Some like it hot...

The evolution and genetics of temperature dependent body size in *Drosophila melanogaster*

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Cottelstone, Cottelstone, Cottelstone Pie
A fly can't bird, but a bird can fly
Ask me a riddle and I'll reply
Cottelstone, Cottelstone, Cottelstone Pie

Nyáron nyaralok, télen telelek,
a Trotechnikus, az mindig Elek,
kérdezz valamit, s én megfelelek,
nyáron nyaralok, télen telelek.

Hedeblij, heideblé, bleedehei, blij
Een vlieg kan niet bijen, maar een bij kan wel vliegen.
Ik laat me niet door raadsels bedriegen.
Hedeblij, heideblé, bleedehei, blij.

A.A.Milne

General Introduction

1 The Science of Biology - *Order without law?*

When saying the famous, or rather infamous, words "there will never be a Newton of the blade of grass" Immanuel Kant refused to accept biology as a real science (quote adapted from the Dutch translation of the German original, Theunissen and Visser 1996). His main argument was the lack of measurable and repeatable patterns in the living world, caused by a universal mechanism independent of the system under study. As a leading scientist of his time he knew that gravity not only will *always* make an apple fall down instead of upwards, but will do so *everywhere* in the known universe. Given the mechanistic view of the world that was then prevalent, physics was considered a science because it deals with natural "laws", which can be empirically demonstrated and theoretically proven. In physics, universal forces apply to all matter. First principles shape the world in a predictable manner.

The biology Kant knew was mainly about trying (and often failing) to classify all the strange beings brought back from the new worlds that were being discovered. Even more troublesome was the notion that mechanistic principles of causation would fail to apply to living beings. Animal behaviors are not only unpredictable but seemingly purposeful, which results in circular causation. The first action (muscle contraction for foraging) is not only the cause of the next one (digestion) but ultimately the consequence of itself (energy intake needed for muscle contraction)(Theunissen and Visser 1996). To Kant, no universal force was known that could have shaped such seemingly purposeful life; life that at the same time lacks predictability. Some truth must rest in his claim.

In the post-Darwin era, modern biologists befell the luxury of not having to bear sleepless nights in search of proper retribution, as they might have done in Kant's time. Evolution itself is the fundamental pattern and the universal mechanism. All biologists will agree, yet it is unlikely that Kant would be convinced. For him, the concept of evolution by natural selection would have

only doubtful merits as a science. Our fate is now ruled by stochasticity; our survival a faint probability and the world we inhabit just a historic mishap. Life is still unpredictable. We have uniqueness and history vs. natural laws. There might be some pattern but never without exception. There seems to be order but where is the law?

The search for biological "laws" did reveal several interesting patterns that are at best descriptions and generalizations, rarely quantifiable and never without exception. The search for robust biological laws against the odds of natural variation is even known to have produced Haeckel's "forgery" of the phylotypic stage (Richardson 1995; Richardson et al. 1997), with drawings a bit too flattering for the pattern that otherwise does exist (Gerhart and Kirschner 1997). Inevitably, unaccounted variation will always obscure the view. In biology often "a "law" is formulated, exceptions are found, and one ends up with natural history" (van der Steen and Kamminga 1991). Frankly, nature doesn't give a damn about "laws". If so, then where does the order come from? Even if it does encompass minor variations on the theme?

Before an attempt will be made to answer these questions, one might wish to put the previous remarks in more scientific and contemporary terms. We are facing "the factual situation that the biological sciences have comparatively few universal (even terrestrial-universal) statements of their own. Moreover, any general theories and laws that biology does have do not yield lower-level statements without the addition of very elaborate and highly specific descriptions of initial and boundary conditions, about which more often than not only incomplete information is available" (van der Steen and Kamminga 1991). According to this comprehensive review of the still ongoing philosophical debate (van der Steen and Kamminga 1991), a general theory of biology needs to explain the overall pattern and at the same time account for the "exceptions", without unduly complicating the issue at hand. Coincidentally, the two examples they give are excellently suitable to discuss here. First, the authors describe non-genetic adaptations to cold exposure, i.e. acclimation. Although the direct physiological cause to induce the acclimation is similar, i.e. reduced metabolic rate, the range of possible responses is so wide that in fact completely opposite mechanisms can be found. Consider the intuitively most logical response, increasing basal metabolic rate compared with hibernation. The authors state that to account for all these "exceptions" one would need to formulate such very complex generalizations that in fact one would end up with a description of each possible case and not with a general law. Next, a physiological model of animal growth is introduced (see section 3.2.2), that is not unduly complex and has been successfully applied to hundreds of species from very different taxa without convincing exceptions. The general message seemed to be that as long as the extensions to the theory

were not "unduly" complicated, proper biological "laws" were feasible that could accommodate natural variability.

My concern with this issue of extending the theory to accommodate "exceptions" lies in the conviction that all such "exceptions" are in fact examples of the natural variability that is intrinsic to living systems. If so, they are no aberrations to be smoothed out but the very essence of life that needs to be understood. From this perspective, evolution by natural selection seems sufficient to account for, not only the patterns but also for the variability. The very force that drives evolution, and thus creates order, is by its nature a diversifying agent. Random divergence underlies any adaptive (i.e. directional) change. Evolution of any property is conditional upon heritable variation for the trait itself. Natural selection *is not the consequence* of heritable variation in fitness. Heritable variation in fitness *is* natural selection (Stearns 1992). A biological pattern and variation in it cannot be viewed separately. It is not just signal and noise; it is how nature works. In biology there are no exceptions that need complicated extensions of the theory to be accounted for, only natural variation that is an intrinsic and necessary component of life. The emergence of any *phenotypic* pattern is dependent on the presence of *genetic* variation for it to be selected upon. To understand life one needs to understand the sources of the variability rather than smooth out the exceptions. This is why there are no biological "laws". This is why "nothing in biology makes sense except in the light of evolution".

2 Natural variation in body size

2.1 Clinal variation in body size - *Bergmann's "rule" rather than "law"*

One natural pattern that has been proposed as a biological "law", when such terminology was fashionable, was recognized long ago and became known as "Bergmann's rule". Clinal variation in body size of endothermic animals has been frequently reported: larger animals (measured as mean body size in populations within species or of species within a genus) are generally found at higher latitudes and altitudes (Mayr 1963). It has been suggested that such a trend would be the result of selection on the surface area to volume ratio, hence on the rate of heat exchange with the environment (Mayr 1963). This explanation seems plausible for endothermic animals that generate their own body heat, however a similar pattern has been found in ectotherms as well. Ray (1960) proposed that ectothermic animals might also follow Bergmann's rule, deduced from two independent observations. First, average temperature decreases with increasing latitudes and secondly, ectothermic animals reared at

lower temperatures typically mature at larger sizes when compared with conspecifics reared at higher temperatures. The latter observation was confirmed by Atkinson (1994), who gathered a large sample of examples, establishing such a trend in phylogenetically distinct ectotherms. He showed that in 83.5% of all cases investigated, the reduction of body size with higher rearing temperature could be demonstrated. Taken together with the decreasing average temperature at increasing latitudes, this finding predicts a phenotypic body size cline in ectotherms. Because body size is known to be strongly associated with the fitness of an animal (Roff 1992) and because it has been demonstrated that the optimal measure of body size might differ between environments (Roff 1981), the existence of geographical (i.e. genetic) variation in ectotherm size is not unlikely. However, a thorough understanding of temperature dependent body size variation in ectotherms is still lacking.

One well-studied model organism is particularly suitable for addressing this intriguing phenomenon in more detail. Next to the advantages offered by a short generation time, accessibility to experimental manipulations and the availability of sophisticated genetic tools, *Drosophila melanogaster*, as well as other *Drosophila* species, has been used extensively to describe the pattern of geographical variation in body size. Comparison of natural populations revealed such geographic variation: body size increases with latitude and altitude. It has been known for a long time that part of this variation is genetic, and not only the consequence of the phenotypic pattern described above (Reed and Reed 1948). When *Drosophila* sampled from different populations along a latitudinal gradient are kept under the same conditions, the difference in body size persists. High latitude genotypes always produce larger adults, hence there is a genetic basis for the pattern. Clinal variation in size has been reported for various species of *Drosophila* and on different continents (Stalker and Carson 1947; Stalker and Carson 1948; Prevosti 1955; Misra and Reeve 1964; David et al. 1977; Coyne and Beecham 1987; James et al. 1995; van 't Land et al. 1999). The repeatability of such clines implies that they are caused by natural selection. The most convincing evidence for this conclusion comes from a recent study on the colonization of the America's by *Drosophila subobscura*. It has been shown that in less than 20 years from independently introducing the species into South and North America, geographical clines have evolved that are morphologically identical to the "founder" cline in Europe (Huey et al. 2000).

Several climatic factors vary with latitude and altitude, such as temperature, rainfall and relative humidity. These factors may be involved in the processes shaping the clines. Laboratory thermal evolution has established temperature as the most likely selective agent. Replicated laboratory populations of several *Drosophila* species revealed that adaptation to lower

temperatures results in increased body size (Anderson 1966; Anderson 1973 – *D. pseudoobscura*, Cavicchi et al. 1989; Partridge et al. 1994a – *D. melanogaster*), in agreement with the observed geographic clines. Geographical clines in *Drosophila* body size are the result of adaptation to different temperatures.

2.2 Phenotypic plasticity of body size

The vast majority of living organisms are faced with varying environmental conditions. In response to these variable factors, individual organisms display morphological, physiological and behavioral changes. Traditionally, quantitative geneticists considered environmental effects as a nuisance, obscuring genetic patterns and reducing selection responses (Falconer 1981). However, as early as in 1909, Woltreck had introduced the term "Reaktionsnorm" (reaction norm) of a genotype (Woltereck 1909). He defined the reaction norm as the continuum of phenotypes produced by one genotype, when exposed to different environments. Another definition of what is often called *phenotypic plasticity* is "the amount by which the expressions of individual characteristics of a genotype are changed by different environments" (Bradshaw 1965). Today, understanding phenotypic plasticity is recognized to be necessary to achieve a coherent evolutionary theory. It is the focus of a relatively new but growing field of research (Schlichting and Pigliucci 1998; Pigliucci 2001).

Temperature not only affects *Drosophila* body size through evolutionary adaptation to different climates. The direct effect of rearing temperature results in a similar pattern. Any genotype, whether cold or warm adapted, realizes a higher body size when reared at lower temperatures (David et al. 1994; Chakir et al. 1995; Noach et al. 1996; David et al. 1997; Karan et al. 2000). This phenotypic plasticity is just as ubiquitous as the genetic divergence between geographical populations, and the presence of genetic variation for plasticity itself has been demonstrated (Noach et al. 1996). As a consequence, natural variation in body size is the result of at least two separate effects of ambient temperature. Genetic differentiation between populations adapted to different thermal regimes alters mean adult body size. Cold adapted, high latitude genotypes are always larger, at identical rearing temperature. At the same time, variation in environmental temperature will affect body size in all populations or genotypes. Lower rearing temperature will result in a higher genotype specific mean body size.

3 Understanding temperature specific body size variation

The actual phenomena of clinal variation in and phenotypic plasticity of body size are undisputed and have been described in detail. The similarity between the patterns suggests some common underlying process. However, these repeatable and very similar size clines are known to be the result of different cellular and genetic mechanisms. Measurements on wing epithelial cells have shown that the difference in body size between natural populations and between laboratory thermal lines is attributable to variation in both the size and number of cells (Partridge et al. 1994b; James et al. 1995; de Moed et al. 1997; Zwaan et al. 2000). In most studies, both variation in size and number of cells was detected but their relative contribution varied. In addition, the genetic architecture of body size clines are known to be rather complex, with evidence for dominance, epistatic and maternal contributions to the phenotypic value (Gilchrist and Partridge 2001). More importantly, when three independent body size clines from different continents were compared, they showed differences in the relative contribution of each of these factors (Gilchrist and Partridge 1999).

These findings establish a link between details of cellular growth/differentiation and organismal size, and show that different cellular and genetic mechanisms can lead to the same phenotypic pattern. However, the question remains: how and why does this phenotypic pattern evolve so precisely and repeatably? Is a large body only advantageous at low temperatures? Are there some physiological constraints that prevent building a large body at high temperatures even if it were advantageous? Do high temperature specific costs exist that prevent more effective growth to a larger adult? Or rather, are cold adapted genotypes overtly sensitive to high temperature and fail in some other function than growth? Determining the adaptive value of a specific body size and unraveling the proximate mechanisms that generate that specific size must be combined to enhance our understanding of temperature specific body size variation.

3.1 Adaptive thermal evolution of *Drosophila* body size

Body size is an important life-history trait, strongly correlated with fitness (Roff 1992). In laboratory studies, a positive correlation between body size and fitness components (eg. fertility, longevity and lifetime mating success) has been reported (eg. Robertson 1957; Tantawy and Vetukhiv 1960; Partridge and Farquhar 1983; Wilkinson 1987). The results of these experiments suggested that large body size (or a genetic correlate of it) is advantageous to adult *Drosophila*. Without a counterbalancing disadvantage, evolution towards

a large adult body would be expected, at all temperatures. However, such evolution does not seem to be in progress, suggesting a compensating disadvantage (Partridge and Fowler 1993). Indeed, basic life-history theory predicts that body size will be optimized rather than maximized to establish the highest possible fitness. Stearns (1992) and Roff (1992) devote a whole chapter to discussing age and size at maturity, being a pivotal issue in life-history theory. Within this framework, life consists of only two stages: "preparation" and "fulfilment", separated by maturation defined as the first reproduction. The age and size at maturation is determined such that fitness is maximized, taking contradictory interests into account. For details see Stearns (1992) and Roff (1992); in short, size at maturation depends basically on pre-adult survival and size specific adult fitness.

Ambient temperature might influence the life-history strategies outlined above. In particular, temperature has an enhancing effect on metabolic rates (Berrigan and Partridge 1997; de Moed et al. 1998). This brings age-related mortality forward. Conditions that bring aging and mortality risks earlier should generally favor precocious maturation. The benefits of earlier reproduction leading to higher lifetime reproductive success should outweigh the disadvantages of a smaller size. Put more broadly, higher risk of environmentally induced mortality should favor rapid growth and reproduction at the expense of maintenance of somatic tissue (Kirkwood and Rose 1991). At higher temperatures, growth rates are enhanced as well. This should allow individuals to realize larger bodies. However, rapid growth is not without cost and evidence has been put forward that organisms do not always maximize, but rather optimize their growth. The disadvantage of not achieving a particular size is outweighed by the reduction of growth related costs (Sibly et al. 1985; Sibly and Callow 1986).

The implications of the previous paragraphs for temperature specific body size are evident. On the one hand, size specific fitness must be determined, at different temperatures. Adult body size could be the target of thermal selection if the fitness advantage of larger body size (or a correlate, such as starvation resistance) is greater at lower temperatures. Bigger is often thought to be better, but maybe only at low temperatures. Alternatively, details of pre-adult development should reveal differences in temperature specific mortality or temperature associated costs to growth.

3.1.1 Temperature and adult fitness

Both patterns, geographical variation and phenotypic plasticity, are repeatable and proposed to be of selective advantage. Should temperature induced variation in body size be adaptive, that would imply that no constant correlation exists between size and fitness. Smaller flies must have a higher

fitness in warmer climates and lower fitness in colder climates. If "big is not always better", fitness traits correlated to adult size must respond differently to changing temperatures than body size itself. Yet, few studies have directly measured fitness differences associated with temperature dependent alterations in body size.

One set of studies explored the possibility of adaptive phenotypic plasticity. It was shown that male territorial success was highest when flies were allowed to compete at the temperature they were reared at, with smaller adults being more successful at higher temperatures despite of their smaller size (Zamudio et al. 1995). In addition, early fecundity was shown to be higher when rearing temperature and test temperature were the same, supporting the idea of adaptive plasticity of body size (Nunney and Cheung 1997).

Another group of studies explored differences in fitness when tested at low and high temperatures, using different genotypes that had been selected for large or small body size. Artificial selection on body size performed at 25°C, resulted in large females having higher fecundity and longevity only at lower rearing temperature (McCabe and Partridge 1997). In a similar experiment it was shown that larger males have higher mating success only when tested at a low temperature (Reeve et al. 2000). These studies indicated that being genetically large for reasons other than adaptation to a cold thermal regime offers a selective advantage in cold environments. All studies agree that a large adult body conveys a higher fitness only at low environmental temperatures.

3.1.2 Temperature and larval growth and survival

Following up on the issue of temperature dependence of pre-adult mortality, an experimental evolution study provided some interesting results. Laboratory lines maintained at low and high temperatures for several years, produced differences in fitness related characters next to divergence in body size. Cold adapted lines were more sensitive to larval crowding in their survival compared to warm adapted lines even when tested at low temperatures (Partridge et al. 1994a). In this case adaptation to a warm thermal regime resulted not just in a smaller adult body but also in a seemingly increased larval competitive ability. An environment that may increase pre-adult mortality perhaps selects for some sort of increased larval vigor. If a cold environment is more permissive for larval survival, genotypes adapted to such conditions could be expected to show lower larval competitive ability. The results on the thermal evolution lines discussed above point towards this scenario, but note that in at least one study on natural populations no obvious differences in larval competitive ability were found within an Australian body size cline (James and Partridge 1998). One possible reason for the difference between

laboratory lines and natural populations might be that in the study on laboratory evolution cited above, larval density was not controlled and the increased larval competitive ability might be the result of the intrinsically higher populations density at higher temperatures. Still, theory predicted (as reviewed by Sibly and Atkinson (1994), see also below) and at least one empirical study suggested that temperature might affect pre-adult survival and, consequently, optimal body size.

A related issue is that of growth associated costs, i.e. the efficiency of growth. It has been shown that growth might be costly (Sibly et al. 1985; Sibly and Callow 1986; Chippindale et al. 1998). One possible mechanism that could be responsible is the efficiency of food conversion into body mass, and the temperature sensitivity of this process has been addressed. A cold evolutionary history has been shown to result in a more efficient conversion of food to adult body. For a given amount of larval food, larger adults emerged (Neat et al. 1995; Robinson and Partridge 2001). Conversely, a warm evolutionary history led to a less than maximal growth efficiency. Whether this finding reflects some physiological/biochemical constraint or in fact results from different allocation of resources, for example to increased larval competitive ability at cost of adult size, is not clear.

3.2 Proximate mechanisms of larval growth

Theoretical models have been developed to identify possible physiological determinants of body size variation. Next to offering a possible proximate mechanism for size variation, these mechanistic models of growth have implications for the proposed selective advantages of the different life-history strategies, i.e. they might distinguish between biochemical constraints and adaptive variation in growth.

Empirical evidence had shown that temperature induced differences in growth efficiency underlie variation in body size (Neat et al. 1995; Robinson and Partridge 2001). In addition, development time is known to be correlated to body size and is subject of thermal evolution, both in the laboratory and in natural populations (Anderson 1966; Partridge et al. 1994a; James and Partridge 1995). These results suggested that some aspect of growth and development could be responsible for variation in body size. However, it would be reassuring to have confirmation that temperature dependent growth rate and development time *can* alter adult body size in the predicted manner, before more detailed studies were to be performed. Van der Have and de Jong (1996) and Atkinson and Sibly (1997) have developed such models.

3.2.1 Van der Have-de Jong model

Based upon the Sharpe-Schoolfield equation connecting enzyme kinetics and biological rates (Sharpe and DeMichele 1977; Schoolfield et al. 1981) van der Have and de Jong (1996) proposed a proximate model to describe the temperature modulated variations in growth rate and development rate in ectotherms. Based on the assumption that growth and development (i.e. differentiation) have different temperature coefficients, the model was used to predict temperature dependent size variation in adult ectotherms.

Both growth rate and development rate are assumed, without loss of generality, to be limited by the activity of one enzyme. For one enzyme the temperature dependence of its activity can be described very accurately (Sharpe-Schoolfield equation). Adult size is thought to be determined by an interaction of growth and differentiation. If growth rate increases while differentiation rate is kept constant, the resulting organism should be larger due to larger cells. In contrast, if differentiation rate should increase while growth rate is kept constant, the resulting adult will be smaller having smaller cells. Both growth and differentiation rates are temperature dependent, and if they have different temperature coefficients, one of them will change faster with changing temperature than the other. Experimental data revealed that differentiation rate increases steeper at high temperatures than growth rate does, resulting in the predicted smaller adult body size at higher temperature (van der Have and de Jong 1996; Gibert and de Jong 2001). The model very precisely describes adult body size variation from the temperature sensitivity of only two physiological processes: the rate of cell growth and cell differentiation. Interestingly, variation in both cell size and cell numbers has been shown to contribute to the observed differences in size at the organismal level, in laboratory thermal lines and in natural populations as well (Partridge et al. 1994b; James et al. 1995; de Moed et al. 1997; Zwaan et al. 2000). Moreover, a comparison of related *Drosophila* species that differ in geographical distribution and adult size, showed that species differ in the parameters of temperature sensitivity for the van der Have-de Jong model (Gibert and de Jong 2001). The different temperature sensitivities of growth and development explain the species differences in body size. Consequently, selection on the temperature sensitivity of pre-adult growth and development seems potentially capable of inducing evolved variation in adult body size.

3.2.2 Sibly and Atkinson: the modified von Bertalanffy model

Another approach consists of a new interpretation of the von Bertalanffy model (Atkinson and Sibly 1997). In this view, growth is the result of the difference between anabolism and catabolism; the rate of these physiological processes is sensitive to temperature. A smaller increase of the rate of processes

that result in the acquisition of resources (anabolism), relative to the increase of the rate of loss of energy and mass through catabolism will result in a smaller adult. This is what might happen at increasing temperatures, as metabolic rates (i.e. enzyme activities) are known to be strongly enhanced by temperature. In this view, a higher temperature would select for mechanisms that enhance resource acquisition. This hypothesis is in line with the data on enhanced resistance to larval crowding in warm adapted thermal lines (Partridge et al. 1994a), because one important consequence of crowding is scramble competition for food. At the same time, a larger capacity of resource acquisition might pose a cost to growth, and Atkinson and Sibly (1997) suggested that adaptation to low temperatures could remove the superfluous capacity and associated costs, and in turn increase growth efficiency. The data provided by Neat and colleagues (Neat et al. 1995) is in line with this interpretation.

The same authors have also provided a more general framework for explaining temperature specific body size variation in ectotherms (Sibly and Atkinson 1994). By incorporating the effect of temperature in a life-cycle analysis, they showed that reduced adult body size is selected for by increased pre-adult mortality. In addition, they reviewed data from eight previous empirical studies to show that in six of these an increased environmental temperature increased juvenile mortality and decreased adult size, as expected. These studies combine a life-history and a more physiological approach, to suggest that temperature induced body size variation may be the consequence of adaptive evolution of pre-adult resource acquisition and juvenile survival, the two likely being related.

3.3 Clinal variation in functional enzyme polymorphisms

Both theoretical models hypothesize variation in (the temperature sensitivity of) enzyme activities. The results on variation in efficiency of larval food processing are also very suggestive (Neat et al. 1995; Robinson and Partridge 2001). A very different line of research might shed some light on these issues. Allozyme frequencies of many enzymes in basic metabolic pathways vary in parallel with the body size clines (Eanes 1999). To our knowledge, these allozyme frequency clines have never been discussed in connection with the cline in body size; the question whether both types of clines are related has not yet been asked. Indirect evidence for a functional link may come from data on the *Pgm* (phosphoglucomutase) locus. The extensive amino-acid variation at the *Pgm* locus (Verrelli and Eanes 2000) was shown to be clinally distributed (Verrelli and Eanes 2001a) and has a functional impact on glycogen synthesis. At higher latitudes, higher frequency of an intrinsically more active PGM allozyme has been found, apparently responsible for the higher glycogen content (Verrelli and Eanes 2001b). To further

underline the involvement of variation in enzyme activities in geographical adaptation, clinal variation in the activity of G6PD (glucose 6 phosphate dehydrogenase) was shown to be opposite to the PGM cline, with lower activity at high latitudes. As higher G6PD activity is responsible for higher pentose shunt flux, and consequently lower glycogen synthesis, the observed patterns of opposing PGM and G6PD activity clines jointly contribute to natural variation in glycogen storage (Eanes 1999; Verrelli and Eanes 2001b). Because variation in the conversion efficiency of food to adult body mass could be expected to result in increased nutrient storage pools and because glycogen was already known to be the most important compound used as "fuel" during pupation (Butterworth et al. 1988), the data on the allozyme clines might be relevant for the geographical variation in body size.

In addition, genetic studies indicated a causal relationship between energy metabolism and organismal traits, supplementing the data on clinal variation in allozyme frequencies. The size of lipid and glycogen storage pools, in second chromosome replacement lines, was shown to be dependent on the variation in the activity of 11 enzymes (Clark and Keith 1988). In addition, significant broad-sense genetic covariance was detected between viability and fecundity on the one side and glycogen content and several enzyme activities on the other side (Clark 1989). The presence of additive genetic variation for all of the 11 enzyme activities under study (Clark 1990) underlined the potential of metabolic traits to be the raw material for natural selection. Interestingly, both PGM and G6PD were included in this study, and both were shown to contribute to functional variation between natural populations (Eanes 1999; Verrelli and Eanes 2001b). Within-population genetic variation in enzyme activity and between-population change in allozyme frequency combine to suggest adaptive evolution at the metabolic level by natural selection of enzyme activity.

4 An overview of the present approach

Clinal variation in body size is adaptive, as shown by the repeatability of the pattern (Stalker and Carson 1947; Stalker and Carson 1948; Prevosti 1955; Misra and Reeve 1964; David et al. 1977; Coyne and Beecham 1987; Huey et al. 2000), laboratory thermal lines (Anderson 1966; Anderson 1973; Cavicchi et al. 1989; Partridge et al. 1994a) and studies of temperature specific fitness of adult size (Zamudio et al. 1995; McCabe and Partridge 1997; Nunney and Cheung 1997; Reeve et al. 2000). At the same time, similar body size clines are the result of varying genetic architectures (Gilchrist and Partridge 1999; Gilchrist and Partridge 2001) and different cellular basis (Partridge et al. 1994b;

James et al. 1995; de Moed et al. 1997; Zwaan et al. 2000). Temperature induced variation in larval growth and development is theoretically capable of producing such divergence in size (van der Have and de Jong 1996; Atkinson and Sibly 1997), and empirical evidence supports this scenario (Gibert and de Jong 2001). Thermal evolution was shown to result in differences in larval efficiency of converting food to adult body mass (Neat et al. 1995; Robinson and Partridge 2001), and probably also in larval competitive ability (Partridge et al. 1994a; but see James and Partridge 1998). Clinal variation in enzyme polymorphisms parallel the body size clines and probably represent functional latitudinal variation in enzyme activity (Eanes 1999; Verrelli and Eanes 2001b). Ample additive genetic variation is present for many enzymes involved in basic carbohydrate and lipid metabolism (Clark and Keith 1988; Clark 1989; Clark 1990) and this variation results in different glycogen and triglyceride storage pools.

These findings not only provide some insight in the various aspects of the phenomenon under study, but also demonstrate the complexity of the problem. The variety of the studies cited above underlines the necessity for an integrative approach. In short, the decision was made that both ultimate and proximate mechanisms must be addressed but in a manner that should allow for as seamless an integration as possible. More precisely, this thesis is based on the underlying conviction that in fact no such dichotomy (ultimate vs. proximate) exists; a thorough understanding of the selective forces that shape body size evolution (ultimate reason) automatically yields the actual trait (or physiological process) that is selected on (proximate mechanism).

To embody this approach, this thesis offers the results of seven studies, progressively more reductionist in experimental design, but continuously aimed at the adaptive pattern of phenotypic variation that needs to be resolved. The first two chapters describe evolved variation in a variety of fitness-related traits. Chapter One offers the largest survey to date of divergence in life-history traits between geographical populations, while testing the effect of a serious challenge to the developing larvae: crowding. In agreement with data from the literature (both theoretical and empirical) the results showed that pre-adult survival (under challenge) is probably an important effector of natural body size variation. Chapter Two more specifically addressed the effects of temperature and larval food quality (without deleterious side effects of crowding) on evolved variation in food processing and the resulting adult size and pre-adult survival. This experiment confirmed the co-evolution of pre-adult survival with the thermal (laboratory) evolution of body size, and suggested that larval food processing is indeed an important proximate correlate. Chapter Three is an introduction to the physiological approach to investigate the involvement of larval energy metabolism in variation in food

processing. To our knowledge, it is the first study to demonstrate the dependency of adult size on pre-adult energy storage. In addition, pre-adult survival emerged to produce the opposite pattern compared to body size variation between populations. In Chapter Four, the presence of a phenotypic life-history trade-off between adult size and pre-adult survival has been formally shown, after all previous results were already suggestive of such a pattern. In addition, we could demonstrate a physiological basis for this evolved life-history variation, being the same larval energy storage compound that was already implicated in the geographical divergence of body size. Furthermore, the results are compatible with the hypothesis that variation in the utilization of storage pools is of importance, as opposed to the size of the energy reserves only. Chapter Five continued to present evidence for the genetic basis of the trade-off between adult size and pre-adult survival, and suggested that selection on larval enzyme activities may underlie the adaptive variation in the utilization of energy storage. In Chapter Six, a slightly different approach is adapted, aimed to continue the study of the genetic variation in cellular metabolism and growth, that might underlie body size evolution. This study identified gene expression profiles that, in a statistical sense, explained a large proportion of the natural variation in body size. A closer examination of the known function of the genes identified showed that the process of cellular growth is likely to be involved. The main contribution of the final chapter, Chapter Seven, is to show that the trade-off between size and survival can be described in terms of gene expression profiles. This trade-off was previously shown to have a genetic basis and probably the result of the utilization of resources. The results in the final chapter showed that an adaptive life-history trade-off could potentially be the result of alterations of cellular metabolism.

As stated before, the aim of the present thesis was to unravel some of the underlying determinants of an adaptive pattern of phenotypic variation. To make reference to section one, much of the confusion around explaining biological patterns seems to come down to a complication intrinsic to biology: our poor understanding of how different levels of organizations are integrated. Lewontin has stated some time ago (1974), that a more precisely defined link between genotype and phenotype is needed for biology to advance (actually he referred to populations genetics). Indeed, if a better understanding of the *process generating the phenotype* were available, many "exceptions", i.e. phenotypic variability (see section 1), were prone to be easily clarified. This work was intended to contribute to this long standing objective.

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Chapter 1

Temperature dependence of fitness components in geographical populations of *Drosophila melanogaster*: Changing the association between size and fitness

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Abstract

Contrary to the conventional wisdom "bigger is better", evolution at high temperature invariably leads to small individuals in *Drosophila melanogaster*. Natural selection is known to be responsible, meaning that genotypes that are small because of adaptation to high temperature must have some temperature dependent fitness advantage. In this study we consider both pre-adult and adult fitness components, and show that large adults from a cold adapted population significantly outperform small adults from a warm adapted population only when tested at low temperature and low larval density. In all other conditions "bigger is not necessarily better", meaning that environmental influences are capable of altering the association between size and fitness. Yet, "smaller wasn't better either" under any condition, when considering the overall measure of fitness. Examining the individual fitness components revealed population by temperature interaction in pre-adult survival, potentially capable to explain the temperature specific advantage of small adult body size. At high temperature, the warm adapted population exhibits superior pre-adult survival while producing small adults. Geographical variation in adult body size seems to be the result of selection on larval growth and competitive strategies, resulting in alterations in the association between fitness components.

Keywords: "bigger is better", correlated selection response, life-history traits, temperature induced change in trait association

Introduction

Temperature has been implicated as an important environmental factor that influences natural variation in *Drosophila melanogaster* body size in two profound ways. Populations adapted to different climatic conditions, i.e. geographical populations from tropical vs. temperate regions, exhibit large and genetically determined differences in adult body size when kept under standard laboratory conditions. Tropical *Drosophila melanogaster* are smaller than their cold adapted counterparts on all continents investigated (Coyne and Beecham 1987; David et al. 1977; Gilchrist and Partridge 1999). The importance of temperature as the selective agent is underlined by results of laboratory thermal evolution. Populations maintained for several years under lower or higher temperatures show similar divergence in body size as the geographical populations (Cavicchi et al. 1989; Partridge et al. 1994a).

In addition to the evolutionary effect of temperature, *Drosophila melanogaster* exhibits developmental sensitivity to larval rearing temperature, resulting in phenotypic plasticity of adult body size. Again, colder rearing temperature yields a larger adult (David et al. 1997; Zamudio et al. 1995). All genotypes, whether genetically large or small increase their size with decreasing rearing temperature but the extent of the temperature sensitivity may differ between genotypes (Noach et al. 1996).

Both patterns, geographical variation and phenotypic plasticity, are repeatable and proposed to be of selective advantage. However, such selection implies that no constant correlation exists between size and fitness, smaller flies having a higher fitness in warmer climates and lower fitness in colder climates. If "bigger is not always better," fitness traits must respond differently than body size under alternative thermal regimes. Yet, few studies have directly measured fitness differences associated with temperature dependent alterations in body size.

One set of studies explored the possibility of adaptive phenotypic plasticity. It was shown that male territorial success of warm reared (small) flies was always higher, but these smaller adults were relatively more successful at the higher temperature despite of their smaller size (Zamudio et al. 1995). In addition, early fecundity was shown to be higher when rearing temperature and test temperature are the same, supporting the idea of adaptive plasticity of body size (Nunney and Cheung 1997, but see Huey et al. 1995).

Another group of studies explored differences in fitness when tested at low and high temperatures, using different genotypes that had been selected for large or small body size. Artificial selection on body size performed at 25°C, resulted in large females having higher fecundity and longevity only at

lower rearing temperature (McCabe and Partridge 1997). In a similar experiment it was shown that larger males have higher mating success only when tested at a low temperature (Reeve et al. 2000). These studies indicated that being genetically large for reasons other than adaptation to a cold thermal regime offers a selective advantage in cold environments.

Laboratory lines maintained at low or high temperatures for several years, produced differences in fitness related characters next to divergence in body size. Survival of cold adapted lines was more sensitive to larval crowding compared to warm adapted lines even when tested at low temperatures (Partridge et al. 1994b). In this case adaptation to a warm thermal regime resulted not only in a smaller adult body but also in a seemingly increased larval competitive ability. Perhaps a trade-off existed between body size and pre-adult competitive ability leading to larval survival. More importantly, this result shows that thermal adaptation of body size was directly linked to an evolved difference in a fitness component.

These studies suggested that temperature induced alterations of body size may in general be adaptive. However, they did not show whether size itself is under thermal selection, or whether any change in body size is a correlated response to selection on other traits or fitness components. The studies cited consider one or a few of many possible traits and significant effects have been found for almost all of them. No studies systematically explored Genotype by Environment interaction for a variety of fitness components in geographical populations reared at different temperatures.

Selection on different traits under low vs. high temperature could account for the lack of a consistent relationship between body size and overall fitness. Understanding thermal evolution of body size therefore necessitates a survey of Genotype by Environment interactions of all relevant fitness traits. Correlated fitness traits should exhibit complementary patterns of temperature dependence, for us to explain geographical variation in body size. Fitness traits that are positively correlated with size in one geographical population might correlate negatively in the other geographical population, depending on the environmental conditions.

In this experiment a systematic approach is attempted in order to identify fitness traits correlated with body size, in a pattern that might explain the selective advantage of adaptation to different climates. Larvae from both a temperate and a tropical population of *Drosophila melanogaster* were reared at low and high temperatures and low and high larval densities. Different larval densities were used to induce variation in adult body size by other means than temperature alone. Larval and adult body weight and several life-history traits were measured, representing both pre-adult and adult fitness components.

Genotype by Environment interaction was found for most traits suggesting that differences in body size between populations were truly adaptive to the different "native" climatic conditions.

Materials and Methods

Populations used

Two different geographical populations of *Drosophila melanogaster* were used, one originating from Panama (collected in 1998) and one from Denmark (collected in 1997). The populations were maintained on standard corn medium at 17.5°C until spring 2000 when the experiment started. A mutant stock, *white*, was used in the competition experiment.

Experimental conditions

To neutralize possible effects of parental rearing temperature, flies used to lay eggs were reared at the experimental temperature. Four days before egg laying, the flies were transferred to new bottles with fresh medium. Several hundred females oviposited for 3 hours, at room temperature. Egg laying took place in empty jars covered with watch glasses containing 4 ml of a 1.9% agar-medium and a drop of a thick yeast suspension. After one hour, the watch glasses were replaced with new ones to remove eggs that were first laid. These eggs are often much further in development as the ones still to be laid.

From the eggs, larvae were reared at temperatures representing the high (27.5°C) and low (17.5°C) regions of the physiological range for *D. melanogaster*. Two different densities were used. Vials (2 ml medium) contained either 24 eggs (low density) or 130 eggs (high density). All eight combinations of rearing temperature, density and population were used.

Measurements

Adult body weight

Fresh weight of adult flies was determined individually, two days after eclosion. Male and female were scored separately. For each treatment fifteen males and females were used. All measurements took place on Mettler ME 22 microbalance to the nearest 0.01 µg.

Development time to adult

Development time was measured in days after egg laying. Three times a day, emerged flies were collected and males and females were counted separately. These animals were used for determining longevity and fecundity. At 17.5°C, 15 replicates were used at low density and 8 replicates were used

at high density. At 27.5°C, 20 replicates were used at low density and 9 replicates were used at high density. The time at which half the adults had emerged was calculated for each vial and gender.

Pre-adult survival and competitive ability

Survival was measured as the percentage of eggs reaching adulthood. The same vials were used as in determining development time. Survival of the *white* stock was determined as well, to verify that it is a viable stock and can be validly used as a standard competitor.

To measure competitive ability for both the Panama and Denmark stocks eggs were collected and placed in vials together with egg from the standard competitor *white*. The ratio *white*:geographical population was 2:1. Adult flies were removed and counted as they emerged. Competitive ability was measured as the percentage red-eyed flies that emerged. The experiment was first done using six replicate vials and then repeated using six more vials for the low density and three more vials for the high density treatment.

Fecundity

The number of eggs laid per female per day was determined. Adults were collected as they emerged and five males and females were put together in a vial (2,3 cm × 8 cm) containing a sugar (1.6%)/yeast (5.4%)/agar (1.9%) medium topped with a drop of life yeast suspension. A different type of food was used because eggs are better visible on this surface and larvae have more difficulty burrowing in it. Vials were kept at the same temperature as the adults emerged at. At 17.5°C the number of eggs laid was determined from days five to seven, at 27.5°C from day four to five. The different "absolute" ages at the different temperatures represent similar "physiological" ages. At the beginning of this period the flies were transferred into new vials and after removal the eggs were counted. Ten replicates were used except for 17.5°C and high density. For Panama seven replicates were then used and for Denmark five. The number of eggs laid in 24 hours by one female was calculated.

Longevity

For measuring longevity, virgin females and males were collected as they emerged. Flies were put in vials with ten animals each, females and males separately. The experiment was conducted at the temperature corresponding to the rearing temperature the flies had experienced. At 17.5°C and low density five replicates were used. For all other treatment groups six replicates were used except for Panama at low density and 27.5°C where eight vials were employed. At the lower experimental temperature adults were transferred to fresh vials every two weeks, at the high temperature every week. If a vial

contained any fungal or bacterial contamination, vials were changed as needed. Every two to three days the number of animals that died was counted. The time at which half of the animals had died, LA50, was determined, by calculating the weighed average of the intervals weighed by the number of animals that died since the last measurement.

Statistical analysis

The Analyses of Variance were performed with SPSS 10.0. From the measurements on the individual fitness components, an overall measure of fitness was constructed. This composite measure, r_c, was defined as:

$$\frac{\text{Pre-adult Survival (fraction)} \times \text{Fecundity (eggs/female/day)} \times \text{Mean Female Lifespan (days)}}{\text{Development time (days)} + \text{Mean Female Lifespan (days)}}$$

This fitness measure, r_c, has the same unit (Δ individuals/generation) and interpretation as r, the intrinsic rate of populations growth. Because the measurements on the individual fitness components have been conducted on different samples, an empirical distribution of r_c has been generated by resampling the data with replacement (bootstrapping). 4000 bootstrap estimates were generated, sufficient for estimating approximate 95% confidence intervals of r_c from the empirical distribution.

Another bootstrap analysis was performed to recreate the positions occupied by the rearing conditions in the character space defined by the difference between populations in female weight and the difference in pre-adult survival. This analysis allows for visualization of environmentally induced shifts in the association between adult size and pre-adult survival between the populations. Within the four combinations of rearing temperature and density, the difference in female weight between the Panama and Denmark populations was determined for 4000 randomly drawn values from the data. If the mean female weight would be the same in the two populations, the expected mean of the resampled differences would be zero. If Panama were larger, the values would tend to the positive, if Denmark were larger they would tend to the negative. The same procedure was used on the pre-adult survival data. The randomly created differences in female weight and pre-adult survival were plotted in a scatterplot. If there were differences between the populations in female weight and pre-adult survival *and* if these differences were not constant across the rearing conditions, the resampled data for the different rearing conditions are expected to occupy different quadrants of the character space.

To test this association, Odds Ratios were calculated from the frequencies of data points in the quadrant of interest vs. all other quadrants. This procedure

quantifies how more likely it is that data expected to be in a certain quadrant actually are in the right quadrant vs. data that are expected to be in another quadrant are wrongfully in the quadrant of interest. Under the null hypothesis, the resampled differences should be centered randomly around (0,0), for all conditions. This means that 25% of the data points are expected to lie in any quadrant for all groups. The Odds Ratio (OR) would then be 0.25/0.25 = 1. An OR significantly higher than 1 would mean that one group is more likely to occupy a certain quadrant than another.

To estimate the distribution of all possible ORs from the resampled data, 80 random groups of 50 data points were chosen from the original 4000. From these 80 groups, the highest and lowest frequencies of "being in the quadrant of interest" were selected. These values were used to calculate the most extreme ORs possible from the resampled data, by taking the lowest frequency of the "expected" group vs. the highest frequency of the "unexpected" group for the lower bound and the opposite combination for the upper bound.

Results

Adult weight

When the data on adult weight were analyzed in a four-way ANOVA (Population=P, Rearing temperature=T, Larval density=D, Sex=S), a significant four-way interaction was found ($p=0.022$). The data were split and the analysis repeated for the two rearing temperatures separately. At both temperatures females were larger than males ($p<0.001$), flies reared at low density were larger than flies reared at high density ($p<0.001$) and Denmark adults were larger than Panama adults ($p<0.021$). No population × sex interaction was found ($p_{17.5°C}=0.901$ and $p_{27.5°C}=0.190$). However, the population × density interaction was significant at the lower rearing temperature ($p<0.001$) (fig. 1). When reared at 17.5°C, Denmark adults of both sexes declined stronger in weight as a result of high larval density, compared to Panama adults. When the data were split according to larval density, a significant population × temperature interaction ($p<0.001$) was detected at high density. Denmark adults again declined strongly in weight.

Development time

Development times were shorter at 27.5°C than at 17.5°C ($p<0.001$), at low density compared to high density ($p<0.001$) and in the Panama vs. the Denmark population ($p<0.001$). However, all two-way interactions and the three-way interaction were significant as well ($p<0.001$). The data were

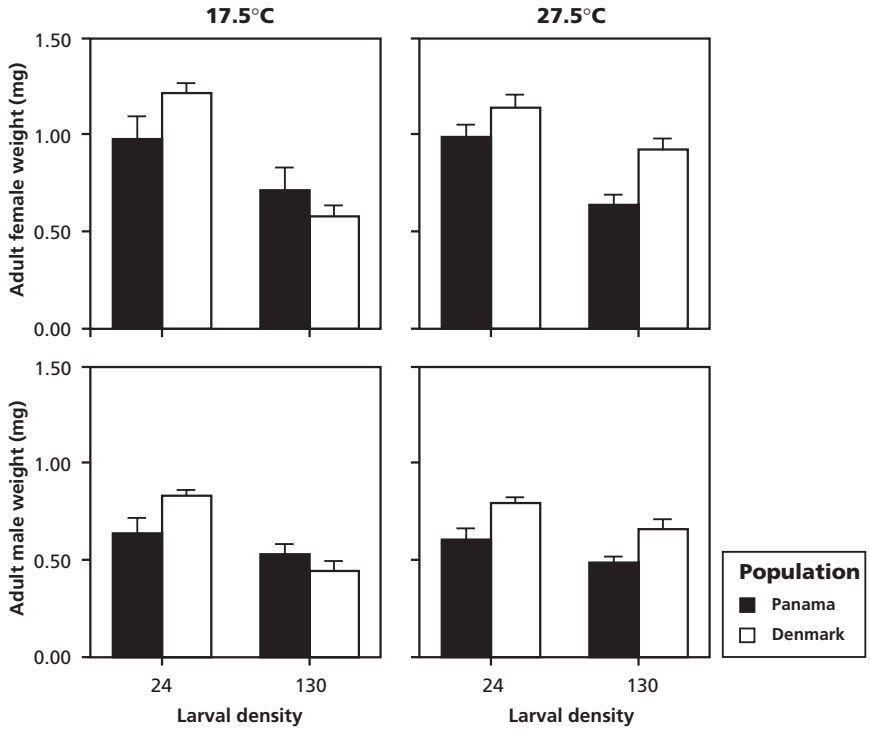


Fig 1. Male and female adult weight (mg) of a temperate and a tropical population of *Drosophila melanogaster* reared at 17.5°C and 27.5°C and low and high larval density. Means and 95% confidence intervals are depicted.

analyzed for the rearing temperatures separately. High density led to a longer development time at both temperatures ($p<0.001$), but the populations only differed at the lower rearing temperature ($p<0.001$) due to the significant interaction between population and density ($p<0.001$). At 17.5°C Denmark flies had an extended pre-adult development at high larval density compared to Panama (fig. 2). At 27.5°C, this pattern was not present. When split for larval density, a significant population × temperature interaction ($p=0.004$) was found at the high density. The data on development time mimicked those on adult weight, but as longer development is considered detrimental for fitness (Krebs and Loeschcke 1999) the fitness implication is actually opposite to the phenotypic pattern.

Pre-adult survival

More animals survived at low density than at high density ($p<0.001$) and the Denmark population had a higher survival than the Panama population ($p=0.002$). However, the three-way interaction (P×T×D) was significant as well

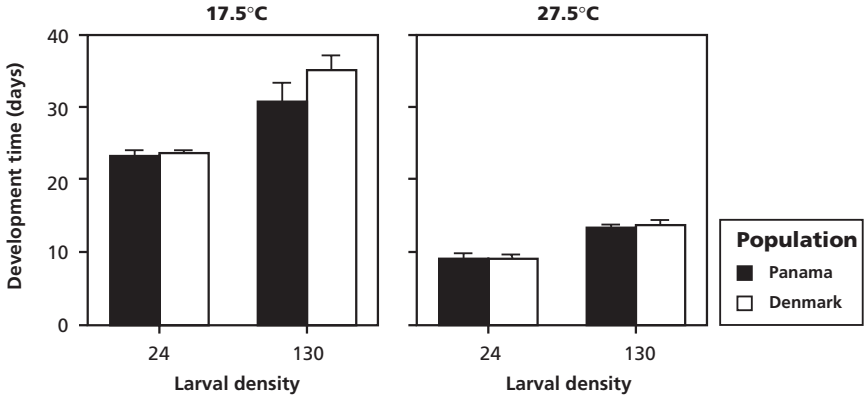


Fig 2. Development time (days) of a temperate and a tropical population of *Drosophila melanogaster* reared at 17.5°C and 27.5°C and low and high larval density. Means and 95% confidence intervals are depicted.

(p=0.012), and the data were analyzed for the rearing temperatures separately. High larval density reduced pre-adult survival at both temperatures ($p<0.001$), but the populations differed significantly only at 17.5°C ($p<0.001$). At 17.5°C the Denmark population had a higher pre-adult survival due to very good performance at low density (fig. 3), but declined stronger in survival at high density than the Panama population ($p<0.001$). When the data were split according to larval density, the Denmark population had a higher larval survival ($p<0.001$) at the low density. More importantly at both densities a significant population × temperature interaction was found ($p_{17.5°C}=0.001$ and $p_{27.5°C}=0.033$). Therefore, the geographical populations survive best at their "native" temperatures and at low densities.

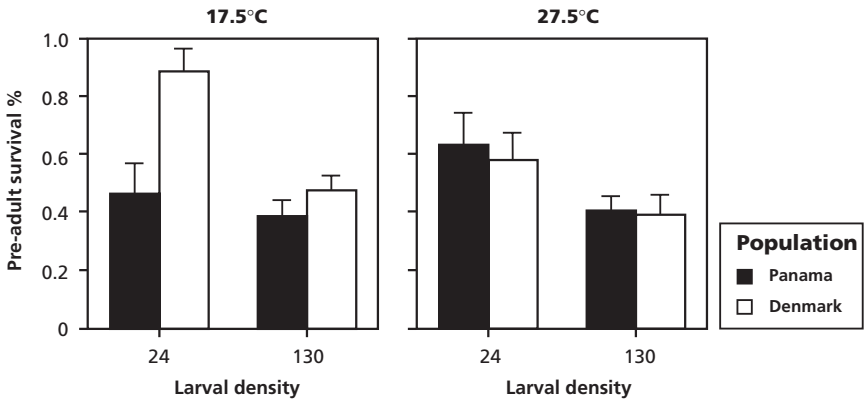


Fig 3. Pre-adult survival (%) of a temperate and a tropical population of *Drosophila melanogaster* reared at 17.5°C and 27.5°C and low and high larval density. Means and 95% confidence intervals are depicted.

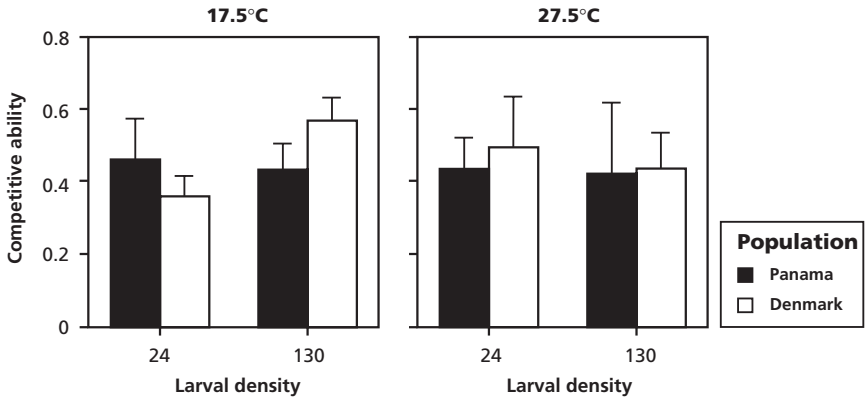


Fig 4. Larval competitive ability measured as the relative survival percentage to adult with a competitor strain present. Means and 95% confidence intervals for a temperate and a tropical population of *Drosophila melanogaster* reared at 17.5°C and 27.5°C and low and high larval density.

Competitive ability

Considering larval competitive ability, only the three-way interaction (P×T×D) was significant. When analyzed for the rearing temperatures separately, the populations did not differ at either temperature. At 17.5°C a significant density ($p=0.008$) and a significant populations × density interaction ($p<0.001$) was found. However, the pattern was the opposite to the one found for absolute survival (fig. 4). At high density a higher competitive ability was detected, due to a relative increase in the performance of the Denmark population. At their "native" rearing temperature (17.5°C), the Danish larvae outperform the Panamanian larvae, when tested against a competitor strain. Possibly they do so by competing in a different way which might be more suitable for lower temperatures. When split to larval density no effect was found to be significant.

Fecundity

Fecundity was measured as the number of eggs laid per day. Temperature and density had a significant effect ($p<0.001$, both) on the number of eggs laid per day. Larvae raised at high temperature and low density laid more eggs as adults. There was no significant difference between the populations and no interactions were found (fig. 5).

Longevity

The age at which 50% of the adult cohort had died, LA50, was used as a measure of longevity. The four-way interaction (P×T×D×S) was significant ($p=0.028$), and consequently the data were analyzed for the rearing temperatures separately. At the high rearing temperature, only a marginally

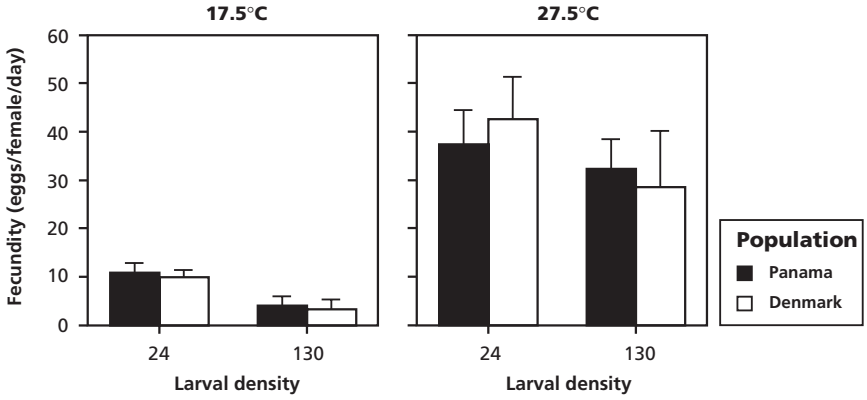


Fig 5. **Female fecundity measured as the number of eggs laid per day. Means and 95% confidence intervals for a temperate and a tropical population of *Drosophila melanogaster* reared at 17.5°C and 27.5°C and low and high larval density.**

significant (p=0.046) three-way interaction (P×D×S) was found, but no further effects. At 17.5°C, the Denmark population exhibited a significantly longer LA50 (p=0.003) compared to the Panama population; also a population × density interaction (p=0.039) was found (fig. 6). Longevity in the Denmark population declined at high larval density whereas for the Panama females longevity slightly increased with density. In summary, the high longevity of the Denmark population at its "native" temperature was more sensitive to the detrimental effect of high density than the longevity of the Panama population. When the data were analyzed for each larval density, low rearing temperature increased (p<0.001) LA50 at both densities. The populations differed (p<0.001), and the populations × temperature interaction (p=0.007) was significant only at low density and in females.

Bootstrap estimates of overall fitness: r_c

From the individual fitness components, a composite measure of fitness (r_c) has been constructed (see Statistical Analysis). This fitness measure is an approximation of r, the intrinsic rate of population growth. From the empirical distribution of bootstrap estimates of r_c, the mean and approximate 95% confidence intervals were derived for the eight groups under consideration (fig. 7A: 17.5°C, fig. 7B: 27.5°C). Using this measure of fitness, the Denmark population significantly outperformed the Panama population at 17.5°C combined with low larval density. Under these conditions, r_c of the Denmark population was the double of that of Panama. Note that, the Panama population performed better at 27.5°C, most notably when combined with high larval density, although not significantly so.

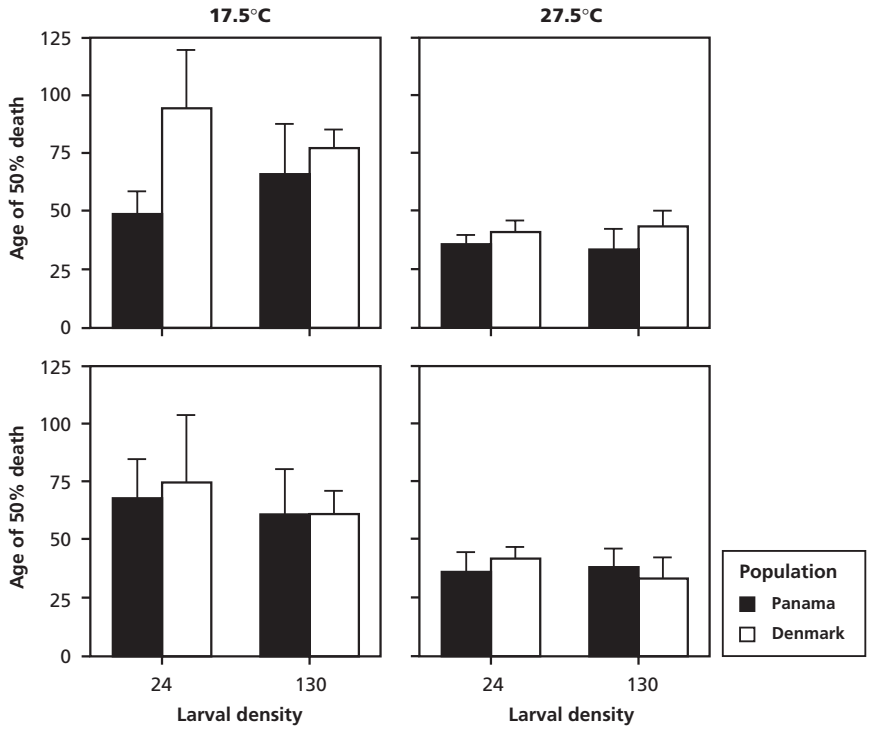


Fig 6. Longevity measured as the age when 50% of the adults decease. Means and 95% confidence intervals for a temperate and a tropical population of *Drosophila melanogaster* reared at 17.5°C and 27.5°C and low and high larval density.

Bootstrap analysis of the association between pre-adult survival and female weight

Pre-adult survival was the only fitness component that exhibited a population by rearing temperature interaction at both densities. An additional analysis was performed, to study the temperature-induced shifts in the association between pre-adult survival and adult weight. The difference in pre-adult survival between Panama and Denmark at the four temperature-density combinations were used, as well as the difference in female weight between Panama and Denmark, raised under similar temperatures and densities. Pre-adult survival and female weight had been measured independently from each other. Possible combinations were generated by resampling both pre-adult survival and female weight, and assigning random pairs (see Statistical Analysis).

In general, temperate adults are larger, consequently the resampled difference in weight (Δ=P-D) is expected to tend to negative values under all conditions. From the population × temperature interaction in pre-adult

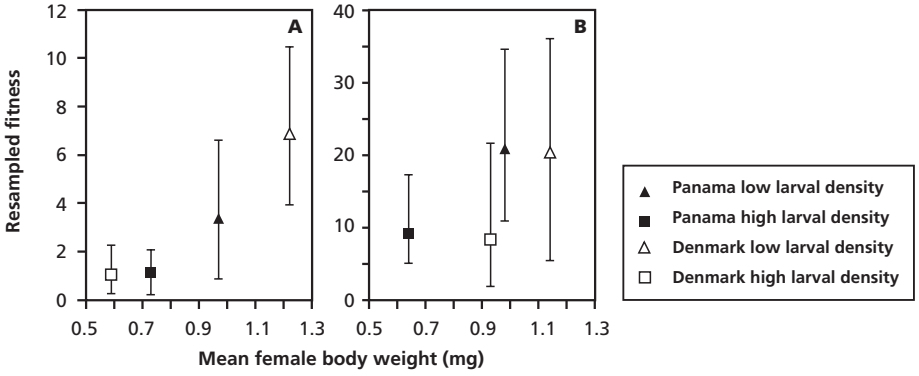


Fig 7A and B. **A composite measure of fitness (r_c) constructed from the individual fitness components. Means and 95% confidence intervals were estimated by resampling the data. The data on a tropical and a temperate population, reared at low and high density is presented separately for the rearing temperatures (17.5°C in panel A, 27.5°C in panel B).**

survival, we expect the resampled difference in pre-adult survival to tend to negative values at 17.5°C but to positive values at 27.5°C, where the tropical population survives best. In a scatterplot, we expect the resampled data from 17.5°C to occupy the lower left quadrant of the plot, whereas the resampled data form 27.5°C should occupy the lower right quadrant.

At low density (fig. 8A) the 17.5°C resampled data almost exclusively occupied the lower left quadrant. This means that the Denmark population reared at 17.5°C and low density is capable of obtaining high pre-adult survival *and* large female weight. The 27.5°C resampled data overlapped with the other temperature but extended into the lower right quadrant. This means that the Panama population at 27.5°C and low density outperforms the Denmark population in pre-adult survival at a smaller female weight.

To quantify the probability that the observed pattern is due to chance only, the Odds Ratio was calculated, comparing the low vs. the high temperature at low density (see Statistical Analysis). This statistic quantifies how more likely the 17.5°C data are to be in the lower left quadrant (where expected) vs. the 27.5°C data (which are expected in the lower right quadrant). The mean OR was 7.32, standing out from OR=1 if no pattern was present. To estimate the robustness of this value, the most extreme ORs possible from the resampled data were calculated. The highest value was 38.5 and more importantly the lowest value was 1.75 which is larger than the value 1 expected under the null hypothesis. This result shows that Panama flies can significantly outperform Denmark flies in an important fitness component, while being small. There is a significant difference in the association between adult weight and a fitness component between the tropical and temperate population.

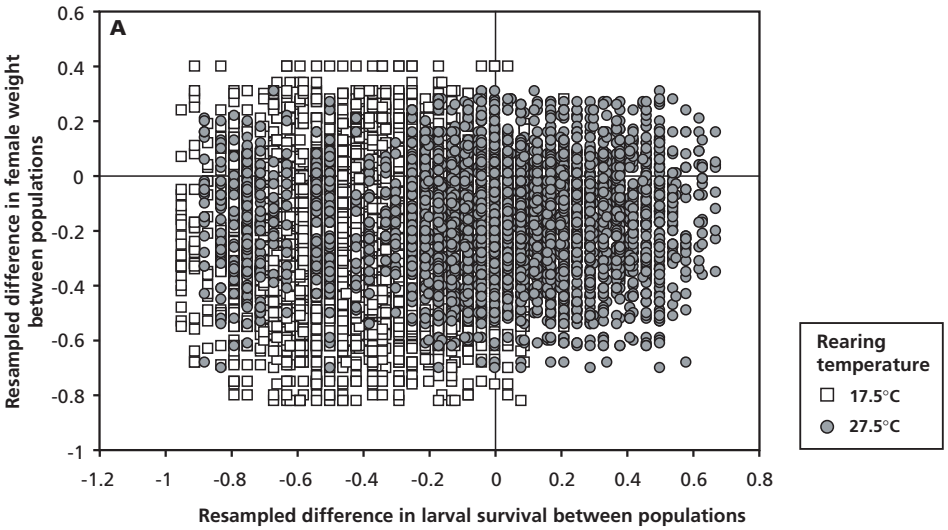


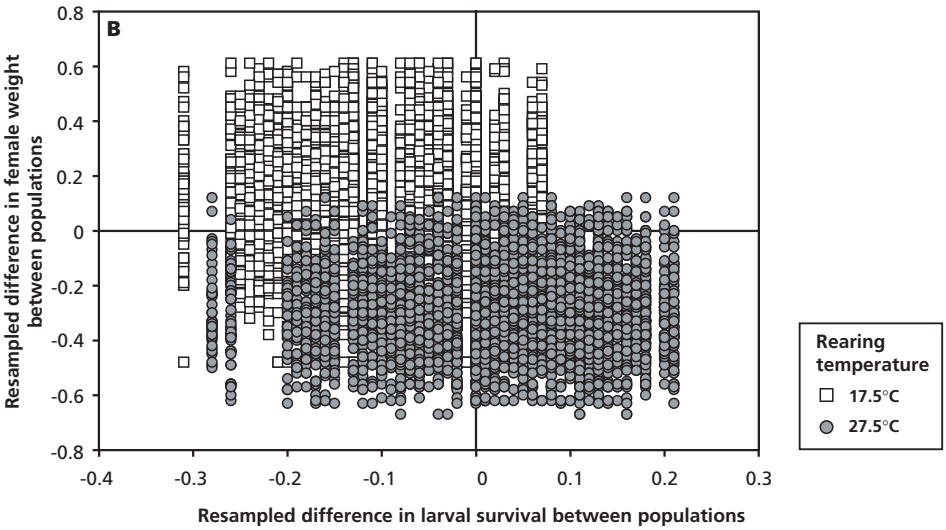
Fig 8A and B. Scatter plot of the resampled differences between the populations in female weight vs. the resampled differences between the populations in larval survival for 17.5°C and 27.5°C. The plots depict the distribution of the possible associations between adult size and larval survival across

Figure 8B shows that at high density there was considerable overlap between the temperatures. Opposite to the well documented pattern of larger adult weight in temperate populations, we found significantly lower female weight in Denmark compared with Panama at 17.5°C and high density. Due to this result, the resampled data from 17.5°C did not show any pattern (fig. 8B), and further statistical analysis was not pursued at high density. However, note that the 27.5°C data did tend to occupy the lower right quadrant, with virtually no data points from 17.5°C in that region.

Discussion

Temperature dependent fitness of large body size

Drosophila melanogaster adults are smaller in the tropics than in populations in temperate climates. The repeated pattern (Coyne and Beecham 1987; David et al. 1977; Gilchrist and Partridge 1999) argues for warm temperature specific selection resulting in a small adult. Our cold adapted population, from Denmark, outperforms the warm adapted populations from Panama at the lower temperature and low density only. In all other comparisons between the populations, a significant difference in body weight did not coincide with a significant difference in overall fitness. "Big is only better" in a cold environment and without extensive interactions with conspecific larvae. Why warm adaptation consistently leads to a small adult body, when in general



populations. The resampled data is presented separately for the rearing densities (low density in panel A, high density in panel B).

a larger size coincides with greater fitness (reviewed by Blanckenhorn 2000), remains to be unraveled.

Temperature dependent trait associations

One part of the explanation for the size difference between tropical and temperate populations emerging from these data is that large size does not necessarily coincide with a higher fitness. In fact, in most comparisons between the two populations, a significant difference in body size was not accompanied by a significant difference in fitness (measured as the constructed fitness r_c), other than at 17.5°C and low density. Temperature and larval density seem capable of altering the association between fitness components.

Such shifts in trait associations induced by temperature have been documented before. Thermal stress has been shown to induce shifts in the genetic correlations between life-history traits in *D. buzzatii* (Krebs and Loeschcke 1999). In general, thermal stress decreased life-history correlations. Other studies in *D. melanogaster* showed that the ability to express higher levels of heat shock proteins reduce larval survival (Krebs and Feder 1997) but increase adult longevity (Tatar et al. 1997). The physiological mechanism to cope with thermal stress altered the association between fitness components. Seemingly, thermal stress selected for an adult vs. a pre-adult fitness component, by increasing longevity at cost of larval survival.

However, the use of stressful environmental temperatures may not reflect possible naturally occurring shifts in trait associations. In another study,

selection lines maintained at temperatures within the normal thermal range of the species also demonstrated shifts in the association between body size and longevity. The major conclusion by Norry and Loeschcke (2002) was that the topography of the selection surface relating body size to longevity was temperature dependent and differed between lines. The positive correlation between thorax size and longevity found in their control lines at 25°C contrasted with the negative correlation between size and longevity in their cold stress selected lines tested at 14°C.

Complementary pattern of temperature sensitivity of a fitness component between populations

Our results suggest that temperature induced shifts in the genetic associations between body size and fitness components underlie the pattern of geographical variation in body size. Evolutionary adaptation to a temperate (Denmark) vs. a tropical (Panama) climate resulted in a cold adapted population specialized to grow efficiently large, have an increased larval survival and higher adult longevity. However, this specialized population seemed to be sensitive to environmental perturbations and lost its advantage at high temperature or high larval density, even though in general still larger. Although our cold adapted population did loose its advantage, it did not perform significantly worse under non-native conditions, in agreement with earlier studies comparing cold adapted vs. warm adapted populations (McCabe and Partridge 1997; Reeve et al. 2000). In other words, although big was not fitter in all conditions, small did not seem to be fitter under any conditions. Some important aspect of adaptation to high temperature remains to be uncovered.

In agreement with our previous finding (Chapter 3), we detect adaptive variation in pre-adult survival at both larval densities, signified by a population × rearing temperature interaction. The temperate population survived best at 17.5°C and the tropical population survived best at 27.5°C, at low and high larval density alike. This pattern of P×T interaction might account for the adaptive variation in body size. Tropical larvae seemed to survive best at 27.5°C while producing a smaller adult whereas temperate larvae survived best at 17.5°C while producing a large adult.

Thermal evolution of larval traits may alter the association between size and fitness

Our impression is that thermal selection might act on larval traits and the correlated response in adult size might be shaped by the temperature dependent association between adult size and larval fitness components. Larval traits are plausible targets of thermal selection as geographic populations and/or laboratory thermal selection lines have been shown to diverge in two

fundamental aspects. Cold adaptation seemed to involve more efficient growth (Neat et al. 1995; Robinson and Partridge 2001; Chapter 2 and this study) while warm adaptation seemed likely to result in increased tolerance to biotic interactions as high larval density (Partridge et al. 1994b). An interesting question remains: why should selection on different aspects of larval life by different thermal regimes affect trait associations?

An explanation might lie in the possible physiological mechanism of cold adaptation. Clinal variation in body size, and thus growth efficiency, parallels the clinal variation in enzyme polymorphisms. For metabolic enzymes, higher frequency of more active alleles towards colder regions have been reported (Eanes 1999; Verrelli and Eanes 2001). The net effect of possessing intrinsically more efficient enzymes when adapted to a cold climate would be to maintain physiological homeostasis against the direct effect of colder environmental temperature. The polymorphisms in enzyme activity show countergradient evolution. Countergradient variation is a well established geographical pattern of genetic variation, in which genetic influences on a trait oppose environmental influences, thereby minimizing phenotypic change along the gradient (Conover and Schultz 1995). As the environmental effect of low temperature is to reduce the rate of enzymatic reactions, countergradient evolution predicts selection for intrinsically more efficient metabolism.

However, a more efficient metabolism evolved to cope with a restrictive abiotic environment might have a biological side effect. Competitive ability can be achieved by two different means (Joshi and Thompson 1995): either by (1) increased capability of reducing the competitor numbers (effectiveness) or by (2) increased tolerance to the inhibitory effect of others (resistance). Enhanced metabolic efficiency seems more capable of increasing resistance than increasing effectiveness. Cold adapted genotypes of high intrinsic metabolic efficiency might be indifferent to biotic interactions and capable of both good survival and efficient growth, until larval density becomes too high. If this hypothesis is correct, larvae of cold adapted genotypes would perform poorly when biotic interactions would become too extensive to be tolerated.

In contrast, warm adaptation might lead to increased effectiveness in competition and effectiveness might be associated with less efficient growth. Physiological mechanisms of competition, i.e. some stress, are likely to consume energy or other resources and allocation to stress resistance has been shown to be costly in terms of correlated fitness traits (Chippindale et al. 1998). Such "effective" genotypes would fail to grow efficiently to build a large adult body but could prevail under conditions of extensive biotic interactions such as high density.

The hypothesis outlined above is consistent with data on the pattern of body size variation and sensitivity to larval crowding between temperature selected populations. Additionally, it assumes differences in larval competitive strategies, probably as a result of different physiology to regulate resource management. Consider larval competitive ability: in this study the temperate population outperforms the tropical one only at 17.5°C. This suggests that the two populations compete in different ways, i.e. that larval competition is according to different strategies. Therefore, the main conclusion is a question: is the key to thermal evolution of body size to be found in evolved differences in larval growth and competitive strategies, rather than in direct selection on adult size itself?

Acknowledgements

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Chapter 2

Experimental evolution in *Drosophila melanogaster*: interaction of temperature and food quality selection regimes

Zoltán Bochdanovits and Gerdien de Jong

Evolution (in press)

Abstract

In *Drosophila*, both the phenotypic and evolutionary effect of temperature on adult size involves alterations to larval resource processing and affects other life-history traits, i.e. development time but most notably, larval survival. Therefore, thermal evolution of adult body size might not be independent of simultaneous adaptation of larval traits to resource availability. Using experimental evolution lines adapted to high and low temperatures at different levels of food, we show that selection pressures interact in shaping larval resource processing. Evolution on poor food invariably leads to lower resource acquisition suggesting a cost to feeding behavior. However, following low temperature selection, lower resource acquisition led to a higher adult body size, probably by more efficient allocation to growth. In contrast, following high temperature selection, low resource acquisition benefited larval survival, possibly by reducing feeding-associated costs. We show that evolved differences to larval resource processing provide a possible proximate mechanism to variation in a suite of correlated life-history traits, during adaptation to different climates. The implication for natural populations is that in nature thermal evolution drives populations to opposite ends of an adult size vs. larval survival trade-off by altering resource processing, if resource availability is limited.

Keywords: experimental evolution, thermal evolution, body size evolution, larval resource processing, *Drosophila melanogaster*

Introduction

In ectotherms, development time and body size are intimately connected, but food shortage and low temperature cause opposite developmental correlations (Atkinson and Sibly 1997). At lower food supply, slower development often leads to smaller adult body size, which is interpreted as a consequence of limited resource availability. At lower temperatures, slower development goes together with larger adult body size suggesting an alteration in the processing of resources between temperatures. This opposite pattern in the relation between body size and development time seems to be general in ectotherms (Atkinson and Sibly 1997). Both these phenotypic patterns are clearly present in *Drosophila melanogaster* and other *Drosophila* species (Gebhardt and Stearns 1988).

Apart these phenotypic effects, adaptation to the laboratory thermal environment influences body size and development time in populations of *Drosophila melanogaster.* After several years of culturing at constant temperatures, body size in cold adapted populations proved larger than body size in populations kept at higher temperature, at every rearing temperature tested (Cavicchi et al. 1989; Huey et al. 1991). This indicates that laboratory thermal evolution induced genetic changes between the lines resulting in the observed difference in body size, in addition to and in the same direction as the phenotypic effect of developmental temperature. The proximate mechanism of this evolutionary divergence was proposed to be a higher conversion efficiency of food to adult body mass by cold adapted larvae both in the laboratory (Neat et al. 1995) and in natural populations (Robinson and Partridge 2001). This hypothesis is in line with the result showing that cold adapted populations that have a genetically determined larger adult body size also exhibit a slightly faster pre-adult development (Partridge et al. 1994). *Drosophila melanogaster* populations from temperate regions too have both genetically larger body size and faster larval development (de Moed et al. 1998; Robinson et al. 2000) than tropical populations (but see van 't Land et al. 1999).

In addition to divergence in adult body size and pre-adult development time, laboratory thermal selection has been shown to result in differences in sensitivity to larval crowding (Partridge et al. 1994). Larval survival in populations adapted to lower temperatures proved higher compared to survival of larvae from high temperature populations, when tested at low temperatures and low larval density. However, the relative survival of cold adapted larvae declined as larval density increased. This pattern suggested that the mechanism that enabled cold adapted larvae to convert food more efficiently to adult body mass increased sensitivity to larval crowding, i.e. decreased larval competitive

ability (Partridge et al. 1995). This finding agrees with the common observation that populations of *D. melanogaster* stabilize at lower densities when maintained at lower temperatures.

Thermal evolution of *D. melanogaster* seems to involve changes in pre-adult resource utilization resulting in co-ordinated alterations in adult size, larval development rate, and pre-adult survival. Variation in resource availability during selection could be expected to alter the outcome of thermal evolution. To understand body size evolution under different thermal regimes, it is imperative to disentangle the effects of selection by ambient temperature and by availability of resources. Availability of resources and larval density are not identical: larval density should be kept constant while resource availability varies. We are not interested in direct larval interference, or in different sensitivities to larval waste products (Botella et al. 1985).

The aim of this study is to investigate how thermal selection in combination with selection on larval resource availability shapes adult body size and correlated traits. Evolution of larval resource acquisition or evolution of efficient resource usage conceivably underlies any changes in body size.

We used four experimental evolution lines. Stocks were maintained at high and low constant environmental temperatures, and on standard food or on a medium containing half of the regular nutritional value. This set-up yields the following four combinations: regular food, low temperature (RL), regular food, high temperature (RH), poor food, low temperature (PL) and poor food, high temperature (PH). Larval density was low, preventing direct interference due to crowding of larvae. Following 10 generations of laboratory natural selection, the four lines were tested at both low and high ambient temperatures on regular food, for development time, feeding rate, larval survival and larval and adult size.

The expectation is that both adaptation to lower temperature and adaptation to poor resource availability involve efficient resource management. Efficient resource management could involve enhanced acquisition, as a higher feeding rate, or more efficient food processing, leading to decreased development time. However, the main question is whether evolved efficiency in resource management would have different effects on organismal traits (i.e. adult size and larval survival) when it is the result of different selection pressures. We attempt to investigate three issues. Would an increased capacity for resource management increase adult body size, when evolved to cope with a cold thermal regime? When an increased efficiency in resource management is an adaptation to poor food quality, would it benefit larval survival rather than adult size? Most importantly, will adaptation to low temperature and low food quality be independent from each other? That is: if lower temperature would lead to larger adults and poor food to higher larval survival, would the

low temperature/poor food environment select for both relatively large adults *and* high survival? Or would some sort of interaction or trade-off appear?

Materials and Methods

Experimental evolution lines

Wild *Drosophila melanogaster* were collected in Houten (the Netherlands) during summer 1998. From this large stock, four experimental evolution lines were set up in 10 vials each; a vial has a diameter of 2.5 cm and contained 10 ml medium. Larval density was kept at 20 larvae per vial throughout the experiment: no interference competition between the larvae is intended. Four combinations were used of two different rearing temperatures, 17.5°C and 27.5°C, and two larval food qualities, standard corn medium (R) and one with half of the nutrient content (P). The low nutrient content represents an environment where resource availability to the developing larvae is limited without the deleterious effects of larval crowding. These lines will be referred to as PL (poor food, 17.5°C), RL (regular food, 17.5°C), PH (poor food, 27.5°C) and RH (regular food, 27.5°C), respectively. Adults were collected immediately upon emergence from the 10 vials of a line. These adults were pooled over vials, and were kept in bottles at 22.5°C, on standard medium and low density. After all flies had eclosed and aged for at least two days, adults of each line oviposited for 3 hours at room temperature. The next generation of each line was started with 10 vials at 20 eggs per vial. This approach allows for selection on larval resource acquisition at different temperatures, without the confounding effect of adaptation by the adults. Selection on development time from adult egg-laying time is avoided. After ten generations the lines were compared for development time, feeding rate, larval survival and larval and adult size, at two test developmental temperatures, 17.5°C and 27.5°C and under regular food conditions.

Experimental conditions

To control for possible non-genetic parental effects, flies were reared at the experimental temperature for one generation before egg-laying. Up to one hundred females from a selection line oviposited for 3 hours, at room temperature. Egg-laying took place in empty bottles covered with watch glasses containing 4 ml of a 1.9% agar-medium and a drop of a thick yeast suspension. Thirty eggs were put into a glass jar of diameter 3.5 cm containing 15 ml of standard corn medium.

From these eggs, larvae were reared on standard corn medium at temperatures representing the high (27.5°C) and low (17.5°C) regions of the physiological range for *D. melanogaster.* For measuring recovery percentage, weight and feeding rate, larvae were gathered at several times during development. Larvae were collected at 17.5°C after 98, 113, 122, 135, 148, 158 hours and at 27.5°C after 39, 51, 63, 75 and 87 hours of development. In addition, adults were allowed to emerge and were collected.

To collect the larvae from the medium a saturated sugar solution was poured in the jars which were placed on an electric heater. After 5 minutes, the solution was poured through a sieve and larvae from 3 replicate vials were handpicked using a small paintbrush. Larvae were collected on ice in a petri-dish moist with a solution iso-osmotic to the larvae. This procedure was repeated three times until no further larvae were recovered.

Measurements

Weight

Fresh weight and dry weight of 20 to 60 larvae, and 60 males has been determined in groups of five to the nearest 0.01 µg, using a Mettler ME 22 microbalance. For drying the specimens a Virtis 5L freeze drier has been used.

Feeding rate

For each experimental group the feeding rate of fifteen individual larvae was measured at the last three time points considered. Larvae were tested on a petri dish filled half way with 5% agar overlaid with a thin layer of a suspension of living yeast. Larval feeding involves scooping or rasping with the chitinous mouthhooks. The semi-liquid food medium is ingested by a pumping action of the muscular pharynx. The scooping and pumping actions are made simultaneously and the retraction rate is visible through the translucent head region. This was quantitatively estimated for individual larvae during 30 seconds. The larvae were collected as described above. Fifteen larvae were randomly sampled. The feeding rate was scored under a low power microscope.

Development time to pupa and emergence

From each experimental line eggs were collected as described above. At both experimental temperatures 10 vials were filled with 20 eggs each. The number of pupae were counted at 8 hour intervals at 27.5°C and at 12 hour intervals at 17.5°C up to six times until no new pupae could be found. When adults started to emerge they were collected at the same intervals. Development time to pupa and adult was calculated as the average time, weighed by the number of pupae or adults found.

Statistical analysis

To investigate adult fresh weight and adult dry weight, larval survival, larval feeding rate and development time, three-factor analysis of variance, or three-factor analysis of covariance with larval weight as covariant, was used with selection temperature, selection food quality and developmental temperature as fixed factors. Significant interactions between the factor development temperature and the other factors led to separate tests per development temperature. The recovery percentage of larvae had a normal distribution and was subjected without further transformations to the analysis. The relationship between larval survival and feeding rate was investigated by a partial correlation test, correcting for the effect of larval weight. Larvae for the correlation analysis had been collected at three independent time points for each experimental line. The mean values of the traits did not differ between the different age classes within a line and the correlation coefficients were calculated across the time points.

Results

Adult body size

In the three-way ANOVA developmental temperature had a significant effect on male adult body weight. Larger adults emerged at 17.5°C compared to 27.5°C regardless of the evolutionary history of the stocks (fig. 1). A cold selection regime also yielded larger males ($p<0.001$). The effect of larval food quality during selection remained marginally non-significant ($p=0.053$), with poor food quality yielding smaller adults.

All two-way interactions between the main factors were significant. Therefore, the data were analyzed separately for the two development temperatures.

At the development temperature of 17.5°C, male body weight did not differ significantly between selection temperatures ($p=0.633$) or food quality regimes ($p=0.905$). The food quality regime by selection temperature interaction was however highly significant ($p<0.001$). That is, a strong interaction between selecting environments is evident at 17.5°C (fig. 1). Males of the PL and RH lines were significantly heavier than males of the PH and RL lines.

At the development temperature of 27.5°C, male body weight differed significantly between selection temperatures ($p<0.001$) and marginally between food quality regimes ($p=0.056$). Low temperature and a regular quality food regime led to higher male body size (fig. 1). Moreover, the food

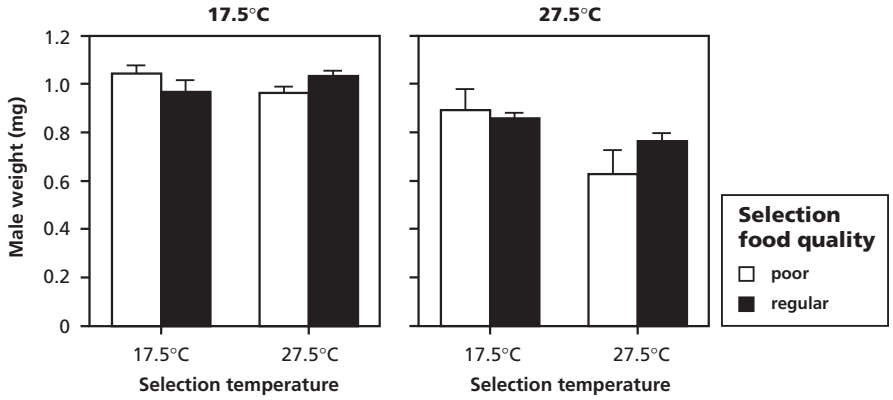

Fig 1. Male weight in mg of the four experimental lines reared at 17.5°C and 27.5°C. Means and 95% confidence intervals are shown.

quality regime by selection temperature interaction was significant ($p=0.005$).

Tests were done approximating RL (17.5°C) and RH circumstances (27.5°C), but RL and RH males did not have largest body size under their own conditions. Low selection temperature leads to efficient larvae and high adult body size, but this is more visible at the higher test temperature. The factors poor food quality and low selection temperature interact in the resulting male body size, as is evident at both test temperatures (fig. 1). At both test temperatures, PL>RL and PH<RH. In conclusion, *simultaneous* adaptation to a cold environment and poor food quality seemed most permissive for the evolution of large adult body weight.

Larval survival

Using the recovery percentage of larvae as a measure of larval survival, only a significant main effect of selection temperature ($p<0.001$) was detected, not of development temperature or food quality. A cold evolutionary history led to higher pre-adult survival (RL&PL>RH&PH). This result was accompanied by an overall significant interaction between selection temperature and larval food quality during selection ($p=0.027$). At both test temperatures food quality by selection temperature interactions were present. Associated with a cold thermal regime, selection on poor food decreased survival (PL<RL) but accompanying evolution at high temperature, selection on poor food slightly increased it (PH>RH) (fig. 2). This pattern is the exact opposite of the pattern found for male body size. Evolution at high temperature seemed permissive for maintaining pre-adult survival when adapting to poor food quality.

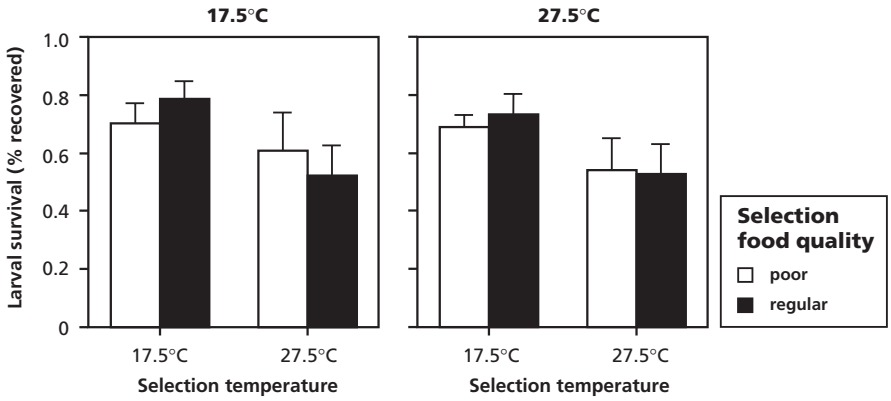

Fig 2. Larval survival as percentage of larvae recovered of the four experimental lines reared at 17.5°C and 27.5°C. Means and 95% confidence intervals are shown.

Larval feeding rate

To assess the level of resource acquisition by the developing larvae, feeding rate of larvae collected at 63, 75 and 87 hours at 27.5°C, and of larvae collected at 135, 148, 158 hours at 17.5°C -presumably late second instar - had been determined. The mean feeding rate within a group did not differ between the age classes for any combination of the three factors and the data were treated as independent samples for all subsequent analysis. Significant main effects ($p<0.001$) of selection temperature, food quality and developmental temperature were found (all $p<0.001$). Overall, the selection lines from 17.5°C showed lower feeding rates than the selection lines of 27.5°C. Selection on regular food resulted in a significantly higher feeding rate than selection at poor food. Lower development temperature is associated with slower feeding. Selection temperature as well as developmental temperature had a significant interaction with larval food quality during selection ($p<0.001$). For both selection temperature and developmental temperature, the higher temperature was associated with a higher difference in mean feeding rate between the two food conditions (fig. 3).

At both development temperatures the interaction between selection temperature and food quality is significant. However, the interaction pattern is clearly different from, and not related to, the interaction patterns of adult body weight and larval survival.

Larval survival and feeding rate

In an attempt to reveal any influence of variation in resource acquisition on survival, partial correlation coefficients between these variables were calculated, corrected for larval weight. Over all factors a significant negative correlation between feeding rate and larval survival was detected ($\rho=-0.525$,

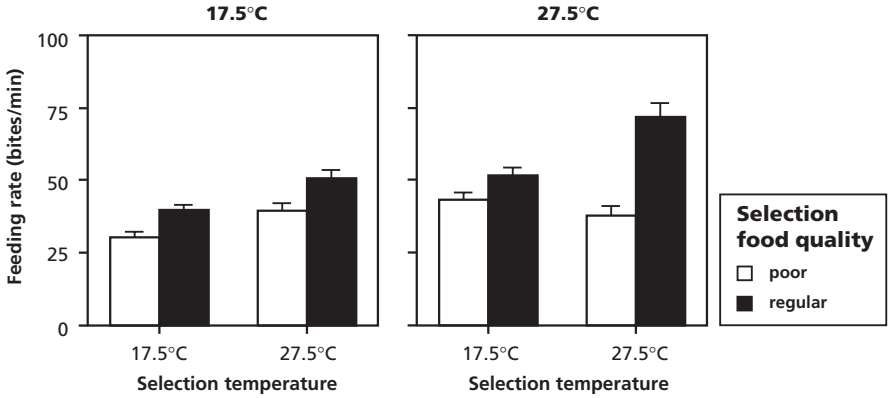

Fig 3. Feeding rate in bites per minute of the four experimental lines reared at 17.5°C and 27.5°C. Means and 95% confidence intervals are shown

$p<0.010$, df=21) (fig. 4). When the data were split for the three factors, this negative correlation was found to be predominantly present at the lower rearing temperature ($\rho=-0.619$, $p=0.042$, df=9) and within the rich food selection lines ($\rho=-0.767$, $p=0.006$, df=9).

Development time

As expected a significant effect of developmental temperature was found on development time both to pupa ($p<0.001$) and to adult ($p<0.001$). At high temperatures development is faster. Larval food quality during selection also resulted in significant differences in development times ($p<0.001$). The rich food lines showed a longer development than the poor food lines

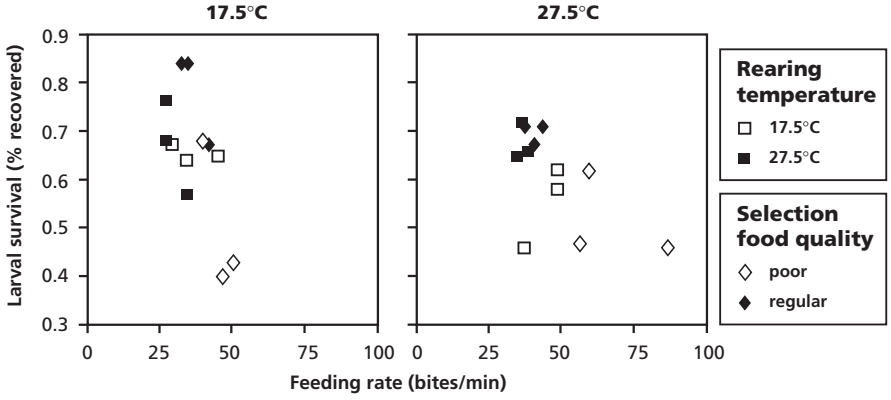

Fig 4. Scatter plot of larval survival and larval feeding rate for the four experimental groups at both rearing temperatures. Within each group, up to three different larval stages were used (see M&M, Statistical Analysis). Note that the graph shows the plain correlation between the variables, not the partial correlation corrected for larval weight as calculated.

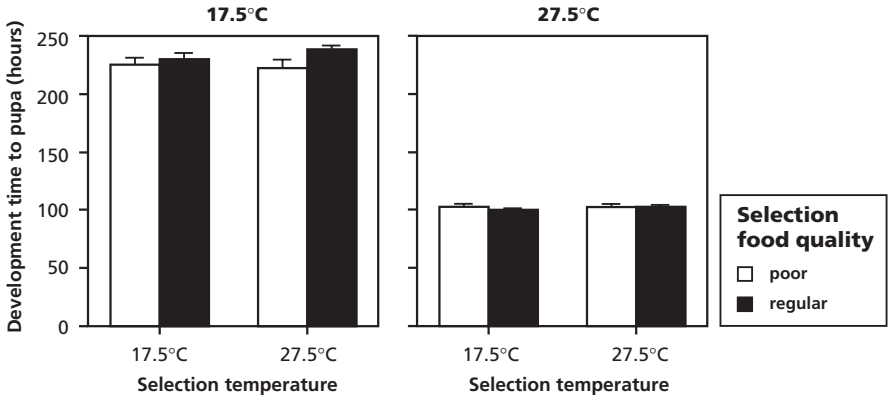

Fig 5. Development time to pupa in hours of the four experimental lines reared at 17.5°C and 27.5°C. Means and 95% confidence intervals are shown.

(RH+RL>PH+PL). Selection temperature did not bring about a difference in development time.

Interactions in development time to pupa were found between food quality during selection and developmental temperature ($p<0.001$), and between food quality and selection temperature ($p<0.012$). The same interactions were significant for development to adult as well (both $p<0.001$). At low developmental temperature the rich food selection lines showed an increase in development time over the poor food selection lines; at high rearing temperature no difference in development time was found between selection lines from poor food and rich food (fig. 5). Interestingly, this pattern was reversed when the interaction between selection temperature and selection food quality is considered. High selection temperature and rich food select to extend development. When following a cold evolutionary history the effect of food quality during selection on present development time is negligible (fig. 5).

To further analyze the interaction between the selection conditions temperature and food the data were split according to developmental temperature. At the lower rearing temperature, the interaction between selection temperature and selection food quality was just non significant in the development time to pupa ($p=0.054$). At the higher temperature significant interaction between selection temperature and selection food quality was found ($p=0.007$). Selection on poor food at low temperature results in the longest development time whereas selection on rich food at low temperature yields the fastest development time. Development time to adult showed a significant interaction between selection temperature and selection food quality at both rearing temperatures ($p=0.003$ for 17.5°C and $p<0.001$ for 27.5°C). At the lower rearing temperature we found that the high temperature

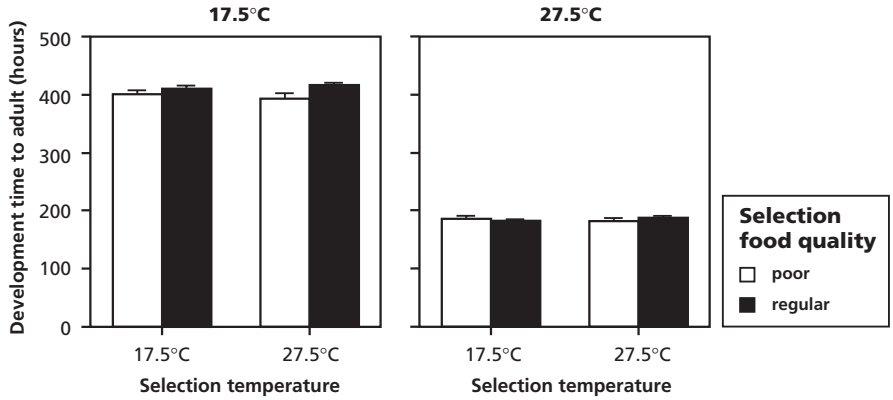

Fig 6. Development time to adult in hours of the four experimental lines reared at 17.5°C and 27.5°C. Means and 95% confidence intervals are shown.

selection lines are more sensitive to differences in food quality than the low temperature selection lines. Adaptation to poor food always leads to faster development. In contrast, at the high rearing temperature, a cold evolutionary history yields a longer development when selection has been on poor food quality (fig. 6). This is the same pattern as found for the development time to pupa as well.

Discussion

Ambient temperature and food availability interact in experimental evolution of *Drosophila melanogaster*

In this study we investigate the outcome of thermal selection taking variation of resource accessibility into account. Both adult body size and larval survival are known to respond to thermal selection and the availability of resources is likely to affect life-history traits in general (Atkinson and Sibly 1997). At the phenotypic level, variation in resource availability has been shown to affect life-history trade-offs in *Drosophila*. Enhanced resource acquisition was shown to lead to increased fecundity at cost of reducing longevity, in spite of only a small redirection of resources from storage to egg production (Simmons and Bradley 1997). Here we search for an effect of resource availability on the outcome of thermal evolution. For all traits considered, an interaction between selection temperature and nutrient quality during selection was found. The outcome of thermal selection is clearly dependent on the accessibility of resources.

Thermal evolution of body size and larval survival at varying nutrient levels

A larger adult body size coincided with a lower larval survival at both selection temperatures. This is not surprising as a trade-off between size and survival has been shown previously (Chapter 4)(Stevens et al. 1999; Stevens et al. 2000). However, coinciding with the low selection temperature, evolution on poor food yielded larger flies with a lower larval survival under the test conditions compared to evolution on regular food. At the high selection temperature, the stocks selected on regular food became larger and produced lower survival opposite to the evolutionary effect of poor food. This means, that thermal selection caused marked differences in the way resources were obtained and utilized. A similar phenotypic pattern has been described in the Antarctic fish *Notothenia coriiceps*. Seasonal variation in growth rate was shown to be mediated by seasonal variation in resource utilization rather than direct effect of resource availability or temperature (Coggan 1997). In parallel, we seek an explanation for the evolved patterns of body size and larval survival in changes to components of resource management (i.e. development time and feeding rate).

Thermal evolution of resource management at varying nutrient levels

An appropriate measure of resource acquisition is larval feeding rate. It has been established as a major component of larval competitive ability (Bakker 1961). Genetic variation in feeding rate has been described before (Sewell et al. 1975; Ohnishi 1979), and coincided with variation in body size, development time and larval viability (Ruiz-Dubreuil et al. 1996; Santos et al. 1997; Borash et al. 2000). The emerging consensus reveals that faster feeding rate facilitates larval viability under crowded conditions.

Interestingly, both development time and feeding rate showed a rather similar response to these selection pressures. In the low temperature selection lines, large adult size and lower larval survival was achieved by a slightly lower feeding rate in the same development time. Total resource acquisition slightly decreased in the poor food vs. rich food lines at low selection temperature. This suggested that cold adaptation combined with low resource availability selected for an increased efficiency of resource allocation to adult size. This result is in line with previous findings (Neat et al. 1995)(Chapter 4). In addition, natural selection by larval crowding has been shown to result in a trade-off between food acquisition and utilization (Joshi and Mueller 1996; Joshi 1997). Our results suggest the same trade-off, as a higher efficiency of utilizing resources to build the adult body coincided with a slightly lower resource acquisition.

In contrast, at the high selection temperature large adults and decreased larval survival were the result of largely enhanced feeding rate and a small, but significant, increase in development. Total resource acquisition was greatly enhanced in the rich food vs. poor food lines at the high selection temperature. This pattern also showed that selection on poor food at high temperatures leads to a lower capacity of resource acquisition leading to an increase in larval survival. Seemingly, resource acquisition is costly. Indeed, increased resource acquisition has been shown before to reduce larval survival (see below) (Chippindale et al. 1998). In summary, when resource availability is limiting, evolution at low temperature enhances adult size in contrast to evolution at high temperature when larval survival is increased, both by decreasing resource acquisition. This pattern equals the evolution of a life-history trade-off when resources are limiting.

A proximate mechanism of evolved life-history variation?

The actual mechanism behind the evolution of the complementary patterns of body weight and larval survival must involve the observed changes in resource processing. The evolved variation in development time seems negligible. However, at both selection temperatures, poor food caused an evolved decrease in resource acquisition. The observed decrease in the capacity for resource acquisition when the availability is low may be expected. Attempts to acquire larger amounts of resources when they are less accessible may require a disproportionate investment of effort, thus resulting in less net nutrient gain. Chippindale et al. (1998) have already suggested that pre-adult resource acquisition may be costly as adult desiccation resistance mediated by larger larval carbohydrate stores leads to lower larval survival and longer development.

We detect costs to increased resource acquisition as well; however, the different selection regimes seem to impose different costs. A cold thermal regime yields a lower adult body size coinciding with enhanced feeding rate, while under a warm thermal regime increased feeding rate lowers larval survival. Note the difference between our results considering the effect of food quality and results cited above showing that increased feeding rate enhances larval survival under crowding. When selection does not involve competitive ability, rather efficiency, enhancement of resource acquisition does not seem to be univocally beneficial. This pattern resembles the well known phenomenon of lower caloric intake increasing longevity in a variety of organisms (Gerhard 2001).

In summary, considering the possible mechanism of thermal evolution of body size, the conclusion is that evolved decrease in resource acquisition

lowers the associated costs, and allows for investment in the trait most strongly increasing fitness under the selection conditions. When nutrient availability is low, simultaneous adaptation to a cold environment leads to a more efficient allocation of resources to adult size. Different studies (Zamudio et al. 1995; McCabe and Partridge 1997; Nunney and Cheung 1997; Reeve et al. 2000)(Chapter 1) have previously suggested that a large adult body size enhances fitness at low temperatures only; the observed investment in size in our study is in line with these findings. When nutrient availability is low, simultaneous adaptation to a warm environment leads to increasing larval survival by reducing the feeding associated costs.

The implication of this study for natural populations is that it seems that both in temperate and tropical regions *Drosophila* face low nutrient availability and evolve to the opposite ends of the adult size vs. larval survival trade-off.

Acknowledgements

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Chapter 3

Temperature dependence of larval resource accumulation and adult body size in *Drosophila melanogaster*

Zoltán Bochdanovits and Gerdien de Jong

Abstract

To identify a proximate mechanism for the evolution of adult body size in *Drosophila melanogaster* we performed an analysis of the temperature sensitivity of larval resource allocation. Both geographical origin and environmental temperature influence the mass-specific accumulation of nutrients by the larvae. Differences in the amount of stored triglyceride and glycogen and in the dynamics of storage were found between populations and between rearing temperatures. Larval nutrient levels predict adult size and larval survival. Genotype by environment interaction for larval survival indicates adaptive plasticity in resource accumulation and adult size. Thermal evolution of body size may be mediated by different patterns of resource allocation in the developing larvae.

Keywords: geographical cline, phenotypic plasticity, resource allocation, size, temperature

Introduction

Geographical variation in body size

Geographical variation in body size has been demonstrated in *Drosophila melanogaster* (David et al. 1977; Coyne and Beecham 1987; van 't Land et al. 1999; Gilchrist and Partridge 1999). Larger animals are found in populations from higher latitudes on all continents. The repeatability of this cline over different continents suggests that it is the result of natural selection. Temperature has been put forward as the most likely selective agent as underscored by the results of replicated laboratory populations of *D. melanogaster* kept at different temperatures for many generations (Cavicchi et al. 1989; Huey et al. 1991). The pattern of body size evolution has consistently been shown, but clines from different continents are known to have different genetic architectures, involving dominance and epistatic interactions (Gilchrist and Partridge 1999). Variation in both cell size and cell number have been implicated in underlying the thermal evolution of body size (de Moed et al.1997; French et al. 1998). In *D. subobscura* a size cline evolved in only a few decades after introduction into North America, but involved a different mechanism than the ancestral cline in wing size (Huey et al. 2000). The exact nature of the evolutionary process that consistently leads to a conserved pattern, though seemingly through alternative mechanisms, remains to be uncovered.

These results clearly show that specific adult body sizes are favored by natural selection under particular climatic conditions; however, a certain size can be achieved by different means. New mutations set aside, the presence of alternative, genetically determined, mechanisms for producing adult body size may be the result of either historic differences in genetic variation of founder populations or selection on traits closely correlated with size. Since, similar body size clines have repeatedly and independently evolved several times, it is not likely that genetic constraints play a major role. Similar size might, however, be the result of combinations of selection pressures on other characters, i.e. life-history trade-offs (Atkinson and Sibly 1997).

Developmental plasticity and energy constraints

In addition to the evolutionary response, *D. melanogaster* exhibits developmental sensitivity, realizing a larger body when reared at lower temperatures (Coyne and Beecham 1987; David et al. 1994; Partridge et al. 1994a; Noach et al. 1996). This phenotypic plasticity of adult size may be the result of adaptive evolution. Alternatively, constraints imposed by temperature on growth and/or development during the larval stage might be responsible. Following a novel interpretation of von Bertalanffy's hypothesis, Atkinson and

Sibly (1997) argue that plasticity of adult body size is the result of a differential effect of environmental temperature on pre-adult anabolism (or resource acquisition) and catabolism (or the loss of mass to maintenance or activity). A similar logic is applied by van der Have and de Jong (1996), showing that differences in the temperature sensitivity of growth rate and differentiation rate can lead to divergence of body size. The basic prediction of both models is that relatively faster development at high temperature will lead to smaller adults, as the rate of processes consuming energy increases faster with temperature than the growth rate, the rate of biomass increase. These mechanistic explanations imply that temperature specific size is the result of physical and/or energy constraints. However, as geographic populations differ in plasticity as well as in overall size (Noach et al. 1996), it seems that the temperature sensitivity of metabolic/physiologic processes underlying the construction of adult size might be selected for. In fact, species differences in parameters of temperature sensitivity for the van der Have-de Jong model have been found (Gibert and de Jong 2001). Selection on the temperature sensitivity of pre-adult energy metabolism seems potentially capable of inducing variation in adult body size.

Temperature sensitivity of resource utilization

The assumption that selection on energy metabolism during pre-adult development underlies variation in adult body size allows us to formulate testable hypotheses on how shifts in larval resource allocation may underlie the thermal evolution of adult body size.

It could be argued that thermal evolution leads to an altered temperature sensitivity of larval resource acquisition vs. utilization. Different ratios of acquiring and utilizing resources by the developing larvae may lead to variation in adult body size. If cold adaptation leads to a relatively larger accumulation of resources compared to the consumption of resources, that will result in a seemingly more efficient construction of a larger adult body. Experimental data point towards this scenario, as an evolutionary history of laboratory cold adaptation has been shown to result in more efficient conversion of larval food into adult body mass (Neat et al. 1995). For the same amount of larval food a larger adult emerged.

This effect of cold adaptation could be the result of two different mechanisms. Either a genetic increase in resource acquisition and accumulation took place without necessarily a change in the utilization of the accumulated resources. Or a cold temperature specific increase in resource allocation to adult size may be responsible. In the latter case one may find either an increase in the efficiency of pupation or a trade-off with larval fitness-associated traits.

Larval resource accumulation and adult body size

The main question of this study is whether variation in adult body size is the result of shifts in larval resource allocation. Selection pressures altering the ratio of resource acquisition vs. utilization may yield different adult body sizes. At the same time, a given body size may be the result of different larval growth strategies resulting in the same ratio of acquiring and consuming resources.

The main goal of this experiment is to identify geographical differences in the accumulation of resources by developing larvae. If the first of the two mechanisms proposed above is true, larger adult size in cold adapted populations is the result of a higher energy content of larval tissue due to an increased accumulation of resources. Therefore, it must first be established whether larvae have a higher mass-specific content of energy storing metabolites. If so, this higher energy content of larval tissue must predict future adult size. For the second scenario to be true, the same energy content of larval tissue should predict a larger adult body size in temperate populations compared to tropical populations. This would indicate a more efficient pupation. To address the possibility of a trade-off between investment in adult size and larval fitness associated traits, larval survival will be measured. First geographical variation in larval survival should be demonstrated, before further investigations on a possible trade-off is meaningful.

Two temperate and two tropical populations will be used, reared at low and high temperatures. At several stages during development, larval size, triglyceride and glycogen content and larval survival will be measured. In addition adult size will be recorded. The data will be analyzed to yield an explanation for the geographical variation in body size in terms of evolution of the temperature sensitivity of larval resource accumulation and utilization.

Materials and Methods

Populations

Wild living *Drosophila melanogaster* have been caught in Panama (9 °N, 80 °W), Congo (4 °S, 14 °E), Denmark (56 °N, 9 °E) and Sweden (60 °N, 15 °E) between 6 to 12 months before the beginning of the experiment. These populations have been cultured at large population sizes under constant light at 17.5°C on a standard corn medium.

Experimental conditions

Egg-laying females had been reared at their experimental temperatures, in order to control for possible maternal effects. Several hundred females

oviposited for 3 hours, at room temperature. Egg laying took place in empty jars covered with watch glasses containing 4 ml of a 1.9% agar-medium and a drop of a thick yeast suspension. Thirty eggs were put into a glass vial containing 5 ml of standard corn medium.

From the eggs, larvae were reared at 17.5°C and 27.5°C. These temperatures are well within the physiological range for *D. melanogaster* but represent its high and low regions. At 72, 120, 168, 184, 200 hours for the 17.5°C group, and at 48, 72, 80, 88, 96 hours for the 27.5°C group, larvae have been collected. These timepoints represent similar developmental stages at the different temperatures, corresponding to the first, second and three different stages of the third larval instar, with the fifth timepoint being the larva just about to pupate. In addition, adults have been allowed to emerge and were collected one day after emergence. For collecting the larvae, a saturated sugar solution was poured on top of the medium, placing the vials on an electrical heater. Between 15 and 45 minutes after adding the solution the floating larvae were collected from 3 replicate vials, using a paintbrush after pouring the solution through a sieve. The number of larvae recovered has been scored. The recovery percentage represents survival from egg till the respective stage and will be used as a measure of larval survival.

Measurements

Weight

Depending on the recovery percentage, fresh and dry weight of 20 to 60 larvae and 60 adults has been determined in groups of five to the nearest 0.01 µg, using a Mettler ME 22 microbalance. For drying the specimens a Virtis 5L freeze drier has been used.

Biochemical assays

The glycogen and triglyceride contents of larvae have been determined. Groups of 10 larvae were homogenized in 300 µl of homogenization buffer (0.01 M KH_2PO_4, 1 mM EDTA pH 7.4) using a motorized Microfix morter and a molten pipette tip as a pestle. The homogenates were centrifuged at 13000 rpm for 3 minutes in order to pellet cellular and cuticular debris. Samples from the same homogenate were used for all assays.

Glycogen

Reagentia as available form the Sigma glucose determining kit (PGO enzyme, catalog no. 510-6) were utilized. The reagents were complemented by 0.1 U/ml amyloglucosidase, for converting glycogen into glucose. A 30 µl sample of the homogenate was added to a total volume of 1 ml test reagents. Following 30 minutes of incubation at 37°C, the absorption at 450 nm was measured.

Triglycerides

Reagentia as available from the Sigma triglyceride determining kit (catalog no. 336-20) were used for the colorimetric determination of the triglyceride content. A 30 µl sample of the homogenate was added to a total volume of 1 ml test reagents. Following 30 minutes of incubation at room temperature, the absorption at 500 nm was measured.

Statistical analysis

Two and three factor Analyses of Variance were used to investigate the fresh and dry weight of larvae and adults, with geographical origin, rearing temperature and gender (for adult weight) treated as fixed factors. Triglyceride and glycogen levels were analyzed by analysis of covariance, in order to control for the differences in weight between the larvae. Unless stated otherwise, all levels refer to mass-specific levels of resources. Bonferroni post-hoc analysis was performed to disentangle the effect of the different populations, following the two- or three-way ANOVA's. The relationship between larval glycogen levels and adult body weight was investigated by means of the non-parametric Spearman correlation test. The effect of environmental temperature on this correlation was analyzed by a multiple regression analysis, with glycogen level and rearing temperature as a dummy variable. Recovery percentages of the larvae were pooled over the different timepoints; an analysis of variance was used to investigate the influence of the fixed factors, geographical origin and rearing temperature. The relationship between larval survival and lipid levels was investigated by the non-parametric Spearman correlation test. An analysis of covariance was used to unravel the relationships between lipid levels and larval survival for different populations and rearing temperatures.

Results

Body size: geographical variation and phenotypic plasticity

Using 3 way ANOVA, we found, as expected, both geographical variation and phenotypic plasticity for adult size (for mean dry and fresh weight within the population × temperature combinations see table 1). Fresh weight ($p<0.001$) and dry weight ($p<0.005$) were larger in adults from the two populations from higher latitude. This difference in size (both fresh and dry weight) was predominant at the lower rearing temperature, where the two tropical populations were smaller then their cold adapted counterparts. At 27.5°C the Denmark populations reached higher body size than the two tropical populations ($p<0.05$); adult fresh weight of the Swedish population

Table 1. Fresh and dry weight of adult *D. melanogaster* and larvae prior to pupation in mg +/- S.E.M.

			Congo	Panama	Denmark	Sweden
17.5°C	adult	fresh weight	1.14 (.06)	1.16 (.10)	1.25 (.07)	1.31 (.12)
		dry weight	.33 (.02)	.34 (.03)	.39 (.02)	.38 (.04)
	larval	fresh weight	1.66 (.14)	1.80 (.04)	2.03 (.07)	1.97 (.10)
		dry weight	.47 (.05)	.48 (.01)	.58 (.03)	.48 (.04)
27.5°C	adult	fresh weight	1.06 (.09)	1.04 (.09)	1.14 (.09)	.99 (.06)
		dry weight	.33 (.03)	.29 (.03)	.32 (.03)	.33 (.03)
	larval	fresh weight	1.31 (.15)	1.34 (.11)	1.64 (.03)	1.80 (.08)
		dry weight	.33 (.03)	.35 (.04)	.46 (.01)	.48 (.02)

was lower than expected on the basis of the larval fresh weight and not significantly different from the tropical populations. Considering the phenotypic plasticity of adult size, fresh weight is higher at the lower rearing temperature ($p<0.05$), dry weight produces the same, near significant, trend ($p=0.055$).

Geographical variation and phenotypic plasticity were also found in larval fresh and dry weights prior to pupation. Larvae from populations from higher latitudes produced larger fresh ($p<0.003$) and dry weight ($p<0.005$). At the lower rearing temperature, larvae grew larger (fresh weight: $p<0.001$, dry weight: $p<0.001$).

Resource accumulation at different larval stages

Effect of rearing temperature on resource accumulation at different larval stages; main environmental effect

Triglyceride and glycogen levels responded to rearing temperature. Overall, larvae reared at 17.5°C accumulated larger levels of both resources towards the end of the larval period (fig. 1). The effect of environmental temperature on glycogen storage was brought about at an earlier age than on lipids. Significant differences between rearing temperatures were found at timepoints three and four ($p<0.001$), corresponding to the early part of the third larval instar. The cold induced increase in glycogen levels dropped back, but remained significant ($p<0.05$), prior to pupation. Triglyceride levels diverged significantly only at timepoint five ($p<0.001$). This response appeared in the Congo, Denmark and Panama lines, but not the Sweden line. At 17.5°C, Swedish larvae accumulated lower triglyceride levels throughout their development and increased rather then decreased the levels of glycogen prior to pupation.

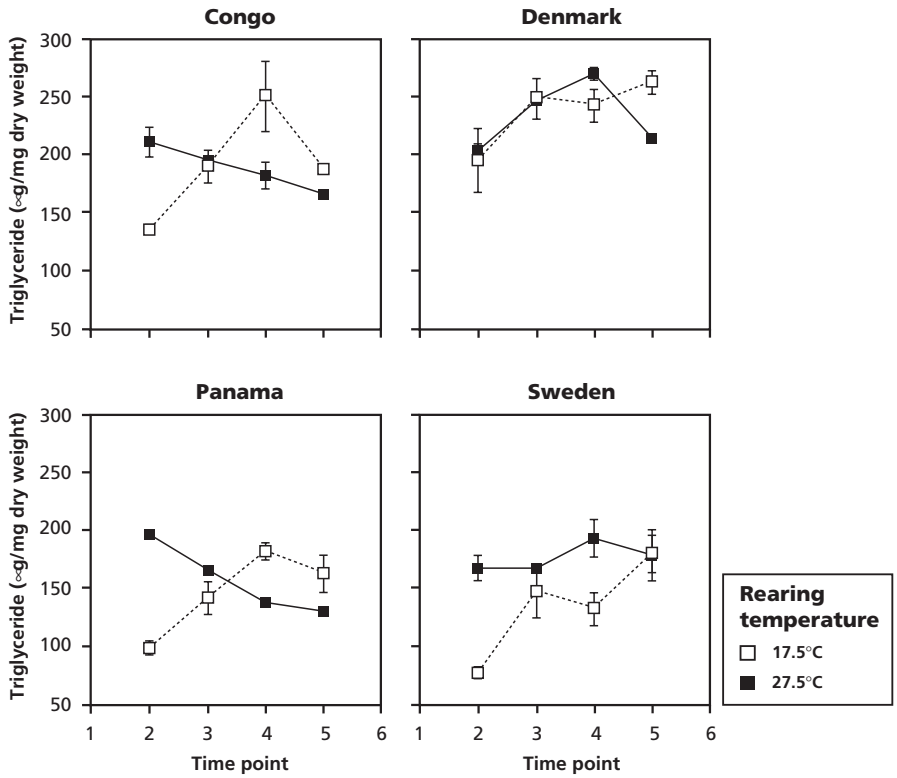
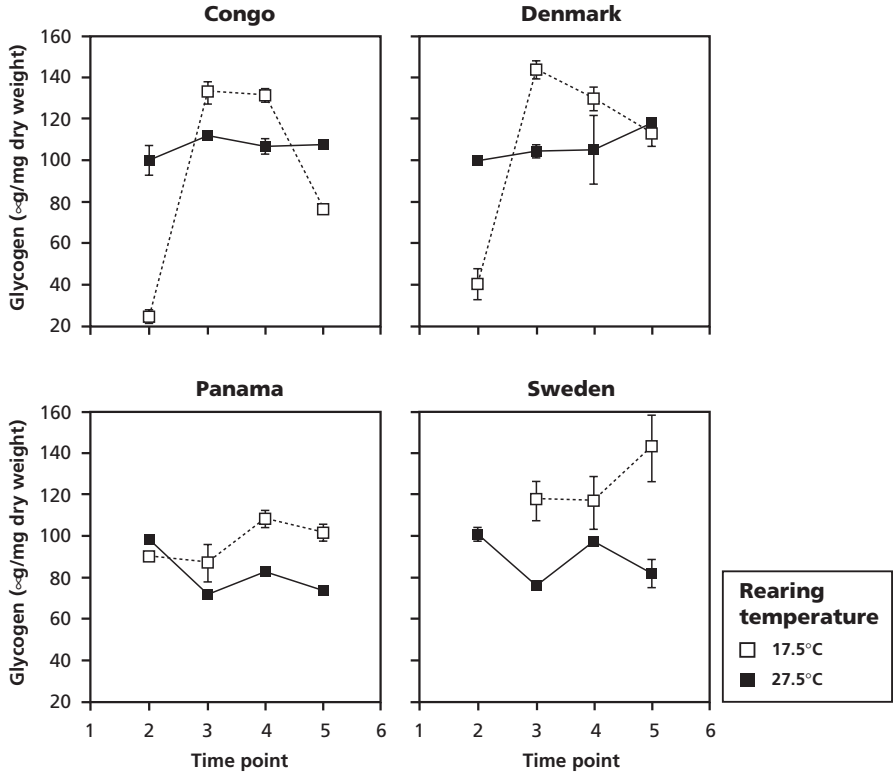


Fig 1. Pattern of mass-specific triglyceride (4 left panels) and glycogen (4 right panels) levels of larvae during development +/- SEM. Mass-specific levels are given in µg triglyceride or glycogen per mg dry weight of the larvae. Time is expressed in timepoints rather then hours to allow for

Effect of geographical origin on resource accumulation at different larval stages; main geographical effect

Differences in triglyceride and glycogen levels were found between populations. Triglyceride levels differed between the populations throughout the last three timepoints, corresponding to the third larval instar ($p<0.001$), owing to higher levels in larvae from Denmark. Swedish larvae did not differ form their warm adapted counterparts. Similarly, glycogen levels differed at timepoints three to five ($p<0.001$), due to higher levels in the Denmark and Congo larvae (only timepoint three and four). Considering the *total* amount of glycogen stored prior to pupation, both populations form higher latitudes show increased storage ($p<0.02$) compared to the two populations from tropical regions.



comparison between the rearing temperatures. These timepoints have been chosen to represent corresponding developmental stages at low and high rearing temperature.

Effect of geographical origin on the pattern of resource accumulation; Interactions

We found differences in the dynamics of resource accumulation between populations at different temperatures (fig. 1). The triglyceride content of larvae from both tropical populations, reared at 27.5°C, decreased towards the end of development. In contrast, larvae from both populations from higher latitudes, grown at 17.5°C, steadily increased their lipid reserves up till pupation. This pattern was accompanied by a geographical origin × rearing temperature interaction at timepoints three to five ($p<0.05$). Larvae from Sweden produced this dynamics as well, even though they stored surprisingly low levels of triglyceride. Additionally, when populations were reared at the temperature not corresponding to the climate they are adapted to, they all produced the same trend. Both populations from higher latitudes reared at 27.5°C and both tropical populations reared at 17.5°C, initially increased triglyceride levels but exhibited a sharp decrease prior to pupation.

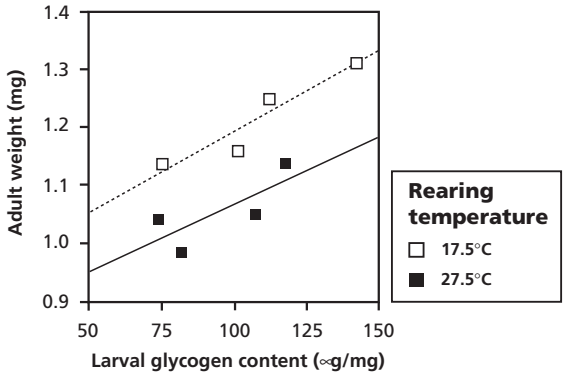


Fig 2. Relationship between larval mass-specific glycogen levels just prior to pupation and adult fresh weight. Group means of the four populations each at two experimental temperatures are plotted.

Glycogen levels remained constant throughout development when larvae are grown at 27.5°C. At 17.5°C, following an initial increase in all populations, the stored amounts diverged prior to pupation.

Larval resource accumulation and adult body size

Phenotypic plasticity and geographical variation in adult body weight was preceded by phenotypic plasticity and geographical variation in larval weight. Significant correlations between adult dry weight and larval fresh ($\rho=0.90$, $p<0.001$) and dry weight ($\rho=0.76$, $p<0.03$) underlined this result. Moreover, we found a significant correlation between the glycogen levels prior to pupation and adult body size (fig. 2). Mass-specific glycogen content related to adult fresh weight ($\rho=0.81$, $p<0.02$). This result indicated that, for a given larval size, the amount of resources stored during development predicts adult fresh weight. A multiple regression analysis using glycogen content and rearing temperature as predicting variables yielded an adjusted R^2 of 0.88. The model

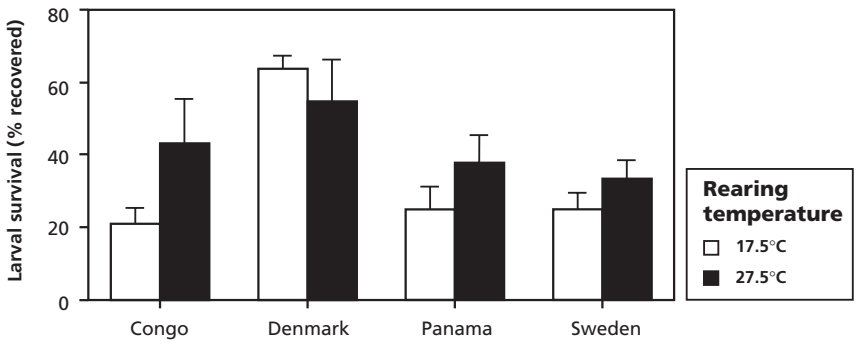


Fig 3. Larval survival measured as the percentage of larvae recovered +/- SEM.

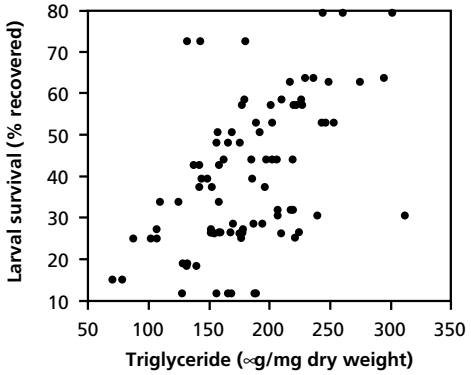


Fig 4. Relationship between mass-specific triglyceride levels of larvae and larval survival.

including glycogen content alone explained a significantly smaller amount of variation (R^2=0.49, F=13.55, $p<0.05$), suggesting that the same glycogen content may have different effects on adult size at different temperatures. Lipid levels showed no correlation with adult body size.

Larval survival

A significant geographical origin × rearing temperature interaction has been found for larval survival ($p<0.001$). Except for Sweden, larvae from all populations showed higher survival at the temperature corresponding to the climate of their origin (fig. 3). This result indicated that the developmental sensitivity of larvae to rearing temperature is adaptive. Averaged over the different rearing temperatures, both tropical populations had lower survival than Denmark ($p<0.001$). Larval triglyceride content correlated with survival (ρ=0.68, $p<0.001$) (fig. 4). The relationship between lipid levels and larval survival was significant at both low (ρ=0.62, $p<0.02$) and high (ρ=0.70, $p<0.004$) rearing temperatures.

Significant interaction ($p<0.01$) between the fixed factor, rearing temperature, and the covariate, triglyceride level, has been found. Such interaction indicates differences in the effect of lipids on survival at different temperatures. Indeed, the positive correlation between triglyceride levels and survival failed to hold when considering the two tropical populations. In spite of significantly higher triglyceride levels accumulated at the lower rearing temperature by both the Panamanian and Congolese larvae, they exhibited a lower survival at lower temperatures. Glycogen content did not reveal any association with larval survival at either experimental temperature.

Discussion

Larval resource allocation and adult body size

Aim of the present study was to examine how evolution of temperature sensitivity of larval resource allocation could possibly account for geographical and phenotypic variation in adult body size in *D. melanogaster.* Two scenarios were proposed to explain the finding that cold adaptation leads to a higher efficiency of converting larval food into adult body mass (Neat et al. 1995; Robinson and Partridge 2001).

The first scenario focuses on larval resource accumulation. An increase in the available resources per mg larval weight might be responsible, without any actual improvement of the efficiency of utilizing those resources during pupation. This increase might be due to enhanced ability for resource acquisition, more efficient enzymes for processing the accumulated nutrients (i.e. a lower mass specific metabolic rate) or go at a cost of other fitness related traits. To assess the latter possibility, an analysis of larval survival has been carried out in parallel.

The second scenario considers variation in pupal efficiency. Cold adaptation might lead to increased efficiency of pupation, leading to the emergence of larger adults given the same mass-specific resources of the larvae.

Geographical origin, environmental temperature and nutrient storage

Geographical origin and environmental temperature both affected larval resource allocation in *Drosophila melanogaster.* The differences were reflected in the resource levels accumulated prior to pupation as well as in the dynamics of storage during larval development. The two temperate populations realized higher nutrient storage at pupation compared to the two tropical ones. High rearing temperature allowed for fast initial accumulation of both lipids and carbohydrates but equal or decreasing levels towards the end of the larval stage. At low environmental temperatures the final resource levels accumulated at pupation were higher. This finding is in line with studies on clinal variation in functional differences in PGM and G6PD allozymes (Verrelli and Eanes 2001). Higher phosphoglucomutase (PGM) and lower glucose-6-phosphate dehydrogenase (G6PD) activity is associated with higher latitudes and results in increased glycogen storage in adults. The same enzyme activities may be expected in larval metabolism as well, resulting in the pattern of glycogen storage as found in this study.

Nutrient storage and body size

The nutrient levels accumulated by the larvae were expected to be proportional to the amount of resources allocated to the establishment of the adult. This expectation is in line with previous results on the relationship between storage of nutrients by adults and adult body size. Selection for starvation resistance produced a correlated increase in both body size and lipid content (Harshman and Schmid 1998). Selection on total lipid content (but not on mass-specific levels) produced a positively correlated response in size (Clark et al. 1990). The same experiment revealed an allometric relationship between body size and lipid content. Variations in nutrient storage and the associated changes in body size are thought to be the result of variation in enzyme activities involved in processing lipids and carbohydrates (Clark 1989). Larval efficiency of processing resources has been implied to be an important factor in establishing adult body size, in particular with respect to temperature (Neat et al. 1995).

The data presented here showed that the mass-specific glycogen content of larval tissue indeed predicts adult weight. Low environmental temperature and an evolutionary history of cold adaptation both facilitated an increase in the mass-specific amount of resources allocated to growth. Consequently a larger body size was realized as proposed in scenario one. The finding that it might be mass-specific carbohydrate content that determines future adult size is not surprising as it is known that during metamorphosis decrease of glycogen content is more pronounced compared to the decay of lipids (Butterworth et al. 1988).

The alternative explanation has been examined as well. Although at low rearing temperatures the same glycogen levels predicted a higher adult body size than at high rearing temperature, in accordance with Neat (Neat et al. 1995) and Robinson (Robinson and Partridge 2001), the geographical populations did not seem to differ in this respect. Scenario two does not seem to be involved in the thermal evolution of adult weight, but may be of relevance for giving a proximate explanation to the phenotypic plasticity of body size. Our findings do imply that resources are utilized in a different manner under distinct thermal regimes.

Nutrient storage and fitness components

Differential utilization of resources in different environments may be of importance for larval survival as well, as suspected before. Accumulation of resources has been shown to correlate not only with size but also with fitness components. Longevity in *A. tabida* (Ellers 1996) and viability (Clark 1989) and starvation resistance (Djawdan et al. 1998; Harshman et al. 1999) in *D. melanogaster* correlated positively with adult lipid content. Storage of

resources in the adult proved, however, plastic. In response to increased amounts of dietary yeast, *D. melanogaster* females decreased accumulation of nutrients but increased the production of eggs (Simmons and Bradley 1997). The trade-off between longevity and fecundity was at least partially mediated by the plasticity in nutrient storage. When supply is not limiting relatively more of the available resources are allocated to reproduction. In caddis flies, strategic allocation of resources has been shown to mediate developmental trade-offs and fine tuning life-histories (Stevens et al. 1999; Stevens et al. 2000). How plasticity in larval resource accumulation relates to fitness components in *Drosophila* is yet unknown.

We present evidence for adaptive plasticity in larval survival. We conclude that the different growth strategies adopted by populations with different geographical origin are most effective in the thermal regimes they have been selected under, as the triglyceride levels are shown to correlate with survival. The result is not entirely surprising as independent studies on laboratory lines of *D. melanogaster* have provided evidence for the adaptive nature of thermal evolution (Cavicchi et al. 1989). As larval survival is an important component of lifetime reproductive success, our results show that thermal evolution has been adaptive in natural *D. melanogaster* populations.

Thermal evolution of body size

In conclusion we would like to argue that the geographical variation in adult body size of *D. melanogaster* is the result of thermal selection on larval resource allocation. We showed that following a cold evolutionary history higher mass-specific levels of resources became dedicated to building the adult body, mimicking the effect of cold rearing temperature. Contrary to the phenotypic effect of temperature, thermal selection did not seem to influence the actual efficiency of the pupal transformation. Such pattern could be the result of different selection pressures, or combinations of them. Evolution of enhanced resource acquisition is likely in temperate regions where developing larvae probably face less abundant and qualitatively inferior food especially in early spring. At the same time, a relaxation of selection on investment in larval survival is possible as cold temperature seems more permissive for pre-adult survival. Adaptive variation in resource acquisition and allocation among latitudinal populations has been shown before in the Atlantic silverside (*Menidia menidia*) (Billerbeck et al. 2000). Given unlimited nutrients, tropical populations show submaximal growth rate but increased sustained and burst swimming capability, by different allocation of resources. In an insect system, genetic differences in lipid reserves between flightless and flight capable morphs of the cricket *Gryllus firmus* were shown to provide the metabolic

basis of life-history variation, i.e. underlie the trade-off between activity and early reproduction (Zera and Larsen 2001).

However, several issues remain unsolved. The observed increase in mass-specific glycogen levels yielding a larger adult in temperate populations can be the result of different phenomena. Cold adaptation may have resulted in increased resource acquisition without altering the allocation of nutrients to storage vs. maintenance. Alternatively, resource allocation to future adult size may trade-off with the maintenance the larvae itself. Laboratory cold adaptation has been shown to result in reduced competitive ability of *D. melanogaster* larvae (Partridge et al. 1994b; Partridge et al. 1995). A trade-off between future size and larval survival, mediated by the accessibility of resources may be involved. Geographical variation in enzyme activities in biochemical pathways directing resources to future size vs. maintenance of larval tissue may be ultimately responsible.

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Chapter 4

Temperature dependent larval resource allocation shaping adult body size in *Drosophila melanogaster*

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Abstract

Geographical variation in *Drosophila melanogaster* body size is a long standing problem of life-history evolution. Adaptation to a cold climate invariably produces large individuals, while evolution in tropical regions result in small individuals. The proximate mechanism was suggested to involve thermal evolution of resource processing by the developing larvae. In this study an attempt is made to merge proximate explanations, featuring temperature sensitivity of larval resource processing, and ultimate approaches focusing on adult and pre-adult life-history traits. To address the issue of temperature dependent resource allocation to adult size vs. larval survival, feeding was stopped at several stages during the larval development. Under these conditions of food deprivation, two temperate and two tropical populations reared at high and low temperatures produced different adult body sizes coinciding with different probabilities to reach the adult stage. In all cases a phenotypic trade-off between larval survival and adult size was observed. However, the underlying pattern of larval resource allocation differed between the geographical populations. In the temperate populations larval age but not weight predicted survival. Temperate larvae did not invest accumulated resources in survival, instead they preserved larval biomass to benefit adult weight. With other words, larvae from temperate populations failed to re-allocate accumulated resources to facilitate their survival. A low percentage of the larvae survived to adulthood but produced relatively large flies. Conversely, in tropical populations larval weight but not age determined the probability to reach adulthood. Tropical larvae did not invest in adult size, but facilitated their own survival. Most larvae succeeded to pupate but then produced small adults. The underlying physiological mechanism seemed to be an evolved difference in the accessibility of glycogen reserves as a result of thermal adaptation. At low rearing temperatures and in the temperate populations, glycogen levels tend to correlate positively with adult size but negatively with pupation probability. The data presented here offer an explanation of geographical variation in body size by showing that thermal evolution of resource allocation, specifically the ability to access glycogen storage, is the proximate mechanism responsible for the life-history trade-off between larval survival and adult size.

Keywords: geographical cline, phenotypic plasticity, resource allocation, size, temperature

Introduction

Thermal evolution of body size

Temperature is almost certainly an important selective agent in shaping *Drosophila melanogaster* body size. Large body size has repeatedly been found to concur with a cold evolutionary history (Huey et al. 1991). Geographical variation in *D. melanogaster* body size is also well documented; larger animals are found at higher latitudes (David et al. 1977; Coyne and Beecham 1987; Gilchrist and Partridge 1999).

A concise explanation for the thermal evolution of body size has, nevertheless, proved difficult to formulate. Larval growth and resource allocation is regarded as an important proximate determinant of variation in adult body size. Theoretical models predict that if a decrease of environmental temperature leads to a stronger decrease of the rate of processes consuming energy (i.e. metabolic rate) than of the growth rate, a larger adult can be realized (van der Have and de Jong 1996; Atkinson and Sibly 1997). Extension of this rationale to comparisons between populations and species is straightforward and has received empirical support, as temperature coefficients of growth rate and development rate has been shown to differ between *Drosophila* species (Gibert and de Jong 2001).

Empirical data support the notion that the evolution of resource processing by developing larvae is responsible for variation in adult body size. For a given amount of food and larval size, larger adults emerged in cold adapted laboratory lines (Neat et al. 1995; Robinson and Partridge 2001). Larger body size at low temperature might be the result of increased resource allocation to the adult body mediated by the accumulation of higher mass-specific levels of glycogen in larvae (Chapter 3).

In searching for an evolutionary explanation of geographical variation in body size, the most important issue is to determine what traits are under direct thermal selection. Evidence exists for evolved differences in both adult and larval fitness related traits. For both males and females it has been shown that larger animals have a higher fitness only at low temperatures. Artificial selection on body size, performed at 25°C, resulted in large females that had higher fecundity and longevity but only at low rearing temperature (McCabe and Partridge 1997). In a similar experiment it was shown that larger males have higher mating success only when tested at a low temperature (Reeve et al. 2000a). Conversely, in another study male territorial success was highest when males reared at high temperature were competed at high temperature. Smaller adults reared at high temperature were more successful at higher temperatures despite their smaller size (Zamudio et al. 1995). Moreover, early fecundity was higher when rearing temperature and test temperature were the

same, supporting the idea of adaptive plasticity of body size (Nunney and Cheung 1997). This evidence suggested a selective advantage for larger body size at lower temperatures, but not necessarily at higher temperatures.

Variation in pre-adult fitness related traits has been found in geographical populations and in laboratory selection lines maintained for years at high or low temperatures. Larval survival was higher at the rearing temperature corresponding to the evolutionary history of three of four geographical populations tested (Chapter 3). In selection lines, larval competitive success was higher at the rearing temperature corresponding to their evolutionary history (Partridge et al. 1994; Partridge et al. 1995). These results indicate adaptive thermal evolution. However, the superior pre-adult survival of cold-adapted lines at low rearing temperatures diminished when larval crowding increased (Partridge et al. 1994; Partridge et al. 1995). At high larval density warm-adapted lines performed equally to the cold-adapted lines when tested at low rearing temperature. This finding suggested that the evolution of smaller body size at higher temperatures was accompanied by increased pre-adult competitive ability relative to the cold-adapted lines. An analysis of geographical variation in larval competitive ability performed on populations from an Australian north-south cline failed to support this hypothesis conclusively (James and Partridge 1998). The role of competitive ability of larvae in temperature adaptation is not yet fully established; yet, selection on pre-adult traits could well be of high importance for understanding the outcome of thermal evolution, including variation in adult body size.

Thermal evolution of resource allocation; a trade-off between size and survival

A major challenge in explaining adult body size remains to combine proximate explanations featuring resource allocation and ultimate explanations focusing on adult and pre-adult life-history traits. The scenario that we propose here is that thermal evolution alters resource allocation to favor adult body size in cold environments and larval survival under warmer climates. At low temperatures, larval resource allocation might be tuned to favor investment in the future adult body if larger adult body size is advantageous and maintenance of larval survival does not require large investments. Evolution at cold temperatures would then result in preferential investment in adult body size by the developing larvae. Conversely, if at high temperatures investment in adult body size is not imperative while facilitating larval survival requires ongoing nutritional input, it could be expected that evolution at high temperature would result in preferential investment into traits ensuring maturation.

Our previous results indicated an evolved ability of temperate populations of *D. melanogaster* to accumulate higher mass-specific levels of resources and build larger adults at a given larval size (Chapter 3). In the same study variation in larval survival was found. However, in that experiment the results might have been due to differences in resource acquisition between tropical and temperate populations. To investigate resource allocation per se, an experimental design needs to control for resource acquisition. Larvae at several stages of development are removed from their feeding medium and the resulting adult body size determined. This design allows to estimate larval survival as the probability of larvae to pupate after being removed from the feeding medium prematurely. To prove the existence of a trade-off between adult body size and larval survival under these conditions, it is necessary to demonstrate that both traits depend on the allocation of the same limiting resource. Therefore, storage of metabolic reserves will be measured as well. The results will be discussed in the light of a possible explanation of the geographical variation of body size, in terms of evolved differential allocation of resources to larval and adult fitness associated traits.

Materials and Methods

Populations

Wild living flies have been caught in Panama (8.5 °N, 79.3 °W; Barro Colorado), Congo (4.5 °S, 11.5 °E; Pointe Noire), Denmark (56.28 °N, 9.25 °E; Wyborg) and the Netherlands (52.02 °N, 5.1 °E; Houten) between 6 to 12 months before the beginning of the experiment. Approximately one hundred females were collected from each of the sites. These populations had been cultured at large population sizes under constant light at 17.5°C on a standard corn medium, prior to the beginning of the experiment.

Experimental conditions

Egg-laying females had been reared at their experimental temperatures, in order to control for possible maternal effects. Several hundred females oviposited for 3 hours, at room temperature. Egg laying took place in empty jars covered with watch glasses containing 4 ml of a 1.9% agar-medium and a drop of a thick yeast suspension. Thirty eggs were put into a glass jar of height 5 cm and diameter 3.5 cm containing 15 ml of standard corn medium.

From the eggs, larvae were reared at 17.5°C and 27.5°C. These temperatures are well within the physiological range that allows for normal development of *D. melanogaster* but represent its high and low regions. At several stages during development, when larvae were still feeding and normally

would not yet pupariate, the larvae were removed from the standard corn medium. Development was interrupted at **45**, 53, **61**, **69**, **81**, **93**, 102 hours for the 27.5°C group and at **80**, **118**, 130, **140**, 154, **178**, **200** hours for the 17.5°C group. These timepoints were chosen to represent a similar range of development at the different temperatures to allow for comparison across temperatures. They cover all of the three different larval stages; the final timepoint printed in bold represents the beginning of the pre-pupal stage, when undisturbed larvae start to pupariate. For statistical analysis, where data were pooled over the developmental temperatures, only the ages printed in bold were taken into account as they represent similar developmental stages (labeled as stage 1 through 5). For all populations, rearing temperatures and timepoints, larvae have been collected from eight replicate vials. Larvae collected from the eight replicate vials were pooled. Three groups of 10 larvae per timepoint were weighed, freeze-dried and stored at -30°C for the biochemical assays. Five groups of 20 larvae were sorted for average size by eye, weighed and transferred to vials containing 2 ml agar medium for each timepoint. This procedure results in variation in larval weight between vials of the same stage. These larvae, that were prematurely deprived from further feeding, were allowed to pupate and the numbers of pupae were scored. The adults were collected one day after emergence and weighed. For collecting the larvae, a saturated sugar solution was poured on top of the medium, placing the vials on an electrical heater. Between 15 and 45 minutes after adding the solution the floating larvae were collected from the replicate vials, using a paintbrush after pouring the solution through a sieve.

Measurements

Weight

Fresh and dry weight of larvae destined for the biochemical assays were determined in groups of 10, fresh and dry weight of larvae transferred to vials without food in groups of 20. Males and females were weighed per vial in the numbers as they emerged varying from one to twenty. Fresh weight and dry weight were determined to the nearest 0.01 μg, using a Mettler ME 22 microbalance. For drying the specimens a Virtis 5L freeze drier has been used.

Biochemical assays

The glycogen and triglyceride contents of larvae have been determined. Groups of 10 larvae were homogenized in 300 μl of homogenization buffer (0.01 M KH_2PO_4, 1 mM EDTA pH 7.4) using a motorized Microfix morter and a molten pipette tip as a pestle. The homogenates were centrifuged at 13000 rpm for 3 minutes in order to pellet cellular and cuticular debris. Samples from the same homogenate were used for all assays.

Table 1: Critical age and critical weight for pupation for the different geographical populations pooled over the corresponding developmental stages (timepoints) across rearing temperatures.

	Critical age (stages)	95% CIV	Critical weight (mg)	95% CIV
Panama	1.42	0.42-2.11	0.28	-0.09-0.54
Congo	2.25	1.49-2.82	0.77	0.48-0.99
Denmark	1.46	0.49-2.11	0.47	0.11-0.72
Netherlands	2.39	1.56-3.03	0.85	0.54-1.10

Glycogen

Reagentia as available form the Sigma glucose-determining kit (PGO enzyme, catalog no. 510-6) were utilized. The reagents were complemented by 0.1 U/ml amyloglucosidase, for converting glycogen into glucose. A 30 µl sample of the homogenate was added to a total volume of 1 ml test reagents. Following 30 minutes of incubation at 37°C, the absorption at 450 nm was measured.

Triglycerides

Reagentia as available from the Sigma triglyceride determining kit (Catalog no. 336-20) were used for the colorimetric determination of the triglyceride content. A 30 µl sample of the homogenate was added to a total volume of 1 ml test reagents. Following 30 minutes of incubation at room temperature, the absorption at 500 nm was measured.

Statistical analysis

Probit regression analysis was performed on the pupation probabilities with larval weight or larval age as independent variables to compare critical weight and age (weigh and age at which 50% of the larvae successfully pupate) between the geographical populations. These statistics are measures for the size and age of larvae necessary to reach the developmental stage that allows for pupation. The age of the larvae is expressed in stages (1 through 5) rather then hours to enable pooled analysis of data from the different rearing temperatures. Pupation probabilities were probit transformed prior to further analysis. Partial Pearson correlation coefficients were calculated between pupation frequencies and adult body size controlling for larval size and age and between pupation frequencies and larval size or age adjusting for the other variable. The former analysis allows for estimating a phenotypic trade-off between adult body size and pre-adult survival. The latter procedure allows for estimating the effect of larval size and of developmental stage on pupation probability (i.e. pre-adult survival), independently from each other. On the data aggregated over vials, a

partial correlation test was used to detect a relationship between probit transformed pupation frequencies, adult fresh and dry weight and larval mass-specific triglyceride and glycogen levels. In vials with only very few larvae emerging, adult weight was higher than the average weight of the larvae placed in the vial, despite the absence of food. The few survivors were probably cannibalizing on the others. These vials were removed from the analysis.

Results

Larval weight, age and probability to pupate

Probit analysis of the data on larval weight, age and pupation probability did not reveal a significant difference in critical weight or age (weight or age at which 50% of the larvae pupate) between the populations (Table 1). The populations did differ, however, in the variable explaining most of the variation in pupation probability. Both size within age and age of the larvae influence pupation probability (fig. 1) but in a different manner for the tropical vs. the temperate populations. Calculating partial correlation coefficients allows for quantifying the contribution of each variable individually.

For the tropical populations, the partial correlation of larval weight within age with pupation probability is appreciable and near significant (Congo: ρ=0.473, p=0.011; Panama: ρ=0.375, p=0.065). In contrast temperate populations showed no correlation at all (Denmark: ρ=-0.013, p=0.949; Netherlands: ρ=0.06, p=0.791). Considering the partial correlation of larval age with pupation probability corrected for the effect of weight, the temperate populations showed moderately large and near significant correlation (Denmark: ρ=0.456, p=0.015; Netherlands: ρ=0.412, p=0.056) opposite to the tropical populations (Congo: ρ=-0.157, p=0.425; Panama: ρ=0.136, p=0.516). The partial correlation coefficients of age and weight within age with pupation probability are summarized in figure 2 to quantify the alternative patterns of tropical vs. temperate populations also depicted in figure 1. Briefly, the tropical populations rely on converting accumulated larval body mass to pupation probability against the temperate populations which do not enhance larval survival by utilizing accumulated resources.

Pupation probability and prospective adult body size

A negative partial correlation was found between pupation probability and adult fresh weight for all populations, controlling for larval size and age and calculating over rearing temperatures (fig. 3). However, this correlation was strong and significant only for the temperate populations (Denmark:

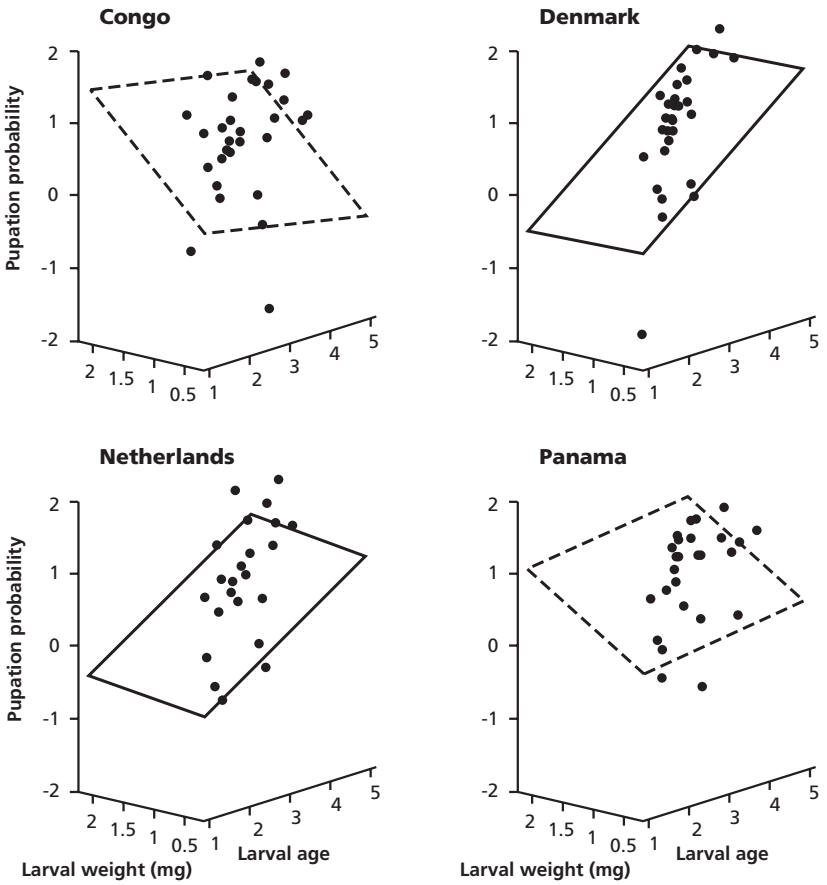

Fig 1. 3D regression planes for larval weight, larval age and pupation probability for the four geographical populations. Larval weight is presented on reverse axes.

ρ=-0.557, p=0.003; Netherlands: ρ=-0.629, p=0.002). In Congo it was not significant (ρ=-0.174, p=0.385) and in Panama the correlation coefficient was significant, but lower compared to the temperate populations (ρ=-0.406, p=0.048). Considering the rearing temperatures separately revealed that this negative relationship was present predominantly at 17.5°C (ρ=-0.632, p<0.001), resembling the pattern produced by the geographical populations. At 27.5°C the correlation was negative but non-significant (ρ=-0.162, p=0.197).

These data again suggest a difference between the geographical populations in the details of determining not only pupation probability but adult body size as well. The temperate and tropical populations again exhibit an alternative pattern when considering the influence of pupation probability on adult weight at different stages of larval development as illustrated in figure 4.

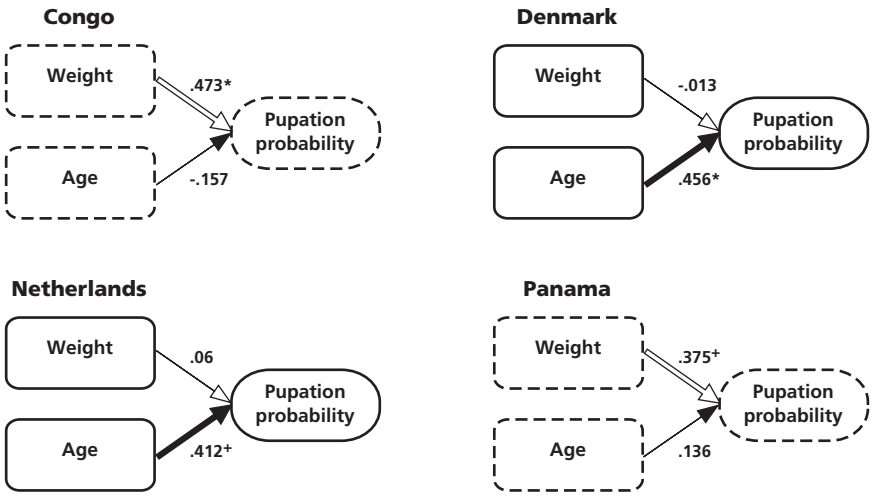

Fig 2. Partial correlation coefficients for larval weight and pupation probability corrected for larval age and larval age and pupation probability corrected for larval weight. * denotes a significant partial correlation coefficient at the 5% level, + denotes a marginally non significant effect.

Larval glycogen content, pupation frequencies and prospective adult body size

Larval mass-specific glycogen levels were partially correlated to both adult body size and pupation probability revealing an interesting pattern (fig. 5). At the lower rearing temperature, the partial correlation between glycogen level and adult body size, controlled for age of the larvae (in hours) and calculated over the geographical populations, was strongly positive (ρ=0.713, p=0.001) but the partial correlation between glycogen level and pupation probability negative (ρ=-0.575, p=0.012). At the high rearing temperature the pattern was reversed, with glycogen level negatively correlated with adult body size (ρ=-0.514, p=0.014) and near significantly positively associated with pupation probability (ρ=0.339, p=0.122).

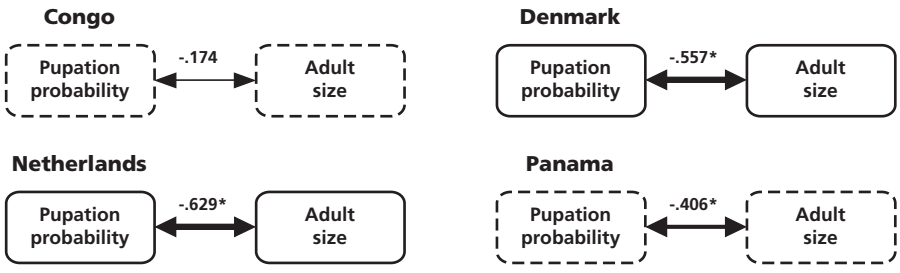

Fig 3. Partial correlation coefficients for pupation probability and future adult body size controlled for larval age and weight. * denotes a significant partial correlation coefficient at the 5% level.

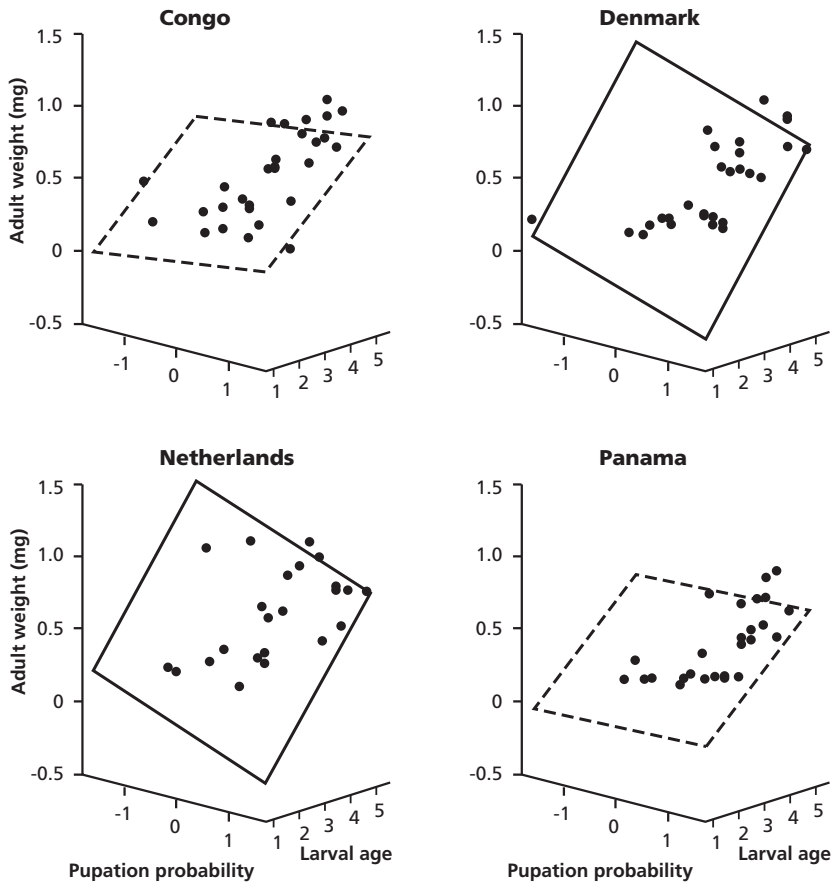

Fig 4. 3D regression planes for larval age, pupation probability and adult weight for the four geographical populations.

A similar, though less robust, pattern was found analyzing the different geographical populations (fig. 6). The tropical populations showed no significant partial correlation between glycogen level and either adult body size or pupation probability. However, the Netherlands population exhibited a

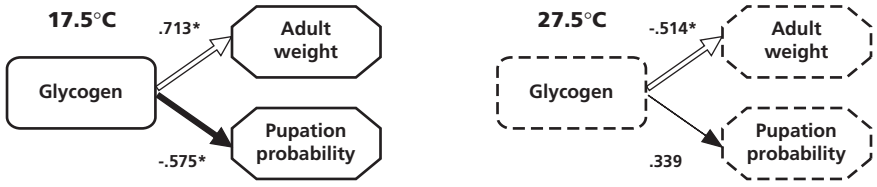

Fig 5. Partial correlation coefficients for larval mass specific glycogen level and both pupation probability and adult body size, controlled for larval age at the different rearing temperatures. * denotes a significant partial correlation coefficient at the 5% level.

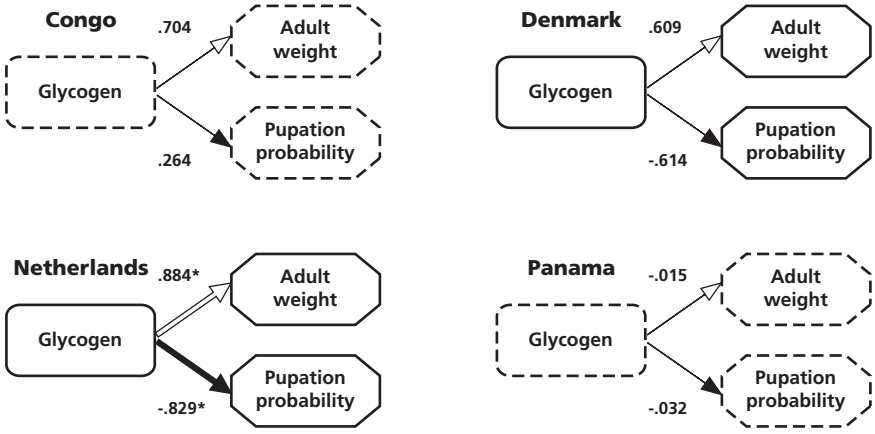

Fig 6. Partial correlation coefficients for larval mass specific glycogen level and both pupation probability and adult body size, controlled for larval age for the different geographical populations.

strong positive partial correlation between glycogen level and adult body size (ρ=0.884, p=0.008) and a strong negative partial correlation between glycogen level and pupation probability (ρ=-0.829, p=0.041). Note that although the Denmark population did not exhibit any significant correlation (both p=0.19) the pattern was similar to the one found in the Netherlands population and the correlation coefficients were sizeable.

In summary, the pattern of utilization of glycogen seemed to be identical at 17.5°C compared the two temperate populations. Glycogen seemed to be invested in adult body size at cost of larval pupation probability. At 27.5°C and in the two tropical populations no significant patterns are found.

Discussion

Adult size and pupation probability: a life-history trade-off

The aim of this study was to examine whether geographical variation in body size could be interpreted as a result of thermal evolution of resource allocation. A life-history trade-off mediated by adaptive resource allocation would provide an ultimate explanation. A physiological mechanism of allocation would provide a proximate explanation. A difference in resource allocation between geographic populations would be the starting observation.

The data showed a negative partial correlation between adult size and pupation probability when larvae of the same age and weight are considered. Higher probability to pupate was associated with lower adult size once feeding was stopped.

This pattern represents a life-history trade-off between pre-adult survival and adult size. Many life-history trade-offs between pre-adult survival and adult size are known. In caddis flies (trichoptera), increased investment in larval defense has been shown to result in smaller adults (Stevens et al. 1999; Stevens et al. 2000). In these studies experimental manipulation of resource availability for pupation allowed the manifestation of a trade-off between pre-adult survival and adult size. Fellowes et al. (1999) found a similar trade-off. *Drosophila melanogaster* larvae that survived being parasitized by *Asobara tabida* emerged as smaller adults than unparasitized larvae. In both *Drosophila* and caddis flies, larval investment in defense decreased adult size. However, in our study no larval defense was involved. The present trade-off between larval survival and adult body size might be more comparable to a trade-off between early fecundity and adult longevity, under stress or without stress (Djawdan et al. 1996; Djawdan et al. 1998). This is a trade-off between a present fitness component and a reservation towards a future fitness component.

Determinants of pupation probability: geographical variation in resource allocation

Life-history trade-offs have often been thought to be the result of adaptive allocation of limiting resources (Atkinson and Sibly 1997; Zera and Harshman 2001). Our data supported distinct thermal evolution of resource allocation. Populations from different geographical origins differed strikingly in the variable explaining most of the pupation probability. Larval weight explained most of pupation probability, but not adult size, in tropical populations. We interpret this as allocation of accumulated resources to survival rather than adult size. A minimal size is needed to pupate (Bakker 1961) and this might vary between genotypes within populations, in our case within the tropical populations. A possible difference between populations would be that critical weight is lower in tropical populations than in temperate populations. An earlier study on variation in critical weight for pupation showed lower critical weight in a tropical population than in a temperate population (de Moed et al. 1999), but in our study no difference in critical weight was found (Table 1). In fact, within the temperate populations variation in larval weight did not explain variation in pupation probability, but rather correlated with adult weight. These larvae depended on age for their survival. The interpretation is that temperate populations preferentially allocate accumulated resources to adult size opposed to larval survival. It seems unlikely that this result was due to lower availability of resources to larvae from temperate populations. Larval weights were not lower (data not shown) and previous results showed that temperate larvae accumulated larger lipid and triglyceride stores (Chapter 3) (Verrelli and Eanes 2001). A possible interpretation might

be that tropical larvae are able to mobilize their resources more easily to facilitate pupation, compared to temperate larvae of similar age. If temperate larvae have been selected for producing large adults, they might fail to reallocate resources for survival once these resources are designated for future adult size. A similar energy reallocation hypothesis has been put forward to explain the trade-off between survival and reproductive investment in aphids (Stadler 1995). When under food stress, young embryos might be reabsorbed by the mother, but once an embryo reaches a certain developmental stage it competes for resources, i.e. the mother is no longer capable of reallocating the energy previously designated for reproduction. In addition, adaptive variation in allocation among latitudinal populations has been shown before, in fish. Individuals from tropical populations of the Atlantic silverside (*Menidia menidia*) show submaximal growth rate but increased sustained and burst swimming capability, reducing predation risk by different allocation of resources (Billerbeck et al. 2000; Billerbeck et al. 2001; Lankford et al. 2001). In parallel with our findings, warm adapted genotypes *M. menidia* reduce investment in growth to increase survival.

Physiological basis of adaptive resource allocation

Evidence to support the argument of temperature dependent resource allocation might come from glycogen content in larvae at the weight and age they stop feeding. Larval mass-specific glycogen content correlated positively with adult size and negatively with pupation probability, at the lower rearing temperature. At the higher rearing temperature, the pattern was reversed. The correlation with glycogen content was interpreted as glycogen usage. If the interpretation of the correlations as glycogen usage were correct, glycogen usage might explain the trade-off between adult body size and survival. Glycogen would be the resource the two traits depend on.

The data also provide some support for evolved differences of resource allocation patterns. Although the two tropical populations produce no significant correlation between glycogen level and adult size or pupation probability, the temperate populations approximate the expected pattern. In the Netherlands a significant and strong negative correlation between larval glycogen level and pupation probability could be found, coinciding with a significant and strong positive correlation between larval glycogen level and adult size. The Denmark population produced the same trend. Larvae from temperate populations do not invest resources to propagate their own survival; instead they seem to invest in adult size. A similar metabolic basis of life-history variation has been documented in a wing-polymorphic cricket *Gryllus firmus*. Genetically determined higher lipid accumulation in flight capable morphs

facilitates flight by providing fuel at cost of ovarian development compared to obligatory flightless morphs (Zera and Larsen 2001).

The correlations with glycogen level are less conclusive for the geographical populations than for the rearing temperatures, but seem to support the hypothesis that differences in the ability to mobilize resources exist between populations. Lower temperature and temperate populations show a positive correlation between glycogen level in larvae and adult body size. The negative partial correlation between pupation probability and adult size was found predominantly at the low rearing temperature and for the temperate populations. The mechanisms of temperature related developmental plasticity in adult body size and temperature related geographic variation in body size might well be similar.

Why larger size when cold?

Cold adapted genotypes or cold reared larvae tended to maintain a higher prospective adult size at a cost of larval survival probability, and seemed to use stored glycogen preferentially to increase adult size. This suggests that a cold environment might select for larger adult size, while a warmer environment might select for higher larval survival. The trade-off seems understandable as large adult body size has been shown to confer higher fitness at low temperature only but not at high temperature. In a comparison of lines selected for body size, McCabe and Partridge (1997) found that the females from the large selection lines were relatively fitter at the colder temperature. At both experimental temperatures, especially the lower one, the small-line females rescheduled their progeny production to later ages. Reeve et al. (2000) found for the same lines that large-line males were fitter than controls at both temperatures. Interestingly, the difference in fitness was greater at the lower experimental temperature, showing genotype by environment interaction indicating a synergistic effect of larger body size and lower temperature. Larger body size may have evolved at temperate latitudes because of the fitness advantages of being larger at lower temperatures (McCabe and Partridge 1997; Reeve et al. 2000b). Nunney and Cheung (1997) compared early fecundity of female *D. melanogaster*. Test temperature for early fecundity was varied, as well as development temperature. Early fecundity was highest if the test temperature was equal to the development temperature, indicating that the developmental response to temperature was adaptive (Nunney and Cheung 1997) (see also Chapter 1).

The reason why temperate populations accumulate larger glycogen stores per unit weight (Chapter 3) (Verrelli and Eanes 2001) but seem to invest less resources in larval survival may be that temperate larvae are unable to access their reserves as quickly as tropical larvae. Adaptation to a warm climate may

result in a larval physiology that allows for quick access of metabolic stores. The resulting pattern of resource allocation would be the one proposed, with temperate populations investing in size and tropical ones investing in survival.

This finding is in line with studies on clinal variation in functional differences in PGM and G6PD allozymes. Higher phosphoglucomutase (PGM) and lower glucose-6-phosphate dehydrogenase (G6PD) activity is associated with higher latitudes and results in increased glycogen storage in adults (Eanes 1999; Verrelli and Eanes 2001). The same enzyme activities may be expected in larval metabolism as well, suggesting a possible metabolic mechanism for the observed physiological trade-off.

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Chapter 5

Larval energy metabolism and a life-history trade-off in *Drosophila melanogaster*

Zoltán Bochdanovits and Gerdien de Jong

Abstract

Natural populations of *D. melanogaster* exhibit a pronounced north-south cline in body size, with smaller animals being more equatorial. This cline is possibly part of an adaptive life-history strategy involving a trade-off between adult size and larval survival. Corresponding north-south clines in enzyme polymorphism allele frequencies have been found and differences in larval metabolism between geographical populations might underlie the thermal adaptation of body size. Adult weight, larval survival, larval enzyme activities and metabolite pool size were determined for crosses between isofemale lines within two temperate and two tropical *D. melanogaster* populations at two temperatures. A genetic trade-off between adult size and pupation probability was evident at low temperature and in the temperate populations. The first and second principal components extracted from larval metabolic traits correlated in the opposite direction with adult weight and pupation probability. The pattern of genetic variation in enzyme activities and metabolite pool size suggested past selection on these traits. Thermal adaptation of body size might involve genetic variation in larval metabolism affecting a life-history trade-off.

Keywords: life-history trade-off, physiological genetics, enzyme activity, adult size, larval metabolism

Introduction

Geographical variation in *Drosophila melanogaster* body size evinces a very specific and repeatable pattern: body size increases in populations at higher latitudes (Coyne and Beecham 1987; David et al. 1977; Gilchrist and Partridge 1999). Such geographical clines have evolved independently on all continents, and laboratory thermal evolution lines pointed to temperature as the most likely selective agent in shaping natural body size variation (Cavicchi et al. 1989; Huey et al. 1991). The adaptive significance of temperature-specific size variation has been shown in several studies that dissected the association between size and adult fitness components. The emerging consensus identified large adult size to be most advantageous at low temperatures, by conveying increased female fecundity (McCabe and Partridge 1997), male territorial success (Reeve et al. 2000) and male mating success (Zamudio et al. 1995). At higher temperatures, a large body size does not seem to convey any specific advantage to the adults. In contrast, pre-adult viability of cold-adapted genotypes that produce large adults seems lower when tested under non-permissive conditions, such as crowding (Partridge et al. 1994; Chapter 1) or low food quality (Chapter 2).

These observations led to the identification of a phenotypic trade-off between pre-adult survival and adult weight (Chapter 4). When developing larvae face conditions of limiting resource availability, adaptive allocation of stored glycogen results in enhancing either future size or larval survival. The proximate mechanism of body size variation was proposed to be an evolved resource allocation strategy that predisposed cold-adapted genotypes to maintain future size at a cost in pre-adult survival, while warm-adapted genotypes seemed to maintain larval survival, at a cost in adult size. Because adult size has been shown to confer less fitness advantage at high temperatures (see references above), this physiological mechanism might well be adaptive. Variation in larval efficiency of resource processing has been shown to underlie adult body size variation (Neat et al. 1995), and adaptation to different climates might be the outcome of selection for differences in metabolism (Berrigan and Partridge 1997).

Allozyme frequencies of many enzymes in basic metabolic pathways vary in parallel with the body size clines (Eanes 1999). These allozyme frequency clines do not seem to have been linked the cline in body size; to our knowledge, the question whether both types of clines are related has not yet been asked. Indirect evidence for a functional link may come from data on the *Pgm* (phosphoglucomutase) locus. The extensive amino-acid variation at the *Pgm* locus (Verrelli and Eanes 2000) was shown to be clinally distributed

(Verrelli and Eanes 2001a) and has a functional impact on glycogen synthesis. At higher latitudes a higher frequency of flies with an intrinsically more active PGM allozyme was found, and this was correlated with an elevated glycogen content (Verrelli and Eanes 2001b; Chapter 3 and 4). Further evidence indicating the involvement of variation in enzyme activities in geographical adaptation, could be found in the clinal variation in the activity of G6PD (glucose 6 phosphate dehydrogenase). The G6PD cline runs opposite to the PGM cline, with lower activity at high latitudes. As higher G6PD activity is responsible for higher pentose shunt flux, and consequently lower glycogen synthesis, the opposing PGM and G6PD activity clines jointly contribute to natural variation in glycogen storage (Eanes 1999; Verrelli and Eanes 2001b; Chapter 3 and 4), possibly linking allozyme clines and body size.

Genetic studies indicate a causal relationship between energy metabolism and organismal traits, supplementing the data on natural variation in adult size and allozyme frequencies. The size of lipid and glycogen storage pools, in second chromosome replacement lines, was determined by the variation in the activity of 11 enzymes (Clark and Keith 1988). In addition, significant broad-sense genetic covariance was detected between viability and fecundity on the one hand and glycogen content and several enzyme activities on the other hand (Clark 1989). The presence of additive genetic variation for all of the 11 enzyme activities under study (Clark 1990) underlined the potential of metabolic traits to be the raw material for natural selection. Both PGM and G6PD were included in this study, and both are known to contribute to variation between natural populations (Eanes 1999; Verrelli and Eanes 2001b). Within-population genetic variation in enzyme activity and between-population change in allozyme frequency combined to suggest adaptive evolution at the metabolic level by natural selection of enzyme activity.

A major challenge in explaining clinal variation in body size remains in merging genetic data on standing variation in enzyme activities with the observations on different glycogen utilization that might underlie the life-history trade-off between adult size and larval survival. The hypothesis to be addressed is the following: genetic variation in the activity of enzymes closely involved in glycogen processing underlies variation in glycogen utilization and causes a genetic trade-off between adult size and larval survival. To allow for a meaningful analysis from an evolutionary perspective, temperate and tropical extremes of two independent body size clines have been sampled. Because the expression of genetic variation can be affected by environmental conditions (Noach et al. 1996), all populations were reared and tested at both low (18°C) and high (28°C) temperatures. These temperatures approximated the thermal conditions the populations were adapted to. Within each population ×

temperature combination, crosses were used to assess the presence of additive, maternal and dominance variance and to detect broad-sense genetic covariance between adult size, larval survival and the metabolic traits. Three separate questions were posed. (1) Does a genetic trade-off between adult size and larval survival exist? (2) How much genetic variation exists for the traits under study within natural populations adapted to different climates? (3) Can the claim of differential glycogen utilization be supported by genetic evidence at the level of enzyme activities? By comparing the different geographical populations and rearing temperatures in addressing each of these questions, the data should (i) provide insight on the genetics of life-history evolution (1), (ii) reveal signatures of natural selection in shaping life-histories if they exist (2&3) and (iii) offer support for the proposed physiological mechanism of body size evolution (3).

Materials and Methods

Drosophila stocks

Two temperate and two tropical strains of *Drosophila melanogaster* were used, originating form Seattle (Washington, USA), Houten (the Netherlands), Congo (Pointe Noire) and Zimbabwe, respectively. For each population eight isofemale lines were used, where each isofemale line was initiated with one inseminated female caught in the wild. In case of the Congo population these lines were "pseudo"- isofemale lines, being the offspring of one inseminated female from a large laboratory population previously caught in the wild. Except for the Zimbabwe lines, all strains were collected six to twelve generations before the start of the experiment and kept on standard corn medium at 17.5°C in standard size bottles. Apart from the starting generation, the lines were kept at large numbers in multiple bottles, to avoid further inbreeding. No heterosis between inbred lines of low fitness is expected that might bias the estimates of genetic variance components. The Zimbabwe lines have been in the lab for approximately ten years on standard corn medium at 25°C in standard size bottles.

Crosses and experimental conditions

Crosses were set up between the isofemale lines to determine whether significant levels of genetic variation were expressed within the geographical populations for the different metabolic and life-history traits under study. To allow for meaningful interpretation of the data, two tropical and two temperate populations were used, reared at 18°C and 28°C. This design allows for

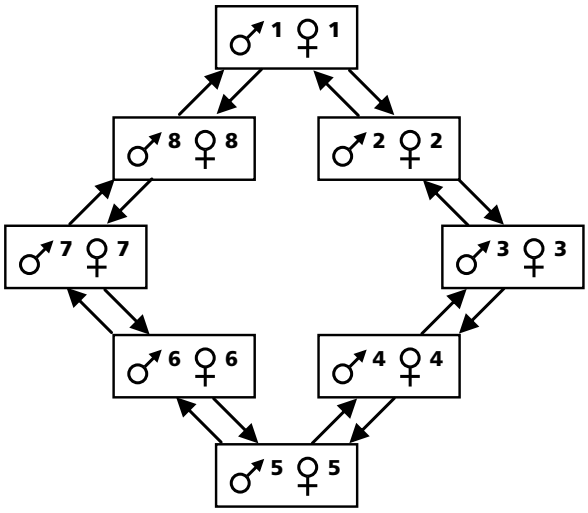

Fig 1. **Crossing scheme applied to generate the progeny that has been analyzed. The sex and isofemale line number are presented in the boxes. Each randomly numbered isofemale line has been crossed to two other isofemale lines with adjacent numbers, i.e. line 2 has been crossed to lines 1 and 3. All reciprocal crosses have been included, i.e. $♂^{2}$ $♀^{3}$ is different from $♂^{3}$ $♀^{2}$. This crossing scheme results in 16 different types of progeny. The data can be analyzed with two nested analyses of variance to yield estimates of additive, maternal and dominance variance.**

deducing the effect of both evolutionary history and genotype x environment interaction on the expression of genetic variation. However, replication of geographical origin and testing different environmental conditions resulted in a limitation on the number of crosses that could be performed, within each line. Consequently, a modified North Carolina cross II has been applied with the smallest possible number of crosses that still allowed for determining the amount of additive, maternal and dominance genetic variance. Each of the eight isofemale lines was crossed to two other isofemale lines in a circular chain cross design (fig. 1). For further details see the Data Analysis section.

All isofemale lines have been maintained at the experimental temperature for one generation, to exclude the possible effect of parental environment on offspring phenotype. Thus, each isofemale line has been maintained at both 18°C and 28°C in standard size bottles on standard corn medium. The crosses were set up by collecting virgin males and females (approx. 50 females) from each isofemale line and combining the samples to yield the crosses described in figure 1. All crosses were allowed to mate for three to five days. Egg laying took place in inverted empty bottles covered with petri dishes filled with 4 ml of 2% agar and a drop of a thick yeast suspension. After up to five hours of egg laying, the petri dishes were removed and thirty eggs were put into

each of six plastic jars of diameter 3.5 cm containing 15 ml of standard corn medium. From these jars, larvae have been collected at 70 hours after egg laying at 28°C, and at 160 hours after egg laying at 18°C. These larvae are in a comparable developmental stage at the two rearing temperatures, in mid third instar. This stage has been chosen to represent the late exponential phase of larval growth, when much metabolic activity is expected. Furthermore, this stage allows for the detection of a larval survival-adult size trade-off. When larvae are removed from the medium, they must complete larval development without further feeding. The variation in the percentage larvae that succeed in pupariating under these conditions, and the variation in the body weight of the adults that subsequently emerge, can be used to measure the correlation between these traits. Previous data have indicated that, at the phenotypic level, a negative correlation, i.e. a trade-off, exists between larval survival and adult size (Chapter 4).

Measurements

Larvae were collected at 70 hours at 28°C and 160 hours at 18°C by pouring a saturated sugar solution on top of the medium and gently heating the jars from below on an electric heater. Under these conditions, larvae surface from the medium and float on top to be poured over a sieve. Larvae are collected using a paintbrush, and temporarily stored in a petri dish on ice, moistened with chilled Ringer solution. Three random groups of five larvae were used for the enzyme assays and determining the glycogen, triacylglycerol and protein content. Three random groups of exactly twenty larvae were placed in standard vials containing 2% agar instead of corn medium. Under these conditions, larvae are provided moisture to prevent dehydration, but cannot feed.

Larval and adult weight

All measurements were done on a Sartorius microbalance to the nearest 0.01 µg. All larvae were weighed before further treatment, in groups of five (for enzyme assays) or twenty (for pupation percentage and adult weight). Males and females that emerged from the vials containing 2% agar were weighed in groups of five for each vial.

Percentage pupation

When apparently all larvae that were able to had pupated, the number of pupae were counted in each vial and the percentage pupation was calculated.

Larval glycogen, triacylgycerol and protein content and enzyme assays

After weight measurement, three groups of five larvae were homogenized in a 1.5 ml eppendorf tube in 200 µl pre-chilled standard phosphate buffer (0.01 M KH_2PO_4, pH 7.4) and diluted to 1000 µl. All samples were centrifuged for 5 minutes at 10000 rpm in a table centrifuge in a 4°C room. 20 µl of the homogenates was applied to 96-wells microtiter plates in a 4°C room. Several microtiter plates were filled, one for each assay, and stored at -80°C until the assays were performed. Due to the size of the experiment, the gathering of the larvae and the plating was done in four blocks. Each block consisted of *all* crosses within one temperate and within one tropical population at one rearing temperature. Thus, each assay was performed on four plates, on the same day using the same batch of chemicals. The activity of glycogen synthase (GS), glycogen phosphorylase (GP), phosphoglucoisomerase (PGI) and glyceride dehydrogenase (GPDH) were determined. The raw data on metabolite storage pool sizes and enzyme activities were normalized for larval weight, i.e. all subsequent analyses were performed on content and/or activity per mg fresh weight. All enzyme assays (but not the measurements of the glycogen, triacylgycerol and protein content) were performed at the temperature at which the crosses had been reared. This procedure allows for the proper comparison of enzyme activities and the phenotypes. All biochemical assays have been performed following the protocols as described by Clark and Keith (1988).

Data analysis

All phenotypic and genetic correlations between the metabolic and life-history traits were calculated as Pearson product moment correlations. Genetic correlations between traits were calculated as correlation between the means of half-sib families, i.e. on the data aggregated across sires. Estimates for the significance of additive, maternal and dominance variance were obtained from combining two nested analyses of variance, first using sire and dam within sire and secondly using dam and sire within dam as random factors. The causal variance components represented by the expected mean squares are

Table 1. Causal variance components associated with the expected mean squares in the two nested analyses of variance (adapted from Table 10.4 (Falconer 1981)).

Expected MS	Variance component	***Expected MS***	Variance component
Sire	$1/4\ V_A$	Dam	$1/4\ V_A + V_M$
Dam w. Sire	$1/2\ V_D + 1/4\ V_A + V_M$	Sire w. Dam	$1/2\ V_D + 1/4\ V_A$

summarized in Table 1, for both analyses. A significant sire effect points to the presence of significant levels of additive genetic variation. The significance of the maternal effect was estimated by comparing the significance of sire and dam effects. A significant dam effect accompanied by a non-significant sire effect points to a significant maternal contribution. The significance of the dominance variance was estimated by comparing the sire and sire within dam effect. A significant sire within dam effect in the absence of a significant sire effect points to the presence of significant levels of dominance variance. A summarized measure of metabolic state per population × temperature was obtained by performing a Principal Component Analysis and extracting the first two Principal Components. The data on pupation percentage have been probit transformed prior to further analysis, to insure a normal distribution of the data. All statistical analyses were performed with SPSS 10.0.

Results

Phenotypic and genetic trade-off between larval survival and adult size

A phenotypic trade-off between larval survival and adult size had been found previously in other populations (Chapter 4). The aim here is to show that such a trade-off has a genetic basis. Consequently, correlation coefficients between probit transformed pupation percentage (i.e. pupation probability)

Table 2A: Phenotypic correlations between larval survival (pupation probability) and female and male weight (mg). Correlation coefficients significant at the 5% and 1% level are marked by * and **.

Phenotypic correlations				
Geographical origin	Rearing temperature		Female weight	Male weight
Congo	18°C	Larval survival	-0.67**	-0.64**
	28°C	Larval survival	-0.15	-0.18
Zimbabwe	18°C	Larval survival	-0.46*	-0.54**
	28°C	Larval survival	-0.20	-0.16
Houten	18°C	Larval survival	-0.64**	-0.80**
	28°C	Larval survival	-0.43**	-0.36*
Seattle	18°C	Larval survival	-0.75**	-0.79**
	28°C	Larval survival	-0.39*	-0.43**

and male and female weight were calculated within each population at each rearing temperature. First the genetic structure of the data was disregarded, yielding phenotypic correlations (Table 2A). In both temperate populations at both temperatures, the expected trade-off was detected. Note that the correlation coefficients were more negative at the lower rearing temperatures. In both tropical populations, a significant negative correlation between pupation probability and adult weight was only found at the lower rearing temperature. In total, six out of eight cases showed the phenotypic trade-off. Secondly, the analysis was repeated on half-sib family means, i.e. on data aggregated over the paternal isofemale line. This analysis identified genetic correlations (Table 2B). The pattern found here resembled the results of the phenotypic correlations. All correlation coefficients were negative, even the non-significant ones. The tropical populations, however, did not produce a significant negative correlation, although note the marginal but strong negative correlation in the Zimbabwe population at low temperature. In contrast, both temperate populations produced very strong and highly significant correlation between pupation probability and both male and female weight at the lower temperature. At 28°C only female weight in the Houten population was significantly correlated with larval survival. Note that the overall trend was similar between the phenotypic and genetic correlations, even if significance levels were lower for the genetic correlations. The data point to a genetic basis for this life-history trade-off.

Table 2B: Genetic correlations between larval survival (pupation probability) and female and male weight (mg). Correlations coefficients significant at the 5% and 1% level are marked by * and **.

Phenotypic correlations

Geographical origin	Rearing temperature		Female weight	Male weight
Congo	18°C	Larval survival	-0.35	-0.17
	28°C	Larval survival	-0.28	-0.21
Zimbabwe	18°C	Larval survival	-0.38	-0.67
	28°C	Larval survival	-0.51	-0.27
Houten	18°C	Larval survival	-0.86**	-0.83**
	28°C	Larval survival	-0.78*	-0.55
Seattle	18°C	Larval survival	-0.85**	-0.95**
	28°C	Larval survival	-0.44	-0.33

Quantitative genetics of larval survival, adult size and metabolic traits

Previous work has identified glycogen as the most likely limiting resource that underlies the larval survival-adult size trade-off (Chapter 4). At the physiological level, variation in the accessibility of metabolite pools may underlie the alternative resource allocation patterns produced by populations with different evolutionary histories. The hypothesis is, therefore, that genetic variation in enzyme activity of enzymes closely involved in glycogen processing may underlie the observed variation in glycogen utilization. To identify the presence of additive, maternal and dominance variance, two nested analyses of variance have been performed on all traits under study, including enzyme activities, metabolite pool size and adult weight and larval survival. Dominance and maternal variance components can be calculated from subtracting the appropriate expected mean squares from both analyses (see Data Analysis).

In the enzyme activity data, only GPDH expressed additive genetic variance (Table 3) in the Seattle population at 28°C, but support was only marginal ($p=0.044$). Glycogen levels showed additive genetic variance in the Seattle population at 18°C ($p=0.001$). Also in the Seattle population, both male and female weight expressed additive genetic variance at low temperature ($p=0.037$ and $p=0.017$) and female weight did so at high temperature as well ($p=0.006$). Pupation probability was shown to exhibit additive genetic variance in the Houten population at low temperature ($p=0.036$). Overall, barely any additive genetic variance could be detected in the data (Table 3). Note though, that the very traits that previously have been shown to be of interest, namely pupation probability, adult weight and glycogen content, were fundamentally the only traits that showed some additive genetic variance. If additive genetic variance was found, it occurred in temperate populations and almost exclusively at the lower rearing temperature, in parallel with the results on the phenotypic and genetic correlations (Table 2A and B).

Dominance variance was expressed in many traits under most conditions (Table 3). No particular pattern could be discovered, except for considerable differences between traits. Triglyceride and protein content and glycogen synthase activity produced an almost complete lack of dominance variance; in fact these traits did not seem to show any variability at all. In contrast, dominance variance was present in phosphoglucoisomerase activity in all samples. Some traits produced maternal variance but overall the data do not provide evidence for large maternal contributions (Table 3). In summary, significant amounts of dominance variance and some maternal variance have been detected.

Genetic correlation between metabolic traits and organismal phenotypes

Variation in the accessibility of glycogen might be the underlying physiological mechanism for the larval survival-adult size trade-off. Finding genetic correlations between metabolic traits and pupation probability and/or adult weight would strengthen this hypothesis and could suggest that the observed trade-off would be the result of natural selection on larval physiology. Detecting significant amounts of dominance variance but no additive variance might suggest that natural selection has been acting upon enzyme activities and metabolite pools. To facilitate the analysis, data on all metabolic traits (enzyme activities and storage pools) were summarized by extracting the first two principal components within each population at each temperature. Consequently, the principal components are not strictly comparable between populations, but offer the best available measure of metabolic state for that populations (Table 4). PC1 and PC2 jointly explained between 55% and 86% of the variation in the larval metabolic trait data. Correlation coefficients between PC1, PC2, pupation probability and male and female weight have been determined for all populations at both rearing temperatures. Because a significant genetic trade-off between pupation probability and adult size has been detected only in the temperate populations at 18°C (Table 2A and B), evidence for adaptive resource allocation by involvement of larval glycogen processing should come from these samples. Within the Seattle population reared at 18°C, PC1 showed significant and strong negative correlation with pupation probability and significant and strong positive correlation with both male and female weight (fig. 2A). Within the Houten population reared at 18°C, PC2 showed significant and strong positive correlation with pupation probability and significant and strong negative correlation with both male and female weight (fig. 2B). Because the principal components were extracted for each population separately, and thus are not strictly comparable, these results are not contradictory. In both temperate populations reared at 28°C, the sign of the correlation coefficients between the principal components and pupation probability was opposite to the sign of the correlation coefficients between the principal components and adult weight. These correlations among PCs and pupation probability were not significant, but in the light of the small sample size, the pattern might be telling. Within the tropical populations less pattern could be seen, as might be expected from the results on the trade-off. However, within the Zimbabwe population reared at 18°C, PC1 correlated strongly and significantly with pupation probability ($\rho=0.83$, $p=0.01$), while showing a sizeable but non-significant negative correlation with male weight ($\rho=-0.52$, $p=0.18$). At the same time pupation probability and male weight

Table 3: The presence or absence of significant (5%) levels of additive, dominance and maternal variance denoted by + or -. The results are given for each trait for every population × temperature combination.

Trait	Geographical origin	Rearing temperature	V_A	V_D	V_M
GP	Congo	18°C	-	+	-
	Zimbabwe	18°C	-	+	-
	Houten	18°C	-	+	-
	Seattle	18°C	-	-	-
	Congo	28°C	-	+	-
	Zimbabwe	28°C	-	+	-
	Houten	28°C	-	+	-
	Seattle	28°C	-	+	-
GPDH	Congo	18°C	-	+	-
	Zimbabwe	18°C	-	+	-
	Houten	18°C	-	+	-
	Seattle	18°C	-	-	+
	Congo	28°C	-	+	-
	Zimbabwe	28°C	-	+	-
	Houten	28°C	-	-	-
	Seattle	28°C	+	-	-
GS	Congo	18°C	-	-	-
	Zimbabwe	18°C	-	-	-
	Houten	18°C	-	-	-
	Seattle	18°C	-	-	-
	Congo	28°C	-	-	-
	Zimbabwe	28°C	-	-	-
	Houten	28°C	-	+	-
	Seattle	28°C	-	-	-
PGI	Congo	18°C	-	+	-
	Zimbabwe	18°C	-	+	-
	Houten	18°C	-	+	+
	Seattle	18°C	-	+	-
	Congo	28°C	-	+	-
	Zimbabwe	28°C	-	+	-
	Houten	28°C	-	+	-
	Seattle	28°C	-	+	-
GLY	Congo	18°C	-	+	-
	Zimbabwe	18°C	-	+	-
	Houten	18°C	-	+	-
	Seattle	18°C	+	-	-
	Congo	28°C	-	+	-
	Zimbabwe	28°C	-	+	-
	Houten	28°C	-	-	-
	Seattle	28°C	-	-	-

Trait	Geographical origin	Rearing temperature	V_A	V_D	V_M
TRI	Congo	18°C	-	-	-
	Zimbabwe	18°C	-	-	-
	Houten	18°C	-	-	-
	Seattle	18°C	-	-	-
	Congo	28°C	-	-	+
	Zimbabwe	28°C	-	+	+
	Houten	28°C	-	-	-
	Seattle	28°C	-	-	-
PRO	Congo	18°C	-	-	-
	Zimbabwe	18°C	-	-	-
	Houten	18°C	-	-	-
	Seattle	18°C	-	-	-
	Congo	28°C	-	-	+
	Zimbabwe	28°C	-	-	-
	Houten	28°C	-	-	-
	Seattle	28°C	-	-	-
Larval survival	Congo	18°C	-	-	-
	Zimbabwe	18°C	-	-	-
	Houten	18°C	+	-	-
	Seattle	18°C	-	+	-
	Congo	28°C	-	-	-
	Zimbabwe	28°C	-	-	-
	Houten	28°C	-	+	-
	Seattle	28°C	-	+	-
Male weight	Congo	18°C	-	-	-
	Zimbabwe	18°C	-	-	+
	Houten	18°C	-	-	-
	Seattle	18°C	+	-	-
	Congo	28°C	-	-	-
	Zimbabwe	28°C	-	+	+
	Houten	28°C	-	+	-
	Seattle	28°C	-	+	-
Female weight	Congo	18°C	-	+	-
	Zimbabwe	18°C	-	+	-
	Houten	18°C	-	-	-
	Seattle	18°C	+	-	-
	Congo	28°C	-	-	-
	Zimbabwe	28°C	-	+	-
	Houten	28°C	-	+	-
	Seattle	28°C	+	-	-

Table 4: Loading of the Principal Components within population and temperature.

		18°C		28°C	
		PC1	PC2	PC1	PC2
Congo	GP	0.75543	-0.60815	0.73077	0.624215
	GPDH	0.87118	0.333169	0.749869	-0.51414
	GS	0.872916	-0.16702	-0.04623	0.979103
	PGI	0.822087	-0.38873	0.938718	0.209909
	GLY	0.935946	0.284374	0.854618	0.195225
	TRI	0.373819	0.153957	0.897851	-0.25328
	PRO	0.371366	0.836949	0.864807	-0.18713
Zimbabwe	GP	0.681922	0.209591	0.94337	0.017702
	GPDH	-0.65541	0.382831	0.768506	-0.56151
	GS	0.890849	-0.1046	0.557431	0.568927
	PGI	0.91111	0.030025	0.411063	-0.88233
	GLY	0.451854	0.768352	0.818022	0.476498
	TRI	0.590874	0.045543	-0.24771	0.845336
	PRO	-0.24169	0.828665	0.840461	0.33314
Houten	GP	0.904517	0.092243	0.468102	0.303128
	GPDH	0.264666	0.294747	-0.6389	0.651504
	GS	0.663069	-0.25563	0.736966	0.235267
	PGI	0.728558	0.534702	-0.40783	-0.32967
	GLY	0.776939	0.058771	0.808277	0.03432
	TRI	-0.24952	0.479686	0.154096	0.763292
	PRO	0.419302	-0.7333	0.714481	-0.25031
Seattle	GP	0.016119	0.577585	0.085168	0.416849
	GPDH	0.525428	-0.75991	0.968743	-0.13299
	GS	0.741227	0.443405	-0.34567	0.615783
	PGI	0.980319	0.010983	0.937333	0.303435
	GLY	0.849209	0.376287	0.387001	0.421007
	TRI	0.232446	0.854166	-0.18047	0.953108
	PRO	-0.69324	0.674449	-0.73622	-0.04189

were weakly negatively correlated (ρ=-0.67, p=0.067). Overall, but especially for the cold reared temperate populations, the interpretation is evident: a combined measure of relevant larval metabolic traits was oppositely associated with pupation probability compared to adult size, while the two life-history traits were themselves negatively correlated.

Discussion

Trade-offs between life-history traits are known to be an important mechanism of maintaining genetic variation under selection (Stearns 1992). Their existence has been documented (Atkinson and Sibly 1997; Zera and Harshman 2001), and adaptive resource allocation is the most plausible mechanism that could cause this pattern (Christians 2000). Previously, a phenotypic trade-off between adult weight and pre-adult survival had been

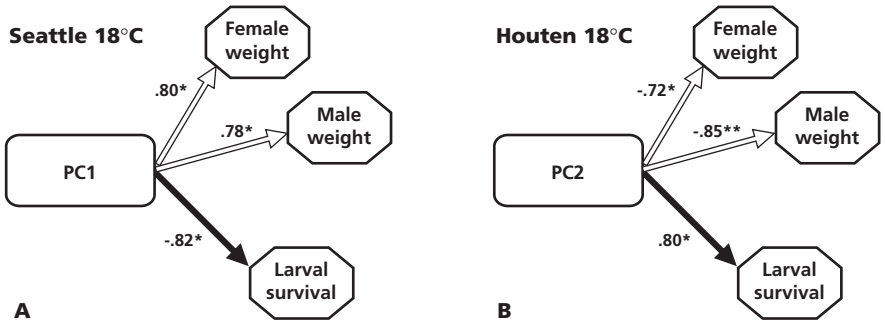

Fig 2. Genetic correlations between a measure of metabolic state and larval survival or adult weight. Panel A shows the correlation coefficients between PC1 and larval survival and PC1 and both female and male weight for the Seattle population reared at 18°C. Panel B shows the correlation coefficients between PC2 and larval survival and PC2 and both female and male weight for the Houten population reared at 18°C. Correlations coefficients significant at the 5% and 1% level are marked by * and **.

identified in *Drosophila melanogaster* (Chapter 4). Here, the phenotypic trade-off has been reproduced, and evidence is presented for a genetic basis of this pattern. This life-history trade-off might conceivably be the result of adaptive evolution, rather than the result of some physiological constraint. Adaptive evolution of a life-history trade-off, probably based on differential resource allocation, implies underlying physiological mechanisms in variation in metabolite pools and in variation in enzyme activities affecting the flux to and from these storage pools. In agreement with this hypothesis, larval glycogen was found to be the common limiting resource for both larval survival and adult size (Chapter 4). Temperate and tropical populations differed not only in the expression of the trade-off itself, but also in the requirement for glycogen to establish the pattern. This again suggested that adaptation to different climates induced genetic changes between populations in the utilization of resources.

Additive genetic variance in enzyme activities has been found (Clark 1989; Clark 1990; Clark and Keith 1988). The challenge remains in detecting additive variance in metabolic traits within natural populations, and possibly differences in the pattern of variation between populations. Such analyses could possibly indicate tentative targets of natural selection, and the physiological genetic basis of adaptation to different climates. Our data showed high prevalence of dominance variance in all traits considered, but virtually no additive genetic variance. One potential source of experimental error that might have caused such a result would be high levels of inbreeding within the isofemale lines. Because three out of the four populations used in this study have been sampled less than 12 month prior to the beginning of the experiment, and were kept in multiple bottles at high numbers, it is not very

likely that such bias was introduced into the results. Actually, the genetic architectures of body size clines are known to be rather complex, with evidence for dominance, epistatic and maternal contributions to the phenotypic value (Gilchrist and Partridge 1999; Gilchrist and Partridge 2001). Our results agree with their conclusion that significant amounts of non-additive effects most be involved in determining the phenotypic value for body size. Moreover, the lack of additive genetic variation is a strong indication that natural selection has been acting upon these traits, including the metabolic traits. Remember that additive variance has been found for *all* enzyme activities studied in laboratory populations (Clark 1990). To detect the dominance variance but little or no additive variance in geographical populations is plausibly a signature of natural selection.

Physiological genetics of a life-history trade-off

Our previous results have suggested that the larval survival-adult size trade-off might be the result of differences between the geographical populations in their ability to mobilize glycogen reserves (Chapter 4). Because the size of metabolite pools has been shown to depend on the activity of relevant enzymes (Clark 1989), one might expect the geographical populations to differ in enzyme activity in enzymes determining the flux to and from glycogen storage. Geographical populations are known to differ in the activity of at least two of such enzymes (Eanes 1999; Verrelli and Eanes 2001b). Variation in enzyme activities contributes to the genetic divergence *between* populations adapted to different climates. In addition, we found no additive genetic variance *within* natural populations for several metabolic traits (although tentatively present in non-selected populations). This result corroborates a potential involvement of larval metabolism in thermal adaptation. What remains to be investigated is whether genetic variation in larval energy metabolism is responsible for the trade-off between larval survival and adult size. This trade-off was (both here and in Chapter 4) most predominant in the cold adapted populations reared at the lower temperature, and the data offered evidence for larval metabolism being the proximate cause of the pattern. In both temperate populations, some measure of metabolic state correlated oppositely with adult size and larval survival. Seemingly, within these populations there is (broad sense) genetic variation for the utilization of energy stores by means of differing enzymatic activity. Genetic variation for resource allocation, involving larval metabolism at the physiological level, seems to exist and to contribute to the geographical variation in adult size.

A similar metabolic basis to life-history variation has been put forward before, in fish, crickets and beetle. The Atlantic silverside (*Menidia menidia*) produced adaptive variation in resource allocation among latitudinal

populations. Individuals from tropical populations show submaximal growth rate but increased sustained and burst swimming capability to reduce predation risk (Billerbeck et al. 2000; Billerbeck et al. 2001; Lankford et al. 2001). Similar to our findings, tropical genotypes of *M. menidia* exploit an alternative resource allocation strategy to enhance survival at cost of investment in growth. Life-history variation in a wing-polymorphic cricket *Gryllus firmus* also has been documented to have a physiological genetic basis. Genetically determined higher lipid accumulation in flight-capable morphs facilitates flight by providing fuel at cost of ovarian development compared to obligatory flightless morphs (Zera and Larsen 2001). Sierra Nevada populations of the Willow Beetle *Chrysomela aeneicollis* (Schaeffer) were shown to differ in phosphoglucose isomerase (PGI) allele frequencies. Different PGI genotypes differ in expression of a heat shock protein (Hsp70) and in tolerance of thermal extremes. The persistence of the PGI polymorphism was suggested to be the result of a trade-off between thermal tolerance and energy allocation (Neargarder et al. 2003).

In this study, we offer evidence for the hypothesis that thermal evolution of body size in *Drosophila melanogaster*, might have a physiological genetic basis involving larval metabolism. There is evidence that the trade-off between adult size and larval survival has a genetic basis, and it seems likely that natural selection has been acting on body size, larval survival but also on metabolic traits during adaptation to different climates. The geographical cline in body size might be the result of selection for adaptive resource allocation at the physiological level.

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Chapter 6

Co-variation of larval gene expression and adult body size in natural populations of *Drosophila melanogaster*

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Abstract

Understanding adaptive phenotypic variation is one of the most fundamental problems in evolutionary biology. Genes involved in adaptation are most likely those that affect traits most intimately connected to fitness: life-history traits. The genetics of quantitative trait variation (including life-histories) is still poorly understood, but several studies suggest that (1) quantitative variation might be the result of variation in gene expression, rather than protein evolution and (2) natural variation in gene expression underlies adaptation. The next step in studying the genetics of adaptive phenotypic variation is therefore an analysis of naturally occuring co-variation of global gene expression and a life-history trait. Here we report a microarray study addressing the co-variation in larval gene expression and adult body weight, a life-history trait involved in adaptation. Natural populations of *Drosophila melanogaster* show adaptive geographic variation in adult body size (Noach et al. 1996; Robinson and Partridge 2001) with larger animals at higher latitudes. Conditions during larval development also affect adult size with larger flies emerging at lower temperatures. We found statistically significant differences in normalized larval gene expression between geographic populations at one temperature (genetic variation), and within geographic populations between temperatures (developmental plasticity). Moreover, larval gene expression correlated highly with adult weight, explaining 81% of its natural variation. Of the genes that show a correlation of gene expression with adult weight, most are involved in cell growth, cell maintenance or are associated with growth pathways.

Keywords: global gene expression, larval gene expression, adult body weight, quantitative trait evolution

Introduction

The molecular basis of quantitative traits remains elusive (Stern 2000; Tautz and Schmid 1998). Quantitative traits are known to have complex genetic architecture, with many genes contributing to the phenotypic variation. Statistical population genetic studies have identified Quantitative Trait Loci (QTLs) for a number of different traits, but limitations on the resolution of these techniques proved prohibitive for a detailed dissection of the molecular determinants (MacKay 2001). The outcome of phenotypic evolution depends on details of the genetic architecture, i.e. the nature of the causative mutations and as a result the nature of the interactions between gene products. Recent studies show that non-coding variation might be important to evolutionary change. Natural variation in quantitative traits might involve differential gene expression, as underlined by sequence divergence in promoter regions (Purugganan 2000) and QTLs that map to non-coding sequences (MacKay 2001). The genes involved seem to possess important regulatory functions during development both in animals and plants (Barrier et al. 2001; Oldham et al. 2000). Moreover, variation in gene expression between natural populations has been proposed to underlie adaptation (Barrier et al. 2001; Oleksiak et al. 2002). However, a direct comparison of global gene expression and an adaptive quantitative phenotype has never been conducted. Such an analysis seems the logical next step in both quantitative genetics and genomics, merging the two fields. In this study we attempt to quantify the amount of quantitative phenotypic variation explained (in a statistical sense) by variation in gene expression.

Analysis of global gene expression using microarrays has proven successful in comparing the mean transcriptional state of two or more distinct groups (White 2001). To obtain a more comprehensive picture of the biological variability in gene expression, recent studies have explored within and between group variation of global transcription using Analysis of Variance (Jin et al. 2001; Oleksiak et al. 2002; Pletcher et al. 2002). However, a major challenge remains detecting a quantitative relationship between variation in gene expression and variation in an adaptive quantitative phenotype. If quantitative trait variation is caused by variation in gene expression (Purugganan 2000) rather than by protein evolution, a correlation between transcript levels and trait value could be expected.

Natural populations of *Drosophila melanogaster* occur over a wide geographic range and are known to be adapted to local conditions (Gilchrist and Partridge 1999; Noach et al. 1996), most notably temperature. Many traits diverge between populations adapted to different climates, such as enzyme activity (Eanes 1999), development time (Robinson and Partridge 2001),

critical larval weight for pupation (de Moed 2000) and adult body size (Robinson and Partridge 2001). Adult weight is the most extensively studied life-history trait in *Drosophila,* is known to be determined during larval development (Chakir et al. 2002), and to be affected by developmental temperature (Noach et al. 1996). If adaptive natural variation in adult body size is attributable to changes in gene expression, a significant co-variation between larval gene expression and adult weight is expected.

We examined global gene expression using Affymetrix GeneChips on staged third instar larvae of a tropical and a temperate population of *D. melanogaster* reared at 17.5°C and 27.5°C. From each population we used five isofemale lines known to differ significantly in adult body size. Across the rearing temperatures, the "broad sense" heritability for adult weight was 0.24 for the temperate population and 0.39 for the tropical population. The experimental design equated to a two-way analysis of variance (ANOVA) with 20 measurements for global gene expression across the four population-temperature combinations, and adult weight. The aim was to detect four (possibly overlapping) classes of genes. Two-way ANOVA allows identification of genes, whose expression differs between populations, differs between temperatures or exhibits population by temperature interaction (P × T). In addition, we applied a non-parametric correlation test on gene expression and adult weight. This analysis identifies a quantitative association between larval gene expression and adult weight, determined as isofemale line means.

Materials and Methods

Fly stocks

Five isofemale lines from Wenatchee (47.26 °N, 120.20 °W; WA, USA) and five isofemale lines from San José (9.59 °N, 84.04 °W; Costa Rica) collected in the summer of 2001 were kept on standard corn medium at 17.5°C until the start of the experiment. Isofemale lines descended from a single inseminated female and represent genetic variation within the population. Prior to the experiment the lines were reared at the experimental temperature for one generation to rule out possible effects of maternal rearing temperature. Larvae were raised at low density under unlimited food conditions. The experiment was conducted at 17.5°C and 27.5°C on standard corn medium stained with 0.05% bromophenol blue. This medium has no effect on larval growth and allows for accurate staging of third instar larvae just prior to pupariation at their maximum size (Andres and Thummel 1994). Adult males and females that emerged from the vials the larvae were previously collected from were weighed in groups of five to the nearest 0.01 μg on a

Sartorius microbalance. Adult weight was averaged over the sexes for subsequent analysis.

Gene expression analysis

Using a paintbrush, larvae with dark blue guts that stopped feeding and started to wander were manually collected from the surface of the medium and were immediately frozen in liquid nitrogen. Approximately 20 larvae from each isofemale line were used for isolating mRNA with Qiagen Direct mRNA kit. From approximately 3 µg of mRNA, Bio-11-CTP and Bio-11-UTP labeled aRNA were prepared using standard Affymetrix protocols (http://www.affymetrix.com/Download/manuals/expression_ever_manual.pdf). The labeled aRNA were applied to 20 Affymetrix Drosophila Genome Arrays. Hybridization and scanning was performed on Affymetrix Fluidics Station 400 and GeneArray(r) Scanner at the Leiden Genome Technology Center. The raw data were subjected to global normalization per GeneChip on Microarray Suite 5.0 prior to further analysis. The data acquired from these procedures are relative measures of gene expression independent from the original larval weights.

Data analysis

We detected the expression of 7652 of more than 13500 genes (56%) represented on the Drosophila Genome Array on at least one GeneChip. There was a systematic difference in the percentage of genes detected between the 17.5°C and 27.5°C reared samples. In the 17.5°C reared samples of the same isofemale lines a smaller percentage of all genes was detected. This may be expected as in ectotherms the "rate of living" is negatively correlated with ambient temperature. For two 27.5°C samples we found the opposite pattern due to very low percentage (approx. 9%) of genes detected. These two arrays were excluded from further analysis. For the remaining arrays at 17.5°C between 13% and 30% and at 27.5°C between 16% and 41% of the genome has been detected. To allow for meaningful statistical analysis of the data we selected only probes that have been detected on at least four microarrays in each of the four population-temperature combinations. This filtering resulted in 1134 probes for further analysis. SAM (Statistical Analysis of Microarrays)(Tusher et al. 2001) was performed on an Excel plug-in (http://www-stat-class.stanford.edu/~tibs/clickwrap/sam.html). In all SAM analyses a False Positive Rate of one was chosen, meaning that in the resulting set of significant genes no more than one false positive is expected. The two-way ANOVA, the Spearman non-parametric correlation test and the Principal Component analysis were performed in SPSS 10.0. PCA summarizes correlated data in a small number of uncorrelated derived variables with little

loss of information and allows for a graphical representation of a large dataset. Hierarchical cluster analysis of gene expression was performed in Cluster freely available from http://rana.lbl.gov/EisenSoftware.htm. Because only genes already found to be significant in SAM have been submitted to the ANOVA and due to the high level of correlation between the expression of the different genes, presumably as a result of co-regulation, no Bonferroni correction was applied. Although the same rationale applies to the non-parametric correlation test as well, due to the large number of genes found to be significant at the 0.05 level, a Bonferroni correction was applied in this analysis. Due to this overtly conservative significance threshold on a reasonable sample size (18 isofemale line means), it is unlikely that the results would be seriously biased by experimental variation between GeneChips. The measure of "broad sense" heritability in body size was calculated using an Analysis of Variance approach. An estimate of the between isofemale line variance is a measure of "broad sense" genetic variance. The sum of the genetic variance and the within line variance (the error variance) is an estimate of the phenotypic variance. The ratio of these two quantities gives a measure of heritability.

Results

Detection of statistically significant differences in gene expression from the two-way ANOVA and correlation analyses. Normalized measures of relative gene expression were acquired for each isofemale line at both temperatures. After filtering of the data 1134 genes were allowed for analysis. To avoid common problems associated with the analysis of such a large number of variables in classical statistical procedures (Benjamini and Hochberg 1995) we first applied a permutation test to the data. Two types of permutation tests as implemented in SAM were used. The first SAM test allowed for comparison of two group means, and therefore could not detect interactions between the main factors, Population and Temperature. This analysis was used as a preliminary test only, for allowing the data to the ANOVA. Four comparisons were conducted, i.e. comparison of the temperatures within the two populations, and of the populations within the two temperatures. The second SAM test was a quantitative test performed with isofemale line mean weight as covariate, an equivalent of a correlation test. These preliminary analyses resulted in a list of 275 probes significant in at least one test. These data were subjected to standard two-way ANOVA and correlation tests.

From the ANOVA we identified 45 genes differing in gene expression between populations, 200 genes differing in gene expression between the temperatures (of which 41 in both populations), and 31 genes with different

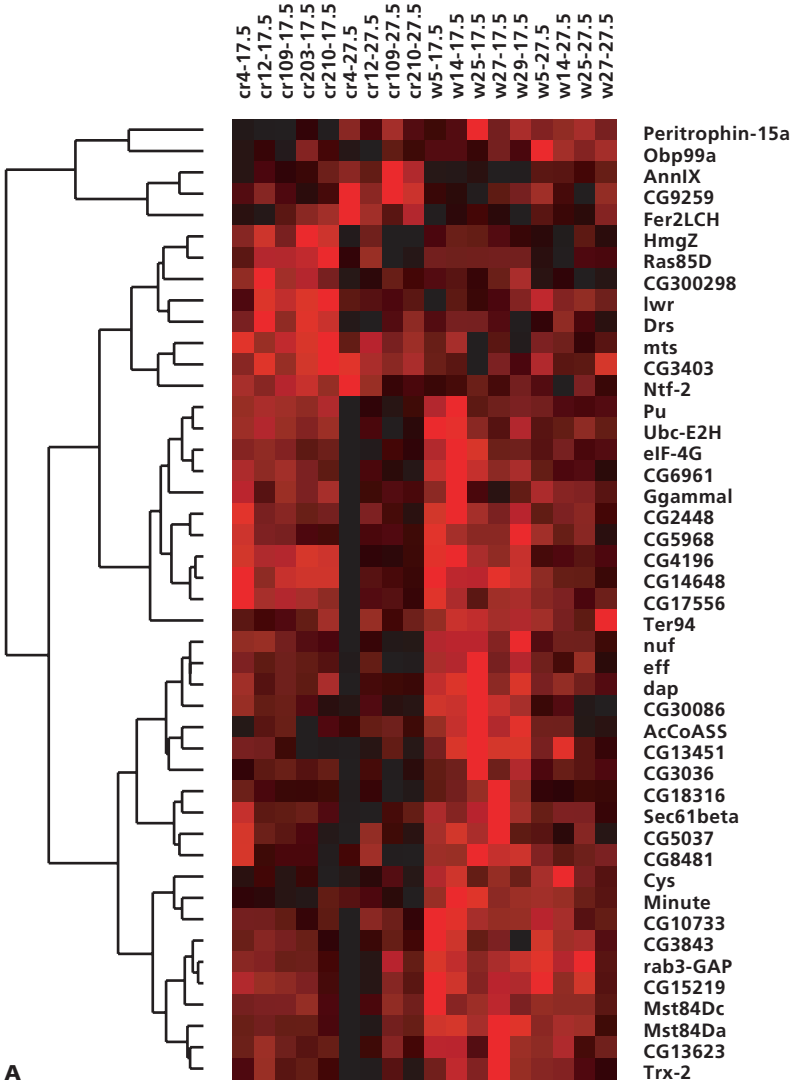
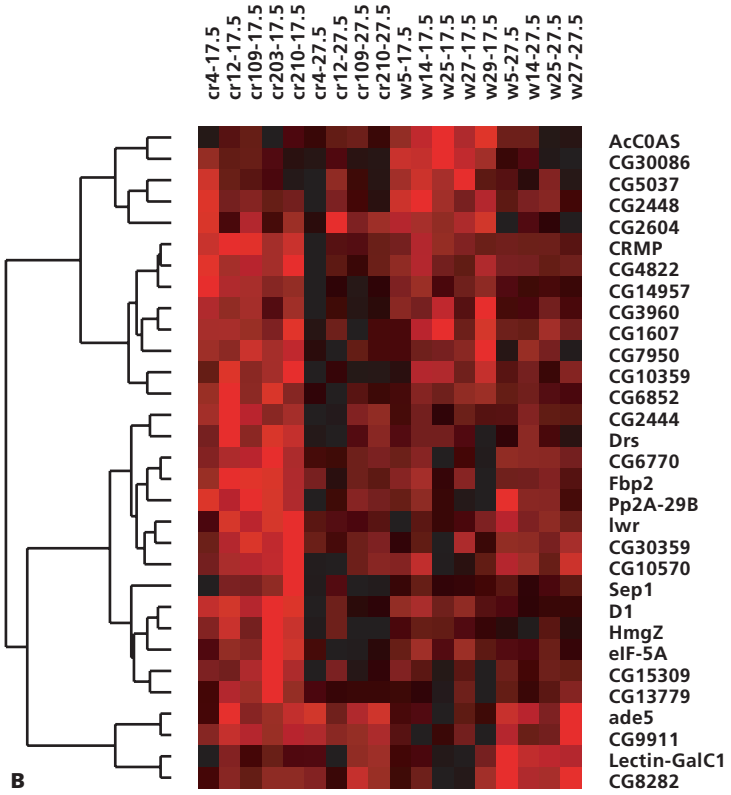


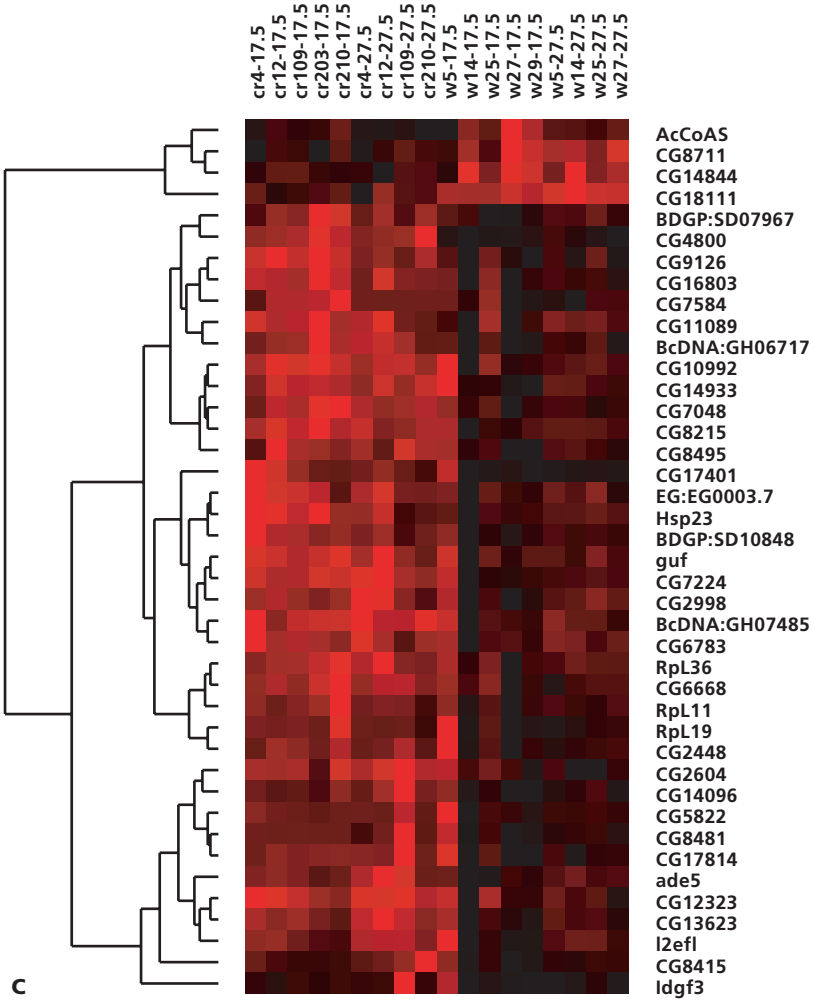
Fig 1. Patterns of transcript abundance of genes found to differ significantly in a two-way ANOVA. The intensity of the red color represents the relative mRNA levels; only for the illustrations the values were standardized to range between 0 and 100, within each gene. The data represents isofemale lines from the tropical (cr) and temperate (w) populations, reared at 17.5°C and 27.5°C. The dendograms on the left group genes with similar expression between natural populations and rearing temperatures. **A**, 45 genes with different expressions between the natural populations. **B**, 31 genes with expression showing population by temperature interaction. **C**, 41 genes with different expressions between the rearing temperatures.

sensitivity for ambient temperature between the populations, i.e. exhibiting Population by Temperature interaction (fig. 1). Seven genes exhibited both a population effect and a P×T interaction. Expression of 19 genes highly correlated with adult body weight (fig. 2). Twelve out of the nineteen "weight"



genes also differed in expression between the geographical populations (Supplementary Information Table 1 and 2). To underline the validity of the statistical procedure used, note that from the 19 genes detected to have a significant correlation -after Bonferoni-correction- between their larval expression and adult body weight, 18 have already been detected by the SAM quantitative analysis (data not shown). Such an overlap between the results from the different analyses clearly demonstrated the robustness of the procedure.

Factor Analysis of high dimensional gene expression data. Because expression of genes was highly statistically correlated, presumably due to co-regulation, we performed several Principal Component Analyses to reduce the number of independent variables. The first two Principal Components (PCs) of gene expression from the 45 "population" genes separated the populations and the rearing temperatures (fig. 3). PC1 demarcated the tropical population reared at 27.5°C vs. the temperate population reared at 17.5°C (compare cr27.5 and w17.5 in fig. 1a). Reared at native conditions one composite measure of gene expression was sufficient to discriminate between natural



populations adapted to different geographical regions. PC2 distinguished the populations reared at non-native conditions (compare cr17.5 and w27.5 in fig. 1a). The first two Principal Components of gene expression from the 31 "interaction" genes were also sufficient to discriminate between the populations and temperatures (fig. 4). PC1 separated the rearing temperatures within the tropical strain (cr17.5 and cr27.5 in fig. 1c) and PC2 separated the rearing temperatures within the temperate population (w17.5 and w27.5 in fig. 1c). The expression of a small set of genes was sufficient to describe genetic differentiation between natural populations and developmental plasticity. Although these statistics do not necessarily imply causality, gene expression in these genes might well be involved in adaptation to geographical and developmental temperatures.

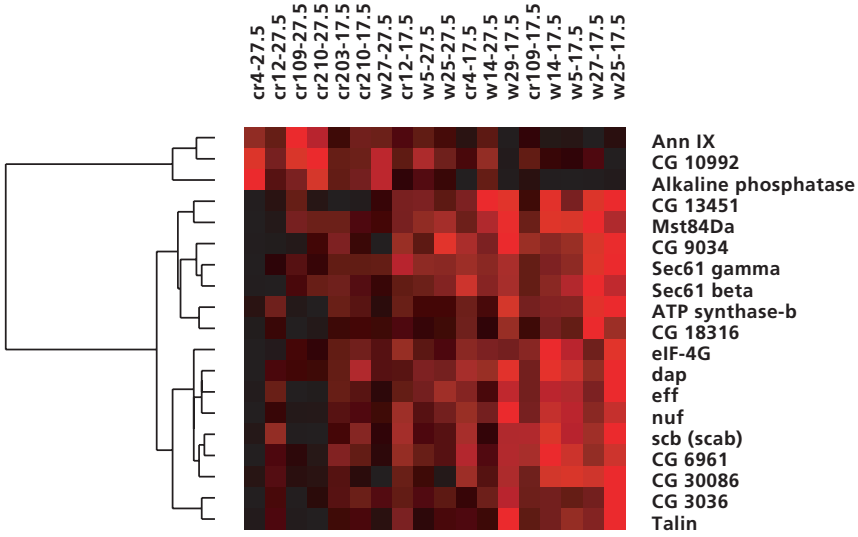


Fig 2. Patterns of transcript abundance of the 19 genes with expression that shows a significant correlation with adult body size. The intensity of the red color represents the relative mRNA levels; only for the illustrations the values were standardized to range between 0 and 100, within each gene. The data represents isofemale lines from the tropical (cr) and temperate (w) populations, reared at 17.5°C and 27.5°C. Arrays are order for ascending mean isofemale line weight. The dendogram on the left groups genes with similar expression across isofemale lines of increasing mean body weight. Expression of three genes shows a negative correlation with body size, the expression of 16 other genes shows a positive correlation.

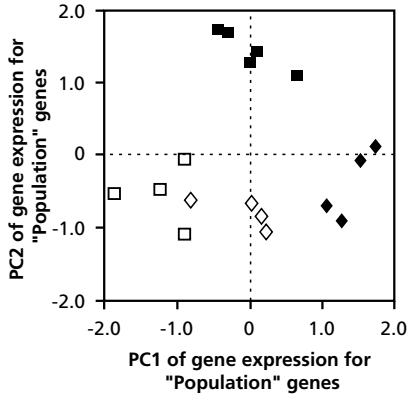


Fig 3. Scatter plot of the first and second Principle Component from gene expression, based upon genes that show a significant difference between populations in a two-way ANOVA. The data represents the tropical population raised at 17.5°C (■) and 27.5°C (□) and the temperate population raised at 17.5°C (◆) and 27.5°C (◇). PC1 (46.10% of variance in gene expression) separates the geographical populations at their native developmental temperatures, i.e. ◆ vs. □. PC2 (18.85% of variance in gene expression) separates the geographical populations under non-native conditions, i.e. ■ vs. ◇. Note that the 45° and 315° diagonals separate the geographical populations and developmental temperatures respectively.

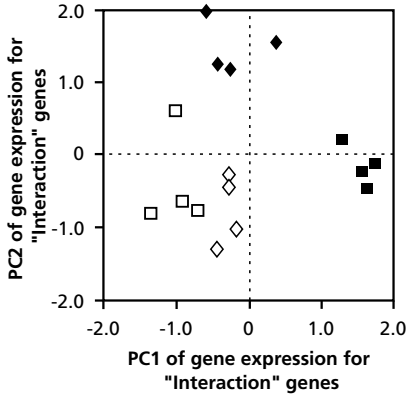


Fig 4. Scatter plot of the first and second Principle Component from gene expression, based upon genes that show a significant population × temperature interaction in a two-way ANOVA. The data represents the tropical population raised at 17.5°C (■) and 27.5°C (□) and the temperate population raised at 17.5°C (◆) and 27.5°C (◇). PC1 (46.36% of variance in gene expression) separates the developmental temperatures within the tropical population. PC2 (26.46% of variance in gene expression) separates the developmental temperatures within the temperate population. Note that the 45° and 315° diagonals separate the geographical populations and developmental temperatures crosswise, opposed to the pattern found in the Principle Components from gene expression of "Population effect" genes.

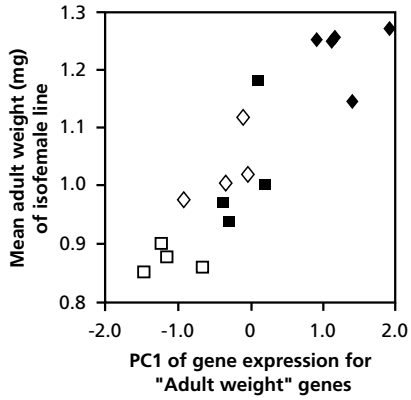


Fig 5. Scatter plot of the first Principle Component from gene expression, based upon genes that show a significant correlation with adult weight and mean adult weight of isofemale lines. The data represents the tropical population raised at 17.5°C (■) and 27.5°C (□) and the temperate population raised at 17.5°C (◆) and 27.5°C (◇). The plot depicts the correlation (R^2=0.902) between the composite measure of expression of 19 genes (74.11% of variance in gene expression) and adult weight. The correlation is present within the populations (rectangle, R^2=0.857 vs. rhombus, R^2=0.867) and within the developmental temperatures (closed, R^2=0.767 vs. open symbols, R^2=0.81).

PC1 summarizing gene expression of the 19 "weight" genes (fig. 2) was subjected to a correlation test with adult weight. Mean adult weight of isofemale lines correlated up to 90% with the derived measure of gene

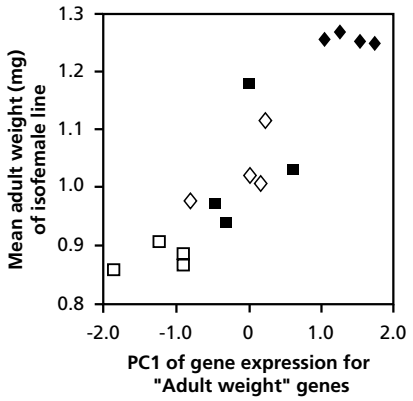


Fig 6. Scatter plot of the first Principle Component from gene expression, based upon genes that show a significant difference between populations in a two-way ANOVA and mean adult weight of isofemale lines. The data represents the tropical population raised at 17.5°C (■) and 27.5°C (□) and the temperate population raised at 17.5°C (◆) and 27.5°C (◇). The plot depicts the correlation (R^2=0.907) between the composite measure of expression of 45 genes and adult weight. The correlation is present within the populations (rectangle, R^2=0.734 vs. rhombus, R^2=0.931) and within the developmental temperatures (closed, R^2=0.853 vs. open symbols, R^2=0.867).

expression (fig. 5). Note that isofemale lines from the tropical population at 27.5°C did not vary much in adult weight or gene expression; neither did isofemale lines from the temperate population at 17.5°C. Native conditions were not very conducive to the expression of genetic variation. However, considerable variation in both weight and gene expression was detected under non-native conditions. It has been reported before that populations express more genetic variation when tested under stressful or non-native conditions (Hoffmann and Merila 1999) (Noach et al. 1996). Our results are in line with these observations. In summary, difference in gene expression and adult weight was highly significant and strongly correlated both within the populations over temperatures (plasticity) and between the populations at both temperatures (genetic variation). Finding that significant correlation between larval gene expression and adult size was present within populations and not only across populations, showed that detecting "weight" genes was not an artifact of population differences, even considering the large overlap between the "population" genes and "weight" genes. In fact, PC1 derived from the "population" genes correlated with body size equally well (fig. 6), suggesting that geographical adaptation and variation in body size were very closely related.

Discussion

The search for the molecular determinants of adaptive variation in quantitative traits is just beginning, but it is already clear that this effort will merge developmental genetics with quantitative genetics (Gibson 2002; White 2001). A major challenge remains in deducing causal mechanisms from statistical associations. We described a pattern of co-regulated gene expression sufficient to distinguish geographical populations and explained, in a statistical sense, variation in adult body size from variation in larval gene expression. One way to assess the genes' possible role in adaptation or in determining body size is to elaborate on their known cellular and biological functions. If variation in body size were a causal component of geographical adaptation, genes differently expressed between the populations might have functions such as cell growth, proliferation and morphogenesis relevant for establishing adult body size. The considerable overlap between the "population" and "weight" genes already suggested that this might be the case.

A total of 69 genes were detected for differing expression between the populations or for exhibiting Population by Temperature interaction. 39 of them are of unknown function (http://flybase.bio.indiana.edu), the remaining 30 could be assigned to a cellular function using the Gene Ontology database (http://www.ebi.ac.uk/ego). 16 of these genes (53%) have functions in "cell growth and maintenance" including cell proliferation. In addition, *Ras85D* a known oncogene involved in morphogenesis was detected (Oldham et al. 2000). Four genes seem to be involved in energy metabolism. Out of four other genes involved in cell communication, two are known stress response genes (Khush and Lemaitre 2000): *Lectin-GalC1* was highest expressed in the temperate population reared at 27.5°C, but *Drosomycine* levels were highest in cold reared tropical larvae. Both cases represented non-native conditions for the geographical populations. Being reared under conditions not resembling the environment the genotypes are presumably adapted to might be experienced as stressful.

Nineteen genes were highly correlated with adult weight; twelve of these could be assigned to a cellular function. Eleven (92%) were involved in "cell growth and maintenance". Several of these genes could be directly linked to morphogenesis. *Scab* has known major effect mutants altering cell movement and disrupting morphogenesis (Stark et al. 1997). *Sec61β* is involved in the protein translocation of *Gurken* and interacts with *Ras85D* (Ford 2002; Valcarcel et al. 1999). *Talin* is an integrin binding molecule and contains an insulin receptor substrate domain. Integrins are known mediators of morphogenesis and the insulin receptor pathway is known to regulate body

size (Brogiolo et al. 2001; Brown et al. 2000; Oldham et al. 2000). *eIF-4G* codes for a eukaryotic initiation factor involved in protein biosynthesis and was suggested to regulate growth (Galloni and Edgar 1999). *eff* is a dominant modifier of the *polyhomeotic* extra sex combs phenotype (Fauvarque et al. 2001). CG 10992 is a Cathepsin B molecule. Cathepsin B is known to be involved in the activation of latent TGF-β in a parasite living in host macrophages (Somanna et al. 2002). Where molecular function could be assigned it seemed that many genes detected in this study were modifiers of "larger" effect genes involved in fundamental stages of morphogenesis, and in pathways affecting growth (Oldham et al. 2000). To confirm this intuitively appealing result, an additional analysis was performed. Experimentwise, we seemed to detect an overrepresentation of genes involved in cellular growth. To address this issue a chi-square association test was performed; we tested whether falling into the category "cell growth and/or maintenance" increased the probability of being called significant in our analyses. Approximately 13500 genes are represented on the Affymetrix GeneChip, 2575 *Drosophila* genes are involved in cellular growth (according to GO). As 24 out of 76 genes called significant in our analyses belonged to that category, it could be shown that genes in cell growth are significantly ($p=0.005$) overrepresented in our sets of candidate genes. That evolution of body size should involve alterations to basic cellular processes is not surprizing. Studies on geographical variation have shown that body size variation can be the result of differences in cell numbers and/or cell size (de Moed et al. 1997; Zwaan et al. 2000). Either mechanism must involve alterations to fundamental processes in cellular growth.

A large fraction of the genes detected in this study are potentially causative to the observed genetic variation in body size, because they fall in a plausible functional category. However, an important question remains in deducing whether the expression profiles of these nineteen genes have changed independently from each other and thus contribute equally to the body size variation. Alternatively, a smaller number of causative changes may coordinately regulate the expression of these genes. Figure 2 shows the results of a cluster analysis on the expression data. Apart from the obvious dichotomy between gene expressions positively vs. negatively correlating with body size, there are at least two additional subsets of genes showing very similar expression profiles. Although speculative, this result suggests that as few as three causative changes may coordinately regulate the expression of a small number of genes to induce large and presumably adaptive variation in a life-history trait.

The pattern of gene expression at different environmental temperatures differentiated the geographical populations, pointing to gene regulation as a mechanism for adaptation (Barrier et al. 2001; Crawford and Powers 1992;

Oleksiak et al. 2002). We found that expression of a small set of genes, already known to be involved in morphogenesis and growth control (Oldham et al. 2000), statistically explained up to 81% (R^2 = square of correlation coefficient) of the variation in adult body size. Both the variation between natural populations and the developmental plasticity was explained. Differential larval gene expression was highly associated with geographical adaptation exemplified by the populations and with evolved developmental adaptation to temperature.

Supplementary Information accompanies the paper on the Molecular Biology and Evolution website.

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Chapter 7

Antagonistic pleiotropy for life-history traits at the gene expression level

Zoltán Bochdanovits and Gerdien de Jong

Abstract

Life-history trade-offs prevent different components of fitness (i.e. reproduction and longevity) to be maximized simultaneously. Overall fitness is maximized by maintaining the optimal combination of negatively correlated life-history traits. Although the existence of trade-offs (i.e. negative correlations between life-history traits) has been clearly demonstrated, the "classical" mechanism of adaptive resource allocation that should underlie them has recently received criticism. In this study we explore the molecular mechanisms of life-history trade-offs applying a quantitative genomic approach. We measured global gene expression in larvae of nine isofemale lines of *Drosophila melanogaster*, and found 34 genes with expression that coincided with the genetic trade-off between larval survival and adult size, an important life-history trade-off. The joint expression of these candidate "trade-off" genes explained 86.3% of the observed trade-off at the organismal level. 14 genes have known functions and suggested that the larval survival-adult size trade-off could be the result of resource allocation at the organismal level, but at the level of cellular metabolism would reduce to a shift between energy metabolism vs. protein biosynthesis, regulated by the RAS signaling pathway.

Keywords: life-history evolution, genetic trade-off, antagonistic pleiotropy, quantitative genomics, global gene expression

Introduction

Life-history traits are often genetically negatively correlated (Barton and Turelli 1989; Bell and Koufapanou 1986). This can be seen as the result of simultaneous selection on two correlated traits. A positive genetic correlation between life-history traits will soon lead to fixation of any particular allele. Only negative correlations between life-history traits persist, and maintain genetic variation (Rose 1983). The loci involved in life-history trade-offs are postulated to show antagonistic pleiotropy: each locus influences both traits, but does so in opposite direction (Stearns 1992). Consequently, fitness will be maximized but a further increase in one trait (i.e. fecundity) will decrease the other (i.e. longevity). The physiological mechanism underlying this pattern should involve competition for some common limiting substance. In other words, life-history trade-offs are the result of adaptive resource allocation of limiting resources (Zera and Harshman 2001).

This explanation of the maintenance of genetic and phenotypic variation in life-histories has some empirical support (Zera and Harshman 2001), but no specific molecular mechanisms have been put forward. No actual loci are known that exhibit antagonistic pleiotropy on two correlated life-history traits. The most specific data are QTLs for ovariole number and for male longevity that have been mapped to the same chromosomal region, and these two traits did show a negative genetic correlation (Wayne et al. 2001). In contrast, developmental genetic studies in *C. elegans* have suggested that trade-offs could be the result of variation in molecular signaling, and not be caused by resource allocation (Leroi 2001). Leroi argues that removing reproduction increases longevity not by removing "a sink for some resource but because it removes the source of some signal", and offers components of a signal transduction pathway as possible candidates. In the light of these divergent approaches to life-history trade-offs, the problem that needs attention is thus the following: does negative pleiotropy in genes affecting life-history traits exist? If so, are these genes involved in resource allocation or rather in signal transduction?

The way to address this issue is to search for genes that oppositely affect two life-history traits known to be negatively correlated. Adaptive variation in quantitative traits has been suggested to be the result of variation in gene expression rather than allelic differences (Barrier et al. 2001; Mackay 2001; Purugganan 2000; Chapter 6). Consequently, in this study we explored global gene expression in staged third instar larvae of nine isofemale lines of *Drosophila melanogaster*. For the same strains two life-history traits, pre-adult survival and adult body weight, have been measured. These traits are expected to exhibit a genetic trade-off, i.e. genotypes that have a higher pre-adult

survival are expected to have a lower adult size and the other way around. If quantitative trait variation is the result of variation in transcript (Barrier et al. 2001; Mackay 2001; Purugganan 2000; Chapter 6) rather than of allelic differences, *and* if the genes involved exhibit antagonistic pleiotropy, we may expect to find genes whose expression correlates positively with one trait but negatively with the other. Such genes would qualify as "trade-off" genes. The question then remains whether they are involved in resource processing or in molecular signaling.

Materials and Methods

Fly stocks and experimental conditions

Five isofemale lines from Wenatchee (47.26 °N, 120.20 °W; WA, USA) and four isofemale lines from San José (9.59 °N, 84.04 °W; Costa Rica) collected in the summer of 2001 were kept on standard corn medium at 17.5°C until the start of the experiment. Isofemale lines descended from a single inseminated female and represent genetic variation within the population. Prior to the experiment the lines were reared at the experimental temperature for one generation to rule out possible effects of parental rearing temperature. Larvae were raised at low density under unlimited food conditions. The experiment was conducted at 27.5°C on standard corn medium stained with 0.05% bromophenol blue. This medium has no effect on larval growth and allows for accurate staging of third instar larvae just prior to pupariation at their maximum size (Andres and Thummel 1994). Adult males that emerged from the vials the larvae were previously collected from were weighed in groups of five to the nearest 0.01 μg on a Sartorius microbalans. In a separate experiment larvae from the same isofemale lines were raised at low density (exactly 50 eggs/jar) and under unlimited food conditions. From these vials *all* larvae were collected at the same time as the staged larvae used for the gene expression analysis. The larvae were collected by pouring a saturated sugar solution over the medium. Larvae surface from the medium and float on top of the saturated sugar solution. The number of larvae collected added to the number of the occasional pupae already present in the vials was taken as a measure of pre-adult survival.

Gene expression analysis

Using a paintbrush, larvae with dark blue guts that stopped feeding and started to wander were manually collected from the surface of the medium and were immediately frozen in liquid nitrogen. Approximately 20 larvae from each isofemale line were used for isolating mRNA with Qiagen Direct

mRNA kit. From approximately 3 μg of mRNA, Bio-11-CTP and Bio-11-UTP labeled aRNA were prepared using standard Affymetrix protocols (http://www.affymetrix.com/Download/manuals/expression_ever_manual.pdf). The labeled aRNA were applied to 10 Affymetrix Drosophila Genome Arrays. Hybridization and scanning was performed on Affymetrix Fluidics Station 400 and GeneArray(r) Scanner at the Leiden Genome Technology Center. The raw data were subjected to global normalization per GeneChip on Microarray Suite 5.0 prior to further analysis. The data acquired from these procedures are relative measures of gene expression independent from the original larval weights.

Data analysis

Overall, we detected the expression of 7652 of more than 13500 genes (56%) represented on the Drosophila Genome Array, with up to 41% detected on individual arrays. To allow for meaningful statistical analysis of the data we selected only probes that have been detected on at least eight of the nine microarrays. This filtering resulted in 1670 probes for further analysis. In this dataset correlation coefficients were calculated between the measure of gene expression of each gene and both adult weight and pre-adult survival. Genes that showed antagonistic pleiotropy were defined as those genes that occupied the opposite tails of the two frequency distributions of correlation coefficients, i.e. exhibited strong negative correlation with one trait but strong positive correlation with the other trait. Due to chance alone, there will always be genes that are present in the two extremes at the same time. These genes are False Positives and their number depends on how wide the "lower" and "upper" tails are set. The cutoff values could in principle be set arbitrarily, but for each cutoff value a certain number of expected False Positives (FP) will be present in the set of candidate genes (CG) detected. The *expected* probability of one gene to be present in the opposite tails of the two distributions simultaneously just by chance alone is the product of the *observed* frequencies of coefficients equal to or more extreme than the cutoff value. This expected probability multiplied by the number of genes in the dataset (1670) gives the expected number of False Positives (FP). To allow for an objective decision on choosing the cutoff value, the number of expected FPs and the number of CGs were determined for a series of cutoff values. Lower FPs coincided with a lower ratio of FP/CG but the relationship was not linear. At low FPs (less than approx. 4) the ratio FP/CG decreased only slightly with a further decrease of FP, from 0.15 (FP=4.2) to 0.1 (FP=1). For this analysis we chose to except FP=2, FP/CG=0.12. A further decrease in FP did not have a substantial effect on FP/CG but would decrease the number of CGs from

34 to 20. This would probably result in losing relevant information without a substantial improvement of sensitivity. Given 1670 probes, this procedure results in considering genes that occupy the lower and upper 3.5% of the distributions ($0.035 \times 0.035 \times 1670 = 2.04$). The cutoff values for the correlation coefficients between gene expression and male weight were -0.62 and 0.74, and for the correlation coefficients between gene expression and pre-adult survival were -0.62 and 0.59. The cumulative Poisson probability for finding 34 hits when 4 were expected was calculated in Excel 97. A Principal Component analysis on the gene expression data was performed in SPSS 10.0. Correlation tests between (1) pre-adult survival and male weight and (2) PC1 and the two life-history traits were performed in SPSS 10.0. A two-way analysis of covariance (SPSS 10.0) was used with PC1 as dependent variable and pre-adult survival and male weight as covariates to measure the percentage explained variance in the data.

Results

Detection of genes with antagonistic pleiotropy

A negative correlation was observed between the family means of pre-adult survival and male weight ($\rho = -0.683$, $p = 0.04$, $n = 9$), showing the expected trade-off. Normalized measures of relative gene expression were acquired for each isofemale line. The correlation coefficients between gene expression and both male weight and pre-adult survival were calculated. Most genes showed no or very low correlation between gene expression and either trait, but some genes showed very strong positive and some genes showed very strong negative correlation. The two distributions of the correlation coefficients were very close to normal (fig. 1A and B). Genes that showed antagonistic pleiotropy were those genes that occupied the lower tail of one distribution *and* the upper tail of the other distribution simultaneously. For every cutoff value of the tails, a set of Candidate Genes (CG) could be found containing a certain number of expected False Positives (FP). The absolute number of expected FP and the ratio FP/CG could be used as objective criteria to decide on the cutoff values to be used (See Materials and Methods). We chose to except two False Positives. In a dataset consisting of 1670 values, two genes were expected to be present in the lower 3.5% of the first *and* the upper 3.5% of the second distribution, by chance alone ($0.035 \times 0.035 \times 1670 = 2.04$). If the observed number of genes present in the lower 3.5% of one distribution *and* present in the upper 3.5% of the other would be considerably higher than 2, those genes would qualify as "trade-off" genes. Using this approach we detected two

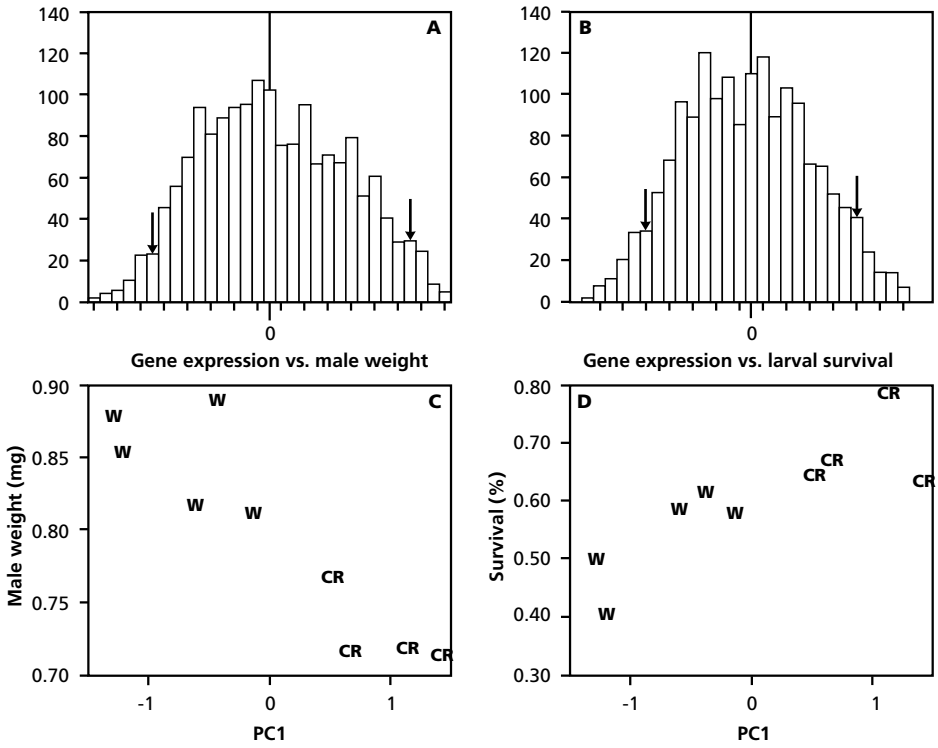

Fig 1. A and B represent the frequency distribution of the correlation coefficients between gene expression of 1670 genes and male weight (A) and gene expression of 1670 genes and larval survival (B). The arrows indicate the cutoff values for the 3.5% most extreme correlation coefficients, that result in 2 expected False Positives for a dataset of 1670 values. The cutoff values for the correlation coefficients between gene expression and male weight were -0.62 and 0.74, and for the correlation coefficients between gene expression and pre-adult survival were -0.62 and 0.59. C and D represent the scatter plots between the first principal component derived from the gene expression data on the 34 candidate "trade-off" genes and male weight (C) and larval survival (D). W represents isofemale lines from Washington, CR represents isofemale lines from Costa Rica.

categories of genes, the "low weight, high survival" genes and the" high weight, low survival" genes. In both categories we expected two False Positives, and detected fifteen and nineteen genes respectively (Table 1). The probability of getting 34 (or more) hits when 4 are expected can be calculated from the Poisson distribution and equals 1.08×10^{-10}. Hence it was extremely unlikely that this set of candidate genes was detected by chance.

Quantifying antagonistic pleiotropy at the gene expression level

These 34 genes exhibited antagonistic pleiotropy in gene expression on two life-history traits in isofemale lines from natural populations. To quantify the joint effect of variation in expression of these 34 candidate genes on the phenotypic variation in the two life-history traits, we extracted the

Table 1. 34 candidate "trade-off" genes from the categories "Low weight, high survival" and "High weight, low survival". Description of the genes can be found in the Flybase database, annotation of molecular and biological functions were derived from the Gene Ontology and KEGG databases.

	Name	Molecular function	Biological function
Low weight, high survival	CG3244	Larval cuticle protein	Contributes to the structural integrity of the cuticle of an insect larva
	Lcp1		
	CG15308		
	CG17738		
	CAH1	Carbonic anhydrase 1	Energy Metabolism
	CG7567		
	CG10622	Citrate cycle; succinate-CoA ligase	Carbohydrate Metabolism
	CG14454		
	CG10311		
	CG15353		
	CG11300		
	CG2233		
	CG7834	Electron transport flavoprotein, works in conjunction with acyl-CoA dehydrogenase	Mitochondrial electron transport system
	CG8661		
	CG8145	nucleic acid binding	
High weight, low survival	Smt3	Protein tagging; ubiquitin-like protein	Cell growth and maintenance
	CG1249		
	CG14957	mRNA splicing	Cell growth and maintenance
	BcDNA:GH02250		
	CG12581		
	CG18239		
	Arf51F	ADP ribosylation factor 51F	Cell growth and maintenance
	CG12868		
	CG3164	ATP-binding cassette (ABC) transporter	Nucleotide binding and transport
	Est-P	Carboxylesterase	
	CG14483		
	BcDNA:GH09045		RAS protein signal transduction
	CG5739		
	CG15219		
	CG3843	Ribosomal protein L10A; structural constituent of ribosome	protein biosynthesis
	CG18449		
	ImpE2	Ecdyson inducible gene E2	imaginal disc eversion - imaginal disc morphogenesis
	CG5171	Trehalose phosphatase	
	CG18078	RNA-binding	

information contained in the data in one derived variable, the First Principal Component (See Methods). PC1 contained over 70% of the variation present in the expression of the 34 genes and was subjected to correlation tests with both pre-adult survival and male weight. This combined measure of gene expression showed significant and very high correlation with pre-adult survival ($r=0.830$, $p=0.006$, $n=9$) and male weight ($\rho=-0.906$, $p=0.001$, $n=9$) (fig. 1C

and D). PC1 explained 86.3% of the observed trade-off, as measured in a two-way analysis of covariance. Overall, gene expression of these selected genes correlated better with both life-history traits, than they did amongst each other.

Discussion

Resource allocation or molecular signaling?

The first question asked in this study was whether genetic trade-offs are the result of genes exhibiting antagonistic pleiotropy on life-history traits. We detected 34 genes with expression exhibiting antagonistic pleiotropy. These genes explained most of the genetic trade-off between pre-adult survival and male weight. The next issue is to decide what the probable mechanism might be. Evidence from statistical population genetics and physiological studies indicated that genetic trade-offs are the result of resource allocation (Christians 2000). However, valid observations have suggested that apparent trade-offs could be induced by molecular signaling (Leroi 2001).

In this study two classes of "trade-off" genes were detected, the "low weight, high survival" genes and the "high weight, low survival" genes. Available data on the annotation of these genes (Gene Ontology database, http://www.ebi.ac.uk/ego; KEGG database, http://www.kegg.org) is summarized in Table 1. Four out of fifteen (26.7%) "low weight, high survival" genes and ten out of nineteen (52.6%) "high weight, low survival" genes have known molecular and/or biological function. All four annotated "low weight, high survival" genes seemed to be causally involved with the observed trade-off. Larval cutical protein 1 is a structural component of the larval cuticle. High expression of this protein might be an investment in larval defense. Genotypes that did so produced higher pre-adult survival at cost of adult weight, in agreement with the predictions of adaptive resource allocation. The three other genes were all enzymes involved in energy metabolism. Genotypes with increased energy metabolism were predisposed to have a higher larval survival at cost of adult size. This finding supported adaptive resource allocation to underlie life-history trade-offs.

Three "high weight, low survival" genes had known molecular functions but no obvious involvement with any biological processes, according to the Gene Ontology database. High expression of an ecdyson-inducible gene (*ImpE2*) is probably not related to the trade-off, but in fact confirmed that the intended developmental stage, i.e. just prior to pupation, has been used. The other six genes had biological functions that seemed relevant for

determining adult weight. Two genes had functions in protein metabolism, a third was involved in mRNA splicing and a fourth was a structural constituent of ribosomes (L10A). The first three were categorized as "cell growth and/or maintenance" and all are involved in protein biosynthesis. In addition one nucleotide transporter gene was detected, plausibly related to DNA synthesis, thus cell division and/or growth. These results suggested that genotypes with enhanced larval protein biosynthesis and/or cellular growth were predisposed to grow large at the cost of their larval survival, in line with the hypothesis of adaptive resource allocation.

However, the last annotated "high weight, low survival" gene adds an interesting complexity to the issue. BcDNA:GH09045 is part of the RAS signaling pathway, which was already known to be associated with growth regulation (Ayllon and Rebollo 2000). In our data, naturally occurring genotypes with higher RAS signaling activity were larger, at cost of larval survival. The most obvious interpretation would be the following. Adaptive resource allocation does underlie the larval survival vs. adult size trade-off, and is caused by differences in cellular metabolism, i.e. protein biosynthesis (growth) vs. energy metabolism (survival). However, this shift might be regulated by the RAS signaling pathway. In this view BcDNA:GH09045 would not be a "death signal" as proposed in *C. elegans* (Leroi 2001) but would simply shift the focus of cellular metabolism, affecting organismal traits. It has been suggested before that molecular signaling could coordinate cellular metabolism (Britton et al. 2002). Taken together with the data presented here we suggest that life-history trade-offs could be the result of adaptive resource allocation at the organismal level, mediated by signal transduction pathways at the level of cellular metabolism.

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Summary and concluding remarks

Clinal variation in body size is a long standing puzzle in evolutionary biology. Body size is the most obvious and the most important characteristic of any organism. A thorough understanding of how and why a certain individual obtains a specific body size, given its evolutionary history and ecological context, is a fundamental question in biology. Besides being of interest in its own right, understanding this very specific and repeatable pattern of natural body size variation also exemplifies a more general problem: how to integrate proximate (mechanistic) and ultimate (evolutionary) approaches to explain as much as possible of the natural variability in an adaptive, phenotypically plastic, quantitative trait?

In the preceding seven chapters, the results of markedly different experiments have been presented. Reductionist in experimental methodology, all studies were aimed at a vertical integration of relevant pieces of the puzzle; both *top-down* and *bottom-up*. A mere description of evolved divergence in body size is no more (or less) interesting than a list of genes found to differ in transcript levels. One by one, the experiments performed were probably just the usual and the expected. The main challenge was to produce a sensible attempt to interpret results from one type of experiment to "explain" patterns found in another. Such an approach seems reasonable if we were to uncover an integrated and comprehensive picture of a very specific instance of adaptive life-history evolution. An overview of the results was already given at the end of the introduction. I propose to limit the discussion here to a clarification of the connections between the various experiments.

Chronologically, the experiments reported in Chapter Three were the first to point towards two important issues; these issues were subsequently elaborated on in completely different contexts. The first result showed that accumulation of metabolites by the larvae takes effect on adult size. This suggested that larval physiology could be meaningfully analyzed as a mediator of resource allocation (i.e. processing). Secondly, Genotype by Environment interaction in larval survival suggested, that thermal evolution need not directly

affect body size, but might induce an adaptive change in a suite of correlated traits, i.e. lead to alternative life-history strategies. Specifically, this result suggested that there could be a trade-off between adult size and larval survival, that might contribute to the maintenance of natural variation in body size.

Chapter One followed up on the notion that concentrating on body size only would not be satisfactory, and a larger suite of correlated life-history traits need to be analyzed to obtain a more precise understanding of the effects of thermal evolution. At the same time, an experimental condition was added, known to most directly affect larvae. If there was indeed a trade-off between size and larval survival, one might expect that non-permissive conditions, such as larval crowding, could illuminate any such effect. Chapter Two was specifically aimed at dissecting the effects of evolutionary temperature per se and the evolution of larval resource acquisition. By combining two selection pressures, the temperature dependent evolution of larval resource acquisition could be addressed. At the same time, the larval traits that were analyzed, most notably feeding rate, could be expected to be mediators of a potential trade-off with adult size, exactly by virtue of their involvement in resource processing. Chapter Four was specifically designed to detect a phenotypic trade-off between pre-adult survival and adult size and show the involvement of larval energy metabolism. Chapter Five was meant to produce evidence for the genetic basis of the life-history trade-off and to produce a physiological explanation for the observed (evolved) variation in glycogen utilization, by focusing on genetic variation in larval enzyme activities. Chapters Six and Seven are obviously related, and combine genetic data (variation in larval gene expression between natural genotypes) with some insights on cellular physiology (annotation of gene functions) to "explain" body size variation, and the genetic trade-off between size and larval survival, from possible shifts in cellular metabolism.

In conclusion, let me offer a brief summary. Geographical variation of adult body size in *Drosophila melanogaster* is plausibly the result of thermal evolution of a genetic trade-off between adult size and larval survival. Because a cold environment seems to select for a large adult body and a warm environment seems to select for increased larval vigor to resist challenges to pre-adult survival, alternative resource allocation strategies have evolved. The physiological basis of these alternative strategies is plausibly variation in the utilization of glycogen induced by variation in the enzyme activities. The actual molecular determinants of this divergence in cellular metabolism might be effectors and regulators of cell growth and differentiation through variation in the activity of signal transduction pathways.

Ultimately, clinal variation in body size is just the façade to be noticed at first glance. The real difference between geographical populations is not a

quantitative but a qualitative one. Warm and cold adapted genotypes are fundamentally different in their cellular metabolism and physiology. This underlying divergence allows them to exhibit alternative resource allocation strategies that result in adaptive life-histories. The result, but not necessarily the target, of this particular instance of life history evolution is the observed clinal variation in adult body size.

Having said this, it is obvious that much of these optimistic conclusions are plausible hypotheses for further research rather than established facts. As ever, more questions remain open at the end of this study than were originally intended to be answered. It was in the year that I was born that Lewontin has already realized that should populations genetics be a mature discipline it needs a more thorough integration of the genotype and phenotype. 29 years later, a true genotype to phenotype map of an adaptive, phenotypically plastic, quantitative trait that is under strong natural selection is still far away. At best, we are at the "end of the beginning" rather than at the "beginning of the end". My hope is that the present thesis has contributed to bring this ideal nearer. In any case, if 29 years from now anyone should know about the mere existence of the present work, I would be more than satisfied.

Zoltán Bochdanovits

Utrecht, May 2003

Samenvatting

Geografische variatie in lichaamsgrootte, ofwel verschil in grootte naar land van herkomst, is een verschijnsel dat al heel lang bekend is in de biologie. Zweden en Nederlanders hebben nu eenmaal een andere gemiddelde lichaamsgrootte dan Italianen of Eskimos. Aangezien grootte een van de meest in het oog springende en belangrijke kenmerken is van elk organisme, is het van groot belang om dit verschijnsel te doorgronden. Een interessant en speciaal geval van geografische variatie in grootte betreft de zogenaamde "clinale variatie". Het verschil in grootte tussen individuen (of populatie-gemiddeldes) uit verschillende gebieden verloopt niet willekeurig: grootte neemt toe met de breedtegraad. Met andere woorden, grotere beesten komen over het algemeen in koudere streken voor. Dit geldt zowel voor warmbloedige als koudbloedige dieren. In dit proefschrift zullen we ons toespitsen op variatie in lichaamsgrootte van een (koudbloedig) insect: de fruitvlieg. De vraag is *hoe* en *waarom* fruitvliegen die aangepast zijn aan een kouder klimaat over het algemeen groter zijn.

Hoe en *waarom* een bepaald individu een bepaalde grootte krijgt hangt af van de omgeving waarin het organisme zich moet handhaven en de *erfelijke* aanleg voor de eigenschap, in dit geval lichaamsgrootte. Omdat "clinale variatie" in lichaamsgrootte van de fruitvlieg zo vaak en zo herhaalbaar voorkomt gaat het waarschijnlijk om een *evolutionair adaptief* patroon. Waarschijnlijk geeft het een voordeel in termen van overlevings- en/of voortplantingskansen (lees: fitness) om juist *die* bepaalde grootte te hebben. De vragen *hoe* en *waarom* zijn in dat geval nauw met elkaar verweven.

Directe omgevingsinvloeden (denk aan te weinig voedsel, heel koud of juist heel warm) hebben een invloed op de lichaamsgrootte van de fruitvlieg, bovenop de genetische aanleg van het betreffende individu. *Hoe* de omgevingseffecten in combinatie met de erfelijke gesteldheid van een individu zich vertalen naar een bepaalde grootte geeft het *proximate mechanisme* van het verschijnsel. Denk hierbij aan de efficientie van het verteren van voedsel bij verschillende temperaturen.

Dezelfde omgevingsfactoren (voedselbeschikbaarheid, klimaat) veroorzaken echter ook selectiedrukken. Ze maken uit welk individu (en dus welke lichaamsgrootte) de meeste kans heeft om te overleven en zich voort te planten. Het antwoord op de vraag *waarom* een individu een bepaalde lichaamsgrootte heeft is de *ultimate verklaring* en moet duidelijk kunnen maken *dat*, en het liefst ook *hoe*, het hebben van een *bepaalde* grootte juist onder *die* omstandigheden fitness *maximalizeert*. Het is namelijk ook denkbaar dat efficient verteren tot een maximale fitness leidt terwijl je klein blijft. De extra energie die je hebt t.o.v. soortgenoten die hun voedsel minder goed verteren kan ook anders besteed worden dan aan groei, zolang het maar je fitness ten goede komt bijvoorbeeld doordat je actiever of zelfs aggressiever bent.

In dit proefschrift heb ik geprobeerd proximate en ultimate verklaringen zo goed mogelijk te verenigen bij het verklaren van een heel oud en vasthoudend probleem, de geografische variatie van lichaamsgrootte van de fruitvlieg. De achterliggende gedachte was om verschijnselen op het niveau van de fysiologie, zoals variatie in de hoeveelheid opgeslagen vetten en koolhydraten, te interpreteren in termen van hun mogelijke verband met fitness, bv. als reserve voor bouwstoffen (groei) of energievoorraad (activiteit). In zeven hoofdstukken zijn de resultaten van zeven experimenten beschreven waarbij patronen op een steeds lager (lees: moleculair) niveau in verband worden gebracht met de adaptieve variatie in lichaamsgrootte.

In hoofdstuk Een zijn de verschillen tussen een noordelijke en een zuidelijke fruitvliegenstam in kaart gebracht, voor een groot aantal kenmerken waarvan bekend is dat ze rechtstreeks invloed hebben op de fitness van de fruitvlieg. Alle experimenten zijn zowel bij een koude als bij een warme temperatuur uitgevoerd zodat elke stam zowel bij de "juiste" als bij een "vreemde" temperatuur getest is. In dit experiment is bovendien ook gebruik gemaakt van lage en hoge larvale dichtheid. Hieruit bleek dat zelfs bij hoge larvale dichtheid en warme test temperatuur er eigenlijk maar één kenmerk is waarin de grote, koudaangepaste stam niet altijd beter was: larvale overleving. De conclusie was dat de stam met de erfelijke aanleg om groot te worden er evolutionair voordeel van heeft als de larven zich in alle rust en het liefst bij een lage temperatuur mogen ontwikkelen. Het vermoeden bestaat dat dit verschil te maken kan hebben met de keuzes die larven maken bij het besteden van hun energie.

In experiment Twee zijn dan ook selectielijnen onderzocht die voor tien generaties niet alleen bij verschillende temperaturen maar ook op verschillende kwaliteit voer zijn gekweekt. De verwachting was dat na tien generaties selectie de fruitvliegen in juist die kenmerken zouden gaan verschillen die ertoe doen voor hun fitness bij die verschillende omstandigheden. Wat bleek was dat de

combinatie van lage selectietemperatuur en slechte voedselkwaliteit maakte dat de fruitvliegen groter werden. De combinatie hoge selectietemperatuur en slechte voedselkwaliteit zorgde juist voor een betere overleving. Afhankelijk van het heersende "klimaat" gaan fruitvliegen dus anders om met voedselschaarste. Bij koud proberen ze ondanks alles hun groei in stand te houden, bij warm zorgen ze voor goede overleving.

In hoofdstuk Drie is er in meer detail gekeken naar de opslag van reservestoffen. Koudaangepaste stammen en bij kou opgroeiende individuen lijken meer glycogeen op te slaan als larf, en wat belangrijker is, het percentage glycogeen per larf (dus de hoeveelheid glycogeen per gram larf) bepaalt de grootte van de adult. Het glycogeengehalte is dus op te vatten als investering in grootte. Tevens is weer gebleken dat de overleving van de geografische stammen (koud- of warmaangepast) het beste is bij de testtemperatuur die overeenkomt met het klimaat waar ze vandaan komen. Koudaangepaste vliegen overleven het beste bij kou, warmaangepaste vliegen overleven het beste bij warmte.

In hoofdstuk Vier is specifiek gezocht naar een verband tussen larvale overleving, adulte grootte en energieopslag, door larven voortijdig te doen stoppen met eten. Onder die omstandigheden moesten larven het zien te redden met de reserve opslag die ze tot op dat moment verzameld hadden. Het bleek dat koudaangepaste vliegen groter werden dan warmaangepaste vliegen, maar ze overleefden de behandeling in een lager percentage, gegeven precies evenveel reserve stoffen (lees: glycogeen). De interpretatie was dat het erom gaat hoe de reserves besteed worden en niet alleen om de hoeveelheid glycogeen per gram larf. Eenzelfde hoeveelheid glycogeen kan of in groei of in overleving geïnvesteerd worden en dat verklaart de verschillen in grootte tussen de geografische stammen. Koudaangepaste vliegen kunnen groot worden ten koste van overleving, warmaangepaste vliegen overleven en blijven klein.

In hoofdstuk Vijf is er een poging ondernomen om de fysiologische oorzaak van het verschil in "glycogeenbesteding" te vinden in de activiteiten van enzymen die glycogeen aanmaken dan wel afbreken. De resultaten wezen erop dat er waarschijnlijk selectie is geweest op de activiteiten van enzymen die glycogeen verwerken. Het leek erop dat de activiteit van enzymen die redelijk nauw betrokken zijn bij de verwerking van glycogeen inderdaad bijdragen tot het verschillend besteden van glycogeen en dus verantwoordelijk zouden kunnen zijn voor de "keuze" die de vliegen maken tussen overleving en groei.

Hoofdstukken Zes en Zeven zijn bedoeld om nog dieper en vooral breeder te kijken naar mogelijke verschillen tussen stammen op een moleculair genetisch niveau. De verschillen in gen-expressie voor alle op dat moment

beschikbare genen zijn in kaart gebracht. De expressie van een betrekkelijk klein aantal genen bleek voldoende om de natuurlijke variatie in grootte te verklaren. De meeste van deze genen bleken betrokken te zijn bij celgroei en celontwikkeling. Een hogere activitiet van genen die celgroei stimuleren leidt tot grotere adulten. Tevens lijkt het ook mogelijk de "keuze" tussen groeien of je energie direct aan overleving te besteden, terug te vinden op het niveau van cellulaire metabolisme.

Samenvattend zijn dit de conclusies. Geografische variatie in adulte grootte van de fruitvlieg is waarschijnlijk het resultaat van temperatuurs-afhankelijke selectie op zowel grootte als larvale overleving. Een koude omgeving lijkt voor een grote vlieg te selecteren en een warme omgeving lijkt voor goede larvale overleving te selecteren. Dit wordt bewerkstelligd door geëvolueerde verschillen in de besteding van reservestoffen. De fysiologische basis voor de verschillende bestedingspatronen is waarschijnlijk variatie in de activiteit van enzymen die glycogeen verwerken. De onderliggende moleculair genetische factoren zijn waarschijnlijk betrokken bij de regulatie van celgroei en celontwikkeling.

Uiteindelijk lijkt natuurlijke variatie in lichaamsgrootte slechts de uiterlijke verschijningsvorm van een diepergelegen verschil tussen warm- en koudaangepaste fruitvliegen. Afhankelijk van het heersende klimaat is het van meer of minder belang om groot te worden. Aangezien groot worden ten koste gaat van de larvale overleving zijn verschillen ontstaan in het bestedingspatroon van de stammen als het gaat om investering van schaarse reservestoffen. Het resultaat van dit specifieke stukje evolutie, maar niet noodzakelijkerwijs het doelwit van selectie, is de geografische variatie in lichaamsgrootte.

Összefoglalás

Előfordulási helyhez kapcsolodó testsúly- és mértebeli különbségek nagyon gyakran fordulnak elő. A svédek, de a hollandok is köztudottan magassabbak mint például a pigmeusok vagy az eszkimók, bár ugyanannak a fajnak (*Homo sapiens*) tagjai. Mivel a testméret az egyik legszembetünőbb és legfontosabb tulajdonsága bármelyik élölénynek (a méret bizony számít), a populációk közötti természetes variáció értelmezése (nemcsak a testsúlyban de más tulajdonságokban is), egy jelentős probléma a biologiában. Az előfordulási helyhez kapcsolható méretbeli különbség egy nagyon érdekes és különleges formája, az úgynevezett "geographical cline", ami nagyjából azt jelenti hogy "földrajzi folyamatosság". Az egyedek (és populáció átlagok) közötti mértebeli különbségek nem véletlenszerüen fordulnak elö: csökkenő szélességi fokon (az egyenlítőhöz közelebb) az átlagos testméret egyre kissebb. Fordítva, hidegebb vidéken többnyire nagyobb állatok fordulnak elő (természetesen egy fajon vagy családon belül hasonlítva). A jegesmedve a legnagyobb medvefaj, de a sajnos már kihalt szibériai tigris is nagyobb volt mint a közismert bengáli rokona. Melegvérű állatokra nézve ez a jelenség már a 19. század végén ismert volt, de ma tudjuk, hogy hidegvérű állatokra, többek között a muslicára is vonatkozik. Ezért lett ennek a diszertációnak a témája a következő kérdés: *miért* és *hogyan* van az, hogy az egyenlítőhöz közelebb élő, vagyis meleg éghajlathoz szokott muslicák kissebbek mint hidegebb vidékhez szokott rokonaik?

Az, hogy egy bizonyos egyednek *miért* és *hogyan* lett egy bizonyos testmérete, az függ a *környezeti* viszonyoktól és a testsúlyra vonatkozó *öröklött* hajlamtól. Mivel ez a földrajzi folyamatosság a muslica testméretében (az egyenlítőhöz közelebb egyre kissebbek) ismételten és egymástól függetlenül előfordul az összes kontinensen, nagyon valószínű hogy ez a jelenség valami *evolúciós előnynek* tudható be. Elképzelhető, hogy számít a túlélési vagy szaporodási esélyeknél, hogy *pont* az a testmérete legyen a muslicának ami van egy *adott* környezetben. A *miért* és a *hogyan* ebben az esetben nagyon közel áll egymáshoz.

Az örökletes hajlam mellett, környezeti behatások (például túl kevés élelem, vagy szélsőséges hőmérsékletek) közvetlenül befolyásolják a muslica fejlődését és kihatnak az egyed testmérettére. Gondoljunk például arra hogy az anyagcsere gazdaságossabban folyik alacsonyabb hőmérséklet mellett és esetleg ez okozza a testmérettbeli különbségeket. Ez egy lehetséges fiziológiai mechanizmus és a *hogyan*-t válaszolja meg. Ugyanakkor a hideghez szokott, átlagosan nagyobb testmérettü muslicáknak lehet *örökletes* hajlamuk arra hogy gazdaságosabban emésszenek. Ilyenkor *ugyanabban* a környezetben (akár hideg akár meleg) mégis nagyobbak lesznek mint az egyenlítöi rokonaik. Hogy *miért* kellene nekik más örökletes hajlamuk legyen, ahoz megint a környezeti behatásoknak van közük.

A környezet nemcsak közvetlenül befolyásolja az egyedek viselkedését, fiziológiáját stb. hanem ugyanakkor a *természetes szelekció* eszköze is. A környezet (élelemhozzáférhetőség, éghajlat) szabja meg azt, hogy melyik muslicának (a kicsinek vagy a nagynak) van nagyobb esélye a túlélésre és a szaporodásra. Ahoz, hogy megértsük miért pont az egyenlítő környékén kissebbek a muslicák, ki kell tudni mutatni azt, hogy pont *ebben* a környezetben az olyan muslicának kissebb a túlélési vagy szaporodási esélye, amelynek az örökletes hajlama azt diktálná hogy nagyobb legyen. Hiába lenne elvileg nagyobb, ha közben elpusztul, az egyenlítői vidéket mégiscsak olyan muslicák fogják benépesíteni amelyek nem kinlódnak ilyen haszontalan dolgokkal, mint a nagyra növés.

Mindez viszont nem jelenti azt, hogy nem jó dolog olyan örökletes hajlammal bírni ami gazdaságos emésztés (anyagcserét) okoz. Az ebből származó előny (több felhasználható energia ugyananyi élelemből) ugyanis nemcsak arra használható fel, hogy nagyobb legyen az ember (vagy a muslica). A termeszétes szelekció ugyanis nem (csak) a testmérettre figyel hanem a túlélési és szaporodási esélyekre. Ezekre az esélyekre a testmérettnek is van hatása, de nem csak annak. A lényeg az, hogy a sok különböző tulajdonságból összeálló túlélési esély legyen a lehető legnagyobb, nem a testmérett. Ha pont meleg éghajlati viszonyok mellett többet ér (a túlélés szempontjából) az energiát például versenygésbe fektetni és nem növekedésbe, akkor az egyenlítő környékét kissebb, de esetleg aktívabb, talán agresszívabb muslicák fogjak benépesíteni. A nagyobb, de lomha rokonok maradnak a hidegen.

Ebben a diszertacióban a *miért* és a *hogyan* kérdéseket egyszerre próbáltam megválaszolni, abból indulva ki, hogy a közvetlen, fiziológiai mechanizmus (a *hogyan*) egyben a múltban fellépett termeszétes szelekció (a *miért*) eredménye. Az egyik megközelítés automatikusan fényt vet a másikra is. Esetleges fiziológiai különbségeket, például felhalmozott zsír- vagy cukortartalékokban amiknek ugy-e könyen lehet közük a testsúlybeli különbségekhez, meg kell próbálni úgy értelmezni, hogy milyen módon lehet

ezeknek kihatasa a túlélési és szaporodási esélyekre. A cukor- és zsírtartalék felhasznalható növekedésen kivül másra is (pl. versenygésre), a kérdés az hogy melyik *beosztás* adja a legnagyobb túlélési esélyt. Ebben a hét felyezetben, hét kisérletnek az eredményeit közlöm, amik egyre mélyebbre merülve probálják meg a fiziológiai és molekuláris tulajdonságokból megmagyarázni ezt a jelentős evolúciós elönyökkel rendelkező testmérettbeli variációt.

Az első felyezetben egy északi (hideghez szokott) és egy déli (meleghez szokott) muslica törzset hasonlítottunk ossze, az eddig egy kisérletben legtöbb különböző tulajdonságra nézve. Olyan tulajdonságokat figyeltünk meg amikről tudott, hogy közvetlenül befolyásolyák a túlélési és szaporodási esélyeket. Ezt, és a többi kisérletet két különböző hőmérsékleten vegeztunk el, hidegen és melegen. Ezáltal az északi és a déli populácio is, egy "ismerős" és egy "idegen" környezetben lett tartva, ami kizárja azt hogy esetleges különbségek egy "idegen" hőmérséklet által okozott stressz erdeményei lennének, mivel megvan az összehasonlítási alap. Emellett, az első kisérletben, alacsony és magas "népsűrűséget" használtunk, hogy egyben egy olyan környezetet teremtsünk (magas népsűrűség) ami erős versenygést vált ki. Kiderült, hogy csak egy olyan tulajdonság van amiben a hideghez szokott, "örökletesen" nagyobb, muslicák nem mindig jobbak. A többi tulajdonságra nézve, még a melegben és a magas népsűrűség mellett sem gyengébbek az egyenlítői muslicákhoz képest, pedig a meleg és a sokaság valószínüleg jobban hasonlít a délvidéki körülményekhez, ami ugy-e az északiaknak "idegen". Az egyetlen tulajdonság amiben a déliek jobbak voltak az a lárvák túlélési esélye. Ez azt jelenti, hogy a hideghez való alkalmazkodással jaró nagyobbá válás jó addig amig a lárváknak nem kell túl erősen versenygeni egymással a tulélésért. Ennek a jelenségnek az oka valószínüleg a felhasználhato energia *beosztasában* áll.

Ezért a második kisérletben olyan muslicákat használtunk amik először tíz generáción keresztül a laboratoriumban alacsony és magas hőmérsékleten és normális és gyenyge élelem mellett voltak tartva. Tíz generácio elég ahoz hogy csak azoknak a muslicáknak az utódjai maradjanak meg a kisérletben amiknek a örökletes tulajdonságaik lehetővé teszik az adott környezetben való tulélést. Ezt hívják laboratoriumi termeszétes szelekciónak. Természetes mert nem mi szabjuk meg hogy kinek szabad túlélni, azt ők harcoljak ki egymás között, de laboratoriumi, mert pontosan tudjuk, hogy milyen ráhatás váltotta ki a szelekciót. Kiderült, hogy ha alacsony hőmérséklet mellett kellett nekik megszokni a gyenge élelmet az a nagyobb testmérettet szelektálta ki. Ha viszont magas hőmérséklet mellett kellett nekik megszokni a gyenge élelmet, akkor a jobb túlélési esélyel rendelkező muslicák maradtak meg. A hőmérséklet függvényében a gyenge élelem minőseg más tulajdonságra szelektál. Ha nincs elég, a hideghez szokott muslicák mégis inkább a növekedésre szánjak a kevés elelmet ami van. Azok a muslicák amik nem ezt tették, kihaltak. Ugyanakkor

a melegben, a keves élelmet a muslicák inkább a (lárva) tulélésre szánják. Amelyek nem ezt tették, szintén kihaltak. Igy lehet az, hogy alig tíz generáció alatt két különböző törzs alakul ki; ezek abban az örökletes hajlamban különböznek, hogy melyik tulajdonságba fektetik be legszivesebben a kevéske energiájukat.

A harmadik felyezetben, megprobáltunk jobban betekinteni azokba a fiziológiai különbségekbe amiknek esetleg közük lehet az élelem *felhasználásában* való különbségekhez. Kiderült, hogy az északi törzsek lárvái nemcsak magassabb zsír- és cukortartalommal rendelkeznek, de *ugyanakkora* cukortartalom mellett *nagyobb* felnőtt muslicává képesek válni. A lárva cukortartalmát tehat fel lehet fogni a növekedésre fordított befeketésként, és ez a növekedés gazdaságosabban történik a hideghez szokott törzsekben (vagyis ezeknek a törzsek örökletes hajlama, hogy gazdságosabban növekedjenek). Ugyanakkor kiderült, hogy az északi törzsek lárvái a déliekhez képest jobban bírják a hideget (a túlelés szempontjából) és a délik meg pont jobban bírják a meleget.

Ebből kifolyólag, a negyedik felyezetben, a növekedés, a lárvák túlélése és a cukortartalom közötti összefüggést tanulmányoztuk, úgy hogy nem teljesen kifejlett lárvákat különitettünk el élelem nélkül. Ilyen körülmények között kell nekik felnőtt muslicáva válni azzal az energia (cukor) tartalékkal ami az elkülonitéskkor éppen van. Kiderült, hogy az északi, hideghez szokott lárvák kissebb arányban élték ezt túl, de amelyik túlélte, viszonylag nagy lett. A délieknek nagyobb arányba sikerült felnötté válni, de többnyire kissebbek maradtak. Mindezt ugyananyi cukortartalom mellett tették, ami azt jelenti, hogy az északiak a növekedésbe fektetnek be, a déliek meg a túlélésbe.

Az ötödik felyezetből az derült ki, hogy ezek a "befektetésbeli" különbségek valoszínüleg onnan erednek, hogy a különböző törzsek cukorfeldolgozó enzimei más hatékonysággal müködnek.

A hatodik és hetedik felyezet egy más oldalról közelitette meg a problémát és a sejtek szintjén való gén aktivitásbeli különbségeket tárta fel. Viszonylag kevés gén (egy pár tucat a 13500-ból) aktivitása mutatott különbséget a törzsek között, de ezek a különbségek elegendők voltak ahoz, hogy a törzsek közötti felnőtt testsúlykülönbség jelentös százalékát megmagyarázzák. Érdekes módon, azok között a gének között amelyeknek az aktivitása magassabb volt az északi törzsekben, nagy arányba olyan gének voltak amikről tudott, hogy a sejtek növekedéséhez van közük. Ennek a kisérletnek az eredményeként a kifejlett egyed szintjén kimutatható méretbeli különbséget sikerult a sejtek növekedésének a szintjén megmagyarázni. Ugyanakkor, a növekedésbe vagy a túlélésbe való befektetés jelei is kimutathatók a sejtek szintjén megfigyelhető génaktivitásbeli különbségekben.

A fentiek alapján az alábbi következtetésre jutottunk: a muslica testsúlyába fellépő természetes variáció nagy valószínüséggel a hőmérséklet hatására, a növekedésre és a lárvák túlélésere kiváltott szelekció eredménye. Egy hideg környezet nagy muslicára szelektál, a meleg környezet pedig jó felnőttéválás elötti túlélesre. A jelenség közvetlen okozója az energia beosztásban fellépett változás, ami valószínüleg a cukorfeldolgozó enzimek hatékonyságával függ össze. A sejtszinten fellépő génaktivitásbeli különbségek, amik egybeesnek a testméretbeli különbségekkel, valószínüleg a sejtek növekedését befolyásolják.

Úgy néz ki, hogy a testméretbeli különbség csak a kivülröl könnyen megfigyelhető megjelenési formája egy mélyebben fekvő különbségnek a hideghez és a meleghez szokott muslica törzsek között. Az éghajlattól függően fontosabb vagy kevésbé fontos nagynak lenni. Mivel a nagyra növés csak a lárvák túlélési esélye árán lehetséges, az evolució során a törzsek különbözőve váltak az energia beosztása szempontjából. Ennek a folyamatnak a következménye, de nem feltétlenül a célja, az előfordulási helyhez kötötdő testmérettbeli variáció.

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Curriculum Vitae

Zoltán Bochdanovits werd op 16 juli 1974 geboren in Oradea (RO). Na het behalen van het VWO eindexamen in 1993 aan het Skinlecollege te Schijndel, heeft hij deelgenomen aan de eindronde van de Nationale Biologie Olympiade in Wageningen en is vervolgens begonnen aan zijn studie biologie aan de Universiteit Utrecht. Tijdens zijn studie heeft hij zes maanden doorgebracht in Budapest aan de Eötvös Loránd Tudományegyetem, waar een eerste kennismaking plaatsvond met de vakgebieden theoretische ecologie en evolutionaire genetica. De eerste onderzoeksstage werd voltooid bij het Hubrecht Laboratorium, waarbij gekeken werd naar moleculaire mechanismen betrokken bij de vroege pootontwikkeling bij de muis. Tijdens de tweede onderzoekstage aan het Rudolph Magnus Instituut is onderzoek verricht naar de neuro-endocriene aspecten van emotionele stress in ratten, met als doel het ontwikkelen van een modelsysteem voor het onderzoek naar post traumatische stress syndroom. In de aanloop naar zijn afstuderen, heeft hij zijn scriptie geschreven aan de Universiteit Utrecht, afdeling Evolutionaire Populatiebiologie, bij Gerdien de Jong over de "Temperatuursafhankelijkheid van lichaamsgrootte bij *Drosophila melanogaster*" om vervolgens vanaf september 1998 een promotieonderzoek te verrichten naar hetzelfde onderwerp.