compare the expression with our study. This method allows to us to answer the question whether our genes that represent enriched GO terms in our study are also up or down regulated in these other studies.

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

Food: high food vs low food			
Samples	Genes significant	Up on high	Down on high
All samples (32)	5946	3150	2796
Control samples (16)	3914	2341	1573
Yoyo samples (16)	2722	1490	1232
Yoyo low start (8)	2004	1155	849
Yoyo high start (8)	495	244	251
Time: first time point vs second time point			
Samples	Genes significant	Up in first	Down in first
All samples (32)	4424	2848	1576
Control samples (16)	5251	3425	1826
Yoyo samples (16)	0	0	0
High food (16)	4561	2919	1642
Low food (16)	0	0	0
Control vs yoyo lines			
Samples	Genes significant	Up for yoyo	Down for yoyo
All samples (32)	74	26	48
yoyo on low food vs control on low food (16)	747	483	264
yoyo on high food vs control on high food (16)	0	0	0
Yoyo high start vs yoyo low start			
Samples	Genes significant	Up for yoyo low	Down for yoyo low
yoyo high start vs yoyo low start (16)	0	0	0
Yoyo low start on low food vs yoyo high start on low food (8)	0	0	0
Yoyo low start on high food vs yoyo high start on high food (8)	0	0	0

Results

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

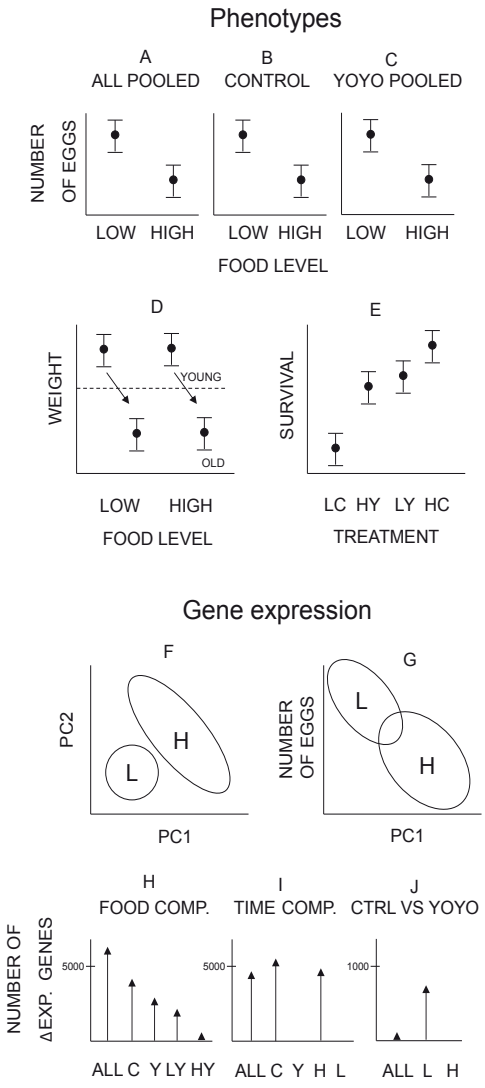

Figure 2. Schematic overview of the results. A. Survival is lowest for the low control line, and highest for the high control line. The low and high yoyo lines are significantly different from the low control, but only the high yoyo line has a significant lower survival rate compared to the high control. B. In time the weight of flies decreased, but with a similar amount between treatments and food types. Flies in different food thus do not differ in weight. C. Egg production is higher at the low food level when all samples are pooled. D. Egg production is higher for the control flies on low food compared to the ones on high food. E. Egg production is higher for yoyo flies when they are on low food. F. PC1 and PC2 of the PCA on gene expression of all genes can separate the low (L) and high (H) food samples. G. Number of eggs counted per sample correlates negatively with PC1, the low food samples overlap slightly with the high food samples. H. Number of differently expressed probes on the y axis dependent on the food contrast. If all (ALL) samples are taken, 5946 probes are tested to be significantly differently expressed. Within the control sample (C) and yoyo samples (Y) this is less. More genes are differently expressed between high and low food when this was compared within the low yoyo treatment (LY) compared to the high yoyo treatment (HY). I. Number of differently expressed probes on the y axis dependent on the time contrast pooled for all samples (ALL), control samples (C), yoyo samples (Y), high food samples (H) and low food samples (L). The highest number of probes differently expressed was found in the control contrast (C). There is a big difference in number of genes expressed differently when the control contrast is compared to the yoyo contrast and when the high food contrast is compared to the low food contrast. J. Number of differently expressed probes on the y axis dependent on the control against yoyo treatments contrast pooled for all samples (ALL), low food samples (L) and high food samples (H). A very low number of probes is found differently expressed when all samples were pooled. A much larger number of probes were differently expressed the low food samples were tested compared to high food samples (0 probes differently expressed) and to all samples pooled.

Phenotypes

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

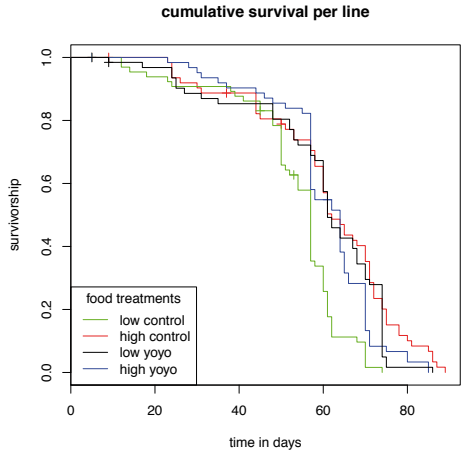

Figure 3A. Survivorship for the four different food treatments lines (green indicates low control, red high control, black low yoyo, blue high yoyo). Y axis represents proportion of flies alive.

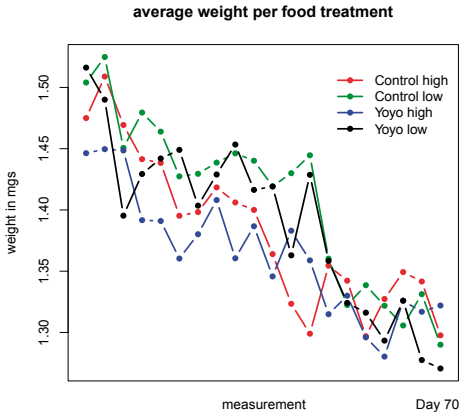

Figure 3B. Average weight dependent on food treatment and time (measurement). The first 20 measurements are shown. At the first measurement flies are 4 days old, at the last in this figure 70 days.

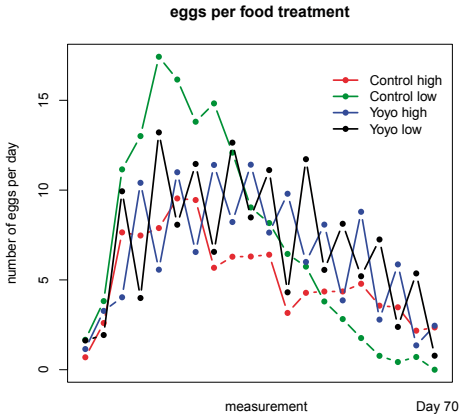

Figure 3C. The average number of eggs for different food treatments and at different time.

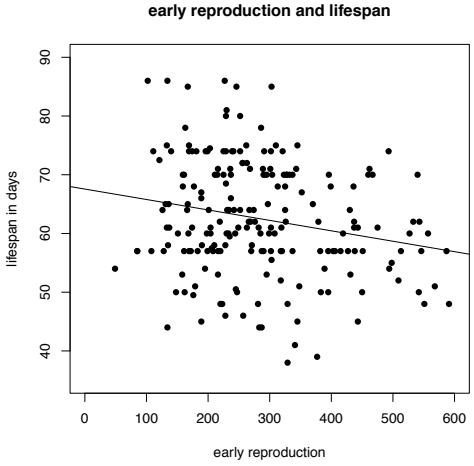

Figure 4A. Early reproduction and lifespan in days for all individuals that survived beyond the first five weeks.

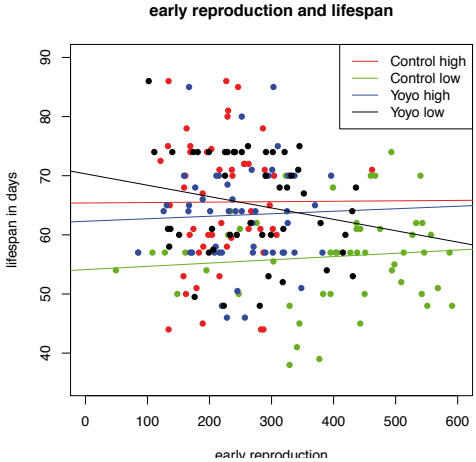

Figure 4B. Relationship between early reproduction and lifespan for individuals colored by food treatment.

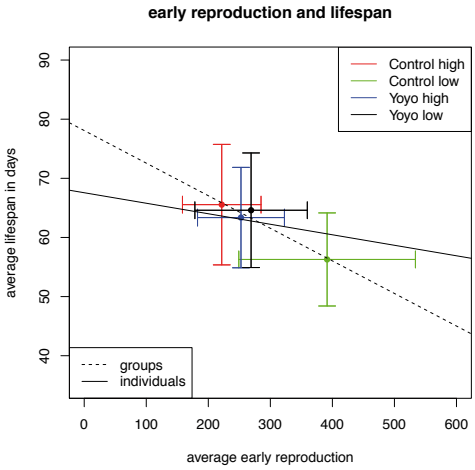

Figure 4C. The relationship between average early reproduction and lifespan (and their standard errors) of the different food treatments. Lines indicate the relationship when the lifespan is regressed on early reproduction for every individuals without taking treatment into account (solid line) and the regression through the averages of the food treatments (dashed line).

Gene expression

Overview Microarrays

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

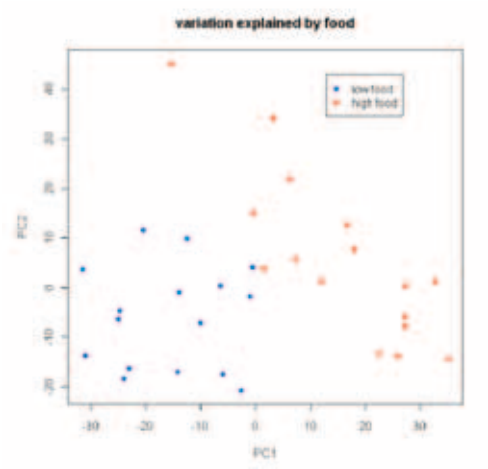

Figure 5a. Variation in gene expression along PC1 and PC2. Samples are colored by the food they were on before sampling.

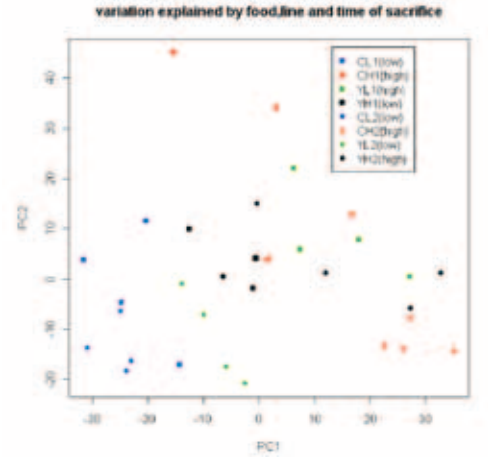

Figure 5b. PC1 and PC2 plotted samples, indicated per line and timing of sacrifice.

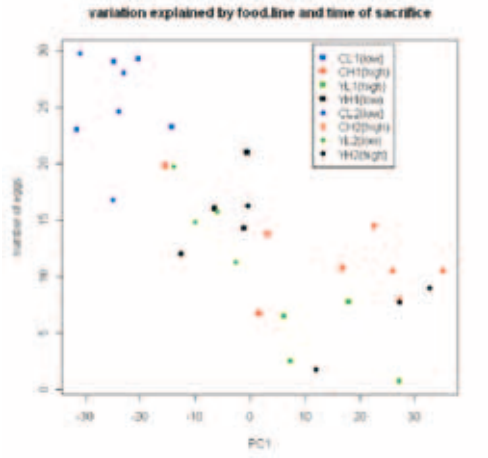

Figure 5c. PC1 and the number of eggs laid before sacrifice. Again line and time at sacrifice indicated.

Diet effect

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

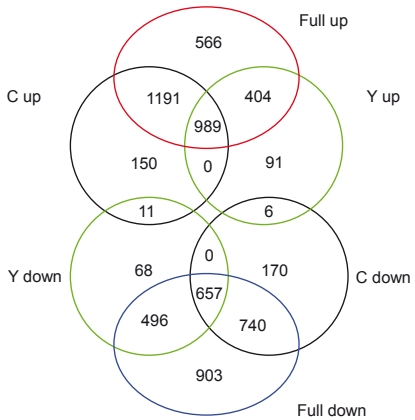

Figure 6A. Venn diagram of up and downreglated genes by comparison.

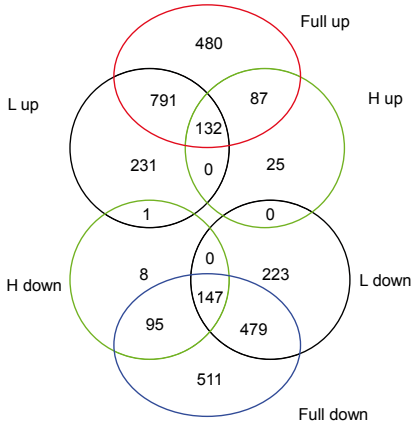

Figure 6B. Venn diagram of gene differently expressed within both yoyo lines (full up or down) or the ones which started low (L up, L down) or high (H up, H down). Up and down signify genes that are up or down regulated on high food.

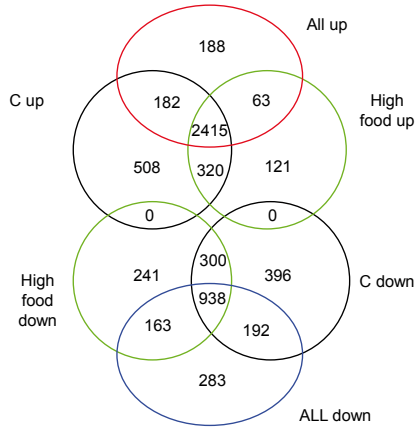

Figure 6C. Venn diagram of genes in time.

Age effect

Despite the fact that the time points over age were separated only by four days, the effects age were pronounced (fig. 6C, also see Appendices D1 and D2). When all the samples were grouped and the two time points where compared, 2848 probe sets were down-regulated in the later age class, while 1576 were up-regulated at a later age (see table 1). In control samples the effect of age is pronounced (see Fig. 5B); 3425 probe sets are down-regulated, while 1826 are up-regulated at the later age class. Interestingly, no such effect is measured in yoyo flies, where 0 probe sets are either up- or down-regulated (see table 1). Upon inspection of the PC analysis plot, time did seem to have a similar effect in the yoyo samples compared to the control samples (lower values PC2, see Fig. 5B) but these changes are not significant. The effect of time was much larger on high diet than on low diet; 2919 probe sets were down-regulated at the later age, while 1643 were up-regulated on high diet, compared to 0 on either up- or down-regulated on low diet. Compared to the association of probe sets differently affected by diet, the association with

specific biological processes is much more apparent. For the gene ontology analysis, the 2415 and 938 (see figure 6C) that were up and down regulated in all, the control, and the high diet samples were used. Genes that are down-regulated in the second age class are associated with *DNA metabolism, repair and replication, cell cycle, cytoskeleton and chromosome organization* and *transcription* and many other processes (see Appendix D2). Genes that are up-regulated in the second age class are associated with *oxidation reduction, proteolysis* and *fatty acid metabolism* (see Appendix D1). Again, this resembles age effects in other studies, which used much larger differences in time between age classes (see Appendix G1 & G2).

Overlap diet and age effect

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

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

		High food	
		3150 up	2796 down
First time Point All samples	2848 up	213	712
	1576 down	427	335
First time All, high food and control samples	2415 up	113	644
	938 down	318	194

Expression differences between control and yoyo flies

A small number of genes (74) are differentially expressed between yoyo flies and control flies when all samples are used for comparison (see table 1). Only three of these showed a similar expression between the two control fly groups (high and low constant) and therefore a consistent difference between control and yoyo samples. More genes are differently expressed between control and yoyo samples on low diet (747), but 0 on high food. These genes up-regulated for yoyo treatments are associated with *odorant binding proteins* and *abiotic and visual perception*, possibly indicated an increased perception of the environment in yoyo flies. These processes are often indicated in studies considering responses to nutrition (Pletcher *et al.* 2002; Libert *et al.* 2007; Doroszuk *et al.* 2012). Some up-regulated genes are also associated with the breakdown

of proteins, while the breakdown of large carbohydrate molecules is down regulated, indicating a different metabolism in yoyo flies compared to control flies on low diet (see Appendix E1 & E2). Both the survival and the reproduction at the time of sampling of the yoyo samples resembled the high control flies when on high diet, compared to the low control flies on low diet. This is therefore associated with the difference in number of probe sets differently expressed.

Variation in gene expression within yoyo samples

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

Gene expression associations with number of eggs

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

Discussion

In this study we set out to investigate the effect of cyclic nutrition variation that is sustained throughout the life of a fly on life history traits and gene expression. Egg production varied with diet level, increasing at low diet, which is relatively similar to the differences between control fly level egg production. Survival of the yoyo flies was intermediate of the two control groups, but more equal to the high control fly survival. The expression of a large part of the genes in the genome showed a plastic response and also here expression of yoyo flies on high diet resembled that of the control high diet flies. A difference in gene expression between yoyo flies and control

was only found on low diet. This aligns well with the fact that in survival the yoyo treatment flies resemble the high constant treatment flies more than the low constant treatment flies. Furthermore, the relationship of the phenotypes, especially egg production, related in a similar way to the gene expression profile when compared to other gene expression studies, although the relationship between diet and life history traits was different.

Phenotypes of control flies

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

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

Phenotypes yoyo flies

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

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

Trade offs

With all the pooled data, we found a negative relationship between early egg production (first five weeks) and lifespan. When the relationship was investigated within treatment groups,

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

Gene expression response on diet

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

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

Besides quantifying the plasticity in gene expression, biological processes that are up- or down-regulated in our study are also of interest. Flies lived longer on high diet, while they reproduced more on low food, which is represented in the gene expression differences. Biological

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

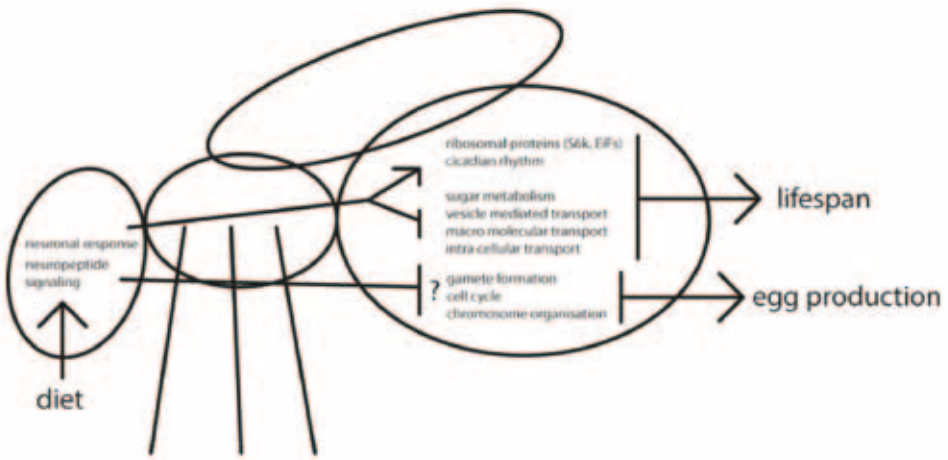

Figure 7. Overview of how diet affects groups of genes with specific biological processes which are known to influence lifespan and egg production. One of the groups of genes affected by nutrition contains neuropeptides signaling proteins, which are known to influence several functions in many organisms.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

The effect of age on gene expression

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

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

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

This study shows that flies cope with cyclical diet variation by changing the regulation of genes, resulting in rapid repeated changes in their phenotypes. Our model of yoyo fruit flies could be used to indicate which mechanisms play a role since the life history parameters (especially reproduction) do change quickly and towards the control fly phenotype. Neuropeptide signaling came up as one of the overrepresented group of genes and is therefore one of the candidate groups of genes because they are known to regulate many life history traits (Alstein and Nassel

2010). The insulin signaling pathway genes are often associated with ageing phenotypes and the effect of dietary restriction. In our study, insulin signaling was not overrepresented in up- or down-regulated genes. Two reasons might be 1) insulin signaling mainly works on the post translational level or 2) insulin signaling can be up- or down-regulated by up-regulating some and down- regulating other genes. The latter should have led to an overrepresentation of the insulin pathway signaling when a GO enrichment analysis was done without separating up- or down-regulated genes (data not shown). Furthermore, insulin signaling pathway genes did not show coherent expression between several microarray experiments (see Appendix G1) and also the separate *Drosophila* insulin like peptides, which are part of the neuropeptide group, did not show differential expression levels in our study.

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

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

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

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Modeling the relationship between age specific fecundity and lifespan in a cohort of fruit flies

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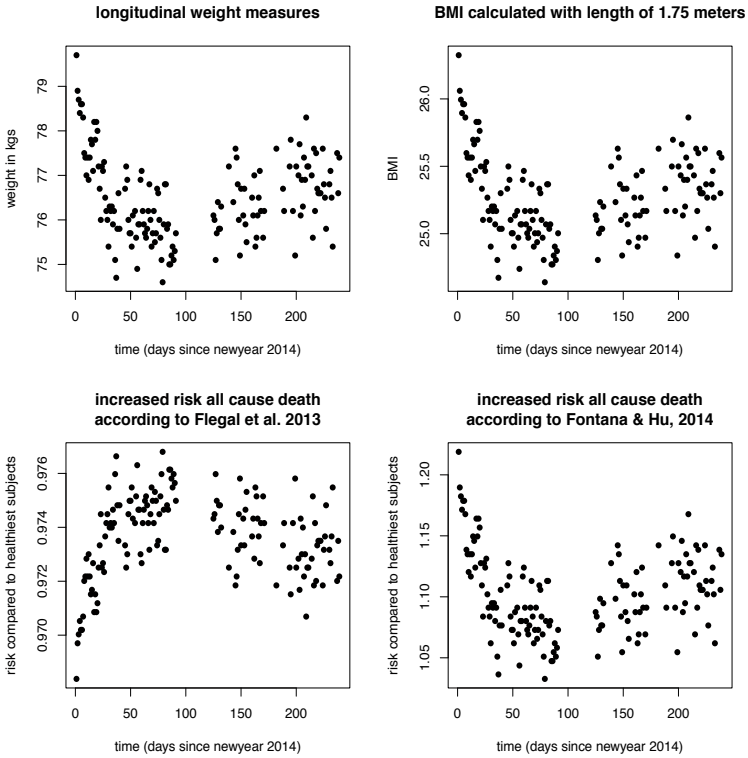


Longitudinal weight data (left above) and the resulting BMI (1.74 meter from head to toe) of an individual human being (subject Joost van den Heuvel, *Homo sapiens*) during the first part of 2014. According to two different studies the estimated hazard ratio for death (y axis lower panel) is different. Flegal et al (2013,left) estimated a higher risk for the lower weight period later in the year of 2014 for Joost, while Fontana and Hu (2014, right) predicted a higher risk for the same period.

Manuscript

Abstract

Populations of laboratory animals that are selected for increased lifespan often show correlated negative responses in early fecundity. However in some cases late fecundity, or total lifetime fecundity, is higher in the populations selected for increased lifespan. By some, this has been interpreted as a falsification of the disposable soma theory. According to the Y–model, in which the effects of variation in allocation and acquisition on life histories are studied, an alternative is suggested. A negative relationship between lifespan and reproduction can be viewed as variation in allocation, whereas a positive relationship is the result of variation in acquisition. A frequently neglected complication of the allocation and acquisition theory is that older individuals often show a decline in acquisition. Therefore, allocation to maintenance and survival might induce a difference in future acquisition which feeds back to increased resource availability. We show that a model which incorporates the ideas of the Y-model, the disposable soma theory and an age-related decrease in resource acquisition, i.e. feeding senescence, can explain how the relationship between fecundity and lifespan changes with age. Furthermore, we modeled different environments in which there is variation in extrinsic mortality rates. In high mortality environments there was selection for high early fecundity, low late fecundity and low lifespans, whereas the opposite was true for low mortality environments. We have analyzed age specific fecundity and lifespan in a cohort of *Drosophila melanogaster* flies which were individually housed. Early fecundity related negatively with lifespan, while late fecundity related positively with lifespan in the same cohort. Our laboratory population of *D. melanogaster* was founded from a combination of flies from different areas in Europe and therefore is heterogeneous in genotype. We conclude that the differences in life history strategies found in one cohort of laboratory flies are the result of a mosaic of selection on the relationship between fecundity and lifespan over age.

Keywords: Trade-off, disposable soma theory, energy allocation model, dynamic programming, lifespan, reproduction

Introduction

Life history traits such as size and age at maturity, age-specific investment in reproduction and lifespan collectively determine the fitness of an organism (Stearns 1992) and are therefore important for the study of evolution. Organisms vary in these traits, because of genetic, environmental or physiological differences. This leads to statistical relationships between traits that range from positive to negative. To understand fully how organisms adapt to their environments, knowledge about these relationships is crucial. How variation in the shape of these traits relates to fitness and thus to their evolution can be described using a demographic, genetic or resource allocation framework (Stearns 1992). The last two frameworks are more appropriate to describe the nature of the relationships between life history traits, while the first is useful to measure the effect of variation in specific traits with respect to fitness and population growth.

One way to assess the degree of genetic variation and covariation of life history traits is by using natural populations that are spatially differentiated. Along the North American cline of *Drosophila melanogaster*, functional pathways involving factors such as insulin/TOR, ecdysone and others were found to be overrepresented in genes likely to be affected by selection (Fabian *et al.* 2012). In a second approach, long term selection experiments have revealed genetic relationships between lifespan and other traits (Leroi *et al.* 1994; Zwaan *et al.* 1995). Overlapping this variation with QTL analysis of these life history traits in *D. melanogaster* (Leips *et al.* 2006; Wilson *et al.* 2006; Lai *et al.* 2007) could indicate which allele frequencies covary with which traits in these populations.

Both positive and negative relationships between life history traits can be explained via the resource acquisition and allocation framework (Van Noordwijk and De jong 1986). One unit of resource can only be allocated to one function (Kirkwood 2005), and therefore if extra resource is invested for instance in reproduction, other traits such as survival or storage will be decreased, if nothing else alters. Recent experimental work has suggested, however, that addition of resource components to *Drosophila* food can increase one trait while other traits remain unaffected (Grandison *et al.* 2009), showing that variation in acquisition can lead to a non-negative relationship between the life history traits lifespan and reproduction. Whether the manipulation of resource will result in direct effects on relationship life history traits may depend on whether and how these traits are physiologically coupled. It is therefore important to understand the shape of the selection forces that can be expected to act on the relationships between such traits, in order to produce the requisite plasticity.

The evolution of ageing itself can be explained using the resource allocation framework (Kirkwood 1977; Kirkwood and Holliday 1979). Furthermore, this framework can be used to predict how organisms are expected to allocate their energy in variable environments, leading to increased lifespan at the costs of reproduction for instance for calorie-restricted mice (Shanley and Kirkwood 2000) or other organisms which also experience variation in food availability (see chapter 3).

Resource allocation frameworks can predict outcomes similar to those of quantitative genetic models. Where the disposable soma theory explains the evolution of ageing from the relationship of physiological functions such as maintenance and repair and reproduction, the antagonistic pleiotropy hypothesis (Williams 1957) predicts that specific genes act in an age dependent manner. Because in the disposable soma theory age is the physiological trait

that changes in time, the antagonistic pleiotropy theory can be seen as a specific result of the disposable soma theory. Examples of genes that are described as behaving in an antagonistically pleiotropic fashion are the APOE locus in humans (Corbo *et al.* 2008) and growth hormone in mice (Bartke 2011). To explain fully the variation of life history traits and its evolution, specific mechanisms need also to be taken into account (Flatt and Heyland 2011). An example is that physiological decline in time, as a consequence of ageing, might cause organisms to acquire less resources (Shanley and Kirkwood 2000; Wong *et al.* 2009).

In this study we first develop a state-dependent model based on allocation theory, with an allocation trade-off between reproduction and maintenance and repair. Added to this is feeding senescence, which might interfere with how acquisition and allocation determine the relationship between reproduction and survival. Furthermore, with this model we can calculate, using dynamic programming, how individuals from different theoretical populations are selected to have different optimal allocation strategies, in response to different extrinsic mortality rates. Then we use a longitudinal study of individually kept, virgin female flies, in which we monitor both survival and reproduction. We test thereby if the basic behavior can model the physiology of the population and whether differences between individuals can be explained by heterogeneity in environments where extrinsic mortality rates are different. Although in the experiment adults are separated in two groups, on low and high food, in the model this type of variation in nutrition is not present. Through feeding senescence, variation in acquisition due to variation in allocation strategies is modeled, which is not due to the environment itself. We test whether the modeled behavior is present in both low and high food groups to be able to see whether the effect is general, and not special for a specific level of food.

Method

Theoretical Models of Resource Allocation

A detailed mathematical description of the model is given in the appendix, and will be briefly described here. The model is state-dependent, which means that depending on the biological age of an individual its strategic choices, and also its condition with respect to survival and acquisition of resource, are affected. At every time step an individual can allocate a proportion of ingested food to reproduction, while the other part is allocated to maintenance and repair. The more an individual allocates to maintenance and repair, the lower the rate of increase of damage (biological age), and, therefore, intrinsic mortality. Using the model we can follow individuals longitudinally and monitor reproduction and survival. We do this by simulating populations of flies with variable allocation patterns around an average (see results). This simulates the result of variation in allocation between individuals. According to the logic of the disposable soma theory (Kirkwood 1977; Kirkwood and Holliday 1979) individuals that are long-lived should have a different age specific pattern of reproduction where early life reproduction should be lower. Therefore, we look at the age specific reproduction of long-lived compared to short-lived individuals, separated by median lifespan within the simulation.

Then we use dynamic programming to calculate what the optimal strategic allocation decisions of individuals are in environments differing in extrinsic mortality. This is similar to flies evolving in different environments with various levels of extrinsic mortality rate. After this we

simulate their life courses in a laboratory context and follow age specific reproduction and survival. Because in reality flies in the laboratory experience very low extrinsic mortalities compared to the field situation, for simplicity, we simulate individuals by putting extrinsic mortality rate parameter (*P*) at zero. Total mortality at the start of life is not zero though. Other mortality parameters (m_1 and m_2) describe mortality in a laboratory context (see Appendix). Variation of the value of P therefore describes different situations with added extrinsic mortality, which is the case if individuals are transferred between laboratory and field situations. These simulations will then indicate what kind of variation is expected to be present if individuals have evolved in a heterogeneous environment or if individuals were taken from various places, which vary in extrinsic mortality. Flies used in the experiment were taken from various places in Europe and then crossed to maximize the amount of genetic variation. Hence, the resulting flies might be genetically variable for allocation strategies.

Note that in the model we focus on the possibility that individuals vary from their strategic decisions based on differential extrinsic mortalities. In the experiment this will be tested by looking at differences between individuals. An important feature of the model is feeding senescence. Because individuals that have an increased allocation to maintenance and repair age slower, some individuals have a higher acquisition late at live compared to individuals of the same chronological age. This leads to an increased variation in acquisition via this feeding senescence. We therefore quantify how variation in allocation might lead to variation in acquisition. Furthermore, we test whether individual differences are in the model are comparable to those in the experiment and do this on two different food levels, to test whether the patterns between individuals is robust. Therefore, the variation between individuals is expected to be similar on both low and high food. Nevertheless, theory has been developed to predict how organisms should optimize their allocation pattern and therefore their traits on different levels of food. In general, it is expected that flies increase reproduction at the cost of lifespan on higher food levels (Shanley and Kirkwood 2000).

Experiment

Flies

Flies (*Drosophila melanogaster*) were wild-caught from six different populations along a transect between Vienna and Athens in the summer of 2008. Once established in the laboratory, they were crossed in a scheme that ensures a balanced contribution of each source population to the newly established outbred population. This latter population was reared in half-pint bottles for 50 generations with at least 300 individuals per generation on 1x medium before the experiments were started (as in chapter 4).To avoid larval and maternal effects on adults and we reared the flies for at least three generations on intermediate food. The larvae were reared in vials with 6 ml of intermediate (2x) food, with a density of 50 eggs per vial. After eclosion each fly was randomly put in either a low food vial (6 ml of food throughout the experiment) or a high food vial (6 ml of food throughout the experiment) without using anesthesia. The single housed flies were all checked for sex and possible fertilized eggs in the first three days, where males and mated females were omitted. Therefore, all flies in this experiment were thus single housed, virgin female flies (similar to chapter 4).

Food

Different food levels were used in this experiment, indicated by 1x (low), 2x (intermediate) and 5x (high) food. The food level varies in sugar (50, 100 and 250 gram per liter in 1x, 2x and 5x) and yeast (35, 70, 175 gram per liter in 1x, 2x and 5x). Furthermore food contains agar (20 gram per liter) nipagine (15 ml of 100 g 4-methyl hydroxy benzoate per liter alcohol) and propionic acid (3 ml per liter). As adults female flies were either on high or low food throughout live. For every treatment we started with 65 flies. After the first transfer moment the number of days they were in a vial varied as following 4,3,3,4,4,3,3,4,4,3,3 etc. to keep in synchrony with the week.

Measurements

The flies were anesthetized with ice (a maximum of 5 minutes) and because flies were weighted to the nearest 0.01 mg before every transfer (which is described in another paper / chapter 4). After weighing, flies almost always directly revived in the new vial. Every vial was kept to count the number of eggs laid by each female. Every day we checked for survival. Flies were censored when they were found death between the stopper, crushed by scientist or escaped from scale because it was not properly anesthetized.

Statistics

We first fitted a generalized linear mixed model (GLMM) using an Poisson error distribution (Poisson 1837) using the lmer function in R (Zuur *et al.* 2009). We used time as measurement, food and lifespan (separated by median in short- and long- lived) as factorized fixed explanatory factors and individual fly as random factor. We started with 65 flies per cohort, but some flies we removed because of wrong sex or being killed accidently. Then we estimated p values for every main factor and every possible interaction using the R package car (Fox and Weisberg 2011). We also tested for significant relationships of cumulative egg production and lifespan at measurement times 5, 9 and 14 (indicating transfer vial number). We used food level as well as main effect and modeled the interaction between cumulative egg production and food level.

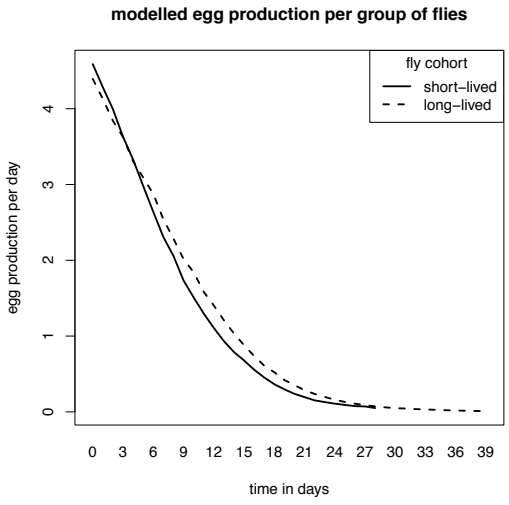


Figure 1. Egg production is modeled organisms where short- and long-lived are separated. Solid line indicates short- lived fly cohort, while the dashed lines indicate the individuals with a higher lifespan than median.

Results

Model

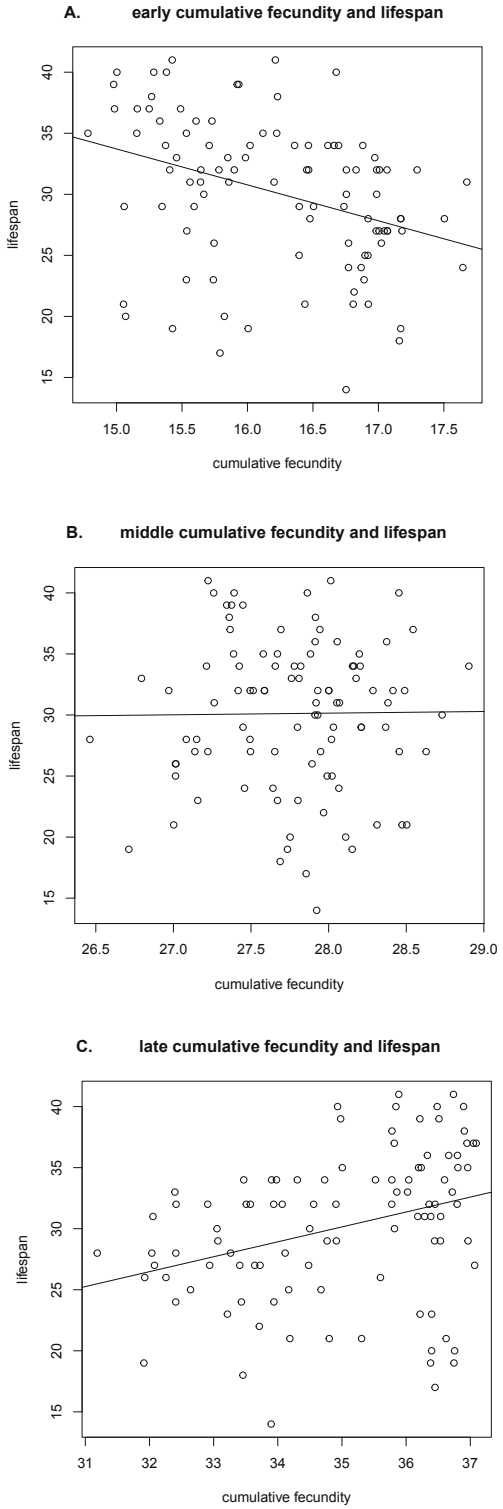
First we modeled a mixed population of flies (N=100) in which half of the flies allocated each day a normally distributed part of their resource income to maintenance and repair with an average of 0.2 and a standard deviation of 0.05, while the other half invested on average 0.3 with a similar standard deviation. Every day within the life of an individual this allocation was allowed to vary, with only the average of the normally distributed value being similar. Then we separated, as in the experiment, the short- from the long-lived individuals. As in the experiment, we found that the individuals that were short-lived produced more eggs during early life, and fewer late in life (see fig. 1). In this modeled cohort of flies the relationship between cumulative fecundity on day 4 was positive, neutral on day 8 and positive on day 12 (see fig. 2).

Next we let four model populations of flies evolve such that each population had a different level of extrinsic mortality. Then we simulated a laboratory experiment in which a group from each of these populations was raised in conditions where the added field extrinsic morality (*P*) was reduced to 0. The daily reproduction early in life was found to be higher for individuals that had previously evolved in the environments with higher extrinsic mortality. This reproduction decreased faster with age, however, for the group from high extrinsic mortality backgrounds, so that late reproduction ended up being lower than for the populations that evolved in lower extrinsic mortality rate environments (see fig 3A). In summary, survival was highest in the populations that evolved in the low extrinsic mortality rate environment (see fig. 3B), while their cumulative reproduction was also highest in the end (see fig. 3C).

Experiment

In the experiment three explanatory variables were taken into account. First the age of the fly was very important. Secondly, we tested whether flies that were relatively short- lived compared to long-lived (lifespan cohorts) were different in numbers of eggs they laid. Lastly, we tested whether age and lifespan cohort affected egg production similarly on high and low food (food treatment), in order to test whether the effect of allocation, acquisition and feeding senescence interacted with the two previously described explanatory variables.

Individuals exposed to the high food level produced fewer eggs ($F_{1,115} = 64.75$, $p<0.05$). There was also a very significant effect of age of the fly ($F_{13,115} = 7251.71$, $p<0.05$) while the effect of lifespan cohort (separated in two group by median lifespan) was not significant as a main effect ($F_{1,115} = 1.56$, $p=0.22$). The interaction of lifespan cohort with food level was also not significant ($F_{1,115} = 1.46$, $p=0.23$). This indicates that time of death was not related to number of eggs and that this was not different between food treatments. The interaction between age and food was very significant ($F_{13,115} = 534.42$, $p<0.05$) indicating how the age-related change in the number of eggs depended on food level. Lastly, both the interaction between age of the fly and lifespan cohort ($F_{13,115} = 631.06$, $p<0.05$) and the three way interaction age, cohort and food ($F_{13,115} = 138.62$, $p<0.05$) were significant. Individuals that lived longer (separated by median lifespan per food group) produced less eggs early in life but more eggs later in life compared to the shorter lived ones (see fig 4.) but this pattern was significantly different between the food types (although only in time).



Cumulative early egg production, measured as egg production from measurement 1 to 5, related negatively to lifespan, but only very marginally so ($F_{1,106}$ =3.88, p=0.05, fig. 5A). When cumulative egg production is calculated from measurement 1 to 9, it did not relate to lifespan at all ($F_{1,97}$ = 0.40, p=0.53, fig. 5B) while food level did ($F_{1,97}$ = 17.37, p<0.05, fig. 5B). The difference was not due to a difference in power, since only 9 more individuals had died at this stage, and therefore were removed from the analysis, and 98 individuals were still left in the analysis. Lastly, if we calculated egg production from measurement 1 to 14, the relationship between egg production and lifespan was positive, and significant ($F_{1,83}$ = 6.04, p<0.05,fig 5C). Lifespan of the individuals at high food was significantly higher than those at low food ($F_{1,83}$ = 24.23, p<0.05, fig. 5C). Therefore this indicates indeed that very early on, egg production related negatively with lifespan, while later egg production related positively with lifespan.

Figure 2. The relationship between cumulative reproduction and lifespan for (A) on day 4, (B) on day 8 (C) on day 12.

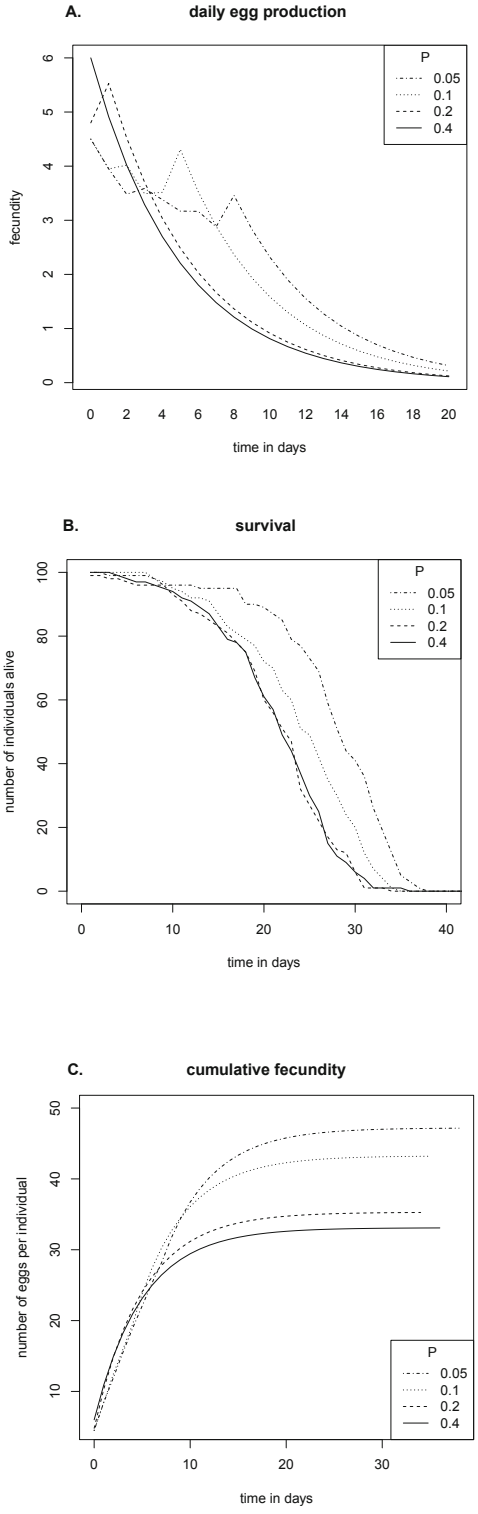


Figure 3. Outcome of theoretical laboratory experiment with 100 individuals from four different populations differing in extrinsic mortality (P). (A) indicates the daily reproduction per populations. (B) indicates the survival and (C) indicates the cumulative reproduction.

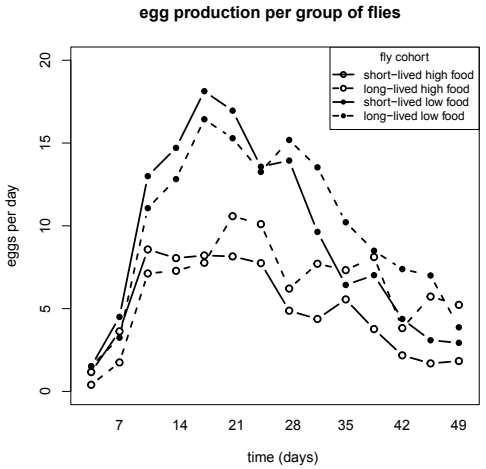
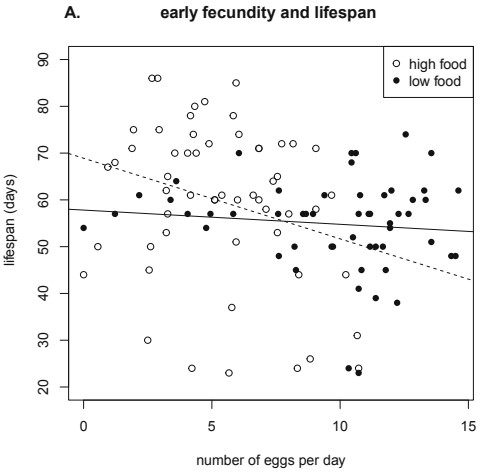
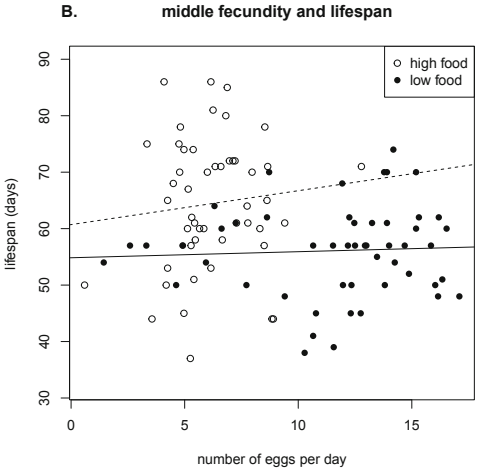


Figure 4. Number of eggs per day for the four groups of flies. Solid lines indicate the short lived group of flies within each food regime, while long lived flies are indicated by the dashed lines. Egg production of flies on high food is shown in open circles, on low food in closed circles. Time goes from measurement 1 to 14 (first seven weeks).





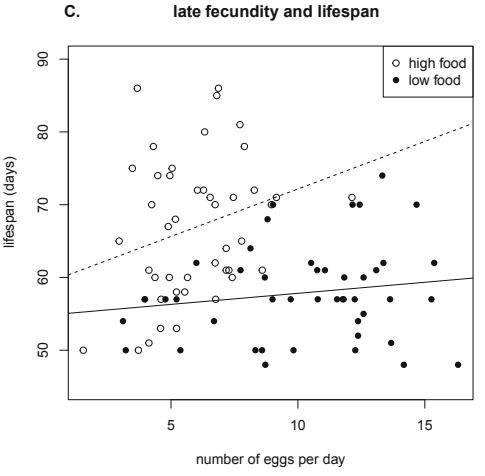


Figure 5. Relationships between number of eggs produced per day and lifespan for (A) egg production from measurement 1 to 5, (B) egg production from measurement 1 to 9 (C) egg production from measurement 1 to 14. Lines indicate separate linear fits between trait per food treatment, but none of these lines are significantly different.

Discussion

We used a state dependent resource allocation model to study the relationship between reproduction and survival using a model that is based on the disposable soma theory (Kirkwood 1977; Kirkwood and Holliday 1979). In this study, the implied feeding senescence, as previously modeled for mice (Shanley and Kirkwood 2000) and shown for flies (Wong *et al.* 2009) was important for the change in relationship from early variation in allocation towards a shift in variation in acquisition. Therefore we conclude that in cohorts of organisms, late life positive relationships between reproduction and lifespan do not have to be the resultant of genetic variation in acquisition from early genetic factors. Rather, late life variation in acquisition could be the resultant of early life variation in allocation.

We modeled four environments varying in extrinsic mortality. According to both the antagonistic pleiotropy theory and the disposable soma theory there should be a relationship between extrinsic mortality and age specific life history parameters (Kirkwood and Rose 1991). Individuals from populations from higher extrinsic mortality rates allocated less to maintenance and repair early in life and therefore reproduced more at lower ages. Later in life the negative relationship between reproduction and survival changed to a positive one. This is mirrored by our experimental finding where individuals that produced more eggs early in life had a lower cumulative egg production late in life and also had a lower lifespan. Because the individuals used for the experiment were bred from several populations from across Europe, a possible explanation for this variation is therefore that individuals that lived longer contain alleles from populations that were selected in an environment with lower extrinsic mortality rates. This study would link the genetic level of the antagonistic pleiotropy theory with the physiological model of the disposable soma theory.

An alternative explanation for variation in strategic allocation patterns might be that selection around the fitness optimum in one environment shows a very low slope. It has been shown that especially for short lived organisms such as *Drosophila* the variation in fitness for strategic differences in fecundity and lifespan is comparatively low (Lewontin, 1965). This would mean that strategies close to the optimum have an almost equal success. Hence, selection is unlikely to have a very strong effect on optimizing strategies around the fitness optimum. This could potentially also lead to variation in life history strategies, but probably of a different kind compared to variation in mortality. A more detailed examination of the fitness landscape around the optimal strategy is needed to estimate the actual differences between genetic variation due to a lack of selection gradient compared to variable optimal strategies from different environments.

In this study we followed cohorts of flies longitudinally and could thereby estimate age specific reproductive rates and survival. We found that within a cohort of flies that fed on one food type early high production of eggs related to a lower production of eggs late in life, as well as a lower survival rate. Early in life the relationship between cumulative egg production and survival was negative, while late in life this was positive. According to the Y- model (Van Noordwijk and De jong 1986) negative relationships between resource-competing traits results from a variation in allocation, while positive relationships between traits are the result of variation in acquisition. Because the individuals were on one food type, variation in acquisition is less expected if individuals have similar conditions. Our model indicates that the variation in acquisition late in life could have been caused by variation in allocation early in life. Our results are therefore

not in contrast with the Y model (Van Noordwijk and De Jong 1986), but can be considered an extension.

Although in the model we only considered individuals that acquired similar amounts of resource early in life, the experimental data consists of individuals kept at relatively high and low medium. Unexpectedly, reproduction was higher on low food, while lifespan was increased at high food, something which is different from other studies of fruit flies (Chippindale *et al.* 1993; Pletcher *et al.* 2002; Mair *et al.* 2003; Doroszuk *et al.* 2012) and also different from theoretical approaches examining the evolution of dietary restriction (Shanley and Kirkwood 2000). In all the experimental cases flies were kept in groups, while we kept them individually, to monitor individual life histories longitudinally. Possibly, an interaction of the number of individuals in a vial, which relates to total intake of food (Wong *et al.* 2009) with the effect of sugar and yeast in the medium, has led to different results from expected. This indicates that in these cases possible mechanistic effects need to be considered, next to the evolutionary ones, to account for the differences between average effects between food levels. This does not however alter the fact that both in the high and low food cohorts of flies the modeled effects were visible, and therefore that the experimental data from both cohorts support the outcome of the model.

To verify whether indeed the genetic variation in this population was large enough to show this response several steps could be taken from this point. One way is to use parent offspring regressions for age specific traits. Variation for age specific fecundity in *D. melanogaster* has been described (Leips *et al.* 2006). Furthermore selection experiments with the population from our experiment as the starting population might show what genes are involved in the age specific physiology of these life history pattern (see May, unpublished). Lastly, if the populations from which the flies were founded are different in the traits studied, modern genomics tools could be used to verify the findings of a possible selection experiment by sequencing the populations (Fabian *et al.* 2012). Although this is all beyond the scope of this study, we still perceive this study is as a first step forward towards unifying the antagonistic pleiotropy theory (Williams 1957) with the disposable soma theory (Kirkwood 1977; Kirkwood and Holliday 1979).

Acknowledgements
We thank Jelle Zandveld and Maarten Mulder for assistance with measuring the phenotypes and Kees Koops for practical assistance. This work was supported by the Network of Excellence LifeSpan (FP6/036894), the European Union's Seventh Framework Program (FP7/2007-2011) under grant agreement number 259679 and the European Research Council.

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Appendix: model equations

The aim of the model is to explore the effect of feeding senescence on the relationship of lifespan and reproduction. In a quantitative genetics framework (Van Noordwijk and De Jong 1986) have already described a model for how acquisition and allocation can affect relationships between life histories. Since we want to explore this addition of feeding senescence in a manner in which biological age can affect feeding rate, we model ageing as a process that can affect the physiological state of the organism, and allocation to maintenance and repair can affect how this parameter, biological age, increases. By modeling a state dependent energy allocation model, we will ultimately use dynamic programming (Mangel and Clark 1988; Houston and Mcnamara 1999; Clark and Mangel 2000) to estimate how optimal allocation patterns vary in environments that have different values of extrinsic mortality. This model can therefore be envisioned as the combination of the Y model (Van Noordwijk and De Jong 1986) and the disposable soma theory (Kirkwood 1977).

Juvenile stage

The organism we model lives in two stages, a juvenile and an adult stage. The juvenile stage is modeled as the developing stage and has a length (*L*) that is updated by 1 every time step, where *L(0)*, length at birth, is 1, and varies between 1 and 11 (see below). We therefore assume that increase in size is constant, which is unrealistic, but since we do not model any decisions for juveniles and since survival is constant over the development of the juvenile there is no need to model development in a more complicated manner. If ten time steps have passed, development is completed and the juvenile becomes an adult. Because the environment at which selection takes place varies in adult survival only, juvenile mortality must have a separate parameter value. Mortality rate, and therefore the chance to survive a time step are constant and we therefore assume that survival is independent of development or size. Therefore, length of the juvenile is modeled as,

$$L(t+1) = L(t) + 1 \tag{1}$$

And survival is modeled as,

$$S(t) = \exp\{-\mu_J\} \tag{2}$$

where μ_J represents juvenile mortality rate. Although we could consider a mortality rate for the total developmental period, to run the dynamic program, where the adult makes strategic decisions per time step, it is easier to model juvenile mortality and development in similar time steps. Because we use dynamic programming to optimize the strategies, the developmental equations are updated in similar time steps as the adults. This is needed to be able to adjust the maximum accumulated fitness every step of the backwards iteration (see below). We consider *F(t,l)* as the maximum accumulated reproductive success of an adult that ultimately develops from a juvenile with length *l*, at time *t*. For a juvenile that has a length smaller than 11, it will remain a juvenile and *F(t,l)* is,

$$F(t, L(t) < 1\ ) = F\{t+1, L(t+1)\}\ S(t) \tag{3}$$

We consider *F(t,d)* to be the maximum accumulated reproductive success of an adult with damage *d*, at time *t*. When the juvenile has completed development, when *L(t)* equals 11, it will develop into an adult and therefore another fitness equation is needed. We therefore assume that size as an adult is fixed, which is reasonable in this model as we assume a homogeneous environment with respect to food. The future fitness of such a juvenile is therefore,

$$F(t, L(t) = 1\ ) = F\{t+1, D(t+1) = 0\} S(t) \tag{4}$$

where *D(t+1)* denotes damage of an adult that just developed from a juvenile, which is 0.

Adult stage

During the adult stage food is ingested every time step; this is then allocated to maintenance and repair, or reproduction. We assume that individuals age, which is modeled via damage (biological age). Although ageing, and therefore increase in damage on the molecular level is in reality a stochastic process, in the model damage increases with a constant rate, if damage is not repaired. Damage affects two processes directly in the model. Most importantly, we assume that individuals with more damage (greater biological age) are less capable of feeding (called feeding senescence). Furthermore, mortality is dependent on damage directly. Individuals that invest less in maintenance and repair are therefore more likely to die every time step and they feed less efficiently per time step.

We consider *A(d,t)* as the acquired resource of an adult that has accumulated amount of damage *d*, at time *t*. We assume that resource from the environment is homogeneous. The potential acquired resource is therefore constant which is modeled as,

$$A(D(t), t) = \frac{a}{\exp\{bD(t)\}} \tag{5}$$

where *a* is the constant resource availability, but the realized acquired resource depends on damage. When damage increases, the intake of food decreases, hence, *b* is a positive constant. This is a realistic manner to model intake for fruit flies (Wong *et al.* 2009), and also for other animals (Shanley and Kirkwood 2000). The mechanisms behind feeding senescence are not yet completely explained. Potentially it could be related to senescence of the feeding apparatus or perception changes which results in the behavioral differences. Otherwise it could be related to ageing of the gut or to mitochondria working less efficiently. Ageing of the ovaries may also lead to fewer eggs that can possibly be produced, all leading to fewer resources needed and therefore causing behavioral differences. In any of these cases equation (5) can describe mathematically what happens, since all the processes are related to ageing, but since both feeding behavior and mortality are influenced by parameter *D*, representing damage, it does lead to the assumption that several organs age at a similar pace.

Organisms require resources to produce both juvenile as well as to repair damage as well as many other functions (Kirkwood 2005). In this model we will only focus on reproduction and maintenance of the soma. Therefore, during the adult stage, all acquired resources are divided between reproduction and maintenance and repair, which is the basic trade-off for one unit of energy proposed by the disposable soma theory (Kirkwood 1977) and Y model (Van Noordwijk and De Jong 1986). This allocation of resource is the only decision made in the model, and this

decision depends on state of the individual. The number of eggs of an individual is limited so it cannot produce eggs throughout its lifespan. Increase in damage is modeled as,

$$D(t+1) = D(t) + c(1 - \frac{qA(D(t),t)^2}{qA(D(t),t)^2 + e^2}) \quad (6)$$

Where *q* is the amount of resource allocated to maintenance and repair and therefore determines efficiency of repair (Mangel and Munch 2005), and can take a value of a minimum 0 and a maximum of 1. When organisms do not allocate to maintenance and repair damage increases by the value that equals *c*. When *qI(D(t),t)* equals *e*, half of the damage is repaired and therefore repair is more costly when *e* increases. In reality damage always increases, as the numerator is always smaller than the denominator, because of the positive value of e^2, which is also biologically realistic for active organisms.

Fitness as an adult is determined by both survival and reproduction. Survival is influenced by biological age and predation level, where mortality is modeled as,

$$M(t) = P + m_1 \exp[m_2 D(t+1)] \quad (7)$$

where *P* is the extrinsic mortality rate and constants m_1 and m_2 link biological age to mortality. In the optimizations of the strategies of allocation in different environments with different extrinsic mortalities, *P* will vary between runs of the optimizations. In virtual experiments where individuals are simulated to be in the laboratory, extrinsic mortality is zero, and therefore absent. Survival is then calculated as,

$$S(t) = \exp[-M(t)] \quad (8)$$

In the forward simulation we can simulate individuals by assuming a value for *q*, which is the proportion of ingested resource allocated to maintenance and repair (eq[6]). To infer what the optimal life history strategies are we have to calculate what strategy maximizes fitness. To do so we use dynamic programming to calculate the maximum value of *F(t,d)*, representing fitness, which is the accumulated reproductive success of an adult at time *t*, with damage *D(t)* and can be described as,

$$F(t,D(t)) = \max[\{(1-q)I(D(t),t)F[t+1,L(t+1)=1] + F[t+1,D(t+1)]\}S(t)] \quad (9)$$

which is taken over *q*, where the value of *q* that maximizes *F(t,d)* is saved. The value *(1-q)* is the portion of acquired resource that is allocated to reproduction, which is multiplied by the fitness of a larva with length 1 as calculated by equation 4, and *F[t+1, D(t+1)]* is the fitness value of an adult in the next time step, a damage of *D* (eq. [6]), given it survives (eq. [8]).

Backward iteration

Using a dynamic programming algorithm (Mangel and Clark 1988; Houston and Mcnamara 1999; Clark and Mangel 2000), equations (3), (4) and (9) can be solved, initiating all fitness values at the time horizon (*t=T*) as 1 and working backwards from that time point. Juvenile

length and adult damage are discretized using steps of 1, with minimum of 1 and maxima of 11 for development and 0 and 499 for damage. Because fitness values can increase or decrease rapidly, dependent on the parameters used for constants, relative fitness is calculated by dividing all absolute fitness values by the maximum for every time step, so that the highest fitness value is 1. This does not change the outcome of the model and the decisions of what strategies are optimal. The algorithm is stopped when all strategies for organisms in all state combinations do not change anymore. From that point optimal strategies for every state combination are saved to be able to run forward simulations. The values of the parameters are given in table 1. The environments differ in extrinsic mortality rates. This is modeled using parameter *P*, essentially representing predation pressure. Other parameters, such as acquisition of resources will be stable. Hence, every environment is completely homogeneous for all parameters, but between environments only extrinsic mortality varies.

Table 1. Parameter values

Parameter	Description	Value	From equation
a	Resource acquisition	1	5
b	Age dependent acquisition decline rate	0.01	5
c	Maximum increase of damage	25	6
e	Half saturation constant for repair	3	6
m_1	Age independent mortality rate	0.001	7
m_2	Age dependent mortality rate	0.01	7
P	Rate of extrinsic mortality	Variable. In four different environments 0.05, 0.1, 0.2, 0.4. In simulations forward 0.	7

Theoretical individual simulations

To perform theoretical experiments, we simulate 100 adults that have an initial biological age of 0. Furthermore, because we mimic experiments, the extrinsic mortality parameter (*P*) is kept 0 when theoretical experiments are done. In the first simulations forward, we do not include optimal decisions in the model, but just simulate the individuals while half are performing with the strategy allocation maintenance and repair with an normally distributed value with an average of 0.2, and a standard deviation of 0.05, hence *q=N(0.2,0.05)*. For the other half of the population *q=N(0.3,0.05)*. In the experiments with real flies the relationship between reproduction and lifespan on two types of food. We did not set out to test the differences between food types and therefore do not simulate organisms with different values of *a* (eq. [5]) but instead set out to test whether the effect of feeding senescence is similar for different food types.

Linking the life history phenotype to genetic variation using a candidate gene approach in the least killifish *Heterandria formosa*

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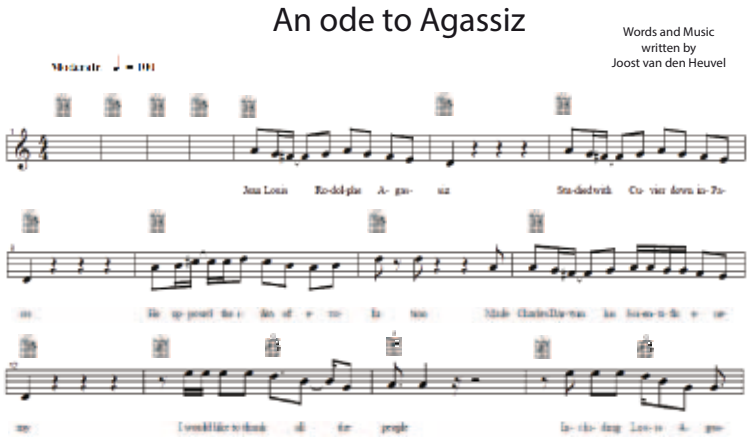

Fragment from the score “An ode to Agassiz” written by Joost van den Heuvel in August 2014. Agassiz was a nineteenth century geologist and naturalist, first to suggest that geological formations have been shaped by past ice ages. He described many fossil and living organisms (especially fishes) including *Heterandria formosa*.

Manuscript

Abstract

Rationale - In ageing research mutants with a large effect have helped to understand the mechanistic underpinning of the studied traits. Especially the conserved pathways such as the growth hormone axis and insulin signaling have received much attention. These genetic variants have such major phenotypic effects (for some traits positive but other negative) that it is unlikely that they represent phenotypic variation seen in an ecological setting. Indeed in humans, besides some major (disease) mutations for growth or ageing, the quantitative effects of genes are small. Here we present a study in which we study a well characterized ecological model organism, the least killifish (*Heterandria formosa*), and we characterize variation in candidate genes between populations for which consistent phenotypic variation is found. To perform such an analysis we first assembled the genome of *H. formosa de novo*, after which we sequenced a small proportion of the genome (0.33%) for eight well-studied populations. This small proportion consists of 75% candidate genes (GH hormone axis, insulin signaling, fish reproduction, telomere biology) and 25% of randomly chosen scaffolds as control regions from the assembled genome.

Results - We found that in general the population structure inferred from the sequenced regions were similar when using control or candidate regions, indicating that many loci where selectively neutral in both these classes of genes. Furthermore, in both the candidate as well as the control region many loci could be shown to correlate with the phenotypes, although there were a significant higher number of loci in the candidate regions that related to the phenotypes "fecundity" and "standard body length" compared to the control regions. Especially a scaffold containing an IRS exon had a very high number of loci associated with these phenotypes of which some were in linkage disequilibrium.

Conclusion - We conclude that for many loci allelic frequencies covary with life history phenotypes in natural population of *H. formosa.* Candidate genes for life history associated more often with the traits compared to control region. Thus, for the first time in a vertebrate, our results indicated that for major mutations discovered via mutant screens, subtle quantitative genetic variation exist that has contributed to shaping the life history phenotypes via natural selection in nature. Further verification of the loci that were related to phenotypes for both the candidate and control regions, using more populations that are studied in the field, will indicate which of the discovered possible loci underpins the phenotypic variation in life history traits. Moreover, such analysis will help discern possible patterns in variability of loci in relation to the pathway topology, that has the potential to guide genetic analysis of variation in lifespan, ageing, and health in humans.

Keywords: genome sequencing, candidate gene approach, life history, ageing, natural populations; natural selection and life history evolution

Introduction

The genetic mechanisms underlying variation in life history traits that have thus far been discovered are mainly based on loci with major effects. Many of these genetic variants affect multiple traits such as growth and lifespan in *growth hormone*, *prolactin* and *thyroid-stimulating hormone* deficient Ames dwarf mice (Brown-Borg *et al.* 1996). Snell dwarf mice (Snell 1929) also lack these hormones and show similar phenotypes, which is also true for mice that have a mutation in *growth hormone releasing hormone* or are *growth hormone receptor* or *insulin life growth factor 1 receptor* knockouts (Bartke and Brown-Borg 2004). Genetic manipulation of genes further downstream such as the *insulin receptor substrate 1* (Selman *et al.* 2008; Selman *et al.* 2011) and *ribosomal protein S6 kinase* (Selman *et al.* 2009) also show these effects indicating that these genes (from *growth hormone releasing hormone* to *S6 kinase*) regulate similar processes. Furthermore, genetic manipulation of insulin signaling genes in flies (Clancy *et al.* 2001; Tatar *et al.* 2001; Tu *et al.* 2002; Hwangbo *et al.* 2004; Bai *et al.* 2012) and nematodes (Kenyon *et al.* 1993) also produce similar lifespan phenotypes.

Because these pathways are evolutionary conserved between species and seem to be involved in lifespan regulation, it has been proposed that they also regulate lifespan in humans (Barbieri *et al.* 2003; Longo and Finch 2003). Similar growth hormone deficiency is present in humans (Donaldson *et al.* 1980) but, because of the rarity of this condition it is unlikely that similar allelic variants influence life history parameters in the general public. Small effect associations have been found between height and *GH1* polymorphism (Audi *et al.* 2007), adiposity and polymorphism in *growth hormone receptor* (Chan *et al.* 2010), insulin resistance and hyperandrogenemia and *insulin receptor* (Mukherjee *et al.* 2009). *Insulin like growth factor 1* variation has been associated with aging phenotypes (Mora *et al.* 2011), while birth weight (Adkins *et al.* 2010) and breast cancer (Neuhausen *et al.* 2011) to variation in *insulin like growth factor 2* and its receptor. Most of these polymorphisms are placed outside exons, while only one out of the three exonic polymorphisms causes a non-synonymous change (i.e. an amino acid change in the respective protein). Thus, these associations suggest that they are more likely to be involved in the regulation of expression of the genes, rather than causing a difference in protein structure.

The specific genes included in these studies are sometimes only associated with traits in specific populations. A genome wide linkage analysis combining data from 11 different countries indicated significant association between 7 loci and human longevity (Beekman *et al.* 2013). This study verified the *APOE* locus as an important gene affecting longevity in humans, while insulin signaling pathway gene *FOXO* failed to give a consistent association (Beekman *et al.* 2013). While the genome wide studies often lack the power to find relationships between genetic and phenotypic variation, the gene specific studies lack an overarching view on where variation in whole pathways associated with life history traits such as lifespan. This may be important if genes are mechanistically related to each other in a pathway, since mutation in different genes could produce a similar effect, when these genes interact.

One of the goals in evolutionary biology and ecology is to understand the genetic underpinnings of natural phenotypic variation. Such genetic underpinning has been revealed with variable success. Analysis of populations along clines of *Drosophila melanogaster* has revealed relationships between phenotypes and genetic variation using a quantitative trait locus approach

(Calboli *et al.* 2003). A whole genome approach using pooled sequencing of three populations has revealed what pathways are overrepresented from all the divergent loci (Fabian *et al.* 2012). Interestingly, these pathways partly overlap with the conserved pathways which have been associated with variation in life history traits using large effect mutants. Footprints of selection for life history traits along a cline have also been found using a much smaller design for the butterfly *Bicyclus anynana*, testing 19 non-synonymous single nucleotide polymorphisms (de Jong *et al.* 2013). However, using complete genome sequencing does not always results in finding the relationship between genetic variation and phenotypes. In a study of the great tit (*Parus major*) a genome wide association study and a quantitative traits locus analysis did not reveal any significant relationship with loci in relation to clutch size and egg mass in a wild population (Santure *et al.* 2013). Furthermore, percentages of the chromosomes from high trait value populations explained more variation between diverse individuals than single polymorphisms, indicating that many genes of small effect are likely to be underlying the phenotypic variation (Santure *et al.* 2013).

Generally, linking phenotypic variation between populations to genes is more likely to be successful if, (i) the selection pressures are clearly definable and related to the ecology of the populations, and (ii) candidate genes are studied and/or when the genome is sufficiently sampled for genetic variation. Therefore, in this study we sequence genes from candidate pathways found in the laboratory and in human populations in a small vertebrate species, *Heterandria formosa,* that lives in populations divergent for life history traits. We choose this species because life history traits have been extensively studied in the field in many populations in a longitudinal way (Schrader and Travis 2012). Notably, female fecundity and offspring size are related to predation pressure and population densities (Schrader and Travis 2012). Furthermore, for traits such as total number of broods and offspring weight significant heritibalities were recorded within a population (Henrich and Travis 1988). The latter traits also show variation between populations (Leips *et al.* 2000; Schrader and Travis 2008; Schrader and Travis 2009) which is the case for size at maturity as well (Leips *et al.* 2013). Average adult female size varies much more than size at maturity, where at low density populations females become much larger, while their average and maximum lifespan is lower (J. Travis, unpublished data). In addition, allozymes where used to construct a biogeography of the species (Baer 1998), and heterozygosity at microsatellite loci correlates to population density estimates in field (Soucy and Travis 2003; Schrader *et al.* 2011). All in all, this species shows well-studied and heritable population differentiation in life history traits related to the ecology of these populations.

We study whether candidate genes and pathways which affect life history traits in laboratory model organisms also do so in ecological field settings. Firstly, we summarized phenotypic data from eight populations of *H. formosa* that differ in four traits which are important for fitness (female standard length, embryo weight, number of broods, female fecundity). Secondly, to test whether selection is acting on candidate genes, we performed a *de novo* assembly of the genome to enable efficient identification of relevant candidate genes. Because we performed genome sequencing, and not for instance exome sequencing, we are able to assess variation which is represented in protein coding differences, but also in possible regulation of genes. This is important because in most cases in which associations with genetic variation and phenotypes are found in the targeted genes, associations are found in intronic, promoter or flanking regions. Thirdly, after the *de novo* assembly a set of scaffolds was chosen as candidate scaffolds (1.6 MB), while control regions were randomly picked (0.4 MB), to be able to test whether there

is enrichment in the candidate loci for relationships between genetic variation and population phenotypes. We sequenced pooled DNA from roughly 50 individuals per populations to estimate allele frequencies. Lastly, we analyzed whether phenotypic variation is related to genetic variation of genes influencing life history traits.

Methods

Field life history traits

Individuals of *Heterandria formosa* where caught from eight populations (Gambo Bayou [GB], Little Lake Jackson [LLJ], McBride Slough [MBS], Moore Lake [ML], Sint Marks River [SMR], Trout Pond [TP], Tram Road [TR] and Wacissa River [WR], see Figure 1, Table 1). Fish were caught, handled and measured as explained before (Schrader and Travis 2012). In short, fish were caught and killed immediately by ms222 and stored in 4% formaldehyde. For the phenotypic data only females are used. Standard length is measured with marking gauge at the nearest tenth of an mm from head-tip to the start of the tail fin. Fecundity was taken to be the total number of eyed offspring in the ovary, while superfetation was defined as the number of broods (which varied between 0 to 5, following staging by Reznick 1981). Offspring weight was measured as the average freeze dried weight of the most developed stage of offspring (either stage 4 or 5, following Reznick 1981). For the latter the whole brood was weighed. For this study data for the years 2000, 2001, 2002 and 2003 were used as sufficient measurements were available for the Spring and the Autumn; life history differences among these populations have been stable for over 20 years (Leips and Travis 1999; Schrader and Travis 2012). Spring samples were taken to be in the months March, April and May, while autumn samples were taken to be caught during September and October. We used a mixed model with year, season and population as explanatory variables.

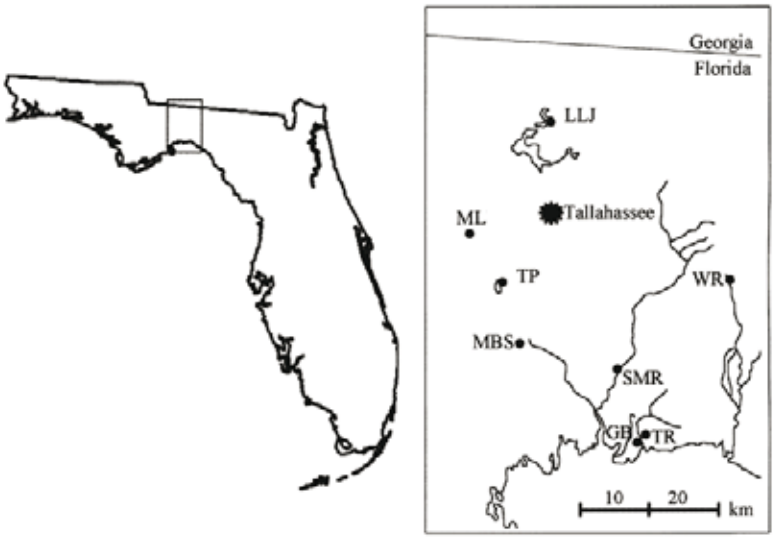

Figure 1. Locations of the sampled fish.

Table 1. Number of individuals and their sex per population used for resequencing.

	GB	LLJ	MBS	ML	SMR	TP	TR	WR
Females	25	40	24	42	33	40	36	25
Males	19	9	18	12	8	6	5	28
Juveniles	3	1	0	0	4	1	0	14
Total	47	50	42	54	45	47	41	67
Date captured	18 – 8 -2011	9 – 3 -2012	18 – 8 2011	5 – 3- 2012	5 – 3 – 2012	5 – 3 -2012	5 – 3- 2012	18 – 8 -2011

Statistical analysis life history traits

We performed analyses on standard length (normal error distribution), superfetation (Poisson error distribution), average weight of the offspring (normal error distribution) and fecundity (Poisson error distribution). The basis of the model was a fixed effects ANOVA with season and population as explanatory variables. Then a mixed effects model was fitted as well to correct for the random effect of year. We compared the AIC's of those two models per trait, where in all cases, except for average weight, the model was improved by adding year as explanatory variable. For offspring weight there was not enough sampling in every year to perform the model with year.

De novo assembly genome

We sequenced the genome of a *H. formosa* F3 female from Wakula Springs, reared in a population tank. DNA was isolated from the liver using the DNeasy kit from Qiagen according to the manufacturers' protocol. 1000ng of DNA was sheared to a 100–800 bp range using a Covaris S-series sonicator. Genomic fragments were fitted with adapters using the Paired-End DNA Sample Preparation Kit PE-102-1002 (Illumina Inc.) and size-selected for 500 bp. Concentration and size profiles were determined on a Bioanalyzer 2100 using a High Sensitivity DNA chip. Paired-end sequencing was performed on an Illumina HiSeq 2000 Sequencing System (Illumina Inc.) using the HiSeq Paired-End Cluster Generation Kit (PE-401-1001) and HiSeq Sequencing Kit (FC-401-1001). Images were processed using Pipeline v1.9. For *de novo* assembly, we used SOAPdenovo, which applies a de Bruijn graph algorithm (Luo *et al.* 2012). We generated assemblies for a range of k-mer values. We used the assembly generated with k-mer = 25 for further analysis.

Choice of candidate genes

We chose 47 genes that we expected to be associated with life history variation. Using evolutionary conserved sequence regions form the NCBI website for other species of fish which are relatively closely related (*Oryzias latipes, Oreochromis niloticus, Takifugu rebripes, Tetraodon nigroviridis*), we extracted the matching scaffolds in our *de novo* assembled genome. At the time the scaffolds were selected, the genome of *Xiphophorus maculatus* was not published yet (Schartl *et al.* 2013) therefore, we did not use information of this species to select scaffolds. Next to candidate genes we also sequenced randomly selected control scaffolds. These were selected

by randomly taken a number between 1 and the maximum number of scaffolds. The scaffolds were only included when they contained coding sequences of genes and did not contain candidate genes. A short overview of candidate genes is given below.

The candidate genes include 26 genes drawn from four groups in the insulin pathway (insulin and insulin-like growth factors and the upper, middle, and lower insulin pathways), 9 genes associated with reproductive output in *Danio rerio*, 5 genes in the growth hormone pathway, 5 telomere genes associated with longevity in humans, and 2 genes involved with lipid metabolism.

GH and *GHRH* (and homolog *PACAP*), via their receptors *GHR*, *GHRHR* and *PACAPR*, both affect insulin (like growth factor) signaling, partly in an independent manner. Insulin (*INS*) and insulin like growth factors (*IGF1* and *IGF2*) have different effects, but all bind as ligands to their own, but also to each other's receptors (*INSR*, *IGF1R* and *IGF2R*). The insulin signaling pathway contain kinases and phospathases, which convey a signal via genes such as *IRS*, *PTEN*, *PDK1* and *TSC* which alter the activity of the *mTOR* complexes (consists of *mTOR, RAPTOR, LAMTOR, PROTOR, RHO* and other genes) as well as *PRAS40*, *S6K*, *FOXO* and *4EBP*. Alternatively, *AMPK* responds to AMP/ATP ratios and also affects this pathway via *mTOR* and *TSC*. *GHRH* has direct effects on follicle stimulating hormone (*FSH*), which affects reproduction. Other reproduction genes are also known to be affected by *GHRH* (such as *COX*) and the expression of genes such *as PGES, STAR, LHR, GTR*, *AROMAT*, *BHSD* and *SCC* are correlated with reproductive output of fish such as *Danio rerio*. Genes which are associated with longevity in genome wide association studies in humans such as *APOE* and *LDLR* regulate lipid metabolism, which is also regulated by insulin. Lastly, genetic variation in the telomere gene *TERF2*, is associated with longevity, and a couple of telomere genes such as *TERT, GAR1, POT1* and *NOP10* were included in this study as well. A complete list of these genes along with a full description of the sequencing effort of all these genes is presented in appendix A. In total, we resequenced DNA aiming for 201 candidate scaffolds with a total of 1.6 million nucleotides. The randomly chosen control genes consisted of a total of 61 scaffolds with an estimated total of 0.4 million nucleotides. In this study we will relate allele frequencies of candidate and control genes with life history traits. The expectation of this study is that the candidate genes will be more associated with the traits than control genes.

Resequencing of the populations

As part of the ongoing longitudinal field study in Florida (Schrader and Travis 2012), a subset of individuals were used to isolate DNA for further sequencing purposes. The number of females, males, and juveniles are given in Table 1. DNA was isolated using the DNA easy kit from Qiagen using the manufacterers' protocol. Where possible, muscle tissue was used to isolate DNA. Reproductive tissue of females was never used in order to avoid contaminating the females' DNA with DNA of males, via the fertilized eggs and embryos. After DNA isolation quality and quantity of DNA was analyzed and measured (nanodrop). DNA was then pooled by adjusting the concentration of DNA to 50 ng / uL with in total volume of 2 ML per population.

To enrich the whole genomic DNA for candidate and the control regions the Agilent Sure Select protocol was used according to the manufactures' protocol. Eight DNA libraries were prepared, and two flow cells of HiSeq (Illumina, pair-end, aiming at a 300 sized amplicon) were used to sequence selected fragments. On both the flow cells all eight libraries were sequenced, to prevent differences between flow cells affecting sequencing depth between populations.

The sequenced reads were mapped unto the scaffolds, and SNPs, insertions, and deletions

were called using the mapping function in CLC workbench (CLC bio, Aarhus, Denmark). Since we used about 50 individuals per population, frequencies lower than 1% or differences between populations are not informative, because only 100 alleles were sequenced. By performing the Agilent Sure Select we expected a 30x coverage per sequenced allele (3000x coverage per population to estimate allele frequency). Therefore possible errors in estimating allele frequencies would be most likely to be a results of the pooling of the DNA and not due to low sequencing effort.

Statistics

To estimate the population structure among the eight populations, we estimated pairwise Nei's genetic distances (Nei 1972, see appendix B) to create a distance dendogram (see appendix B for details). For every variable site, F_{st} values were calculated according to eq. 7.1 from Charlesworth and Charlesworth (2010), which is further explained in appendix C. As stated above, because we used material from 50 individuals (on average) per population, it cannot be expected that a measurement of very low population divergence (low F_{st} values) are reliable. Hence, before testing the associations between allele frequencies and life history traits we removed all variable sites with an F_{st} value of 0.05 or lower. Although this is a huge number of variable sites, in other populations differentiation studies the cut off for F_{st} is taken to be higher, also because high F_{st} values are expected to results from natural selection. As we used a candidate gene approach and we compare candidate against control genes we used an F_{st} limitation to reliably measure small differences in allele frequencies, rather than for statistical power argumentation. Unfortunately, we could not perform a region wide F_{st} calculation, although sometimes this might be better, because the quality of the assembly is relatively low (i.e. many loci are located near places which still have undetermined nucleotides, Ns). Therefore a sliding window approach, calculating F_{st} values over a large range of nucleotides, is not feasible.

Non coding, synonymous and non synonymous variable sites

To estimate whether a variant site was located in a coding region or not, and, if so, whether substitutions are synonymous or non-synonymous, the sequences of the scaffolds were compared to estimated gene products from *Xiphophorus maculatus* using peptide data from the ensembl (www.esembl.org) website. Because *X. maculatus* is the species which is the closest relative to *H. formosa* and one of the completely sequenced fishes (Schartl *et al.* 2013), it can be expected that comparison with this species gives the best estimates for coding regions. When the coding region is known, the possible variable sites that produce synonymous or non-synonymous changes could be easily obtained. We performed analyses of associations of sequence variants with life history traits for each of the three types of variants: non-coding, synonymous and non-synonymous.

Relationships with traits

For every variable site we detected, we calculated the p value from a linear regression among the eight populations of allele frequency (the response variable) against the individual life history trait (the predictor). In the absence of any associations, the distribution of p-values among loci should be uniform. This procedure tests for simple linear associations of allele frequencies without accounting for population structure. To examine associations in the context of the existing population structure, we also tested the relationships of all variable sites using the Bayenv protocol (Coop *et al.* 2010). This Baysian statistical approach first estimates the population structure with a

randomly chosen set of loci (5000 in this case). This population structure is considered as the null model for testing the associations between allele frequencies and life history traits. The p values obtained from the linear regression and the Bayes factors obtained from the Bayenv program were then used to examine whether variants in candidate genes are more strongly associated with life history traits than control genes.

Because as we sequenced roughly 6x the amount of nucleotides in candidate regions, we could not simply compare the most significant loci between candidates and controls. To account for the difference in sequencing effort, we adopted a bootstrap method and sampled the p (or Bayes factor) value distribution and sorted the outcome per sample. We then took 2000 of these samples which led to a distribution of p values and Bayes factors which could be compared between candidate and control regions. When the candidate and control regions differed, we expected the lowest p value distributions (because sample were sorted) and highest Bayes factor distributions (high Bayes factor indicates significant different from null model) to be most different between the two groups of statistics (see Appendix D for further information about the bootstrap method).

Number of loci in a scaffold

When p- or Bayes factor values were calculated, we could test whether there were genes that were overrepresented in the most significant variable sites. For instance, if we were to find scaffold 1 represented ten times among the 25 most significant loci, we can estimate how often one would find a specific scaffold more than ten times or more when we randomly sample 25 variants. We performed this calculation only for the candidate genes for each type of variant, non-coding, synonymous and non-synonymous.

Results

Field life history traits

Life-history traits varied widely among populations (Figure 2). The average body length varied least, with the highest population average (TP) being about 27% higher than the lowest average (TR). At the other extreme, the level of superfetation (number of simultaneous broods) varied most, with the highest average (TP) being about three times the lowest (WR). These population differences were, statistically, highly significant no matter which other terms were included in the model (Table 2). For standard length and fecundity, population, season and the interactions affected the data significantly. For superfetation, season, but not year, affected the outcome, while population was significant, although only weakly so (p=0.033). Offspring weight was affected by population, but not by the main effect of season, although there was a strong significant interaction between population and season. Seasonal variation was much lower than population variation for all traits except the level of superfetation, for which the two sources of variation were comparable in importance.

Assembled de novo *genome*

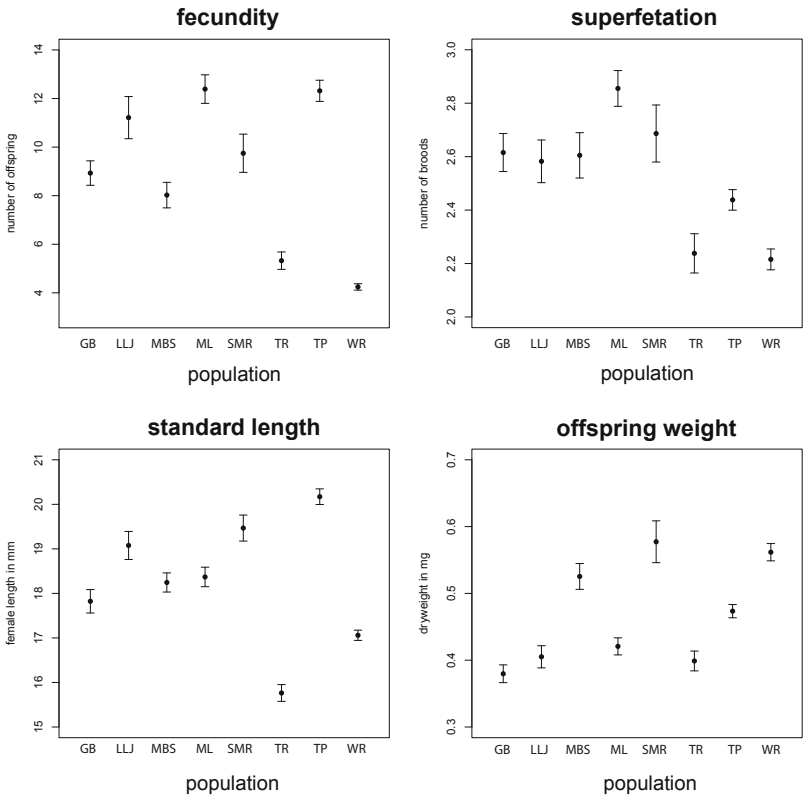

Figure 2. Average and standard error of traits from the field for the eight difference populations.

Table 3 lists the characteristics of the genome for the different k-mer values. Based on the combination of scaffold n50 and assembly size, we chose the genome produced by a k-mer of 25 to search for genes of interest and the random picked scaffolds with genes. This assembly consisted of over 140.000 scaffolds. Appendix A is an overview of the scaffolds, those that contain genes of interest and random chosen scaffolds with their characteristics. The average size of the candidate scaffolds was larger (and significantly so) with an average of 10.1 kb (sd = 8.9 kb), while the average size of the randomly chosen control scaffolds was 6.7 kb (sd =7.4 kb). Since in this dataset the size of the scaffolds related negatively to the proportion of coding region and the number of variable sites, we found less variable sites per sequenced nucleotide in control regions.

Table 2. Analysis of variance on four traits.

	Standard length				Superfetation			
	Fixed effects		Mixed effects		Fixed effects		Mixed effects	
	Chisq	Pval	Chisq	Pval	Chisq	Pval	Chisq	Pval
Population	2408	<0.001	336	<0.001	17	0.019	15	0.033
Season	480	<0.001	59	<0.001	23	<0.001	20	<0.001
Interaction	344	<0.001	44	<0.001	3	0.882	3	0.87
AIC	5240		5231		3304		332	
Relative likelihood	0.01202				<0.001			
	Fecundity				Average weight			
	Fixed effects		Mixed effects		Fixed effects		Mixed effects	
	Chisq	Pval	Chisq	Pval	Chisq	Pval	Chisq	Pval
Population	1713	<0.001	1450	<0.001	7	<0.001	NA	
Season	441	<0.001	300	<0.001	0	0.15		
Interaction	115	<0.001	114	<0.001	1	<0.005		
AIC	7927		3870		NA			
Relative likelihood	<0.001				NA			

Table 3. Characteristics of genome assemblies using different values of K-mer.

K-mer	Contigs >100	Total bp	Longest contig	Contig N50	scaffolds	Scaffold N50
21	1586309	520 MB	8114	440	150322	7492
23	1305399	550 MB	13393	638	134767	8451
25	1231948	560 MB	12224	739	140855	8042
27	1227524	570 MB	16750	799	150778	7441
31	1333019	600 MB	23536	852	175817	6255
33	1333019	600 MB	23536	852	175817	6255
35	1333019	600 MB	23536	852	175817	6255

Description of variation

In total 71,999 loci were found to be variable. Since in total there were 2,025,672 sites sequenced, we found a variable site per every 28.13 nucleotides. Table 5 lists the types of variation (SNP, N=46,807, insert N=8,454 or deletion, N=16,738), number of populations in which this locus was variable (from 0 to 8), and whether the locus was a candidate or control locus. For every variable site we calculated the heterozygosity per populations. Then we tested using a glm with binomial error distribution to test if the populations differed in heterozygosity for the total 71,999 variable loci. The heterozygosity of populations TP, ML and LLJ was significantly lower than the rest, where TP < ML < LLJ. This pattern of relative genetic variation is the same as reported for microsatellite data (Soucy and Travis 2003). The number of variable loci per population and the statistical estimates of heterozygosity are shown in Figure 3. Because in some cases there were more than 2 alleles, heterozygosity can be higher than 0.5. Almost 20.000 loci were variable in only one population, while between 5.000 and 10.000 loci were variable in 2 to 8 populations (see figure 4). The coverage of the variable sites showed two peaks, with the second peak indicating a coverage of higher than 4000x (see figure 5). We also calculated F_{st} values for all variable sites. Frequencies of six possible alleles were used, A, T, G, C, insertion or deletion. Most F_{st} values (62.2%) are below 0.05 (see also Figure 6) and 5.8% of the variable loci have an F_{st} value above 0.25. For the analysis of the relationships between traits and allele frequencies, only the sites that have a F_{st} value higher than 0.05 were used (see Method section).

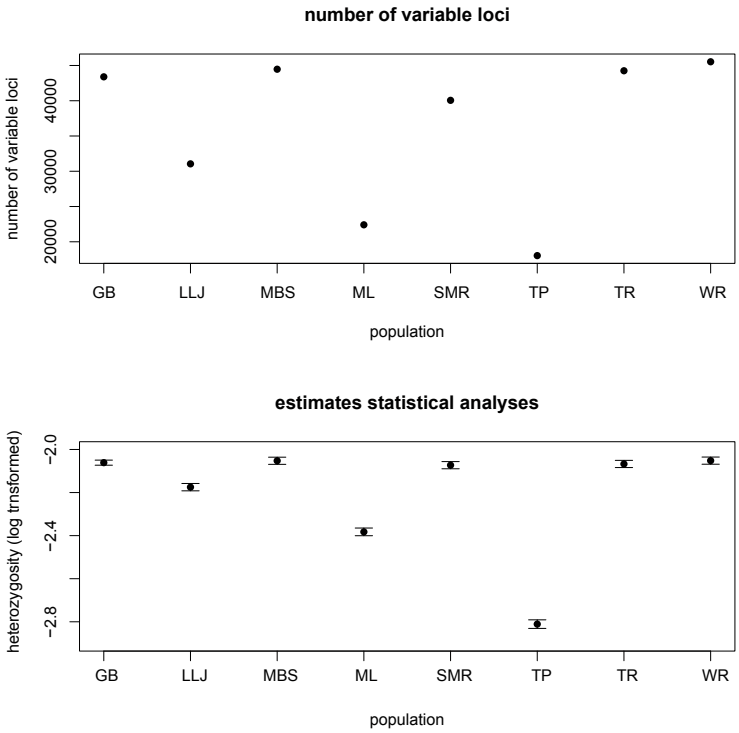

Figure 3. Variability measured as the number of variable sites (above) and estimates in glm for heterozygosity (below) per population.

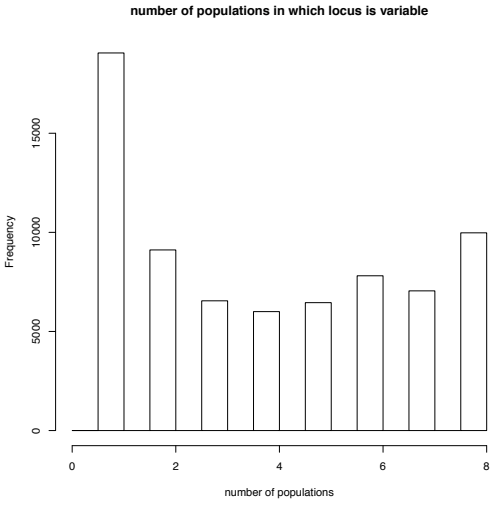

Figure 4. Histogram of the frequencies in what number of populations they are variable. For instance, a little bit more than 10000 loci are variable in 8 all the eight populations.

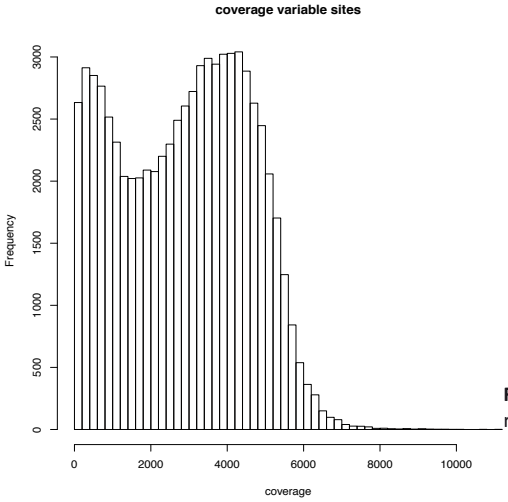

Figure 5. Coverage of the sites as mapped variable against the reference genome.

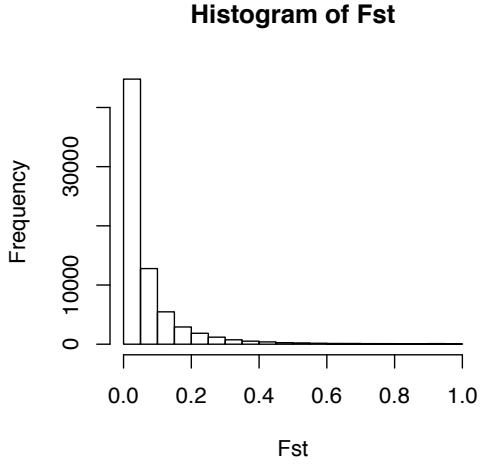

Figure 6. Distribution of Fst values.

Coding regions and variable sites

Because the first draft of the genome contained many scaffolds, the sequenced genes were distributed over several scaffolds. Because we searched for the genes using coding regions of other fishes, we only obtained scaffolds with coding regions so these is clearly an overrepresentation of coding region within our study. In total 11.4% of the sequenced scaffolds were coding regions, which was higher than for a comparable fully assembled genome (SCHARTL *et al.* 2013), probably due to lack of intronic regions. For candidate regions the coding regions were 10.6% while 23.4% of the control scaffold sequence was coding. The percentage variable sites within coding regions in the two types of genes was 5.5% and 10.7%. Of the 3359 variable sites in coding region in candidate scaffolds, 1522 were non-synonymous. This was 613 of the 1220 in control scaffolds (see Table 4).

Table 4. Total number of basepairs resequenced and the distribution among candidate and control region scaffolds.

Region	Number of scaffolds	Total basepairs	Number of Ns	ATGC's
Candidate	201	2.025.672	327.983	1.697.689
Control	61	408.002	77.367	330.635
All	262	2.433.674	405.350	2.028.324

	Candidate	Control	Can/con
Total atgcs	1.697.689	330635	5.13
Conding regions	180.002	77.367	2.33
Total variable site	60.651	11.348	5.34
Coding region variable sites	1837	607	3.03
Non synonymous variants	1522	613	2.48

Table 5. Total number of SNPs, insect and deletions in candidate and control genes split for the number of populations in which such a variable site is variable. Only for two variable sites (two inserts) was the site variable between populations but not within any.

	Candidate genes				Control genes				Total
Nvar	SNP	Insert	del.	Total	SNP	Insert	del.	Total	
0	0	1	0	1	0	1	0	1	2
1	11168	1825	2958	15951	2159	342	599	3100	6058
2	5439	843	1391	7673	1010	153	276	1439	9112
3	3983	580	934	5497	756	98	195	1049	6549
4	3662	505	873	5040	720	88	154	962	6002
5	3896	528	992	5416	771	90	176	1037	6453
6	4589	650	1390	6629	841	97	242	1180	7809
7	3670	672	1666	6008	608	115	317	1040	7048
8	2932	1595	3909	8436	592	282	666	1540	9976
	39339	7199	14113	60651	7457	1266	2625	11348	71999
	Candidate + control genes								
Total	40605	9824	25461	71999					

Population structure

There is substantial population structure related to spatial proximity. The dendogram (see Fig. 7) places closely adjacent populations together (ML and TP, GB and TR, compare fig. 1 with fig. 7) and separates groups of populations found in three physiographic regions (LLJ in the Ochlockonee River drainage, ML and TP in the Munson Hills, GB, TR, SMR, MBS, and WR in the Tertiary limestone south and east of Tallahassee). The dendogram remained similar whether based on candidate genes sequences or control gene sequences (see appendix B). The dendogram was also robust to using loci that were variable in many populations or, few populations as well as to using non- coding or coding sequences (see appendix B). This robustness indicates that there are many variable sites which are not affected by selection, even for coding region sequences.

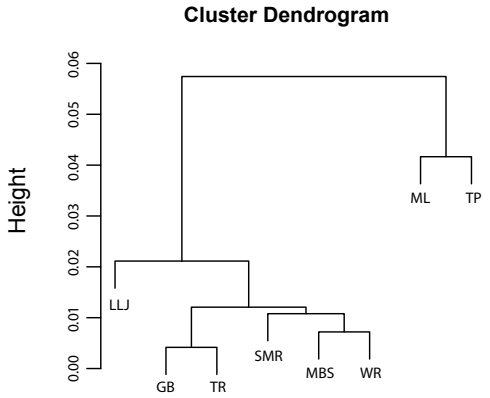

Figure 7. Cluster cladogram of genetic distances between populations.

Bootstrap tests of p-values for associations of allele frequencies and life history variation

We examined the p-values for the regressions of allele frequency on each of the four life-history traits for non-coding, synonymous and non-synonymous variants. The difference between candidate and control region variants was most pronounced for fecundity (see Fig. 8). For synonymous and non-synonymous coding variants the p-values in the lowest range were significantly lower for the candidate genes ($p<0.05$). For non-coding variants candidate genes also showed higher associations, but not for the lowest p values, but for p values that were a bit higher (see Fig. 8). In contrast, the difference between candidate and control region variants was least evident for superfetation (Fig 8); there was no difference in the p-value spectrum.

The patterns for standard length and offspring weight were more complicated. For standard length, p-values non-coding variants were lower for candidate genes in the higher range of p-values (see Fig. 8). The pattern for offspring weight was different. For the lower p-value range, candidate loci were significantly more associated for synonymous loci, but significantly less for non-synonymous loci (see Fig. 8).

In general, associations of life history traits with allele frequencies in non-coding regions were different between candidate and control genes at higher p-values and the differences were spread over broader range of p-values than was the case for synonymous and non-synonymous variant associations. This pattern indicated that for non-coding variants, the associations with life history traits were smaller in magnitude and spread over a larger number of loci, while the associations of life history traits with coding variants were larger and distributed over a smaller set of loci, representing an enrichment of a small number of very low p-values (see appendix D).

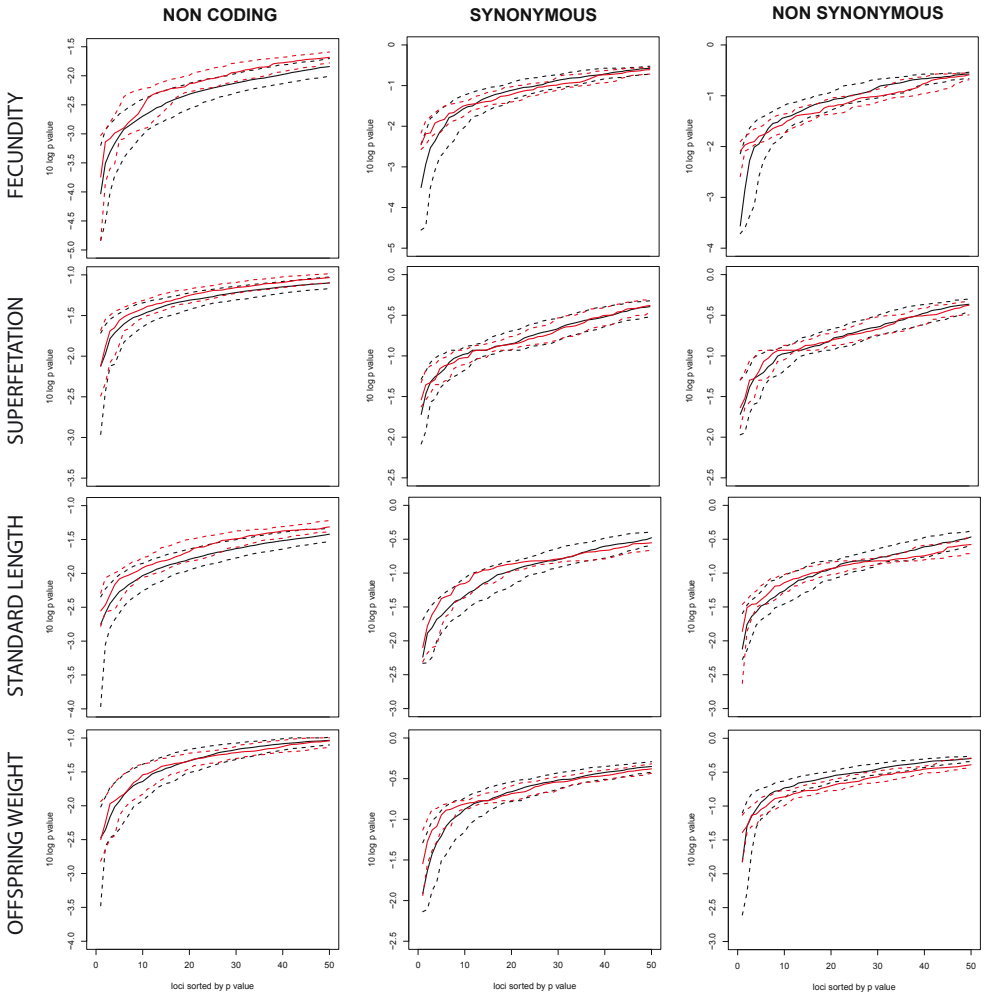

Figure 8. Bootstrapped p value of loci with different effects, from the linear regression with fecundity, superfetation, standard length and offspring weight in different row, while column represent non-coding, synonymous and non-synonymous variants. In black, lines indicate the 95% interval (dashed) and median (solid) of the bootstrapped sorted p value for candidate loci, in red for control loci.

Bootstrap tests of association with Bayes factors

We also performed a bootstrap method on the calculated Bayes factors. In theory, for the loci for which it is most likely that selection has acted will have Bayes factors that approach a value of 0.5 while the loci for which no support for selection is found have a value of 0. The results of this analysis are shown in Figure 9, for fecundity, superfetation, standard length and offspring weight. Only for fecundity and standard length were there significant differences, where candidate loci showed high Bayes factor values for non-synonymous variants. These overrepresentations where found in the range of the highest values for Bayes factors (0.4 < Bayes factor < 0.45) but also at the lower Bayes factor values. Similar to the p value analysis, it seems that the associations with the traits were higher (for fecundity and standard length) for non-synonymous variants.

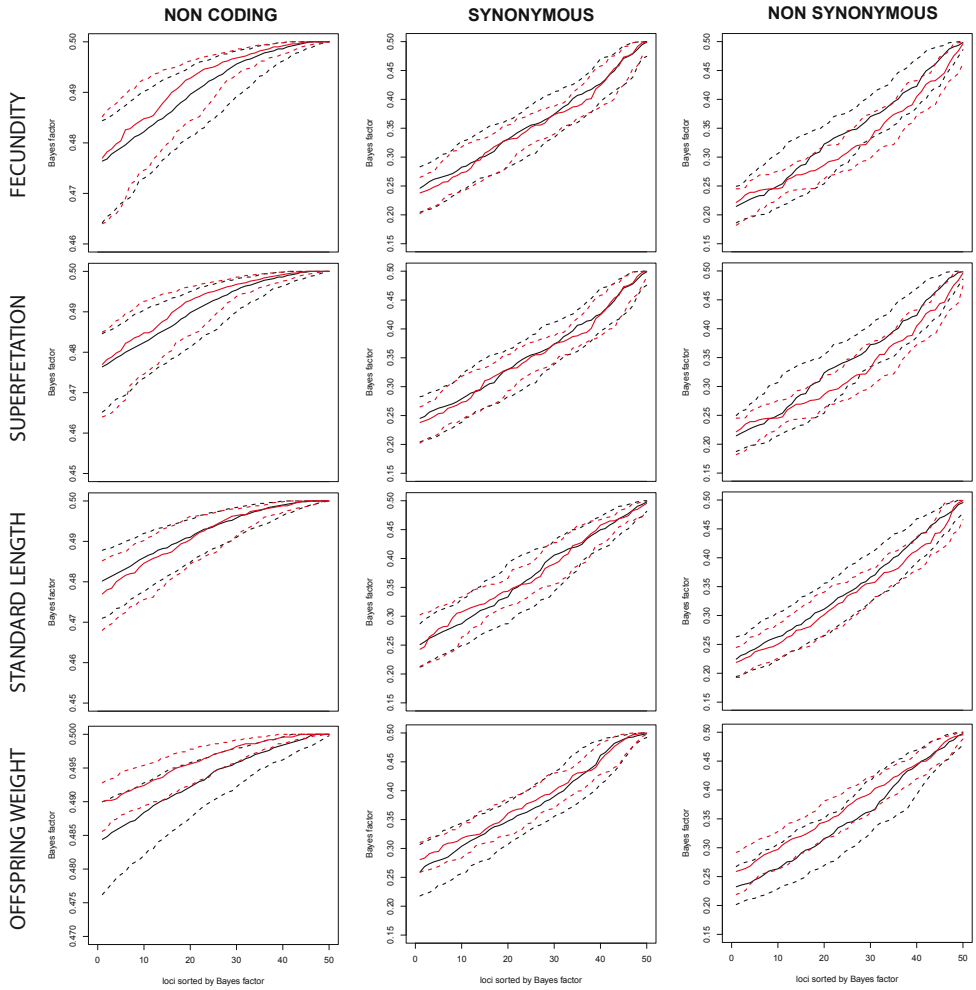

Figure 9. Bootstrapped Bayes factors of loci with different effects, from the analysis of fecundity, superfetation, standard length and offspring weight in different row, while different columns represent non-coding, synonymous and non-synonymous variants. In black, lines indicate the 95% interval (dashed) and median (solid) of the bootstrapped sorted Bayes factors for candidate loci, in red for control loci.

Scaffolds with more than a random number of significant loci

We listed the scaffolds that were among the most significant p- and Bayes factor values for fecundity and standard length for non-coding, synonymous and non-synonymous variants (table 6). A scaffold containing exon 2 of IRS (IRS, scaffold number 16743) was represented most often in the most significant p-values for fecundity and standard, as well as the Bayes factor values for standard length. Among the 25 most significant non-coding, synonymous and non-synonymous variants were 7, 6 and 4 variants of IRS scaffold 16743. In less than 1 in 10,000 random samples of 25 random scaffolds were more than 6, 5 and 3 variants of the same scaffold found. Therefore this indicates that among the most significant loci, IRS (scaffold number 16743) was represented more than expected. This was also true for this scaffold for p-values for standard length (<0.002

times per sample) and for Bayes factors related to standard length (<0.002 times per random sample). In addition FOXO (scaffold number 99512) was represented three times among the four lists of the significant loci most often represented; the expected number of times sampled in this scaffold was much higher than for IRS scaffold 16743, namely 0.6, 0.002 and 0.01 among the p-values for fecundity, Bayes factor values for fecundity, and Bayes factor values for standard length. A third gene that was represented three times was AMPK (0.07, <0.002 and 0.01 times per sample for p-values for standard length, Bayes factor values for fecundity and Bayes factor values for standard length respectively).

Table 6. Scaffolds which are most represented in the most significant loci for fecundity and standard length for the linear and Bayesian analysis. Gene name and scaffold number is indicated to illustrate that although some scaffolds represent similar genes, they do not represent similar scaffolds. The expected values (Exp) are indications of how often one would find genes with those distributions when scaffolds would be sampled at random. For instance 0.60 for Foxo (99512) indicates that 0.6 times of every sample of 25 genes, a scaffold is represent more than 2 times in the sample of the non-synonymous variants.

P values fecundity					P values standard length				
Gene	NC 25	SY 25	NS 25	Exp	Gene	NC 25	SY 25	NS 25	Exp
Foxo (99512)	0	0	3	0.60	IGF2R (65415)	1	2	0	0.43
IRS (8517)[1]	1	2	0	0.43	IRS (16743)[1]	0	5	4	<0.002
IRS (16743)[1]	7	6	4	<0.0001	Pi3k (36974)	2	1	0	0.59
LDLR (115790)	0	2	1	0.93	S6k (80639)	5	1	0	<0.002
Pi3k (36974)	0	2	1	0.93	AMPK (48469)	0	3	1	0.07
Bayes factors fecundity					Bayes factors standard length				
Gene	NC 137	SY 25	NS 25	Exp	Gene	NC 139	SY 25	NS 25	Exp
ApoE (62803)[2]	0	0	4	0.13	ApoE (34143)[2]	0	2	3	0.09
Foxo (99512)	4	2	3	0.002	Foxo (99512)	2	3	2	0.01
Pi3k (36974)	6	2	0	0.02	IRS (3149) [1]	10	0	0	0.05
AMPK (48469)	3	2	5	<0.002	IRS (16743)[1]	7	0	3	<0.002
RhoA (100262)	2	3	0	0.11	AMPK (48469)	4	0	3	0.01

[1]These three scaffolds containing coding regions for IRS, are most similar to three different coding regions, namely XM_004082003.1, XM_005813319.1 and XM_004079882.1 of *Xiphophorus maculatus* for scaffold numbers 3149, 8117 and 16743 respectively and therefore it is very likely that these different scaffold represent different genes.

[2]There two scaffolds containing coding regions for ApoE, are most similar to two different coding regions, namely XM_005799446.1 and XM_005802834.1 of *Xiphophorus maculatus* for scaffold numbers 62803 and 34143 respectively and therefore it is very likely that these different scaffold represent different genes.

RS allele frequencies

Figure 10 gives an overview of the SNPs found in exon 2 of IRS, which are listed in Table 6. Of the ten listed, five of them were non-synonymous. Nine of the SNPs were among the most significant variants for both fecundity and standard length. Two of these nine SNPs were also among the most significant Bayes factor values. Only one of the SNPs was a very significant Bayes factor, without also being significant in the linear regressions. This SNP, for which the base was either a C or T, was very divergent between populations; three populations were fixed for T whereas the other populations had frequency of C that varied between 0.965 and 0.99. Most SNPs that were significant in the IRS scaffold varied in relative frequency of the major allele between 0.7 and 1.0. Some of the SNPs were physically closely linked, so we tested for the presence of linkage disequilibrium. SNPs 15534 and 15542 were under-represented for the combination of the major and minor alleles (0 DNA molecules found that contain both the minor alleles). For

SNPs 15542 and 15671 the combination of both the minor alleles were also not represented. In both these cases this was significant (Chi square test values 5.58 and 4.89 respectively, $p<0.05$ for both, see Table 7).

Table 7. Observed and expected number, and the chi square computation $(O-E)_2/E$ per cell, of alleles estimated from single sequencing reads from the Wacissa River populations for SNPs 15534, 15542 and . Number of sequenced reads are corrected for number of individuals from which DNA was isolated, which was 67 individuals, and therefore, 134 alleles.

15542	G/A pos. 15534		15671	C/T pos. 15542	
Observed	G	A	Observed	C	T
C	88.59	22.57	A	90.96	20.54
T	22.84	0	G	22.50	0
Expected	G	A	Expected	C	T
C	92.44	18.73	A	94.41	17.09
T	18.99	3.85	G	19.05	3.45
$(O-E)^2/E$	G	A	$(O-E)^2/E$	C	T
C	0.16	0.79	A	0.13	0.70
T	0.78	3.85	G	0.62	3.45
Chi $sq._{d.f.=1}$ = 5.58, $p<0.05$			Chi $sq._{d.f.=1}$ = 4.89, $p<0.05$		

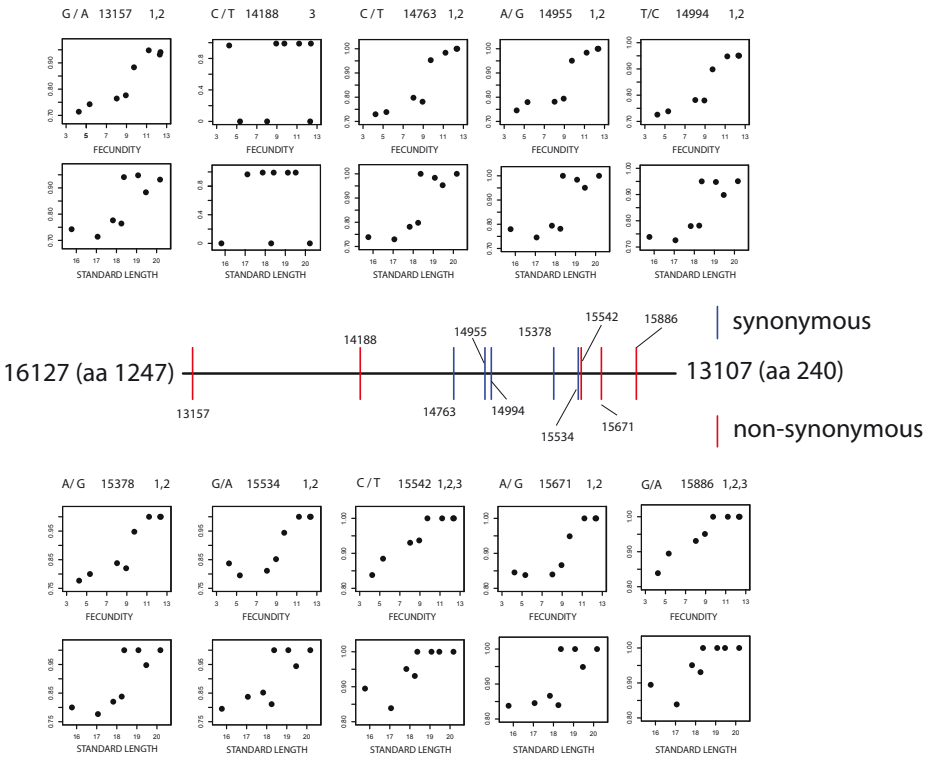

Figure 10. Exon 2 of IRS and the position in exon two of the most significant variable sites. Colors indicate whether a SNP was synonymous (blue) or non-synonymous (red). Above the subgraphs is from left to right the major / minor allele is given, the number of the locus on the exon and with 1, 2 and 3 whether the locus is significant for p value with fecundity, p value with standard length or Bayes factor for standard length.

Discussion

Candidate genes associate with population differentiation for life history traits

In this study we tested whether genes that were found in mutational screens in model organisms and that affect life history traits, explained natural phenotypic population differentiation in a small live bearing fish, *Heterandria formosa.* Indeed, candidate genes were more often highly associated with the traits fecundity and standard length compared to control loci. Although we tested genes in several pathways (GH, insulin, reproduction, telomeres, lipid metabolism), we only found associations with the traits for insulin signaling, or insulin regulated genes. Therefore it seems that although candidate genes were more associated, not all candidate genes contributed equally to this difference.

The power to detect footprints of selection

In this study we used pools of individual DNA samples and therefore tested allele frequencies rather than individual genotyping. Several studies have been using a similar type of approach such as for Atlantic herring (Lamichhaney *et al.* 2012). (Günther and Coop 2013) showed that for this species a genome wide approach using Baysian statistics, partly correcting for populations structure could be used, even at a coverage of 40x per population estimating allele frequencies, to identify allele frequencies shaped by local adaptations. Instead of using an ecological variable, to test for selection, we used the estimates of the traits as covariate in the analysis. Population structure indeed seemed to indicate the need for a proper control for populations structure. The high predation pressure populations, and therefore low density populations, where also the three populations that grouped together in the dendogram (Fig. 7). Therefore, it was quite likely that population structure would affect the significance of the variant loci. Indeed the Bayes factor analysis was not completely similar to the linear relationships (an analysis which does not take population structure into account). Roughly though, the genes which were most significant in the linear regression analysis were also most significant in the Bayes factor analysis. In general, the Baysian factor analysis showed fewer differences between candidate and control genes. Most of the highly significant loci in the linear regression, were still very significant when the low density populations (TP, ML, LLJ) were omitted from the analysis (data not shown, see Figure 12 for the relationship of the high density populations with traits). This indicates that between the high density populations, the significant genes related well to the traits tested.

The Heterandria *genome, evolutionary conservation and genetic variation calling*

In this study we used a *de novo* assembly of the genome, based on one illumina Hiseq sequencing of a F3 female fish (from a ninth population that was not re-sequenced). Therefore, this individual was very heterozygous. This has probably led to much more scaffolds per gene than what would have been the case if we would have used an inbred individual. This also means that it is relatively difficult to annotate the genome and therefore to choose proper controls for this study. As a results, candidate genes such as the insulin receptor were not matched by a control gene with a similar receptor function. Also we did not match the control genes in coding region or length of the whole scaffolds. The variation found in control regions was larger than in candidate regions, which might have led to higher associations with the traits. In general this would also mean that the candidate genes were more conserved. This would make it more likely to pick up signals of selection if selection would have been taken place, and also would makes it less likely to

conclude that traits relate to allelic variation, while it did not. In general, it is better to first relate the variation in the genes between species to better match the controls with the candidate genes rather than taking random control genes as we did.

To estimate the coding regions in the *H. formosa* genome we used a genscan from the ensembl website of *Xiphophorus maculatus*. This is a library of predicted coding region with a messenger RNA and protein expectations of similar genomic DNA regions. Since *X. mculatus* is the only fully sequenced live bearing fish (Schartl *et al.* 2013) this was a good option. Nevertheless, a better option would be to use a RNA sequencing assembly of *H. formosa* itself. This is not available yet. Although coding regions between the different sequenced fishes are very well conserved, because fish such as *Oryzias latipes*, *Xiphophorus maculatus* and *Heterandria formosa* are very closely related, still it is possible that these coding regions have been changed due to frame shift (because of insertions and deletions). Indeed the number of insertions and deletions are high between populations of *H. formosa* (see Table 5). A possible reason for the last three SNPs in exon 2 of IRS to be called non-synonymous might indeed be the fact that they are called so wrongly, because of a possible frame shift (see below). On the other hand, the alleles (amino acid variation) found between populations are also found between the protein sequences of fishes (see Appendix E). Therefore it seems quite likely that these differences are true differences. Interestingly, both the majority of synonymous and non-synonymous significant loci were found in the exon of IRS which is less conserved between fishes, and fishes and mammals (see Appendix E). The protein sequences between live bearing fishes *X. maculatus* and *H. formosa* do vary. The evolutionary changes between genera and species within genera first have to be shaped on the small scale between and even within populations (Darwin 1859) and therefore it would be interesting to look at allelic variation between *X. maculatus* individuals or populations as well.

IRS as a potential regulatory gene for suits of life history traits

In particular one scaffold representing the insulin receptor substrate (IRS) gene showed a high number of significant loci, which were partly in linkage disequilibrium. It is not necessarily surprising that candidate genes are more related to certain traits in natural populations, but, the specificity of the associations in the upper part of the insulin pathway suggests that there are genes in the topology of a pathway that have more "evolutionary degrees of freedom" to regulate those traits. In particular, genes downstream of IRS are more often associated with single traits, such as growth, stress responses and reproduction. Crucially, IRS regulates the actions of these genes, and thus natural selection mediated genetic population differentiation for IRS will affect whole suite of traits at the same time.

The IRS gene varies over its exons in how conserved the gene is between fish species. The part of the IRS gene which is highly conserved is the part that binds to the insulin receptor (see appendix E). Comparing the structure of the gene from rat (Gual *et al.* 2005) with the found changes in this study indicated that the non-synonymous significant variants might affect the degradation of the IRS protein (see appendix E). In a study in which this region of the protein was modified or truncated, it was shown that this affected the degradation significantly (Boura-Halfon *et al.* 2010). Furthermore, the part of the protein in which we found non-synonymous SNPs in are associated with several health parameters in human populations (Bacci *et al.* 2013; De Cosmo *et al.* 2013; Lim *et al.* 2013; Vats *et al.* 2013; Alharbi *et al.* 2014) Therefore it is plausible that the differences found in life history traits are underpinned by structural differences in the protein

that affect post translational regulation of the IRS gene.

The differences between sequenced populations in this study are caused by a combination of predation pressure and density, which themselves vary inversely (Schrader and Travis 2012; Macrae and Travis 2014). The expectation for high predation pressure populations is that they mature earlier and that they live shorter, everything else being equal (Kirkwood 1977). (Reznick *et al.* 2004) showed that under common garden situations high predation guppies matured earlier, but grew larger, reproduced more and lived longer, under two different feeding regimes. (Schrader and Travis 2012) also showed that the density of the populations related negatively with predation pressure. Guppies from low predation populations are heavily regulated by density (Bassar *et al.* 2013). Therefore, the amount of nutrition might be much higher for the low predation pressure populations, leading to an ability to increase reproduction, growth rate and lifespan. Indeed, feeding is found to be different between the populations of the high and low predation guppies (Bassar *et al.* 2012). Although females of *H. formosa* mature at a smaller size at low density populations, average adult female size is much larger, while average and maximum lifespan are shorter, suggesting indeed that also females of *H. formosa* show similar patterns compared to guppies (J. Travis, unpublished, data). Therefore, we would expect the variation of the IRS gene, which is also an important candidate genes for ageing and lifespan, to relate to lifespan between populations as well. To verify this, either common garden experiments monitoring lifespan using populations or samples of high and low mortality experiments such as (Schröder *et al.* 2009) could be used to verify the variation found in this study to directly relate mortality with variation in allelic variation.

The significant loci in exon 2 of IRS shown in Figure 10 were significant for both fecundity and standard length. These traits were related to each other, although not significantly, when population averages were used. It is very likely though that an increase in fecundity is physically and evolutionary correlated with an increased size at maturity, and therefore, average standard length as used in this study. Therefore, this analysis was sensitive for type I error. A multivariate (MANOVA) type of analysis might have been more appropriate here. Nevertheless, the significance found for both traits on similar loci probably shows that selection has shaped the allelic variation. If many loci would have been significant for one trait and many others for another trait, type I error might have caused inflation in the number of significant loci. Because at least in the most significant genes there is an overlap between fecundity and standard length, it makes it more plausible that these loci are really affected by past evolution (most notably natural selection), although verification needs to be performed. Also, if these errors might have occurred, it does not explain the difference found between candidate and control genes, but could have only affected the number of significant loci found in both.

Relevance and outlook

For the first time in a vertebrate species, genes from candidate pathways have been sequenced for populations that differ markedly in life history traits. Significant relationships were found in some candidate genes, but not all genes showed significant loci. Therefore, this indicates that it is better to use multiple genes in the same pathway, rather than using a single gene representative of several pathways that is common in candidate gene approaches (Nielsen *et al.* 2009)..

We related allele frequency differences between the populations with average trait

values, measured in many years from field samples, rather than using estimates of environmental parameters. Since phenotypes do not resemble environmental variation per se, it is likely that in our study the successful findings of loci under selection can be attributed to the use of phenotypes as covariates, rather than environmental gradients. Furthermore, in our study the life history traits are highly differentiated between populations, while the geographic distances between populations are small.

It is possible that variation in phenotypes relate to different loci in the same pathways in another group of populations, more geographically distant from the populations around Tallahassee. For instance, in human GWAS studies, a gene variant relating to longevity in one population, might not relate to longevity in another populations (Beekman *et al.* 2013). The pathway though, in which these genes are involved in, could be affected in several populations, but through variation in different genes or loci. Another reason might be that such human GWAS studies look at similar relationships between phenotypic variation and genetic variation between individuals within populations, while we have looked for the relationship between genetic and phenotypic variation between populations. This approach, using a set of candidate pathways, has proven to be successful in the case of populations divergent for crucial life history traits. Linking phenotypic variation with allele frequency differences between human populations might improve the GWAS studies in humans, compared to the often used approach where consistency between studies of within population relationships are tested.

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Appendix A1.

Overview of genes and scaffolds in which coding sequences of the genes were found. The scaffold for more detailes, for every scaffold additional information is given in table 2.

Pathway	Gene name	Scaffolds
Growth hormone pathway	GHRH	scaffold18799_GHRHcompleteCDS
	GHRHR	scaffold9205GHRHRexon6_10 scaffold64582GHRHRexon1 scaffold94137GHRHRexon2_5
	PACAPR	scaffold100603PACAPRexon1_7
	GH	scaffold45149_GHexon1_2 scaffold90146_GHexon3 scaffold27040_GHexon4_5
	GHR	scaffold70377_GHRI_exon1_7 scaffold20586GHRII_aa24_535_7exons
Insulin (like peptides) and their receptors	INS	scaffold64807INS_exon1_2
	INSR	scaffold5149INSRIexon1_3 scaffold12593INSRIIexon20_22 scaffold33012INSRIexon4_21 scaffold73871INSRIIexon1_3 scaffold76037INSRIIexon15_18 scaffold85304INSRIIexon9_14 scaffold97472INSRIIexon4_8 C18043827INSR
	IGF 1	scaffold23080IGF1exon1 scaffold5100IGF1exon2
	IGF1R	scaffold4331IGF1R2_exon1 scaffold13048IGF1R2_ex2_10 scaffold23570IGF1R1_ex1 scaffold44758IGF1R1_ex2_4 scaffold46413IGF1R_ex1_6 scaffold84898IGF1R1_ex5_16 scaffold128065IGF1R1ex17
	IGF2	scaffold50023IGF2_completecds
	IGF2R	scaffold2732IGF2R_6 scaffold21134IGF2R_1 scaffold33658IGF2R_9 scaffold49604IGF2R_2 scaffold65415IGF2R_7 C17458382IGF2R_8 C17554774IGF2R_4 C17654682IGF2R_3 C17714660_IGF2R_5
Upper insulin signaling pathway	IRS	scaffold3149IRS2a_ex1_2 scaffold8517IRS2ex1_3 scaffold16743IRS1bex2_3 scaffold59183IRS2bex1_3 scaffold85425IRS1ex2_3 scaffold88129IRS1ex1 scaffold90335IRS1bex1
	Pi3K and PtdIns4,5	scaffold716Pi3kC2catdex1_4 scaffold7121Pi3kC2gamex1_12 scaffold7610Pi3kC2catgamex12_19 scaffold8454Pi3kC2catbex1_21 scaffold9792Pi3kC2cataex9_20 scaffold23084Pi3kC2cataex1_5 scaffold26630Pi3kC2bex1_22 scaffold34501Pi3ktype3ex10_11 scaffold34882Pi3ktype3ex12_13 scaffold36974Pi3kC2cataex5_20 scaffold40169Pi3kC2aex1_3 scaffold54907Pi3kC2aex4_26 scaffold65050Pi3kC2catgamex1_10 scaffold73625Pi3kC2catgamex11 scaffold79605Pi3ktype3ex14_18 scaffold84418Pi3kC2aex27 scaffold85524Pi3ktype3ex5_9 scaffold135832Pi3kC2cataex7_8 scaffold140063Pi3ktype3ex2_4 C17248961Pi3ktype3ex19 C18124401Pi3ktype3ex1 C18389018Pi3kC2cataex6 scaffold9699catgammalikeex1_10 scaffold83777Pi3kcatgammaex10b
	Pten and PDK1	scaffold5933Pdk1ex1_3 scaffold8208Pdk1ex7_12 scaffold101472Pdk1ex6 C18189222pdk1ex4_5 scaffold64448PtenB4 scaffold68317PtenA1 scaffold108273PtenB1 scaffold119906PtenB2 scaffold120886PtenB3
	AMPK	Scaffold26467_AMPKgam2 Scaffold41126_AMPKgam1 scaffold59695_AMPKb1 scaffold92890_AMPKb2 C17317651_AMPKb3 C18136237_AMPKgam3 scaffold48469A_SMPKb4PLA2 scaffold28114B_AMPKgam4

Middle insulin signaling pathway	TSC	Scaffold39338_TSC2ex1_4 scaffold95279_TSC2ex5_7 scaffold135669_TSC2ex8_12 scaffold24639TSC5 scaffold29846TSC1 scaffold57979TSC3 scaffold90520TSC2 scaffold106997TSC6 C17680850TSC4 C18096177TSC7
	Akt, SGK	scaffold49274SGK1aex1_8 Scaffold35644SGK1bex1_7 scaffold87705SGK1cex1_5 Scaffold64956SGK3ex1_11 Scaffold8797RAC_beta1 Scaffold10203RAC_alpha2 Scaffold10493RAC_gamma3 Scaffold20420RAC_gamma2 Scaffold44857RAC_gamma1 Scaffold89783RAC_gamma4 C18281712RAC_alpha1 C17126849RAC_beta2
	Rheb	Scaffold62725Rheb1 Scaffold40933_Rheb2 C17213715Rheb2
	mTor	Scaffold8397Tor8 Scaffold22984Tor12 Scaffold27836Tor13 Scaffold32842Tor3 Scaffold45151Tor10 Scaffold48798Tor6 Scaffold54558Tor4 Scaffold60499Tor9 Scaffold61483Tor7 Scaffold63736Tor11 Scaffold64134Tor5 Scaffold93786Tor2 Scaffold95849Tor1
	Lamtor, Protor, Raptor	Scaffold22096p14 Scaffold18272Raptorex24_31 Scaffold19440Raptorex1 Scaffold22372Raptorex16_19 Scaffold28068Raptorex6 Scaffold29265Raptorex9_15 Scaffold50473Raptorex5 Scaffold77620Raptorex23 Scaffold79291Raptorex7_8 Scaffold94351Raptorex20_22 Scaffold95635Raptorex4 Scaffold96103Raptorex2_3 Scaffold61098Protorex1_6
	Rho	Scaffold31275RhoAex2_3 Scaffold54074RhoAex1 Scaffold100262RhoAlex1_3 Scaffold41868CRhoCex1_2 Scaffold43374RhoClex1_3 scaffold19499RhoF scaffold112757RhoG

Lower part insulin signaling pathway	S6K	Scaffold20381S6K2 Scaffold117614S6K1 Scaffold47290_s6k1b Scaffold70024_s6k2 Scaffold80639_s6k1c C16993135_s6k1a
	PRAS	Scaffold25352PRAS40ex3_5 Scaffold91433PRAS40ex1_2
	Foxo	scaffold10318Foxo12 scaffold13611Foxo14 scaffold14531Foxo10 scaffold21673Foxo9 scaffold22176Foxo5 scaffold24016Foxo1 scaffold28134Foxo2 scaffold40974Foxo6 scaffold58537Foxo13 scaffold59408Foxo8 scaffold64856Foxo11 scaffold99512Foxo4 scaffold120610Foxo7 C17664408Foxo3
	4ebp	scaffold140524ebp2ex1_2
Lipid metaboslism	ApoE	scaffold62803ApoEex1_2 scaffold34143ApoEbex1 scaffold96268ApoEbex2_3
	LDLR	scaffold2436LDLRex1_6 scaffold57795LDLRrp4ex2_13 scaffold77455LDLRlike scaffold81980LDLR1like scaffold90419LDLRrp1 scaffold93431LDLRrp4 scaffold115790LDLRex7_12
Telomere biology	Terf2	scaffold1924_TERF2
	Gar1	scaffold24927_GAR1
	Pot1	scaffold31859_POT1
	Tert	scaffold31393_TERT
	Nop10	scaffold128178_NOP10
Reproduction genes	PGES	scaffold4101PGES2ex2_3 scaffold104035PGES2ex1 C16859708PGES2ex4 C17894137PGES2ex5
	Cox	scaffold41763coxaex1_6 scaffold55466Coxbex1 scaffold113411cox1bex2_8 scaffold122096cox2ex1_9 C17962623cor1bex9
	BHSD	scaffold689853BHSDex2_3 scaffold436023BHSDex
	LHR	scaffold21713LHRex1_8 scaffold75694LHRex9
	GTR	scaffold34891GTRIIex1 scaffold46854GTRIIex2_8
	FSH	scaffold7796FSHRex1_9
	Aromat	scaffold18527AROMATex1_3
	SCC	scaffold34491SCCex1_7
	STAR	scaffold51555ASTARex1_5

Appendix A2: scaffolds and their traits

Columns indicate name of the scaffold, length of the scaffold, number of A, T, G or Cs, number of Ns, type of scaffold (CA=candidate, CO= control), number of A, T, G or Cs which are in coding regions, number of variable sites which are not in coding region (non-cod). number of synonymous (syn) variants, number of non synonymous variants (nons) in coding region and total number of variable sites in scaffold (tot).

Scaffold	length	atgc	Ns	type	Sizecod.	Non-cod	variants	nons	tot
scaffold140524ebp2ex1_2	8424	7183	1241	CA	529	284	2	7	293
scaffold8797RAC_beta1	5257	4518	739	CA	1053	141	4	6	151
scaffold10203RAC_alpha2	5073	4042	1031	CA	447	101	1	7	109
scaffold10493RAC_gamma3	5517	4740	777	CA	418	171	4	7	182
scaffold20420RAC_gamma2	15107	11852	3255	CA	689	365	5	7	377
scaffold44857RAC_gamma1	12326	9129	3197	CA	1014	340	11	6	357
scaffold89783RAC_gamma4	5021	4419	602	CA	354	181	3	2	186
C18281712RAC_alpha1	995	995	0	CA	345	19	0	0	19
C17126849RAC_beta2	222	222	0	CA	75	3	0	0	3
Scaffold62803ApoEex1_2	4129	3370	759	CA	771	116	10	12	138
scaffold34143ApoEbex1	9685	7920	1765	CA	5064	181	58	103	342
scaffold96268ApoEbex2_3	4584	3710	874	CA	159	151	2	2	155
scaffold10318Foxo12	20231	16171	4060	CA	1542	440	7	11	458
scaffold13611Foxo14	16407	14662	1745	CA	1287	434	5	7	446
scaffold14531Foxo10	50482	44722	5760	CA	551	1783	8	12	1803
scaffold21673Foxo9	910	659	251	CA	426	11	0	0	11
scaffold22176Foxo5	571	339	232	CA	102	0	0	0	0
scaffold24016Foxo1	27933	24653	3280	CA	1056	888	4	10	902
scaffold28134Foxo2	14002	12213	1789	CA	1032	359	14	11	384
scaffold40974Foxo6	1151	860	291	CA	306	7	2	4	13
scaffold58537Foxo13	6346	5532	814	CA	801	143	5	7	155
scaffold59408Foxo8	16632	14264	2368	CA	564	505	3	3	511
scaffold64856Foxo11	17244	14721	2523	CA	1536	348	6	15	369
Scaffold99512Foxo4	14427	13324	1103	CA	1506	437	12	21	470
scaffold120610Foxo7	952	423	529	CA	351	0	0	0	0
C17664408Foxo3	411	411	0	CA	300	0	2	1	3
scaffold45149_GHexon1_2	2933	2211	722	CA	564	30	8	16	54
scaffold90146_GHexon3	2444	1137	1307	CA	153	5	0	1	6
scaffold27040_GHexon4_5	1986	1319	667	CA	159	27	0	1	28
scaffold18799_GHRHcompleteCDS	15231	11857	3374	CA	453	464	5	6	475
scaffold9205GHRHRexon6_10	17009	15096	1913	CA	471	724	1	6	731
scaffold64582GHRHRexon1	6404	5129	1275	CA	183	254	2	0	256
scaffold94137GHRHRexon2_5	9063	7295	1768	CA	507	339	9	10	358
scaffold100603PACAPRexon1_7	12452	10551	1901	CA	852	380	12	9	401
scaffold70377_GHRI_exon1_7	25945	23080	2865	CA	1680	826	8	22	856
scaffold20586GHRII_aa24_535_7exons	5742	4325	1417	CA	1248	105	14	24	143
scaffold23080IGF1exon1	13944	10826	3118	CA	195	312	0	0	312
scaffold5100IGF1exon2	9931	8010	1921	CA	444	233	1	1	235
scaffold4331IGF1R2_exon1	10161	9113	1048	CA	807	296	8	10	314
scaffold13048IGF1R2_ex2_10	17548	14568	2980	CA	1374	445	11	9	465
scaffold23570IGF1R1_ex1	17540	15205	2335	CA	561	607	3	11	621
scaffold44758IGF1R1_ex2_4	6821	4760	2061	CA	585	153	7	5	165
scaffold46413IGF1R_ex1_6	47349	43451	3898	CA	1197	2114	8	8	2130
scaffold84898IGF1R1_ex5_16	16355	13182	3173	CA	2313	367	8	11	386
scaffold128065IGF1R1ex17	5481	5175	306	CA	429	197	0	5	202
scaffold50023IGF2_completecds	34433	31034	3399	CA	1014	892	6	8	906
scaffold2732IGF2R_6	2652	1614	1038	CA	495	27	3	11	41
scaffold21134IGF2R_1	23080	18317	4763	CA	1413	738	9	26	773
scaffold33658IGF2R_9	11756	9406	2350	CA	2460	272	14	32	318

scaffold49604IGF2R_2	6412	4520	1892	CA	552	122	4	8	134
scaffold65415IGF2R_7	12546	9545	3001	CA	3164	197	28	42	267
C17458382IGF2R_8	322	322	0	CA	114	5	2	6	13
C17554774IGF2R_4	360	360	0	CA	105	5	0	0	5
C17654682IGF2R_3	406	406	0	CA	189	1	2	1	4
C17714660_IGF2R_5	437	437	0	CA	204	6	1	8	15
scaffold64807INS_exon1_2	4121	3321	800	CA	288	32	1	3	36
scaffold5149INSRIexon1_3	27252	24661	2591	CA	648	1031	5	8	1044
scaffold12593INSRIIexon20_22	18414	14293	4121	CA	1293	437	9	13	459
scaffold33012INSRIexon4_21	19414	17963	1451	CA	2982	748	26	20	794
scaffold73871INSRIIexon1_3	12518	10414	2104	CA	576	317	1	2	320
scaffold76037INSRIIexon15_18	4725	3754	971	CA	507	135	4	7	146
scaffold85304INSRIIexon9_14	9169	8365	804	CA	1110	301	9	14	324
scaffold97472INSRIIexon4_8	5652	4071	1581	CA	813	61	8	8	77
C18043827INSR	674	674	0	CA	231	14	1	2	17
scaffold3149IRS2a_ex1_2	35212	30021	5191	CA	3282	953	18	36	1007
scaffold8517IRS2ex1_3	19983	17107	2876	CA	3108	453	22	28	503
scaffold16743IRS1bex2_3	18047	15899	2148	CA	3120	509	21	34	564
scaffold59183IRS2bex1_3	8296	7785	511	CA	3648	205	41	46	292
scaffold85425IRS1ex2_3	14881	12706	2175	CA	2850	349	22	23	394
scaffold88129IRS1ex1	2560	1875	685	CA	480	63	2	3	68
scaffold90335IRS1bex1	3316	3126	190	CA	348	148	1	1	150
scaffold2436LDLRex1_6	2636	1924	712	CA	810	23	4	10	37
scaffold57795LDLRrp4ex2_13	10631	9084	1547	CA	1932	306	9	31	346
scaffold77455LDLRlike	6078	4296	1782	CA	318	127	2	3	132
scaffold81980LDLR1like	15901	13707	2194	CA	2286	550	11	27	588
scaffold90419LDLRrp1	12742	11649	1093	CA	852	495	3	6	504
scaffold93431LDLRrp4	6686	5778	908	CA	405	256	2	4	262
scaffold115790LDLRex7_12	3699	2990	709	CA	1008	58	6	17	81
scaffold5933Pdk1ex1_3	17961	14390	3571	CA	834	455	8	10	473
scaffold8208Pdk1ex7_12	11979	9676	2303	CA	558	298	5	4	307
scaffold101472Pdk1ex6	2951	2375	576	CA	78	82	0	0	82
C18189222pdk1ex4_5	844	844	0	CA	309	22	1	3	26
scaffold716Pi3kC2catdex1_4	16829	13661	3168	CA	828	609	7	21	637
scaffold7121Pi3kC2gamex1_12	14792	10446	4346	CA	2331	276	16	28	320
scaffold7610Pi3kC2catgamex12_19	6262	4719	1543	CA	945	124	5	10	139
scaffold8454Pi3kC2catbex1_21	36281	32163	4118	CA	3246	1154	18	22	1194
scaffold9792Pi3kC2cataex9_20	11061	8883	2178	CA	1698	276	14	8	298
scaffold23084Pi3kC2cataex1_5	6500	4166	2334	CA	1410	60	8	12	80
scaffold26630Pi3kC2bex1_22	17059	14423	2636	CA	3992	336	31	45	412
scaffold34501Pi3ktype3ex10_11	1450	672	778	CA	222	14	1	1	16
scaffold34882Pi3ktype3ex12_13	1132	886	246	CA	228	15	0	2	17
scaffold36974Pi3kC2cataex5_20	27744	24098	3646	CA	2370	1162	18	37	1217
scaffold40169Pi3kC2aex1_3	16312	14359	1953	CA	1251	209	7	9	225
scaffold54907Pi3kC2aex4_26	20377	16858	3519	CA	3114	238	8	12	258
scaffold65050Pi3kC2catgamex1_10	16723	14043	2680	CA	1989	463	25	20	508
scaffold73625Pi3kC2catgamex11	891	588	303	CA	171	3	4	1	8
scaffold79605Pi3ktype3ex14_18	1925	1684	241	CA	525	32	6	2	40
scaffold84418Pi3kC2aex27	6870	4102	2768	CA	180	42	0	0	42
scaffold85524Pi3ktype3ex5_9	3908	2705	1203	CA	546	67	6	8	81
scaffold135832Pi3kC2cataex7_8	1387	987	400	CA	282	13	1	1	15
scaffold140063Pi3ktype3ex2_4	1653	1425	228	CA	489	29	5	7	41
C17248961Pi3ktype3ex19	254	254	0	CA	165	4	1	2	7
C18124401Pi3ktype3ex1	761	761	0	CA	66	39	0	0	39
C18389018Pi3kC2cataex6	1269	1269	0	CA	153	44	1	0	45
scaffold9699catgammalikeex1_10	19192	15110	4082	CA	1509	685	13	22	720
scaffold83777Pi3kcatgammaex10b	1994	1576	418	CA	87	71	1	0	72

scaffold1924_TERF2	11595	10402	1193	CA	981	346	7	19	372
scaffold24927_GAR1	4048	3249	799	CA	240	116	0	2	118
scaffold31859_POT1	10065	7664	2401	CA	705	190	6	10	206
scaffold31393_TERT	15438	13283	2155	CA	2994	431	33	53	517
scaffold128178_NOP10	4540	3727	813	CA	948	115	10	24	149
scaffold64448PtenB4	7322	5342	1980	CA	579	156	3	3	162
scaffold68317PtenA1	17947	15814	2133	CA	1053	208	1	3	212
scaffold108273PtenB1	3520	2851	669	CA	222	68	1	3	72
scaffold119906PtenB2	5300	4304	996	CA	177	158	1	1	160
scaffold120886PtenB3	1834	1655	179	CA	147	94	1	1	96
scaffold4101PGES2ex2_3	2038	1395	643	CA	555	23	4	5	32
scaffold7796FSHRex1_9	18952	13946	5006	CA	1614	505	14	22	541
scaffold18527AROMATex1_3	13966	11729	2237	CA	1873	394	20	25	439
scaffold21713LHRex1_8	9676	6624	3052	CA	708	158	7	9	174
scaffold34491SCCex1_7	24433	22245	2188	CA	1485	847	12	14	873
scaffold34891GTRIIex1	12310	9106	3204	CA	327	355	0	3	358
scaffold41763coxaex1_6	23268	18606	4662	CA	1344	757	11	11	779
scaffold436023BHSDex1	1218	1133	85	CA	150	16	1	1	18
scaffold46854GTRIIex2_8	18461	15621	2840	CA	1491	715	16	14	745
scaffold51555ASTARex1_5	12800	11393	1407	CA	1056	409	9	11	429
scaffold55466Coxbex1	2632	1668	964	CA	129	43	0	0	43
scaffold689853BHSDex2_3	12150	10236	1914	CA	981	382	21	12	415
scaffold75694LHRex9	5186	3924	1262	CA	1215	98	12	27	137
scaffold104035PGES2ex1	4378	2914	1464	CA	558	80	4	6	90
scaffold113411cox1bex2_8	6737	5839	898	CA	1182	239	9	15	263
scaffold122096cox2ex1_9	16345	15121	1224	CA	1854	592	11	11	614
C16859708PGES2ex4	170	170	0	CA	168	0	0	1	1
C17894137PGES2ex5	549	549	0	CA	102	10	0	4	14
C17962623cor1bex9	602	602	0	CA	351	11	2	4	17
Scaffold20381S6K2	38987	31677	7310	CA	974	1220	6	12	1238
Scaffold62725Rheb1	12190	9559	2631	CA	597	356	1	3	360
Scaffold117614S6K1	4147	3852	295	CA	114	145	0	0	145
C17213715Rheb2	244	244	0	CA	102	7	0	2	9
Scaffold26467_AMPKgam2	16803	13760	3043	CA	1173	545	8	11	564
Scaffold39338_TSC2ex1_4	4543	3882	661	CA	810	158	11	9	178
Scaffold40933_Rheb2	7818	6922	896	CA	564	249	4	3	256
Scaffold41126_AMPKgam1	5678	3733	1945	CA	1050	103	16	12	131
scaffold47290_s6k1b	4910	4128	782	CA	636	109	6	9	124
scaffold59695_AMPKb1	9194	7483	1711	CA	771	312	2	5	319
scaffold70024_s6k2	10884	8165	2719	CA	1619	298	15	19	332
scaffold80639_s6k1c	11023	9891	1132	CA	1353	349	20	21	390
scaffold92890_AMPKb2	1806	1421	385	CA	417	36	6	4	46
scaffold95279_TSC2ex5_7	5946	4234	1712	CA	810	211	9	9	229
scaffold135669_TSC2ex8_12	3977	3452	525	CA	931	136	12	11	159
C16993135_s6k1a	194	194	0	CA	66	10	0	1	11
C17317651_AMPKb3	274	274	0	CA	129	2	0	1	3
C18136237_AMPKgam3	775	775	0	CA	210	19	1	5	25
scaffold48469A_SMPKb4PLA2	22260	19642	2618	CA	1851	751	21	35	807
scaffold28114B_AMPKgam4	11606	10008	1598	CA	1449	330	11	17	358
scaffold8397Tor8	15878	10918	4960	CA	762	128	0	6	134
scaffold22984Tor12	8760	6520	2240	CA	138	90	0	0	90
scaffold27836Tor13	5053	4069	984	CA	735	24	2	1	27
scaffold32842Tor3	3758	3456	302	CA	846	27	1	2	30
scaffold45151Tor10	3552	2618	934	CA	415	40	6	12	58
scaffold48798Tor6	11054	9329	1725	CA	1138	118	5	4	127
scaffold54558Tor4	7800	4993	2807	CA	129	68	0	0	68
scaffold60499Tor9	4655	3136	1519	CA	270	39	1	4	44

scaffold61483Tor7	20620	17562	3058	CA	963	260	1	4	265
scaffold63736Tor11	5111	3267	1844	CA	453	24	1	1	26
scaffold64134Tor5	8754	6823	1931	CA	519	77	2	2	81
scaffold93786Tor2	4432	2833	1599	CA	678	26	3	1	30
scaffold95849Tor1	8551	6371	2180	CA	1116	87	5	4	96
scaffold24639TSC5	3562	2719	843	CA	285	157	1	2	160
scaffold29846TSC1	10739	7729	3010	CA	909	189	5	12	206
scaffold57979TSC3	1937	1283	654	CA	600	11	5	21	37
scaffold90520TSC2	2505	1831	674	CA	81	107	0	0	107
scaffold106997TSC6	1794	1396	398	CA	312	58	4	4	66
C17680850TSC4	419	419	0	CA	48	12	0	0	12
C18096177TSC7	729	729	0	CA	186	22	0	5	27
scaffold18272Raptorex24_31	28162	25709	2453	CA	1200	1223	16	23	1262
scaffold19440Raptorex1	21066	18315	2751	CA	585	777	3	2	782
scaffold19499RhoF	21586	19892	1694	CA	2619	603	18	31	652
scaffold22096p14	15583	14892	691	CA	679	171	1	4	176
scaffold22372Raptorex16_19	11080	10158	922	CA	519	491	4	6	501
scaffold25352PRAS40ex3_5	4770	3352	1418	CA	315	98	4	3	105
scaffold28068Raptorex6	14660	13420	1240	CA	615	677	8	13	698
scaffold29265Raptorex9_15	20813	18036	2777	CA	489	756	5	4	765
scaffold31275RhoAex2_3	5940	4780	1160	CA	429	145	2	3	150
scaffold35644SGK1bex1_7	5952	4753	1199	CA	1092	118	8	9	135
scaffold41868CRhoCex1_2	13812	12998	814	CA	282	582	1	4	587
scaffold43374RhoClex1_3	6040	5438	602	CA	747	189	2	7	198
scaffold49274SGK1aex1_8	14754	13672	1082	CA	1659	549	15	13	577
scaffold50473Raptorex5	7795	6470	1325	CA	294	311	4	5	320
scaffold54074RhoAex1	9457	7597	1860	CA	763	289	5	11	305
scaffold61098Protorex1_6	20067	16358	3709	CA	855	547	6	10	563
scaffold64956SGK3ex1_11	21915	18228	3687	CA	1221	646	4	12	662
scaffold77620Raptorex23	632	551	81	CA	111	18	2	1	21
scaffold79291Raptorex7_8	14909	13030	1879	CA	309	557	2	4	563
scaffold87705SGK1cex1_5	5301	4319	982	CA	1023	105	11	16	132
scaffold91433PRAS40ex1_2	1624	906	718	CA	360	13	1	2	16
scaffold94351Raptorex20_22	11594	10033	1561	CA	471	404	1	5	410
scaffold95635Raptorex4	7741	6627	1114	CA	351	269	8	9	286
scaffold96103Raptorex2_3	4000	3555	445	CA	189	173	0	0	173
scaffold100262RhoAlex1_3	18010	15854	2156	CA	2220	518	15	28	561
scaffold112757RhoG	18257	16446	1811	CA	573	693	5	17	715
scaffold1441control1	29227	24587	4640	CO	1059	1011	10	11	1032
scaffold6741control2	7957	7251	706	CO	2223	226	22	32	280
scaffold24556control3	22374	15074	7300	CO	3435	435	29	52	516
scaffold28358control4	21009	17220	3789	CO	3393	571	23	37	631
scaffold41792control5	2486	1628	858	CO	75	51	3	0	54
scaffold49633control6	15880	13651	2229	CO	1350	137	7	4	148
scaffold51968control7	4560	3468	1092	CO	204	137	1	3	141
scaffold53887control8	8111	7060	1051	CO	462	212	3	8	223
scaffold57901control9	8267	6339	1928	CO	1478	240	31	32	303
scaffold62278control10	1760	1171	589	CO	105	20	0	0	20
scaffold71501control11	6139	4361	1778	CO	253	138	2	1	141
scaffold74869control12	22973	19545	3428	CO	6102	580	65	92	737
scaffold90704control13	5790	4650	1140	CO	612	164	5	6	175
scaffold91780control14	6071	4776	1295	CO	330	194	4	2	200
scaffold116281control15	7793	6658	1135	CO	456	347	4	1	352
scaffold118958control16	3715	3135	580	CO	438	103	4	5	112
scaffold122179control17	2022	1722	300	CO	165	34	0	0	34
scaffold127172control18	3727	3423	304	CO	536	137	5	9	151
scaffold128799control19	5551	5075	476	CO	1698	157	17	21	195

scaffold134449control20	2111	1847	264	CO	177	114	2	1	117
scaffold135207control21	1126	893	233	CO	282	22	3	7	32
scaffold140180control22	2421	1968	453	CO	582	13	4	5	22
C16756477control23	156	156	0	CO	90	0	0	0	0
C16856826control24	170	170	0	CO	138	0	0	0	0
C17604996control25	383	383	0	CO	114	10	0	2	12
C17758274control26	461	461	0	CO	123	8	0	2	10
C17766170control27	466	466	0	CO	288	4	1	1	6
C18269016control28	971	971	0	CO	878	4	6	14	24
C18293126control29	1017	1017	0	CO	282	46	3	1	50
scaffold3817control30	14341	11162	3179	CO	2736	285	35	47	367
scaffold3867control31	15471	12974	2497	CO	981	533	6	9	548
scaffold14792control32	11739	9061	2678	CO	1371	293	21	23	337
scaffold14803control33	12352	9498	2854	CO	1032	349	9	12	370
scaffold15392control34	6736	5334	1402	CO	303	138	3	0	141
scaffold20006control35	15931	13820	2111	CO	402	440	1	1	442
scaffold22939control36	2474	1162	1312	CO	201	0	0	2	2
scaffold33230control37	3682	2343	1339	CO	375	58	0	0	58
scaffold39948control38	24987	21010	3977	CO	5856	626	54	103	783
scaffold41999control39	7088	5632	1456	CO	1353	178	8	16	202
scaffold44579control40	15700	11975	3725	CO	297	370	2	11	383
scaffold59898control41	14767	10394	4373	CO	366	178	0	2	180
scaffold62708control42	1429	1108	321	CO	78	21	1	0	22
scaffold66120control43	16078	13855	2223	CO	813	474	4	10	488
scaffold75613control44	1307	888	419	CO	126	39	4	0	43
scaffold76918control45	19446	16287	3159	CO	1263	263	4	5	272
scaffold78018control46	3842	2581	1261	CO	1068	73	13	12	98
scaffold82051control47	2307	1600	707	CO	144	142	6	11	159
scaffold89408control48	3232	2254	978	CO	538	21	1	8	30
scaffold93428control49	827	671	156	CO	252	12	4	2	18
scaffold96869control50	10010	9202	808	CO	1935	316	25	27	368
scaffold99071control51	2381	1902	479	CO	453	58	5	13	76
scaffold113847control52	2354	2023	331	CO	402	75	2	3	80
scaffold140653control53	1890	1836	54	CO	1629	23	31	37	91
C16740587control54	154	154	0	CO	66	0	0	0	0
C16838076control55	167	167	0	CO	87	0	0	0	0
C17260889control56	257	257	0	CO	99	4	1	0	5
C17353477control57	285	285	0	CO	123	5	1	3	9
C17391509control58	298	298	0	CO	153	3	1	2	6
C17730606control59	446	446	0	CO	243	7	1	7	15
C18015641control60	647	647	0	CO	438	4	3	5	12
C18052991control61	683	683	0	CO	202	25	0	0	25

Appendix B: populations structure for subsets of data

Nei's pairwise genetic distance is calculated by dividing the sum of squared differences between allele frequencies of specific alleles on a specific locus, by the multiplication of the square root of the squared allele frequencies of the two populations considered. If we let, $p_{i,j,k}$ be the frequency of the *i*th allele, of the *j*th lous of the *k*th populations, then if we consider the pairwise genetic difference between the Moore Lake (ML) and Trout Pond (TP) population, we would calculate the genetic distance by,

$$d = -\ln\left(\frac{\sum_{i=1}^{n}\sum_{j=1}^{m} p_{i,j,ML}\,p_{i,j,TP}}{\sum_{i=1}^{n}\sum_{j=1}^{m} p_{i,j,ML}^{\,2}\sum_{i=1}^{n}\sum_{j=1}^{m} p_{i,j,TP}^{\,2}}\right)$$

Where *d* is the genetic distance, and *n* and *m* are the number of allele and loci considered, respectively.

All loci

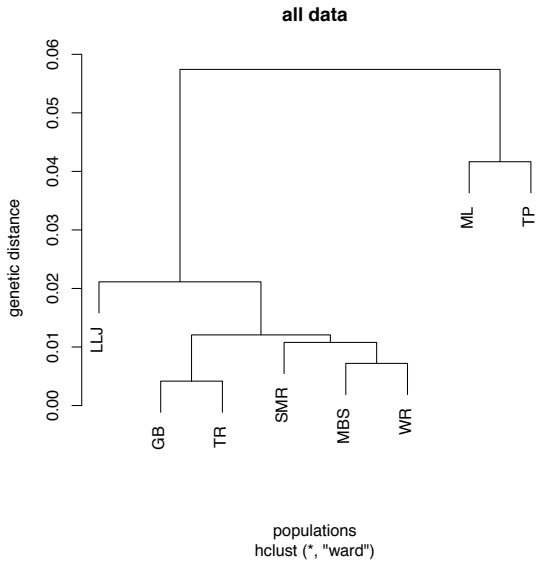

Figure B1. Dendogram using the Nei's genetic distance over 71999 variable loci.

Candidate versus control loci

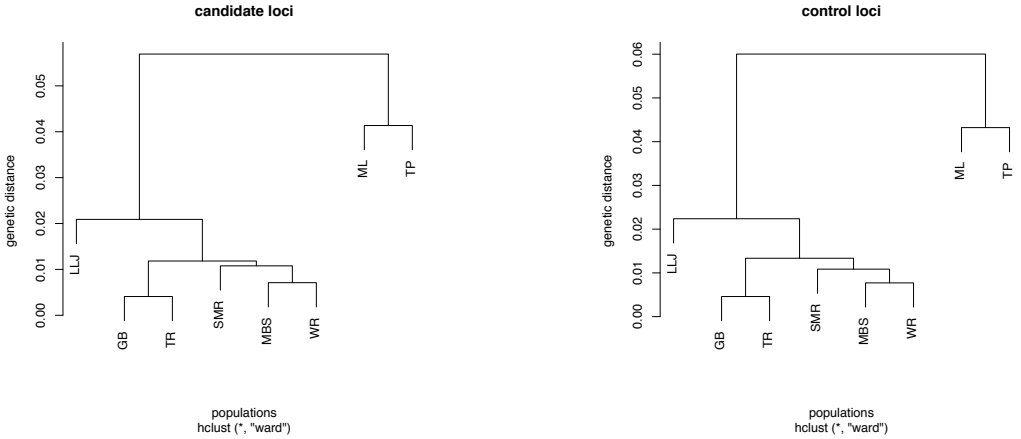

Figure B2. Dendograms of genetic distance separated between the 60.651 candidate and 11.348 control loci.

SNPs, inserts and deletions

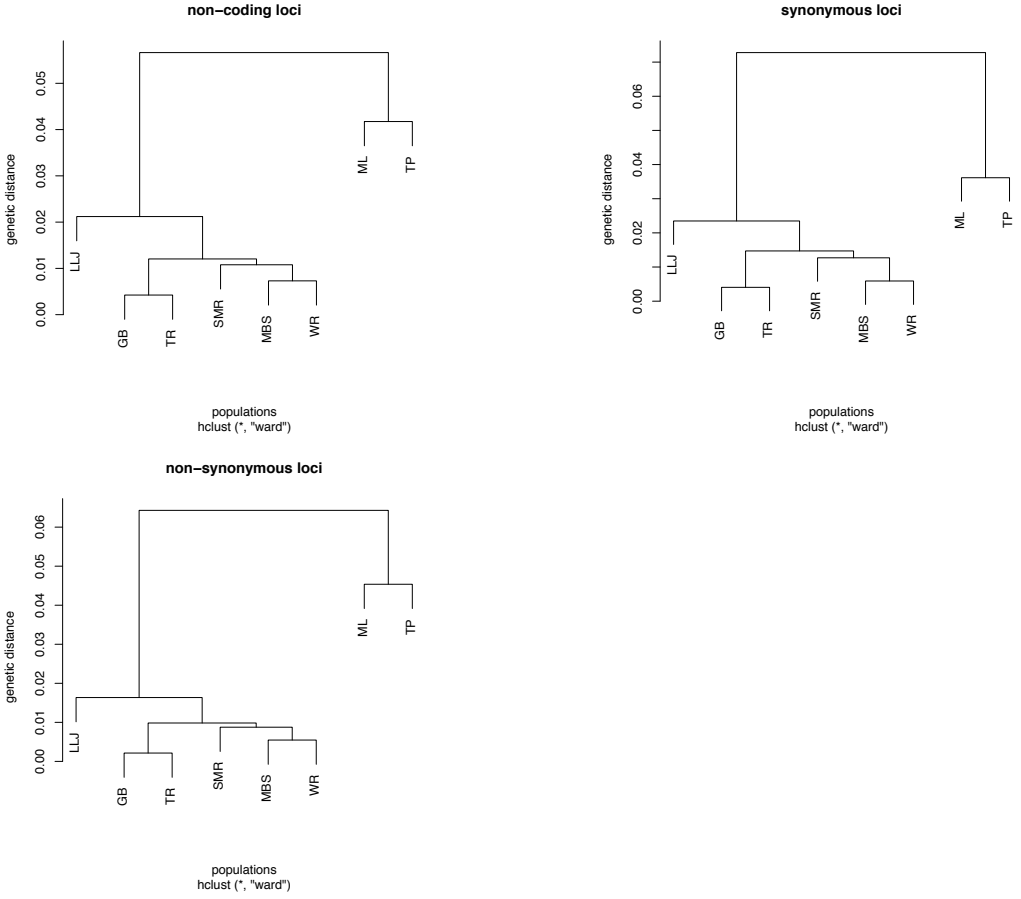

Figure B3. Dendograms of loci that are non coding (67.420 loci), synonymous (2444) and non synonymous loci (2135).

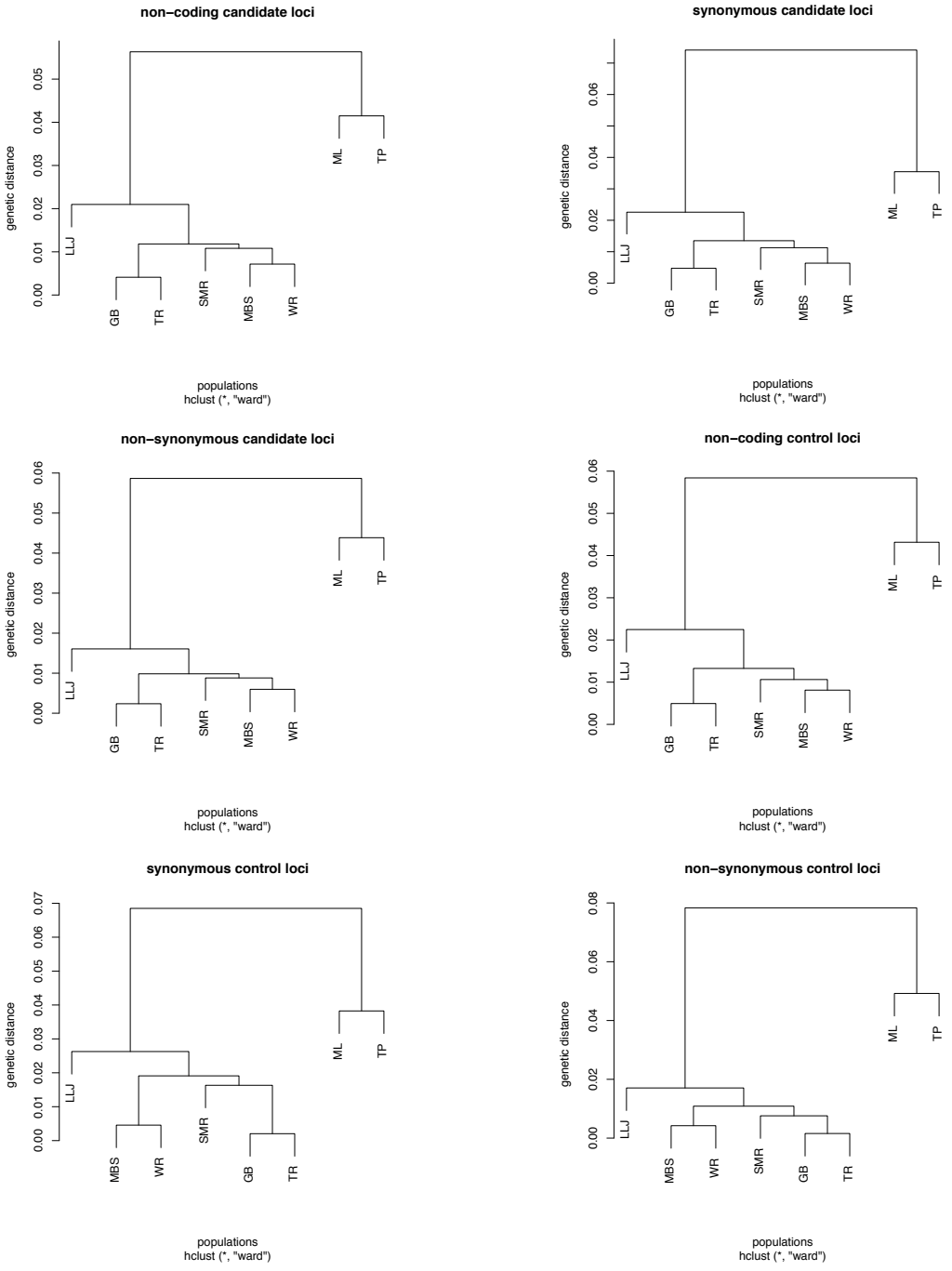

Figure B4. Dendograms of genetic distance for non coding candidate loci (57292), synonymous candidate loci (1837), non synonymous candidate loci (1552), non coding control loci (10128), synonymous control loci (607) and non synonymous control loci (613).

Appendix C: F_{st} calculations

Pop	Allele freq	H
GB	p_{GB}	$2p_{GB}(1-p_{GB})$
LLJ	p_{LLJ}	$2p_{LLJ}(1-p_{LLJ})$
MBS	p_{MBS}	$2p_{MBS}(1-p_{MBS})$
ML	p_{ML}	$2p_{ML}(1-p_{ML})$
SMR	p_{SMR}	$2p_{SMR}(1-p_{SMR})$
TP	p_{TP}	$2p_{TP}(1-p_{TP})$
TR	p_{TR}	$2p_{TR}(1-p_{TR})$
WR	p_{WR}	$2p_{WR}(1-p_{WR})$

Fst = [2*mean(p)*{1-mean(p)} – mean(H)]/ [2*mean(p)*{1-mean(p)}]

If there are more than two alleles, the calculation is different. Now we consider three allele frequencies, *p*, *q* and *s*, where $p + q + s = 1$. First, heterozygosity per population will be calculated as,

$$H_{Si} = 1 - p_i^2 - q_i^2 - s_i^2$$

Where *i* denotes population identity. Then we take the average over the eight populations, which we write as

$$\overline{H}_S = \sum_{i=1}^{n} \frac{H_{Si}}{n}$$

To estimate the heterozygosity between populations, we first average the frequencies of the alleles over the eight populations and then calculate, the total heterozygosity as,

$$H_T = 1 - \overline{p}^2 - \overline{q}^2 - \overline{s}^2$$

Then F_{st} will be calculated as

$$Fst = \frac{H_T - \overline{H}_S}{H_T}$$

Appendix D: hypothetical outcomes bootstrapping p values

In our study we re-sequence roughly six times more loci from candidate genes than from control genes. This resulted also in a roughly six times higher number of variable sites found in candidate scaffolds. To compare the p values associated with relationship with traits we cannot compare the lowest p values of these candidate and control regions, because the number of p values is unequal. By bootstrapping the two p value dsitrbutions, these distributions can be compared. In this appendix possible distribution differences and the outcome of bootstrapping are shown.

Comparison 1: two uniform distribution with different numbers

First the bootstrapping method will be performed on two uniform distributions, as if we would get from a statistical test in which the null hypothesis is true. Then two distributions are compared for which one distributions 60000 tests are done, and 10000 for the other, just like our example of candidate and control loci. From these two distributions 1000 p values are sampled. These are sorted and put as a row in a sorted matrix. This is done 2001 times. When this is ready, the first column holds 2001 values of the lowest p values per sampling. When this column is created from two equal distributions these two columns will overlap. When the median p value from one column is outside 95% of the sorted other column, there can be called significantly different with an *a* of 0.05. Therefore we plot the median and 95% confidience intervals over columns 1 to 50 of the two uniform distributions (see Fig. 1). Indeed the distributions of two uniform distributions are alike and therefore overlapping.

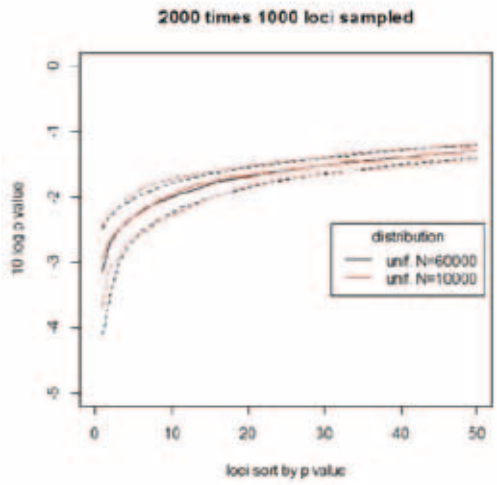

Figure D1. Median and 95% confidence intervals of samplings which are sorted per random draw from uniform p value distribution with in black 60000 p values and in red 10000 p values.

Comparison 2: differences in sampling effort

We can perform a similar test but then take 3000 p values from one distribution every time a sample is taken and 500 for the other. If this is done, then the lowest p values of the samples taken with greater number are lower. Therefore the first 50 lowest have a lower p value distribution (see Fig. 2). This indicates that taking different number of p values in a sample does have a major influence on the outcome. Also the lowest p values (left on axis) are overlapping and therefore the lowest p values of the higher sampling is not significantly different, while that of the higher p values are.

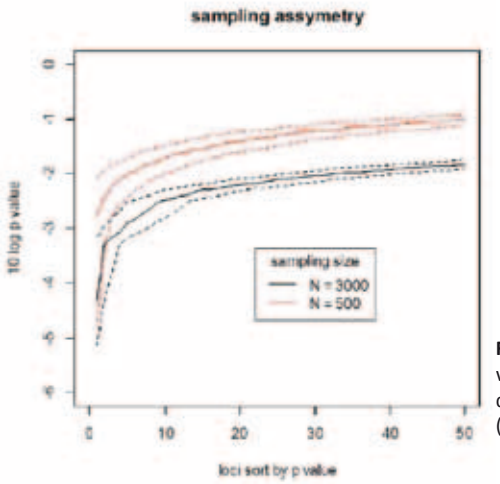

Figure D2. Median and 95% confidence intervals of samplings which are sorted per random draw from uniform p value distribution with high sampling (3000) and in red low sampling (500) of p values.

Comparison 3: Negative exponential and uniform

When many low p values are present at the cost of higher p values, are negative exponential p value distribution can emerge. A bootstrap method was performed on two of these distributions as well. The results is that over the whole range of p value (from lowest to higher) the negative exponential is enriched for lower p values. This means that if the candidate loci would have a negative exponential distribution, while the control do not, we would find a enrichment in a large region over the x axis.

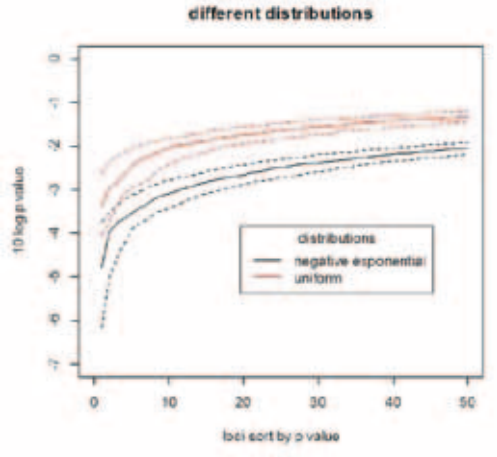

Figure D3. Median and 95% confidence intervals of samplings which are sorted per random draw from a negative exponential p value distribution in black and uniform in red.

Comparison 4: enriched uniform against uniform

Lastly we compare the outcome of a distribution while is uniform, but enriched with 1% lower p values. We have taken 10000 p values from a t test testing two normal distributions with averages of zero and standard deviation of 2 compared to a distribution with an added 100 p values of t tests with a difference in average of 0.5 and for both groups (N=50) a standard deviation of 2. This would be the case if in candidate loci most where neutral, and therefore, for these loci the null hypothesis of no effect would be true, but for a few cases (100 out of 10000 in this example) the p values would be lower than expected from the null hypothesis. The result is that bootstrapping both these distributions lead to a significant enrichment of lower p values (left on x axis, see Fig. 4), while for higher p values this enrichment is not present.

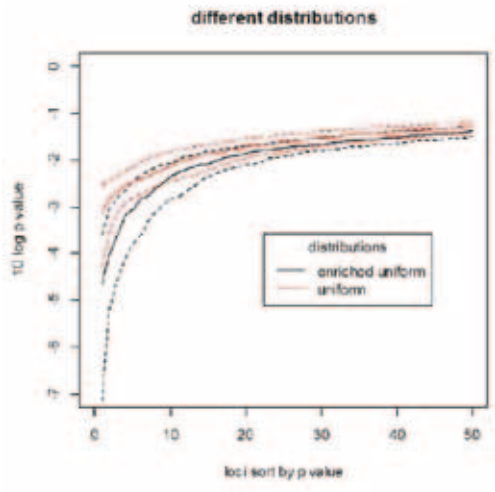

Figure D4. Median and 95% confidence intervals of samplings which are sorted per random draw from a low p value enriched uniform p value distribution in black and uniform in red.

Appendix E: IRS gene alignment and positions non-synonymous SNPs from exon 2

```
65_IRS1B_Xiphophorus      MEGQAGELHGNEDVRKSGYLRKQKSMHRRYFVLRAASERGPARLEYYESEKKFRGKAPVP 60
65_IRS1_Heterandria       MEGQAGEPHGNEDVRKSGYLRKQKSMHRRYFVLRAASERGPARLEYYESEKKFRGKAPVP 60
65_IRS1_Oryzias           MNVQKKLRGSGEDVRRSGYLRKQKSMHRRYFVLRAASERGPARLEYYESEKKFRGKAPVP 60
65_IRS1_Oreochromis       MESQAGEQHSHEDVRKSGYLRKHKSMHRRYFVLRAASERGPARLEYYESEKKFRGKAPVP 60
65_IRS1_Takifugu          MENQAGD--SCEDVRRSGYLRKQKSMHRRYFVLRAASERGPARLEYYESEKKFRGKAPVP 58
65_IRS1B_isoX2_Danio      MENHMELQNSTEDVRRSGYLRKQKSMHRRYFVLRAASERGPARLEYYESEKKFRGKAPVP 60
                          *: :     . ****:******:************************************

65_IRS1B_Xiphophorus      KKAVALETCFNINKRADS------KNKHMIVLYTRAESFVVAAENEEDQDEWFQAMVELQ 114
65_IRS1_Heterandria       KKAVALETCFNINKRADXXXXXXXKNKHMIVLYTRAESFVVAAENEEDQDEWFQAMVELQ 120
65_IRS1_Oryzias           KKALALETCFNINKRADA------KNKHMIVLYTRAESFAVAAESEADQDEWYQAMVELQ 114
65_IRS1_Oreochromis       KKVVALETCFNINKRADS------KNKHMIVLYTRAESFAVAAENEADQDEWYQAMVELQ 114
65_IRS1_Takifugu          KKAVALETCFNINKRADS------KNKHMIVLYTRAESFALAAENEADQDEWYQAMVDLQ 112
65_IRS1B_isoX2_Danio      KKALALETCFNINKRADS------KNKHMIVLYTRAESFVVAAENEADQEEWYQAMVELQ 114
                          **.:*************       ***************.:***.* **:**:****:**

65_IRS1B_Xiphophorus      CKSK-PQTRSPRPHAQALGWWMRRNVAVAAVALCRAARDMFHWCFGLKHTLSSCFLLMLQ 173
65_IRS1_Heterandria       CKSKLADTARPRPHAHALG------------------------------------------ 139
65_IRS1_Oryzias           CRSKESNMCARGGTWGGGG------------------------------------------ 133
65_IRS1_Oreochromis       CRSK--------------------------------------------------------- 118
65_IRS1_Takifugu          CKSKCTCTLTCMHAG---------------------------------------------- 127
65_IRS1B_isoX2_Danio      CKSK--------------------------------------------------------- 118
                          *:**

65_IRS1B_Xiphophorus      NRTVGGGGSEVGKRGSKVEIGDFKSKNPNDSASSGDYGAPNPGPAFKEVWQVKVWPKGLG 233
65_IRS1_Heterandria       -------------------------KNPNDSASSGDYGAPNPGPAFKEVWQVKVWPKGLG 174
65_IRS1_Oryzias           -------------------DNCSSISALYDSGATGDYGVPNPGPAFKEVWQVKVWPKGLG 174
65_IRS1_Oreochromis       --------------------------NPNDGSGAGDYGVPNPGPAFKEVWQVKVWPKGLG 152
65_IRS1_Takifugu          -------------------------NPTEGGALGDYGVPNPGPAFKEVWQVKVWPKGLG 161
65_IRS1B_isoX2_Danio      --------------------------ALCENANGGDYGVPSPGPAFKEVWQVKVWPKGLG 152
                                                   :..  ****.*.********************

65_IRS1B_Xiphophorus      QLKNLVGIYRLCLTEKTVNFVKLNSDAAAVVLQLMNVRRCGHSENFFFVEVGRSAVTGPG 293
65_IRS1_Heterandria       QAKNLVGIYRLCLTEKTVNFVKLNSDAAAVVLQLMNVRRCGHSENFFFVEVGRSAVTGPG 234
65_IRS1_Oryzias           QAKNLVGIFRLCLTDKTVNFVKLNSDAAAVVLQLMNVRRCGHSENFFFIEVGRSAVTGPG 234
65_IRS1_Oreochromis       QAKNLVGIYRLCLTDKTVNFVKLNSDAAAVVLQLMNVRRCGHSENFFFVEVGRSAVTGPG 212
65_IRS1_Takifugu          QAKNLVGVYRLCLTEKTVNFVKLNSDAAAVVLQLMNVRRCGHSENFFFVEVGRSAVTGPG 221
65_IRS1B_isoX2_Danio      QAKNLVGIYRLCLTEKTVNFVKLNSDAVAVVLQLMNVRRCGHSENFFFVEVGRSAVTGPG 212
                          * *****::*****:************.********************:************

65_IRS1B_Xiphophorus      EFWMQVDDSVVAQNMHETLLEAMKALSEEFRQRTKSQS-NSGPGGGATASNPISVPSRRH 352
65_IRS1_Heterandria       EFWMQVDDSVVAQNMHETLLEAMKALSEEFRQRTKSQS-NSGPGGGATASNPISVPSRRH 293
65_IRS1_Oryzias           EFWMQVDDSVVAQNMHETLLEAMKALSEEFRQRSKSQS-NSGPGGGATASNPISVPSRRH 293
65_IRS1_Oreochromis       EFWMQVDDSVVAQNMHETLLEAMKALSEEFRQRSKSQS-NSGPGGGATASNPISVPSRRH 271
65_IRS1_Takifugu          EFWMQVDDSVVAQNMHETLLEAMKALSEEFRQRSKSQS-NSGPGGGATASNPISVPSRRH 280
65_IRS1B_isoX2_Danio      EFWMQVDDSVVAQNMHETLLEAMKALSEEFRQRSKSQSTAAGAGGGTTASNPISVPSRRH 272
                          ***********************************:****  :*.***:**********

65_IRS1B_Xiphophorus      HPNPPPSQVGFLRRPRTEPTLGS-------------------------------------- 375
65_IRS1_Heterandria       HPNPPPSQVGFLRRPRTEPTLGANVGAPLGSSSASPTPRHSFPRSRTASDGGKSEDGIAG 353
65_IRS1_Oryzias           HPNPPPSQVGFLRRPRTEPPGGANCGSAVNSGSASPTPRPNFPRSRTASDGGKSEDGVAG 353
65_IRS1_Oreochromis       HPNPPPSQVGFTRRPRTEPPGGTNSGAPINCANASPTPRHSFPRSRTASDGGKGEDGITG 331
65_IRS1_Takifugu          HPNPPPSQVGFTRRPRTEPPGGANGSVGVSSASASPTPRHSFPRSRTASDGAKVEEGMIG 340
65_IRS1B_isoX2_Danio      HPNPPPSQVGFTRRPRTEPPGSTAG-----CNNTSPASRHSFPRTRTSSDGAKVDDGGCA 327
                          *********** *******. .:

65_IRS1B_Xiphophorus      ------------------------------------------------------------
65_IRS1_Heterandria       STPLHGVNTSPSTNGSCSTTPILRSKSARSVPTTAAKTSFGLMRSISTPAPSPSPAPSLS 413
65_IRS1_Oryzias           STPLQIGNSRASANGSCSTTPILRSKSARSAPNSGAKTPLGLMRSISTPAPSP--APSLS 411
65_IRS1_Oreochromis       ATPIHGVNSSPSTNGSCSTTPILRSKSARSAPTTTAKTPLALMRSISTPAPSP--APSLS 389
65_IRS1_Takifugu          TTPLQGPCSSPSSNGACSTTPVLRSKSARSAPTTTTKTPLGLMRSISTPAPSP--APSLS 398
65_IRS1B_isoX2_Danio      TTGEIMPCSSPTTNGSVSNTPILRSTSVRAS--TPVKAQHALMRSTSTPAPSA--APSMP 383


65_IRS1B_Xiphophorus      -------------------------GAYTRVASHHTSVSCSPSDYGSSDEYGSSPGDHTV 410
65_IRS1_Heterandria       SSSGHGSEFGGITS----VGPAPGSGAYTRVASHHTSVSCSPSDYGSSDEYGSSPGDHTV 469
65_IRS1_Oryzias           SSSGHGSEFGGVAP----TATVQGTSAYSRVLSHQTSVSGSPSDYGSSDEYGSSPGDHAH 467
65_IRS1_Oreochromis       SSSGHGSEFGGLTS----CAGAPGSGAYSRIPSHRASVSGSPSDYGSSDEYGSSPGDHTV 445
65_IRS1_Takifugu          SSSGHGSEFGGITS----SATSGPSGAYSRIPSHHTSISGSPSDYGSSDEYGSSPGDHTL 454
65_IRS1B_isoX2_Danio      SGSVHGSEFCATGTGTGNSGGGANGSMYSRLPLRQPSVSGSLSDYGSSDEYGSSPGEHSL 443
                                                . *:*:  ::.*:* * ***************:*:

65_IRS1B_Xiphophorus      LPSPSLPGSSISSTGSQCLGEDGANYILMGQRGGTSSSVSNSNQVNMMSSSQPVPGTPTN 470
65_IRS1_Heterandria       LPSPSLPGSSVSSTGSQCLGEDGANYILMGQRGGTSS-VSNSNQGNMMSNSQPVPGTPTN 528
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65_IRS1_Oryzias          LPSPTLTGDSAGSISSQSLGDDGTNYILMGHSGGDGN----NNQRNLMSSSQPPPGTLAF 523
65_IRS1_Oreochromis      LPSPSLPGSSVGSVSSQSLSEDGANYILMGQRGSSGG-----GNSNQGSGSQPTPGTPTS 500
65_IRS1_Takifugu         LPSPSLPGSSVGSMSSQSLGEEGANYILMGQRRNSIEG----GVSNHGAS--PAPGTPTY 508
65_IRS1B_isoX2_Danio     LT-PTMQAGFTASSGSNSLGDEASNYILMSQRGSSKS-------------------QTSQ 483
                         *. *:: ..  .* .*:.*.::.:*****.:  .                      :

65_IRS1B_Xiphophorus     GSLPQTRRVLRRSSSRECEAERRLLSKRASLPPMALERLAPHQRRAEEPTDEDSADYAIM 530
65_IRS1_Heterandria      GSLPQTRRVLRRSSSRECEAERRLLSKRASLPPMALERLAPHQRRAEEPTDEDSADYAIM 588
65_IRS1_Oryzias          GSQAVTRRVLRRSSSRECEAERRLLSKRASLPPMALERLAPRQRRAEQPGDEDSADYAIM 583
65_IRS1_Oreochromis      GSQ--TKRVLRRSSSRECEAERRLLSKRASLPPMALERLAPRQRRAEEPADDDSADYAIM 558
65_IRS1_Takifugu         GSHPQSRRMVRRSSSRESEAERRLLSKRASLPPMALERLAPRQRRAEEPADEDSADYAIM 568
65_IRS1B_isoX2_Danio     NALPQTRRVLRRSSSRECEAERRIMSKRASLPPMALERLAPLRRAGEEAVEED--DYAIM 541
                         .:   ::*::*******.*****::**************** :* .*:. ::*  *****

65_IRS1B_Xiphophorus     SRSTSRESFASTCSSVQRESAL--GTCVGGGGYLDVAGELKTDGGP------GAIAG--- 579
65_IRS1_Heterandria      SRSTSRESFASTCSSIQRESTL--GSCVGGGGYLDVAGELKTDGGP------GAVAG--- 637
65_IRS1_Oryzias          SRSTSRELFASSCSSTQGEQAG--GAGTGGGGYLEVAGELKSDGGA------GARSGADL 635
65_IRS1_Oreochromis      SRSTSRESFTSTCSSTQKESAMSAGSAGGGGGYLDVAGEFKAEGSV------GAGGSVDL 612
65_IRS1_Takifugu         SRNTSHESFTSTASSSQRECALSTGSGGAGGGYFDVAGELKADATG------VPGGGGDL 622
65_IRS1B_isoX2_Danio     NQTSSRESFVARQDS---------GPSTSGTGYLEVAAEIKGDGGSSDTGITAVIGGAKG 592
                         .:.:*:* *.:  .*         *.  .* **::**.*:* :.           ..

65_IRS1B_Xiphophorus     -VDNGYMSMLPGVTQPPVSSCQTMTVCIPESDSKPADDYMAMTPNNSVSPPQQIRPLPVS 638
65_IRS1_Heterandria      -VDNGYMSMLPGVTQPPVSSCQTMTVCVPESDSKSADDYMAMTPNNSVSPPQQIRPLPVS 696
65_IRS1_Oryzias          GIDNGYMSMLPGVTHPPVSLSQSLAVSFPDTDFKPTDDYMAMTPNNSVSPPQQIHPPPAS 695
65_IRS1_Oreochromis      GVDNGYMSMLPGVTQPPTSLSQSLAVSVPDSESRPADDYMAMTPNNSVSPPQQIRPPPTS 672
65_IRS1_Takifugu         GVDNGYMSMLPGVTQSPVSLAQSLSVSVPDSDSKPADDYMAMTPNNSVSPPQQIRPPLTS 682
65_IRS1B_isoX2_Danio     AVDNGYMSMLPGVTAPPVSLSHSLSVTVSDSDSKSADDYMAMTPNNSISPPRQIRPPSGT 652
                          :************ .*.* .::::* ..::: :.:***********:***:**:*   :

65_IRS1B_Xiphophorus     DGYMIMSPNSSCSPDQRGSLSEGAWVG--SADSRAGSDYMNMSPISAPSVNGSPPQNEHT 696
65_IRS1_Heterandria      DGYMIMSPNSSCSPDQRGSLSEGAWVG--SADSRAGSDYMNMSPISAPSVNGSPPQNEHS 754
65_IRS1_Oryzias          DGYMIMSPNSSCSPDQRGGLSGGTWQGSGSADSRAGSDYMNMSPISTRSVNGSSPPPDNT 755
65_IRS1_Oreochromis      DGYMIMSPNSSCSPDQRGGLSGGTWVGSDSADSRPGSDYMNMSPISARSVNGSPPPPEHS 732
65_IRS1_Takifugu         DGYMIMSPNSSCSPDQRASLSGGAWVGSGSADSRAGSDYMNMSPISARSVNGSPPPPEHT 742
65_IRS1B_isoX2_Danio     DGYMMMSPNSSCSPDQRG--VPTAWIGSSSADSRTGSDYMNMSPISARSANSTPPPPEPH 710
                         ****:************.     :* *  *****.***********: *.*.:.*  :

65_IRS1B_Xiphophorus     CHLDTSLQQPPPKMVYSYYSLPR------------------------------------ 719
65_IRS1_Heterandria      CHLDTSLQQPPPKMVYSYYSLPRSYKHNPTSGLFDDGPGRGRRPNGNCSRGGGGSRNSGG 814
65_IRS1_Oryzias          SHLEASLQQAP-KMVYSYYSLPRSYKHNPSSVHFDSGPGRGRKSNGSCSKGTGDGRTNGR 814
65_IRS1_Oreochromis      SHLETSPQQHGPKMVYSYYSLPRSYKHSPSTGHFDDGPGRGRRPNGSSRRGMGGGKPIRG 792
65_IRS1_Takifugu         SHSETHSQQQAPKMVYSYYSLPRSYKHNPTTGHFDDGPGRGRRHNGSFSRGMGGGRSMSV 802
65_IRS1B_isoX2_Danio     TPSDQHSNQPQPKMVYSYYSLPRSYKHN-TSTHFEEGPGRGKHLVNG-GKGIGGGRGLCN 768
                            :   :*   ***********

65_IRS1B_Xiphophorus     -------------------------SSASSESLGENDDRTNQALGNVTTG-SQPK----- 748
65_IRS1_Heterandria      HLEQ-PTGSSGTGRHQSLSSSSYSSSSASSESLGENDDRTNQALVNVTSG-SKPKEVSKI 872
65_IRS1_Oryzias          CQEQPAASGSGVGRHLSLSSSSYSSSSASSESLGETDDRTSQALSNLLSG-SQLKDRSKL 873
65_IRS1_Oreochromis      LQEQAAVGNAVVGRHLSLSSSSYSSSSASSESLGESDERTTQALSNATGG-TQSKDGSKQ 851
65_IRS1_Takifugu         HQEQQVTVTSMVGRHLSFSSSSYSSSSASSESLGENE---AGGGGTVPGG-TPSKDASKP 858
65_IRS1B_isoX2_Danio     SKSK--HQEPLVGRHLSLSSSSYSSSSASSESLGEGEEKAIKGTGGAAGVTAKDIERGSL 826
                                               ********** :     .       :

65_IRS1B_Xiphophorus     ---------------------------EANTLPRVRENPLPPEPKSPGEYVSIEFKGEQ 780
65_IRS1_Heterandria      AQRRGSGGLTKQGNHFRSRPVSLFVDVSKANTLPRVRENPLPPEPKSPGEYVSIEFKGEQ 932
65_IRS1_Oryzias          TEKRGCGGLSKQGSHARSRPVSLFVDVSKANTLPRVRENPLPPEPKSPGEYVSIEFKGEK 933
65_IRS1_Oreochromis      QQRRGSGGLSKQGSYTRSRPVSLFVDVSKANTLPRVRENPLPPEPKSPGEYVSIEFKGEK 911
65_IRS1_Takifugu         QQRRGSGGLSKQSNHSKTRPVSLFVDVSKANTLPRVRENPLPPEPKSPGEYVSIEFKGEK 918
65_IRS1B_isoX2_Danio     QQRQGSGRAKQGHSGHRGRPLSLFVDVNKANTLPRVRETPLLPEPKSPGEYVSIEFKGER 886
                                                 :*********.** *****************:

65_IRS1B_Xiphophorus     CSQTGVGVGRG------------------------------------------------- 791
65_IRS1_Heterandria      CSRTGVGIGRGRGLRHGSLLNHGSSHQHTQNRPTPSLETFIPLSHSPSAPVSPPTPSEYV 992
65_IRS1_Oryzias          CNYPGGTAGRGRGLRYGSSSPHGSSSQHSRSRPTSSVGNINPDSRSPTTPISPPTASEYV 993
65_IRS1_Oreochromis      GNPTGIGLGRGRGLRHGLSLPHGSSSQQPQNKPSSSFGNFIPLSRSPSAPITPPAASEYV 971
65_IRS1_Takifugu         CSQAGVGGGCSRGLRHGLSFPHSSGSKHSQHRPTSCLGNLMPLSRSPSAPITPPAVSEYV 978
65_IRS1B_isoX2_Danio     NQKAEGNGTRG--FRQDASLP--PSSHRHLPRPTSCVAGFLQFSQSSSTPIPPSTASEYV 942
                          . .      .

65_IRS1B_Xiphophorus     -------------------------------------------TDSLCQGTVEVAEPPLRK 809
65_IRS1_Heterandria      NMDPGPSPSPSPHSNTQLVFPPFHTPPTPPVLAHAPKTCMEGTDSLCQGTVEVAEPPLRK 1052
65_IRS1_Oryzias          NMDLGPSPSPSPLSATPLVFPSFHTPPTPPTLVHVPKICNEGAINPRDKGAQLAEAPLRK 1053
65_IRS1_Oreochromis      NMDLGPSPSPSPLSLTPLVFPSFHTPPTPPTLAHAPKPCNEDNTRPHEDEVEVAEAPLRK 1031
65_IRS1_Takifugu         NMDLGPSPSPSPVSHNPLVFPAFHTPPTPPTLAHAPKAHEEATSGLGEEGAPMAEPPLRK 1038
65_IRS1B_isoX2_Danio     NMELGVSLSPSPISLTPLGFPSFPTPPVTP--AAAPKAHDEHRSTVLNDIGAEREMAQRK 1000
                                                          :     * . **
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65_IRS1B_Xiphophorus      SRDSVPSVIETESPTSCGDYTEMAFSLSNNTAPRSSPSVSPKVPSPTRTDPSVSVLSRGL 869
65_IRS1_Heterandria       SRDNVPSVTESESPTSCGDYTEMAFSLSNNTAARSSPSVSPKVPSPTRTDPSVSVLSRGL 1112
65_IRS1_Oryzias           SRVGVLSATDSESPTSCADYTEMAFSLNNNTVPLSSSSVSPKVTSPTLIDSSVSVLSRGL 1113
65_IRS1_Oreochromis       SRESVPSVTKSESPTSCGDYTEMAFSLNNNPVPRSSSSVSPKGPSPTRDDT--SVLPRGL 1089
65_IRS1_Takifugu          IKENVPSVTESESPPSSGDYTEMAFSMNTDPVPRSSSSVSPKAPSPTRTDPSAPTLSRGL 1098
65_IRS1B_isoX2_Danio      SRQ--GSVPQSGDSPTCGDYTKMAFSLNSGSTLSSTSPKSPLLDQPESVVP-ALGLGLGL 1057
                           :    *. .: ...:..***:****:.....  *:.. **   .*    .    *  **


65_IRS1B_Xiphophorus      DFPLSKSGLNPDQGAKVIRADPQGRRRHCSETFVATPSLAQPG----------------- 912
65_IRS1_Heterandria       DFSLGKSGLNPDQGAKVIRADPQGRRRHCSETFVATPSLSTSASTSSSTASLFPEHTQAV 1172
65_IRS1_Oryzias           DFSLGKSGTNPDQGAKVIRADPQGRRRHCSETFVASPSLPSTS-TSSSTASLFPEHAQAV 1172
65_IRS1_Oreochromis       DFPLSKTGPNPDQGAKVIRADPQGRRRHCSETFLASTSLSTSTSTSSSTASLFPEH-QSV 1148
65_IRS1_Takifugu          DFPLPKSGPNPDHGAKVIRADPQGRRRHCSETFLAAPSSSASSTTSSSTVSLFPEHTQPV 1158
65_IRS1B_isoX2_Danio      DFPLAKVP-NPDHGAKVIRADPQGRRRHCSETFQAPSSLLACPSTSSS--SAFPDHTQVI 1114
                          **.* *   ***:******************** *..*

65_IRS1B_Xiphophorus      ----------------------CFPPPRL--------------------------------- 919
65_IRS1_Heterandria       SRRLGFESMLWGNGAVTDPPTQFPLPAQQSLPTNNQTLSTEQGLNYIDLDLVNKETPYAG 1232
65_IRS1_Oryzias           ARRLGFESMLWGNGAMTDAPMPCPLPGQQSLPANNPATPPERGLNYIDLDLVNKESPHTD 1232
65_IRS1_Oreochromis       ARRLGFESMLWGNGVVTDSPTQFPLPGQQSLPTNAQTSSAEQGLNYIDLDLANKESPHVG 1208
65_IRS1_Takifugu          SRRLGFESMLWGNGAVTDNPPQFTLPGQQPSPANTQTASTEQGLNYIDLDLASKECPHLD 1218
65_IRS1B_isoX2_Danio      SRRLGLDGMLWGNSSSSDVSSQYINPGLSSLSVS-QTSSMEQGLNYIDLDLANKESSHAS 1173
                                                *

65_IRS1B_Xiphophorus      ------------------------------------------------------------
65_IRS1_Heterandria       LEGATGGQ-PQSRLFS-VLGSGSVVGGTGGSAGGSSSSS---LNTYAS---------IDF 1278
65_IRS1_Oryzias           LDGASGSQ-PSSRLFS-VVGGGSVVGGVGSAVGGNNNSS---LNTTRMNTLQRNTTRIDG 1287
65_IRS1_Oreochromis       LDGPSGSQ-APSRLFS-VLGSGSGVGAAGASVGSSGSSS---LNTYAS---------IDF 1254
65_IRS1_Takifugu          LDGSAGLQ-AASRLFS-VLGGGPVVGGLSGAVGSSSSSSSSSLNTYAS---------IDF 1267
65_IRS1B_isoX2_Danio      TDGQAAVHTPASRIFSSVLGVGGATGGLVGTGGSGSNASN--LNMYAS---------IDF 1222


65_IRS1B_Xiphophorus      ------------------------------------------------------------
65_IRS1_Heterandria       YKSEELRTHQNGNKEGT---------------------------------------- 1295
65_IRS1_Oryzias           KQQKQILRGCNGCVIANGEKKEHTLTAACPVIVP----SICSQMEKRCAEMNEF------ 1337
65_IRS1_Oreochromis       YKSEELRTHQNGSKEGTEC------------------------------------- 1273
65_IRS1_Takifugu          YKSEELRTHQNGNKEGKVKHEVNDSTAAQTSAAEGLRGSTRTLLEGSYGEQFEFVREGEI 1327
65_IRS1B_isoX2_Danio      YKSEELRTHQGSSSNSRKDGTAL---------------------------------- 1245


65_IRS1B_Xiphophorus      ------
65_IRS1_Heterandria       ------
65_IRS1_Oryzias           ------
65_IRS1_Oreochromis       ------
65_IRS1_Takifugu          YLEKGE 1333
65_IRS1B_isoX2_Danio      ------
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Figure 1. Alignments of several IRS proteins from *Xiphophorus maculatus*, *Oryzias latipes, Oreochromis niloticus, Takifugu rubripes, Danio rerio and Heterandria formosa.* In red are the non-synonymous changes from exon 2 of nucleotide positions 15886 (in VGAPL into VGSPL), 15671 (KTSFG into KTPFG), 15534 (GSGAY into GSSAY) and position14188 (TPYAG into TPHAG) as indicated in red.

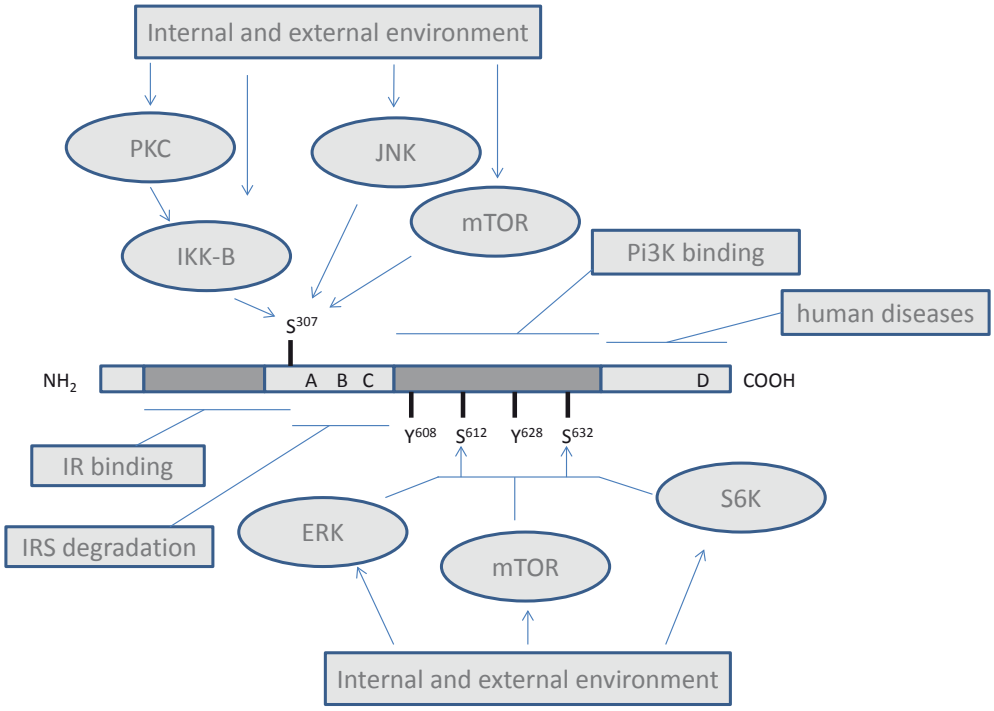
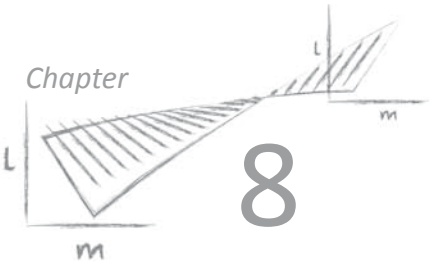


Figure 2. Schematic overview of the IRS gene (redrawn from (Boura-Halfon *et al.* 2010)). Letters A, B, C and D represent the nucleotide change positions found (15886, 15671, 15534, 14188 respectively) as described above. At several sites there are posttranslational modifiable places, affected by genes PKC, IKK-B, mTOR, JNK, ERK and S6K. The protein consists of an insulin receptor binding domain (IR binding) and then a part which determines the degradation (IRS degradation). One phosphorylation site partly determines the degree to which the protein is degraded and how much it associates with the insulin receptor. The three non-synonymous SNPs found are within the domain that determines how fast the protein is degraded (Boura-Halfon *et al.* 2010). After these regions there is a domain that binds to the Pi3k (Pi3K binding), which again can be heavily modified by other insulin signaling pathway members. In the last domain again we have found a non-synonymous SNP, but no information is present on the possible effects of this SNP. Nevertheless, SNPs associated with diseases in human populations have been found in this region (human diseases



Discussion

Joost van den Heuvel

"To make a theory of some phenomenon followed by a clever calculation and then finally to have the result confirmed by an experiment provides a tremendous source of satisfaction. In some instances the experiment takes place before the calculation, in which case it's not predicting but, rather, explaining a result, and it's almost as good."

Leonard Susskind

Synopsis of the thesis and outline of discussion

Life history traits evolve under variation in nutrition. The resource acquisition and allocation framework is useful at predicting and explaining variation in life history strategies. When variation in nutrition is experienced within or between generations in a predictable manner, plasticity to these conditions can evolve. Such plastic responses have received a lot of attention as ways to increase health and prolong lifespan. Specific models can be constructed to test ideas on how these plastic responses have evolved. This again leads to certain predictions, but also offers explanations for responses measured in the laboratory. Three of these models have been described in this thesis.

To study the effect of variation in nutrition in the laboratory, organisms need to be reared on different types of nutrition. Although in the field organisms might experience continuous variation, because of spatial or temporal changes in nutrition, the effect of continuous variations in the laboratory is not often studied. I reported on such a study in the second part of this thesis. The variation in traits was also linked to gene expression differences, and individual variation was also modeled.

Genetic interventions, next to dietary interventions, are very powerful tools to study how variation in life histories evolved. Both type of studies have shown that a few signaling pathways are very important. Studies relating genetic variation with phenotypic variation often suffer from lack of power (when too many variable sites are sequenced) or a narrow view (where only a couple of genes are studied). Furthermore, variation between individuals within populations is studied, and therefore, genetic variation is relatively low. A combination of a candidate gene approach with many genes involved in life history variation and ageing was studied in a livebearing fish, on which I reported in the last part of this thesis. In the rest of this discussion I will summarize and form a synthesis of the presented chapter, after which I will discuss future directions for ageing focused life history research.

Synthesis of multiple types of studies involving several different organisms and various experimental and analytical tools

In **chapter 2** we verified whether the observed increase in flight stress response of adult *Bicyclus* butterflies that were stressed as larvae (Saastamoinen *et al.* 2010) would evolve in the environment as explored in the theoretical model. This increased resistance came about by an increased pupal allocation to thorax tissue, at the cost of storage of (nutritional) reserves and of the potential number of eggs. This change in allocation was not present in adults that developed from larvae from optimal conditions, which were also not flight stress resistant.

Theoretical models applied to specific life courses have shown that state-dependent models can be used to test how organisms would respond if present in the evolutionary natural situation, for instance as done for sticklebacks (Lee *et al.* 2011) and butterflies (Kivelä *et al.* 2013). Firstly, the ability to integrate prediction from theory with data from experiments critically depends on measuring the appropriate type of traits. In the case of the predictive adaptive response (PAR) in butterflies this was allocation to flight, which in the laboratory was measured by the relative thorax size (Saastamoinen *et al.* 2010). In the case of the sticklebacks this was age specific growth rate of the fish (Lee *et al.* 2011). Secondly, one needs to manipulate the organism in a way that represents challenges in the field. Sticklebacks are restricted in the time they can spend growing before the unset of spawning, and therefore the increase in growth rate at the

cost of somatic maintenance (Lee *et al.* 2011) upon photoperiod variation was measured. In general it is beneficial to be able to quantify the state-dependent changes organisms go through and to have knowledge of the ecological background, to make relevant evolutionary models. The disposable soma theory of ageing is also a good basis for such models (Kirkwood 1977; Kirkwood and Holliday 1979) and can be used within the ecological evolutionary framework (Calow and Townsend 1981; Calow 1987; Sibly and Calow 1987) to better understand the specific outcomes of experiments. A valuable general extension of these models is to predict the parameter space within which the responses evolve or change, to be able to make predictions about other species or situations. Furthermore, modelling whole life histories, rather than a single stage or trait can be time consuming, but these models seem to represent the decisions organisms make better than more simplified versions (see especially the different versions of the stickleback model (Lee *et al.* 2011)).

Dietary restriction increases lifespan in many species (McCay *et al.* 1935; Klass 1977; Muller *et al.* 1980; Weindruch *et al.* 1986; Chippindale *et al.* 1993) but not in all organisms (Nakagawa *et al.* 2012). In the model presented in **chapter 3**, the relationship between variation in success of reproduction and the resource that can be allocated to maintenance was very important. In organisms for which resource can dramatically alter within a reproductive bout and against which no buffering can be applied, dietary restriction is unlikely to be an adaptive response. This is the case for instance when a mouse-like animal has fertilized a number eggs and would need resources to develop them all. Were resource level to suddenly drop, the development of the embryos would suffer. Therefore, we would expect the increased lifespan to evolve for a mouse-like organism in an environment in which nutrition varies mostly seasonally, and to a lesser degree spatially. For organisms such as short-lived fruit flies it would be impossible to respond to seasonal variation in nutrition, since they would not live long enough to extend lifespan upon DR in such a way as to benefit from it. Rather, we expect flies to respond very quickly to variation in nutrition. In relation to the PAR hypothesis, this DR modelling approach sheds light on the applicability of PAR to organisms such as mice and flies, and thus has ramifications for the use of the PAR hypothesis to explain variation in human life histories.

Indeed as predicted from my DR model, in **chapter 4** I show that flies respond very quickly to variation in nutrition, and are able to do this throughout their entire lifespan. On the other hand, early life experience also affects the plasticity and average of life history traits throughout life. The simulations performed in **chapter 3** were not suited to verify whether a continued effect of early life would be present. In general early life effects on life histories are widely present (Metcalfe and Monaghan 2001). In the model from **chapter 3**, information on the current patch (in the spatial version where flies evolved lifespan extension upon DR) did not have any predictive value for the patch in the next time step. It is plausible that information gained from the environment as a larva is not up to date when the fly hatches. Therefore, it might need some setting points for life course fixation during the first early days of adult life. This means that in contrast to the expectations of the model, fruit flies can at least use information to make some predictions over the short period the fly lives. Thus, the short term changes in the model of **chapter 3** did not fully capture the changes experienced in the life of a fly, and that patch variability is much more temporarily correlated than modeled. This feature could be added to the model so that instead of temporal and spatial variation we would model increased or decreased autocorrelation.

In **chapter 5** we have seen that not only the phenotypes change very fast when flies are

transferred from high to low, or low to high food, but the transcriptome does as well. We have not been able to identify what the regulators of these rapid changes were, because fruit flies alter thousands of genes within a couple of days. This variation represents similar patterns in gene expression and life history trait differences between cohorts constantly kept on high or low food, which allows for the model ('yoyo model') be used to improve our understanding of the mechanisms that regulate their life histories. To understand how these large trascriptome differences come about, flies need to be sampled at times when these changes occur. When flies on constant food are sampled somewhere during their life course, these changes have already occurred. Therefore, flies should be sampled in time series just after changes in food till all the transcrptome variation is fixed. Within such time series, it should be possible to measure which changes cause other differences. Furthermore, these type of studies will benefit from measurements on multiple levels such as the combination of steroid hormones, post translational status of important pathways and the effect of single transcription factors such as Foxo on many genes (Alic *et al.* 2011).

In our study, the type of genes and functions directly regulated by Foxo were differently expressed, as well as the expression of Foxo itself (data not shown). However, the actual causality between these correlations can only be proven by using measurements of transcription factor binding on the DNA of these target genes (Alic *et al.* 2011). In this chapter we showed that the number of eggs produced by flies related closely to the variation in expression of a large number of genes. Many studies perform whole genome expression arrays of flies for which phenotypic data are not present. Since in our study flies on high food counterintuitively produced a lower number of eggs, it was very important to have properly measured multiple phenotypes, instead of just survival.

In **chapter 6** we used the individual based data from the constant nutrition treatments of the fly experiment to test whether they would fit a model-derived prediction. A general prediction from both ecological evolutionary physiology (Calow and Townsend 1981; Calow 1987; Sibly and Calow 1987) and quantitative genetics models (Van Noordwijk and De Jong 1986; De Jong and Van Noordwijk 1992) is that positive relationships between traits can be caused by variation in acquisition and that trade-offs are due to variation in allocation. In **chapter 6** we studied the age-dependent correlation between survival and egg production and concluded that there is age specificity in the relationship between these traits. As age within a cohort progresses, the relationship becomes more positive. Modelling state-dependent ingestion rate based on data from flies (Wong *et al.* 2009) led to these predictions. If a fly allocates less to maintenance early in life, it will produce more eggs, but will also age faster. This increased ageing rate will lead to a faster decrease in feeding rate. Variation in early allocation therefore leads to variation in late acquisition rate. A similar pattern between early fecundity and survival, and a change in age-specific egg production has been found in other studies in flies (Curtsinger 2013). The explanation given for the change in relationship between fecundity and survival in the latter study is the fact that very late in life only the individuals are left that live a long time and therefore reproduce longer. In other words, reproductive lifespan is related to total fecundity and lifespan, and therefore late in the cohort, relationships become more positive (Curtsinger 2013). This explanation is dependent on the fact that individuals die, and are therefore not represented anymore at later time points. In our study, and in the other studies cited above, the rate of fecundity also changes earlier, thus well before flies start to die. Typically, age-specific reproduction between high and low reproducing

flies cross at the age of 30 days (Curtsinger 2013; and see **chapter 6**), and our model therefore explains how the relationship changes throughout the whole life course, and thus also why the fecundities of short- and long-lived individuals differ. In contrast to the conclusion of (Curtsinger 2013), I conclude that allocation (spiked with acquisition) theory is needed to understand the whole life course of individual flies.

In **chapter 7** we sequenced candidate genes for life history regulation in natural populations of a small livebearing fish, *Heterandria formosa.* The populations vary on a continuous scale in fecundity, standard length, offspring size, and superfetation (number of broods are carried simultaneously). Candidate genes more often showed higher levels of association with the traits fecundity and standard length. Moreover, a high number of significant loci were found in one scaffold, representing an IRS gene. This association was found over the whole scaffold, and especially over a stretch of 7000 nucleotides. Currently, it is difficult to assign the non-synonymous SNPs within this gene to specific effects on the insulin pathway. Roughly, exon 2 consists of three parts in the protein (see Fig. 10, **chapter 7**). When the protein was compared to a rat protein model, most non-synonymous SNPs were found in parts that do not associate with other proteins in the signaling pathway (insulin receptor or post translational modifiable sites that associate with Pi3k, Gual *et al.* 2005; see Zoncu *et al.* 2011 for pathway details). Studies have shown that the non-synonymous SNPs that we found were in a region which is associated with changes in protein degradation (Boura-Halfon *et al.* 2010) and with health traits in human populations (Bacci *et al.* 2013; De Cosmo *et al.* 2013; Lim *et al.* 2013; Vats *et al.* 2013; Alharbi *et al.* 2014). A problem with the protein sequences is, however, that the conservation of the protein between fishes and mammals is low, and therefore, the experiments and the models made of the proteins in mammals are difficult to interpret for a fish, or at least needs to be interpreted with caution. Therefore, it would be better to perform similar mechanistic studies based on *Heterandria* proteins, for instance using manipulation in *Heterandria* cell lines. Before that, and probably a better option, would be to verify these SNPs and their phenotypic associations in additional populations within the river delta, but also in a separate group of populations distant to the studied sites. This would mean estimating the trait variation in these populations.

In general, there is a lack in understanding about how the differences between species, and their variation in the evolutionary conserved pathways, leads to variation in how the pathways work. Although this information is absent, as in the DR discussion, the literature is over-confident in revealing similarities, while the differences are much more obvious. Nevertheless, relying on information of other species, the non-synonymous changes found here in regions have an impact either on the functioning of the protein in other systems, or variation in allele frequencies associate with health traits.

Have we met our aims?

Has this thesis given new insights in the study of life histories and ageing? Have the various studies been integrated effectively? In general the thesis has pointed at how evolutionary theory can help to guide through various types of experiments that are published in the literature. In two chapters experimental data were directly compared to theoretical models. Experiments can be done, and verbal explanations can be used to point to possible evolutionarily plausible impacts. With models we can quantify whether suggested evolutionary backgrounds can explain the specific patterns, and whether, were we to extrapolate them, could we have used these models

to discover when or where we would find these evolved pattern. This work has the potential to strengthen or weaken our verbal explanations. Clearly the models point at where we must look. For instance, in the *Bicyclus* system, one needs to compare more plastic with less plastic species and see how the results match the environmental variation.

We concluded that the variation between individuals found in the *Drosophila* experiment was due to variation in acquisition of resources late in life after variation in allocation early in life. Therefore, the next step is to quantify how much resource flies must have acquired later, to have longer lifespans and a higher egg production. The problem is that the early time differences are small. But, in general, the factors that make organisms unhealthy late in life are initiated when they are still happily flying, swimming or walking around. Therefore, understanding how very small differences early in life play out to become larger differences late in life is going to be a difficult but nevertheless, important and interesting task. Within a heterogeneous cohort of flies this could be measured as egg production. One future aim is to mechanistically quantify what underlies the smaller differences early in life and how these smaller differences become larger ones later in life.

Comparisons in biology always reveal similarities and differences. The question is do we learn more from differences or more from similarities? We know that evolution produces the differences and similarities. Therefore, how evolution works can teach us how we should interpret the differences and similarities in one unitary framework. We can call this framework anything we like, eco-evo-devo or eco-evo-phrame, but we need to decide what is in it and what is not and how the separate parts relate to one another. Let's begin with what we do know.

The DR model showed us two important things. The DR responses of organisms tend to be different, but if they are similar, and if organisms respond in a similar way, they do so, because in their evolutionary background DR has different meanings to the various organisms. Restriction in diet for a fly means bad patch, let's move on. For a mouse it means it might progress into a time period of worsening conditions and it needs to prepare for winter. Flies also survive the winter, but do not do so during most dietary restriction experiments in the laboratory. Flies can be made to hibernate, but this involves using photoperiod and cold temperatures, not food. Therefore, the input signals are different, but the output can be similar. But for all these organisms, specific cues lead to specific evolutionary important outcomes, which are very different evolutionarily, but can seem similar on the mechanistic or phenotypic level.

We have also found that genes of the insulin signaling pathway seems to associate most closely to traits in the field in yet another species, *Heterandria formosa*. But the specific variable sites are different compared to other species and the part of the gene IRS of *Heterandria* where these sites were found, are not evolutionary conserved between most fishes and mammals. Furthermore, even the relatively highly conserved regions in the protein are not conversed, and the mechanistic relationships between IRS and other genes in the pathway are likely to be different (such as specific post translational modifiable places). Interestingly, the many dimensions the DR response represents to various organisms can be mirrored by the many dimensions the insulin pathway can take (Zoncu *et al.* 2011, see also Smith and Shanley 2010; Dalle Pezze *et al.* 2012). Genetic variation, but also the number of copies of genes - such as one insulin and two insulin like growth factors in vertebrates (but also relaxins) to seven *Drosophila* insulin-like peptides and 40 or more in *C. elegans*; as ligands for three receptors in mammals (five receptors in fishes, depending on the fish) and one receptor in flies and worms - plays a role. The insulin pathway interacts with other pathways (MAPK, TOR), but basically they are so intertwined that separating

them does not appear to make much sense.

A quantitative model needs to be produced in which we have on the top level an ecologically informed evolutionary resource allocation model. For instance, *Drosophila melanogaster* is active in the summer, finding patches of fruit on the ground where they will feed, mate and lay eggs. The combination of photoperiod, temperature, resource intake, presence or absence of eggs in the fruit, the size of the fruit and the condition of the fruit will lead to a decision of the female to lay one or more eggs. An evolutionary model could predict, depending on the state of the environment how often a female with a specific internal state would lay how many eggs in this fruit. In general these models can predict to which functions resource needs to be allocated depending on the state of the organisms and of the environment. A seasonal model (such as the *Bicyclus* model) would also predict that a fly during this season (summer, autumn) should invest in reproduction, but how many eggs depends on the state of the fruit. When it becomes colder, and the fruit fly dry season begins (dry in terms of number of eggs), flies would not consider laying eggs as they prepare to hibernate. Both state changes, the difference between dry and wet season, winter and summer, and whether to reproduce or not, depending host plant or fruit availability, are partly regulated via the insulin signaling pathway (Mair and Dillin 2008; Flatt *et al.* 2013). As genetic variation within a species affects how environmental variation leads to phenotypic variation (such as in the *Heterandria* case) clearly the fruit- and butterflies make their decisions (optimal life history strategies) differently. While different species might cope with the environment in various ways, to some extent the mechanisms might be similar (Flatt *et al.* 2013). Cases where decisions are slightly different in these pathways are important to understand differences between populations and between species. Variation within populations (**chapter 6**) but also genetic variants within species (**chapter 7**) or closely related species are interesting to study to ultimately understand how life histories and ageing evolved and are regulated. But the selection that leads to the evolution of these alternative states can be modelled using evolutionary models. Both types of studies therefore inform each other.

The *Drosophila* literature also contains ecological interesting features. For instance, there are marked differences in life histories along a cline. Within these clines 'summer phenotypes' such as the response to DR, but also the seasonal differences such as diapause-incidence can be studied. Because both are regulated via similar pathways, their interaction and whether they are involved in resolving possible genetic constraints are interesting future directions. Furthermore, the variation of life histories along a cline can be studied with comparison to differences between continents, but also the lack of diapause in African populations is interesting (Schmidt *et al.* 2005; Schmidt *et al.* 2008; Schmidt 2011), especially since both European and American populations have evolved this trait. Obviously there is a lot to learn.

Drosophila, because it is a model organism, is also a very appropriate for which to study the mechanisms of gene expression, post translational modifications, possible epigenetic and gene expression differences between tissues and variation in these traits between closely related species, which all have completely sequenced genomes. Understanding how these populations and species differ will be improved by the mechanistic models of specific pathways, leading to changes in the activity of transcription factors. Needless to say, the genetic variation in these pathways, but also the transcription factors and promotor regions of the target genes, the target genes themselves, and their expression differences will inform us about how genetic variation is translated into phenotypic variation. None of these techniques or measurements alone will

bring us to a better understanding. The large numbers of genes differently expressed in the variable flies were summarized as GO terms. Although this conveniently summarizes the response, this does not fully capture the biology, since sometimes biologically different genes are put in one category, while sometimes genes coding for proteins of similar processes (but differently regulated genes) are included in different GO terms. Nevertheless, it can be seen that at the level of resource allocation, where for instance growth or reproduction is increased on high nutrition, on the level of GO terms genes can represent these targets of allocation. Decisions to allocate resource upon variation in food can be represented mechanistically by measurements on, and the modelling of, the nutrient sensing pathway. The latter ultimately leads to activities of transcription factors that regulate large sets of genes, representing functions (growth, survival, reproduction) as summarized by GO terms. Therefore, in our unitary life history model, containing ecology, evolution and mechanisms, these features are the parts which are integrated as such (see Fig. 1).

In addition, the large clinal differences and variation between continents in *Drosophila* populations and the ecological differences with respect to other biotic players in the ecosystem are not very well understood. Species such as *Bicyclus anynana* and *Heterandria formosa* are better suited for making progress on the ecological evolutionary part of the biology of life histories. In general, life history theory does not include density dependence very well (Bassar *et al.* 2013). The livebearing fish are a family of organisms that might be well suited to use as an experimental group for studying more ecological density dependent life history biology. Another reason for choosing to perform this work on fish, is because modelling approaches integrating several trophic levels and their interactions have been performed on fish (mostly for fishery type of ecologies) and have been tested on *Heterandria formosa* (Van Leeuwen *et al.* 2008; Schröder *et al.* 2009). This will also increase our understanding how organisms with very divergent life histories can be compared and can differ, and will ultimately improve our understanding of the evolution and plasticity in life histories.

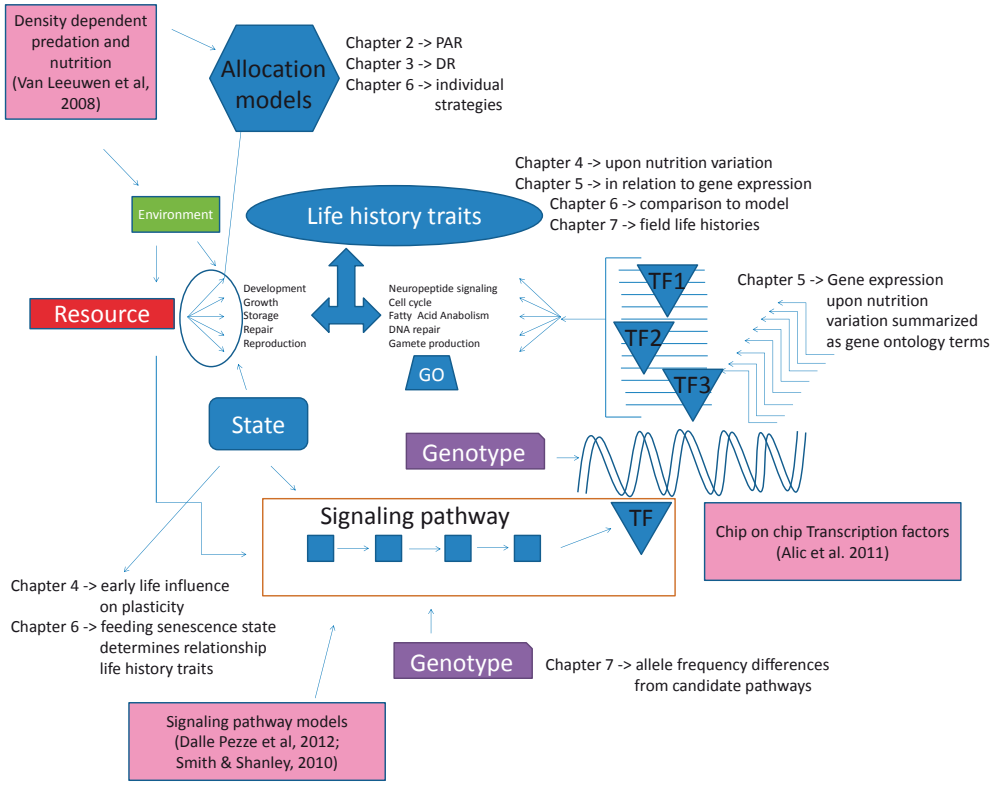


Figure 1. Overview of the unitary framework for ecologically informed life history research, with a basis in mechanisms of life histories. At some places, points are indicated where this thesis studies some aspects. In pink, studies from literature are indicated that show what kind of studies could be added to integrate the whole set of studies even more. Counterclockwise from left above, allocation models indicate to what functions resource needs to be allocated to maximize fitness, dependent on the environment (extrinsic mortality, nutrition, temperature, and variation in these (spatial and/or temporal). In chapters 2, 3 and 6 we constructed such models. (Van Leeuwen *et al.* 2008) show how densities of higher and lower trophic levels can affect life histories, which affect possibly optimal decisions, but this density dependence, which might be important for seasonal variation, is often not included in life history models. Allocation to these functions is expressed finally as phenotypes, i.e. life history traits. The life history traits that depends on plasticity in response to variation in nutrition was studied in chapters 4, 5 and 6 with *Drosophila melanogaster,* but also for field phenotypes for *Heterandria formosa.* The allocation decisions do not only depend on the environment, but also the internal state of the organism (such as age, fatness, size, as studied in chapters specifically in chapter 4 and 6 experimentally). In reality, the internal and external state, and how resource is allocated to certain functions is determined by signal transduction pathways such as the insulin, Tor, MAPK and RAS / RAF pathway). Genetic variation in these pathways might be associated with life history traits (chapter 7). The output of such a pathway is typically differential expression of many genes, upon an external and/or internal change in state and can be mechanistically modeled (Smith and Shanley 2010; Dalle Pezze *et al.* 2012). The expression of target genes is realized through the activity of transcription factors. One transcription factor can regulate many genes. Nevertheless, differential expression of thousands of genes is regulated by cascades of transcription factors, and specific transcription factors regulate a set of genes which represent certain functions (Alic *et al.* 2011). Interestingly the whole sets of genes differently expressed are often summarized in biological functions, such as fatty acid anabolism, DNA repair and gamete production, which again can be related via biological functions of the resource allocation model to life history traits. Such an association was performed in chapter 5. One would be able to study all these parts and integrate them by using the type of studies as performed in this thesis, added by the studies in pink, preferably within one system or species.

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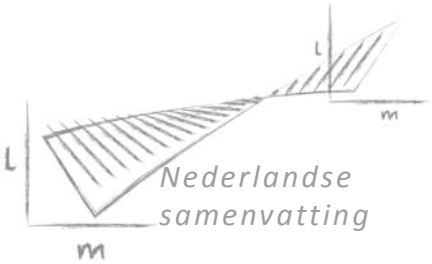

Nederlandse samenvatting

if (phenotypic variation==TRUE){
if (genetic – phenotypic covariation ==TRUE){
if (phenotypic – fitness covariation == TRUE)
[look for descent with modification by natural selection]}
else[look for evolution by neutral processes]}
else[look for phenotypic plasticity]}
else[boredom, look somewhere else]

Inleiding

De beschrijving van de timing en hoeveelheid van alle eigenschappen die het reproductief succes van een organisme beïnvloeden noemen we de levensgeschiedenis van een organisme. De belangrijkste levensgeschiedeniseigenschappen zijn grootte (van het ei, of lichaam bij maturatie), ontwikkelingstijd, fecunditeit (aantal nakomelingen) en levensduur. Een belangrijk concept in de theorieën die levensgeschiedenissen beschrijven is het idee dat een organisme een beperkte hoeveelheid voedsel tot zich kan nemen, en de vergaarde nutriënten moet verdelen over functies, zoals reproductie, overleving, immuun respons en metabolisme.

Volgens de disposable soma (Kirkwood 1977) theorie wordt veroudering veroorzaakt door fouten die ontstaan in het somatische gedeelte van het lichaam (gedeelte die geen reproductie cellen produceert), en een gebrek aan het herstellen van deze fouten. Moleculair ontstaan deze fouten door schade aan het DNA, verkorting van de telomeren, dysfunctionele mitochondriën en ophoping van kapotte eiwitten. Organismen kunnen de mate van fouten beperken, door meer nutriënten (of energie) te investeren in somatisch herstel. Echter, dit is kostbaar, waardoor er bij een hogere mate van herstel minder gegroeid of gereproduceerd kan worden. Volgens deze theorie zal er in verschillende omgevingen selectie zijn voor verschillende balansen van investering in reproductie en overleving (herstel). In omgevingen waarin het organisme een lagere kans heeft lang te leven door externe factoren (predatie, honger, bevriezing) zullen organismen minder investeren in langer leven, maar meer in reproductie. De evolutietheorie kan gebruikt worden om voorspellingen te doen over de mate van veroudering van organismen afhankelijk van omgevingsomstandigheden waarvan naast extrinsieke mortaliteit (externe factoren die mortaliteit veroorzaken) de aanwezigheid van nutriënten en de variatie van deze omstandigheden in seizoenen en locaties belangrijke factoren zijn.

Door natuurlijke selectie zullen de gemiddelde eigenschappen tussen populaties variëren (Darwin 1859), die deels genetisch bepaald zijn. Bovendien evolueert ook de mogelijkheid voor individuen binnen een populatie om plastisch te reageren op variatie van de omgeving. Hierdoor zullen individuen met eenzelfde genotype (totaal aan erfelijk materiaal) verschillende fenotypen (verzameling van alle waarneembare kenmerken van een individu) laten zien, afhankelijk van de omgevingsvariatie. Als generaties lang de gemiddelde temperatuur omhoog gaat, zal bijvoorbeeld in een populatie fruitvliegen de gemiddelde lichaamsgrootte omlaag gaan (omdat kleinere individuen een hogere fitness hebben). Maar in bijvoorbeeld een Europees klimaat zijn er ook verschillen tussen seizoenen. Zo zullen aan het einde van de lente vliegen larven langzamer maar langer ontwikkelen, en uitgroeien tot grotere adulten in vergelijking met de zomer, waarbij ze sneller ontwikkelen en kleiner blijven. Dit soort plastische responsen zijn ook geëvolueerd, en de mate van plasticiteit hangt af van de voorspelbaarheid van de variatie in de omgeving, hoe vaak een bepaalde omstandigheid voorkomt (bijvoorbeeld bepaalde temperatuur) en of de verschillende fenotypen door een enkele genotype gerealiseerd kunnen worden. In verouderingsonderzoek is een vaak bestudeerde plastische respons het langer leven onder dieet restrictie. Bijvoorbeeld, mensen met een BMI tussen de 20 en 25 leven langer dan wanneer ze een BMI van boven de 25 hebben. Bij muizen, vliegen, wormen en zelfs bij gist, leidt dieet restrictie tot een langere levensduur ten koste van de reproductie.

Methoden en resultaten

In dit proefschrift bestudeer ik veroudering en toets ik theorieën en ideeën die gangbaar

zijn in het verouderingsonderzoek. De eerste theorie is die van de voorspellende adaptieve respons (de VAR). Gluckman en Hanson (2004) hypothetiseren dat tijdens de ontwikkeling in de baarmoeder, menselijke foetussen een voorspelling kunnen doen van de omgeving en de mogelijke voedselomstandigheden in de toekomst, ver na de geboorte. Embryo's maken gebruik van deze informatie om een adaptieve (evolutionair voordelig) respons te genereren (de VAR). Wanneer moeders weinig voedsel hebben stellen embryo's zich in op een voedselschaarste na de geboorte. Als de voorspelling niet uitkomt, dan ontstaan er verhoogde kansen op ziektes zoals arteriosclerose en diabetes type 2. De kans dat een VAR evolueert is afhankelijke van hoe goed de voedselcondities in de toekomst te voorspellen zijn tijdens de korte periode waarin de foetus zich ontwikkelt. In hoofdstuk 2 test ik of een VAR is geëvolueerd bij een organisme waarvan de omgeving zeer voorspelbaar varieert (het bruin tropisch zandoogje, *Bicyclus anynana*), en waarvan de levensduur kort is. Ook hebben eerdere experimenten (Saastamoinen *et al.* 2010) al laten zien dat adulten beter tegen stress kunnen als ze als larve al gestrest zijn. In een theoretisch model laat ik zien dat in de omgeving van *B. anynana* het inderdaad mogelijk is dit soort voorspellingen te doen aan de hand van larvaal voedsel. Larven die beperkt voedsel ervoeren in het model, investeerden meer in borstspieren waardoor ze beter en efficiënter konden vliegen. Hiermee was het makkelijker om weg te vliegen uit de plaatselijke slechte omgeving als adult. In het veld verhoogde dit het reproductieve succes en daarom evolueerde er een VAR. In een gemodelleerde laboratorium omgeving verhoogde dit de vliegstress resistentie van adulten wanneer ze als larven minder te eten hadden gekregen. De conclusie van hoofdstuk 2 is dat in een voorspelbare omgeving waarin *B. anynana* leeft, een VAR inderdaad kan evolueren.

In hoofdstuk 3 bekijken we een ander idee dat vaak genoemd wordt in de literatuur. Milde dieet restrictie leidt tot een toename van levensduur bij zeer verschillende organismen zoals ratten, muizen, vliegen, wormen en gist. Omdat deze organismen zeer verschillende plaatsen innemen in de stamboom van het leven, hetgeen de evolutie van de organismen op aarde representeert, wordt vaak gedacht dat de levensduur verlengende werking van dieet restrictie een evolutionair geconserveerde eigenschap is. Dit suggereert dat deze respons of een gefixeerde onveranderlijke eigenschap is van alle organismen (niet veranderbaar door mutatie of andere omgevingsfactoren) of dat het evolutionair voordelig is voor alle organismen om deze respons te laten zien. Omdat het eerste niet waar is (de plastische responsen van levensduur en reproductie zijn zeer makkelijk te evolueren, genetisch variabel en verschillen erg binnen en tussen soorten), testen we in hoofdstuk 3 met een model, gebaseerd op een eerder model (Shanley and Kirkwood 2000), wat de evolutionair meest voordelige respons is op voedsel beperking voor verschillende typen organismen. Het resultaat was dat alleen voor organismen waarbij voedsel restrictie ook de juveniele overleving sterk verlaagde, een hogere mate van investering in langer leven ten koste van reproductie kon evolueren. Verder was dit verschillend voor zeer kort levende dieren in vergelijking met langer levende dieren. Kort levende dieren reageerden op plaatselijke verschillen in voedselhoeveelheid in de omgeving terwijl langer levende dieren ook rekening hielden met seizoensverschillen. De conclusie in hoofdstuk 3 is dat levensduur verlenging bij voedsel restrictie niet een evolutionair adaptieve respons is voor alle organismen en dus niet evolutionair geconserveerd zou moeten zijn.

In hoofdstuk 4 beschrijf ik een experiment waar we testen of vliegen die een jojo dieet krijgen verschillen in levensduur ten opzicht van vliegen die altijd hoog concentratie voedsel krijgen (hoog voedsel) of altijd laag concentratie voedsel (laag voedsel) te eten krijgen. Verder

beschrijf ik de variatie in gewicht en reproductie op het constant en jojo dieet. Dit onderzoek was opgedeeld in twee experimenten. In experiment 1 hebben we het effect van jojoën getest met 5 vliegen in een buisje, omdat we hiermee grotere aantallen konden houden. In exp. 2 bekeken we individuele levensgeschiedenissen, waarbij per vlieg het gewicht en reproductie werd bepaald. In exp. 1 leefden jojo vliegen niet significant korter dan langlevende vliegen (namelijk vliegen die constant hoog voedsel kregen). Ze leefden wel langer dan vliegen die laag voedsel aten. Verder waren ze aangekomen na drie of vier dagen op hoog voedsel en verloren ze weer gewicht op laag voedsel. In exp. 2 zagen we dat vliegen meer eitjes legden op laag voedsel, en eenzelfde respons zagen we binnen de jojo vliegen. Na drie dagen op laag voedsel hadden ze meer eitjes gelegd dan jojo vliegen op hoog voedsel. We vonden echter geen verschillen in gewicht. De overleving van de jojo vliegen in exp. 2 leek dan echter veel op die van exp. 1; jojo vliegen leefden langer dan vliegen op laag voedsel, maar bijna net zo lang als vliegen op hoog voedsel. Een opvallend resultaat in zowel exp. 1 en 2 was dat de concentratie voedsel die vliegen kregen aan het begin van het adulte leven een langdurig effect had op het gemiddelde en de variatie van de eigenschappen tijdens het jojoën. In het experiment begonnen we altijd een groep jojo vliegen op laag en een groep jojo vliegen op hoog voedsel zodat ze altijd direct jojo vliegen op hoog en laag voedsel konden vergelijken. De uiteindelijke conclusie was dus dat jojoën voor vliegen een relatief klein negatief effect heeft op de overleving van vliegen en dat de vliegen zeer plastisch zijn in gewicht (exp. 1) en reproductie (exp. 2). Echter, het effect van ervaring vroeg in het leven beïnvloedde de levensgeschiedenis eigenschappen tot laat in het leven.

Omdat de vliegen zo snel reageerden op voedsel vroeg ik me af of op het niveau van genexpressie de vliegen ook zo snel veranderde. Daarom hebben we de genexpressie gemeten van jojo vliegen op hoog en laag voedsel op hetzelfde moment om deze te vergelijken met vliegen die continu hoog of laag voedsel kregen. Ook hebben we een groep jojo vliegen vier dagen langer laten leven en de vliegen opgeofferd als ze gewisseld waren van voedsel. Op dit tijdstip hebben we ook vliegen opgeofferd die constant voedsel kregen. Hierdoor konden we de genexpressie patronen beschrijven van jojo vliegen ten opzicht van de constante vliegen, maar ook tussen tijdstappen in het experiment van vliegen die van laag naar hoog of van hoog naar laag werden verplaatst. Net als de levensgeschiedeniseigenschappen, was de expressie van meer dan 5000 genen verschillend tussen jojo vliegen op hoog en laag voedsel, zowel op de eerste als tweede tijdstip. Deze genen waren net zo verschillend als de verschillen tussen constante vliegen. Dit betekent dat de vliegen inderdaad duizenden genen anders tot expressie brengen als ze jojoën en dat deze genen op eenzelfde manier veranderden als bij de constant hoog of laag vliegen. Verder waren grote groepen genen goed te relateren aan de eigenschappen, vooral het aantal eitjes dat ze in vier dagen hadden gelegd voordat we ze opofferden. De conclusie was dat vliegen inderdaad tijdens het leven zeer plastisch zijn in een groot aantal groepen genen.

In hoofdstuk 4 en 5 beschrijf ik variatie gemeten op het individueel niveau van een vlieg. Van elke vlieg hebben we vele malen het gewicht en het aantal gelegde eitjes gemeten. Theoretisch gezien leven vliegen langer wanneer ze aantal het begin van het leven minder eitjes leggen, omdat ze de nutriënten die ze niet investeren in eitjes leggen kunnen investeren in onder andere reparatie van het somatisch gedeelte van het lichaam. In hoofdstuk 6 modeleren we het effect van variatie in het investeren in reparatie tussen individuen in een populatie. Het nieuwe in het model is dat deze verschillen in reparatie ook als gevolg heeft dat individuen die biologisch minder oud zijn (minder schade aan somatisch gedeelte van het lichaam), beter zijn in voedsel

opnemen. Zo ontstond er in het model door variatie in investering vroeg in het leven, wat een verschil veroorzaakte in veroudering, variatie in voedsel opname efficiëntie laat in het leven. Dit beïnvloedde dan weer de relatie tussen levensduur en reproductie op verschillende tijden in het leven van de vliegen wanneer individuen vergeleken werden. We hebben daarna getest of dit model klopt met de werkelijkheid met behulp van de data verkregen in hoofdstuk 4, waar we individuele vliegen volgden. Vliegen die vroeg in het leven weinig reproduceerden, leefden langer, maar reproduceerden later in het leven meer. Dit zorgde voor een verandering van relatie tussen reproductie en levensduur van negatief aan het begin van het leven naar positief laat in het leven. Dit patroon was hetzelfde tussen vliegen op hoog en op laag voedsel.

In hoofdstuk 7 beschrijven we de genetische variatie in kandidaat genen voor levensgeschiedenis eigenschappen in relatie met fenotypische variatie in het veld bij een levendbarende vis, *Heterandria formosa*. Van de genen die uitkozen zijn, is bekend dat ze in grote mate groei, reproductie en levensduur bepalen bij model organismen. Eerst hebben we het genoom van *H. formosa* bepaald, waarna we genen uitkozen waarvan we verwachtten dat deze relateerden aan de fenotypen in het veld. Deze fenotypen zijn in de literatuur beschreven en relateren aan predatie druk, waarbij populaties met hogere predatiedruk sneller en groter groeien en kleinere maar meer nakomelingen maken (Schrader and Travis 2012). Een deel van deze variatie is overerfelijk, hetgeen bepaald is in laboratorium experimenten. We vergeleken de kandidaat genen met control gebieden in het genoom, die we random gekozen hebben. Inderdaad correleerde genetische variatie in de kandidaat genen veel beter met de eigenschappen dan de controle genen. Vooral in één gen, de insuline receptor substraat, vonden we vele plaatsen in het gen waarbij variatie correleerde aan de eigenschappen 'grootte van het vrouwtje' en het aantal nakomelingen. Dit laat zien dat kandidaat genen die in het laboratorium levensgeschiedeniseigenschappen bepalen bij organismen als muizen, vliegen, gist ook in een meer natuurlijke omgeving dezelfde eigenschappen bij de levendbarende vis *H. formosa* zouden kunnen beïnvloeden. Verder onderzoek is natuurlijk van belang, om te kijken hoe deze variatie in de genen precies de variatie tussen populaties tot gevolg heeft.

Discussie

In dit proefschrift heb ik een aantal zaken beschreven en getest, op een aantal manieren bij een aantal organismen. De nadruk lag hierbij op het effect van nutriënten op levensgeschiedeniseigenschappen met een focus op veroudering. Sommige ideeën die de ronde doen in de literatuur kloppen wel, anderen lijken een slechte theoretische basis te hebben. De VAR werkt prima bij *B. anynana*, een dier die in een zeer goed voorspelbare omgeving leeft. Het idee dat een VAR ook bij mensen is geëvolueerd hoeft echter niet te kloppen. In hoofdstuk 3 zagen we namelijk dat variatie in voedsel tussen zeer kort levende dieren een andere adaptieve respons veroorzaakt dan bij langer levende organismen. Voor mensen, die weer een stuk langer leven dan muizen, is seizoensvariatie mogelijk weer meer vergelijkbaar met de spatiële variatie, zoals gemodelleerd in hoofdstuk 3. Hierdoor zal de VAR, maar ook voedsel restrictie, andere evolutionaire drukken leggen op de levensgeschiedenis van mensen in vergelijking met muizen, vliegen, gisten en andere soorten. Dit werk zou het besef hiervan moeten vergroten.

In het vliegen onderzoek bekeken we de plasticiteit van vliegen op veel en weinig voedsel. In de eerdere hoofdstukken hadden we al theoretisch bepaald dat kort levende organismen als de vlieg snel zou moeten reageren op voedsel veranderingen (in het model spatiële variatie in

voedsel). Toevallig reageerden de vliegen ook snel, waarbij ze binnen drie tot vier dagen het gewicht en het aantal eitjes veranderden en dat dit gepaard ging met de verandering in regulatie van duizenden genen. Dit laat zien dat een algemeen model (zoals beschreven in hoofdstuk 3) iets kan zeggen over specifieke experimenten. Verder ecologisch onderzoek zou zich misschien moeten richten op de daadwerkelijke variatie in voedsel en effecten van juveniele overleving en succes bij de vliegen. In hoofdstuk 6 vergeleken we een deel van de verkregen data met een model. Dit model verklaard hoe de relatie tussen levensduur en reproductie kan veranderen over de tijd in een populatie van vliegen. Dat voedsel inname lager is bij oudere vliegen is bekend. Als dit werd opgenomen in de berekening van het model, veranderde de relatie tussen reproductie en levensduur, hetgeen vaak wordt waargenomen in experimenten. Dit model leidt weer tot meer hypothesen, namelijk dat selectie op voedsel inname een verandering in de relatie tussen reproductie en levensduur zou veroorzaken (wanneer deze eigenschappen genetisch gerelateerd zijn). Bovendien zouden vliegen die langer leven en gezonder zijn in vergelijking met anderen met dezelfde chronologische leeftijd meer voedsel tot zich moeten nemen.

In het laatste data hoofdstuk beschreven we de correlatie tussen variatie in kandidaat genen en levensgeschiedenis eigenschappen van een levendbarende vis, *Heterandria formosa.* Zowel de individuen waarvan DNA was geïsoleerd als de eigenschappen zijn gemeten van individuen uit het veld. In de literatuur wordt vaak de variatie van deze genen gerelateerd aan variatie in eigenschappen bij organismen in het laboratorium. We concludeerden dat het loont om naar dezelfde genen te bekijken in verschillende omstandigheden bij verschillende organismen. Het effect van deze genen in het lab is veel groter, ook door de het feit dat de eiwitten waarvoor de genen coderen vaak volledig ontbreken bij dieren die mutant zijn in het lab. De kleinschaligere maat van effect is iets wezenlijks anders, maar is blijkbaar toch bepaald door eenzelfde groep genen. Zowel in de theoretische als het genexpressie hoofdstuk heb ik gekeken naar eenzelfde soort vraag, namelijk, kunnen we op eenzelfde manier kijken naar fenomenen in verouderingsonderzoek bij verschillende organismen. Voor zowel de VAR als het dieet restrictie onderzoek bleek dat het zeer belangrijk was wat de evolutionaire en ecologische details van het organisme waren. In het onderzoek naar genexpressie bij de vliegen bleken de expressie van genen op eenzelfde manier relateerden aan de eigenschappen als in andere studies. Hoe vergelijkbaar verschillende organismen zijn in hoe ze verouderen verschilt erg van waar je naar kijkt. Evolutionair gezien zouden ze iets anders moeten doen, maar dit zou gerealiseerd kunnen worden door dezelfde groepen genen, maar waarschijnlijk wel op een andere manier. Alles lijkt hetzelfde, maar overal zijn verschillen te ontdekken.

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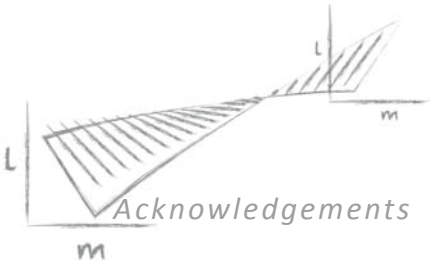

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"Thank you for the music, the songs I'm singing
Thanks for all the joy they're bringing
Who can live without it, I ask you in all honesty
What would life be, without a song or a dance, what are we
So I say, thank you for the music, for giving it to me."

Andersson & Ulvaeus

After a jump through space-time (across many dimensions I cannot comprehend) I find myself again at the Royal Station Hotel in Newcastle, but now in a different room and six years later, and without Marina. She was with me when we would find a place to live for the first three months of my PhD project. I am thinking of all the people that helped me during this very long period that went by in a flash. First there is Marina, thank you for everything. Thanks to my family for the nature and nurture that shaped me into what I am today (pa, ma, Esther, Sjoerd, Judith) and thanks to in-laws (Onno, Jan, Sara, Rene, Paula, Tessa). Thanks nephews Tim, Kas, Daniel. Special thanks to the ACtjes (Kars, Rick, Bert, Sander, Sabrine, Katey, Leonie, Noa) for even more nurture. Very valuable were their questions, especially, when will you finish? I am almost there.

I would like to thank the theoretical biology, animal and plant ecology and evolutionary biology people in Leiden from the past for the education given during my bachelor and master period, which had an enormous effect on being able to start and perform the work I have done. When I visit the university in Leiden nowadays, I cannot imagine a similar person being educated in Leiden. I want to thank the students which I studied with and I still regularly see (such as Daniel, Maarten, Wouter, Willem, Thijs and others, and special thanks to Thijs from the Mr. Jansen for making this possible). Thanks to Wouter for organizing moth catching events that produces new curiosities, which also led to unworkable ideas.

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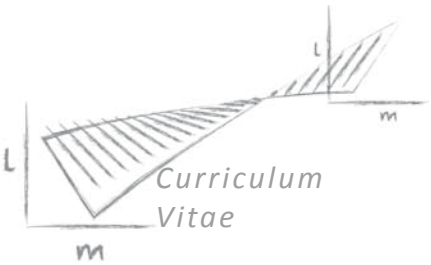
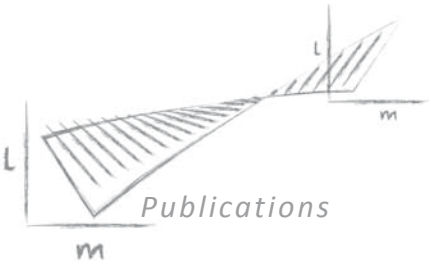


Curriculum vitae

"Maybe I will never be,
All the things I wanna be,
Now is not a time to cry,
Now is the time to find out why,
I think you're the same as me
We see things they'll never see
You and I are gonna live forever"

Noel Gallagher

Joost van den Heuvel was born on the 14th of January 1984 in Rotterdam, the Netherlands. When he was young he was fascinated by professor Barabas' time machine (Suske & Wiske, by Willy Vandersteen) with which he send his friends to past era's, which included visits to the dinosaurs. Furthermore, he very much liked looking at the fish kept at his parental home (especially the guppies). Not able to build anything Joost decided to study biology in 2002, and started to study evolutionary and ecology during his bachelor, combining the time machine and animal fascination. During his master degree projects he focused on modelling and performing selection experiments, focused on the life history traits body size and lifespan. To further study life histories, now specifically focused on ageing, and combine modelling with experiments, in 2008 he started a PhD project with Paul Brakefield and Bas Zwaan in Leiden, and Thomas Kirkwood and Daryl Shanley in Newcastle. The project was funded by the EU Network of Excellence Lifespan, which had as one of the many goals to integrate experiments and modelling, which was realized by Joost, as he combined modelling and experiments. As the project grew older Joost continued in the IDEAL EU project, working on similar topics. Joost published papers in international scientific journals and presented his work at various international meetings. During his PhD Joost participated in teaching in various courses (statistics, evolutionary biology, genomic architecture, plant biodiversity field course) and supervised several students. Currently Joost works as a postdoctoral researcher at the institute of Cell & Molecular Biosciences at the Newcastle University in Newcastle Upon Tyne.



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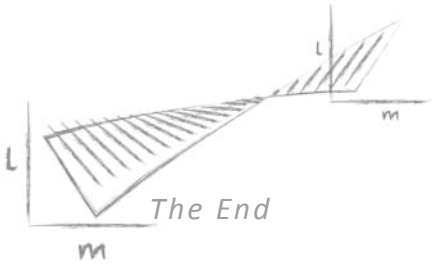
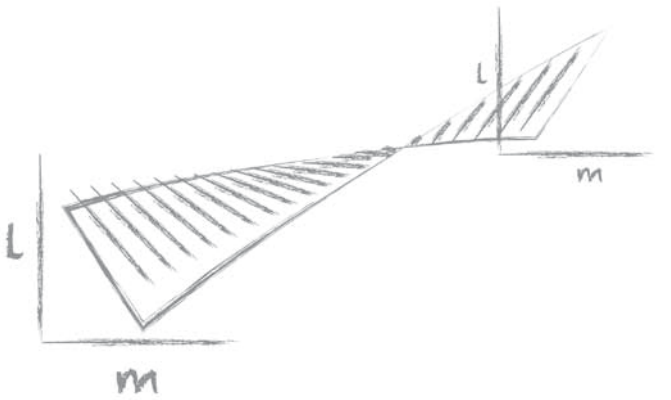


“And in the end, the love you take Is equal to the love, you make”

Lennon & McCartney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