Phenotypic plasticity of phages with diverse genome sizes

by

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Summary

A key factor in studying evolutionary biology is an understanding of the mechanisms organisms utilise in the ongoing process of adaptation. When faced with a heterogeneous and unpredictable environment, we expect organisms to evolve either as specialists or generalists, yet a unifying theory as to which will evolve is still lacking due to conflicting hypotheses based on limited empirical evidence. Phenotypic plasticity allows a single genotype to express different phenotypes, and has been found as an adaptive response to changing environments in all major taxa. With the advent of genomics it has become possible to study the underlying genetics of this phenomenon. It is however becoming clear that there is no single principle governing the plastic response, but rather a complex set of interactions between what appears to be regulatory and structural genes. With empirical data only recently becoming more readily available, the modelling of plastic responses are often still founded on the theoretical predictions and assumptions for which there is little proof. To bridge the gap between theory and nature, the challenge facing scientists today is the construction of experimental systems where theoretical predictions can be scrutinised. Given that phenotypic plasticity is a widespread phenomenon, understanding the magnitude and constraints of this response is an important issue in the study of evolution.

Models have predicted a correlation between genome size and phenotypic plasticity, with increased genome size (complexity) linked to higher levels of phenotypic plasticity. Experimental findings, however, increasingly point to plasticity being governed by complicated sets of interactions between various parts of the genome, the

adaptive landscape, and environmental cues. In the work presented here, a study was designed to test for a correlation between genome size and the level of plasticity by, looking at the fitness response of phages exposed to varying temperature.

Seven phages differing in genome size and genome composition were used. Genome sizes ranged from 5386 bp to 170 000 bp. Taking advantage of the short generation times of phages, fitness could be measured as the growth rate per hour, which was compared among the different phage groups. The growth of large populations within a constant, controlled environment minimized the complications of environmental heterogeneity, and allowed for quantitative measure of the response to different temperatures. This was used to gain insight into how genome size relates to the level of phenotypic plasticity. Limited generation numbers were allowed for, to ensure population growth could be directly related to the plasticity of the genome, since numerous generations would be required for the effects of selection to become apparent. Adsorption rates are influenced by temperature, and were therefore measured to determine if it had a significant effect on the resulting population density. Results showed a marginal interaction between genome size and phenotypic plasticity, with adsorption rate having no significant effect. More experimental work would be required to verify this finding.

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Chapter1

Literature review

Introduction

In 1931 Wright postulated that a population experiencing an unchanging environment, and additive loci, will theoretically move towards the trait value of the highest fitness, with genetic variation becoming non-existent except for mutation events, regardless of stabilising or directional selection. Should the environment be heterogonous over space and time, however, this prediction does not hold (Gavrilets and Scheiner 1993). Environments that are highly variable provide a number of different ecological niches that a population can exploit for survival (Elena and Sanjuan 2003), and since the adaptive landscape reacts dynamically to a changing environment, populations have a range of dimensions available to them on the fitness landscape (Bull *et al*. 2004). When a population faces environmental fluctuations, it could respond with the maintenance of several genotypes, each with a fixed phenotype and maximum fitness for a different environment, or through plasticity, where a single genotype expresses different optimal phenotypes for the different environments (Gavrilets and Scheiner 1993). The response to selection in varying environments therefore depends on the ability of a single genotype to express several different phenotypes. There are many potential responses to changing environments but, as a result of evolutionary constraints, not all these options are open to exploration (Antovics 1976). The potential of organisms to produce a variety of different phenotypes in a range of environments could provide a selective advantage, with such

adaptive phenotypic plasticity providing fitness benefits to the adapting lineages (DeWitt *et al*. 1998).

In recent years the focus has shifted towards understanding adaptation with particular emphasis on the role of phenotypic plasticity (Relyea 2002). An increasing number of studies have aimed to determine constraints on the evolution of plasticity, its inherent costs and the implications for adaptive evolution (Padilla and Adolph 1996; Behera and Nunjundiah 1997; De Witt *et al*. 1998; Bull *et al.* 2000; Relyea 2002; Bull *et al.* 2004). Studying evolution in organisms in their natural habitat is confounded by the presence of trade-offs, environmental gradients, spatial variation in population size, gene flow and competition (Bull *et al*. 2004). A solution to this has been the use of captive populations in the laboratory, specifically microbes, held in a constant, controlled environment, allowing observation of the phenotypic response to selection (Falconer 1989; Bennet *et al.* 1990; Elena *et al*. 1996; Holland 1996; Novella *et al*. 1996; Turner and Chao 1998; Burch and Chao, 1999; Holder and Bull 2001; Rokyta *et al* 2002). In an attempt to better understand the role played by phenotypic plasticity in the initial fitness response to a changing environment, the aim of this study was to generate empirical data to evaluate a possible correlation between genome size and levels of plasticity. With this aim we present data that challenges the preconceived theoretical assumptions that genome size influence phenotypic plasticity in a predictable way. Results indicate a very low level of interaction between genome size and plasticity, with further work required to elucidate this possible relationship.

Phenotypic Plasticity

Phenotypic plasticity has been the subject of much heated debate and many physiological, behavioural and genetic studies, since it was recognised in nature (Via *et al.* 1995). It was originally defined as the variable phenotypic expression of traits in the same genome when exposed to a range of environmental conditions (Gause 1947; Bradshaw 1965), and has since been expanded to include varying phenotypes within the same genotype despite a homologous environment (Behera and Nanjundiah 1995). A reaction norm is defined as the function that quantifies the systematic profile of phenotypes a genotype can produce across a given range of environments (Schmalhauzen 1949) i.e. the pattern of change in phenotypic expression of a genotype across environments (Gavrilets and Scheiner 1993). While phenotypic plasticity is often the term used to describe irreversible variation in traits as a result of a variable environment during development (Piersma and Drent 2003), it could also refer to intra-individual and reversible phenotypic changes (Piersma and Lindström 1997; Starck 1999).

Plasticity deals with a number of processes including reversible changes in physiological processes, changes that are irreversible, and changes which contribute to plasticity as they minimize the effect of the stressor on the internal environment (Via *et al.* 1995). The phenotypic plasticity of a population is an indication of the resistance and tolerance to the stress experienced by the population due to exposure to environmental changes. Plasticity plays an important role in evolution by enlarging the range of selection environments within which a genome can function (Via and Lande 1985; West-Eberhard 1989). The loss of fitness associated with rapidly changing environments could

potentially be offset by increased plasticity of the genome, since this widens the niche breadth of a population (De Witt *et al.* 1998). An increase in genome size and complexity was calculated to lead to a decrease in rate of evolution, a fact that could be combated by increased levels of plasticity (Behera and Nanjundiah 1996). It could therefore be inferred that lager, more complex genomes, would show increased levels in phenotypic plasticity (Behera and Nunjundiah, 1997).

Numerous theoretical models have been published that predict the conditions under which adaptive plasticity should evolve. Prerequisites include populations having to experience heterogeneous environments that favour alternative phenotypes, the presence of reliable environmental cues, low cost of complexity and a single phenotype not having superior fitness in all environments (Via and Lande 1985; Via 1987; Van Tienderin 1991; Gomulkiewicz and Kirkpatrick 1992; Moran 1992; Padilla and Adolph 1996). Since there are many possible ways of attaining plasticity, no one set of underlying rules have proven sufficient in predicting the outcome of such a dynamic set of interactions (Springman *et al*. 2005).

The fitness implications associated with possessing alternative phenotypes in different environments are frequently studied (Moran 1992, Relyea 2002, Scheiner and Lyman 1991). In microbial systems, for instance, it has been experimentally determined that generalists can evolve without having to combat a significant fitness cost compared to specialists (Novella *et al*. 1999; Turner and Elena 2000).There can however also be a fitness cost and limit to being phenotypically plastic, apart from the cost of producing a particular phenotype (DeWitt *et al*. 1998). Such potential plasticity costs include maintenance costs; genetic costs of pleiotropy and epistasis, developmental instability

and information costs of obtaining environmental cues (Charnov and Bull 1977; Getty 1996).Costs of plasticity make adaptation to multiple environments a compromise with selection toward fitness optima in each environment opposed by potential costs of plasticity (Via and Lande 1985).

Few models have focussed on the effect of genetic structure of the genome on the outcome of adaptation through phenotypic plasticity (Scheiner and Lyman 1989). In order to do this, information would be required regarding the existence of loci which differ qualitatively in their phenotypic expression in response to the environment (Scheiner 1998). It has been hypothesised that the observed phenotype is determined by at least two types of loci; plastic loci that are sensitive to the environment, and mean loci that are insensitive to environmental changes (Bradshaw 1965; Gavrilets 1986; Scheiner and Lyman 1989; de Jong 1990; Via *et al*. 1995). The existence of both of these loci has been shown in experiments with *Drosophila melanogaster* (Scheiner and Berrigan 1998), indicating that underlying genetic architecture influences the outcome of selection (Scheiner 1998). This idea was expanded by Behera and Nunjundiah (1997) with the incorporation of gene control in their model of the evolution of phenotypic plasticity. They calculate that regulatory genes at plastic loci, that are sensitive to the environment, regulate the activity of mean loci that are insensitive. Structural genes at the mean loci determine the phenotype being expressed, but the process is controlled by the regulatory genes that react according to environmental input (Bradshaw 1965; Scheiner and Lyman 1991). Selection will act directly on the structural genes and indirectly on the regulatory genes through the interactions between plastic and mean loci (Behera and Nunjundiah 1997) An interaction between such regulatory and structural loci has been described in all

5 major kingdoms (Wu 1998). The plastic response will therefore be reliant on the presence and ratio of plastic- and mean loci respectively as well as the level of complexity of the interactions between them.

The Adaptive landscapes and the cost of complexity

A theoretical explanation for adaptive evolution through mutations of small effect was proposed by Fisher (1930), who in his 1930 work *The Genetical Theory of Natural Selection* used a geometric model as theoretical explanation for the probability that random mutations of a specific phenotypic size would be advantageous. A population starts its adaptive walk a distance from an adaptive optimum, and then travels toward the fitness peak through natural selection. Fisher argued this to be the result of small advantageous mutations, since large mutations are not only less abundant, but also have a small chance of being advantageous and an increased risk of carrying deleterious pleiotropic effects (Burch and Chao 1999). As the number of phenotypic dimensions increase, which is related to the complexity of the organism, mutations of large effect are more likely to have deleterious effects (Burch and Chao 1999). It was therefore suggested that organisms pay a price for complexity; since mutations are less likely to be advantageous in complex organisms. The rate of substitutions of favourable mutations should, all else being equal, be slower in more complex organisms (Orr 2000). Recently, however, this idea of evolution mainly through small mutational steps has been challenged since it has been shown that some adaptations clearly result from a few mutations of large effect as opposed to several mutations of small fitness effect (Bull *et*

al. 2000). It has become clear that Fisher's theory was incomplete; with Kimura (1970) pointing out that Fisher overlooked the fact that slightly favourable mutations must escape stochastic loss when rare. Despite mutations of small effect having greater probabilities of being favourable, they suffer a smaller chance of fixation when only slightly favourable. Therefore, in a population that is large enough to allow advantageous mutations of both large and small fitness effect, evolution could proceed by large steps due to the higher probability of fixation of large mutations (Burch and Chao 1999). In a small population, small mutations sweep to fixation, since they are the only mutation events occurring sufficiently frequent. When corrected for the chance of fixation for each mutation, Fisher's model predicts that step size along the adaptive pathway should be smaller as the size of the population under selection decreases (Burch and Chao 1999). Fisher's conclusions about the relationship between organismal complexity and adaptation rate remains tenuous, however, and the question arises: how much slower do complex organisms adapt when taking into account that adaptation requires not only that the mutation be favourable, but also that it becomes fixed (Orr 2000).

In view of Fisher's geometric model of adaptation Orr (2000) derived a model to calculate the total cost of complexity during adaptation. Particularly, the rate of increase in fitness characterizing adaptation in complex versus simple organisms was modelled based on the assumption that all organisms adapt via mutations of the same size. If fitness is determined by n independent characters, it occurs in n-dimensional space. When the organisms adapt through mutation, a mutation's phenotypic effect is represented by a vector with direction in phenotypic space. Fisher's model allows for a kind of pleitropy where any mutation potentially affects any character, though some characters will be

affected more profoundly (Orr 2000). Orr calculated that the cost of complexity is brought about by a reduction in the rate of adaptation, since most mutations are deleterious. Behera and Nanjundiah's model (1997) provide a possible solution to this cost by calculating increased level of plasticity in larger genomes. For the purpose of this study we measured population fitness as a surrogate to indicate levels of phenotypic plasticity in seven phages, to determine if indeed such a linear correlation exists.

Experimental evolution and modelling

In artificial selection experiments, the aim is to create an environment where the measured fitness is a function of a single trait, and this is achieved through manipulation of the selective pressures applied. Such an experiment which minimizes correlated effects is hard to achieve, but provides valuable tests for the assumptions of modelling theories. These kinds of experiments are fraught with statistical and analytical difficulties for measuring fitness functions, even in single environments (Mitchel-Olds and Shaw 1987; Scheiner 1989). Studying plasticity as a trait is complicated by the fact that plasticity is often not quantifiable on a single individual. It is most often measured across a group of related individuals in varying environments (Scheiner 2002). In quasi-natural protocols, the key element is the predictability of the response. The occurrence of plasticity is predicted to be dependant on the rate at which the environment changes. If the response is immediate in the face of environmental heterogeneity, plasticity will be favoured. In instances where the environmental change is slower than the generation time, specialisation is favoured over plasticity (Orzach 1985).

Construction of an evolutionary model relies heavily on a large body of assumptions, many of which deal with the genetic basis of a trait (Scheiner 2002). Experimental evolution studies have indicated plasticity to be genetically controlled (Schlichting and Pigliucci 1993) with additive genetic variation existing for phenotypic plasticity (Scheiner 1993). In order to simplify an experiment or model, environments are regarded as either fixed or varying cyclically between two extremes (Behera and Nunjundiah 1996), which is rarely the case in nature. Travels along the mutational landscape have been modelled based on the assumptions that organisms of varying complexities undergo mutations with similar fitness effects, as well as experiencing similar fitness decrements when faced with the same environmental disturbance (Orr 2003). This has lead to an increasing number of contradicting models, with little or no empirical evidence used in the formulation of the fundamentals (Scheiner 2002).

Selection experiments allow the testing of such models by quantifying variables and scrutinizing the predictive power of a model as well as the validity of the assumptions. Models that investigate the evolution of phenotypic plasticity can be constructed with the presence of only plastic loci or a combination of plastic and mean loci (Scheiner 1993). As with the plasticity of a given trait, the plasticity of a genome taken as a whole will be determined by the presence and relative abundance of plastic and mean loci. The different types of loci present have been shown to have a profound effect on the resulting level of phenotypic plasticity, with loci influencing not only the slope of the reaction norm, but also the curvature (Scheiner 1998).

To directly test the theoretical predictions of phenotypic plasticity using an integrated quasi-natural selection experiment, information is required about the shape of

the fitness function over a range of environments (Gavrilets and Scheiner 1993b). Ways to generate this kind of data include measuring the fitness in multiple environments.

The advantages of using phages as a study system

Due to the fast generation times, and the relatively simple interactions between phage and host, fairly complex evolutionary parameters have been demonstrated using this system. A system consisting of a single host type and phage strain can evolve a high degree of complexity, with mutations and selection leading to the presence of a number of adaptive variants of both host and phage within a single population (Chao *et al*. 1977). With each variant lineage increasing exponentially, and different lineages increasing at different rates, this leads to the most fit phenotype becoming increasingly frequent. Selection will therefore be for lineages that emerge and become established with greater rates of replication than the ancestors, thereby changing the population permanently (Hoffman and Parsons 1991). A population can be assumed to contain a diversity of sequences, resulting from inaccuracies during self-replication, as well as a diversity of lineages, each of which differs to a greater or lesser extent from the founding individuals (Chao *et al.* 1977).

The interaction between phage and bacterium starts with the random collision of phage with host, which is followed by adsorption of the phage to very specific receptors sites on the host cell, should they be present. The phage genome enters the host cell and after a latent period during which the phage multiplies, the host cell lyses and infective phage particles are released (Chao *et al.* 1977; Lindberg 1973). While phages do encode a

number of proteins, the host cell provides the supply of material required for the phages to replicate their genomes, as well as the apparatus for constructing proteins (Bell 1997).

Despite being organisms containing minimal genomes, it has been shown that the systems are sufficiently complex to generate effects predicted by theories of adaptation (Burch and Chao 1999; Bull *et al*. 2000; Bull *et al*. 2004). Comparisons between species have brought forward evidence that major differences can be the result of few amino acid changes (Dean and Golding 1997; Bradshaw *et al*. 1998; Wu *et al*. 1999). Phage genomes are able to adapt not only through the accumulation of point mutations, but also as a result of exchange between the gene modules that are present in the genomes of different phages. In experiments with viruses, fitness losses were overcome in a variety of ways and through several pathways; the most predominant being recombination between viruses, which has been shown to be a common event, and highly efficient despite the apparent randomness of exchange events (Felsenstein 1974; Haigh 1978; Chao 1990). Through the modular structure of the genome, the level of diversity can increase due to random exchange of modules to facilitate adaptation (Monod *et al*. 1997; Hendrix and Duda 1992).

Although phage evolution is proving to be relatively complex, it is still an ideal system for studying real-time evolution in a controlled environment due to their large population sizes and short generation times. Fitness can easily be determined by measuring the increase/decrease in the reproductive capacity of the population.

Aim

The aim of the study was to use a comparative approach to determine if there is a positive correlation between genome size and the level of phenotypic plasticity found in seven phages.

Strategy

Adaptation of viruses and bacteria through artificial selection has commonly been used in studies of experimental evolution (Elena *et al.* 1996; Holland 1996; Novella *et al.* 1996; Turner and Chao 1998; Holder and Bull 2001; Rokyta *et al*. 2002). The phages chosen represent a variety of genome characteristics, including a small single stranded DNA genome, and six phages with larger double stranded DNA genomes and temperature was chosen as a stressor since previous work has shown phages capable of adapting to higher than optimum growth temperatures, thereby indicating an innate ability to adapt to alternative temperatures (Dowell 1980). Table 1 summarises the physiological and genome characteristics of the seven different phages chosen for the experiments described in the chapters to follow. The two T even phages are closely related with 10-20% sequence divergence between them, and gene numbers in excess of 300. T3 and T7 are related, with similar overall genome composition, but T3 containing 51 genes, whereas T7 contains 56 genes. T1 is not related to any of the other T-phages and it remains the least studied of that group (Roberts *et al.* 2004). ΦX174, comprising 11 genes, uses host DNA polymerases for genome replication and host RNA polymerases

for transcription, with lysis timing dependant on host cell division. The T-phages use phage-encoded polymerases for genome replication, with T3 and T7 utilizing phage encoded RNA polymerases for transcription, and the T-even phages having to rely on host RNA polymerases for transcription. The T phages mentioned below degrade the host genome to enhance the nucleotide pool, and suppress host RNA synthesis, lowering competition for ribosomes (Bull *et al*. 2004).

Changes in the physical conditions were the defining feature of the environments created during experimentation to stress the organism. Temperatures both higher and lower than the optimum were used, with the effect of competition for host being minimized by the host population outnumbering the phage population at all times. With the short exposure of phages to the different environments, changes in fitness trends should not be due to underlying genetic changes. The starting populations were as close to homogeneity as possible.

Using all the phages during each experiment was a necessary step to control for differences between repeats. Replicate lines were genetically identical at the beginning of each experiment, removing a source of experimental variation.

Incubation temperature has been shown to have an effect on the adsorption rate of bacteriophages of *Streptococcus thermophilus* (Binneti *et al.* 2002). Low temperatures were shown to lead to slower adsorption rate, with the maximal rate achieved near the optimum growth temperature. To quantify the contribution of adsorption rates to fitness, adsorption rates of the phages were measured at the optimum temperature as well as the most extreme temperatures used in the plasticity experiments.

TABLE 1.1: Genome properties of the seven phages used in the experiments (Roberts et al. 2004, Desplats and Krisch 2003, Wang *et al*. 2005, Pajunen *et al*. 2002, Springman *et al.* 2005, Kim and Davidson 1974, Bull *et al*. 2004)

Phage class	Name	Genome	Genome size (kb)	Latent period
Isometric	ΦX174	ssDNA	5.4	23 minutes
T- odd	T1	dsDNA	46	13 minutes
T- odd	T3	dsDNA	38.2	13 minutes
T- even	T4	dsDNA	168.895	23. minutes
T- odd	T5	dsDNA	121.3	40 minutes
T- even	T6	dsDNA	170	25.5 minutes
T- odd	T7	dsDNA	39.9	13 minutes

Chapter 2

Materials and methods

Phages, Bacteria and Media

All phages and host bacteria were obtained from the German National Resource Centre for Biological Material (Deutsche Sammlung Von Mikroorganism- DSMZ).

Media

Liquid NZCYM consisted of 10g Casein hydrolysate, 5g NaCl, 1g Casamino acids, 5g yeast extract, 2g $MgSO_4$ x 7 H_2O, 2g Maltose, per litre of water. pH was adjusted to 7.5 before autoclaving, and agar was added at 15g/l for plates and 7g/l for soft agar overlay.

General Growth Media (GGM) was made up using 10 g Tryptone, 5g Yeast extract, and10g NaCl per litre of water, with the final pH adjusted to7.0. Plates were made by adding 15 g/l of agar to the liquid culture prior to autoclaving.

Bacterial Stocks

The chosen phages required two different hosts, with the T phages growing in *E. coli* 613 (DSM), and ΦX174 active only in *E. coli* 13127 (DSM). It was beyond the scope of this study to attempt growing the phages in a single host, and provided the opportunity to

have the phages grow in their natural hosts rather than introducing a possible added stress in the form of a new host. To ensure the integrity of the bacterial stock solutions, separate laminar flow cabinets and equipment were used when making freezer stocks. Host cells were purchased as desiccated cultures, and following re-suspension in GGM, live colonies were obtained by streaking out of the suspension on GGM agar plates and overnight incubation at 37ºC. The remainder of the original suspensions were stored in 15% glycerol at -70ºC. Since phages were required to be grown in NZCYM, it would be crucial that downstream experiments used NZCYM only. Single colonies were picked from the agar plate using a sterile pipette tip, and 5 ml of GGM and NZCYM were inoculated respectively and subsequently incubated at 37ºC for 12 hours. From these overnight cultures, 3ml were used to infect 100ml of GGM and NZCYM followed by incubation at 37ºC to obtain a host culture with cell density of 0.6 at OD_{600}, equivalent to a concentration of approximately 1x 10^{8}cfu/ml. Bacterial cells showed similar growth profiles in both media with similar cell concentrations reached in the same period of time. All further experiments were conducted using host cells propagated in NZCYM. The cells were concentrated 100-fold in NZCYM, and frozen in 15% glycerol at -70 ºC for use in downstream experiments. During experiments, frozen aliquots were thawed and added to 10ml NZCYM, followed by growth at 37 ºC with aeration for approximately 1 hour or till the density reached a density of 0.6 at OD_{600}.

Phage Propagation

Phages were acquired as cell lysates, containing numbers of phage in excess of 1 x 10^{10} pfu/ml, and free from bacterial cells. To obtain live phage cultures, concentrated host cell cultures from the freezer stock were grown to a density of approximately 1 x 10^{8}, 1-2 hours prior to inoculation with10 µl (10^{8} pfu) of phage lysate. The suspension was incubated for 5-6 hour at 37°C with shaking. A clear lysed culture began to form from the top of the solution, signifying phages multiplying and lysing the host cells. Phages were isolated through the addition of chloroform which killed host bacteria, and released the phages. Centrifugation at 4000rpm for 8 minutes in a bench-top centrifuge precipitated the bacterial debris, with the supernatant containing the phages. The phage titre was determined through serial dilution of the supernatant, and plating out the phages using the double agar layer plaque assay (Adams 1959 as referenced in Svenson & Christianson 1991). Dilutions were mixed with soft agar containing a high concentration of susceptible host and then plated on agar plates. Soft agar was kept at approximately 40°C, and prior to plating out on agar plates, the phage suspensions and host cells were added ensuring a multiplicity of infection of at least 0.1. Plates were incubated overnight, and the number of phages per ml of suspension could be determined by counting the clear plaques that formed on the plates against the background of live host cells. The product of the number of visible plaques and the dilution factor was used to calculate the phage titre. For each experiment measuring fitness, serial dilution of the isolated phage suspension was performed in duplicate to determine the average initial and final phage concentration.

To ensure the integrity of the phage lineages, methods were put in place to screen for contamination events. The different phages were characterised by host specificity, plaque morphology, genome size, and restriction enzyme digestions. The host specificity

of ΦX174 did not allow contamination with the T phages, since it could only be propagated in the specified host strain, and experiments proved the T phages incapable of growing on *E. coli* 13127. The size, shape, and clarity or turbidity of plaques has historically been used to characterize different virus strains. T1 formed a large plaque with a small turbid halo surrounding the large glassy centre of the plaque (figure2.1c). T3 and T7 formed large plaques with a broad turbid halo surrounding the glassy centre and making up more than half of the entire plaque (figure 2.1b). T4, T6 (figure 1a) and T5 all shared common plaque morphology of small round glassy plaques forming without any surrounding turbidity. To distinguish between the different phages sharing plaque morphologies, genome size and restriction enzyme digestion patterns were used.

Figure 2.1a

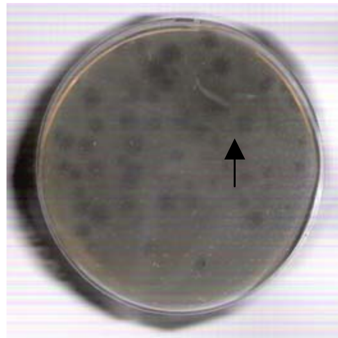
Figure 2.1b

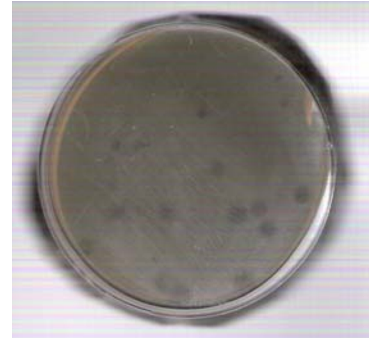
Figure 2.1c

Figure 2.1: Plaque morphologies used to distinguish between phages. Figure (a) shows the small plaques of T6 (similar to T4 and T5). Figure (b) depicts the larger plaques of T3 with a broad opaque "halo" around the clear centre. Figure (c) shows the plaque morphology of T1 with a large clear plaque and very little surrounding turbidity.

DNA extraction and restriction enzyme digestion

A standard phenol-chloroform DNA extraction was done for all phages to extract DNA (Sambrook *et al.* 1989), and a 1.5% agarose gel, stained with ethidium bromide used to visualise the relation between the various genome sizes. Samples were loaded on the gel

and exposed to a 75V current for 1 hour (Sambrook *et al*. 1989). Phage T5 had similar plaque morphology to T4 and T6, but could be distinguished through gel electrophoresis as having a smaller genome size relative to T4 and T6. To distinguish between T4 and T6, restriction enzyme digestions were performed as described in Monod *et al*. (1997). No restriction enzyme maps were available for T4 or T6 and a range of restriction enzymes were used to screen for a distinguishing recognition site. Phage T4 showed no recognition sites for the enzyme *Ava* I, whereas, T6 showed one site. Phage DNA was digested according to New England Biolabs, and DNA fragments were resolved on 1.5% agarose gels containing ethidium bromide. A similar protocol was followed for T3 and T7, with the use of *Xba* I (New England Biolabs). A restriction map for T7 (New England Biolabs) shows three recognition sites for *Xba* I however, only two bands were visible on the agarose gels. T3 had no recognition sites for *Xba* I. In all cases, restriction digestions were performed in water baths at the optimum temperature of 37°C for 1 hour, and exposure to 64°C for 20 minutes stopped the reaction, as per supplier's recommendations. T1 had clearly distinctive plaque morphology which could be used for identification, as it has a similar genome size to T5. Since ΦX174 has a different bacterial host, no cross-contamination was possible and host specificity ensured pure stocks.

Fitness Experiments

Plasticity experiments

Phage stocks were prepared within two days of their use in experiments as described in previous paragraphs. For each experimental repeat all 7 phage were exposed to a range of temperatures simultaneously. A gradient PCR thermocycler, programmed to produce a gradient of temperatures across the heating block, produced a controlled environment allowing consistency between repeats. The extent of the temperature range was determined by the capability of the PCR machine in use. Temperatures centred around 36.2°C, which is as close to the physiological optimum of 37°C as was possible when selecting for a gradient on the heating block. Four other temperatures were selected from the available range, and were chosen to have similar intervals between subsequent temperature classes. The lowest temperature was 27°C, followed by 31.1°C, 36.2°C, 41.7°C and a maximum temperature of 46°C. In a separate experiment the two bacterial host strains were exposed to the range of temperatures for one hour to determine viability. In both cases temperature had a negligible effect on the final number of bacteria, with differences being less than an order of magnitude between populations tested at the various temperatures. For the fitness experiments, bacterial cells were taken from the frozen stock and grown for at least 1 hour till a density of approximately 1 x 10^8 cells/ml was reached. In 0.5ml disposable tubes, 200µl bacterial stock was inoculated with 10^4-10^6 phages for a multiplicity of infection of at least 0.1, and immediately placed on ice to minimize adsorption. For each phage, the fitness would be measured at each of the temperatures during a single reaction and compared to the initial fitness. The phage titre was determined at the start of each experiment by setting up the reaction as mentioned above, followed chloroform extraction and immediate serial dilution and plating of the solution using soft agar overlays. To test the fitness response, the tubes

were placed in the heating block, such that each phage was represented at each temperature, but spaced randomly within the temperature zones. The reaction was allowed to continue for 1 hour, and fitness measured as the rate of change in phage numbers in a liquid culture. Growth was stopped by placing the solutions on ice, followed by the addition of chloroform and subsequent centrifugation to extract the total number of viable phages present after 1 hour. Serial dilutions of the phages were plated out in soft agar with host cells at a multiplicity of infection of at least 0.1. Plates were incubated for 5-6 hours, and the plaques counted to determine the titre at the end of the experiment.

Fitness calculation

Generation times differ between the various phages, and are affected not only by phage metabolism but also by adsorption rate (Bull *et al*. 2004). The comparison of fitness between the phages was standardized using

$$\log 2\ [N(t)/N(0)]/Y$$

where N(0) represent the initial phage number, N(t) the phage present after 1 hour, and Y the number of generations per hour for each phage (Bull *et al.* 2000, and Rokyta *et al*. 2002). This presented fitness on a log scale which was a time independent measure allowing comparisons between phages with different generation times. Each of these experiments was repeated 5 times.

Adsorption Experiments

Since adsorption rate influences generation time and could be affected by temperature, the rate was determined for each phage at the optimum temperature of 36.2°C, and the two extreme temperatures of 27°C and 46°C. Phage and bacterial stocks were prepared as described for the fitness experiments. In a 2ml reaction volume, approximately 10^4 – 10^5 phages were added to bacterial stocks, ensuring that the multiplicity of infection was at least 0.1. After the addition of the phage, a 1 ml aliquot of each culture was serially diluted and plated out immediately in soft agar overlays to determine the concentration of the total phage (N_0). The remaining samples were incubated for 5 minutes at the chosen temperatures, using electronic heating blocks, to ensure stringent control of the temperature. After 5 minutes, the 1ml samples were centrifuged to pellet the bacteria and adsorbed phage. The supernatant was then serially diluted and plated out using soft agar overlays to determine the amount of free phage remaining in culture (N_u). Adsorption rate was determined as described in Bull *et al*. (2004) by solving for k in

$$N_u = N_0 e^{-kCt}$$

$$\text{giving} \quad k = -\ln(N_u/N_0) / Ct$$

where k was the adsorption rate, t the time measured in minutes, and C was cell density. The use of frozen bacterial stocks ensured standardisation of bacterial concentration between repeats. A 5 minute interval was proven to be enough time for a drop of 1 order

of magnitude in free phage for most phages, and corresponds to the time and results of Bull *et a*l. (2004), who determined adsorption rates for a similar group of phages. During the adsorption experiments, assays used a lower density of phage to facilitate titre determination with minimal dilutions. Three separate assays of adsorption rate were performed for all of the phages, during which time all 7 phages were exposed to the temperatures simultaneously.

Statistical analysis

To make fitness values of different phages comparable, all fitness values were divided by the specific phage's average fitness at 36,2 ºC, thereby scaling the fitness of all species to a maximum of 1. An interaction between size and temperature would be expected if plasticity depends on size. To test for this, the following linear model was fitted:

$$\text{fitness} = \text{run} + \text{size} + \text{temperature} + \text{temperature}^2 + \text{size:temperature} + \text{size:temperature}^2$$

Significance of the last two terms would indicate that genome size affected plasticity. However, this required that many parameters be estimated and for a modest data set it is very costly in terms of degrees of freedom. The data around the optimum temperature was only slightly curved which suggested that a straight line might fit the data better. Therefore both a straight line of fitness on difference in temperature from optimum and a curved line were fitted. To reduce the number of parameters that needed to be estimated, the following transformations were done: a) 36.2 was subtracted from the

temperature so that the maximum would be at zero and only temperature^2 on its own would give a curved relationship. b) One was subtracted from fitness so that no intercept needs to be estimated and size could be left out of the model.

For the curved model we fitted the following model to the transformed data:

fitness = run + temperature^2 + size:temperature^2, with no intercept

and for the straight line:

fitness = run + |temperature| + size:|temperature|, with no intercept

To establish if the observed marginal significance was simply the result of small sample size, we did a post hoc power test to detect what size of interaction we would have been able to detect.

To establish if adsorption rate affected the interaction, adsorption was quantified at three temperatures. The adsorption data was not symmetrical around 36 degrees but tended to be higher for deviations to the left than deviations to the right, with the variance increasing as the temperature increased. The transformations used for the fitness data could therefore not be applied to this data set. Equality of variance was achieved through raising Adsorption x 10 to the power 0.125. We started with the model:

$$(\text{Adsorption} \times 10)^{0.125} \sim \text{Size} + \text{Temp} + \text{Temp}^2 + \text{Temp:Size} + \text{Temp}^2\text{:Size}$$

with *Temp* representing temperature. Non-significant terms were dropped to find the minimal adequate model.

Statistics were done in R (R Development Core Team, 2005) and SPSS.

CHAPTER 3:

Does Phenotypic Plasticity of Phages correlate with Genome Size

Abstract

Many traits and mutants are only associated with change under stressful conditions. Plasticity enlarges the range of environments within which a genotype can function effectively without genetic mutations, and increases adaptability in rapidly changing environments. Increased complexity has been shown to decrease the rate of adaptation; a situation which can theoretically be offset by increased levels of plasticity. Phages are an ideal system for studying evolution as they have short generation times. We correlated the degree of fitness drop to genome size to test if genome size affects the level of plasticity. Adsorption rates were measured to see if it affected the measured fitness values, but no significant interaction was recorded. We found only a marginal significant correlation between genome size and the level of plasticity of the genome, indicating that further experimental work is required to test the robustness of this correlation.

Introduction

A population experiencing heterogeneous environments, each with a specific optimal phenotype, could theoretically respond in three different ways in the absence of any constraints: (1) specialists could evolve for the different environments, (2) plastic phenotypes could evolve to match or try to match the optimum in each environment, and (3) a single, fixed phenotype could emerge that has a high fitness when averaged across

all environments (Scheiner 1998). A plastic response would be defined as the ability of the genotype to express alternative phenotypes in response to the environment that it experiences (Ernande & Dieckman 2004), with the range of expressed phenotypes being the reaction norm (Schmalhausen 1949). In environments that change rapidly and are unpredictable, individuals that can express phenotypic plasticity are expected to have a definite fitness advantage, since plasticity enlarges the range of selective environments within which a genotype can function effectively without genetic mutations (Hinton and Nowlan 1987; Padilla and Adolph 1996; Kingslover and Huey 1998). Plasticity provides an immediate solution to the problem posed by changing environments, while selection requires numerous generations for advantageous mutations to become fixed and is vulnerable to high gene flow between different environments.

Studies have found plasticity as an adaptive response in several taxa, and also revealed phenotypically plastic individuals to have a fitness advantage relative to non-plastic individuals in defined environments (Travis1994). On the adaptive landscape of the phenotype, any point will represent one genotype, with plasticity allowing for a single genotype to have the same fitness in varying environments, causing a high plateau on the fitness landscape where a sharp peak used to be. It is generally accepted that possessing various phenotypes, however, can negatively affect fitness; a cost which could limit the evolution of plasticity and lower the overall fitness of plastic individuals (Relyea 2002).

The phenotype of plastic traits can be influenced by at least two types of loci (Behera and Nanjundiah 1997); plastic loci that are sensitive to the environment and code for regulatory genes, and mean loci that code for structural genes and respond to regulatory signals (Bradshaw 1965; Gavrilets 1986; Via *et al.* 1995). The effect of a mean

allele at one locus is therefore modifiable by plastic alleles at other loci (Behera and Nanjundiah 1995). The importance of such genetic architecture on the adaptive process is yet to be fully understood, but it is agreed upon that it would play a critical role in the evolution of plasticity (Scheiner 1998).

In Fisher's geometrical analogy of adaptation (1930), individuals were characterised by the number of phenotypic characters under selection, and a mathematical explanation was given in support of adaptation through many small steps on the adaptive landscape. Further expansions on this model by Orr (2003), predicted a negative correlation between the number of phenotypic characters under selection, and the rate of adaptation, known as the cost of complexity; complexity being the number of characteristics under selection or the number of traits affected by a locus. This prediction that larger genomes generally adapt slower and reach lower overall fitness due to the cost of complexity has been refuted in experiments where fitness recovery after temperature shock was measured for phages representing different genome sizes (Bull *et al*. 2004). Genome size was found to be an unreliable indicator of the maximum fitness that could be reached and the rate of adaptation following environmental shock.

Mathematical models have calculated that the cost of complexity can be offset by the presence of phenotypic plasticity, which leads to larger, more complex genomes having higher levels of plasticity (Behera and Nunjundiah 1996). In the present study we designed experiments to test for the presence and extent of this correlation between genome size and the level of plasticity. Population fitness was used as a proxy for phenotypic plasticity in bacteriophages and was measured over a range of temperatures.

Designing experiments to test experimental evolution is challenging as it requires a system that can be manipulated and monitored over evolutionary time scales (Burch and Chao 1999). Taking advantage of the high genomic mutation rate and short generation time of bacteriophages, many groups have been successful in looking at various aspects that characterize the adaptive landscape during evolution of laboratory populations (Burch and Chao 1999; Bull *et al.* 2000; Wichman *et al*. 2000). Stressful conditions can be simulated using factors such as extreme temperatures, chemical influence of salts, and the presence of unusual organic compounds (Scheiner 2002).

Materials and Methods

We developed a method for measuring fitness in numerous phage strains simultaneously, by exposing the phages to a range of temperatures. Phage density was prevented from exceeding cell density to avoid co-infection and the resulting competition between phages in a cell. NZCYM medium was used for propagation of all phages and cells. NZCYM medium was mixed as 10g Casein hydrolysate, 5g NaCl, 1g Casamino acids, 5g yeast extract, 2g $MgSO_4$ x 7 H_2O, 2g Maltose, per litre of water. pH was adjusted to 7.5 before autoclaving, and agar was added at 15g/l for plates and 7g/l for soft agar overlay. Cells used for the propagation of the phage were *Escherichia coli* 613 and 13127 for T-phages and ΦX174 respectively and were obtained form the German National Resource Centre for Biological Material (DSMZ).

A gradient PCR thermocycler was utilized in creating a standardised temperature-controlled environment. As a measure of initial population density, phages

were added to bacterial hosts for each of the lineages, diluted, and plated out immediately for growth at 37°C. Phage fitness response was then assayed at 27°C, 31.1°C, 36.2°C, 41.7°C and a maximum temperature of 46°C, for 60minutes. The choice of temperatures was governed by the heating capability of the PCR machine used. Following exposure to the various temperatures, phages were extracted using chloroform and serially diluted before being plated out in soft agar overlays. Multiplicity of infection was such that phages were at a low density relative to the host cells. Fitness was calculated as the number of doublings of phage per generation time of the phage as in Bull *et al.* (2000) which is a measure independent of phage generation time:

$$\text{Log}_2 \text{ (titer at the end of the assay/titer at outset)/ (number of generations per hour)}$$

Phages were plated out immediately following the plasticity experiments, so as to avoid a reduction in estimated fitness due to storage. A fitness value of zero indicated that the population size remained constant, whereas a positive value indicated increased titre and a negative value was the result of a decrease in titre. Serial dilutions and plating out was done in duplicate to reduce noise in the fitness values for each replicate. The average values were used.

Adsorption rates were determined for each phage at temperatures of 27°C, 36.2°C and 46°C, using electronic heating blocks due to the requirement for larger reaction volumes. Phages and bacterial stocks were prepared as described for the fitness experiments. Using a multiplicity of infection of at least 0.1, approximately 10^4 – 10^5 phages were added to bacterial stocks to a final volume of 2 ml. Following inoculation,

phages were immediately extracted from a 1ml aliquot of each sample, using chloroform, to determine the total initial phage concentration (N_0). Serial dilutions were used with soft agar overlays to visualise and quantify phage plaques. The remaining sample volumes were incubated for 5 minutes at the specified temperatures, followed by centrifugation of the samples at 4000rpm's for 5 minutes to separate the bacteria and adsorbed phage from unadsorbed particles (N_u). Serial dilutions were plated out with soft agar overlays. Adsorption rates (k) were determined as described in Bull *et al*. (2004) by solving for k in

$$N_u = N_0 e^{-kCt}$$

where k was the adsorption rate, t the time measured in minutes, and C represented cell density. The use of frozen bacterial stocks ensured standardisation of bacterial concentration between repeats. Three separate assays of adsorption rates were performed for all of the phages, during which time all 7 phages were exposed to the temperatures simultaneously. To ensure contamination had not occurred between the various phage, a combination of host specificity, genome size, restriction enzyme digestions and plaque morphology was used.

If plasticity depends on size then there should be a significant size by temperature interaction in the model:

$$\text{fitness} = \text{run} + \text{size} + \text{temp} + \text{temp}^2 + \text{size:temp} + \text{size:temperature}^2$$

where *temp* is temperature. This required the estimation of seven parameters and for a small data set this drastically reduced the number of degrees of freedom. The interaction terms were not significant. To reduce the parameters that needed to be estimated we followed two additional approaches. Both a straight line of fitness on difference in temperature from optimum and a curved line were fitted to the data. The following

transformations were done to reduce the number of parameters to be estimated to three: a) for each phage the fitness values were divided by that phage's average fitness at 36.2°C. b) 36.2 was subtracted from the temperature so that the temperature^2 alone would give a curved relationship. c) 1 was subtracted from all fitness values so that no intercept needed to be estimated and size could be omitted.

For the curved model we fitted the following model to the transformed data:

$$\text{fitness} = \text{run} + \text{temperature}^2 + \text{size:temperature}^2\text{, with no intercept}$$

with no intercept and for the straight line

$$\text{fitness} = \text{run} + |\text{temperature}| + \text{size:}|\text{temperature}| \text{ with no intercept.}$$

A post hoc power test was done to determine the size of interaction we would have been able to detect. To establish if the marginally significant effect could be explained by adsorption, adsorption was quantified at three temperatures. The transformations used for the fitness data was not possible for the adsorption data (see results). Equality of variance was achieved through raising Adsorption x 10 to the power 0.125. We started with the model:

$$(\text{Adsorption x } 10)^{0.125} \sim \text{Size} + \text{Temp} + \text{Temp}^2 + \text{Temp:Size} + \text{Temp}^2\text{:Size}$$

Statistics were done in R (R Development Core Team, 2005) and SPSS.

Results

Estimates of the fitness response showed overall similarity between al the phages in their reaction to temperature variation (Table 3.2.). The highest fitness was measured at 36.2

degrees, with fitness decreasing as temperatures deviated from this optimum (Figure 3.1). Phages differed in their maximal fitness values measured at 36.2 ºC, so to make visual inspection of the data simpler, the data was transformed by dividing the fitness values by the average fitness of the specific species at 36.2ºC. This scaled the fitness of all species so that the maximum fitness was 1(Figure 3.2). These figures show no obvious effect of size on plasticity. Despite a four-fold difference in genome size between T3 and T6 (Table 3.1) there is a striking similarity in their fitness response. If indeed plasticity and size were linked one would expect ΦX174, with the smallest genome size of only 5.4kb, to show the largest fitness decrement due to low levels of plasticity, but this was not clearly evident in the fitness values recorded.

Table 3.1 Genome characteristics of the seven phages used in the experiments.

Phage	Genome	Genome size (kb)
ΦX174	ssDNA	5.4
T1	dsDNA	46
T3	dsDNA	38.2
T4	dsDNA	168.895
T5	dsDNA	121.3
T6	dsDNA	170
T7	dsDNA	39.9

Mean fitness values and adsorption rates are shown in Table 1 for all phages following exposure to the various temperatures. Adsorption rates were multiplied by 10^8 to eliminate the need for the 10^{-9} and 10^{-10} notations.

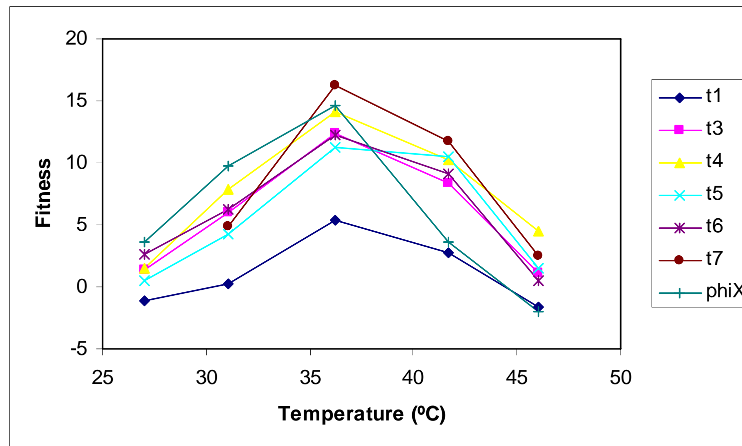

Figure 3.1: Fitness response of different phages to a range of temperatures.

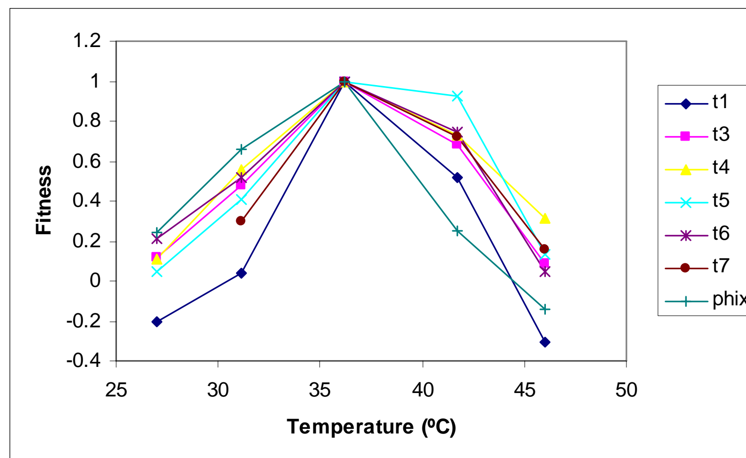

Figure 3.2: Scaled fitness values for all seven phages with a maximum fitness of 1

Table 3.2: Fitness results for plasticity experiments and adsorption rates. Mean fitness was calculated from the results of 5 repeats except for T7 and PhiX174 where only 4 repeats were done. Adsorption rate experiments were done in triplicate for all phages.

Phage	Temperature	Mean fitness	Average Adsorption rate x 10^8
PhiX174	27	-1.11461	0.0692
	31.1	0.193751	
	36.2	5.385406	0.58740
	41.7	2.804505	
	46	-1.6483	0.29167
T1	27	1.43529	0.32333
	31.1	5.962338	
	36.2	12.3666	0.36933
	41.7	8.415602	
	46	1.065141	1.19223
T3	27	1.521121	0.37767
	31.1	7.819596	
	36.2	14.12734	0.67667
	41.7	10.28655	
	46	4.445252	0.22733
T4	27	0.511571	0.086
	31.1	4.305033	
	36.2	11.27981	0.18603
	41.7	10.46593	
	46	1.483728	0.19797
T5	27	2.564645	0.05083
	31.1	6.306194	
	36.2	12.19379	1.29967
	41.7	9.064828	
	46	0.56059	0.52700
T6	27		1.01233
	31.1	4.862291	
	36.2	16.30453	1.45333
	41.7	11.75676	
	46	2.541912	1.346
T7	27	3.59632	0.11677
	31.1	9.688849	
	36.2	14.65492	1.24867
	41.7	3.670469	
	46	-2.02257	0.54567

Since adsorption rate can be influenced by temperature, and higher adsorption rates have been associated with higher fitness (Bull *et al.* 2004), adsorption rates were measured for all the phages at three temperatures, to determine its contribution to the changes in population growth. Considerable variation was seen in adsorption rates across all the phages, yet the observed rates corresponded well to those found in previous studies (Schlesinger, 1932; Bull *et al* 2004). Figure 3.3 shows box plot representations of the adsorption rates at the optimal temperature of 36.2 ºC and the two most extreme temperatures used in the plasticity experiments.

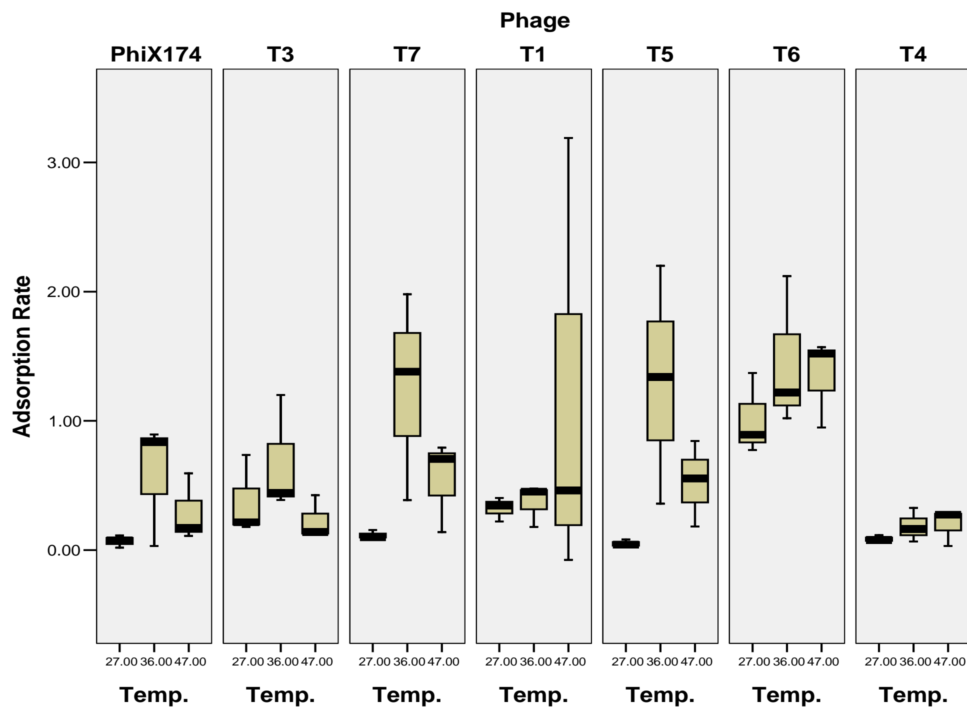

Figure 3.3: Bar graphs representing the adsorption rates of the seven phages as measured after 5 minutes. Phages are indicated at the top of each graph with adsorption rate scaled by multiplication with a factor of $1x10^8$. Temperature is measured in degrees Celsius.

No clear pattern emerged that would suggest adsorption rate was significantly influencing population density. Despite a clear difference in the adsorption rate of T5 at the two extreme temperatures, there is very little difference in the absolute fitness that

was measured at these temperatures. Phages like T6 and T7 had higher adsorption rates at 46°C than 27°C, yet showed lower overall fitness at higher temperatures. No evidence was found to support the idea that higher adsorption rates would lead to significantly increased fitness over the temperature range used here.

Statistical analysis

Table 3.3: Parameter estimates of the curved and straight line models.

Model	parameter	estimate	P
Curved line	run	.	<0.0001
	temperature^2	-0.01064	<0.0001
	size:temperature^2	0.000014	0.0890
Straight line	run	.	<0.0001
	temperature	-0.1121	<0.0001
	size:temperature	0.000134	0.0599

The curved and straight line models explained 74% and 75% of the variance respectively. Table 3.3 and the diagnostic plots (not shown) suggested that both models could be valid. This marginal significance could have been the result of a small sample size, but the post hoc power test showed the experiment had an 80% power to detect an interaction term of 2×10^{-5} and 2×10^{-4} respectively. Thus in both cases the design was sensitive enough to detect an effect twice as large as was observed here.

The adsorption data was not symmetrical around 36 degrees but tended to be higher for deviations to the right than deviations to the left, and the variance increased as the temperature increased. The initial model showed no significance of the interaction terms: Temp:Size and Temp^2:Size. We simplified the model by dropping both interaction

terms and eventually also size, keeping only the significant terms. This gave a very weak minimal adequate model explaining only 16% of the data.

$$(\text{Adsorption x } 10)^{0.125} \sim -0.485 + 0.072\text{xTemperature} - 0.0009\text{xTemperature}^2$$

A far better model was obtained by using Size as a factor. In this case both interaction terms were significant and 55% of the variance is explained.

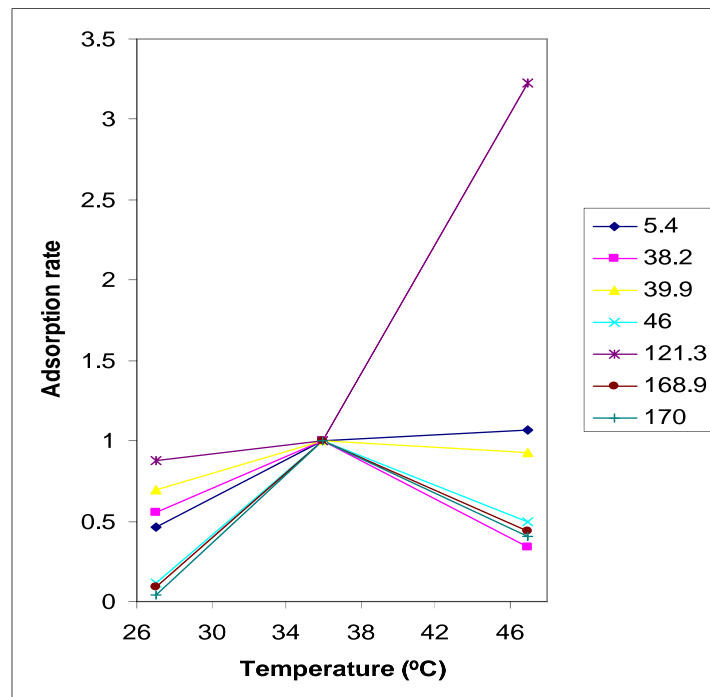

Figure 3.4: Scaled adsorption rates plotted over temperature. Absolute adsorption values were multiplied by 10^8 and then scaled by raising this value to the power 0.125

Discussion

A controlled experimental environment was used to test for the predicted correlation between genome size and plasticity by measuring population fitness. In the presence of phenotypic plasticity, a population can deal with a wider range of environmental variables with lower impact on fitness. The more plastic and robust the genome is, the smaller the fitness decrement should be, since it buffers the population against a large initial drop in overall fitness. A high level of phenotypic plasticity will reduce the initial fitness decrement when a population faces a stressor, since the genome can express various phenotypes that could potentially deal with the new environment. Introducing temperature as an exogenous stress factor to the environment caused an initial fitness decrement in all groups due to high levels of mortality and/or drastic reduction in reproductive output.

Our results, however, point out the potential complexity when attempting to reconcile theoretical predictions with empirical data in evolutionary studies. It appears that unrelated genotypes of similar size such as T1 and T3/T7 may differ in their level of phenotypic plasticity and therefore differ in their ability to accept environmental modification. The plastic response of similar sized, related phages differed in much the same way as unrelated phages of different sizes, with no clear pattern emerging. The fitness decrement measured in the related phages T7 and T3 showed more similarities with phages of different genome sizes (T4 and T6) than T1 which has a similar genome size (Figure 3.2). Based purely on genome size, T5 with its intermediate genome, would be expected to have a higher level of plasticity than T3 and T7, yet lower than that

measured for the larger T4 and T6. The results do not support this prediction however, nor could evidence be found that ΦX174 had the lowest plasticity, as would be expected since is had the smallest genome size. Measured fitness values do not give overwhelming support for the prediction that plasticity is closely linked with genome size, but statistical analysis pointed to a marginal interaction between genome size and plasticity that could not be explained by the adsorption data. To scrutinise this interaction, the experiment needs to be repeated with larger sample sizes and would have to be done on a broader range of genome sizes, with more phylogenetic information available. Exposing phages to a different stressor, such as different salt concentrations or pH, could also be used to create an environment where fitness decrement would typically be buffered through phenotypic plasticity.

Understanding the dynamic interaction between the different components of a genome and how they function, will eventually lead to understanding the effect of pleitropy and the contribution of different genes to phenotypes. As early as 1965 Bradshaw showed that closely related species of plants can show vast differences in their levels of phenotypic plasticity even in similar environments. While genomes may be similar in size and genome architecture, they could differ at the level of gene expression and the ratio of mean loci to plastic loci. Several experiments have pointed towards a complex series of epistatic interactions underlying the phenotypic response (Scheiner and Lyman 1991). For the human pathogen *Staphylococcus aureus*, plasticity through differential gene expression is achieved by global regulatory loci, present in all populations, and forming complex regulatory networks (Cheung and Zhang 2002; Novic 2003). When faced with a new environment, gene inactivation could provide the fitness

advantage required to offset the potential negative effect of the environment. In experiments with *E. coli*, most of the inactivation events were found in operons of genes not required in the environment currently experienced, thereby providing savings in energy and amino acid requirement (Edwards *et al*, 2002). Smaller fitness decrements in the face of changing environments could therefore be facilitated by plasticity in a few crucial genes, rather than the entire genome. If this is the case, then genome size becomes an unreliable measure of plasticity, as it is the plasticity of and epistasis between various components of the genome that plays a role, rather than overall genome characteristics, size or organisation.

The study of phenotypic plasticity is complicated by the level of epistatic interactions between plastic and non-plastic loci and the number of characteristics under selection. The portion of genes that are involved in the response, given a specific set of conditions, is arguably a better measure of "size" rather than the total number of genes. Further experiments with phages will be required to determine the extent to which modularity increases the adaptability of the phages to broader environmental niches, and how this relates to phenotypic plasticity. Much work remains to be done for models to encompass all the current available data, as well as the experimental work required to validate the existing range of models describing phenotypic plasticity. At present there is still very little data available that describes the variability of reaction norm shape, and even less information exists about non-linear reaction norms (Gavrilets and Scheiner 1993b). Empirical information together with theoretical development will be required to shed more light on the importance of phenotypic plasticity in evolution of populations. It is essential that theoretical models, in order to be realistic, be developed in conjunction

with experimental testing, even in the simplest scenarios, if we hope to ultimately understand the high levels of complexity seen in the natural world around us.

Literature Cited

Adams, M. H. 1959. *Bacteriophages*. Interscience Publishers, New York.

Antovics, J. 1976. Nature of limits to natural selection. *Annals of the Missouri Botanical Garden* 63:224-247.

Behera, N., and Nanjundiah, V. 1995. An investigation into the role of phenotypic plasticity in evolution. *Journal of Theoretical Biology* 172:225-234.

Behera, N., and Nanjundiah, V.1996. The consequence of phenotypic plasticity in cyclically varying environments: a genetic algorithm study. *Journal of Theoretical Biology* 178:135-144.

Behera, N., and Nanjundiah, V. 1997. *trans* Gene regulation in adaptive evolution: a genetic algorithm model. *Journal of Theoretical Biology* 188:153-162.

Bell, G. 1997. *The basics of selection*. Chapman and Hall, New York, NY.

Bennet, A. F., Dao, K. M., and Lenski, R. E. 1990. Rapid evolution in response to high temperature selection. *Nature* 346: 79-81.

Binetti, A. G., Quiberoni, A., and Reinheimer, J. A. 2002. Phage adsorption to *Streptococcus thermophilus.* Influence of environmental factors and characterization of cell-receptors. *Food Research International* 35:73–83.

Bradshaw, A. D. 1965. Evolutionary significance of phenotypic plasticity in plants. *Advances in Genetics* 13:115-155.

Bradshaw, H. D. JR., Otto, K. G., Frewen, B. E., McKay, J. K., and Schemske, D. W. 1998. Quantitative trait loci affecting differences in floral morphology between species of monkeyflowers (*Mimulus*). *Genetics* 149:367-382.

Bull, J. J., Badgett, M. R., Springman, R., and Molineux, I. J. 2004.Genome properties and the limits of adaptation in bacteriophages. *Evolution* 58:692-701.

Bull, J. J., Badgett, M. R., and Wichman, H. A. 2000. Big-benefit mutations in a bacteriophage inhibited with heat. *Molecular and Biological Evolution* 17:942-950.

Burch, C. L., and Chao, L. 1999. Evolution by small steps and rugged landscapes in the RNA virus Φ6. *Genetics* 151:921-927.

Chao, L. 1990. Fitness of RNA virus decreased by Muller's ratchet. *Nature* 348:454-455.

Chao, L., Levi, B. R., and Stewart, F. M. 1977. A complex community in a simple habitat: an experimental study with bacteria and phage. *Ecology* 58:369-378.

Charnov, E. L., and Bull, J. 1977. When is sex environmentally determined? *Nature* 266: 828-830.

Cheung, A. L., and Zhang, G. 2002. Global regulation of virulence determinants in *Staphylococcus aureus* by the SarA protein family. *Frontiers in Biosciences* 7:1825-1842.

Dean, A. M., and Golding, G. B. 1997. Protein engineering reveals adaptive replacements in isocitrate dehydrogenase. *Proceedings of the National Academy of Sciences of the United States of America* 94:3104-3109.

De Jong, G. 1990. Genotype-by-Environment interaction and the genetic covariance between environments: multilocus genetics. *Genetica* 81:171-177.

Desplats, C., and Krisch, H. M. 2003. The diversity and evolution of the T4-type bacteriophages. *Research in Microbiology* 154:259–267.

De Witt, T. J. 1998. Costs and limits of phenotypic plasticity: tests with predator-induced morphology and life history in a freshwater snail. *Journal of Evolutionary Biology* 11:465-480.

De Witt, T. J., Sih, A., and Wilson, S. D. 1998. Costs and limits of phenotypic plasticity. *Trends in Ecology and Evolution* 13: 77-81.

Dowell, C. E. 1980. Growth of bacteriophage ΦX174 at elevated temperatures. *Journal of Genetic Virology* 49:41-50.

Edwards, R.J., Sockett, R. E., and Brookfield, J. F. Y. 2002. A simple method for genome-wide screening for advantageous insertions of mobile DNAs in *Escherichia coli*. *Current Biology* 12:863–867.

Elena, S. F., Cooper, V. S., and Lenski, R. E. 1996. Punctuated evolution caused by selection of rare beneficial mutations. *Science* 272:1802-1804.

Elena, S. F., and Sanjuan, R. 2003. Climb every mountain? *Science* 302:2074-2075.

Ernande, B., and Dieckman, U. 2004. The evolution of phenotypic plasticity in spatially structured environments: implications of intraspecific competition, plasticity costs and environmental characteristics. *Journal of Evolutionary Biology* 17:613-628.

Falconer, D. S. 1989. *Introduction of quantitative genetics*. 3rd Edition. Longman Scientific and Technical, Wiley, New York.

Felsenstein, J .1974. The evolutionary advantage of recombination. *Genetics* 78:737-756.

Fisher, R. A. 1930. *The genetical theory of natural selection*. Oxford University Press, Oxford, Dover, New York.

Gause, G. F. 1947. Problems of evolution. *Transactions of the Connecticut Academy of Sciences* 37:17-68.

Gavrilets, S. 1986. An approach to modelling evolution of populations with consideration of genotype-environment interaction (Translated from Russian). *Soviet Genetics* 22:28-36.

Gavrilets, S., and Scheiner, S. M. 1993. The genetics of phenotypic plasticity. V. Evolution of reaction norm shape. *Journal of Evolutionary Biology* 6:31-48.

Gavrilets, S., and Scheiner, S., M. 1993b. The genetics of phenotypic plasticity. VI. Theoretical predictions for directional selection. *Journal of Evolutionary Biology* 6:49-68.

Getty, T. 1996. The maintenance of phenotypic plasticity as a signal detection problem. *American Naturalist* 148:378-385.

Gomulkiewicz, R., and Kirkpatrick, M. 1992.Quantitative Genetics and the evolution of reaction norms. *Evolution* 46:390-411.

Haigh J. 1978. The accumulation of deleterious genes in a population: Muller's ratchet. *Theoretical Population Biology* 14:251-267.

Hendrix, R. W., and Duda, R. L. 1992. Bacteriophage λPaPa: Not he mother of all λ phages. *Science* 258:1145-1148.

Hinton, G. E., and Nowlan, S. J. 1987. How learning can guide Evolution. *Complex Systems* 1:497-502.

Hoffman A. A., and Parsons, P. A. 1991. *Evolutionary genetics and environmental stress*. Oxford Science Publications, Oxford, UK.

Holder, K. K., and Bull, J. J. 2001. Profiles of adaptation in two similar viruses. *Genetics* 159:1393-1404.

Holland, J. J. 1996. Visualizing virus fitness gains. *Nature Biotechnology* 14:431-432.

Kim, J., and Davidson, N. 1974. Electron microscope heteroduplex study of sequence relations of T2, T4, and T6 bacteriophage DNAs. *Virology* 57:93-111.

Kimura, M. 1970. On the evolutionary adjustment of spontaneous mutation rates. *Genetical Research* 9:23-34.

Kingslover, J. G., and Huey, R. B. 1998. Evolutionary analysis of morphological and physiological plasticity in thermally variable environments. *American Zoologist* 38:545-560.

Lindberg, A. A. 1973. Bacteriophage receptors. *Annual Review of Microbiology* 27:205–241.

Mitchel-Olds, T., and Shaw, R. G. 1987. Regression analysis of natural selection: statistical inference in biological interpretation. *Evolution* 41:1149-1161.

Monod, C., Repoila, F., Kutateladze, M., Tetart, F., and Krisch, M., 1997. The genome of the pseudo T-even bacteriophages, a diverse group that resembles T4. Journal of *Molecular Biology* 267:237-249.

Moran, N. 1992.The Evolutionary Maintenance of Alternative Phenotypes. *American Naturalist* 139:971-989.

Novella, I. S., Clinis, M., Elena, S. F., Kohn, J., Moya, A., Domingo, E., and Holland, J. J. 1996. Large population passages of vesicular stomatitis virus in interferon-treated cells select variants of only limited resistance. *Journal of Virology* 70:6414-6417.

Novella, I. S., Hershey, C. L., Escarmis, C., Domingo, E., and Holland. 1999. Lack of evolutionary stasis during alternating replication of an arbovirus in insect and mammalian cells. *Journal of Molecular Biology* 287:459-465.

Novic, R. P. 2003. Auto-induction and signal transduction in the regulation of staphylococcal virulence. *Molecular Microbiology* 48:1429-1449.

Orr, H. A. 2000. Adaptation and the Cost of Complexity. *Evolution* 54:13-20.

Orr, H. A. 2003. A minimum on the mean number of steps taken in adaptive walks. *Journal of Theoretical Biology* 220:241-247.

Orzach, S. H. 1985. Population dynamics in variable environments. The genetics of homeostasis revisited. *American Naturalist* 125:550-572.

Padilla, D. K., and Adolph, S. C. 1996. Plastic inducible morphologies are not always adaptive: the importance of time delays in a stochastic environment. *Evolutionary Ecology* 10:105-107.

Pajunen, M. I., Elizondo, M. R., Skurnik, M., Kieleczawa, J., and Molineux, I. J. 2002. Complete nucleotide sequence and likely recombinatorial origin of bacteriophage T3. *Journal of Molecular Biology* 319:1115–1132.

Piersma, T., and Drent, J. 2003. Phenotypic flexibility and the evolution of organismal design. *Trends in Ecology and Evolution* 18:228-233.

Piersma, T., and Lindström, Å. 1997. Rapid reversible changes in organ size as a component of adaptive behaviour. *Trends in Ecology and Evolution* 12:134–138.

R Development Core Team. 2005. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Relyea, R. A. 2002. Cost of Phenotypic Plasticity. *The American Naturalist* 159:272-282

Roberts, M. D., Martin, N. L., and Kropinski, A. M. 2004. The genome and proteome of coliphage T1. *Virology* 318:245–266.

Rokyta, D., Badgett, M. R., Molineux, I. J., and Bull, J. J. 2002. Experimental genomic evolution: extensive compensation for loss of DNA ligase activity in a virus. *Molecular and Biological Evolution* 19:230-238.

Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual.* 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Scheiner, S. M. 1989. Variable selection along a successional gradient. *Evolution* 43:548-562

Scheiner, S. M. 1993. Genetics and evolution of phenotypic plasticity. *Annual Review of Ecology and Systematics* 24:35-68.

Scheiner, S. M. 1998. The genetics of phenotypic plasticity. VII. Evolution in a spatially structured environment. *Journal of Evolutionary Biology* 11:303-320.

Scheiner, S. M. 2002. Selection experiments and the study of phenotypic plasticity. *Journal of Evolutionary Biology* 15:889-898.

Scheiner, S. M., and Berrigan, D. 1998. The genetics of phenotypic plasticity. VIII. The cost of plasticity in *Daphnia pulex*. *Evolution* 52:368-378.

Scheiner, S. M., and Lyman, R. F. 1989. The genetics of phenotypic plasticity. I. Heritability. *Journal of Evolutionary Biology* 2:95-107.

Scheiner, S. M., and Lyman, R. F. 1991. The genetics of phenotypic plasticity. III. Plasticities and fluctuating asymmetries. *Journal of Evolutionary Biology* 4:51-68.

Schlesinger, M. 1932. Adsorption of bacteriophage to homologous bacteria. II. Quantitative investigation of adsorption velocity and saturation. Estimation of the particle size of the bacteriophage. *Zeitschrift fur Hygenie und*

Immunitaetsforschung. 114:149-160.Translated in G. S. Stent (Ed) *Papers on Bacterial Viruses*. Little, Brown, and Co., Boston Massachusetts.pp 26-36.

Schmalhausen, I. I. 1949. Factors of Evolution. *The theory of stabilizing selection*. Blakiston, Philadelphia.

Schlichting, C. D., and Pigliucci, M. 1993. Control of phenotypic plasticity via regulatory genes. *American Naturalist* 142:366-370.

Springman, R., Badgett, M. R., Molineux, I. J., Bull, J. J. 2005. Gene order constrains adaptation in bacteriophage T7. *Virology* 341:141 – 152.

Starck, J. M. 1999. Structural flexibility of the gastro-intestinal tract of vertebrates – implications for evolutionary morphology. *Zoologischer Anzeiger* 238:87–101.

Svenson, V., and Christiansson, A. 1991. Methods for phage monitoring. *FIL-IDF Bulletin*, 263, 29–39.

Travis, J. 1994. Evaluating the adaptive role of morphological plasticity. Pages 99-122 *in* P. C. Wainwright and S. M. Reilly, eds. Ecological Morphology: *Integrative Organismal Biology*. University of Chicago Press, Chicago.

Turner, P. E., and Chao, L. 1998. Sex and the evolution of intra-host competition in RNA virus phi6. *Genetics* 150:523-532.

Turner, P. E., and Elena, S. F. 2000. Cost of host radiation in an RNA virus. *Genetics* 156:1465-1470.

Van Tienderin, P. H. 1991.Evolution of generalists and specialists in spatially heterogeneous environments. *Evolution* 45:1317-1331.

Via, S. 1987. Genetic Constraints on the evolution of phenotypic plasticity. Pages 47-71 *in* V. Loescheke, ed. *Genetic Constraints on Adaptive Evolution*. Springer, Berlin.

Via, S., Gomulkiewicz, R., de Jong, G., Scheiner, S. M., Schlichting, C. D., and Van Tienderin, P. H. 1995. Adaptive Phenotypic Plasticity: consensus and controversy. *Trends in Ecology and Evolution* 10:212-217.

Via, S., and Lande, R. 1985. Genotype-environment interaction and the evolution of phenotypic plasticity. *Evolution* 39:505-522.

Wang, J., Jiang, Y., Vincent, M., Sun, Y., Yu, H., Wang, J., Bao, O., Kong, H., Hu, S. 2005. Complete genome sequence of bacteriophage T5. *Virology* 332:45–65.

West-Eberhard, M. 1989. Phenotypic plasticity and the origin of diversity. *Annual Review of Ecology and Systematics* 20:249-278.

Wichman, H. A., Scott, L. A., Yarber, C. D., and Bull, J. J. 2000. Experimental evolution recapitulates natural evolution. *Philosophical Transactions of the Royal Society of London, Series B* 355:1677-1684.

Wright, S. 1931. Evolution in Mendelian population. *Genetics* 37:97-159.

Wu, R. 1998. The detection of plasticity genes in heterogeneous environments. *Evolution* 52:967-977.

Wu , G., Fiser, A., Ter Kuile, B., Sai, A., and Muller, M. 1999. Convergent evolution of *Trichomonas vaginalis* lactate dehydrogenase from malate dehydrogenase. *Proceedings of the National Academy of Sciences of the United States of America* 96:6285-6290