Evolution and Plasticity of Body Size of *Drosophila* in Response to Temperature

Ph.D. Thesis

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UMI Number: U602507

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Abstract

Ectotherm body size is positively correlated with latitude, giving rise to body size clines, found in different continents. Ectotherm body size also shows a developmental response to temperature, increasing at lower developmental temperatures. To investigate the effects of temperature in the evolution and plasticity of body size clines, I used two species of the genus *Drosophila* as model organisms.

To investigate the cellular mechanism underlying the evolution of wing size clines the two newly established *D. subobscura* wing size clines in the Americas were compared with the ancestral European cline. Clinal differences in Europe and South America were due to changes in cell number, whereas clinal differences in North America are due to changes in cell area. These results suggest that the cellular mechanism underlying the establishment of wing size clines is contingent and not predictable.

The genetic control of body size in the *D. melanogaster* South American body size cline was investigated by means of QTL mapping. The results found in South America were consistent with those previously found in Australia, and in both continents the inversion *In(3R)P* was associated QTL controlling wing area.

Genes of the insulin signalling pathway, known to affect size, were characterized in their effects under different temperature and larval crowding regimes.

The evolution of plasticity of body size traits was analysed using different thermal selection regimes. The phenotypic plasticity of wing size and its cellular components was examined by rearing flies, selected under fixed or variable thermal environments, at two different experimental temperatures. Plasticity of wing size did not vary among the different selection lines, however, plasticity of both cellular components of body size did. Costs and benefits of adaptation to cyclical thermal environments were assessed with larval competition assays and by assessing size when all lines were reared

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under cyclic thermal conditions.

Acknowledgements

1. General Introduction

1.1 Body size and its relation to fitness.

1.1.1 Life history and life history traits: a definition.

"Life history" is the term used to describe the events marking the life cycle of an

organism, starting from its conception to its death (Peters 1983). For instance, the

description of the life history of an individual *Drosophila melanogaster* would include

events such as the hatching of the larva from the egg, the different larval instars, the

pupation, the emergence of the imago from the pupa, the reproductive life, the

senescence and death of the fly. Most, if not all, of the relevant events of a life history

of an individual are either determined or affected by its phenotype; phenotypic traits

affecting life history are often grouped together and referred to as "life history traits".

Examples of life history traits are growth rate, clutch size, age at first reproduction and

reproductive lifespan (Stearns 1992, Roff 1992).

1.1.2 Body size as a life history trait.

One particularly important life history trait is body size. Body size affects life history by

its influence on, among other things, metabolic rate, energy requirements and

reproductive success (Schmidt-Nielsen 1983; Calder 1984); it is therefore clear that

body size has a pervasive effect on almost all aspects of life history, making it a major

parameter in the life history of every individual.

1.1.3 Fitness: a multifaceted concept.

The analytical study of life history measures the relative success of an organism

throughout its life cycle and measures how variations in life history and life history

traits affect such success. The measure of the relative success of an organism is defined

as the "fitness" of the organism. The concept of fitness implies the evaluation of the

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performance of an organism for a given parameter, but consensus for a single, general

definition of fitness has proven elusive (Murray 1990, de Jong 1994), not for lack of

attempts (see for an example: McGraw and Caswell 1996). A measure of fitness is

generally attributed to every life history component (Prout 1971), but the best measure

of fitness is often dependent on the organism in study and the circumstances of the

analysis.

In general, the concept of fitness is associated with a measure of reproductive

success, usually, but not solely, evaluated as number of offspring produced by one

organism or as a short-term numerical dominance of a genotype (Endler 1986; Stearns

1986), although this is not necessarily universally accepted, (see for instance Eldredge

1995). As the different components of the phenotype affect reproductive success in

different ways, fitness can be measured in two ways: local measures and global

measures. A local measure of fitness takes into account the fitness component

associated with a life history trait, on the assumption that the maximization of the

component under study will increase the overall fitness of the individual. Examples of

traits whose correlation with reproductive success can be used as a local measures of

fitness could include egg size (Azevedo 1997), ovariole number and early fecundity

(Wayne 2001) in *D. melanogaster*, rearing period length in the bird *Rissa trydactila*

(Cam *et al.* 2003), body size in sticklebacks (Candolin and Voigt 2003) and the snake

Vipera aspis (Bonnet *et al.* 2000), and colour pattern in the grasshopper *Tetrix subulata*

(Forsman and Applequist 1999). A global measure of fitness involves the interaction of

all fitness components. Examples of a global measure of fitness are Fisher's Malthusian

parameter r (Fisher 1930) and the net reproductive rate R_0 (Charlesworth 1980). Global

measures of fitness do not imply the concept of "goodness of design", often implicit in

local measures of fitness; global measures of fitness describe the effect of natural

selection (Byerly and Michod 1991).

1.1.4 Body size and fitness.

Body size is often either a direct target of selection (Nagel and Schluter 1998; Bonnet et

al. 2000; Reeve et al. 2000), or it shows a significant correlation with traits under

selection (Gebhardt and Anderson 1993; Barbaud et al.1999). A correlation between

body size and fitness has been demonstrated both in the wild (Hews 1990; Preziosi and

Fairbairn 1996; Wikelski and Trillmich 1997; Milner et al. 1999) and in laboratory

experiments (Conner and Via 1992; Santos et al. 1997; Norry and Loeschcke 2002); due

to such importance in the life history and fitness of an organism, understanding the

genetic architecture of body size and the nature of its interaction with the environment

is paramount to shed light on the forces shaping the variation in body size observed in

nature between and within species.

1.2 Clines as a regular pattern in body size variation.

1.2.1 Clines: a gradual spatial variation in morphometric traits.

The term cline comes from the Greek *"klinein"*, meaning "to lean, to bend, to slope". It

was used first by Huxley (1938) to define "a gradation in measurable characters", and

the concept has been expanded by Endler (1977) to include the notion of continuous and

gradual geographic variation in phenotype, gene or genotype frequency.

In its simplest form, a cline is monotonic geographical variation, such as the *D.*

subobscura body size cline found in North and South America (Huey et al. 2000,

Gilchrist et al. 2001), but clines can be nonmonotonic, such as the *D. melanogaster*

body size cline found on the east coast of North America, where size shows an increase

moving from low to mid latitude, and decreases going from mid to high latitude (Long

and Singh 1995).

Despite the fact that the definition of cline does imply a gradual change in the

trait under analysis, it does not necessarily imply that this change is due to natural

selection: a cline can be either caused by random genetic drift and migration (Falsetti

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and Sokal 1993) or it can be caused by natural selection (Berry and Kreitman 1993), or

a mixture of the two (Lenormand and Raymond 2000; Marshall and Sites 2001). The

genetic nature of a phenotypic cline can be confirmed under laboratory conditions by

sampling populations along the cline and rearing them under standard conditions; if the

phenotypic cline persists in the standard laboratory environment, then the genetic basis

of the cline is confirmed (James et al. 1997). The genetic nature of a phenotypic cline,

and its recurrence with parallel clines in different continents in response to similarly

scaled selective forces, is a strong indication that a cline is created and maintained by

natural selection.

1.2.2 Body size clines.

In 1847 Bergmann formalized, in what was going to be known as Bergmann's Rule, the

observation that, amongst birds and mammals, species from higher latitudes tend to be

larger and heavier than species from lower latitudes. The rule was subsequently

expanded by Rensch (1938) to refer to populations within the same species, rather than

to different species of the same taxa. The physiological explanation for the positive

correlation of size and latitude is quite simple: at higher latitude the average temperature

is lower, increasing heat dispersion. To counterbalance this phenomenon, endotherms

increase in mass at higher latitude, thus decreasing the body surface/body mass ratio

and slowing down heat dispersion; the resulting correlation of body mass with latitude

gives rise to body size clines in endotherms (Meiri and Dayan 2003).

It is therefore quite extraordinary to observe that a similar pattern is found in

ectotherms, where such an explanation is clearly not feasible (Ray 1960; Atkinson

1994; Van Voorhies 1996). Body size clines, positively correlated with latitude, have

been observed, among others, in the bee *Apis mellifera* (Alpatov 1929), the fly *Musca

domestica* (Bryant 1977), the ant lion *Myrmeleon immaculatus* (Arnett and Gotelli

1999), the copepods *Scottolana canadensis* and *Attheyella nakaii* (Lonsdale and

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Levington 1985; Ishida 1994), the snail *Littorina obtusata* (Trussel 2000) and the flies

D. serrata (Hallas et al. 2002) and *Z. indianus* (Karan et. al 2000).

Together with body size, ectotherms show clinal variation in other life history

traits correlated with size. For instance, growth rate varies clinally in several species of

ectotherms, with individuals from higher latitude growing faster that individuals from

lower latitudes. This trend has been found in *Scottolana canadensis* (Lonsdale and

Levington 1985), in *Myrmeleon immaculatus* (Arnett and Gotelli 1999), in several

species of fish, like the Atlantic silverside *Menidia menidia* (Conover and Present 1990;

Billerbeck *et al.* 2000); the striped bass *Morone saxtilis* (Brown et al. 1998); and the

mummichog *Fundulus heteroclitus* (Schultz et al. 1996), and two species of frog, *Rana*

climatans and *R. sylvatica*, (Berven et al. 1979; Berven and Gill 1983; Riha and Berven

1991).

A second trait correlated with body size that shows clinal variation in ectotherms

is development time. Clines where development time is negatively correlated with

latitude have been found in the grasshopper *Caledia captiva* (Groeters and Shaw 1992),

in *Myrmeleon immaculatus* (Arnett and Gotelli 1999), in the water strider *Aquarius*

remigis (Blanckenhorn and Fairbairn 1995), the hemipteran *Panstrongylus megistus*

(Barbosa et al. 2001) and in *Rana climatans* and *R. sylvatica* (Berven et al. 1979;

Berven and Gill 1983; Riha and Berven 1991).

1.2.3 Drosophila melanogaster: a model for the study of clinal variation in body size.

Much work has been done in order to understand why and how Bergmann's rule applies

to ectotherms. Most of this work has been carried out on the common fruit fly,

Drosophila melanogaster.

There are many reasons for the focus on this particular species. First, it is known

that *D. melanogaster*, like other cosmopolitan species of the genus *Drosophila*,

produces parallel body size clines on different continents (for *D. melanogaster*: Watada

et al. 1986; Coyne and Beecham 1987; Imasheva et al. 1994; James et al. 1995; van't Land et al. 1999; Gilchrist and Partridge 1999; Huey et al. 2000; Zwaan et al. 2000; for *D. subobscura*: Gilchrist et al. 2001, Calboli et al. 2003, for *D. serrata*: Hallas et al. 2002).

Second, clines for traits correlated with body size clines have been found for *D. melanogaster* in different continents. Clines for development time, ovariole number and egg size have been found in Australia (James and Partridge 1995, Azevedo et al.1996) and South America (van't Land et al. 1999). Clines for ovariole number have also been found in Europe, Africa (David and Bocquet 1975a, b) and Asia (Watada et al. 1986). In all cases, flies from higher latitudes are bigger, lay larger eggs, develop faster and have more ovarioles.

Third, quite obviously, the wealth of information about the genetics, development and physiology of *D. melanogaster* accumulated since the early twentieth century, and the availability of the published genome sequence, makes *D. melanogaster* particularly amenable to empirical testing and therefore a perfect model for the study of the evolution of body size clines.

1.2.4 Clines and temperature.

The regular pattern of *Drosophila* body clines found on different continents strongly implicates natural selection in their establishment (Partridge and French 1996), as does molecular evidence from the eastern Australian cline (Gockel et al. 2001, see below). Temperature is the most probable major selective factor causing these latitudinal clines in size and development time. Latitude is consistently correlated with average, minimum and maximum temperature but not with other factors that could influence size, such as humidity or rainfall (Zwaan et al. 2000). As well as increasing with latitude, body size in *Drosophila* increases both with altitude (Stalker and Carson 1948) and during the colder period of the year (Stalker and Carson 1949; Tantawy 1964; Kari

and Huey 2000). The hypothesis that temperature is a selective agent for the formation of body size clines in ectotherms is supported by empirical evidence: caged laboratory populations of *D. melanogaster* kept at different temperatures show that flies evolve genetically different size, with larger flies in the "cold" selection lines (Anderson 1973, Cavicchi et al. 1985; Partridge et al. 1994). This implies that temperature, or something correlated with temperature, is the selective agent.

Apart from body size, thermal selection lines and body size clines also show a striking coincidence in other traits: development time (James and Partridge 1995) and egg size (Azevedo et al. 1996). All empirical evidence therefore points to temperature as the major selective agent in the establishment of body size clines, with other factors playing a minor role (Kennington et al. 2003).

Finally, it is important to note that, in *D. melanogaster*, body size changes can be achieved through changes in cell size, cell number or both. Wing area has been especially studied under this light. Several works (e.g. Robertson 1959; Cavicchi et al. 1985; Partridge et al. 1994) have found that laboratory thermal selection lines differ in wing area entirely as a consequence of a difference in cell size. Latitudinal clines, on the other hand, show variation in wing area based mainly on cell number, with cell size contributing at most only a small amount (James et al. 1995, 1997; Pezzoli et al. 1997, Zwaan et al. 2000). Considering the remarkable consistency in other phenotypic responses, such discrepancy between natural occurring clines and thermal selection lines requires further investigation. One possible explanation for this difference could be that the cell size difference that we have seen in thermal selection lines is an early stage in the evolution of body size that eventually will evolve into a cell number difference (see also Partridge and French, 1996).

To test this hypothesis, I examined the cellular basis of three wing size clines in *Drosophila subobscura*: Europe, North and South America. *D. subobscura* is endemic to Europe, where latitudinal clines in several traits, including body size, are observed

(Misra and Reeve, 1964). This species has recently colonised South America, with a first report in 1978 (Brncic, et al. 1981), and North America, with a first report in 1982 (Beckenbach, et al. 1986). While the flies in North and South America rapidly diverged from the European ancestral population in many traits (for a detailed list of such traits I refer the reader to Chapter 3), the first survey on body size, conducted using flies collected in 1986 and 1988, failed to show any latitudinal size cline on either continent (Pegueroles et al. 1995). A second survey conducted by Huey and Gilchrist (Huey et al. 2000, Gilchrist et al. 2001) in North America, with flies collected in 1999, did find a wing length cline, with genetically larger flies at higher latitudes. The two newly established *D. subobscura* clines proved to be a unique chance to measure the cellular basis of the latitudinal variation at an early stage and therefore to determine both whether cell size variationwas characteristic of newly established clines and whether wing size itself or its cellular components are a target of selection.

1.3 Body size as a quantitative trait

1.3.1 Quantitative genetics: an introduction.

The term "quantitative genetics" refers to the branch of genetics used in the study of the genetic architecture of quantitative traits (also known as "metric" or "polygenic"), i.e. traits that show a continuous and gradual variation between individuals rather that falling into distinct classes. Genes regulating quantitative traits are referred to as Quantitative Trait Loci, or simply QTL (Falconer and Mackay 1996; Lynch and Walsh 1998). Due to their continuous variation found in nature, life history traits fall within the definition of quantitative traits, and the study of their genetic architecture is consequently "quantitative genetics of life history traits".

The foundations of the theoretical framework used to study quantitative genetics were laid in the early twentieth century. As early as 1906 G. U. Yule proposed that

quantitative traits could be under the cumulative influence of a discrete number of Mendelian genes; in 1910 T. H. Morgan established that Mendelian genes are linked on chromosomes and in 1918 R. A. Fisher demonstrated that correlation between relatives in outcrossing populations can be explained by segregation of QTL. K. Sax was the first to use phenotypic markers to try to detect QTL for seed size in beans (Sax 1923). By 1932 the work of Haldane, Wright and Fisher had synthesized Darwinism and Mendelism, demonstrating that natural (or artificial) selection can affect the Mendelian factors controlling quantitative traits (Fisher 1930; Wright 1931; Haldane 1932); from the 1930' the progress in the theoretical understanding of quantitative genetics was successfully put into practice in genetic breeding programs of plants and animals. Despite this early start, quantitative genetics found itself hindered in the actual detection of QTL by the insufficient number of markers that can be found for any one organism: the visible markers available at the time were simply not sufficient to give an adequate coverage of the genome.

In 1961 Niemann-Soressen and Robertson were the first to attempt QTL mapping in cattle, using blood groups rather than morphological markers. This work was groundbreaking also because it was the first attempt to detect QTL in an existing population rather than in an experimental population produced especially for QTL detection. It was also the first study that attempted to estimate the power to detect QTL. Shortly after, Law (1966) successfully used substitution lines in wheat to map QTL for vernalisation. In the 1970s advances were also made in the statistical theory of QTL detection and the crossing schemes to be employed (Jayakar 1970; Hanseman and Elston 1972; Soller at al. 1976; Soller and Genizi 1978). In spite of all efforts, the major practical problem in the detection of QTL was still the lack of a sufficient number of codominant markers that covered the entire genome of the organism under study, codominant markers having the obvious advantage that they can be unambiguously traced to the paternal or maternal population.

One first step to obviate this limitation was the use of electrophoretic polymorphisms of proteins in the 1980s. While some success was achieved with plants (Tanksley et al. 1982; Kahler and Wherhahn 1986; Edwards et al. 1987; Weller et al. 1988), it was clear that a much greater number of markers is required. These became available at the DNA level. The first of such markers used in quantitative genetics was the "Restriction Fragment Length Polymorphism" (RFLP). Beckmann and Soller (1982) first proposed the use of RFLP as markers to detect QTL, and this approach was successfully employed by Paterson et al. (1988) to map QTL in tomato. RFLP proved a good marker for quantitative genetic studies in plants; in animals, RFLP did not prove as useful due to a relatively low level of polymorphism.

The major breakthrough in animal quantitative genetics came with the introduction of the "Polymerase Chain Reaction" (PCR) by Mullis et al. (1986), a DNA amplification technique that allows to generate enough copies of a target sequence to detect polymorphism consisting of even one nucleotide. In 1989 the use of PCR allows three labs (Litt and Luty 1989; Tautz 1989; Weber and May 1989) to independently identify the presence of short sequences of highly polymorphic repetitive DNA, normally referred to as "DNA microsatellites" or "microsatellites". Microsatellites are prevalent in all organisms, are neutral and codominant and are normally polyallelic. These characteristics immediately make microsatellites an ideal marker for QTL detection studies (e.g. Routman and Cheverud 1997; Gockel et al. 2002; Zhou et al. 2003). A second important marker type obtained by the further refinement of DNA analysis techniques were the "single nucleotide polymorphisms" (SNPs), which are also employed as markers for QTL detection (e.g. Zimmerman et al. 2000; McRae et al. 2002; Nonneman and Rohrer 2003).

It can clearly bee seen from this brief outline that the progress of quantitative genetics has proven to be dependent on two different fields: the advancement of

molecular techniques, which have seen the recent explosion of genomics development (van Buijtenen 2001; Walsh 2001; Stearns and Magwene 2003), and the advancement of computer hardware and software for the statistical computations needed in QTL analysis, as the progress in the understanding of quantitative genetics at the molecular level increases rather than decreases the need for more complex statistical models employed in quantitative genetics and QTL detection. Since the times of R. A. Fisher, advances in quantitative genetics and advances in statistical methodology have been inextricably entwined; this relationship is going to be maintained in the future.

1.3.2 Quantitative genetics and evolution.

Understanding the genetic architecture of an organism's traits, and in particular of life history traits, is the ultimate goal of evolutionary genetics, as this would be the fist step to understand the way natural selection shapes different organisms and would allow inference of the evolutionary history of a particular trait and its evolutionary potential (Roff 1997). For practical and economical reasons though, the vast majority of studies in quantitative genetics have been limited to a small number of domesticated plants and animals. In particular, the need to perform carefully controlled crosses between parental lines, and the lack of sufficient genetic data on all but a handful of species (either model organisms or species of economic interest), has always hindered the detection of QTL affecting variation in life history traits in species living in the wild.

The first steps to circumvent these problems have come from the application of the theoretical framework developed in animal and plant breeding to natural populations. The earliest theoretical models were based on the assumption of linearity, additivity and normality of distribution of genetic effects, on the assumption of a constant fitness landscape (the fitness landscape, first proposed by Wright in 1932, is the multidimensional space relating phenotypes and fitness) and on the assumption of a

constant genetic variance-covariance matrix **G** (Pigliucci and Schlichting 1997). The assumption of equilibrium, although not fundamental, is a consequence of the assumptions just enumerated. More recently, a wealth of works has been produced addressing epistasis, pleiotropy and Gene x Environment interactions in the genetic effects (Wright 1980; Schlichting 1986; Barton and Turelli 1989; Scheiner 1993; Gimelfarb 1996; Taylor and Higgs 2000; Hermisson 2003). Evidence is increasing of non-linearity of effects (Gifford and Barker 1991; Gross et al. 1998; Klingenberg and Nijhout 1999; Roff et al. 1999). The assumptions of a distribution far from normality (Turelli and Barton 1990), and of evolution far from equilibrium (Akin 1983; Altemberg 1991; Charter and Rogers 1997) have been explored. Frequency-dependent selection models have been developed (Wallace 1989 and 1991; Cosmidis et al. 1999; Sinervo and Svensson 2002; Wolf 2003), and, most crucially, the invariance of the genetic variance-covariance matrix has been questioned, both on theoretical (Schlichting 1986; Turelli 1988; Steppan et al. 2002) and empirical grounds (Wilkinson et al.1990, Mazer and Schick 1991a, b; Campbell 1996; Roff and Musseau 1999; Conner et al. 2003).

From an empirical standpoint, several studies have been performed on various life history traits. In the genus Drosophila alone, several works have focused on the detection of QTL controlling ovariole number (Wayne et al. 2001), cuticular hydrocarbons (Takahashi et al. 2001), male and female lifespan (Vieira et al. 2000; Pasyukova et al. 2001), abdominal bristle number and sternopleural bristle number (Gurganus et al. 1998; Gurganus et al. 1999; Kopp et al. 2003), wing shape (Zimmerman et al. 2000), sex comb number (Nuzhdin and Reiwitch 2000), toxin resistance (Jones 1998), genital lobe shape (Macdonald and Goldstein 1999) and body size (Gockel et al. 2002).

Understanding genetic control of life history traits at the molecular level is

fundamental to gain insight into how selection acts on them. Quantitative genetics will greatly influence the way that we describe the pattern of evolution in nature, thanks to the evolving theoretical rationale and the increasing amount of molecular genetic data obtained by QTL detection studies. In the particular case of thermal selection, identifying the genes underlying clinal size variation is one of the steps necessary to understand how selection acts on size.

1.3.3 Genetic control of body size clines in Drosophila melanogaster.

Several works have investigated the genetic basis of body size clines. The focus of these studies has been on chromosome inversion frequencies and DNA sequence polymorphism. In addition to this, QTL mapping studies for body size have been performed on the Australian (Gockel et al. 2002) and South American clines (Calboli et al. in press and detailed in chapter 4).

Work done by Gockel et al. (2001) on neutral markers, analysed flies from the Australian cline. Gockel and colleagues found that latitude explains 80% of the observed variation in wing area. On the other hand, 14 microsatellites loci analysed in the study did not show clinal variation. Microsatellites are neutral markers primarily influenced in their spatial distribution by demographic factors. The different pattern of latitudinal variation observed between body size and neutral markers strongly supports the hypothesis that the Australian body size cline is caused and maintained by natural selection rather that by population structure or drift.

A second line of investigation has concentrated on chromosome inversions. Drosophila melanogaster shows a high degree of polymorphism for chromosome inversions (Mourand and Mallah 1960; Watanabe 1967; Singh and Das 1990). Parallel geographical clines have been found in both body size and the frequencies of the cosmopolitan inversions In(2L)t, In(2R)NS, In(3L)P and In(3R)P in three continents

(Knibb 1982; van't Land et al. 2000); additionally, the same inversions show frequencies that fluctuate seasonally with temperature, decreasing in the cold "winter" conditions (Knibb 1986). These results are of particular interest because not all inversions show the same pattern of spatial variation. In Australia, the frequency of inversions In(2L)t, In(2R)NS, In(3L)P and In(3R)P does show clinal variation parallel to the one observed for body size, while the frequency of inversions In(3R)C and In(3R)Mo does not show clinal variation (Ashburner and Lemeunier 1976, Knibb 1986).

Recent work focused on the inversion In(3L)P, found on right arm of the 3rd chromosome, established that its relative frequency is inversely correlated with cold resistance in flies from the Australian cline (Weeks et al. 2002). In addition to these results, a QTL detection study, performed by association mapping (Gockel et al. 2002) on the Australian cline showed that a high LOD peak for wing area on the right arm of the third chromosome was associated with reduced recombination rates between the microsatellite markers in the region covered by In(3R)Payne. Empirical evidence supports the hypothesis that some chromosomal inversions are associated with the regulation of body size in size clines. The major limitation of work concentrating on chromosome inversion is the size of the inversions themselves, because chromosome inversions span many millions of kilobases and contain hundreds of genes. Nonetheless, these results give at least an indication of the possible position of QTL controlling body size.

1.3.4 From theory to practice: from phenotype to QTL detection.

The set-up of a QTL detection study is deceptively simple. All that is needed are two populations of the organism in study differing in the trait under analysis and polymorphic for a certain number of codominant neutral molecular markers. Individual

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from the two populations are mated and a recombinant F_1 is generated (Lynch and Walsh 1998; Mackay 2001a, b). After the F_1 is produced, further segregating generations are produced by means of backcross with one of the parental lines or by full sib mating, to the desired n-th generation (where n stands for the number of recombinant generations) to form an Advanced Intercross Line (Darvasi and Soller 1995). The effect of multiple generations of recombination between the two parental genotypes is to break the linkage between parental loci.

Once the desired intercross line is produced, each individual of the intercross population is scored for both the phenotype(s) in study and the genotypic markers. The information gathered is used to create an association map between phenotype and genotype. The association study is performed by specially designed software (Basten et al. 2002, but see also http://biosun01.biostat.jhsph.edu/~kbroman/qtl/ for a more comprehensive list). One of the possible methods employed in QTL mapping is Composite Interval Mapping (CIM; Zeng 1993, 1994). CIM tests the hypothesis of the presence of a QTL in a chromosome interval flanked by two markers, simultaneously controlling for the effect of linked QTL outside the test zone within a predefined interval. CIM also controls for genetic background by including a number of markers in a stepwise elimination procedure. The output of CIM analysis is converted into a LOD score plot (Lander and Botstein 1989), where LOD values "peak" above genomic areas containing one or more QTL. The significance threshold value for CIM is calculated by a resampling procedure. Such a genome-wide scan identifies genomic regions associated with QTL controlling the trait under study.

According to the resolution of the first scan, further fine scale mapping can be performed, limited to the areas of highlighted by the genome-wide analysis. This is step not always necessary, though. The findings of an association mapping are used to restrict the focus to a number of positional candidate (or putative) genes. Putative genes

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need to be screened for their expression pattern, for their affect on development and physiology and for the effect of mutants in order to assess the plausibility of their involvement. Final evidence of involvement of a putative gene would come from linkage disequilibrium analysis (Mackay 2001a, b).

The characterisation of the phenotypic effects of putative genes is a non-trivial step in QTL mapping. Considering that just a fraction of the genes in any given organism has been fully characterized (only about 40% of the 13600 genes of D. melanogaster, one of the best known model organisms, have a known function), it is clear that any attempt to identify a gene affecting quantitative variation would be hindered without some background information about the putative loci under study. As a result of QTL mapping studies (see Chapter 4), attention was drawn to a genomic region containing several genes in the insulin/IGF-like signalling pathway. These genes are known to be involved in the control of growth and size in Drosophila (Chen et al. 1996; Bohni et al. 1999; Verdu et al. 1999; Brogiolo et al. 2001). In order to better characterize the effects of these genes for body size, I analysed the effect of null/hypomorphic mutations in the Drosophila insulin-signalling pathway on larval competitive ability at different temperatures and in relation to larval crowding. Mutants in the insulin-signalling pathway are known to affect body size and development, and could be implicated in the quantitative control of body size along latitudinal clines; nonetheless their effects on survival at different temperature and levels of larval crowding are not well characterized. The results of this work are presented in Chapter 6.

The assessment of which of the putative loci are actually controlling the quantitative trait under analysis can be performed by quantitative complementation (Pasyukova et al. 2000; Robin et al. 2002). Briefly, flies bearing different alleles for a putative locus (ideally introgressed into the same genetic background) are crossed with flies carrying a deficiency or a mutation at the locus of interest. This procedure is

normally carried out testing two alleles for the putative locus at one time, but more alleles can be tested. The resulting progeny can be of four possible genotypes: they can carry one or the other allele for the putative locus against the mutation/deficiency or against the balancer chromosome. A linear model is employed to test whether the phenotypic difference caused by the two alleles for the putative locus is the same in the flies carrying the mutation/deficiency compared to flies carrying the balancer chromosome. If the two alleles do not vary in their degree of dominance, the result is quantitative complementation; if the two alleles do vary in their degree of dominance, the result is quantitative failure to complement, and the locus in study is confirmed as having an effect on the trait in analysis. Quantitative complementation has one major caveat: genome-wide epistatic effects are likely to confound the results obtained (Gilchrist and Partridge 1999) unless quantitative complementation is performed in isogenic lines differing only in the QTL; the mutation/deficiency stock should also be backcrossed into the same genetic background. Due to this severe constraint, quantitative complementation is best used to identify quantitative genetic variation in artificially selected inbred strains.

An alternative method to identify putative loci is QTL cloning. QTL cloning, a very powerful technique first adopted by Frary and colleagues in 2000 to identify the gene fw2.2 as a major source of quantitative variation in tomato fruit size (Frary et al. 2000), involves adding a segment of DNA thought to contain a QTL as an additional copy into the genome of the organism in study (see also Fridman et al. 2000 about QTL controlling sugar content in tomato and El-Assal et al. 2001 about QTL controlling flowering time in A. thaliana), then scoring the phenotype of the transformed individuals to asses the effects of the additional copy of DNA. QTL cloning has a number of caveats as well: gene expression levels in transgenes need not reflect endogenous expression patterns and a simple additive model of QTL action might not apply. In addition, this technique is best suited to estimate QTL of large effects in

inbred lines where replicated QTL genotypes are not necessary to estimate the effect of QTL alleles (Flint and Mott 2001).

In summary, the identification of QTL controlling the trait in study is a multiple-step procedure. Different organisms and inbreeding levels have specific advantages and impose specific constraints that call for the use of procedures that take these differences into account. Molecular techniques and statistical methods for the analysis of quantitative variations are ever advancing (Walsh 2001; Barton and Keightley 2002; Doerge 2002; Lund et al. 2003; Schadt et al. 2003), and guarantee that quantitative genetics will play and increasing role in our understanding of the genetic regulation of phenotypes.

1.4 Thermal selection and plasticity of body size

1.4.1 Plasticity: a definition.

"Plasticity" is the ability of a genotype to give rise to different phenotypes in different environments (Bradshaw 1965; Scheiner 1993). "Norm of reaction" (or "reaction norm") is the range of phenotypes that a single genotype can produce in different environments (Wolterek 1909, Stearns 1989a).

Two important points have to be made about plasticity. First, different environments can be so due to qualitative differences (e.g. presence or absence of predators) or due to quantitative differences (e.g. temperature gradients). The problem from an empirical and theoretical standpoint is in the uncertainty of how the organisms in study perceives the environment: quantitative variations can be perceived as qualitative if a threshold mechanism is in place; qualitative variations can be assessed as quantitative, if the perception of apparently discrete environments is based on a continuous scale determined by, for instance, the quantity of a chemical (Via et al. 1995).

Second is the consideration that plasticity can be considered in two distinct ways: "character state" or "polynomial". The character state approach defines plasticity as difference between mean phenotype expressed in different environments (Via and Lande 1985; Van Tienderen 1991; Gomulkiewicz and Kirkpatrick 1992). The polynomial approach defines plasticity as the polynomial function of the phenotypic values expressed in different environments (de Jong 1995; Gavrilets and Scheiner 1993a, b). For discrete environments, the character state and the polynomial approach are mathematically equivalent, if the environments are ordered according to trait value (de Jong 1995; Van Tienderen and Koelewijn 1994). In continuous environments it is always possible to translate a polynomial model into a character state one, but it is not normally possible to do the reverse, making the two approaches not fully equivalent (Via et al. 1995).

1.4.2 Plasticity and evolution.

The knowledge of the genetic basis of plasticity is, if possible, even poorer than our knowledge of the genetic basis of quantitative genetic variation. However, from a theoretical standpoint it is accepted that two classes of genes influence plastic responses (Schlichting 1986; Via 1993; Scheiner 1993a; Via et al. 1995). The first comprises genes whose alleles are expressed in different environments with varying effect on the phenotype ("allelic sensitivity"). The second is composed by regulatory loci that control the differential expression of other genes according to the environment ("gene regulation"). As a regulatory gene could affect the transcription of a locus, modifying its sensitivity to different environments, the two categories partially overlap. It is important to keep in mind that both classes of genes are not expected to have an effect independent of the mean of the trait they influence (Scheiner and Lyman 1991; Scheiner 1993b; Schlichting and Pigliucci 1993, 1995). The outcome of the plastic genetic

regulation of the phenotype can occur in two ways: graded or discrete responses. Allelic sensitivity is recognized as the basis for graded responses; gene regulation as the basis for discrete responses (de Jong 1995; Schlichting and Pigliucci 1995). Reaction norms involve both classes of gene to different extents due to their partial overlapping effects.

The extent to which natural selection directly affects plasticity and reaction norms is still not clear (Via and Lande 1985; Via 1993; Scheiner 1993a, b; Via et al. 1995), but it is accepted that adaptive plasticity could evolve in populations that encounter predictable environmental change (Schlichting 1986; Via 1987; Stearns 1989b; Scheiner 1993a). Plasticity can be either the main target of selection, or the by-product of selection on the different mean phenotypes in different environments (Via 1993; Scheiner 1993b; Via et al. 1995). In a stable environment, when a trait is invariable during one individual's lifetime, such as adult size in insects, only one component of the norm of reaction is exposed to selection during the lifetime of the organism. In this case, natural selection can act only on the expressed phenotypic value, and evolution in the rest of the norm of reaction can occur only through correlated responses (Via 1993; de Jong 1995).

If the environment encountered by an organism changes during its lifetime, several phenotypic components of the reaction norm could be selected at once, even if each organism can only express one phenotypic value at a time. The outcome of selection could be caused by the temporal sequence of different environments that are experienced by an individual. The expression of a specific phenotypic value at a certain stage in the life of an individual could affect fitness at later stages (Lande and Arnold 1983; Gomulkiewicz and Kirkpatrick 1992). An example of this is the effect on fitness of the correlation, or lack thereof, between temperature during and after development. In addition, if different reaction norms carry different costs or different benefits, reaction norms that minimize such costs, or maximize the benefits, are expected to be

selected for, and this could cause reaction norms to be selected on phenotypes other than the one currently expressed (Van Tienderen 1991).

Costs and limits of plasticity (DeWitt et al. 1998) need further discussion. Maintaining the sensory and regulatory machinery needed by a plastic genotype to produce different (adaptive) phenotypes in different environments is likely to require energy expenses (Futuyma and Moreno 1988; Van Tienderen 1991; Moran 1992; Leon 1993). Producing the same phenotype in the same environment for higher energy expenditure would decrease the relative fitness of a plastic phenotype compared to a non-plastic one. Plasticity is limited when a plastic genotype cannot produce a trait mean as close to the optimum value for a given environment as a non-plastic genotype does. This situation could be caused by unreliability in the environmental cue used to control plasticity itself (Moran 1992; Getty 1996) or by a time lag between perception and response to an environmental cue (Padilla and Adolph 1996). Evolution of plasticity is therefore shaped by the fitness advantages it confers to a plastic genotype, and the costs incurred by the genotype to maintain its plastic prerogatives.

Finally, it is important to remember that natural selection acts on populations with a finite number of individuals carrying a finite number of alleles for any given locus. Demography, migration and rates of mutation are all likely to be important factors in establishing the plasticity of a trait and its norm of reaction. Genetic correlation between traits can also affect plasticity. Ultimately, the fact that plasticity results a target or a by-product of selection could be due to the amount of genetic variation present in the population for the trait in analysis, due to genetic correlation between traits under different selective pressure or due to a mixture of the two.

1.4.3 Temperature and plasticity in Drosophila melanogaster.

Temperature has strong phenotypic effect on body size in almost all ectotherms, with

individuals reared at lower temperatures being of bigger body size (Ray 1960; Atkinson 1994). *D. melanogaster* is no exception, showing a pronounced response of body size to developmental temperature in many life history traits.

In particular, body size and developmental time are affected by temperature, with flies growing at lower temperature showing bigger size and longer developmental time (Azevedo et al. 1996; James et al. 1997). Despite the longer developmental time, flies reared at lower temperature have higher growth efficiency, thus achieving bigger size (Robinson and Partridge 2001). Longevity is also affected, with flies kept at lower temperature having an increased adult lifespan (Loeb and Northrop 1917; Pearl 1928; Alpatov and Pearl 1929; Sohal 1986).

As both thermal plasticity and thermal selection act in the same direction on *Drosophila* body size, with larger size in the cold, it could be possible to suppose that the plastic response is adaptive. Where plasticity is adaptive, several models of the evolution of reaction norms argue that under different environmental regimes, and in the absence of genetic constraints or physiological costs, reaction norms should evolve towards a response that gives an optimal phenotype in each environment (Via and Lande 1985; de Jong 1990. 1999; Gomulkiewiz and Kirkpatrick 1992; Gavrilets and Scheiner 1993). If there is an optimal adult body size that increases with declining temperature, then we mights expect that all genotypes would use some combination of genetic mean body size and plasticity to achieve that optimum. But this is not what is observed. The adult body size achieved at a given temperature differs between genotypes that evolved at different latitudes or at different temperatures in the laboratory (Cavicchi et al. 19985; Partridge et al. 1994; James et al. 1995). The data point to the conclusion that the plasticity of body size to temperature is subject to stabilizing selection or is non-adaptive, or that there is a constraint, perhaps because of a physiological cost (van Tienderen 1991; Agrawal 2001; Releya 2002; Kassen 2002).

Genetic variation has been demonstrated for phenotypic plasticity (Scheiner and

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Lyman 1989; David et al. 1994; Noach et al. 1996) and *D. melanogaster* body size

reaction norm could evolve in response to temperature. Yet, *Drosophila* body size norm

of reaction does not appear to evolve in any consistent way either in response to latitude

(James et al. 1997; Morin et al. 1999), or to laboratory thermal selection (Partridge et al.

1994). The temperature associated with maximum body size shows some evidence of a

small response to thermal selection (Delpeuch et al. 1995; Morin et al. 1997, 1999) but,

both in nature and in the laboratory, the main evolutionary response to temperature is

mean body size, with at most minor effects on the degree of plasticity.

1.5 Outline of Thesis

In this thesis I investigate the evolution and plasticity of body size of two *Drosophila*

species, *D. melanogaster* and *D. subobscura*, in response to temperature, using flies

collected from body size clines found in three continents and flies kept under different

thermal regimes in laboratory conditions. My aim was to increase the understanding of

the genetic basis of body size variation along size clines, of the evolution of newly

established clines and of the relationship between body size plasticity and temperature.

In Chapter 3 I investigate whether cell size and cell area effects in the

establishment of two recent body size clines of *D. subobscura* are predictable. The

discordant results found for cell size and cell number effects in thermal selection lines

and body size clines of *D. melanogaster* could be caused by an evolutionary process or

could be due to some other causes, such as drift or founder effect or a difference in

other aspects of the laboratory and field environments. To address the issue I compared

cell number and cell size in the recently established *D. subobscura* body size clines of

in the Americas with the ancestral European one.

In Chapter 4 I investigate the genetic basis of body size of *D. melanogaster*

along the South American cline using QTL mapping. The results are discussed in light

of the work previously done by Gockel et al. (2002) on the Australian cline.

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In Chapter 5 I investigated whether hypomorphic mutants for the insulin pathway confer a selective advantage in the larval stage in flies reared at different temperature and under different food availability. Mutants of the insulin pathway were chose as they are known to affect body size, and seem to be implicated by QTL mapping in the control of body size differences along the South American and Australian clines.

In Chapter 6 I investigate the effects of adaptation to cycling thermal environment in laboratory flies. The investigation focuses on the evolution of plasticity of body size and its cellular components in variable versus fixed thermal environments. In addition, the investigation tried to highlight possible costs and benefits of adaptation to different thermal regimes.

2. General Materials and Methods

2.1. Fly Populations

2.1.1. Drosophila subobscura *flies (Chapter 3).*

The *D. subobscura* flies used for this study were collected in three continents: North

America, Europe and South America (Table 2.1), and were used to investigate the

cellular basis of rapidly evolving body size clines. North American flies were collected

in 1997 (April and May) from 11 localities by R. B. Huey and G. W. Gilchrist;

European flies were collected in 1998 (May) from 10 localities by R. B. Huey; South

American flies were collected in 1999 (November) from 10 Chilean localities by R. B.

Huey, Gilchrist, M. Pascual and J. Balanya. The flies were raised in population cages

(10 flies per sex from each of 15 to 25 isofemale lines) at Washington University for

five or six generations in common laboratory conditions at 20°C, then one generation

was reared under controlled density of 50 flies per vial. The eclosing flies were

collected and the wings mounted on tape on slides by undergraduate students.

Table 2.1 Localities of collection. Name and latitude (decimal degrees) of each

population.

Europe 1998	Latitude N
Arhus, DK	56.2
Leiden, NH	52.2
Lille, FR	50.6
Gif-sur-Yvette, FR	48.7
Dijon, FR	47.4
Lyon, FR	45.5
Montpellier, FR	43.6
Barcelona, SP	41.4
Valencia, SP	39.4
Malaga, SP	36.7
North America 1997	Latitude N
Port Hardy, BC	50.7
Peachland, BC	49.8
Bellingham, WA	48.7
Centralia, WA	46.7
Salem, OR	44.9
Medford, OR	42.3
Eureka, CA	40.8
Redding, CA	40.6
Davis, CA	38.6
Gilroy, CA	37.0
Atascadero, CA	35.5

South America (Chile) 1997	Latitude S
Coyhaique	45.58
Castro	42.50
Porto Montt	41.47
Valdivia	39.77
Laja	37.17
Chillan	36.62
Curico	34.92
Santiago	33.50
Illapel	32.00
LaSerena	29.92

2.1.2 South American D. melanogaster *flies (Chapter 4).*

Drosophila melanogaster flies used for QTL mapping were collected by I. R. Wynne

(University of Copenhagen) in 1999. Isofemale lines were established from females

collected at two sites on the west coast of South America. Nine lines were established

from flies from Tarapoto in Peru (Peru, 6° 29' S; 76° 21' W) and six from flies from

Puerto Montt in Chile (PM, 41° 28' S; 73° 00' W). These lines were maintained in the

lab at 25°C for 10-15 generations on standard fly food.

2.1.3. Dahomey and Thermal selection lines (Chapter 5 and 6).

The Dahomey lines originated from a collection of *Drosophila melanogaster* made in

Dahomey, West Africa in 1970. The resulting stock was mass-bred in population cage

culture at 25°C until 1994.

To start the thermal selection lines, on 27 September 1994, twelve new cages

were started, each from 9 fly culture bottles containing large numbers of larvae

collected from the Dahomey stock. These cages were allocated to four different thermal

treatments, with three cages assigned randomly to each. The first two treatments were

constant temperatures of 18 and 25°C. Two variable temperature regimes were also

initiated, with the temperature cycling between 18 and 25°C. The cycling lines spent

less time at 25°C, to approximately equalise the physiological time spent at the two

temperatures. The long-cycle regimes were chosen to allow most members of the long-

cycle populations to encounter the same thermal regime in early adulthood, when most

reproduction takes place, as during pre-adult development. One cycling regime

changed temperature each day (short cycle), with 10 hours at 25°C and 14 hours at

18°C. These short-cycle flies were therefore exposed to selection at both 18 and 25°C

throughout their lives. In the long-cycling cycling regime, the proportion of time spent

at the two temperatures was the same as in the short-cycle, with 7.2 weeks at 25°C and

10 weeks at 18°C. The long cycle lines had a mean development time from egg to adult

of 12.55 days at 18°C, and 6.89 days at 25°C, and had an adult lifespan of about 20 days

at 25°C, and about 35 days at 18°C. Hence, approximately 50-60% of long cycle line

flies did not experience a change in temperature during their lifetime.

2.1.4. sparkling poliert (spa^{Pol}) *stock.*

This population was used as a competitor stock for the males of the body size selection

lines (Section 2.1.3). The population was produced by crossing flies bearing the

recessive mutant marker *sparkling poliert (spa^{pol})* in a Dahomey genetic background in

1997. The original *sparkling poliert* population was produced by the back-crossing of a

sparkling poliert mutant into a Dahomey background in 1988.

2.1.5 Insulin/IGF-like signalling pathway mutants stocks.

The insulin/IGF-like signalling pathway mutants used in the experiment described in

Chapter 6 are detailed in Table 2.2. All mutant-carrying stocks were backcrossed to a

standard Dahomey background four times.

Table 2.2

Name	Genetic asset	Provenance	Additional information
InR^{E19}	InR^{E19}/TM3 Sb	R. Garofalo, State Uni. NY	Reported in Chen et al. 1996. Hypomorph mutation of the insulin-like receptor
chico	Chico/CyO	C. Zucker	Reported in Clancy et al. 2001.
p60^A	p60^A/SM6A Cy	D. Weinkove, Neth. Canc. Inst. The Netherlands	Reported in Weinkove et al. 1997. Null mutation.
PKB^{CZ}	PKB^{CZ}/TM6B Hu	C. Zucker	PKB hypomorph

2.2 Culture media

2.2.1. Culture medium.

All flies were kept as base stocks on a cornmeal/sugar/yeast medium. The same medium was used for rearing flies under experimental conditions. The ingredient doses for one liter of culture medium are:

85g sugar

60g maize meal

20g dried yeast

10g agar

25ml of 10% Nipagin solution in ethanol

1 liter of water

2.2.2. Grape juice medium.

Whenever it was necessary to collect eggs or first instar larvae, laying pots were prepared with grape juice medium, to make eggs and larvae easier to see on the medium itself. The ingredient doses for one liter of grape juice medium are:

50g agar

600ml grape juice

1 litre water

42.5ml of 10% Nipagin solution in ethanol

2.3 Fly Rearing

2.3.1. South American D. melanogaster *stocks.*

Flies from the South American clines were kept on vials containing 7 mL of standard medium. For each isofemale line two vials were established, to increase population size. When flies were transferred to fresh medium, for each isofemale line, flies from the two vials were pooled together and randomly assigned to two new vials; this procedure was

carried out to avoid genetic drift. Files were housed at 25°C.

2.3.2. Mutant IIS *flies and* sparkling poliert (spa^{pol}) *stocks.*

Flies of the ISS mutants and *sparkling poliert (spa^{pol})* stocks were kept in vials, in

number variable from four to seven, and were housed at 18°C.

2.3.3. Caged stocks for the thermal selection lines.

Three 1/3 pint bottles containing 70ml of culture medium were added each week to each

cage, and the three oldest bottles were removed. The number of bottles maintained in a

cage depended upon the temperature at which the cage was maintained. Cages

maintained at 25°C were kept on a four-week cycle, so that there were always 12 bottles

in the cage. Cages maintained at 18°C were kept on a six-week cycle, with 18 bottles in

the cage. The short cycling thermal selection line cages were kept on a five-week cycle,

with 15 bottles in the cage. The long cycling thermal selection line cages were kept on a

four-week cycle when they were at 25°C, and on a six-week cycle when they were at

18°C, and the number of bottles in the cages was adjusted accordingly.

2.4 Fly handling

Whenever flies had to be manipulated, they were anaesthetised using carbon dioxide.

However, when flies where handled for the collection of virgin females, they were

anaesthetised by placing them on a glass surface cooled with ice, because carbon

dioxide can cause bubbling of the gut in very young adult flies, causing sterility.

2.5 Standard density culture

Adult flies were placed in laying pots containing grape juice medium with a dab of live

yeast on the surface. After an acclimatisation period of 24 hours, flies were transferred

onto fresh medium for a pre-lay of 24 hour to encourage laying of any retained eggs.

Flies were then transferred onto fresh medium for 3 hours at 25°C for egg collection. First instar larvae were then transferred to vials containing 7mL of culture medium, using a mounted needle, at a standard density of 50 larvae per vial. This procedure was employed for the experiments described in Chapters 4-6.

Eggs were collected using the same procedure, using the standard density of 80 eggs per vial. This procedure was employed for part of the experiment described in Chapter 6.

2.6 *Drosophila subobscura* measurements

In the study presented in Chapter 3, the left or right wings of 20 females and 20 males *D. subobscura* flies were measured for each of the populations described in section 2.1.1. Occasionally fewer flies per population were available for measurement, but always at least fourteen flies per sex were scored. Cell density was measured using a microscope with *camera lucida* attachment and graphic table at 10x40 magnification. Cell density varies across the surface of the *Drosophila* wing. However, concordant differences in the cell area between different parts of the wing blade are found for differences between both individuals and populations (see Delcour and Lints 1966, Partridge et al. 1994, Pezzoli et al. 1997). The proximal and distal part of the vein IV showed a different lengthening pattern with latitude in the European and North American clines (Huey et al. 2000, Gilchrist et al., 2001). For this reason two different sampling areas in the region between the vein IV and V were examined. These areas have been previously used in the analysis of cell size/cell number variation. They can be located independently of wing allometry and wing area changes and are regions of relatively low variation in cell density. The two sampling areas were considered proximal and distal, referring to the crossvein and the landmarks used by Huey and Gilchrist (Huey et al. 2000) (see Fig. 1). The number of trichomes in two 500 μm^2 sampling squares within each sampling area was counted and cell area was calculated as

(500/no. trichomes). Two measurements were taken for each sampling area and the average was used for statistical analysis. Because cell area is variable across the wing blade, it was not possible to infer total cell number in the wing, and a total cell number index was used. The length of the vein IV was used as representing wing length; the index was calculated as (wing length^2/cell area); again two indices were calculated, one using the distal cell area and one using the proximal cell area, on the grounds of the different behaviour of the two segments of the vein IV in Europe and North America. The vein IV itself was measured using an ocular micrometer on a 10x eyepiece on a dissection microscope, at 4x magnification.

Fig. 2.1. *Drosophila subobscura* wing. The two white squares, left and right of the posterior crossvein, represent the area where cell area measurements were taken. The white line is the length of the vein IV. In Huey et al. (2000) the vein IV was measured from the base of the vein to the crossvein and from the crossvein to the wing border.
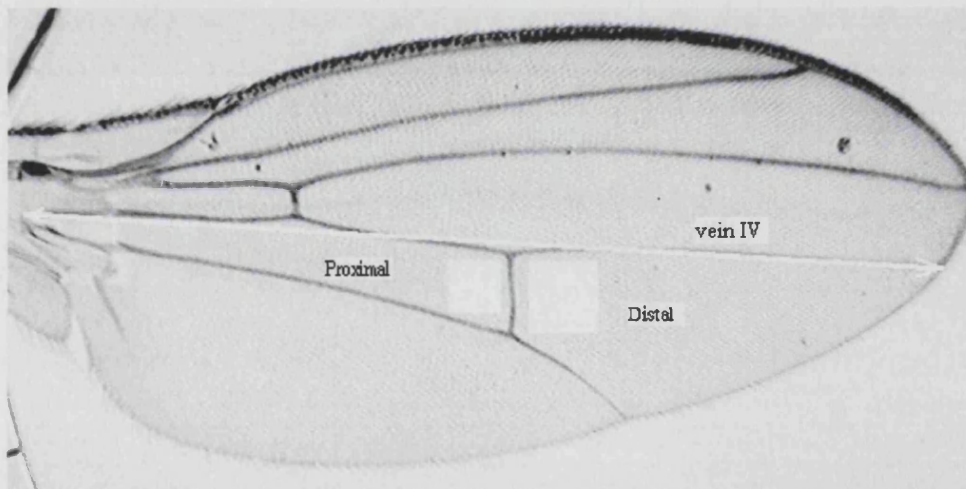
2.7 *Drosophila melanogaster* measurements

Flies kept under standard density conditions (see section 2.5) were collected, after

carbon dioxide anaesthesia, in Eppendorf tubes and frozen. The right wing of adult flies,

normally five females and five males per vial, was removed and mounted on a

microscope slide using propanol and Aquamount. An image of each wing was digitised

using a camera attached to a microscope at x25 magnification. The area within six

landmarks around the edge of each wing (black line, Fig. 2.2) was calculated using

Object-Image software version 1.62 for the Macintosh (an implementation of the public

domain NIH Image program by Norbert Vischer, available at

http://simon.bio.uva.nl/object-image.html).

To assess the cell density in fly wings, two images in an area of the wing (within

the white rectangle in Fig 2.2) were captured using a video camera attached to a

compound microscope at x400 magnification. The images were then examined using

Object-Image software, and the number of trichomes within the image area was

counted. The average cell density of the two images was used to calculate an index of

cell area and, in conjunction with the area of the whole wing, to calculate an index of

cell number for each wing.

the wing within which the two cell density measures per wing were taken.
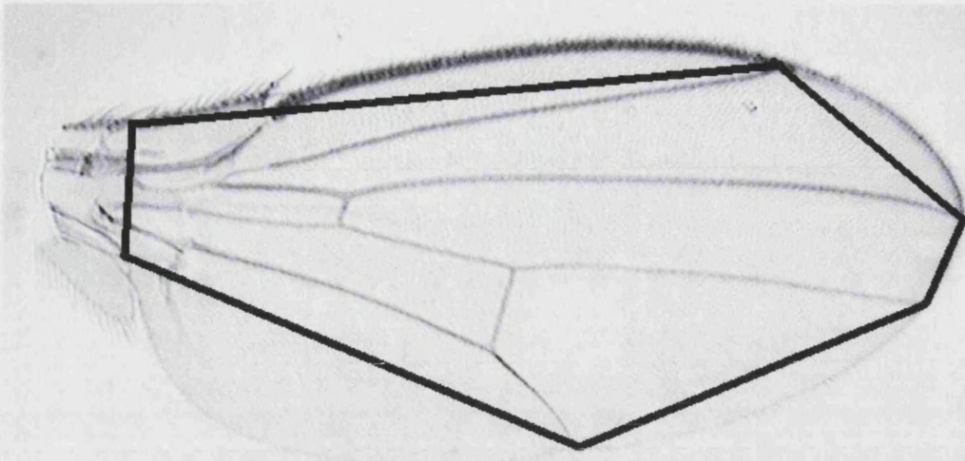
Fig. 2.2. *Drosophila melanogaster* wing. The black outline superimposed on the wing

2.8 Larval competition assays

In order to examine fitness during the pre-adult period, I set up larval competition assays in the experiments detailed in Chapters 5 and 6. Based on a pilot study to determine the effects on egg-to-adult survival rates, four types of food treatment were chosen: 100% food, with 7 mL of standard cornmeal/yeast/sugar/agar fly medium per vial; 50% food, with 3.5 mL of medium per vial, 25% food, with 1.75 mL of medium per vial; 10% food, with 0.7 mL of medium per vial. With the exception of the 100% treatment, fly medium was dispensed on top of a 2.5% agar solution scaled per treatment to bring the final wet mass to 7 mL. The trial had shown that the four food treatments affect larval survival markedly, with reduced survival at lower food level ($F_{3,36} = 14.69$, $P < 0.0001$). This procedure had the effect of confining the larvae into a reduced space, effectively increasing larval crowding with the decreasing of the food quantity dispensed on top of the agar.

2.9 Statistical analysis

All statistical analysis was performed using R 1.5.0 to R 1.7.1 for Linux (Ihaka and Gentleman 1996, obtainable from http://www.r-project.org).

3. Newly established body size clines in *D. subobscura*: are cell size and cell area effects predictable?

3.1 Abstract

Latitudinal genetic clines in body size occur in many ectotherms including *Drosophila* species. In the wing of *D. melanogaster,* these clines are generally based on latitudinal variation in cell number. In contrast, differences in wing area that evolve by thermal selection in the laboratory are in general based on cell size. To investigate possible reasons for the different cellular bases of these two types of evolutionary response, I compared the newly established North and South American wing size clines of *Drosophila subobscura*. The new clines are based on latitudinal variation in cell area in North America and cell number in South America. The ancestral European cline is also based on latitudinal variation in cell number. The difference in the cellular basis of wing size variation in the American clines, clines of roughly the same age, together with the similar cellular basis of the new South American cline and the ancient European one, suggest that the antiquity of a cline does not explain its cellular basis. Furthermore, the results indicate that wing size as a whole, rather than its cellular basis, is under selection. The different cellular basis of different size clines is most likely explained either entirely by chance or by different patterns of genetic variance - or its expression - in founding populations.

3.2 Introduction

As mentioned in the general General Introduction, body size clines, with size positively correlated with latitude, are often found in ectotherms (General Introduction 1.2.1 and 1.2.2). Some cosmopolitan species belonging to the genus *Drosophila* have been found to produce parallel wing area clines on different continents (General Introduction 1.2.3); temperature is the most probable selective factor causing these latitudinal clines in size (General Introduction 1.2.4). This conclusion is supported by laboratory thermal selection experiments, were flies adapted to colder temperature are lager than flies adapted to warmer temperature (General Introduction 1.2.4). A notable difference between thermal selection lines and flies from natural body size clines is the cellular mechanism mediating size differences, with laboratory selection flies differing in wing area through changes in cell size, and flies from clines show variation in wing area based mainly on cell number (General Introduction 1.2.4).

The difference in the cellular basis of wing area differences in latitudinal clines and laboratory thermal selection lines requires explanation, especially if both are due to thermal selection. Is the cell size difference that we have seen in thermal selection lines an early stage in the evolution of body size that eventually will evolve into a cell number difference (see also Partridge and French, 1996)? If this is the explanation, then we should expect to see clines based on cell size in nature when a latitudinal wing size cline is established for the first time.

To test this idea, I examined the cellular basis of three wing size clines in *Drosophila subobscura*: Europe, North and South America. *D. subobscura* is endemic to Europe, where latitudinal clines in several traits, including body size, are observed (Misra and Reeve, 1964). This species has recently colonised South America, with a first report in 1978 (Brncic, et al. 1981), and North America, with a first report in 1982 (Beckenbach, et al. 1986). After colonisation, the North and South American populations underwent significant genetic differentiation from the original European

colonizers in a number of different traits: allozyme polymorphism (Prevosti et al. 1983, Balanya and Serra 1994), lethal allelism (Sole et al. 2000), chromosomal polymorphism (Prevosti et al. 1985, 1988, Ayala et al. 1989, Mestres et al. 1994), DNA polymorphism (Latorre et al 1986, Rozas et al 1990, Rozas and Aguade 1991) and quantitative traits (Budnik et al. 1991). Nonetheless, the first survey, conducted using flies collected in 1986 and 1988, failed to show any latitudinal size cline on either continent (Pegueroles et al. 1995). A second survey conducted by Huey and Gilchrist (Huey et al. 2000, Gilchrist et al. 2001) in North America, with flies collected in 1999, did find a wing length cline, with genetically larger flies at higher latitudes.

Huey and Gilchrist (Huey et al. 2000, Gilchrist et al. 2001) found that the increase in wing length with latitude in the European cline was associated with a relative lengthening of the basal portion of the vein IV, whereas the increase in North America was associated with an increase in the distal portion of the same vein (see Fig. 2.1 in General Material and Methods). Preliminary results (G.W. Gilchrist, unpublished observations) indicate that in South America both segments of the vein IV increase in length with latitude. These findings suggest that total wing size or one of its cellular components, rather than the size of a particular wing region, may be the target of selection. Assessing the cellular basis of the latitudinal variation could provide evidence on whether wing size itself or its cellular components are a target of selection. At present the adaptive significance of evolutionary size increase in the high latitude populations is not understood.

3.3 Materials and Methods

3.3.1 D. subobscura flies.

Drosophila subobscura flies from Europe, North and South America were used for this experiment (General Materials and Methods 2.1.1 and Table 2.1 therein).

3.3.2 Wing length and cell area measurements.

For each wing, a measure of cell area was taken from two standard sampling areas, and the length of vein IV was measured; all measures were taken according to the procedures detailed in the General Materials and Methods (General Materials and Methods 2.6 and Figure 2.1 therein).

3.3.3 Statistical analysis.

The five characters measured (wing length, distal and proximal cell size, distal and proximal cell number index) were analysed separately. For each trait, we used a standard linear model to estimate the regression coefficients simultaneously by nesting latitude inside sex and continent. This yields an estimate of the slope for each continent-by-sex subset of the data. We tested for parallel regression slopes using a standard ANOVA comparison of slopes test. Type III sums of squares were used for all ANOVA's to compensate for the unequal sample sizes. Data were normally distributed in all cases (Shapiro-Wilk W test). In addition, plots of residuals versus latitude revealed homoscedasticy and therefore no transformation was deemed necessary.

3.4 Results

3.4.1 Wing area.

Ln(wing length) was regressed on latitude, nested within sex and continent, to produce individual estimates of the slopes for females and males in North America, South America and Europe. All slopes but one, North American Males, were significant and positive (Fig 3.1 and Table 3.1, 3.2). A comparison of slope test revealed that all the slopes in the three continents were homogeneous; the main effects on size were due to Sex and Latitude. Our data show that, by 1999, a latitudinal wing length cline had evolved in South America.

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Fig. 3.1. Regression of ln(wing length) on latitude. A Females, B Males

(A)
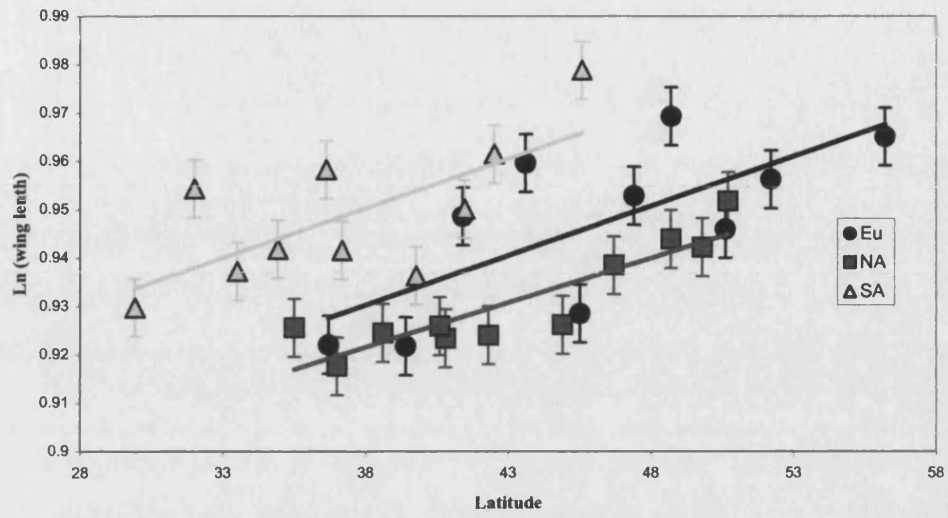
(B)
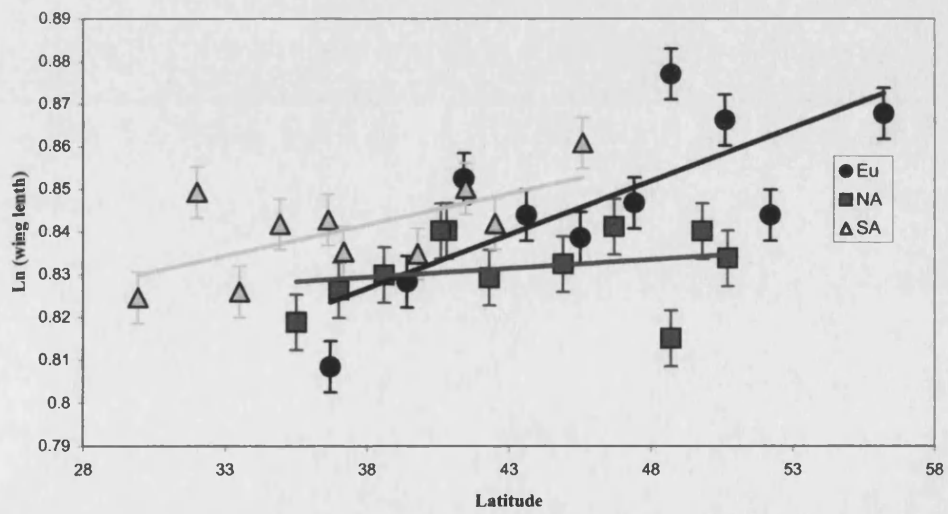
3.4.2 Cell size

The regression of proximal cell size and distal cell size was analysed using the same

nested design as for ln(wing length). For proximal cell size, with the exception of North

American males, no significant regression with latitude was found; the trend for both

sexes in Europe and South America is negative, while it is positive in North America

and a linear model for comparison of slopes revealed significant difference between

continents, detected by significant interaction between Continent and Latitude (Fig 3.2

A, 3.2 B and Table 3.1, 3.2).

Fig. 3.2. Regression of proximal Cell Size (± s.e.) on latitude. Area in square micrometers.

(A)

(B)
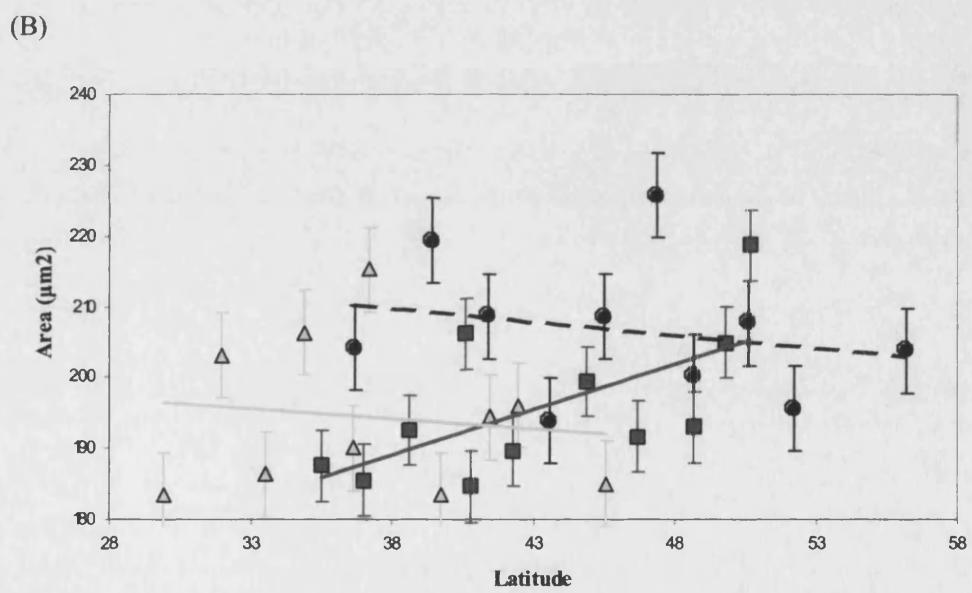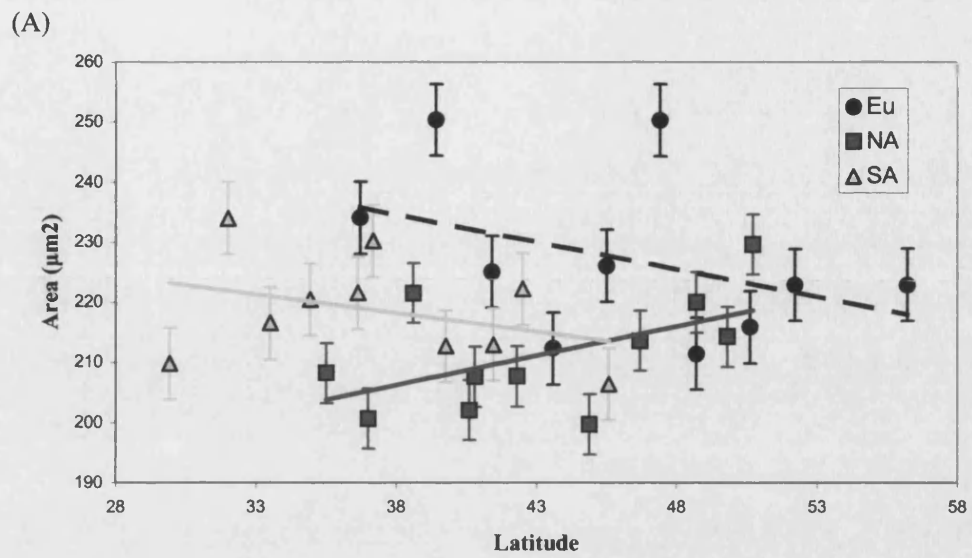
For distal cell size, the regression analysis on latitude (Fig. 3.3 A, 3.3 B and Table 3.1, 3.2) was negative but not significant for European flies. North American females exhibited positive and significant cline, whereas in males the trend is positive but not significant. The South American flies yielded a similar pattern to that in Europe with a negative but not significant trend in distal cell size for both sexes. A comparison of slopes test detected significant difference between continents, revealed by significant interaction between Continent and Latitude. No main effect was applicabl

Fig. 3.3. Regression of distal Cell Size (± s.e.) on latitude. Area in square micrometers.

(A)
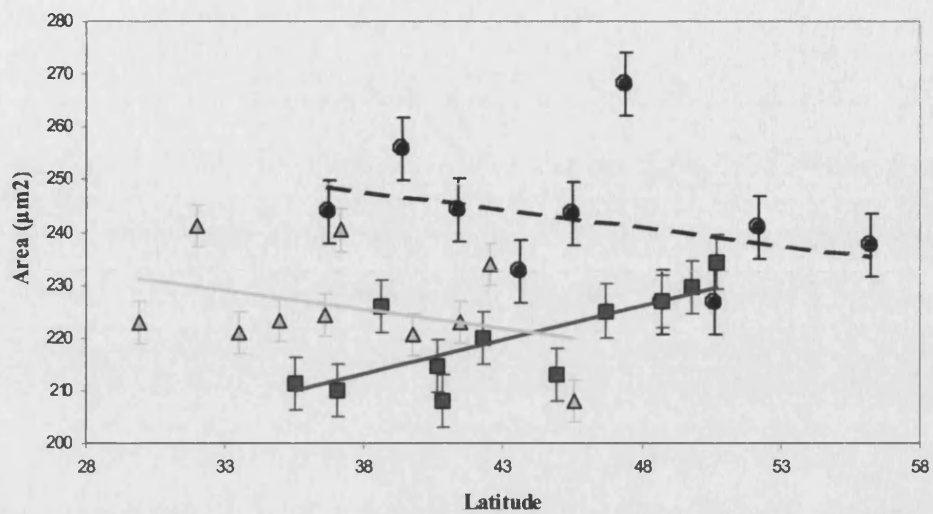
(B)
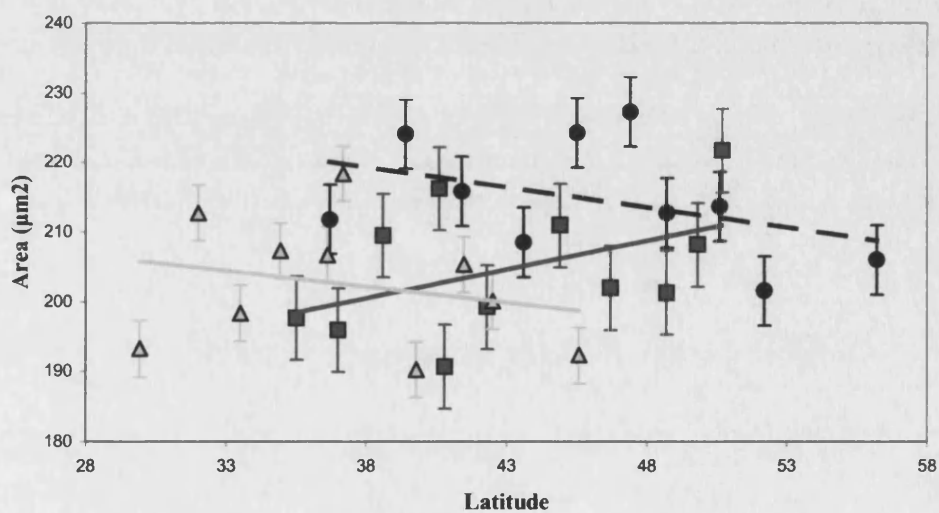
3.4.3 Cell Number Index

The regression slopes for proximal and distal cell number index were estimated using a similar nested model to that for cell area and ln(wing length). For the proximal index (fig 3.4 A, 3.4 B and Tables 3.1 and 3.2) both sexes in Europe and females in South America showed a significant positive regression coefficient. North American flies did not show a significant regression in either sex. A comparison of slopes test showed significant differences between continents revealed by a significant interaction between Continent and Latitude. No main effect was applicable.

Fig. 3.4. Regression of proximal Cell Number Index (± s.e.) on latitude.

(A)
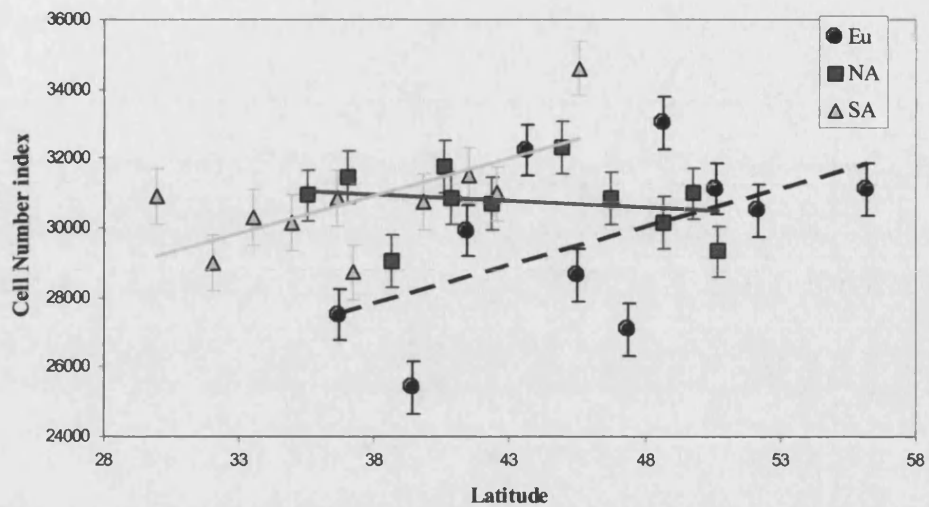
(B)
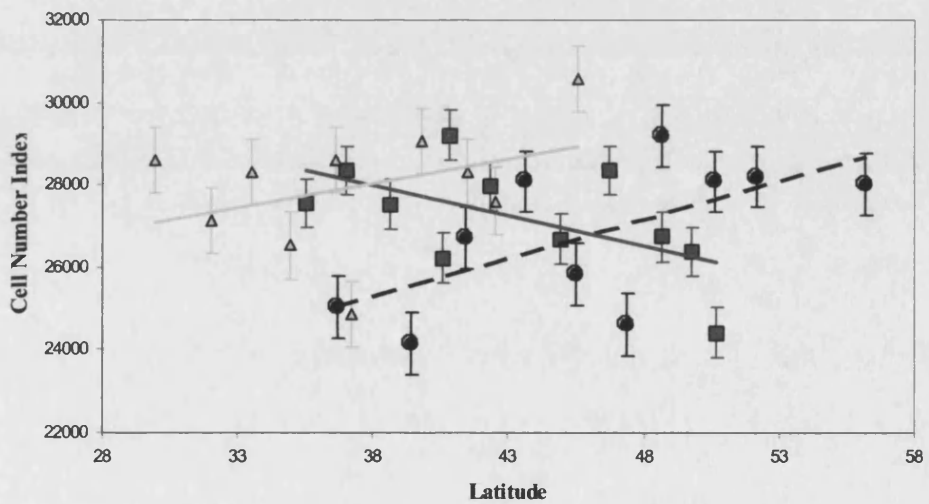
For the distal index we found similar results, with both sexes in Europe and South America giving a positive regression with latitude and North American flies not showing a significant regression with latitude(Figure 3.5; Tables 3.1 and 3.2). The same results were found for the comparison of slopes test, with significant differences between continents due to a significant interaction between Continent and Latitude. Again no main effect was applicable.

Fig. 3.5. Regression of proximal distal Cell Number Index (± s.e.) on latitude.

(A)
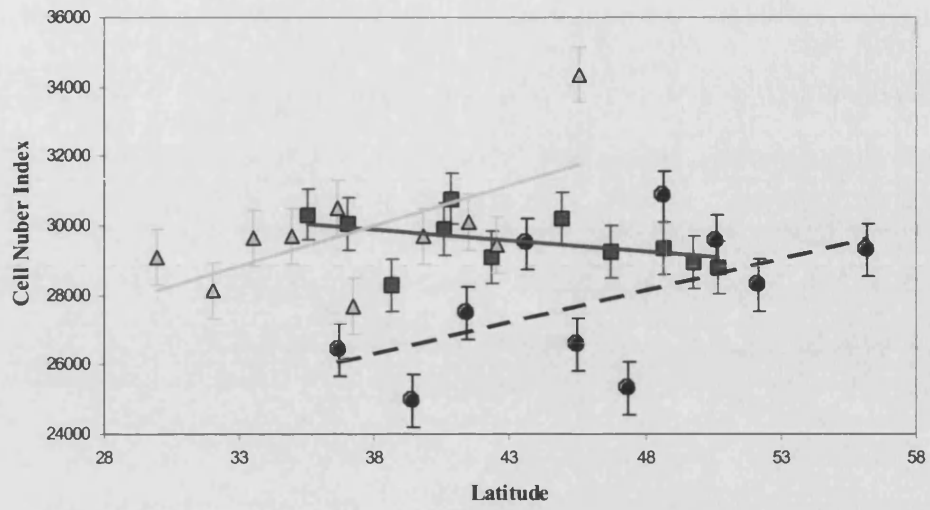
(B)
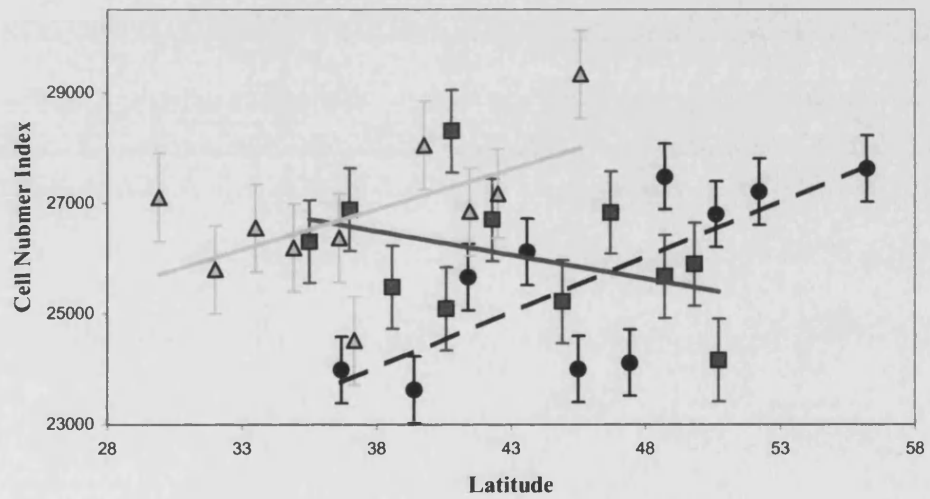
Table 3.1. Linear Model Estimates

Continent	Sex	Intercept ± SE	Slope ± SE	t-value (slope)
Wing Length				
Eur	F	0.85 ± 0.027	0.0020 ± 0.00060	3.52***
NoA		0.85 ± 0.029	0.0019 ± 0.00070	2.82**
SoA		0.87 ± 0.026	0.0021 ± 0.00070	2.96**
Eur	M	0.73 ± 0.027	0.0025 ± 0.00060	4.22***
NoA		0.81 ± 0.028	0.0004 ± 0.00060	0.65
SoA		0.79 ± 0.026	0.0015 ± 0.00070	2.10*
			R^2: 0.9686	
Proximal Size				
Eur	F	268.87 ± 26.204	-0.90 ± 0.564	-1.60
NoA		167.95 ± 28.146	1.02 ± 0.651	1.56
SoA		241.82 ± 25.481	-0.62 ± 0.677	-0.91
Eur	M	225.42 ± 26.651	-0.41 ± 0.573	-0.71
NoA		141.46 ± 27.451	1.26 ± 0.632	2.00*
SoA		204.27 ± 25.481	-0.27 ± 0.677	-0.39
			R^2: 0.6448	
Proximal Index				
Eur	F	19526.89 ± 3717.254	219.71 ± 79.988	2.75**
NoA		32395.61 ± 3992.795	-39.56 ± 92.318	-0.43
SoA		22713.71 ± 3614.786	215.92 ± 96.032	2.25*
Eur	M	18019.56 ± 3780.702	190.24 ± 81.341	2.34*
NoA		33287.16 ± 3894.167	-141.65 ± 89.703	-1.58

SoA		23591.82 ± 3614.786	116.86 ± 96.032	1.22

R^2: 0.6559

Distal Size

Eur	F	272.71 ± 23.935	-0.67 ± 0.515	-1.29
NoA		164.02 ± 25.709	1.30 ± 0.594	2.18*
SoA		253.31 ± 23.275	-0.74 ± 0.618	-1.19
Eur	M	242.48 ± 24.344	-0.60 ± 0.524	-1.15
NoA		170.48 ± 25.074	0.80 ± 0.578	1.39
SoA		219.63 ± 23.275	-0.46 ± 0.618	-0.74

R^2: 0.7290

Distal Index

Eur	F	19450.69 ± 3175.131	181.44 ± 68.323	2.66*
NoA		32159.92 ± 3410.487	-61.28 ± 78.854	-0.78
SoA		21194.66 ± 3087.607	231.43 ± 82.027	2.82**
Eur	M	16328.97 ± 3229.326	202.19 ± 69.478	2.91**
NoA		29611.56 ± 3326.243	-83.83 ± 76.621	-1.09
SoA		21362.75 ± 3087.607	145.37 ± 82.027	1.77*

R^2: 0.7256

Table 3.2. Comparison of slopes test, type III sums of squares. We cannot reject the null model (homogeneity of slopes) for Wing Length, however all other traits show a significant interaction between Continent and Latitude.

Wing Length	Df	Mean Sq	F Value	Pr(F)	
Cont	2	0.0027	1.26	0.292	
Sex	1	0.0284	13.27	0.001	***
Lat	1	0.0911	42.60	0.000	***
Cont:Sex	2	0.0024	1.13	0.331	
Cont:Lat	2	0.0034	1.59	0.214	
Sex:Lat	1	0.0022	1.03	0.314	
Cont:Sex:Lat	2	0.0025	1.19	0.313	
Residuals	50	0.0021			

Proximal Size	Df	Mean Sq	F Value	Pr(F)	
Cont	2	12611.7	6.19	0.004	na
Sex	1	5547.1	2.72	0.105	
Lat	1	5.4	0.00	0.959	
Cont:Sex	2	101.9	0.05	0.951	
Cont:Lat	2	9986.9	4.90	0.011	*
Sex:Lat	1	1021.8	0.50	0.482	
Cont:Sex:Lat	2	44.3	0.02	0.979	
Residuals	50	2036.6			

Proximal Index	Df	Mean Sq	F Value	Pr(F)	
Cont	2	284537902.0	6.94	0.002	na
Sex	1	33044.0	0.00	0.977	

Lat	1	269060107.0	6.56	0.013	na
Cont:Sex	2	2770047.0	0.07	0.935	
Cont:Lat	2	268717224.0	6.56	0.003	**
Sex:Lat	1	45384977.0	1.11	0.298	
Cont:Sex:Lat	2	4780141.0	0.12	0.890	
Residuals	50	40984634.0			

Distal Size	Df	Mean Sq	F Value	Pr(F)	
Cont	2	12246.5	7.21	0.002	na
Sex	1	1585.8	0.93	0.339	
Lat	1	113.0	0.07	0.798	
Cont:Sex	2	683.9	0.40	0.671	
Cont:Lat	2	9454.0	5.56	0.007	**
Sex:Lat	1	20.6	0.01	0.913	
Cont:Sex:Lat	2	380.7	0.22	0.800	
Residuals	50	1699.2			

Distal Index	Df	Mean Sq	F Value	Pr(F)	
Cont	2	249272726.0	8.34	0.001	na
Sex	1	14536132.0	0.49	0.489	
Lat	1	323093344.0	10.81	0.002	na
Cont:Sex	2	4676216.0	0.16	0.856	
Cont:Lat	2	234242077.0	7.83	0.001	***
Sex:Lat	1	6586313.0	0.22	0.641	
Cont:Sex:Lat	2	7432990.0	0.25	0.781	
Residuals	50	29901981.0			

3.5 Discussion

The most important result of this work is the finding that the two newly established North American and South American wing area clines in *Drosophila subobscura* differed in the cellular basis of the latitudinal variation. The North American cline was based on cell size while the South American cline was based on cell number. The ancestral European cline was also based on cell number. Cell size showed a positive regression with latitude in North American female flies, while South American and European flies showed a positive cline with latitude in both cell number indexes. The slopes for ln(wing length), reflecting overall size, were positive and significant, with the exception of North American males. Thus, parallel wing size clines are present on all three continents. The data show that latitudinal size clines in nature can differ in their cellular basis, as previously observed (Zwaan et al. 2000).

The situation presented by the North and South American clines is unusual. The founding populations in the two continents are closely related genetically (Prevosti et al. 1983; Balanya and Serra 1994;Mestres et al. 1994; Mestres and Serra 1995), and the evolution of the clines has been monitored since colonisation. Despite the fact that the establishment of body size clines in the Americas was expected (Pegueroles et al., 1995) and was eventually found, the cellular mechanism underlying wing size differences is not the same in the two continents. The comparison between the Americas and the ancestral European population is also revealing. Comparison of the European and North American clines, with their different cellular basis, is consistent with the idea that the cellular basis of body size variation could change from cell size to cell number with time (Partridge and French, 1996). However, the South American data is not consistent with this hypothesis. The newly established South American cline is based on cell number. Thus the hypothesis that the cellular basis of wing size

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difference evolves over time from cell size to cell number is not supported by our findings.

The relative lengths of the proximal and distal segments of the vein IV differ in Europe and North America (Huey and Gilchrist 2000, Gilchrist et al. 2001). Nonetheless I found that cell size and cell number showed the same clinal pattern in both the proximal and distal segments of the wing. The results hence suggest that thermal selection may target the whole wing rather than just one of its parts.

Wing area is positively correlated with body size as a whole (Reeve and Robertson 1952, Robertson 1959, Misra and Reeve 1964, Wilkinson et al. 1990). However, the cellular basis of variation in wing area is not always the same as that for other anatomical regions. Comparison of cline-end populations of a South American *D. melanogaster* size cline showed that the contribution of cell size differed in different organs (wing, eye and proximal tarsal segments), with size variation between populations attributable to cell number for wing area and to cell size for eye and tarsal segments (Azevedo et al. 2002). The different cellular basis of the two newly established *D. subobscura* clines and the different cellular basis for clinal variation in the size of different body parts within a single cline, all show that, rather than its cellular components, size per se or something genetically correlated with size is the target of selection.

The recent colonisation of the Americas by *Drosophila subobscura* is a singular chance to observe the evolution of metric traits in the field. It has allowed us to discount time since establishment as a likely cause of the different cellular bases of the response of body size to selection in different populations. While we cannot completely rule out the hypothesis that the cellular basis of latitudinal size variation in the North and South American clines may not be caused by the same selective agents, a more parsimonious explanation is that pure chance or

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differences in the genetic composition of founding populations or the way that variation is expressed in different local environments must be responsible.

4. QTL for body size in the *D. melanogaster* South American cline: comparison with the Australian cline and the importance of *In(3R)P* inversion in determining body size.

4.1 Abstract

Latitudinal genetic clines in body size are common in many ectotherm species and are attributed to climatic adaptation. Here, Quantitative Trait Loci (QTL) mapping is used to identify genomic regions associated with adaptive variation in body size in natural populations of *Drosophila melanogaster* from extreme ends of a cline in South America. The results found show that there is a significant association between the positions of QTL with strong effects on wing area in South America and those previously reported in a QTL mapping study of Australian cline end populations ($P < 0.05$). In both continents, the right arm of the third chromosome is associated with QTL with the strongest effect on wing area. I also show that QTL peaks for wing area and thorax length are associated with the same genomic regions, indicating that the clinal variation in the body size traits may have a similar genetic basis. The consistency of the results found for the South American and Australian cline end populations indicate that the genetic basis of the two clines may be similar and future efforts to identify the genes producing the response to selection should be focused on the genomic regions highlighted by the present work.

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4.2 Introduction

Many organisms show latitudinal clines in quantitative traits such as behaviour, stress resistance and morphology that are assumed or demonstrated to be attributable to climatic adaptation (Parson and Hoffmann 1993, Hoffmann and Harshman 1999). One such trait is body size, which frequently shows a pattern of genetically based increase in relation to latitude in both endotherms and ectotherms (General Introduction 1.2.1 and 1.2.2). The reasons for the association between size and latitude in ectotherms are not understood. A clue to the underlying mechanisms could be found from identifying the genes responsible for the latitudinal variation.

The ideal subject for investigation on the genetic basis of latitudinal body size variation is the fruit fly *Drosophila melanogaster. D. melanogaster* has been found to produce parallel body size clines that show a positive relationship with latitude in all continents (General Introduction 1.2.3). Because the temperate and tropical populations from these geographical regions all have different genetically inferred histories (Hale and Singh 1991), natural selection in response to some latitudinally varying environmental factor, most likely temperature, is the best explanation for these clines (General Introduction 1.2.4).

Whole chromosome substitution analysis by Gockel et al. (2002) has shown that the bulk (77%) of the genetic variation for size between cline end populations in Australia and South America is located on the third chromosome, with smaller effects on the second chromosome (13%) and only minor genetic effects on the X chromosome. Gockel et al. (2002) also carried out recombination mapping on Australian cline end populations using microsatellites as neutral markers. Using this approach they found Quantitative Trait Loci (QTL) for body size on the tip of the right arm of the second chromosome and along a large region of the right arm

of the third chromosome, in good correspondence with the information from chromosome substitution.

In the present study, the QTL effects on body size variation in the South American cline were mapped for comparison with the pattern in the Australian cline. In view of the results from that study, an F_{10} generation was used rather than an F_3 to increase the number of recombination events to improve the precision of mapping.

The plateau of high LOD scores for wing area on the right arm of chromosome 3 in the Australian cline was associated with low recombination rates between the microsatellite markers in the region (Gockel et al. 2002). This could indicate the presence of a chromosomal inversion for this genomic region in one of the parental lines. Subsequent examination by Weeks et al. (2002) of patterns of genetic covariation in a mid-latitude population from Eastern Australia revealed strong associations between markers found within the cosmopolitan inversion *In(3R)Payne* and variation in size. This inversion runs between 89C2-3 and 96A18-19, in close correspondence with the region of reduced recombination revealed by QTL mapping. In addition, the frequency of this inversion increases with latitude in Australia (Knibb et al. 1981). In the present study, as well as testing for reduced recombination and an effect on size associated with this chromosomal region, I tested directly for the presence of *In(3R)Payne* in the South American populations by an analysis of polytene chromosomes.

4.3 Materials and Methods

4.3.1 Parental lines for QTL mapping.

The isofemale lines used for this study were collected in South America in 1999 (General Materials and Methods 2.1.2). To maximise the size difference between the isofemale lines used in QTL mapping, wing area was measured (see section 4.3.3 "Size measurement" below) for all isofemale lines. Before being measured, all lines were reared on a standard fly medium at a standard density of 50 larvae per vial at 25°C. Consistent with the clinal pattern, the temperate PM lines were significantly larger than the tropical Peru lines (females, $F_{1,12} = 76.827$, $P < 0.001$; males, $F_{1,12} = 84.706$, $P < 0.001$). Size differences in males ranged between 4.7 and 9.8 standard deviations and differences between females ranged between 4.4 and 11.6 standard deviations. The lines Peru12 and PM1 gave the greatest difference in size across the sexes and were therefore used to produce an F_{10} generation. In using these lines I have assumed that within locality variation in size does not greatly affect the number and position of QTLs detected. The differences in wing area between the parental lines were mediated entirely by changes in cell number (data not shown).

4.3.2 Generation of F_{10}

The mapping population was established by crossing a single virgin Peru12 female with a PM1 male. After the nine non-overlapping generations, eclosing flies were allowed to mature and to mate for three days before being transferred to a cage containing a grape juice agar plate on which they laid eggs for three hours. After 24 hours, first instar larvae were picked from the grape juice agar plate and placed in 24 vials at a density of 50 larvae per vial. These cultures were reared at 25°C and the eclosing flies were frozen for size measurement and genotyping.

4.3.3 Size measurement.

Wing area and thorax length were measured following the protocol established by Gilchrist and Partridge (1999) for wing area and James et al. (1995) for thorax length (General Material and Methods 2.7 and Fig 2.2). A total of 295 F_{10} individuals from 17 different vials were measured for both traits. One-way ANOVA showed there were no significant vial effects for either trait ($P > 0.05$ in both cases) and there were no significant deviations from normality when the measurements for each of the traits were pooled ($P > 0.05$ in both cases).

4.3.4 Molecular markers.

A total of 32 markers were typed in the parents and the offspring (Table 4.1). Markers were distributed on all three chromosomes with seven markers on the X chromosome, 13 markers on the second chromosome and 12 markers on the third. On average, the spacing between markers was 9.7 cM on the X chromosome, 8.4 cM on the second chromosome and 9.3 cM on the third. The largest gap, of 22.0 cM, was on the third chromosome. DNA extraction from individual flies, PCR protocols and allele scoring followed methods outlined in Gockel et al. (2002). Christian Schlotterer, Stuart Macdonald and Julia Gockel designed the primers employed in this work. Jason Kennington and Alice Smith did the PCR and the genotyping.

Table 4.1. Molecular markers.

Marker	Genetic location (cM)	Cytological location	Multiplex	Label	Concentration in PCR. (μM)
AF047180	1-0.0	1B8	A	Fam	0.27
X4364768gt	1-8.0	4C8	B	Fam	0.14
X6213328ca	1-16.0	5F6	B	Fam	0.14
AC004114	1-28.0	8E3-E4	B	Hex	0.14
DROSEV	1-33.4	10A1-A2	C	Hex	0.27
3641.2	1-52.0	14A	B	Hex	0.05
DMARIADNE	1-58.0	16E2-17B1	–	Fam	0.54
AC008318	2-0.0	21A	D	Fam	0.10
DS01340	2-10.5	24A1-A2	E	Hex	0.27
AC004721	2-17.5	25E6	F	Hex	0.13
AC003052	2-22.0	27A2-B2	D	Ned	0.16
AC004722	2-28.0	28C2-C4	G	Hex	0.28
AC005889	2-35.0	30A3-A6	H	Fam	0.16
AC006302	2-48.5	34C4-D2	G	Fam	0.13
AC006472	2-61.0	45E1-46A2	D	Fam	0.05
AC004516	2-76.0	52D2-D15	F	Hex	0.11
AC004641	2-81.0	53D1-E2	H	Hex	0.16
AC004564	2-95.0	57E1-E2	G	Ned	0.13
AC004365	2-97.5	58A4-B1	F	Fam	0.09
DS08011	2-101.0	59A1-B2	E	Fam	0.27
AC004343	3-0.5	62A1-A2	I	Fam	0.27
AC004658	3-8.0	63D2-E1	J	Ned	0.11
AC005814	3-10.0	64A6-B6	K	Fam	0.18

AC008198	3-26.0	66D10-E2	K	Hex	0.18
DM22F11T	3-44.0	73A1-B7	A	Hex	0.27
DROPROSA	3-51.0	86E3	J	Fam	0.11
DMTRXIII	3-54.2	88B3	L	Ned	0.27
AC009394	3-62.0	90E-90F	C	Ned	0.27
DRONANOS	3-66.2	91F13	K	Ned	0.18
DMU25686	3-73.0	93F	J	Hex	0.16
DMU1951	3-81.0	95C	I	Hex	0.16
AF221066	3-103.0	100F5	L	Fam	0.27

4.3.5 QTL analysis.

QTLs were mapped by Composite Interval Mapping (CIM; Zeng 1993, 1994) and

multitrait composite interval mapping (MCIM; Jiang and Zeng 1995) using QTL

Cartographer 1.16 (Basten et al. 2002). For each trait, the CIM procedure was

used to test the hypothesis that an interval between adjacent markers had a QTL

affecting the trait, while accounting for genetic background by including

statistically relevant markers identified with multiple regression as cofactors. All

statistically relevant markers were used as cofactors (eight for wing area and six

for thorax length) and tests were performed over 2 cM intervals with a flanking

window size of 20cM. This window size was chosen because it was the maximum

distance over which complete linkage disequilibrium was observed between

markers. Likelihood ratio test values (the ratio of the likelihood that a QTL is

present relative to the null hypothesis of no QTL) were calculated for each test

interval and, following convention, were converted to LOD scores (Lander and

Botstein 1989). Experiment-wise significance levels were determined by

permutation (1000 replicates), in which phenotypes were shuffled against

genotypes and the analyses redone (Churchill and Doerge 1994).

Because wing area and thorax length were highly correlated ($r = 0.48$; $P <$

0.001), multitrait composite interval mapping (MCIM) was used to jointly map

QTLs affecting both traits. The MCIM procedure is similar to the single-trait CIM

analyses, but it takes into account the correlated structure of phenotypic data,

providing additional power and accuracy for mapping QTLs (Jiang and Zeng

1995). As with CIM, experiment-wise significance levels were determined by

permutation (1000 replicates), in which phenotypes were shuffled against

genotypes so that the correlations between traits were maintained.

To determine if QTL detected by joint MCIM had pleiotrophic effects on

both traits, individual MCIM likelihood ratio test values were examined for each

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position where joint mapping indicated the presence of a QTL. As proposed by Jiang and Zeng (1995), pleiotropy was indicated for a given QTL by the rejection of the null hypothesis of no more than one trait having a likelihood ratio test value greater than a significance threshold value of 5.99 ($\chi^2_{0.05, 2}$). This test requires no correction for multiple testing, because each position was fixed prior to the test (Jiang and Zeng 1995).

4.3.6 Comparison of QTL locations in different continents.

To evaluate quantitatively the similarity of locations of QTL identified for wing area in Australia and South America, a resampling test was performed, as described in Macdonald and Goldstein (1999). To do this, LOD scores were summed at the locations of the highest QTL peaks from Gockel et al. (2002) and compared this to summed LOD scores obtained by placing the same number of points at random in 10,000 simulations.

4.3.7 Polytene chromosome squashes.

Chromosome squashes were prepared from the salivary glands of third instar larvae using the procedure described in Ashburner (1989). In addition to determining the karyotype of each parental line, we also tested whether these lines had the same inverted sequences as the lines used in Gockel et al. (2002). I did this by crossing Peru12 virgin females with males from the northern Australian line (Meg4), and virgin PM1 females with males from the southern Australian line (Dom20), and then inspecting polytene chromosomes in the progeny for inversion loops.

4.4 Results

The LOD profiles for wing area and thorax length based on CIM are shown in

Figure 4.1. For both traits there were two main regions where LOD scores exceeded the permutation-based significance threshold, one on the second chromosome, the other on the right arm of the third. There were no significant QTL peaks on the X chromosome for either trait.

QTL Cartographer identified a total of eight significant LOD peaks for wing area, three on the second chromosome and five on the third (Table 4.2). The peaks on the second chromosome were on the right arm of the chromosome, and on the third chromosome, there were four major peaks clustered together on the right arm and a small single peak on the tip of the left arm. The peaks on the right arm of the third chromosome had the largest LOD scores and explained the largest proportion of the size variation (Table 4.2). However, the valleys between these peaks, and those on the second chromosome, never dipped below the significance threshold level. It is therefore difficult to estimate how many QTL there really are in these regions. The peak ranges given in Table 4.2 were defined as the lowest LOD score adjacent to either side of an identified peak.

For thorax length, there were a total of eight significant peaks identified, three on the right arm of the second chromosome, and five on the right arm of the third. On both chromosomes, the peaks grouped together with the LOD score never falling below significance between them. Thus, as for wing area, it is unknown if these regions contain a single or multiple QTL. All peaks identified for thorax length were found in chromosomal regions matching those found for wing area and had overlapping peak ranges (Table 4.2). LOD scores for thorax length tended to be much lower than for wing area. This could be due to a greater measurement error being associated with thorax length, because the flat surface of the wing is capable of being much more accurately measured than is the length of irregularly shaped thorax.

The resampling test for coincidence between the QTL found by Gockel et al.

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(2002) and those presented here for wing area were significant ($P < 0.05$).

Joint MCIM analysis of wing area and thorax length produced very similar results to those obtained using single trait CIM. No new QTL peaks were detected by MCIM, and the LOD scores at each of the QTL positions detected by CIM exceeded the permutation-based significance threshold. Application of Jiang and Zeng's (1995) pleiotropy test revealed that QTL on the right arm of the second and third chromosomes had pleiotrophic effects on both traits, whereas the QTL on the tip of the left arm of the third chromosome was nonpleiotrophic, affecting wing area only.

The plateau in the LOD score found on the right arm of the third chromosome with both CIM and joint MCIM is indicative of a genomic area of reduced recombination. Of the 296 flies scored, no recombinants were found between markers AC009394 (90E-F) and DMU1951 (95C). The adjacent regions showed reduced recombination as well. To the left only one individual showed recombination between markers DMTRXIII (88B3) and AC009394 (90E-F). To the right only 31 recombinants were observed between marker DMU1951 (95C) and AF221066 (100F5), considerably fewer that the number observed on a chromosomal region of similar size on the left arm of the third chromosome: between markers AC005414 (64A6-B6) and AC008198 (66D10-E6) 91 recombinants were found.

Visual inspection of the polytene chromosome squashes revealed that the Peru12 line had an inversion on the right arm of the third chromosome running between cytological bands 89 and 96, while the PM1 line had a standard sequence. The lack of inversion loops in progeny derived from a cross between Peru12 and the northern Australian line confirmed that these lines have the same inverted sequence, which has been identified as *In(3R)Payne* (see Weeks et al. 2002).

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Figure 4.1. LOD profiles for (A) wing area and (B) thorax length based on composite interval mapping. For both profiles the symbols X, 2, 3 in the box above the plots refer to the three major chromosomes; the horizontal line indicates the threshold value for the trait. The triangles on the x-axis correspond to the locations of markers used in the study and the dots represent the positions of QTL identified with QTL cartographer.
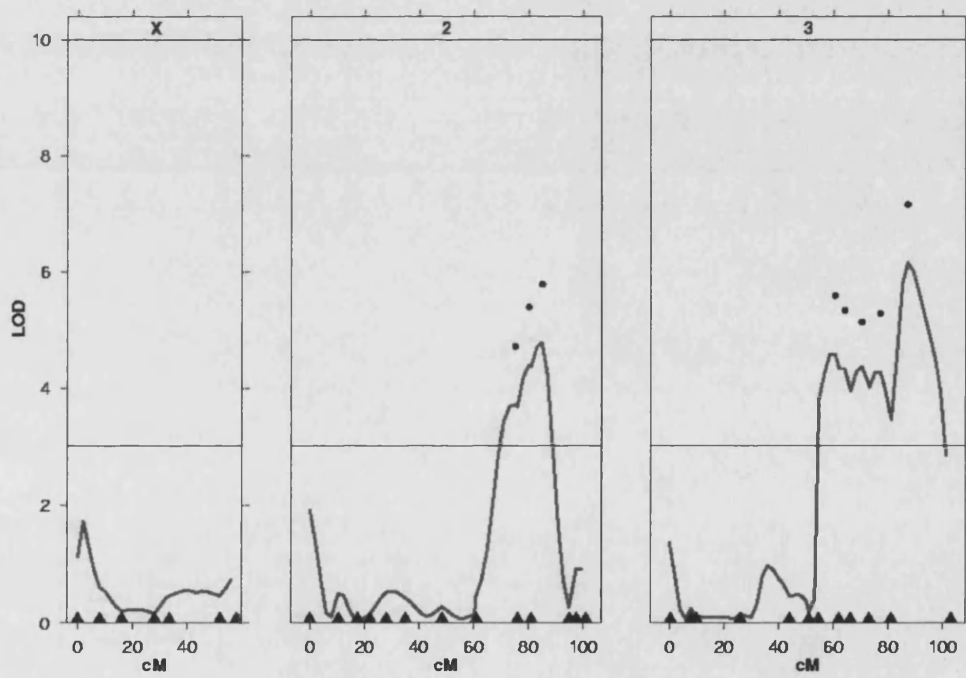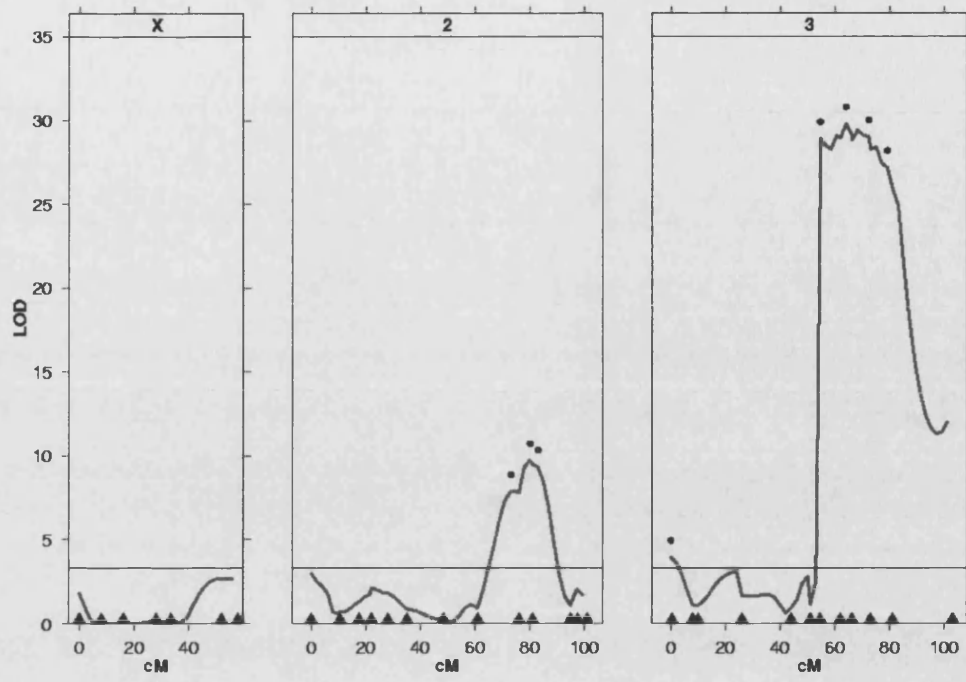
Table 4.2. Locations of QTL peaks, LOD scores and the proportion of the variance (R^2) explained by the QTL conditioned on the background markers.

Chromo some	Map position (LOD peak)		Map position (Peak range)		LOD	R^2
	Genetic (cM)	Cytological	Genetic (cM)	Cytological		
Wing area						
2	73.0	51D	65-76	48B-52D	7.9	0.10
2	80.0	53C	76-81	52D-53E	9.7	0.06
2	83.0	54C	81-90	53E-56E	9.3	0.09
3	0.01	61C	0-3	61C-62E	4	0.04
3	54.5	88A	54-58.5	87F-89B	28.9	0.43
3	64.0	91B	58.5-66	89B-91F	29.8	0.42
3	72.2	93E	66-73	91F-93F	29	0.40
3	79.0	94F	73-101	93F-100A	27.2	0.48
Thorax length						
2	75.0	52B	71-76	51A-52D	3.7	0.07
2	80.0	53C	76-81	52D-53E	4.4	0.07
2	85.0	55C	81-88	53E-56B	4.8	0.16
3	60.5	90A	52-62	87B-90E	4.6	0.15
3	64.0	91B	62-66	90E-91F	4.3	0.10
3	70.2	93B	66-73	91F-93F	4.1	0.14
3	77.0	94D	73-81	93F-95C	4.3	0.17
3	87.0	96C	81-101	95C-100A	6.2	0.53

4.5 Discussion

The most important finding from this study was the strong similarity between the QTL

maps for latitudinal genetic variation for wing area in South America and Australia. The

results found show that, in both continents, the right arm of the second and third

chromosomes are associated with QTL, and the strongest effects are on the right arm of

the third chromosome. The region on the third chromosome with strong effects covers a

significant portion of the genome (at least 6.8%) and the coincidence in localization of

QTL in South America and Australia could have been due to chance alone. However,

the similarity between QTL maps from different continents was confirmed statistically

using the resampling test.

It was interesting to note that the peak found on the distal end of the second

chromosome in Australia was absent in South America, and that the single peak found

on the tip of the left arm of the third chromosome in South American flies replaced by a

more centrally located peak in Australian flies. No QTL were detected on the X

chromosome in either continent.

Noor et al. (2001) have shown that a 'small X-effect' in QTL mapping studies can

result from the lower density of genes per centiMorgan on the X relative to the

autosomes of *D. melanogaster*. However, this 'small X-effect' should not have biased

the whole chromosome substitution studies carried out by Gockel et al. (2002), which

also showed a small X-effect on body size variation in South American flies. Equally,

the region on the right arm of the third chromosome with a strong effect on body size

was not the centromeric region of low recombination and high gene density identified

by Noor et al. (2001). Hence, the consistent pattern across continents is unlikely to be

due to variation in gene density.

The QTL peaks for both traits analysed in the present study fall under highly

correlated genomic regions, indicating that the genetic basis of the body size traits may

be similar. Indeed, based on Jiang and Zeng's (1995) test for pleiotropy it is apparent

that both the major QTL regions on the right arms of the second and third chromosomes were pleiotrophic or contain closely linked QTL with predominant effects on only one trait.

In both Australia and South America the highest LOD scores overlap the right arm of the third chromosome where the inversion *In(3R)Payne* is located. Due to the lack of recombination in this region, it is difficult to know the actual number or precise locations of QTL because the LOD scores never fall below significance threshold and form a broad peak

The strong linkage disequilibrium we found between the neutral microsatellite markers within the inversion implies a lack of multiple crosses over events between the standard and inverted sequences. In terms of future QTL mapping this is problematical because, in the absence of recombination, it will be impossible to gain more fine-scale maps. One way to address this problem might be to use for mapping lines diverged in size, but homosequential over this region. However, before such an approach is taken, it would first be necessary to show that adaptive variation in size and the inversion are not completely linked.

Parallel geographical clines have been found in both inversion frequency and body size in three continents (Knibb 1982), and relative frequency of *In(3L)Payne* is inversely correlated with cold resistance (Weeks et al. 2002) and fluctuates with seasonal changes, decreasing in 'winter' conditions (Knibb 1986). These associations highlight the potential influence of inversions in clinal trait variation and could help understanding the adaptive significance of evolutionary divergence in size.

5. Insulin/IGF-like signalling pathway mutants and larval competitive ability: do hypomorphic/null mutants confer greater fitness at higher temperature?

5.1 Abstract

In the present work I characterize the effects of 5 different mutations in genes encoding components of the Insulin/IGF-like Signalling pathway on larva-to-adult survival at different temperatures and different food levels, chosen to affect larval crowding. Alleles controlling body size in naturally occurring body size clines or in laboratory thermal selection lines produce reduced body size in populations with a hotter thermal history and are expected to increase larval survival at high temperatures and high crowding, or to decrease it at low temperatures; three of the genes characterized did not conform to this pattern, while Inr^{E19} shows a pattern of effects more consistent with theoretical expectations. The results are discussed in the light of the results obtained in QTL mapping efforts done on flies form the Australian and South American body size cline (Chapter 4).

5.2 Introduction

Drosophila melanogaster body size clines are found in all continents, with flies from lower latitudes showing genetically smaller size than flies from higher latitudes (General Introduction 1.2.3). Similar results are found in laboratory thermal selection lines, with lines from "warm" selection regimes being genetically smaller than flies from "cold" selection regimes (General Introduction 1.2.4).

Insulin/IGF-like Signalling (IIS) pathway mutants have been shown to affect size and growth rates in *D. melanogaster*. In particular, hypomorphic mutants of *InR* (Chen et al. 1996; Brogiolo et al. 2001), hypomorphic mutants of *chico* (Bohni et al. 1999), hypomorphic mutants of *Akt/PKB* (Verdu et al. 1999) and hypomorphic mutants of *p60* (Weinkove et al. 1999) are known to reduce size by reducing both cell size and cell number and increase developmental time. The IIS hypomorphs have a similar growth rate, developmental time and size characteristics relative to wild type as tropical populations do relative to temperate populations and "warm" selection lines do relative to "cold" selection lines (Oldham et al. 2000).

Support for the hypothesis that IIS genes could be directly implicated in the control of body size along latitudinal clines comes from QTL mapping studies on body size, performed on flies from the Australian and South American clines (Gockel et al. 2002; Calboli et al. in press and detailed in Chapter 4). These studies indicated the cosmopolitan inversion *In(3R)P* as associated with QTL controlling clinal variation in body size; the inversion *In(3R)P* contains three genes in the IIS pathway, *InR*, *Dp110* and *Tsc1*, and two more, *Akt/PKB* and *FKHR*, are situated in areas where recombination is likely to be affected by the presence of the inversion. In addition, a broad correlation is found between three clinally selected inversions and IIS pathway genes. Parallel geographical clines in body size and inversion frequency are found for the inversions *In(2L)t*, *In(3L)P* and *In(3R)P* in three continents (General Introduction 1.3.4). These inversions contain approximately 27% of the 15393 genes and putative genes of *D.*

melanogaster genome. Of the 19 genes in the IIS pathway, 12 are within inversions, and 4 are in regions were recombination is likely to be affected by the presence of the inversions (Table 6.1). A binomial test gives a probability P = 0.0012 of non-random association, if we consider only the genes directly within inversions, and a probability of P = 3.852 x10^{-7} if considering all the genes likely to be affected during recombination by the presence of inversions. Even using the more conservative estimate, a non-random association seems to exist between clinally selected inversions and the IIS pathway. The data therefore seem to suggest that one or more genes in the IIS pathway could be implicated in the genetic control of body size along latitudinal clines. Nevertheless, little work has been performed to characterize the effects of hypomorph/null alleles of IIS genes on pre-adult survival and fitness at different temperatures.

In order to get a better understanding of the interplay between IIS pathway genes and temperature, I examined the effects of hypomorphic null mutations in genes encoding IIS components on pre-adult survival. I used an F_1 produced by crossing hypomorph/null alleles of five IIS genes to a standard Dahomey stock. Because the mutants were carried on chromosomes that were hold over a balancer chromosome, the relative frequency of adult IIS mutant heterozygotes to balancer heterozygotes could be used as a measure of fitness. All IIS mutant stocks had already been backcrossed four times to the same standard Dahomey genetic background. The F_1 generation flies were tested at three different temperatures. I also used 4 different levels of food supply. The aim was partly to induce mortality to increase any fitness effects (Santos et al. 1994; Santos et al. 1997). In addition, there are some indications that in both field (Nunney 1990; Krebs et al. 1992; James and Partridge 1998) and laboratory (Santos and Partridge, unpublished manuscript; Bochdanovits and de Jong 2003) levels of larval crowding may be greater at higher temperatures.

Table 5.1 Cosmopolitan inversion and IIS pathway genes. The asterisk (*) next to the

cytological location of each gene indicates that the gene is within an inversion. The first

five Dilp genes are grouped together, to reflect their identical cytological position.

Inversion	Cytological Location
In(2L)t	22D3..6 – 34A8..9
In(3L)Payne	63B8..9 – 72E1..2
In(3R)Payne	89C2..3 – 96A18..19

Genes	Cytological Location
Dilp 1; Dilp 2; Dilp 3; Dilp 4; Dilp 5	67C8 *
Dilp 6	3A1
Dilp 7	3E2
InR	93E4 *
Chico	31B1 *
p60	21B8
Dp110	92F3 *
Pten	31B4-5 *
Akt/PKB	89B6
PDK1/Pk61C	61B1
dS6K	64E8-11 *
dTOR	34A4 *
Tsc1	95E4 *
Tsc2	76F2
Foxo/FKHR	88A6-8

Fig 5.1 Insulin/IGF-like signalling pathway.
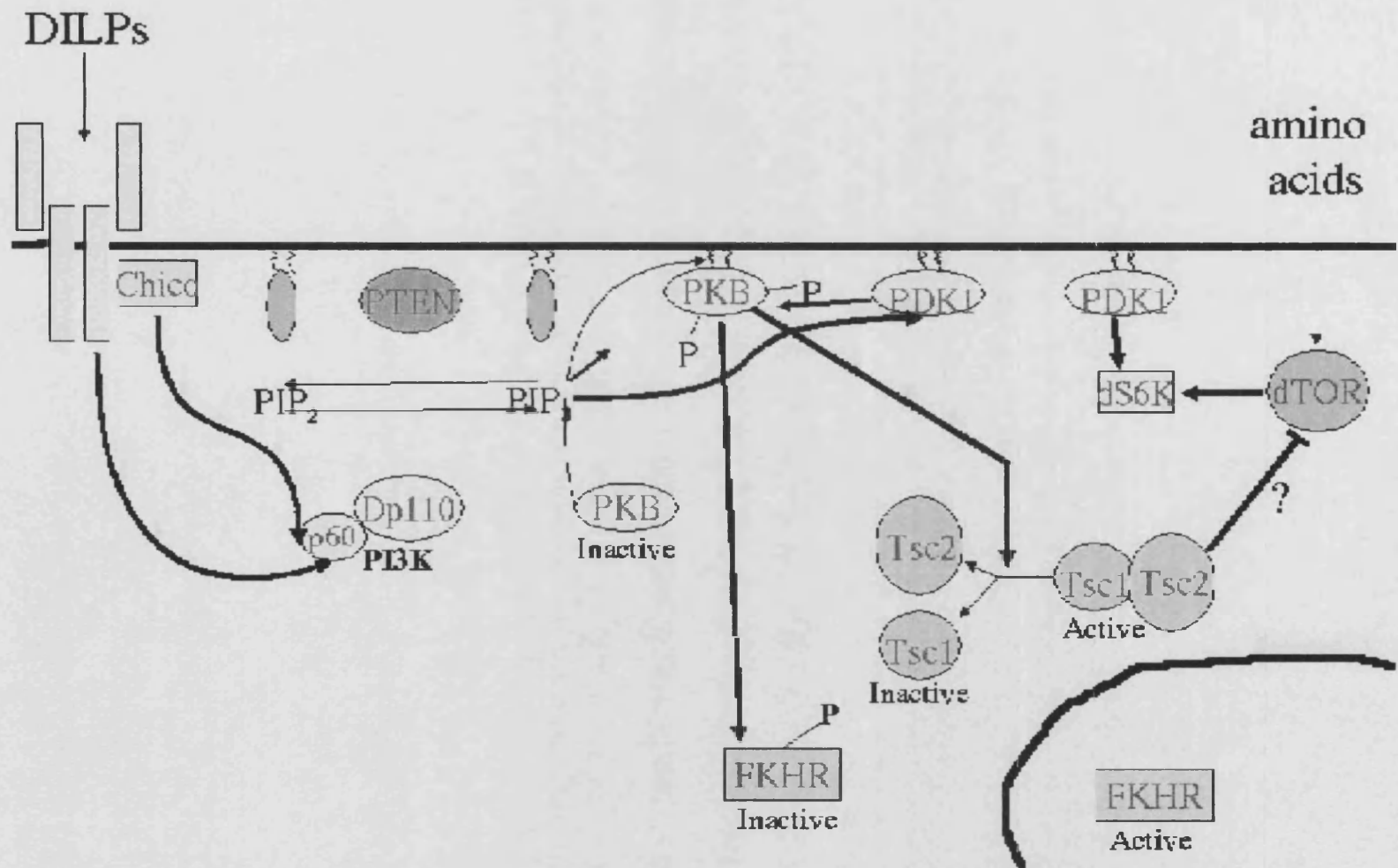
5.3 Materials and Methods

5.3.1. IIS mutant stocks.

IIS mutant stocks used in this work are described in the General Materials and Methods,

section 2.1.5 and in Table 2.2.

5.3.2. Dahomey stock.

The Dahomey stock used for the F_1 crosses is described in the General Materials and

Methods (2.1.3).

5.3.3 Crossing scheme.

Virgin Dahomey females were collected on ice (General Material and Methods 2.4) and

mated with males from the five mutant stocks. All the parental crosses and the F_1

progeny can be summarized by the following scheme:

Parental flies: **Mutant/Balancer x Dahomey/Dahomey**

F_1: **Mutant/Dahomey** and **Balancer/Dahomey**

For each cross, the hybrid F_1 is a mixture of "wild-type" flies carrying the

Mutant/Dahomey genotype, and "balancer" flies carrying the Balancer/Dahomey

genotype.

5.3.4. Larval competition assay.

A larval competition assay was established for each cross according to the protocol

outlined in the general Materials and Methods, section 2.8. The competition assays were

repeated at three temperatures: 18°C, 25°C and 27°C. For each mutant stock 10 vials

were seeded with 50 larvae each per each combination of food by temperature. As

already mentioned in the general Materials and Methods, the procedure followed for the

larval competition assays has the effect of restricting the same number of larvae in a

more and more reduced volume of medium, affecting the level of larval crowding.

5.3.5. Statistical analysis

The four IIS mutant stocks were analysed separately by a GLM model. The number of "wild-type" and "balancer" adult flies was recorded for each vial; the GLM model uses the number of "balancer" flies as a covariate to the number of "wild-type" flies to correct for the overall number of flies eclosing from each vial. Linear contrast analyses were used to compare the interaction between food level and temperature. Food level was contrasted in the following pairs: the 100% versus 50% food treatment, the 25% versus 10% food treatments and the "high" (100% and 50%) versus "low" (25% and 10%) food treatments. The second set of linear contrasts contrasted temperature effects: 18°C versus 25°C and 27°C together and 25°C versus 27°C. The lower temperature of 18°C is thus taken as a control for the effects of the two higher temperatures. Analysis of quantile plots and standardized residuals did not highlight any particular deviation from linearity; no significant overdispersion was found. All the conclusions were drawn from the GLM results, but, in order give the reader a visual representation of the data, boxplots of the percentage of wild-type flies surviving were used.

5.4 Results

5.4.1 chico.

Analysis of deviance shows a significant interaction between temperature and food (Chi Square probability = 0.016). Main effects were therefore not applicable. Contrast analysis on the GLM reveals that flies heterozygote for *chico* show a higher fitness compared to flies heterozygote for the balancer at 25C and 27C under low food dose (z-value = -2.552, p = 0.01) at all temperatures.

Fig 5.2 Proportion of "wild type" *chico* heterozygote flies surviving to adulthood at the four food treatments divided per temperature.
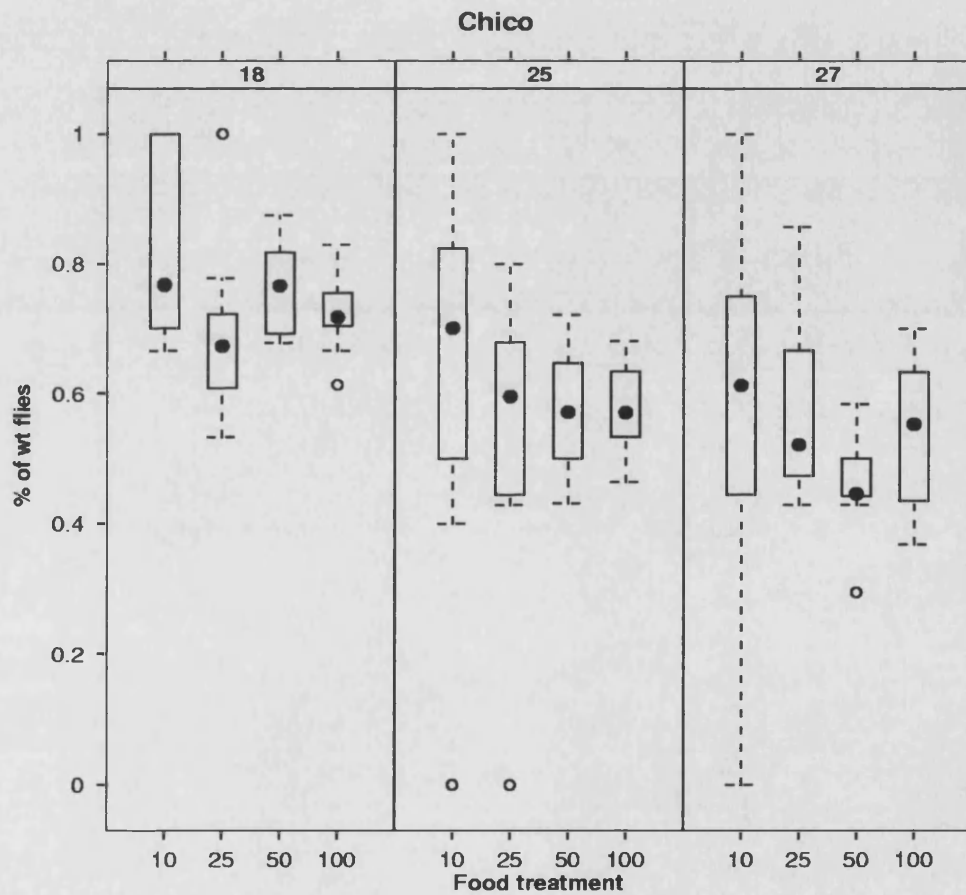
5.4.2 InR^{E19}.

Analysis of deviance shows a significant interaction between temperature and food (Chi Square probability = 0.011). Main effects were therefore not applicable. Flies heterozygote for *InR^{E19}* show a lower fitness at low temperature and low food treatment than flies heterozygote for the balancer. Contrast analysis shows a significant interaction between low food dose and low temperature (18C) compared with 25C and 27C (z-value = -3.824, p = 0.0001).

Fig 5.3 Proportion of "wild type" *InR^{E19}* heterozygote flies surviving to adulthood at the four food treatments divided per temperature.
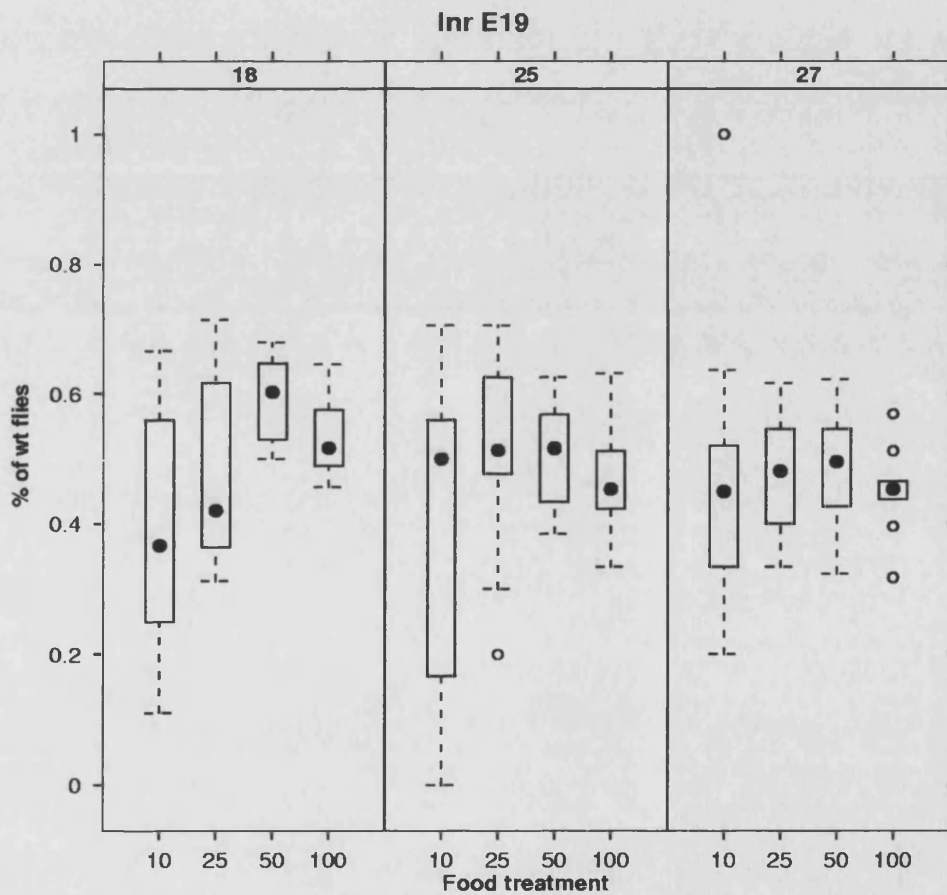
5.4.4 p60.

Analysis of deviance does not show any significant interaction between temperature and

food, but temperature and food show a significant effect on survival (for temperature:

Chi Square probability = 1.1 e-05; for food: Chi Square probability = 7.6 e-09).

Temperature differences were due to an overall higher survival of *p60* heterozygote

flies at 18°C compared to 25 and 27°C (z-value = 4.52 e-07); food differences were due

to overall higher survival of p60 heterozygote flies at the 10% food treatment compared

to the 25% food level (z-value = 5.863, p = 4.55 e-09).

Fig 5.4 Proportion of "wild type" *p60* heterozygote flies surviving to adulthood at the

four food treatments divided per temperature.
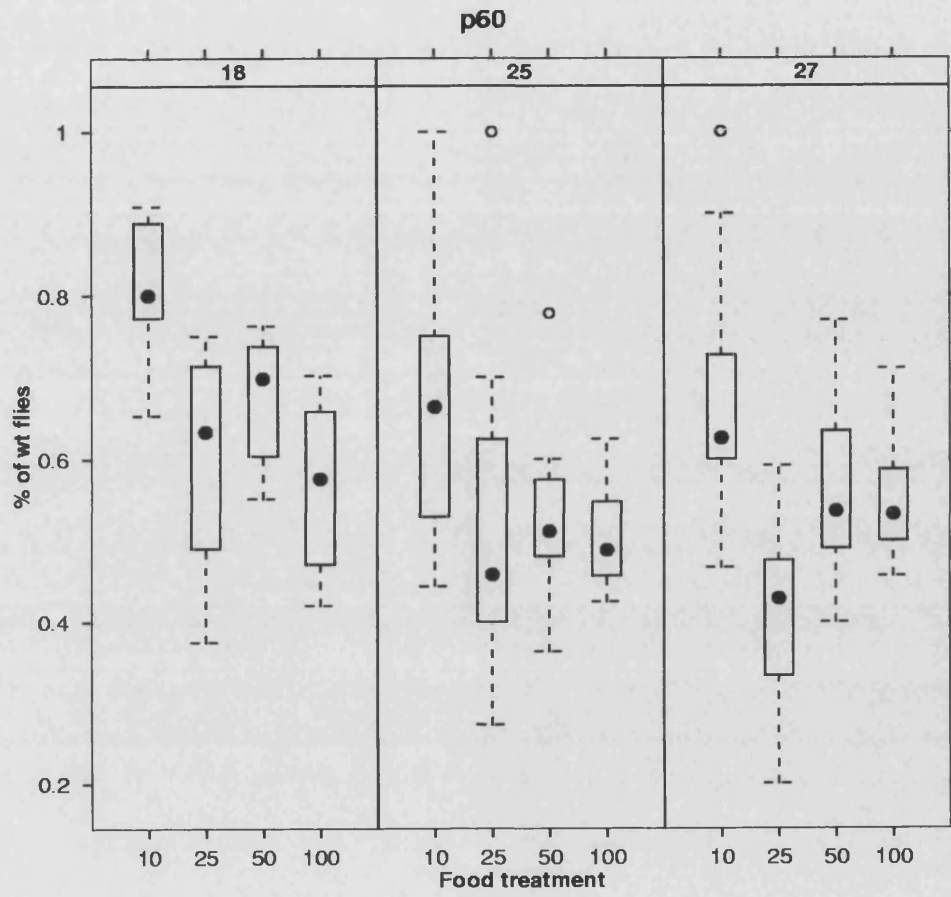
5.4.5 Akt/PKB.

Analysis of deviance shows a significant interaction between temperature and food (Chi

Square probability = 0.003). Man effects were therefore not applicable. Flies

heterozygote for *AktPKB* show higher fitness at lower food treatments, increasing with

decreasing temperature; linear contrasts show strong interaction comparing the "low"

food treatments with the "high" treatments at 18°C versus 25 and 27°C (z-value =

3.787, p = 0.0002).

Fig 5.5 Proportion of "wild type" *Atk/PKB* heterozygote flies surviving to adulthood at

the four food treatments divided per temperature.
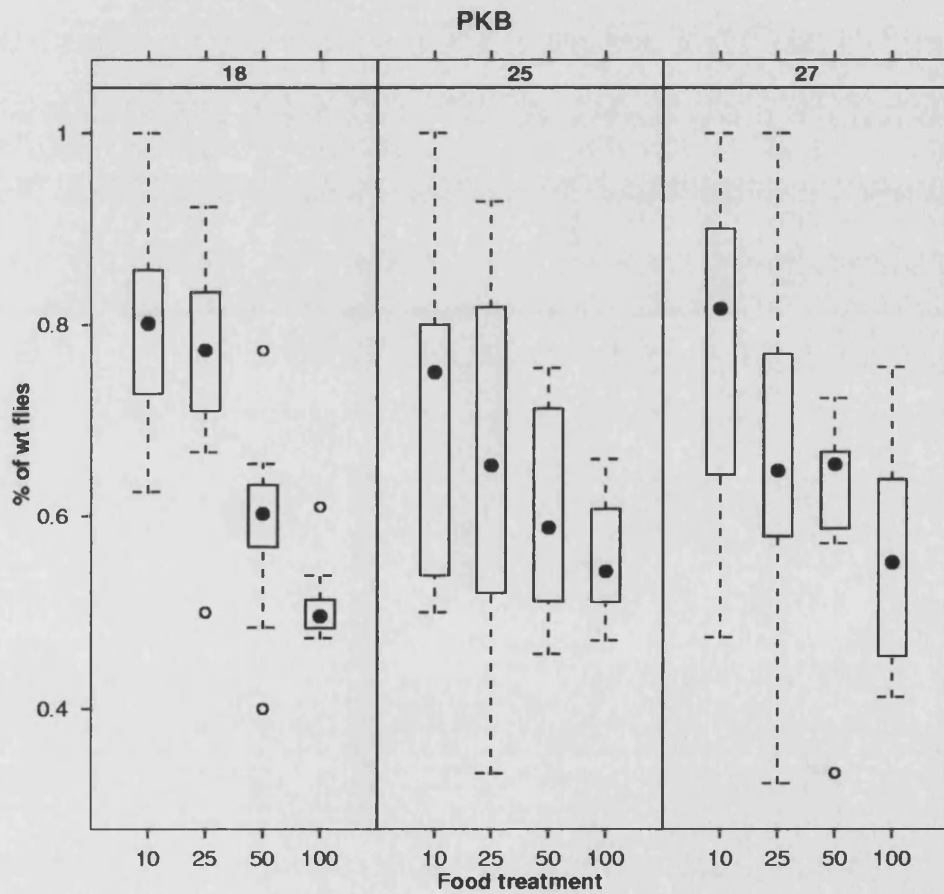
5.5 Discussion

The aim of the work presented in this chapter was to characterise the effects of null and hypomorphic mutations in genes of the IIS pathway on pre-adult survival at different temperatures and levels of larval crowding, and to test if any of the mutants under study showed a pattern of effects similar to the one expected for genes involved in the control of body size variation in thermal selection lines or along size clines. The results showed that all IIS genes mutant alleles interact with crowding and temperature to alter the larva-to-adult survival of heterozygote flies. In three crosses, namely *chico*, *p60* and *Akt/PKB* by Dahomey, flies heterozygous for a hypomorph/null allele showed an increased larval survival under high crowding conditions; in one cross, InR^{E19} by Dahomey, heterozygous flies showed a reduced survival at high crowding and lower temperature. With the exception of the *p60* cross, all crosses show a strong interaction of temperature with food level, with survival of flies heterozygous for the mutant increasing with decreasing temperature coupled with decreasing food level for the chico and *Akt/PKB* crosses and survival of the InR^{E19} decreasing with decreasing temperature coupled with decreasing food level. The *p60* cross was the only cross not showing any interaction; *p60* heterozygous showed an overall increase in survival at 18°C and an overall increase in survival at the 10% food level compared with the 25% food level.

How can we interpret these results in the light of the body size variation measured along natural clines or in laboratory selection lines? As pointed out in the introduction, genes controlling the observed variation in size in nature or under laboratory selection are expected to increase the larval-to-adult survival at high temperature. The crosses of *chico*, *p60* and *Akt/PKB* show that the heterozygote mutant/wild-type flies have the greatest survival at high levels of larval crowding, but this effect is more pronounced at low temperature. This pattern does not seem consistent with what we would expect for genes affecting body size variation under thermal selection.

One mutant genes, InR^{E19}, show patterns more consistent with theoretical expectations. The InR^{E19} cross shows decreased larval-to-adult survival at low temperature, albeit only under high larval crowding. It is therefore possible to hypothesize that InR^{E19} could be, at least in part, responsible for the body size variation observed in selection lines and in size clines.

Larval crowding is known to affect both developmental time and body size, with longer developmental time and smaller body size in flies reared under a high crowding regime, and shorter developmental time and bigger body size under low crowding regime (Bierbahum et al. 1989; Santos et al. 1994; Lazebny et al. 1996; Santos et al. 1997; James and Partridge 1998). Selection lines maintained at different crowding regimes show a size and developmental time differentiation: flies from high crowding lines produce individuals that are smaller and have increased developmental time when flies from all selection lines are tested under standard conditions (Roper et al. 1996). These results are similar to results obtained from temperature selection, with flies form "cold" selection lines showing bigger size and faster development than flies from "warm" selection lines (General Introduction 1.2.4). Support for the importance of crowding in the establishment of body size differences comes from unpublished work by Kennington and Gockel, who did not find any size differentiation after 55 generations of thermal selection under standardized larval crowding (p = 0.866 for females and p = 0.917 for males). Larval crowding is also thought to play a part in the establishment in body size clines, although evidence for an increased crowding at lower latitudes is not conclusive due to the difficulty assesing population density in the field.

The fact that all genes under study affect larval survival at higher levels of larval crowding highlight the need to analyse the relationship between the evolution of body size and thermal and density-dependent selection in *Drosophila*. It is possible that temperature selections acts on body size altering the levels of crowding in culture, and in support to this idea is the fact that "warm" selection lines show a higher population

density than flies from "cold" selection lines. Nonetheless the interplay of effects between crowding and temperature has never been fully addressed and future work should concentrate on a positive resolution of the issue.

6. Thermal selection and plasticity of cell area and cell number:

effects of adaptation to cycling thermal environment.

6.1 Abstract

Phenotypic plasticity is the ability of a genotype to give rise to different phenotypes in response to environmental conditions. Body size shows phenotypic plasticity in ectotherms, with development at colder temperatures leading to an increase in body size. In addition, body size evolves in response to thermal environment, with genetically larger individuals found at lower temperatures, in both laboratory and nature. In this study, I investigated evolution of phenotypic plasticity for body size in *Drosophila*, following adaptation to constant and cycling thermal environments. Mean wing area was greater in the lines from the 18°C thermal regime than in those from 25°C. Mean wing area was at least as great in the lines from variable thermal regimes as in the 18°C lines. No evidence for evolution of phenotypic plasticity for wing area was found in response to variable thermal environments when the selection lines were reared at constant temperature. However, wing cell area, which was entirely responsible for the thermal plasticity of wing area, showed increased phenotypic plasticity in the lines from the variable thermal environments. When all selection lines were raised under cyclic thermal regime, no effects of selection regime on mean wing area could be detected. The 25°C selection lines showed the smallest reduction in wing area when reared at variable temperature as opposed to 18°C. Evolutionary responses in pre-adult competitive ability were also evaluated. When all selection lines were raised under cyclical thermal conditions, the two cyclic lines had a larval viability comparable to the constant 18°C line but lower than the constant 25°C line at lower food doses; when all lines were raised at constant 18°C, the two cyclic selection lines had greater viability than both constant selection lines at lower food doses; no effects of selection could be detected when all lines were raised at constant 25°C. I conclude that the increase in

plasticity of wing cell area in the lines from cyclic thermal regimes may increase their ability to match their growth rate to nutrient-availability.

6.2 Introduction

The term "plasticity" defines the ability of a genotype to give rise to different

phenotypes in different environments; "norm of reaction" (or "reaction norm") defines

the range of phenotypes that a single genotype can produce in different environments

(General Introduction 1.4.1). Empirical evidence indicates that norm of reaction of a

trait can itself evolve, as seen in the bacterium *Escherichia coli* for acclimatation to

temperature (Bennett and Lenski 1997), in butterflies for seasonal polyphenism for

wing phenotype (Kingsolver 1995), in the mouse *Mus domesticus* for body weight and

nesting at different temperatures (Lynch 1992), in *Daphnia gelatea* for the specific rate

of weight gain under different nutritional conditions (Hairston et al. 2001) and the fly *D.

melanogaster* for fecundity in time (Teotonio et al. 2002). The value of a trait is

determined by genetic effects on the trait mean, its norm of reaction and the plastic

response to the environment. An important issue is the factors determining the relative

role of these influences on trait values (Kingsolver and Huey 1998; de Jong 1999).

In almost all ectotherms temperature has strong phenotypic effect on body size,

with individuals reared at lower temperatures being larger (General Introduction 1.4.3).

The plasticity of body size in response to temperature could be a non-adaptive effect.

This hypothesis is unlikely to be correct. Numerous studies have shown both that

growth is costly, both because it can increase the risk of starvation during development

(Chippindale et al. 1996; Blanckenhorn 2000; Prasad et al. 2001) and because it can

delay adulthood (Harshman et al. 1999). Furthermore, larger adult body size is often

associated with increase reproductive success (Robertson 1957; Partridge and Farquhar

1983; Lefranc and Bundgaard 2000; Reeve at al. 2000) and survival (Partridge and

Farquhar 1981, 1983; Partridge et al. 1986). The plastic and the evolutionary effects of

temperature on *Drosophila* body size act in the same direction, with larger size in the

cold, suggesting that the plastic response may be adaptive. The plastic response of

ectotherm body size to temperature during growth could be adaptive, either because larger adult body size is adaptive, and growth at lower temperatures results in greater availability of nutrients for growth, or because larger adult body size is more strongly associated with fitness at lower temperatures.

Where plasticity is adaptive, several models of the evolution of reaction norms argue that, under different environmental regimes and in the absence of genetic constraints or physiological costs, reaction norms should evolve towards a response that gives an optimal phenotype in each environment (Via and Lande 1985; de Jong 1990, 1999; Gomulkiewicz and Kirkpatrick 1992; Gavrilets and Scheiner 1993). Genetic variation for the norm of reaction of *Drosophila* body size to temperature has been demonstrated (General Introduction 1.4.3). However, the norm of reaction of body size to temperature appears not to evolve in any consistent way either in response to latitude, or to laboratory thermal selection, despite substantial evolution in the mean trait value. The data point to the conclusion either that the plasticity of body size to temperature is under stabilising selection, or that there is a constraint on its evolution, possibly because of a physiological cost (van Tienderen 1991; Agrawal 2001; 321; Relyea 2002; Kassen 2002).

In *Drosophila* lower temperatures directly increase growth efficiency, and increased growth efficiency also evolves in colder laboratory thermal regimes and at higher latitudes (Neat et al. 1995; Robinson and Partridge 2001). Plasticity of body size could then be an adaptive, but passive, response to thermal environment and hence availability of nutrients for growth. Under this hypothesis, plasticity could be under stabilising selection, because any change in the reaction norm could either increase the danger of starvation during pre-adult growth or lead to a failure to take advantage of nutrient-availability to maximise size. In contrast, if there is an optimal adult body size that increases with declining temperature, then we might expect that all genotypes would use some combination of genetic mean body size and plasticity to achieve that

optimum. But this is not what is observed. The adult body size achieved at a given temperature differs between genotypes that have evolved at different latitudes or at different temperatures in the laboratory.

The data so far, therefore, suggest that plasticity of body size in response to growth temperature may serve to match growth rate to nutrient-availability. However, plasticity alone apparently cannot achieve this matching, because mean body size evolves in response to different average temperatures. Mean body size may evolve because of a constraint on the amplitude of phenotypic plasticity or because the range of temperature-variation normally encountered does not impose selection for increased plasticity. I have experimentally investigated the effects of variable thermal regime on the evolution of plasticity of wing size and wing cell area in *D. melanogaster* under long-term laboratory natural selection (Rose et al. 1990; Huey et al. 1991). Two cycling thermal regimes regularly exposed the flies to two different temperatures and two constant temperature regimes acted as controls. This allowed us to evaluate whether variable thermal selection acts directly on plasticity itself. If it does, then we might expect it to increase in cyclical thermal environments, where there would be selection both to take advantage of great nutrient-availability in the cold and to avoid starvation at the higher temperature.

6.3 Materials and Methods

6.3.1 Thermal selection lines.

The thermal selection lines used in this experiment are described in the General Materials and Methods, section 2.3.1.

6.3.2 Wing and Cell area assays.

The lines were assayed three times, first in early 1997, approximately two and a half years after they had been established, and second in early 1999, approximately four and

a half years after establishment and third in 2003, about six years after establishment. In 2003 females only were assayed. The raw data for the 1997 assay were collected by Rohema Miah and the raw data for the 1999 assay were collected by Michael Reeve. The effects of selection temperature and experimental temperature on adult body size, measured as wing area, were assessed, by rearing flies from the four selection regimes at both 18 and 25°C. Wing area was used as a measure of body size because genetic and environmental correlations have been shown between the sizes of different anatomical regions of *Drosophila* adults (Cowley and Atchely 1990; Wilkinson et al. 1990). To control for a possible effect of parental thermal environment on offspring performance and size (Huey *et al.* 1995, Crill *et al.* 1996), parents of the experimental flies were also reared at the experimental temperature. Eggs were collected from each population cage by placing yeasted bottles in the cage until a moderate density of eggs had been laid, and these bottles were then placed at the experimental temperature. The eclosing adults were used as parents for collecting eggs and setting up standard density cultures vials, following the procedure detailed in the General Materials and Methods, section 2.5. Twenty vials were set up for each replicate selection line at each rearing temperature in the 1997 assay, 10 vials in the 1999 assay and 5 vials in the 2003 assay.

The right wings of four adults of each sex (five for the 2003 assay) from each vial were mounted and measured. In the 1997 assay only, a *camera lucida* attached to a dissecting microscope and a Quora graphics tablet connected to a computer was used. In the 1999 and 2003 assays, wing area was measured according to the procedure outlined in the General Material and Methods, section 2.7.

Cell density in the wings was measured in the 1999 and 2003 assays, following the procedure described in the General Material and Methods, section 2.7.

6.3.2. Adaptation to cycling thermal regimes assay (2003).

In order to determine whether the cyclical lines are more able to track changes in

temperature with a change in growth rate or efficiency, and hence increase their body size, I measured wing size of flies of all selection lines after they had been reared in five different thermal regimes: the daily cycling regime, a switch from 18 to 25°C after 4 days, a switch from 18 to 25°C after 8 days, a switch from 25 to 18°C after 48 hours, a switch from 25 to 18°C after 84 hours. First instar larvae were collected and five vials with 50 larvae each were set up per cage. The right wing of five males and five females per vial was mounted and measured. Both larval collection and wing mounting and measuring were done following the procedures described in the section 5.3.1.

To examine the effects of thermal selection on fitness during the pre-adult period, I set up a larval competition experiment, following the procedure detailed in the General Materials and Methods, section 2.8. Eggs were collected from all cages and the larvae reared under standard density. The eclosing flies were transferred to laying pots and handled as described in the section "Wing and Cell area assays". For each level of food, ten vials per cage were seeded with 25 first instar larvae from the cage stock and 25 larvae of the mutant *sparkling poliert* (spa^{pol}) eye mutant in Dahomey background used as standard competitor stock. Again, larval collection followed the aforementioned procedures. Competitor stocks act as a yardstick against which to measure the competitive ability of other strains, and give a more sensitive index of competitive ability than do pure cultures (Santos et al. 1992, 1994). The experiment was repeated at 25°C, 18°C and under daily cyclical thermal switch.

6.3.1. Statistical analysis.

In all analyses of wing area, the data were divided according to sex, to eliminate sex and its interaction terms from the analyses, and increase the statistical power available for the other terms. The data for each sex were subjected to a standard linear mixed-effects model, with experimental temperature and selection regime as fixed main effects, and replicate line as a random effect nested within selection regime. Linear contrast analyses

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were used to compare the interaction between selection regime and temperature in the two cycling lines, the two constant temperature lines, and the cycling versus the constant temperature lines. Cell area and cell number data were subjected to an analysis of variance similar to that used to analyse the wing area data. Departures from the assumptions of the F-test were never detected using both a Bartlett test for homogeneity of variances and a Shapiro-Wilk test for normality.

Plasticity was measured as the difference of the mean value of a trait between the lower and the higher temperature. An increase in the difference would reflect an increase in plasticity; a significant interaction term between selection line and raising temperature would indicate significant differences in plasticity between selection lines. Linear contrast analyses were used to compare the interaction between selection regime and temperature in the two cycling lines, the two constant temperature lines, and the cycling versus the constant temperature lines. Again all data was homoscedastic and normally distributed.

In the "cycling conditions" test, the five temperature-switch treatments were analysed separately. Selection regime was the only fixed main effect; replicate line nested within selection regime was the random effect. Again, the F-test assumptions of normal distribution and homogeneity of variances were not violated in any cases. Scope of growth was measured in females as the difference (wing size under thermal switch − wing size under fixed temperature); two differences were computed, one against size at 18°C and one against size at 25°C, using the females flies assayed in 2003 as controls. These data were analysed with a simple one-way ANOVA, because the only comparisons possible were between the mean size value of each replicate under different growing conditions. No random effects were therefore present in this analysis.

The analysis performed for the larval competition test was a Generalized Linear Mixed Model via Penalized Quasi-Likelihood (glmmPQL), using a binomial distribution of errors. This model accommodates both fixed and random effects and

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corrects for the decreasing number of flies eclosing at the lower food percentage treatments. The data from the two temperatures were analysed separately, resulting in a model with food treatment and selection regime as main fixed effects and replicate lines nested inside selection regime as a random effect. Linear contrast analyses were used to compare the interaction between selection regime and food level; selection regime was contrasted in the following pairs: the two cycling lines, the two constant temperature lines, and the cycling with the constant temperature lines. The second set of linear contrasts contrasted food effects: the 100% versus 50% food treatment, the 25% versus 10% food treatments and the "high" (100% and 50%) versus "low" (25% and 10%) food treatments. All the conclusions were drawn from the glmmPQL results but, in order give the reader a visual representation of the data, boxplots of the percentage of wild-type flies surviving are presented.

6.4 Results

6.4.1. Wing area at constant temperature.

As expected, analysis of the wing area data from all three samples revealed that flies of both sexes reared at the lower experimental temperature had significantly larger wings than those reared at the higher temperature (Tables 6.1 and 6.2). The 1997 data indicated that there was a significant effect of selection regime on wing area in both sexes. Linear contrast analysis revealed that the 18°C and the two cyclic lines had evolved significantly larger wings than those that had evolved at 25°C (females: $t\text{-test}_{(8)}$ = 4.85, P = 0.0013; males: $t\text{-test}_{(8)}$ = 4.41, P = 0.0022). For both sexes, the cycling lines showed no differences in wing area between long and short cycle, and between the two cycling lines and the 18°C line. No significant interaction between selection regime and temperature was detected in either sex. Analysis of the 1999 data showed a pattern similar to the 1997 data, but females only showed a significant response to selection.

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Females of the 18°C and of both cycling lines had evolved significantly larger wings

than those that had evolved at 25°C (females: t-test$_{(8)}$ = 4.29, P = 0.027). The linear

contrast between the two cycling lines could not detect any difference in wing area; and

no difference in wing area could be detected between the two cycling lineas and the

18°Cline. Once again, no significant interaction was found between selection regime

and temperature. Analysis of variance for females wing area in 2003 showed a strong

effect of selection (F$_{(3,8)}$ = 14.42, P = 0.0014); linear contrasts showed that the 18°C line

and the two cycling lines had a greater wing area than did the 25°C line (t-test$_{(8)}$ = 6.22,

P = 0.0003). As in 1997 and 1999 linear contrasts could not detect any significant

difference in wing area between the two cycling lines or the two cycling lines and the

18°C line. No interaction could be found between selection and temperature. In

summary, in all three assays the 18°C and cyclic lines were bigger than the 25°C lines,

and most importantly, analysis of all the 1997, 1999 and 2003 data for wing area

revealed no significant interaction between experimental temperature and selection

regime in either sex (see Table 6.2). This result demonstrates that there were no

differences in wing area plasticity between lines from the different selection regimes at

any stage in the experiment.

Table 6.1. Mean wing area ± 95% confidence intervals, measured in mm^2. On the left, values for flies raised at 18°C, on the right values for flies raised at 25°C

1997 Females	18°C	25°C
18°C fixed temp.	1.354 ± 0.009	1.204 ± 0.012
25°C fixed temp.	1.325 ± 0.023	1.187 ± 0.023
Long Cycle	1.353 ± 0.017	1.225 ± 0.012
Short Cycle	1.353 ± 0.027	1.218 ± 0.012
1997 Males	18°C	25°C
18°C fixed temp.	1.238 ± 0.008	0.947 ± 0.012
25°C fixed temp.	1.208 ± 0.031	0.925 ± 0.040
Long Cycle	1.223 ± 0.045	0.957 ± 0.031
Short Cycle	1.235 ± 0.010	0.948 ± 0.032
1999 Females	18°C	25°C
18°C fixed temp.	1.499 ± 0.058	1.246 ± 0.037
25°C fixed temp.	1.450 ± 0.031	1.226 ± 0.042
Long Cycle	1.494 ± 0.013	1.242 ± 0.058
Short Cycle	1.512 ± 0.008	1.256 ± 0.012
1999 Males	18°C	25°C
18°C fixed temp.	1.211 ± 0.036	0.966 ± 0.021
25°C fixed temp.	1.190 ± 0.045	0.955 ± 0.051
Long Cycle	1.190 ± 0.012	0.961 ± 0.053
Short Cycle	1.228 ± 0.037	0.969 ± 0.016
2003 Females	18°C	25°C
18°C fixed temp.	1.728 ± 0.077	1.493 ± 0.033
25°C fixed temp.	1.603 ± 0.073	1.349 ± 0.087
Long Cycle	1.699 ± 0.033	1.431 ± 0.054

| Short Cycle | 1.725 ± 0.062 | 1.437 ± 0.030 |

Table 6.2. Analysis of variance on wing area for thermal selection lines reared and tested at 18 and 25°C. Only fixed effects are shown.

Wing Area. 1997 Females

Effect	Num DF	Den DF	F ratio	P
Rearing temperature	1	8^A	3046.9	<0.0001
Selection regime	3	8^B	7.85	0.009
Rearing temp. x selection	3	8^A	3.39	0.08

Wing Area. 1997 Males

Effect	Num DF	Den DF	F ratio	P
Rearing temperature	1	8^A	3442.59	<0.0001
Selection regime	3	8^B	6.56	0.015
Rearing temp. x selection	3	8^A	1.17	0.38

Wing Area. 1999 Females

Effect	Num DF	Den DF	F ratio	P
Rearing temperature	1	8^A	2656.05	<0.0001
Selection regime	3	8^B	6.94	0.0129
Rearing temp. x selection	3	8^A	2.36	0.1478

Wing Area. 1999 Males

Effect	Num DF	Den DF	F ratio	P
Rearing temperature	1	8^A	3007.17	<0.0001
Selection regime	3	8^B	2.65	0.1206
Rearing temp. x selection	3	8^A	2.12	0.1753

Wing Area. 2003 Females

Effect	Num DF	Den DF	F ratio	P
Rearing temperature	1	8^A	338.38	<0.0001
Selection regime	3	8^B	14.42	0.0014

| Rearing temp. x selection | 3 | 8[A] | 0.62 | 0.62 |

6.4.2 Cell area index.

Cell area was measured in 1999 and 2003. For the 1999 analysis, the cells from wings of flies of both sexes reared at the higher temperature were significantly smaller than those of flies reared at the lower temperature (Tables 6.3 A and 6.4 A). Analyses of variance revealed a significant interaction between selection regime and cell area in both sexes. Linear contrast on the interaction term revealed very significant difference between the cycling lines and the fixed lines (females: t-test$_{(8)}$ = -6.95, P = 0.0001; males: t-test$_{(8)}$ = -11.1, P < 0.0001), with flies from cyclic lines having cells bigger at 18°C and smaller at 25°C than the fixed lines; the observed difference was greater at 18°C. In 2003 the results were consistent with the results of 1999. Analysis of variance revealed a significant interaction between selection regime and cell area. Linear contrast on the interaction term showed again that cell area plasticity in the cyclic lines was greater (t-test$_{(8)}$ = -9.54, P < 0.0001), as a result of flies from cyclic lines having cells bigger at 18°C and smaller at 25°C than flies from the fixed temperature lines; again, the observed difference was greater at 18°C.

In both analyses there was a significant interaction between experimental temperature and selection regime. These results demonstrate that, in contrast to the result for plasticity on wing area itself, plasticity for cell area was greater in the cycling than in the fixed temperature lines in both assays.

6.4.3. Cell number index.

Cell number index was calculated and analysed for the 1999 and 2003 assays only. The plasticity of cell number in response to temperature was in every case of opposite sign to that of wing size, with sell number greater at the higher experimental temperature. There was a significant interaction between experimental temperature and selection line for cell number in both sexes (Tables 6.3 B and 6.4 B). In the 1999 flies linear contrasts on the interaction term was highly significant in both sexes (females: t-test$_{(8)}$ = 4.09, P =

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0.0035; males: t-test$_{(8)}$ = 5.58, P = 0.0005), with flies from cyclic lines having greater

plasticity of cell number index than flies from fixed lines as a result of flies from cyclic

lines having fewer cells than flies from fixed lines at 18°C and more cells at 25°. The

observed difference was greater at 18°C. The results in 2003 were consistent with the

1999 results: a significant interaction term between temperature and selection regime

was found for cell number, and linear contrast on the interaction term showed that

cycling and fixed lined differ significantly (t-test$_{(8)}$ = 4.41, P = 0.0012), with flies from

cyclic lines having fewer cells than flies from fixed lines at 18°C and flies from cyclic

lines having more cells at 25° than the fixed lines.

Once again, the interaction between selection regime and temperature was

significant; and these results demonstrate that plasticity for cell number was greater in

the cycling than in the fixed temperature lines in both assays. Plasticity of both cellular

components of wing area therefore was greater in cyclic thermal environments, but in

an exactly compensatory fashion that resulted in no increase in plasticity for wing area.

Table 6.3. (A) Cell Area Index ± 95% confidence intervals, measured in mm^2, and (B) Cell Number Index ± 95% confidence intervals. For both measure, on the left, values for flies raised at 18°C, on the right values for flies raised at 25°C.

(A) Cell area index

1999 Females	18°C	25°C
18°C fixed temp.	1.978 e^{-4} ± 3.37 e^{-6}	1.338 e^{-4} ± 9.89 e^{-6}
25°C fixed temp.	1.837 e^{-4} ± 2.33 e^{-6}	1.298 e^{-4} ± 6.44 e^{-6}
Long Cycle	2.040 e^{-4} ± 7.37 e^{-6}	1.296 e^{-4} ± 8.69 e^{-6}
Short Cycle	2.030 e^{-4} ± 4.97 e^{-6}	1.316 e^{-4} ± 3.69 e^{-6}

1999 Males	18°C	25°C
18°C fixed temp.	1.554 e^{-4} ± 6.51 e^{-6}	1.089 e^{-4} ± 1.32 e^{-5}
25°C fixed temp.	1.497 e^{-4} ± 4.98 e^{-6}	1.032 e^{-4} ± 1.36 e^{-6}
Long Cycle	1.690 e^{-4} ± 7.24 e^{-6}	1.027 e^{-4} ± 6.76 e^{-6}
Short Cycle	1.651 e^{-4} ± 1.35 e^{-5}	1.033 e^{-4} ± 5.82 e^{-6}

2003 Females	18°C	25°C
18°C fixed temp.	2.243 e^{-4} ± 1.31 e^{-5}	1.918 e^{-4} ± 5.87 e^{-6}
25°C fixed temp.	2.220 e^{-4} ± 7.06 e^{-6}	1.975 e^{-4} ± 1.82 e^{-5}
Long Cycle	2.560 e^{-4} ± 8.81e^{-6}	1.817 e^{-4} ± 4.61 e^{-6}
Short Cycle	2.540 e^{-4} ± 8.87 e^{-6}	1.835 e^{-4} ± 6.65e^{-6}

(B) Cell number index

1999 Females	18°C	25°C
18°C fixed temp.	7594 ± 314	9338 ± 446
25°C fixed temp.	7907 ± 245	9452 ± 745
Long Cycle	7431 ± 380	9603 ± 941
Short Cycle	7460 ± 150	9565 ± 318

1999 Males	18°C	25°C
18°C fixed temp.	7794 ± 214	8898 ± 1075
25°C fixed temp.	7956 ± 102	9273 ± 553
Long Cycle	7056 ± 371	9368 ± 1115
Short Cycle	7455 ± 407	9407 ± 490
2003 Females	18°C	25°C
18°C fixed temp.	7203 ± 497	7789 ± 310
25°C fixed temp.	6738 ± 332	6851 ± 573
Long Cycle	6645 ± 260	7877 ± 256
Short Cycle	6795 ± 442	7841 ± 407

Table 6.4. Analysis of variance on (A) wing cell area and (B) cell number in the wings of the thermal selection lines reared and tested at 18 and 25°C. Only fixed effects are shown

(A) Wing Cell Area.

1999 Females

Effect	Num DF	Den DF	F ratio	P
Rearing temperature	1	8^A	4249.07	<0.0001
Selection regime	3	8^B	21.40	<0.0001
Rearing temp. x selection	3	8^A	20.64	<0.0001

1999 Males

Effect	Num DF	Den DF	F ratio	P
Rearing temperature	1	8^A	4970.15	<0.0001
Selection regime	3	8^B	5.11	0.029
Rearing temp. x selection	3	8^A	42.07	<0.0001

2003 Females

Effect	Num DF	Den DF	F ratio	P
Rearing temperature	1	8^A	480.21	<0.0001
Selection regime	3	8^B	6.23	0.017
Rearing temp. x selection	3	8^A	30.91	0.0001

(B) Wing Cell Number.

1999 Females

Effect	Num DF	Den DF	F ratio	P
Rearing temperature	1	8^A	826.83	<0.0001
Selection regime	3	8^B	0.98	0.45
Rearing temp. X selection	3	8^A	6.22	0.017

1999 Males

Effect	Num DF	Den DF	F ratio	P
Rearing temperature	1	8^A	404.01	<0.0001
Selection regime	3	8^B	1.83	0.22
Rearing temp. x selection	3	8^A	11.45	0.0029

2003 Females

Effect	Num DF	Den DF	F ratio	P
Rearing temperature	1	8^A	31.88	0.0005
Selection regime	3	8^B	12.12	0.0024
Rearing temp. x selection	3	8^A	19.39	0.0005

^{A} Denominator DF corresponding to the DF for the interaction "Temperature x Line in Selection" effect.

^{B} Denominator DF corresponding to the DF for the "Line in Selection" effect

6.4.4 Wing area under variable temperature.

Wing area in flies from the thermal-switch regimes was analysed with a standard linear mixed-effect model. The five thermal switch regimes were analysed separately; replicate line within selection regime was the error term for selection regime. In all cases the only significant effect was that of sex; no selection effects or interactions between sex and selection could be detected. For instance, flies of both sexes showed reduced size differentiation under the daily cycle regime ($F_{(3,8)}$ = 1.356, p = 0.32; Fig 6.1 A); equivalent results were found for the other four temperature switch treatments.

The difference (size under variable regime – size under constant regime) was computed for the five thermal switches compared to both 18 and 25°C fixed temperatures. These differences were analysed with a simple one-way ANOVA. For all five thermal switches, in both comparisons with the two fixed temperatures, orthogonal contrasts indicated that the 25°C lines decreased size less than did the lines from the other selection regimes when size was compared with growth in cyclic conditions with that at 18°C; the 18°C line did not significantly differ from the two cyclic lines, and the two cyclic lines did not differ between themselves. Under daily switch, females of the 25°C line showed the smallest difference in size when compared to the size reached at the 18°C fixed rearing temperature ($t_{(8)}$ = -3.771, p = 0.00546; Fig 6.1 B) and they showed also the greatest difference in size when compared to the size reached at the 25°C fixed rearing temperature ($t_{(8)}$ = -3.334, p = 0.01033; Fig 6.1 C). In the comparison against the size reached at both fixed rearing temperatures, the three remaining lines did not significantly differ. Equivalent results were found for the size comparisons between the selection lines raised under the other four thermal switch regimes.

Fig. 6.1. (A) Boxplot of Body Size under Daily Cycle regime, females on the left, males

on the right. (B) Boxplot of Scope of Growth, as size differences between the daily

cycle size and the fixed 18°C size. Females only. (C) Boxplot of Scope of Growth, as

size differences between the daily cycle size and the fixed 25°C size. Females only.

6.1 A. Body size under daily thermal switch
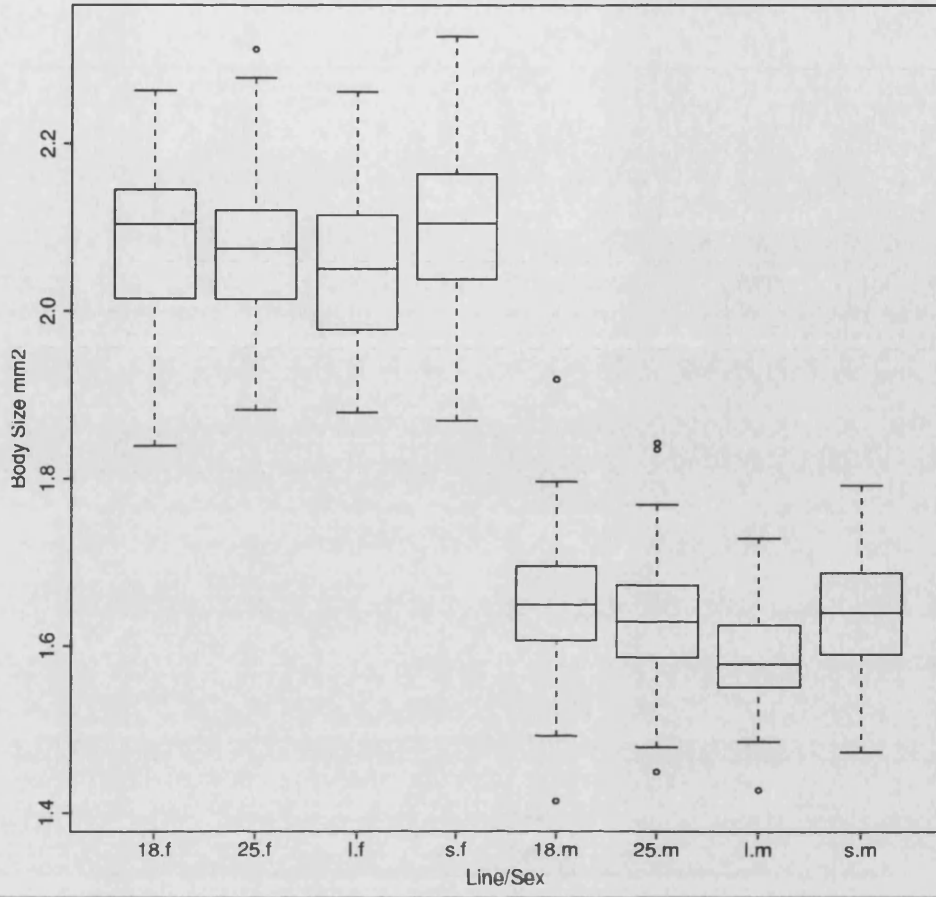
6.1 B. Size differences between daily cycle and fixed 18C temperature
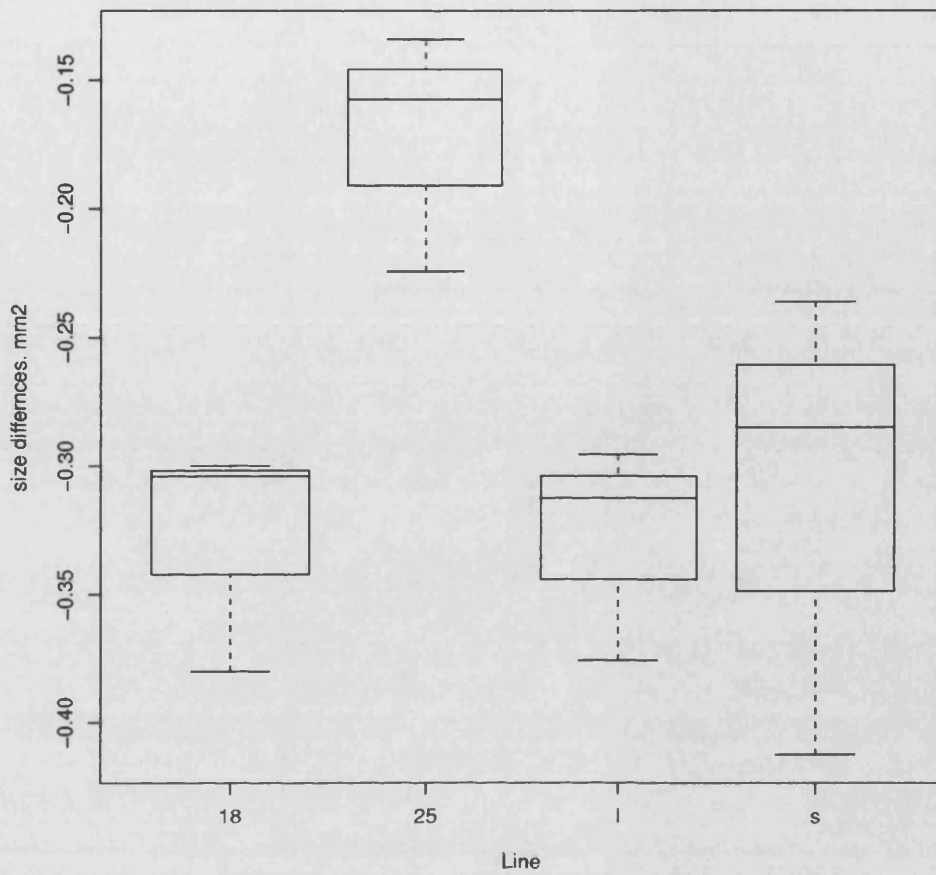
6.1 C. Size differences between daily cycle and fixed 25C temperature
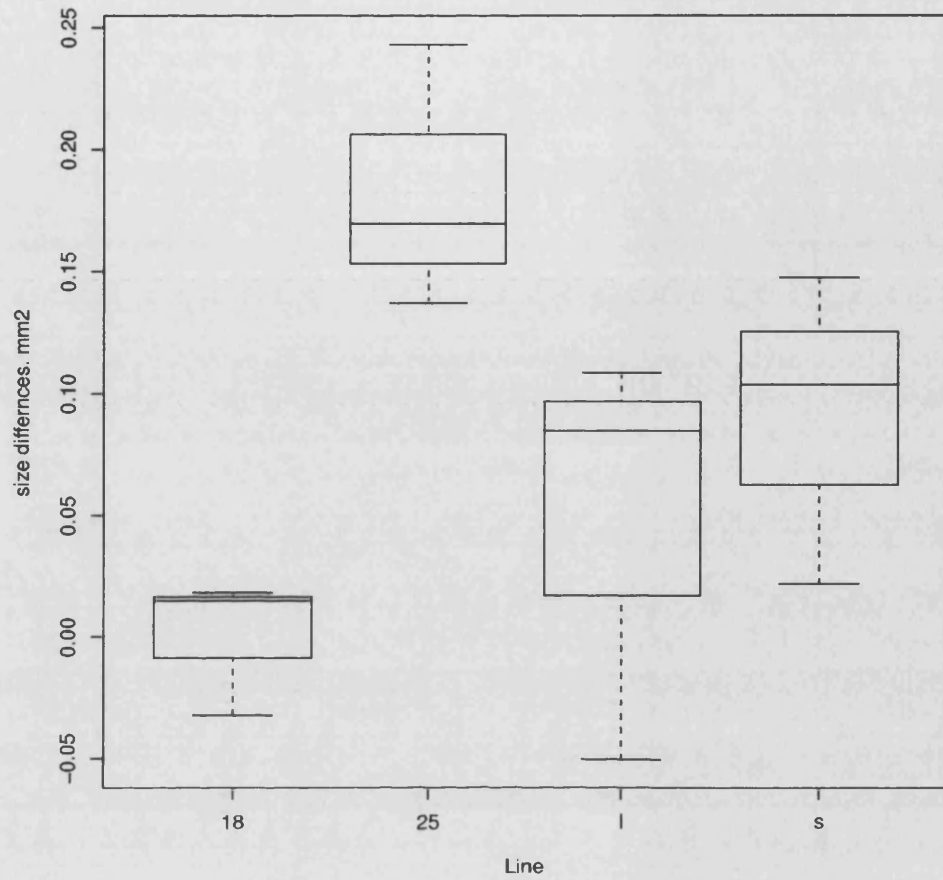
6.4.5. Larval competition assay.

The data for the competition experiments were analysed with glmmPQL, giving

analysis of deviance tables equivalent to the ones used to analyse the relationship

between wing size and temperature (Table 6.5, Fig 6.2). At 25°C the only significant

effect was that of food treatment ($F_{(3,24)}$ = 65.52, P < 0.0001), with a lower survival at

lower food doses. The selection line effect was not significant, nor was the interaction

term between selection and food treatment. At 18°C an interaction between food

treatment and selection line was evident ($F_{(9,24)}$ = 3.06, P = 0.014), main effects were

therefore not applicable. A similar result was found for the daily cycle, with a

significant interaction between food and selection ($F_{(9,24)}$ = 8.65, P < 0.0001). For the

18°C experiment linear contrast analysis showed a strong interaction between food level

and selection regime when cyclic and fixed lines were compared at 25 and 10% food

levels (t-test$_{(24)}$ = -4.18, P = 0.0003), with the cycling lines showing a greater

survivorship (Fig 6.2 B). For the daily cycle experiment linear contrast analysis showed

a marked interaction between food level and selection regime when cycling and fixed

lines went from high to low food levels (t-test$_{(24)}$ = -4.51, P = 0.0001). A second set of

orthogonal contrasts was used for the cycling experiment, using the 25°C line as control

for the other three. These contrasts showed that the 25°C line has a much greater

survivorship than the other lines in the passage from high to low food levels (t-test$_{(24)}$ =

-7.30, P = 0; Fig 6.2 C).

Table 6.5. Analysis of deviance on (A) competitive ability at 25°C and (B) competitive ability at 18°C as measured by a Generalised Linear Mixed Model via Penalised Quasi Likelihood.

(A) competitive ability at 25°C Effect	Num DF	Den DF	F ratio	P
Food treatment	3	24^A	65.52	<0.0001
Selection regime	3	8^B	1.23	0.36
Food treat. x selection	9	24^A	0.3874	0.93

(B) competitive ability at 18°C Effect	Num DF	Den DF	F ratio	P
Food treatment	3	24^A	45.17	<0.0001
Selection regime	3	8^B	2.04	0.19
Food treat. x selection	9	24^A	3.06	0.014

(C) competitive ability under daily cycle Effect	Num DF	Den DF	F ratio	P
Food treatment	3	24^A	23.42	<0.0001
Selection regime	3	8^B	11.88	0.0026
Food treat. x selection	9	24^A	8.65	<0.0001

^{A} Denominator DF corresponding to the DF for the interaction "Food Treatment x Line in Selection" effect.

^{B} Denominator DF corresponding to the DF for the "Line in Selection" effect

Fig. 6.2. Boxplot of the percentage of wild type (wt) flies emerging from each vial at (A) 25°C; (B) 18°C and (C) Daily Cycle. The four selection regimes are compared at the four food treatments. The black dot inside the boxes indicates the median.

6.2 A. Competition at 25C

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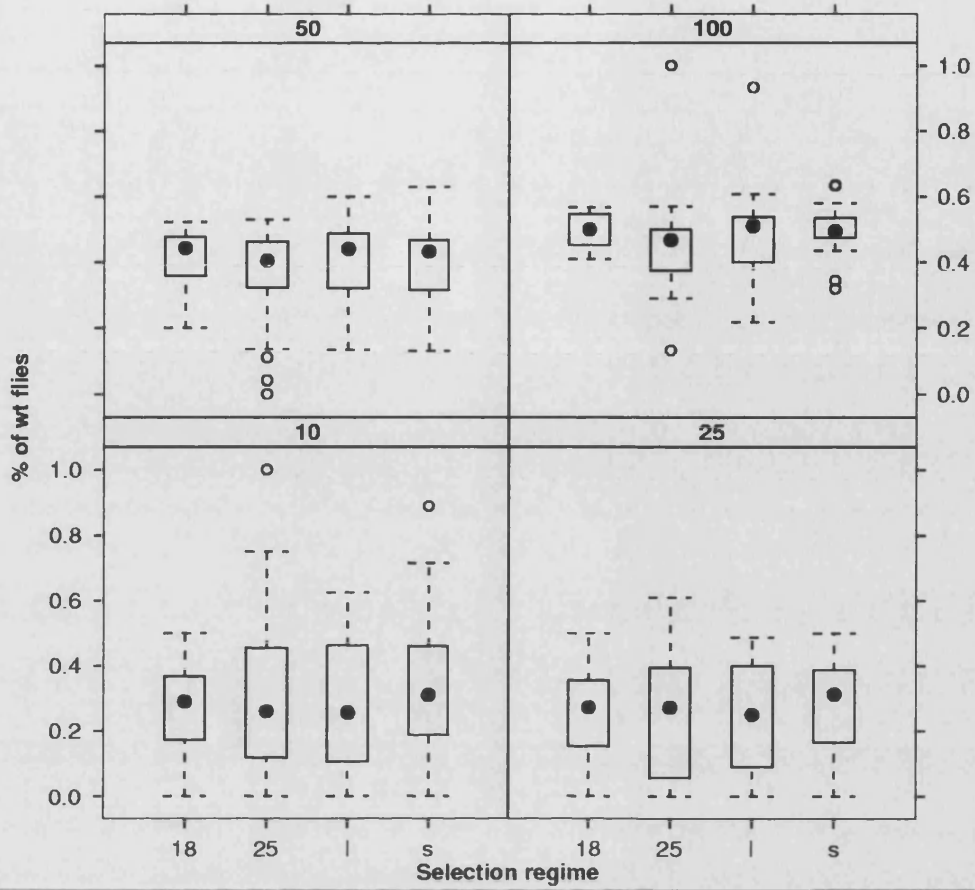
6.2 B. Competition at 18C
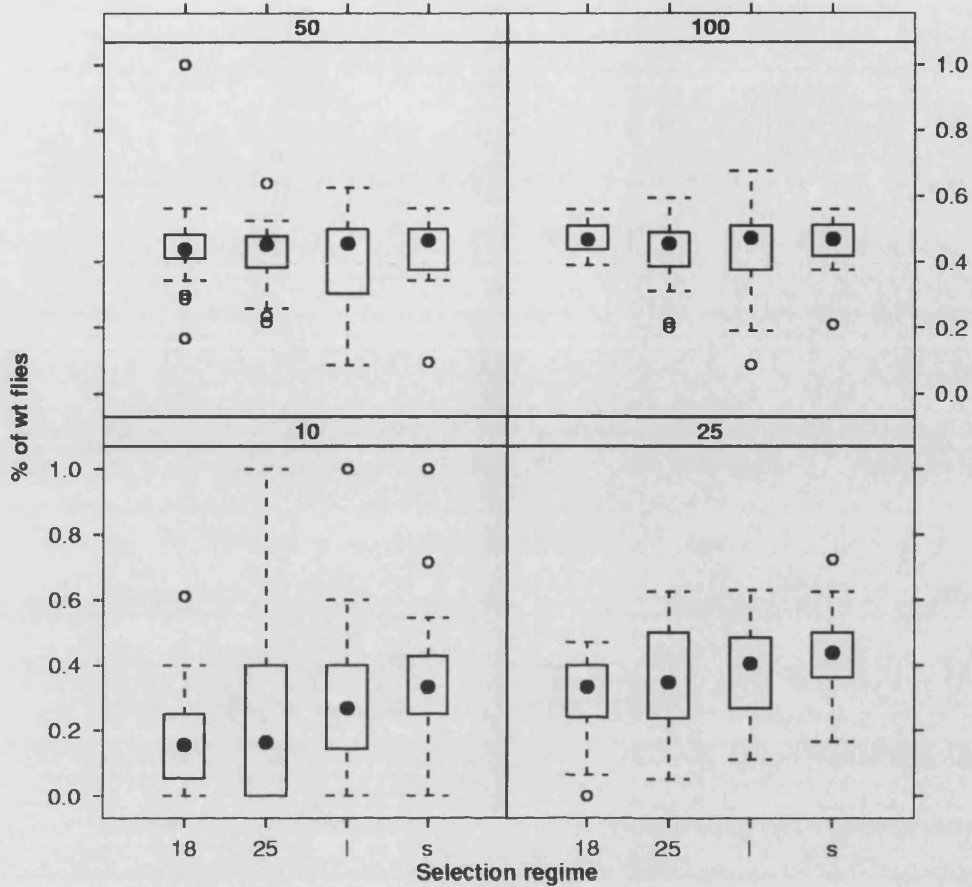
6.2 C. Competition under cyclic switch
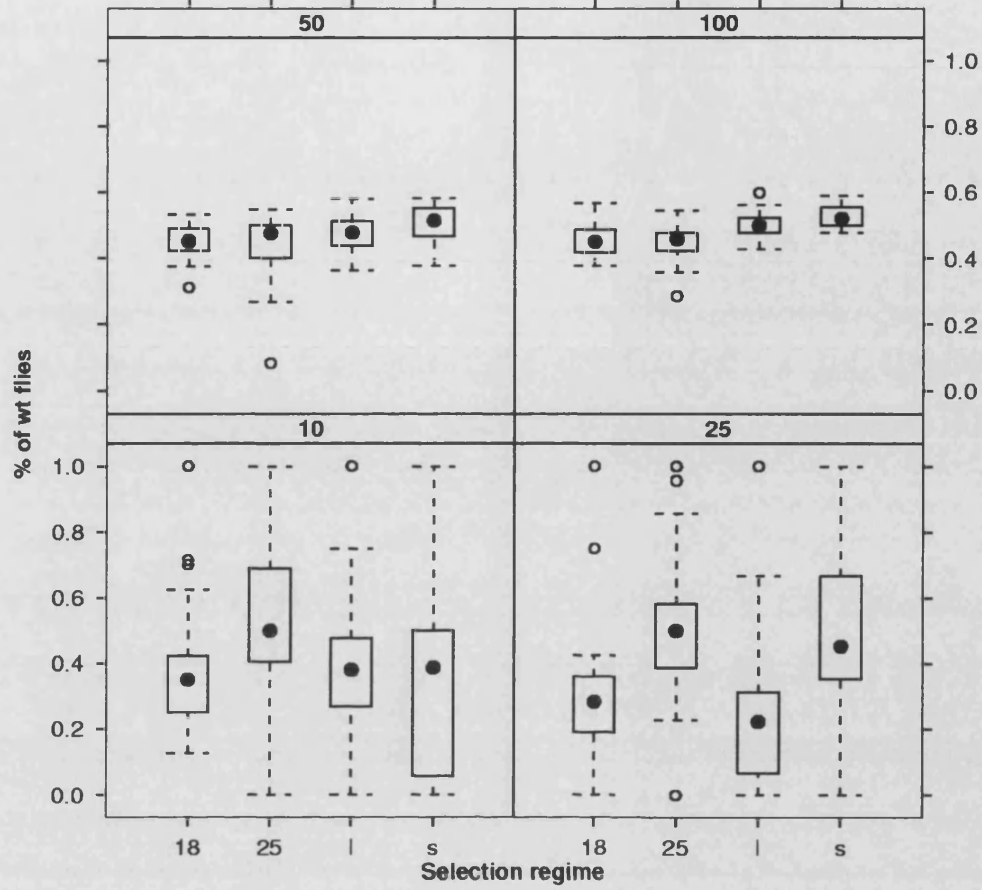
6.5 Discussion

Mean wing area showed a significant evolutionary increase at the lower, constant experimental temperature, in line with previous studies (Anderson 1966; Cavicchi *et al.* 1985; Partridge *et al.* 1994). The direction of evolution can be deduced because the Dahomey base stock had a 24-year history of culture at 25°C. All the lines showed plasticity of wing area in response to temperature and, as previously reported (Cavicchi et al. 1985; Partridge et al. 1994; Azevedo et al. 2001), the plasticity was based solely on plasticity of wing cell area. Plasticity of wing area showed no evidence of evolutionary response to thermal environment. Despite significant evolution of mean wing area over the course of the study, the plasticity of the trait failed to show any evidence of differentiation between lines from different thermal selection regimes. This result suggests that there was either no selection or stabilising selection for the norm of reaction of wing area, or that there was a constraint on the response to directional selection.

Although the norm of reaction of wing area to temperature did not evolve with thermal regime, the norms of reaction of wing cell area and cell number increased in the cyclic thermal regimes, and in an exactly compensatory manner. The plasticity of cell number therefore contributed negatively to the plasticity of wing area. This finding points to two conclusions. First, the evolutionary increase in the plasticity of the cellular components of wing area could be an adaptation, or genetically correlated with an adaptation, to cyclic thermal environments through a mechanism that does not involve changes in wing area. Second, the exact compensation between wing cell area and cell number supports the idea that the norm of reaction of wing area itself is under stabilising selection.

If we assume that plasticity of wing cell area can contribute to fitness either

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because it increases wing area or because it increases pre-adult survival, at least under some circumstances, several of our experimental results help to explain the greater thermal sensitivity of wing cell area in the cyclic selection lines. First, in the constant temperature regimes, the cyclic thermal lines had mean size at least as great as that seen in the selection lines. They therefore showed the full evolutionary increase in mean size, despite their regular and, in physiological terms equal, exposure to the higher temperature. Second, the 25°C thermal selection lines showed the least decrease in wing area in the transition from 18°C to cyclic thermal culture conditions. Third, the cyclic thermal selection lines showed greater pre-adult competitive ability that the lines from the other selection regimes with culture at 18°C and on low food. Fourth, the 25°C thermal selection lines showed greater pre-adult survival than did lines from the other selection regimes under cyclic thermal conditions and on low food.

Opportunity for growth during the pre-adult stages in *Drosophila* increases with rearing at lower experimental temperatures, because a larger adult is obtained from consumption of the same amount of yeast during growth, showing that efficiency of growth increases (Robinson and Partridge 2000). This increase in growth efficiency may account for the evolutionary increase in size at lower temperature if, for instance, the larva allocates more of its nutrients to growth. If adult size is ultimately limited by nutrient-availability, the failure of the 25°C lines to achieve the size seen in the lines from the other selection regimes at constant rearing temperatures suggests that their genotype prevents them from growing at the higher rates that are demonstrably biologically possible. This finding suggests that they are growing conservatively, perhaps to avoid starvation when confronted with food shortage. The invariant nature of the plastic response, in contrast, suggests that it may be a direct response to the availability of nutrients for growth.

The conservative growth trajectory of the 25°C lines may explain both why they could maintain size in cyclic conditions relative to that at 18°C and why they showed greater competitive ability on low food in cyclic conditions. Since their growth trajectory was already low, they were anyway failing to take advantage of the additional nutrients available for growth at the lower temperature, so would be less affected by reduction in exposure to cold conditions. In addition, their low growth trajectory may have explained their increased survival on low food in cyclic conditions. Their low growth rate may have made them less likely to starve in the transition to warm conditions. It is surprising that they did not also have higher survival at 25°C under low food. Possibly it was the challenge of the changes from cold to warm conditions that were crucial for their survival advantage.

Increased plasticity of cell area may have allowed the cyclic lines to take advantage of increased nutrient-availability at the lower temperature for growth while maintaining some of their adaptation to the warmer temperature. The similarity in size of the cyclic lines to the 18°C selection lines is a clear indication that their fixed growth responses had become less conservative than those of the 25°C lines. This increased growth rate did not lead to any competitive survival disadvantage at 25°C, and was associated with an advantage over all other lines at 18°C when food levels were low. The plasticity of cell size was much greater in the cyclic lines than in those from other thermal regimes, and this may have allowed them to keep their options open at the lower temperature. From a developmental standpoint, it is known that wing area and cell area are temperature-sensitive from the early first larval instar to the late pupal stage, in what seems to be a purely additive fashion (French et al. 1998). This may indicate available nutrients are cumulated in the larva in accordance with the thermal regime in which growth occurs. The capacity for cell expansion in the cyclic lines was

increased, and this process occurs at the pupal stage. By making the extent of cell expansion depend upon the nutrients available at that time, the cyclic lines may have been able to achieve the large size seen in the 18°C lines, but to avoid committing the future adult cells to division in the larval period. This may have improved their competitive ability at the low temperature. The cyclic lines did had lower larval competitive ability under cyclic thermal conditions compared to the 25°C lines. Their growth advantage was also less apparent under these conditions. These results seem to indicate that selection on the cyclic lines is stronger during the exposure to the lower temperature, giving the two cycling lines a similar competitive ability to the fixed 18°C line when tested under daily cyclic conditions.

In conclusion, our main finding was that cell area, the cellular basis of wing area plasticity in response to temperature, increased in variable thermal environments, Plasticity of wing area itself, however, was unaffected. The increase plasticity of cell area was associated with an evolutionary increase in mean wing area relative to the constant temperature 25°C lines. It was also associated with increased larval competitive ability at low food levels in the cold. Our results suggest that there is stabilising selection on plasticity of wing area itself, because of the need to match growth rate to nutrient availability. Increased plasticity of wing cell area may allow this matching to occur mainly in the pupal period, allowing overall size to increase without a corresponding decrease in larval competitive ability.

7 General Discussion

7.1 Evolution of body size

Intraspecific variability in body size is observed in a plethora of different species, both in nature and under laboratory condition (General Introduction 1.1. and 1.2). Three major considerations emerge from the study of the variation in body size. First, body size differences are, at least in part, due to genetic differences; second, differences in body size are correlated with differences in fitness; third, differences in body size, both in nature and in the laboratory, do not occur at random but exhibit characteristic patterns of variation. These observation have profound implications for our understanding of the evolution of body size, and, more generally, of life-history traits. The use of model organisms in the study of body size variation is a necessary step to circumscribe and outline the questions we need to answer to understand the evolution of body size, and allows to use them as a stepping-stone to create a more general and inclusive theoretical framework. In the present work I used two species of the genus *Drosophila* as model organisms. In the genus *Drosophila* body size is positively correlated with fitness (Robertson 1957; Tantawy and Vethukhin 1960; Partridge and Farquhar 1981; 1983; Lefranc and Bundgaard 2000; Reeve et al. 2000); at the same time, body size is both target of thermal selection and influenced by temperature, with temperature-mediated size plasticity (General Introduction 1.4). In order to make sense of these observations, and to create a theoretical framework able to explain and predict the evolution of body size, it is fundamental to recognize that three different forces determine the evolution of body size: selection, constraints and trade-offs.

7.2 Body size and selection

7.2.1 Evidences of selection along clines.

The effect of selection on body size is clearly evident in the parallel body size clines

produced by different species of *Drosophila* in different continents. While factors such

as genetic drift or founder effect could play a role in the establishment of these clines,

the simple observation that body size clines are regular occurrences, present for more

than one species and in more than one continent (General Introduction 1.2), effectively

rules out the hypothesis that body size clines are not predominantly shaped by natural

selection. The colonisation of South and North America by *D. subobscura* is

exceptional testimony to the effects of selection on body size because it proves that the

effect of selection on the evolution of body size clines is predictable, to the point that

when *D. subobscura* colonized the Americas, the establishment of size clines, with size

positively correlated with latitude, was predicted and punctually verified after a matter

of years after colonisation (Chapter 3). The synchronicity and consistency of this pattern

proves that selection is the driving force behind the establishment of size clines and that

selection is strong enough to give rise to clines in what would have to be considered to

be an "instant" in evolutionary terms.

Selection along body size clines is not simply evident in the similar pattern of

phenotypic differentiation between populations found in different continents; the

genetic architecture underlying such differentiation is also very similar. The comparison

between the results obtained from QTL mapping efforts in the Australian (Gockel et al.

2002) and South American (Calboli et al. in press and Chapter 4) *Drosophila*

melanogaster clines shows that the same genomic regions are involved in the control of

body size in both clines. In particular, in both clines the inversion *In(3R)P* of Payne is

strongly associated with the observed variation in body size (Introduction 1.3.4 and

Chapter 4).The fact that the results of two independent QTL mapping efforts

consistently implicate the same chromosomal regions in two continents, is again a

strong indication that the observed clinal variation in body size is actually caused by

selection and not simply by random factors. At present, the relationship between

cosmopolitan inversions and QTL controlling body size is not fully disentangled, and

we are not able to judge whether this relationship is of causation (the presence of QTL

on the inversions could turn them into single selection units) or simply correlation

(inversion frequency could be selected independently that body size, but the

independent selection forces could give the overall result of similar clinal trends).

Nevertheless, the clinal variation in frequency of cosmopolitan inversions (Knibb 1982)

is, again, a cosmopolitan and well-defined pattern, much more likely to be caused by

selective forces rather than genetic drift or other random effects.

7.2.2. Thermal selection.

The most important selective agent shaping body size variation along clines is

undoubtedly temperature (General Introduction 1.2.4). Temperature varies gradually

with latitude, whereas other possible selective factors do not show such regular pattern

(Zwaan et al. 2000). Laboratory evidence gives a second indication that thermal

selection is the main driving force behind clinal size differentiation. Thermal selection

has been performed in more than one occasion and more than one *Drosophila* species

(Anderson 1973, Cavicchi et al. 1985; Partridge et al. 1994), with surprisingly

consistent results: "cold" selected flies increase in size compared to "warm" selected

flies. The emergence of the same pattern in different laboratories, at different times,

with different base stocks is compelling evidence that thermal selection has a consistent

and predictable effect on size. The results from laboratory selection can be extended to

naturally occurring clines because body size is not the only trait that shows a similar differentiation between natural and laboratory populations. Parallel differences have also been recorded in egg size, developmental time and larval growth efficiency (James and Partridge 1995; Azevedo et al. 1996). Laboratory thermal selection is therefore an acceptable, albeit not perfect, approximation of the effects of selection in the wild.

7.2.3 Targets of thermal selection.

Despite the evidence that thermal selection acts in the establishment of body size clines and size differences in thermal lines, the actual targets of selection are still somewhat elusive. The traits varying along clines or in thermal selection lines show a correlated trend in variation (James and Partridge 1995; Azevedo et al. 1996; van't Land et al 1999; Wolf et al. 2000; Robinson and Partridge 2001; Hallas et al. 2002), making it difficult to identify what traits are directly under selection and what traits are just showing a correlated response due to pleiotropy. Selection could also act at the same time and in one single direction on more than one trait.

The means by which selection acts on body size are also not perfectly clear. On one hand, body size can be direct target of selection, with increased size correlated with increased fitness (Robertson 1957; Tantawy and Vethukhin 1960; Partridge and Farquhar 1981; 1983; Lefranc and Bundgaard 2000; Reeve et al. 2000). On the other hand, body size has been shown to respond to larval crowding, with both short term plastic and long term genetic responses (Sokolowski et al. 1977; Joshi and Mueller 1996; Santos et al. 1997), with a pattern similar to the response to thermal selection (Roper et al. 1996). This observation is of particular interest, as thermal selection lines normally show a lower population size at lower temperatures (Santos and Partridge, unpublished manuscript; Bogdanovits and de Jong 2003); while the issue is not

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conclusively resolved, differences in population size are thought to play a role in the establishment of body size clines (James and Partridge 1998). The synergic effects of temperature and larval crowding are also evident in the larval competition experiments of Chapters 5 and 6, where an interaction between crowding and temperature was clearly evident. Further work should be done to characterise the relationship between larval crowding and temperature, in order to understand how much of the observed variation is due to a direct effect of thermal selection on adult size and how much is mediated through larval crowding effects.

7.2.4 Selection and reaction norm.

While the effects of thermal selection are directional in the establishment of body size clines and size differences between thermal lines, the effects of selection on reaction norms seems to be stabilizing. In the experiment presented in Chapter 5 the reaction norm for body size did not change with time, irrespective of the thermal selection regime. While this pattern could be also caused by lack of genetic variation for plasticity, the change in plasticity of the cellular components of wing size in the cycling lines does not support such a conclusion. The results presented in this thesis support the hypothesis that body size reaction norm is under stabilising selection, a result that is actually coherent with data obtained from natural populations (Karan et al. 1999), where norm of reaction does not change with time. The reason why the norm of reaction is under stabilizing selection is at present not understood.

7.2.5 Size and selection.

Selection, in the present case, thermal selection, is a force necessary to explain the rapid establishment of *D. subobscura* body size clines in North and South America, and the

genetically based size differences in thermal selection lines, leaving random

demographic effects to a minor role. The cause for selection is to be found in

differences in fitness between individuals; the differences observed between populations

along clines or under different thermal selection are therefore adaptive but, as selection

acts on individuals and phenotypes as unities, it is often difficult to pinpoint which

components of the phenotype are under direct selection and which change because of

pleiotropic correlations.

7.3 Body size and constraints

Constraints affecting body size and its evolution are caused by the genetic architecture

of an organism, its developmental program and by biomechanical and biophysical

factors (likely to be especially important in an organism that flies).

Lack of genetic variation for a trait is the first possible limit that could prevent

response to selection (Roff 1992). Such a situation is more likely to be important in a

trait controlled by a single locus (Roff 1986), as the polygenic architecture of body size

and other life history traits should limit the effects of genetic variability constraints

(Musseau and Roff 1987; Roff and Musseau 1987). Nonetheless, population bottlenecks

or founder effects could reduce genetic variability (Chang et al 1994; Reiland et al.

2002) to the point of constraining or limiting the response to selection.

Additionally, developmental constraints could affect the response to selection.

For instance, it is known that growth of the imaginal discs is under strong cell

autonomous regulation (Garcia-Bellido and Mari-Beffa 1992; Garcia-Bellido et al.

1994; Garcia-Bellido and Garcia-Bellido 1998); empirical evidence shows that, when

wing area is directly selected for increase or decrease, an increase in wing area is

underlined by an increase in cell number, while a decrease in wing area was underlined

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by a decrease in cell area (Partridge et al. 1999). This response to selection could be caused by constraints in the regulation of imaginal disc growth.

Constraints could therefore act on the different cellular mechanism underlying the establishment of the *Drosophila subobscura* body size clines in the Americas in different ways. First, differences in genetic variability for cell number increase and cell area increase in the two founding populations could have created a "preferential" response to selection in either cell area or cell number; second, differences in size in the two founding populations could have caused selection to act in opposite direction in the two clines, with a constrained response in cell area and cell number.

Apart from internal constraints, organisms experience constraints caused by the environment they grow and live in. In particular, constraints caused by the presence of other individual of the same species have been recently addressed, and they can play an important role in the evolution of body size (Wolf 2003). Most importantly, constraints due to the interaction with individuals of the same species have been presented in a thorough mathematical formulation, thus allowing precise predictions that can be empirically tested. One of such prediction is that, whenever body size is positively correlated with competitive ability, a constraint arises in the selection for increased body size because the general competitive ability of the population would increase as well, countering the effects of selection and slowing the response to selection. Such constraint would be proportionally stronger with interactions between relatives.

Finally, maintaining flight ability at different temperatures is likely to apply a number of biophysical/biomechanical constraints on body size. It is known, for instance, that the wing/thorax ratio increases monotonically with decreasing temperature (David et al. 1994; Azevedo et al. 1998; Morin et al. 1999; Moreteauet al. 2003), probably due to the necessity to generate more lift as body size increases with

decreasing temperature. Constraints due to the maintenance of flight ability and performance have not been fully explored.

In conclusion, it is clear that constraints could affect the evolution of body size; their effect can be general, when they are developmental or biomechanical, or particular to a given population, in the case of bottlenecks or founding effects. Unequivocal evidence that constraints are in place and affecting the evolution of life-history traits is intrinsically difficult to collect, due to the contingent nature of some constraint and to the difficulty of proving a negative. Nevertheless, a clearer appreciation of the effects of different constraints is paramount for the understanding of the evolution of body size and life history traits.

7.4 Body size and trade-offs

Trade-offs are present when the maximisation of the relative fitness value associated with one trait produces a decrease in the relative fitness value in a second trait (Stearns 1992).

In the first instance, it is important to notice that dipterans have two significantly different bauplane in the larva and in the imago, and the switch from larva to imago does requires a catastrophic metamorphosis, taking place during pupation. The two different bauplane create a particular state of affairs. Each individual has to survive trough the entire larval and pupation periods in order to transform into an adult and have a chance to reproduce; the imago is therefore directly affected by the ontogenetic and selective history of the larva. At the same time, the imago is selected to successfully find food, a mate and a breeding substrate; the larva is therefore affected by the selective forces exerted on the parental imagines.

The consequences of the life-cycle of dipterans for the adult (or larval,

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accordingly) phenotype have wide implications. First, the "overall" performance of each individual throughout its life cycle will be a compromise between the maximization of fitness for each bauplan and the effects that such maximization has on the overall survival and reproductive success, forcing the resolution of a number of trade-offs. Second, the phenotype does not necessarily reflect the effect of selective forces presently acting on it, if selection is stronger under one stage of the life-cycle than the other. Third, the extent of selection at any moment is a function of the environment, which is not automatically the same in space and time. The relative importance of selection at one or the other stage of the life-cycle therefore changes according to the environment, and the same phenotype can be then caused by a set of different selective forces.

How can we understand then the results observed for body size clines and thermal selection lines, keeping in mind possible trade-offs between selection on the larval stage and selection on the adult stage? As previously mentioned, larval crowding can be an important selective force in shaping the evolution of body size. Whether adult body size mainly reflects selective forces on adult size itself, or selective forces affecting the larval stage is a question still unresolved. Population densities differ between "cold" and "warm" selection lines, and empirical observation of population densities in nature is in accord with laboratory observations (see discussion to Chapter 5). Furthermore, genes located on the right arm of the third chromosome, the same area implicated in the control of clinal size variation in Australia and South America, seem to confer greater fitness at high crowding and high temperature (Chapter 5). Trade-offs in the overall control of adult body size are therefore likely to be present and important. Trade-offs could also be important in the cellular basis of the plastic response to temperature observed in the thermal selection lines (Chapter 6).

7.5 The lesson from *Drosophila*

The use of *Drosophila* as a model organism to understand the evolution of body size and life history traits is exemplary of successes and pitfalls of evolutionary biology. On one hand, *Drosophila* allows the collection of an amount of data, both in nature and in the more controlled conditions of laboratory environment. The data collection, started by T. H. Morgan almost one hundred years ago, has been so fruitful that *Drosophila* species, and especially *D. melanogaster*, are some of the best known model organisms employed by biologists worldwide. The similarity in genetic architecture found between eukaryotes allows to use the results found for *Drosophila* as a stepping-stone for work on different organisms, and allows to discern unifying patterns of variation, expression and evolution. On the other hand, the amount of data collected needs to be clearly set into a sound unifying theoretical framework, in order to make sense of what would otherwise be a mass of disjointed observations. The formulation of a comprehensive and coherent theory on the evolution of life-history traits is the single most important challenge in our understanding of the phenotypic variation and the evolution of body size. Such theory must not simply be limited to a cohesive explanation of the observed variation; it should also make accurate and testable predictions that can be used to verify its validity. Different mechanisms, such as selection, constraints and trade-offs, can be cause of the variation found in nature and in the laboratory, and the theory must be able to explain and predict the relative importance of each under different conditions. Data collection is a fundamental step for scientific discovery, but data, no matter how exciting, acquires a meaning only when in the context of a theoretical rationale. The history of quantitative genetics in the twentieth century is a perfect example of the vital and euristic interplay of theory and practice in the advancement of science; evolutionary biology as a whole must be able to do the same.

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