RIJKSUNIVERSITEIT GRONINGEN

How to be good at being a virus
Biochemical constraints of viral life-history evolution

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Chapter 1
Introduction and aim of thesis

'You can't always get what you want,

... but if you try sometimes you might find, you get what you need'

(The Rolling Stones)

1.1 Biological organization, biological evolution and biological models

How much is the path of evolution guided by its history, i.e. the evolutionary constraints that result from the established organization of an organism? This question has puzzled evolutionary biologist for centuries and spawned a controversy that is as old as evolutionary biology itself (Gould & Lewontin 1979; Charlesworth et al. 1982; Maynard-Smith et al. 1985; Houston 1997; Wagner 1998; Pigliucci & Kaplan 2000; Pigliucci 2007). Until the present day, this controversy seems to be unresolved - maybe because this fundamental evolutionary question has no general answer. Evolutionary constraints seem to depend on the biological context, i.e. the organism in question, the level of organization considered as well as the evolutionary time frame one is looking at.

Nonetheless, the evolutionary consequences of biological organization cannot be ignored. After all, evolutionary contingency implies that new

organisms are descending from existing organisms. The current organization of a biological organism is therefore the raw material for mutational variation and future evolutionary change. The big question is therefore not whether biological organization matters, but how it can be incorporated into evolutionary models in a meaningful way. In its essence organization is the interaction between the parts of an organism. Yet, what are these parts and how can we define interactions between them?

Representing biological organization is difficult, incorporating it into evolutionary thinking even more so. Organisms consist of numerous parts that show complex interactions. Approaches to reduce the complexity have followed different avenues. Generic models reduce the details of interactions and instead use a statistical description subsuming interactions of a similar kind. Specific models reduce the number of interacting parts by focusing on a specific subset of the organisms, like a specific genetic pathway. Generic and specific models both have their benefits and drawbacks. Generic models are largely independent of a specific biological context and therefore applicable to a wide range of biological systems. On the downside, they can only produce generic conclusions and, therefore, have little predictive power for a specific biological context. In contrast, specific models can predict the path of evolution in quite some detail, but they only apply to a specific group of organisms in a limited evolutionary time frame. In this sense predicting the path of evolution is analogous to forecasting the weather – the longer one projects the current situation into the future, the more uncertainty is attached to the prediction.

1.2 Aim of this thesis

How much generality should we trade in to account for a specific biological context? In my opinion, evolutionary models should incorporate specific real world mechanisms in order to be applicable to real world problems. In viral systems these mechanisms are particularly well known. In this thesis I therefore chose to study the evolutionary

limitations of viruses by mathematical models that are based on known molecular genetic mechanisms. In my view, this integration of molecular genetics and evolutionary theory is the most promising avenue to apply evolutionary models to important problems like the evolution of viral drug resistance, changes in viral virulence or the switch between host organisms.

Molecular genetics has opened the possibility to introduce a meaningful representation of biological organization into evolutionary thinking. The well defined concepts of molecular genetics aid the development of evolutionary models that are stated in real world mechanisms, like protein expression or enzymatic rates, and therefore enable a direct experimental test of theoretical predictions. A specific mechanistic underpinning puts a boundary to the process of evolution, as it allows for an a priori definition of feasible strategies in terms of protein expression, enzymatic activity and binding properties. Given the range of possibilities one can then find the best strategy given the biochemical limitations. Models that are framed in terms of a specific mechanistic organization can therefore arrive at testable predictions on the limitations of viral adaptations or in other words explain why a virus cannot always get what it wants. Knowledge of such limitations is of obvious importance for designing approaches to existing problems of viral evolution. In particular, explicit consideration of mechanistic limitations of viral adaptation might challenge the pessimistic premise that viral adaptation cannot be countered in view of the high rate of evolution due to the enormous size of virus populations.

Molecular genetics can benefit from evolutionary thinking, since evolutionary theory necessitates the integration of biochemical aspects into a single overall measure of viral fitness. In other words, evolutionary thinking leads to a consideration of the interaction of biochemical processes throughout the entire life cycle. Tools of evolutionary modeling, in particular life history theory, can therefore provide an integrative approach for novel questions in molecular

genetics, resulting from the study of large scale gene expression profiling. Classical molecular genetics often focuses on cases in which the coupling of a certain biochemical rate has a one-on-one relationship with survival or replication. This understanding can be misleading when one biochemical rate affects multiple aspects of the viral life cycle in opposing directions (Krakauer & Komarova 2003). Even more, the replication rate itself does not need to be an accurate predictor of viral fitness when it has negative side effects on other aspects of the life cycle. For example, fast replicating pathogens that kill their host quickly will not have much opportunity to be transmitted to a new host organism. In such a case, fast replication has a negative side effect on transmission. Selection will therefore not maximize the replication rate. Instead, overall fitness is maximized for a certain intermediate balance between replication and transmission. Evolutionary modeling, and in particular the tools of life history theory are designed to integrate these opposing selective forces throughout the viral life cycle in order to arrive at an overall prediction.

The viral life cycle establishes an important link between viral biochemistry and viral fitness. Gene regulatory interactions of even the simplest organisms are highly complex. Accounting for the full complexity remains a forbidding task for most organisms. Analysis of the viral life cycle breaks down this complexity by focusing on a few components that are crucial for viral fitness. In a first step, a life history model identifies those components of the life cycle that are most relevant for viral fitness. In a second step, these components are then investigated taking account of viral biochemistry. This approach has the advantage that the complexity of the problem is reduced by singling out the biochemical aspects that are most relevant for viral fitness. It is the main aim of this thesis to demonstrate that this modeling approach is capable of identifying and integrating biochemical detail into models of viral evolution in a rather straightforward way. Moreover, these models can arrive at predictions that are stated in biochemical parameters that are accessible to experimental testing.

1.3 The bacteriophage life cycle

Bacteriophages are ideal model systems to investigate the evolutionary limits of viral evolution and their dependence on viral biochemistry. The biochemical key elements shaping the viral life cycle are known in considerable detail. Most globally, the viral life cycle falls into two main steps: replication inside the host cell and transmission between hosts cells. Essentially, viruses are genetic elements that acquired the ability to survive outside of the host cell and to transmit to new host cells by packaging their genome into a protein capsid or lipid vesicle. The ability to survive outside of the host cell is the single property that distinguishes viruses from other selfish genetic elements like transposons or plasmids (Shimotohno et al. 1980; Shimotohno & Temin 1980; Forterre 2006). Phage transmission between host cells depends on diffusion through the medium and adsorption to a new host cell by the aid of surface proteins. In liquid conditions, diffusion is fast and transmission between hosts is proportional to an adsorption rate and the density of host cells and phage particles (Schlesinger 1960). In a spatially structured agar medium viral diffusion can, however, become limiting for transmission to new host cells.

Phage transmission can occur horizontally, i.e. between unrelated hosts, or by vertical transmission from an infected parent cell to daughter cells. Horizontal and vertical transmission each require specific genetic mechanisms for the adaptation of the life cycle. Horizontal transmission requires packaging of the viral genome in a survival stage and mechanisms to leave the host cell. Packaging of the viral genome can either occur in a capsid of viral proteins, or by budding of viral vesicles through the host cell membrane. Assembly of viral particles in the host membrane provides a mechanism for the virus to leave the host cell in a continuous process without destroying it (e.g. Phage M13). In contrast, the release of viral particles which are not assembled in the cell membrane requires the destruction of the host cell through lysis mediated by specific lysis proteins (Young et al. 2000). Viruses that destroy the host cell in order to transmit are called lytic.

The decision to destroy the host cell though the expression of lysis genes is one of the most important aspects of the viral life cycle, since destruction of the host cell also destroys the machinery of phage replication. The time interval between infection of the host cell and its destruction is called lysis time. The number of phage offspring which is produced during that interval is called burst size. The reproductive output of lytic bacteriophages depends on the interplay of lysis time and burst size. During viral replication within the host cell the number of newly produced phage particles increases with time (Doermann 1952; Loeb & Zinder 1961). Prolonged lysis time therefore increases burst size, but at the same time increases generation time and delays viral spread and infection of new host cells. These two consequences of lysis timing have opposing effects on viral fitness. Bacteriophage lysis timing should therefore evolve to an intermediate optimum that balances its cost and benefits.

As an alternative to horizontal transmission, lysogenic bacteriophage can transmit from parent to offspring through vertical transmission. Parent-to-offspring transmission requires the survival and growth of host cells. In order to 'keep its host alive' lysogenic phages therefore actively repress the viral genes for replication and host lysis, keeping the host in a state called lysogenic infection. Additionally, lysogenic phages ensure their vertical transmission through integration of their genome into the host genome by specialized enzymes called integrases (Groth & Calos 2004). A lysogenic phage that is integrated in the host genome is referred to as pro-phage. Lysogenic phage can switch to a lytic cycle upon external triggers that indicate poor survival conditions for the host cell like UV damage, nutrient starvation or general stress response. This switch from the lysogenic to lytic conditions under poor host survival can be compared to a strategy of 'leaving a sinking ship'.

Viral replication within the host cell determines the rate at which the resources of the host cell are exploited. Molecular mechanisms that regulate this stage of the life cycle have important implications for viral

virulence, e.g. for degree of damage a virus causes to host fecundity and longevity. Benign phages like phage M13 and phage λ, for example, which do not lyse the host cell, evolved a suite of mechanisms to control their own rate of replication within the host cell, in order to keep the host alive. Phage M13, for example limits its own replication by the production of large amounts of protein P5 that covers the single stranded form of M13 and prevents a conversion to the double stranded DNA replication stage (Baas 1985). Likewise, bacteriophage λ actively represses its replication genes by the virulence repressor cI in order to ensure host survival during vertical transmission (Oppenheim et al. 2005). These, self-repression mechanisms that regulate of viral genome replication demonstrate that viruses are able to control their own level of virulence and, hence, adapt it to the environmental conditions.

Replication within the host cell falls into two distinct stages that have specific requirements on the timing and balance of viral gene expression. During early infection primarily the viral replication proteins are expressed. Accordingly, there is no production of new viral particles. This period is called eclipse time. During late infection viral packaging and lysis proteins are produced, viral particles are assembled and the number of phage particles inside the host cell increases. This phase is called maturation time. The timing and balance of viral protein expression over the early and late infection cycle is essential for efficient production of viruses. The expression of a single gene can therefore have conflicting consequences for early and late viral infection. This can lead to opposing selection forces on the expression of a particular gene during early and late infection and limit the evolution of viral gene regulation.

1.4 The environment determines selection on the viral life cycle

Viruses are obligatorily dependent on their host. The evolutionary success of viruses is therefore closely linked to the ability to transmit between host organisms. The conditions for viral transmission, in turn, strongly depend on the environment, e.g. the availability of hosts, the structure of the host population and competition for these hosts with other viral strains. The viral environment determines the relative importance of the transmission stage in comparison to other viral life stages. Accordingly, a change in the environment alters this balance and creates selection on viral life-history characteristics like virulence and transmissibility.

An important example is the adaptation of bacteriophage lysis timing to the host density in the environment (Wang et al. 1996). Theory predicts that a change of host density changes the relative contribution of replication and transmission to overall viral fitness over the entire life cycle and should therefore select for altered lysis timing. This adaptation of lysis timing to the host density has indeed been demonstrated in experimental evolution with phage RB69 (Abedon et al. 2003).

Not only the density, but also the structure of the host population markedly influences the evolution of viral life history. In a spatially structured host population transmission to uninfected hosts is limited when infected individuals form spatial clusters (Boots et al. 2004). Furthermore virulent viruses can drive the host locally to extinction and therefore cut of their route for future transmission. A spatially structured host population therefore generally favor the evolution towards lowered pathogen virulence (van Baalen & Sabelis 1995; van Baalen & Rand 1998; Johnson & Boeriijst 2002), but also viral infectivity (Boots 2007).

Competition between viruses is another important factor for the evolution of viral life history. Generally competition of parasites during co-infection of the same host is predicted to select for an increased replicating rate and an associated increase in virulence (van Baalen & Sabelis 1995). However, competition between strains can be affected by mechanisms other then resource competition, like for example direct interference between viral strains. If viruses interfere with each other's replication, the production of viable offspring and the level of virulence may be reduced (Turner & Chao 1999; Chao et al. 2000; Brown et al. 2002).

1.5 Constraints imposed by molecular mechanisms

Viral adaptation to the prevailing environment through changes in the viral life history requires biochemical changes that alter the timing and balancing of the production of viral proteins and their enzymatic activity. Biochemical constraints that limit changes in the timing of the life-cycle can hamper optimal adaptation to a given environment. Constraints occur when the change in a biochemical aspect that is beneficial to one part of the life-cycle has negative side effects on other parts.

Overlapping genes are obvious examples for a mechanistic link between different aspects of the viral life cycle. For instance, gene A of bacteriophage ΦX174 can either be translated as a complete protein, or as a C-terminal fragment of gene A (called gene A*). Even though both proteins are encoded for by a single stretch of the viral genome they fulfil different tasks. Whereas gene A is essential for viral DNA replication, gene A* is affecting lysis and causes the shutdown of host DNA replication (Funk & Snover 1976; Colasanti & Denhardt 1985; Baas 1985). A mutation in gene A can therefore change several parameters of the life cycle simultaneously. In bacteriophage MS2 the lysis gene has an overlapping region with the coat and replication genes. Accordingly, changes in the RNA sequence of the lysis gene can

have consequences for the viral replication and packaging process, simultaneously.

Besides overlapping genes, the nucleotide genome of single-stranded viruses has a secondary structure that affects multiple processes like ribosome binding, gene regulation and the initiation of viral packaging. Mutations that affect viral secondary structure can therefore alter multiple aspects of viral reproduction simultaneously. A particularly illustrative example is the central operator loop of the RNA phage MS2 which is (1) part of the over-lapping lysis and replicase gene, forms (2) the translation termination hairpin for the coat gene which controls the expression of lysis proteins. It furthermore contains (3) the Shine-Dalgarno sequence and start-codon of the replicase gene, is (4) engaged in the control of replicase expression through the Min-Jou RNA-RNA interaction and forms (5) the nucleation point of the viral packaging process (Peabody 1997; Licis et al. 2000). The multifunctionality of the central MS2 operator loop leads to strong selection for the maintenance of the secondary RNA structure. Due to the importance of the secondary structure the realm of viable mutations in sequence space is confined to sequences that maintain this secondary RNA structure (Olsthoorn et al. 1994; Licis et al. 1998). Such feasible mutational paths have been studied extensively in computational models of RNA folding (Fontana & Schuster 1998; Schuster & Fontana 1999). These models predict the occurrence of extensive ‘neutral networks’, e.g. adjacent positions in genotype space that represent a particular RNA fold, and eventual nucleotide changes that radically change the RNA fold (Van Nimwegen & Crutchfield 2000).

Resources of the host cell are finite. Production of viral components is therefore limited by host-cell resources. Over-allocation into the replication of viral genomes, for example can hamper the production of viral proteins and therefore drastically reduce the production of viable viral offspring (Katanaev et al. 1996).

Additional constraints for the viral intra-cellular development result from the competition between the processes of genome replication, genome translation and genome packaging. The processes of genome replication and translation are mutually exclusive since replication moves along the viral genome in the 3' to 5' direction, whereas translation occurs in the 5' to 3' direction. Therefore the queuing of ribosomes on the viral genome interferes with genome replication (Eigen et al. 1991; van Duin & Tsareva 2004; Regoes et al. 2005). Furthermore, the process of genome packaging is competing with genome replication and translation. A rate of packaging that above an optimal level can therefore hamper viral genome replication and reduce viral fitness (Krakauer & Komarova 2003). Due to this interdependence of genome replication, translation and packaging evolutionary change in on aspect of the reproductive cycle will affect other aspects simultaneously. A prediction of optimal viral development therefore relies on the integration of these processes.

Biochemical constraints can also occur when a single protein affects multiple aspects of the life cycle. One example is the function of the virulence repressor protein cI in the life-cycle of phage λ. The cI protein represses the replication and host lysis of the lysogenic bacteripohage λ, after it has integrated into the host genome, thereby enabling vertical transmission (Oppenheim et al. 2005). At the same time the cI helps to defend the host cell against the take-over of a competing λ phage, by repressing the replication of this competitor inside the cell. Such a defence is called superinfection inhibition. The dual role of the repressor protein cI in the control of virulence and superinfection inhibition leads to a mechanistic coupling between these two traits.

Another example in which one protein effects multiple parts of the life cycle is the receptor-destroying enzyme of influenza virus. This receptor-destroying enzyme has the seemingly paradoxical task to destroy the target receptor on the host cell surface that is used for viral

attachment. The activity of receptor destroying enzymes is, however, required to release of viral particle from cell mucus and from the host cell after the process of viral budding. Since the detrimental and beneficial effects are both caused by one and the same protein, the costs and benefits of receptor destruction are unavoidably linked.

1.6 Overview of the thesis: How do genetic constraints limit the adaptation of the viral life-cycle?

In order to illustrate how biochemical constraints affect the path of viral evolution I studied four different classes of viruses and their particular biochemical limitations in the evolution of the viral life cycle.

In **chapter 2** I disturbed viral gene expression balance and investigated how the virus can adapt its life cycle to this disturbance. For this purpose I cloned and overexpressed each of the four genes of MS2 from a plasmid leading to an excess supply of these genes during viral infection. Even though the virus receives one of its proteins 'for free', this disturbance of the viral gene balance is disastrous for the production of viral offspring. However, through adaptation to the conditions of gene over-expression the virus is able to restore some of its reproduction, supposedly through adaptation of its own gene regulation. The outcome of adaptation demonstrates some peculiar consequences of the evolution of viral protein balance. Adaptation to an excess of the coat gene led to an offspring production which exceeds the offspring produced by a wildtype infection in undisturbed condition whereas the adaptation to an excess of the replication protein is particularly difficult for the virus. Adaptation to the over-abundance of the lysis gene enabled the virus to reach a wildtype burst size at a much shorter lysis time and therefore greatly improved viral fitness. This indicates that lysis time of phage MS2 cannot evolve freely but is constrained by the expression and efficiency of the lysis protein.

Whereas chapter 2 studies the balance of viral replication within the host cell, **chapter 3** is focused on the optimal strategy for transmission between host cells in a spatially structured host population. In bacteriophage the strategy of transmission is determined by the rate of attachment to the host cell. In a liquid medium it always pays to attach to a host when it is encountered. Therefore in liquid medium the rate of attachment should increase to its maximum. In a spatially structured host population, however, maximal attachment rate is not necessarily beneficial. When a phage grows on a spatial lawn of host bacteria it kills all bacteria in a focal area that is termed plaque. In the center of the plaque the absence of bacteria prohibits any further replication. Therefore it can pay for a phage to decide 'not to infect' and instead diffuse further out to the border of the plaque. By this mechanism the short-term benefit of immediate replication can be out weighted by the long-term benefit of future plaque growth. For this reason the phage should evolve lowered rates of host attachment in a spatial medium. I investigated this effect in an individual-based computer simulation and in experimental evolution with bacteriophage ΦX174. For this purpose I followed the evolution of attachment rates and plaque size of a phage strain that was previously adapted to a spatially structured host population into the conditions of a liquid environment. As expected adaptation to the liquid environment selected for an increased rate of attachment and at the same time decreased the ability to form plaques in a spatially structured host population. These experimental results were in line with my theoretical predictions. Yet, when I studied the growth curve of the evolved lines I discovered that the lines evolved in the liquid environment had a lower fitness than the ancestor in the liquid environment. An unknown factor must have led to a decrease in fitness during the course of adaptation.

The search for this factor is the topic of **chapter 4**. Ultimately, we had to conclude that the loss in burst size of the evolved ΦX174 strains was caused by a peculiarity in the bacterial host strain that we used for phage evolution. Our search revealed that this strain, E.coli C122 mutT,

harbors a hitherto unknown lysogenic bacteriophage that apparently interfered with the reproduction of phage ΦX174. Although unintended, the presence of this phage illustrates that viral competition can have important implications for the outcome of viral evolution. Viral strategies to deal with such competition are the main theme of chapter 4.

In **chapter 5** I investigate the evolutionary role of molecular mechanisms that defend the host cells of a non-virulent, vertically transmitting virus against the take-over of a virulent, horizontally transmitting mutant. These mechanisms are called superinfection inhibition. In phage λ, superinfection inhibition is closely linked to the control of viral virulence, since virulence of the resident virus and replication of a superinfecting competitor are both controlled by the same protein (the cI protein). Due to this mechanistic link, increased virulence is associated with the cost of increased susceptibility to superinfection. This detrimental side effect of virulence can enable the persistence of vertically transmitting viruses or lead to the evolutionary co-existence of a vertically transmitting defense specialist and a horizontally transmitting attack specialist. Strikingly, the mechanisms that link virulence to superinfection inhibition also occur in other benign viruses of completely different origin (Retroviruses). The link between virulence and superinfection might therefore play a crucial role in the maintenance of the benign viral state in general.

In **chapter 6** we study the constraints in the evolution of influenza virus that result from the opposing effects of the viral receptor-destroying enzyme on the viral life cycle. Influenza virus requires the receptor-destroying activity to prevent the agglomeration of viral particles on the host cell surface and in the cell mucus. However, receptor-destroying activity has an important side effect as it destroys the target receptor of viral binding to the host cell and therefore hampers the infection process. Due to these side effects the activity of receptor destroying enzymes should not evolve to a maximum, but

rather to an intermediate optimum. This optimum depends on the abundance and biochemical properties of the receptor and can lead to conditions that require viral specialization to one tissue or host at the expense of replication in another tissue or host. Since tissue specificity is an important factor for viral virulence and the route of viral transmission, the mechanisms of exclusive tissue specificity can offer a plausible mechanism for the dichotomy between virulent but poorly transmittable strains and non-virulent by highly transmittable strains of influenza virus.

These case studies lead to a row of observations that could lead to applications for to specific problems of viral evolution. Chapter 2, for example, shows that adaptation to the overexpression of the viral replication genes is particularly difficult for the virus. Furthermore, the expression of the lysis gene seems to be an important limitation for the viral life cycle. Knowledge of these bottlenecks can be invaluable in the design of anti-viral drugs. Chapter 3 shows that spatial confinement of epidemics might provide means to select for reduced levels of viral infectivity. Chapter 4 points out that self-imposed limitation of viral genome replication is crucial in the evolution of viral virulence. Chapter 5 describes the limitations of viral adaptation to anti-viral drugs that inhibit the activity of the viral neuraminidase protein. In all four case studies I demonstrate that modeling of the viral life cycle can aid to identify specific molecular targets that are the bottleneck of viral reproduction. Potentially, these targets might become the basis of future anti-viral strategies which are resilient to viral adaptation and drug resistance.

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Chapter 2
Evolution of viral development of RNA phage MS2 under experimentally disturbed gene balance

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2.1 Abstract

Viral development depends on the careful timing and balance of viral gene production. To understand the role of gene balance for viral development and evolution we over-expressed each of the four genes of RNA phage MS2 during the infection cycle, and evolved the phage under these conditions of disturbed gene balance. Even though overexpression provides viral gene products 'for free', this disturbance reduced viral burst size by orders of magnitude and altered the timing of lysis. Phage MS2 was able to adapt its gene regulation to this disturbance in some directions, but failed for others. Adaptation to overexpression of the coat gene led to a burst size above that of the ancestor in the absence of overexpression, but increased lysis time. Adaptation to replicase overexpression shortened lysis time, but resulted in a lower burst size. Viral strains that had adapted to lysis

gene overexpression had only half the lysis time of the ancestor in the absence of overexpression. Surprisingly this had no cost for burst size. The lysis time of MS2 seems therefore to be constrained by lysis efficiency. Our results demonstrate that within limits the shape of the viral growth curve can respond to selection and becomes the target of evolution itself.

2.2 Introduction

Viruses are ideal model systems to study the adaptation of organisms to changes in the environment. The adaptation of the viral life cycle to environmental challenges depends on the availability of genetic variation in viral gene regulatory processes and the biochemistry of the host cell. Optimality models aiming to predict adaptation of the viral life cycle to the environment rarely incorporate such mechanistic details (Bull et al. 2004). A notable exception is the study of bacteriophage lysis timing that incorporates the expression of lysis genes into models for the optimal timing of the viral life cycle (Wang et al. 2000; Young et al. 2000; Heineman et al. 2005; Wang 2006). Here we take this approach one step further and investigate the role of other viral genes for the shape of the viral growth curve and viral lysis timing.

Theory of lysis timing predicts that viruses should evolve a lysis time that balances the increase of viral fecundity due to prolonged within-host replication against the decrease of viral fecundity due to delayed release of viral particles (Abedon 1989; Wang et al. 1996). The optimal lysis time is determined by two factors; the increase of viral particles during replication within the host, given by the viral growth curve, and the average time required to spread to a new host, given by the host density. Optimal viral life history therefore depends on both, the environment and the viral growth curve. Experiments have demonstrated the evolution of lysis time in response to the environment (Abedon et al. 2001), but studies that address the effect of changes in the viral growth curve are lacking.

In virtually all models of viral life history evolution the viral growth curve plays a central role. The viral growth curve integrates the complex dynamics of viral development into a small number of phenotypic parameters (most notably lysis time and burst size) that can be quantified by the classical one-step growth curve essay (Gong et al. 1996; Alexander et al. 1998). These parameters are determinants of viral fitness and therefore crucial ingredients in models that relate viral lysis timing to the evolution of viral virulence (Bull 2006).

However, the shape of the viral growth curve is an evolvable feature itself. In fact, the shape of the viral growth curve reflects the interplay of viral nucleotide replication and the production of viral structural and lysis proteins during intracellular replication. These processes are subject to a number of unavoidable constraints and trade-offs. First, viral genome replication and translation occur in opposite direction along the genome, and can therefore not occur simultaneously (Eigen et al. 1991; Regoes et al. 2005). Second, the packaging process interferes with viral genome replication (Krakauer & Komarova 2003). Additionally, the early stages of development can exhaust resources that are required for later development (Katanaev et al. 1996). Viral gene regulation is predestined to create a balance that optimizes viral growth given these constraints and trade-offs. However, it is largely unknown to what extent the limitations of intra-cellular development constrain the evolution of viral gene regulation and the viral life cycle.

Small bacteriophages contain only few genes and therefore offer an exquisite possibility to study the evolution viral gene balance and the viral growth curve. One example is the single-stranded RNA phage MS2 that contains a genome of only 5.3kb encoding the four genes maturation, coat, lysis and replicase. Regulatory interactions between these genes are well characterized. Coat represses replicase and the expression of coat and lysis are positively correlated by read-through translation. In contrast, expression of maturation is independent of the

expression of other MS2 genes and it does not affect the balance of other MS2 genes (Poot et al. 1997; Van Duin & Tsareva 2004).

The RNA genome of MS2 folds into an intricate secondary structure that is involved in gene regulation by protein-RNA interactions. A key element in viral gene regulation is the central operator loop of MS2 (pos. 1715-1765) that is part of the overlapping replicase and lysis genes, forms the coat termination hairpin, contains the Shine-Dalgarno sequence and the start-codon of the replicase gene (Olsthoorn et al. 1994). Expression of replicase is controlled by two mechanisms: the Min-Jou long-range RNA-RNA fold and the binding of the coat protein to the operator (Van Himbergen et al. 1993; Licis et al. 1998). Coat-RNA binding is at the same time the nucleation point of the viral packaging process (Peabody 1997).

Due to these structural constraints and functional overlap it is obvious that regions like the central operator loop are subject to a multitude of pleiotropic effects that strongly limit sequence evolution (Olsthoorn et al. 1994; Licis & Van Duin 2006). By means of an experimental evolution approach we aim at shedding light on the question how these structural constraints affect the evolution of the viral growth curve. To this end we confronted MS2 with a host environment that disturbs the natural balance of MS2 gene expression during the infection cycle. This was achieved by cloning individual genes of MS2 in an expression plasmid and in-trans production of a surplus of these genes during the infection cycle of MS2. By this approach we investigated the immediate physiological effects of viral gene disturbance on the shape of the viral growth curve. Furthermore, we followed the adaptation of MS2 to these conditions of disturbed gene regulation in order to investigate in how far the virus can evolve its own gene regulation to restore the viral growth curve. Knowledge of this evolutionary flexibility of the viral growth curve is essential for predicting viral evolution, since the shape of the viral growth is a key ingredient for models of optimal viral life history.

2.3 Material & Methods

2.3.1 Strains and growth conditions

We acquired MS2 ancestor by heat induction (10 min 42°C) from the MS2 cDNA plasmid pMS2000 that we received as a generous gift from René Olsthoorn. The host E.coli Top10F' (*Invitrogen*) was used for all experiments under standard growth conditions (37°C, Luria broth LB). The plasmid pBAD and the F' plasmid, required for MS2 infection, were maintained by Ampicillin (Amp) and Tetracycline (Tet) resistance markers, at 100 μg/ml Amp and 60 μg/ml Tet (LB+Amp+Tet) in all growth media.

2.3.2 Cloning

The ORF's of the genes maturation, coat, lysis and replicase were PCR amplified from pMS2000 by forward primers that introduce a NcoI restriction site at the start codon and reverse primers that introduce a HindIII restriction site after the stop codon. The PCR products were sequentially digested by NcoI and HindIII (*New England Biolabs*) and ligated into NcoI and HindIII digested plasmid pBAD (*Invitrogen*). All constructs were verified by sequencing. We refer to the plasmids carrying the maturation, coat, lysis and replicase ORF's as pMat, pCoat, pLys and pRep, respectively. The primers that were used for cloning are summarized in Table 1.

2.3.3 Protein electrophoresis

After induction, the cells carrying pMat, pCoat, pLys and pRep were expected to express an additional protein of molecular weights of 42 kDa, 12 kDa, 8 kDa and 60 kDa (Katanaev et al. 1996), repectively. To verify this, cells carrying pMat, pCoat, pLys, pRep and empty pBAD were diluted 1:10 into LB+Amp+Tet and grown for 4h prior to induction with 10 mM Arabinose (ara). Upon induction the cells were grown at 30°C for additional 4h, paletted by centrifugation and boiled

for 5 min in sample buffer (4 % SDS, 10 % 2-Mercaptoethanol, 20 % Glycerol, 0.1 % Broomphenol blue, 100 mM Tris, pH 8.0) prior to electrophoresis on a 4-20% polyacrylamide gradient gel for 2-4h at 300V. For the cells carrying pMat, pCoat, pLys and pRep we observed bands at the expected molecular weights that are absent in the negative control (Figure 1A). Expression of the smaller lysis protein could not be visualized by protein electrophoresis, but could be demonstrated by its phenotype of cell lysis upon ara induction of a pLys-carrying strain (see Figure 1B).

Table 1: Primers used for cloning of the four MS2 genes

Maturation

	_NcoI___ _____*maturation*_____
Forward	5' GCGCG**CCATGG** CACGAGCTTTTAGTACCCTTG
	_Hind3__ stop ___*maturation*_____
Reverse	5' GCGC**AAGCTT** ***CTA*** TCTAGAGAGCCGTTGCCT

Coat

	_NcoI____ _____*coat*_____
Forward	5' GCGCG**CCATGG** CAGCTTCTAACTTTACTCAGTTCG
	_Hind3__ stop ___*coat*_____
Reverse	5' GCGC**AAGCTT** ***CTA*** TTAGTAGATGCCGGAGT

Lysis

	_NcoI____ ___*lysis*_____
Forward	5' GCGCG**CCATGG** CAGAAACCCGATTCCCTCAG
	_Hind3__ stop ___*lysis*_____
Reverse	5' GCGC**AAGCTT** ***TTA*** AGTAAGCAATTGCTGTAAAG

Replicase

	_NcoI___ _____*replicase*_____
Forward	5' GCGCG**CCATGG** CATCGAAGACAACAAAGAAGTTCA
	_Hind3__ stop ___*replicase*_____
Reverse	5' GCGC**AAGCTT** ***CTA*** CCGAGGAGAGCTCG

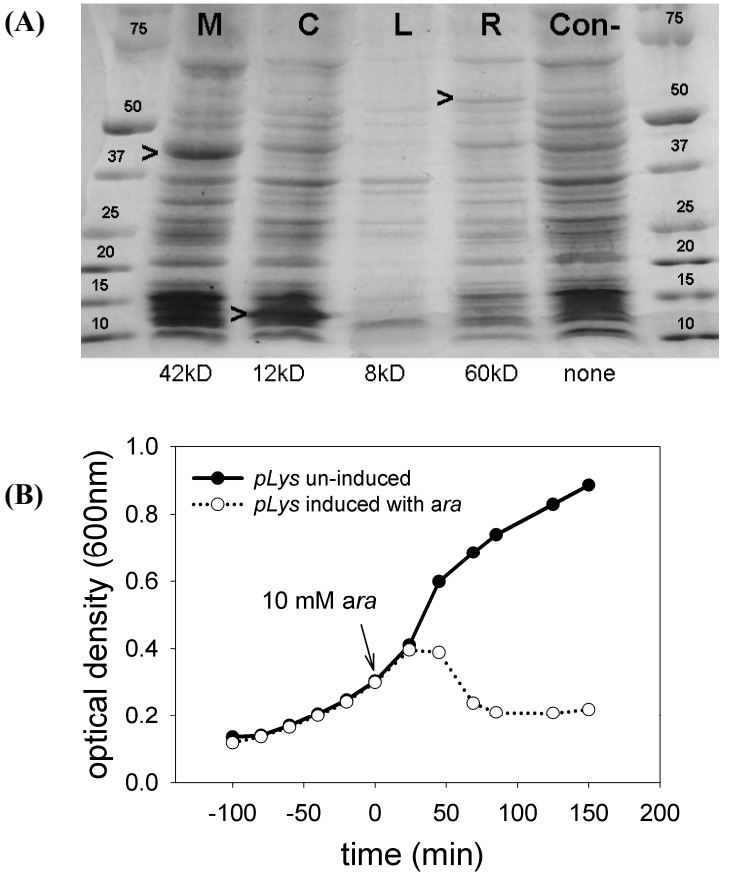

Figure1: Demonstration of gene expression in host cells carrying plasmids with an MS2 gene. (A) SDS-PAGE of total protein of cells carrying the plasmids pMat (M), pCoat (C), pLys (L) and pRep (R) after 2h of induction with 10 mM arabinose (ara). In case of pMat, pCoat, and pRep (R) an extra band is visible that corresponds to the expected size of the maturation (42 kDa), coat (12 kDa) and replicase (60 kDa) proteins, respectively. These bands are absent in cells carrying the empty pBAD plasmid (Con-). (B) The lysis protein (expected size 8 kDa) could not be visualized by SDS-PAGE. Instead expression of pLys was demonstrated by a drop of density in the bacterial growth curve after induction with 10 mM ara.

2.3.4 One-step growth curve assays

With the help of an automated setup (Figure 2) we were able to perform fine-scale measurements of the one-step growth curve of 8 viral strains in parallel. The strains were diluted to a titer of 10^6 pfu/ml. The titers were confirmed by plating and plaque count. Host cell cultures were inoculated in 10 ml LB+Tet+Amp from 100 µl frozen aliquots (in 40% Glycerol at -80°C) and grown aerated in exponential phase for 6 h until OD600=1 (~10^7 cfu/ml). With one exception, plasmid expression was induced 30 min prior to infection by 10 mM ara. In the case of pLys the time for induction was reduced to 5 min prior to infection, since after 30 min of induction of pLys most cells were already lysed (Figure 1B) (The latter effect was not yet known when the experiment underlying Figure 3B was performed. For this reason, the induction time of pLys was 30 min). Induced cells were infected at a multiplicity of infection smaller than one (MOI < 1) and incubated for 20 min at 37°C without shaking. The infected host cells were applied to a filter-holder that carries a 0.2 µm PVDF membrane (Millipore) and pre-rinsed by 20 times the volume of the filter chamber (20 x 0.2 ml) in order to remove free phage. Subsequently, we rinsed the infected cells by a constant flow (8 volume changes per 10 min) of growth medium and collected the filtered fractions in a 96-well block. The 96 phage fractions thus obtain a stable titer since the infected host cells are retained on the filter. The rate of phage production per time interval for each of the 8 growth curves was determined by top-agar plating and plaque count of all 96 obtained fractions (12h incubation at 37°C and saturated humidity).

2.3.5 Estimation of burst size and lysis time

We estimated burst-size as the ratio of the phage fraction with the highest titer and the titer in the initial fraction. Lysis time was estimated by the time point after which the slope of the one-step-growth curve starts to decrease. The reference values of lysis time and burst size of

the ancestor in the absence of overexpression were based on the data underlying Figure 3A (Burst-size 1.6*103 pfu, lysis time 65min).

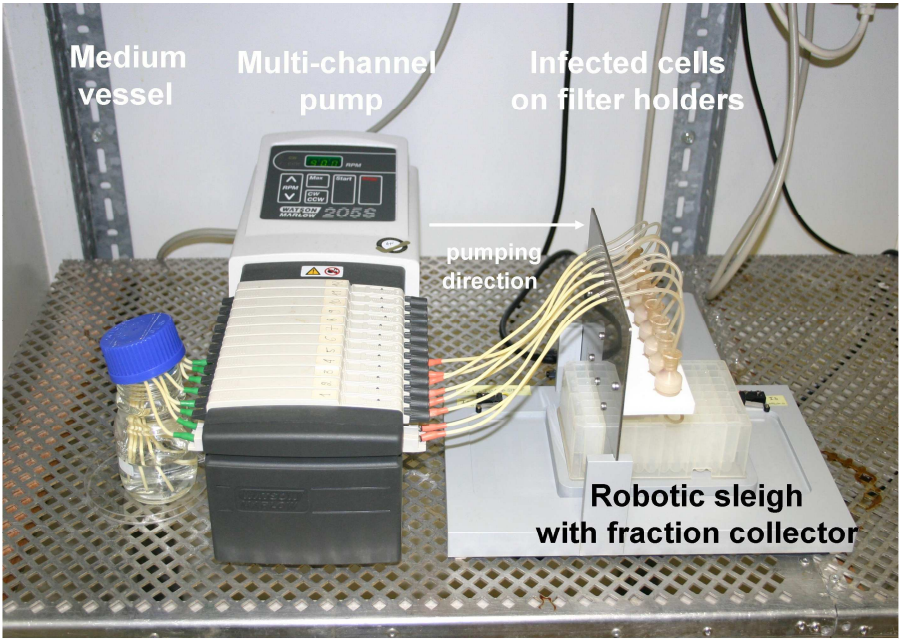

Figure 2: Automated setup for the measurement of the one-step growth curve. Infected host cells are immobilized on 0.2 µm filters that retain infected bacteria but allow newly produced phage to pass through the filter. With the aid of a multi-channel peristaltic pump, sterile medium is pumped through the filters and all newly produced phage are washed through the filter into the tubes of a 96-well fraction collector. The fraction collector is moved to a new position at constant time intervals. The collected fractions of phage lysate per time interval can be used to determine the rate of phage production by plating and counting plaque forming units. By this method the growth curves of 8 viral strains can be assayed in parallel under identical environmental conditions.

2.3.6 Conditions of experimental evolution

Host strains were daily inoculated from frozen aliquots and grown in 6 ml LB in exponential phase for 3h and induced with 10 mM ara, 30 min prior to infection with MS2. As explained above, hosts that carry pLys, were induced 5 min prior to infection. Infected cells were grown for 6h at 37°C. At the end of the growth cycle the remaining host cells were killed by the addition of chloroform and vortexing. Phage were transferred to freshly grown and induced cultures daily, with the aid of sterile toothpicks.

2.3.7 Estimation of viral fitness

A virus that grows with burst size B and generation time τ can go through $\frac{1}{\tau}$ generations per time interval and therefore produces $B^{1/\tau}$ offspring. We take the logarithm of this as our fitness measure

$$W = \log\left(B^{1/\tau}\right) = \frac{1}{\tau}\log\left(B\right).$$

The values in Figure 5 are represented relative to the fitness of the ancestor in the absence of overexpression

$$W_A = \frac{1}{65}\log\left(1.6 \cdot 10^3\right).$$

2.4 Results

2.4.1 Immediate effect of MS2 gene overexpression

The overexpression of all four viral genes negatively affected the growth of MS2, but the magnitude strongly differed between genes (see Figure 3). Whereas the overexpression of *coat*, *replicase* and *lysis* reduced the burst size by two or three orders of magnitude (factor of 1500, 400 and 1000 respectively), the overexpression of *maturation*

reduced phage production only by a factor 3. As indicated above, this mild effect of *maturation* is not surprising, since overexpression of maturation has no known regulatory consequences for other MS2 genes (Poot et al. 1997).

2.4.2 Evolutionary adaptation to overexpression

We evolved viral populations on host cells that carry pRep, pCoat, pLys for 20 consecutive transfers in batch culture, with 4 replicates per treatment. Evolution to pMat-carrying cells was omitted, since expression of maturation showed little effect on the viral growth curve and will therefore only create weak selection. We refered to the 12 evolutionary lines as R1, R2, R3, R4, C1, C2, C3, C4 and L1, L2, L3, L4, respectively. To indicate the cycle numbers we use a notation like C1-20 for transfer 20 of line C1.

Within the first five days of experimental evolution the populations L2 and R2 went extinct. This suggests that selection was stringent. In order to enable survival of the remaining lines in the lysis overexpression treatment (L1, L3, L4) we reduced the induction of pLys from 30 min to 5 min prior to infection (equivalent conditions are used in Figure 4B).

Adaptation of viral strains to the conditions of overexpression substantially improved viral reproduction with respect to the unevolved ancestor (Figure 4). The effect of adaptation differed between the three overexpressed genes, but was higly consistent within the replicate evolution lines per treatment.

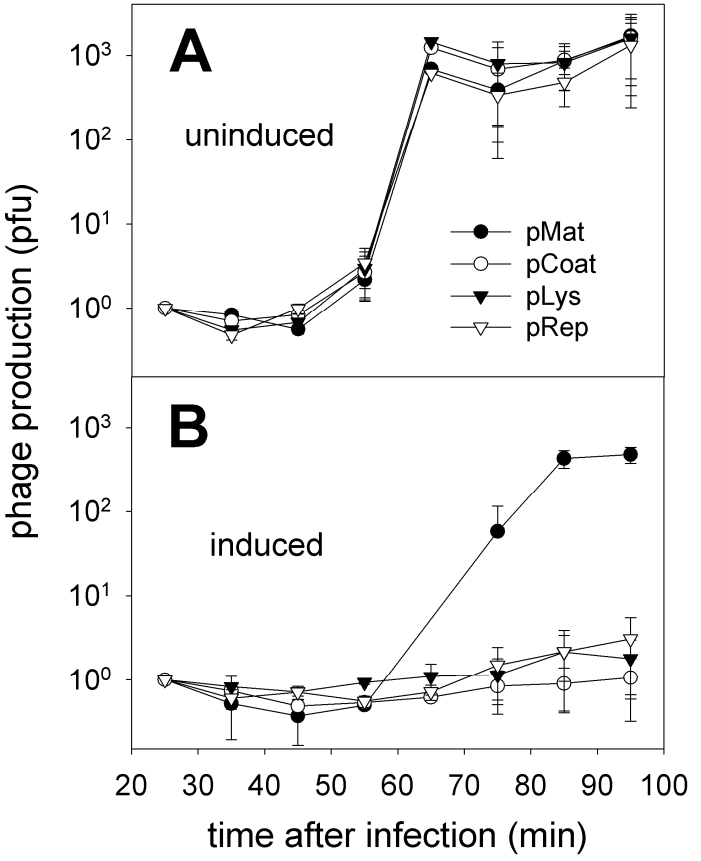

Figure 3: Effect of overexpression of MS2 genes on the one-step growth curve of the wildtype strain. (A) Growth curve of the wildtype strain of MS2 in host cells that carry the plasmids pMat, pCoat, pLys and pRep without the induction of protein expression. Burst size is about 1.6·103 pfu, and lysis time is about 65 min. (B) Growth curve of the wildtype strain after induction leading to overexpression of the maturation, replicase, coat and lysis proteins. Overexpression of replicase, coat and lysis proteins leads to a strong reduction in burst size, while there is only a marginal reduction in burst size in case of maturation-overexpression.

Strains that evolved under coat overexpression (Figure 4 A) recovered a burst size that is comparable to the burst size of the ancestor in the absence of overexpression (Table 2 and Figure 5 A). Surprisingly lines C2-20 and C3-20 even evolved a 3 fold higher burst size than that of 3 fold higher burst size than that of the wild type in the absence of overexpression. Apparently these two strains evolved a strategy to use the surplus of coat protein. However, the strains that evolved under overexpression of coat show a delay in lysis time by 10-20 min relative to the ancestor in the absence of overexpression (Table 2, Figure 5B). This negative effect of increased lysis time more then compensates for the increase in burst size. Hence after 20 generations of adaptation the overall fitness of the evolved lines was still smaller than the fitness of the ancestor in the absence of overexpression.

Table 2: Lysis time and burst size of evolved strains and wild type

Strain	*Lysis time*	*Burst size*	*95% confidence interval of burst size*
Ancestor	*65*	*1553*	*± 161*
C1-20	*75*	*983*	*± 484*
C2-20	*85*	*3196*	*
C3-20	*85*	*5012*	*± 646*
C4-20	*75*	*1418*	*± 249*
R1-20	*55*	*417*	*± 55*
R3-20	*55*	*394*	*± 59*
R4-20	*55*	*661*	*± 181*
L1-20	*30*	*1196*	*± 568*
L3-20	*30*	*905*	*± 79*
L4-20	*30*	*669*	*± 111*

* *no replicate*

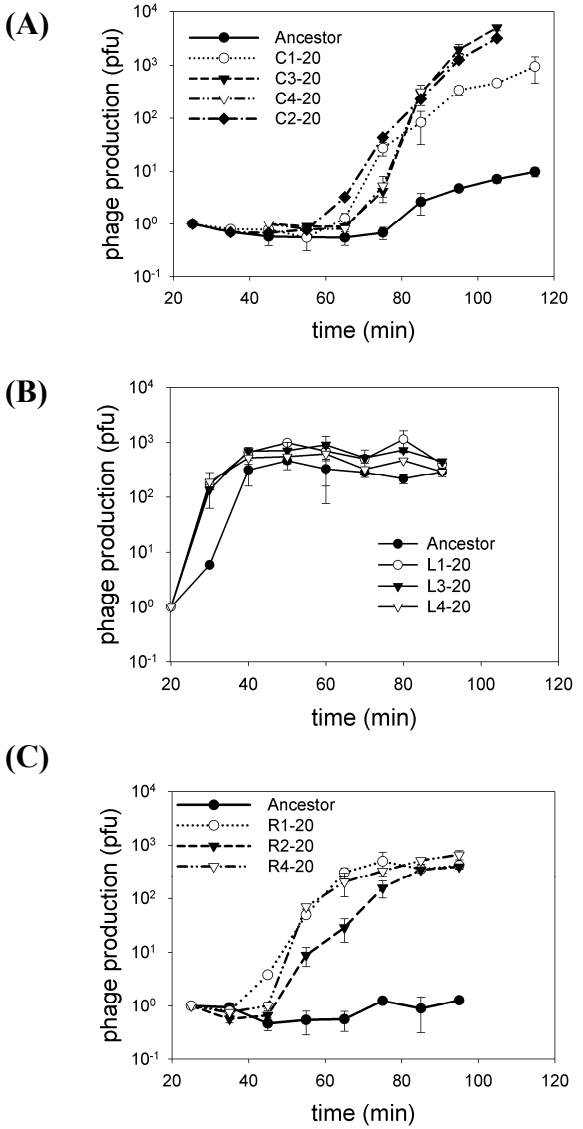

Figure4: One-step growth curves after adaptation to overexpression of the viral genes (A) coat, (B) lysis and (C) replicase. (A) Lines that were adapted to the conditions of coat overexpression (C1-20, C2-20, C3-20 and C4-20)

reach a burst size that is three orders of magnitude higher than that of the unevolved ancestor, but show a delayed lysis time. (B) Lines that were adapted to the conditions of lysis overexpression (L1-20, L3-20 and L4-20) reach a larger burst size and shorter lysis time than the unevolved ancestor. Within a short lysis time these strains produce a substantial burst size. (C) Lines that were adapted to the conditions of replicase overexpression (R1-20, R2-20 and R4-20) reach a burst size that is three orders of magnitude higher than the burst size of the unevolved ancestor and show a shortened lysis time.

Strains that evolved under lysis overexpression (Figure 4 B) reached a burst size that is below that of the ancestor in the absence of overexpression (Table 2, Figure 5 A). However, the relatively mild reduction was associated with a strong reduction in lysis time (30 min in strains L1, L3 and L4 vs. 65 min in the ancestor in the absence of overexpression) (Figure 5 B). Considering that this lysis time allows the strains to complete almost two generations in the time of a single generation of the ancestor, the adapted lines have a much higher fitness than the ancestor in the absence of overexpression (Figure 5C). (Note: Here the burst size of the ancestor under lysis overexpression is larger than in Figure 3B since the expression of lysis is milder. See M&M).

Lines that were adapted to replicase overexpression (Figure 4 C) could partially restore their burst size and showed a shortened lysis time. All the surviving lines R1, R3 and R4 showed a 10 min shorter lysis time than that of the ancestor in the absence of overexpression, at a burst size that is below the burst size of the ancestor in the absence of overexpression (Table 2, Figure 5A). The negative effect on burst size is partially restored by the shortened lysis time and adaption to replicase results in fitness that is comparable to the ancestor in the absence of over-expression (Figure 5C).

2.5 Discussion

The parameters of the viral growth curve are the central ingredients of optimality models that predict the adaptation of the viral life cycle to environmental conditions. The viral growth curve reflects the molecular mechanisms underlying viral development, but the interplay of these mechanisms is largely unknown. Previous experimental work has mainly focused on the effect of lysis gene expression on the timing of lysis (Heineman et al. 2005; Wang 2006). Here we consider the balance of all viral genes that are expressed upstream to the expression of the lysis gene. In order to systematically investigate the fitness consequences of the disturbance of all four genes of the bacteriophage MS2 we overexpressed each of its genes individually and determined the effect of overexpression on the viral growth curve. Furthermore we evolved the virus under conditions of viral gene overexpression enabling us to study viral adaptation to a disturbance of gene balance.

Overexpression of *coat*, *replicase* and *lysis* reduced viral fecundity by orders of magnitude whereas *maturation* had only a weak effect on viral reproduction. The weak effect of *maturation* is not too surprising since maturation has no known regulatory interactions with other viral genes (Poot et al. 1997). Overexpression of *maturation* is therefore not likely to disturb the balance of viral development and has only minor consequences for the viral growth curve. In contrast to *maturation* the genes *coat*, *replicase* and *lysis* are coupled by mutual regulatory interactions that balance genome replication and protein synthesis. In the natural infection cycle of MS2 the expression of replicase is downregulated by two independent mechanisms. The so-called Min-Jou long-range RNA interaction prevents replicase expression in the absence of coat translation (Van Himbergen et al. 1993). In addition coat protein acts as direct translation repression of replicase by binding to the translation initiation site of replicase (Peabody 1997).

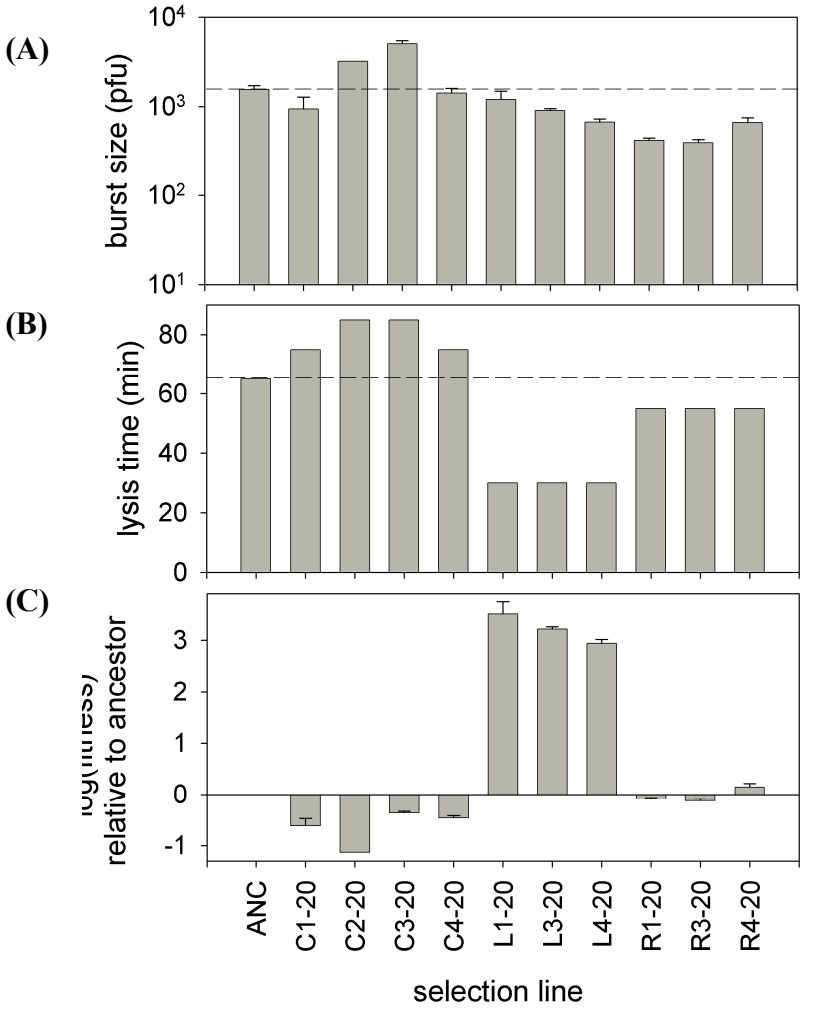

Figure 5: Burst size, lysis time and fitness of evolved lines relative to the ancestor in the absence of overexpression. (ANC represents the ancestor)

This redundancy in the repression of replicase suggests that uncontrolled replicase expression has severe fitness costs. These costs probably arise from a trade-off between the production of viral RNA and the production of viral proteins. Viral RNA replication consumes host resources in the early stage of development that may become limiting during late development. Excessive viral RNA replication would be inefficient since more genome strands would be produced than could be packaged. This effect was demonstrated during the formation of MS2 particles in an in vitro translation system (Katanaev et al. 1996). When increasing amounts of viral RNA are added to an in vitro translation system the production of MS2 particles increases until a maximum in the production of viral particles is reached. When more than this optimal amount of viral RNA was added, the production of viable viral particles sharply declined. In our experiment, the fitness effect of replicase overexpression is likely caused by the same trade-off between the production of viral RNA and proteins. The limited degree of adaptation to *replicase* overexpression achieved in our experiment indicates that this trade-off cannot easily be compensated by changes in viral gene regulation. In view of these results, stimulation of viral genome replication might offer an interesting anti-viral strategy that is resilient to viral adaptation. This is in striking contrast to many anti-viral strategies that aim at the exact opposite – a reduction of viral genome replication (Xu et al. 2007; Paeshuyse et al. 2007; Kong et al. 2007; Vrancken et al. 2008).

Coat is a direct repressor of replicase (Peabody 1997). Overexpression of coat has therefore been proposed as a potential anti-viral strategy in the MS2-related phage Qβ (Lindemann et al. 2002). Experiments designed to test this idea were, however, not able to maintain viral replication repression, since Qβ could readily adapt to coat overexpression by avoiding the repressive action of the coat protein. In our experiments we found the same rapid adaptation to overexpression of coat in MS2. Interestingly, adaptation to overexpression of coat even led to a burst size that by far exceeds that of the ancestor in the absence

of overexpression (C2-20 and C3-20 in Figure 5A). One explanation is that overexpression of *coat* provides a protein that is limiting viral growth during late viral development. For normal viral development the coat protein is required in an amount that is 6 to 10 times higher than that of any other viral gene (Beremand & Blumenthal 1979; Katanaev et al. 1996). During late infection coat might therefore be limiting and external production might relax this limitation. Since coat is a repressor of replication the virus nevertheless needs to avoid the repressive action of coat during early infection in order to utilize it during late infection. Evolution of a complete avoidance of coat-RNA binding is not possible, since the coat-RNA interaction is the starting point of viral packaging (Peabody 1997). For this reason residual replicase repression through the utilization of excess coat might explain the delayed lysis time in the phage that evolved to coat overexpression.

Viral packaging might become a limiting factor for intra-cellular viral development also for another reason. It has been proposed that in single-stranded RNA viruses the rate of packaging could exhaust the pool of replicating RNA genomes and push viral replication to a standstill (Krakauer & Komarova 2003). In principle, this might explain the delayed lysis time in the lines adapted to *coat* overexpression. For two reasons, we consider this explanation unlikely. First, the positive feedback of RNA replication on *replicase* production leads to an increase of RNA genomes at a rate that is proportional to the square of the RNA concentration, whereas the rate of packaging increases only linearly with the RNA concentration (Eigen et al. 1991). It is therefore not easy to perceive how packaging could outcompete RNA replication. Second, plus-stranded RNA (RNA+) viruses like MS2, copy through a RNA+ to RNA- template mechanism (Regoes et al. 2005). Since the RNA- templates are not packaged they accumulate in excess abundance (about 50 times the amount of RNA+ strands). Replication from RNA- templates will therefore continue even if all RNA+ strands are packaged. Accordingly, the exhaustion of the pool of viral RNA genomes through the packaging process is not likely to slow

down the viral production process in the absence of a repression mechanism for replicase.

Adaptation to lysis overexpression reduces lysis time by half at almost no costs for burst size. Halved lysis time allows the phage adapted to *lysis* overexpression to go through two generations in the time frame of one ancestral generation in the absence of overexpression. This greatly increases viral fitness. It is therefore an intriguing question why the virus does not reduce its lysis time during a natural infection. The most straightforward explanation is that in MS2 the achievable lysis time is constrained by the expression level and/or the efficiency of the MS2 lysis protein. Indeed, the single-component lysis system of phage MS2 is known to be less efficient than the two-component lysis system of phages of comparable genome size, like DNA phage ΦX174 or the RNA phage f2 (Loeb & Zinder 1961; Wang et al. 2000; Young et al. 2000). The two-component lysis systems of ΦX174 and f2, allow a lysis time of 25 min and 40 min, respectively, with a burst-size comparable to that of MS2 (Loeb & Zinder 1961; Beremand & Blumenthal 1979; Pepin et al. 2006). The relative inefficiency of the lysis system of MS2 is apparently caused by inefficient *lysis* expression, since artificial expression of the same lysis protein readily lyses host cells within 30 min after induction (Figure 1B). This constraint in lysis time in MS2 contrasts with the general idea that lysis time can evolve freely to match the environmental optimum (Bull et al. 2004).

Our findings demonstrate that the viral life cycle is integrated through the balance between processes during early and late viral development. Both lysis timing and virus production strongly reflect the interplay of viral genome replication and protein production. Models for the evolutionary optimization of the viral life cycle need to consider the finite supply of host resources as well as the trade-offs between replication and protein production. Integration of these molecular trade-offs into current theoretical approaches could provide an important link

between evolutionary modelling and detailed knowledge of viral gene regulation.

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Chapter 3
Spatial structure and the evolution of viral infectivity

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3.1 Abstract:

Virulence and infectivity are the main determinants of the harm imposed by a pathogen on its host population. Both pathogen properties are not static, but can be shaped by selection. The selective forces strongly depend on the spatial structure of the host and the pathogen population. In general, localized interactions are expected to select for a lower virulence and a lower infectivity. Here we apply this general idea to the adaptation of a bacteriophage to various spatial settings. By means of a spatially explicit model we demonstrate that evolution in a medium with low diffusivity leads to intermediate levels of infectivity and, correspondingly, to epidemics resulting in large plaques with fuzzy edges. In a well-mixed environment, infectivity will evolve towards much higher levels. When placed back into a spatially

structured medium, phage adapted to a well-mixed environment form small plaques with sharp edges. We tested this prediction experimentally by placing a strain of bacteriophage ΦX174 that was adapted to a spatially structured Petri dish environment into a liquid environment. As predicted by the model, the phage evolved an increased level of infectivity that was associated with a reduced plaque forming ability. Some of the experimental results are, however, puzzling. During evolution in the liquid environment, the fitness of the evolved lines (quantified by lysis time and burst size) decreased rather than increased in the course of evolution. We discuss potential causes for these unexpected findings.

3.2 Introduction

A large body of theory shows that the spatial structure of host and pathogen populations has major implications for the evolution of pathogen traits like virulence and infectivity (Boerlijst et al. 1993; Sato et al. 1994; van Baalen & Sabelis 1995; Rand et al. 1995; Keeling 1999; Haraguchi & Sasaki 2000; Johnson & Boerlijst 2002; Read & Keeling 2003; Lion & van Baalen 2008). Localized host-parasite interactions are subject to two evolutionarily relevant factors that are absent in well-mixed systems. First, highly infectious and virulent pathogens reduce the local pool of susceptible hosts which, as a consequence, hampers future spread (Sato et al. 1994; Rand et al. 1995; Boots & Sasaki 1999). Second, spatial viscosity leads to the clustering of related individuals. In such a situation, long-term benefits for the cluster of related individuals can outweigh the short-term benefits of high infectivity and virulence, thereby promoting more prudent predation (van Baalen & Sabelis 1995; van Baalen & Rand 1998; van Baalen 2002; Johnson & Boerlijst 2002; Lion & van Baalen 2008). The selective forces in a spatially structured population can therefore be fundamentally different than in a homogeneously mixed host population.

In a series of experimental and modeling studies, Boots and colleagues have argued that spatial structure will select for lower levels of infectivity, i.e. to a lower probability that an encounter of a pathogen with a host leads to infection (Boots et al. 2004; Boots & Mealor 2007). The main mechanism is self-shading, i.e. the phenomenon that a highly infectious pathogen hampers its own (long-term) rate of transmission by surrounding itself by an area of infected host individuals (Boots & Sasaki 2000). Boots and colleagues have developed their ideas for the context of a host population that upon recovery from infection attains life-long immunity (Boots et al. 2004). Experimental evolution of larvae of the moth Plodia interpunctella and their species specific granulosis virus (PiGV) has demonstrated that host motility can also be a mechanism that affects the evolution of infectivity (Boots & Mealor 2007). Here we argue that the same principles apply to the spread of a viral epidemic in a population of bacteria.

Spatial epidemics of bacterial viruses on a lawn of host bacteria, generally known as plaques, create a circular area of host extinction that increases at a rate depending on phage infectivity and virulence. The infectivity of a phage is determined by its rate of adsorption to the host cell, while phage virulence is inversely related to the timing of host lysis (Schlesinger 1960; Wang et al. 1996; Bull 2006). Phage reproduction in a plaque requires replication of phage inside the host cell and phage release through host lysis followed by dispersal though diffusion and adsorption to new host cells (Koch 1964; Yin & Mc Caskill 1992; You & Yin 1999; Fort 2002). High adsorption rate (high infectivity) rapidly exhausts the local pool of available hosts and delays the spread to uninfected regions of the host population (Yin & Mc Caskill 1992). It is evident that the evolution of infectivity will be strongly affected by such spatial aspects of the environment.

In order to investigate the effect of spatial structure on the evolution of phage life history we study the evolution of phage infectivity by a combined theoretical and experimental approach. In our theoretical

study we simulate the process of plaque formation and the evolution of phage adsorption rate with a spatially explicit individual based simulation model. The model enables us to link microscopic parameters like phage adsorption to macroscopic processes like the spread of a phage epidemic during plaque formation. It also yields an explicit prediction of the optimal adsorption rate as a function of the viscosity of the host population. To test the theoretical predictions, we followed the evolution of phage infectivity by experimental evolution of bacteriophage ΦX174. To this end we placed a strain of bacteriophage ΦX174 that was adapted to spatial plaque growth in a Petri dish environment into a liquid environment and followed the evolution of phage adsorption rate, phage plaque sizes and the timing of lysis for several months of serial batch transfer. This way we were able to link the evolutionary changes in infectivity and virulence to the rate of epidemic spread during plaque formation.

3.3 A simulation model for the spread of a phage epidemic

We simulated the process of plaque formation in a square lattice model that was implemented in the C package CASH (http://theory.bio.uu.nl/rdb/software.html). The bacterial host population was initialized by placing bacteria with the initial density b_0 at random locations on a grid of size 256 x 256 or 512 x 512, respectively. Host bacteria were immobile and did neither divide nor decay spontaneously. Viral particles could be in three states: diffusive, adsorbed to a host bacterium or taken up by a bacterial host. Diffusion was simulated by swapping adjacent grid points through the Margolus algorithm (Toffoli & Margolus 1987). The degree of swapping could be changed and reflects the degree of spatial structuring. Well-mixed conditions were simulated by swapping every grid point to a randomly chosen new location.

On a grid point with an alive bacterium, a viral particle adsorbs to the bacterium with rate β. This adsorption rate is the evolutionary parameter under consideration. Adsorbed viral particles can either desorb again (at rate β_{-1}) or they are taken up by the host with the fusion rate f. Infected hosts lyse at a rate $1/\tau$, where τ corresponds to the expected lysis time. Upon lysis, Y viral particles are released that all enter the diffusive state. We quantified viral fitness as the total number of viral particles after 200 and 2000 time steps in the well-mixed and spatially structured environment respectively. Standard parameters of the simulation were $\tau = 100$, $\beta_{-1} = 10^{-3}$, $f = 10^{-2}$, $b_0 = 0.6$, $Y = 10$, grid size 256 x 256 in Figure 1A,B and 512 x 512 in Figure 1C. In order to determine the rate of adoption β^* that maximises viral fitness we simulated the epidemic spread of a range of phages differing in their value of β values on a spatially explicit grid and under conditions of random mixing of the grid.

3.3.1 Simulation results

As expected the phage population grows exponentially under well-mixed conditions (Figure 1A), at least as long as uninfected host bacteria are still abundant. In a spatially structured population, however, the phage density increases at a quadratic rate. Quadratic increase is expected if the infection front corresponds to a travelling wave moving with constant speed (Yin & McCaskill 1992). It is evident from Figure 1A that population viscosity imposes a severe constraint on the growth rate of a phage population.

In addition to the immediate effect of population viscosity on the growth rate of the phage population, viscosity has more indirect long-term implications by affecting the evolution of infectivity. This can be concluded from Figure 1B that shows the effect of the adsorption rate β on viral fitness under both spatially structured and well-mixed conditions. In a well-mixed environment, viral fitness is positively

associated with β, implying that selection should maximize β. In contrast, in a spatially structured environment viral fitness is maximized for an intermediate adsorption rate β^* (here $\beta^* = 0.04$).

It is intuitively obvious that for small values of β an increase of the adsorption rate has a positive effect on viral fitness. But why is viral fitness in a spatially structured population negatively related to adsorption rate at high values of β? The reason can be seen in Figure 1C. For high values of β, many phage are delayed in their diffusion through temporary adsorption to already infected hosts. This loss is several times higher in a spatially structured environment than under well-mixed conditions, since in a spatially structured population newly formed phage particles are released in a partially 'harvested' area with relatively few non-infected host targets. This mechanism is analogous to the process of epidemic self-shading (Boots & Sasaki 2000; Boots et al. 2004). The effects of self-shading are directly visible in the shape of the plaques produced at high and low adsorption rates (Figure 2A). In case of a high adsorption rate, newly formed phage particles tend to harvest all available hosts in the vicinity of their release site. In contrast, a phage population with a low adsorption rate corresponds to a 'prudent predator' (van Baalen & Sabelis 1995) that leaves a large fraction of hosts unharvested. Prudent predation has the advantage that newly formed phage particles move for a long distance before adsorbing to a host. This implies that the infection front moves faster than in case of a population with high infectivity.

As a consequence, highly infective phage populations form relatively small, slowly expanding plaques with sharp edges, while low-infectious populations produce large, more rapidly expanding plaques with sharp edges containing a relatively large fraction of unharvested hosts. The optimal level of β is determined by the trade-off between the intensity of harvesting per unit area (which is high for high values of β) and the

area covered by the epidemic per time unit (which is high for a low value of β).

3.3.2 Model predictions

For the experiments described below our model leads to the following predictions. If a phage population adapted to a spatially structured environment (and, hence, with an intermediate infectivity) is placed in a well-mixed environment, infectivity should evolve to higher levels. When the newly evolved phage are placed back into the ancient spatially structured environment they should produce smaller and less fuzzy plaques than their ancestor. While the fitness of the newly evolved phage should be higher than that of their ancestor under well-mixed conditions, it should be lower than that of the ancestor under the original spatially structured conditions.

3.4 Experimental evolution of phage infectivity

3.4.1 Methods

3.4.1.1 Conditions of evolution

We used strain AP100 of bacteriophage ΦX174 (kindly supplied by Art Poon) which is well adapted to a spatial Petri-dish environment and evolved six replicate lines of this strain (A-F) in liquid host suspension. Phage were evolved on host *E. coli C122 mutT* that was grown from a frozen reference stock daily in LB broth (10 mM $MgCl_2$ and 5 mM $CaCl_2$) in 10 ml at 33 ºC. Using cells from the reference stock prevented the host to coevolve with the virus. After 4h of viral growth 500 µl of culture were chloroformed to eliminate bacteria and 10 µl of a 10^{-4} dilution of the supernatant was transferred into 10 ml of fresh LB broth into which 10 µl of host overnight culture was simultaneously added. By this treatment we evolved six replicate lines (A-F) for 130 cycles of batch transfer.

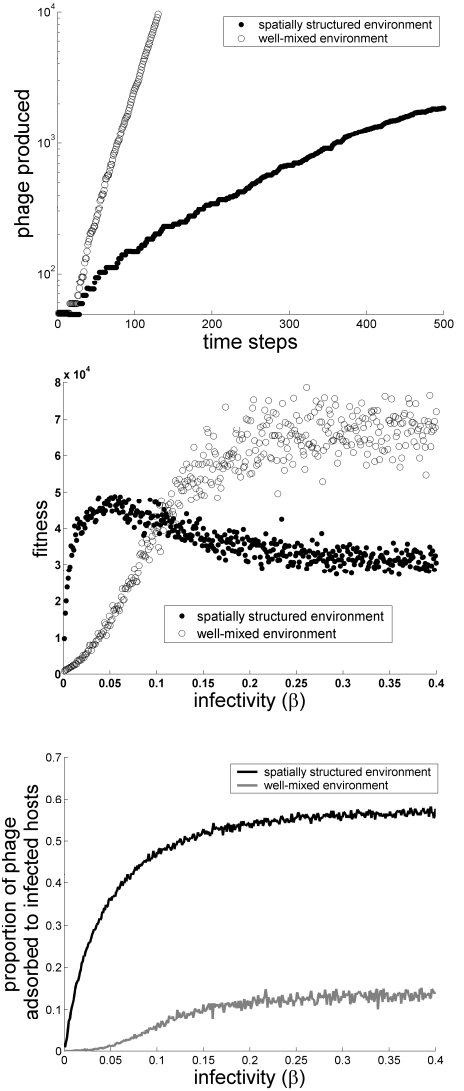

Figure1: Growth characteristics of a viral population predicted by the spatial model. (A) Growth of a viral population in a spatially structured (closed

circles) and a well-mixed (open circles) environment. The population grows at an exponential rate under mixed conditions and at a quadratic rate in a spatially structured host population. (B) Viral fitness (total number of phage particles after 200 and 2000 time steps in the liquid an well-mixed environment, respectively) as a function of the adsorption rate β *under spatially structured (closed circles) and well-mixed conditions (open circles). (C) Proportion of phage that are adsorbed to an already infected host in a spatially structured (closed circles) and a well-mixed environment (open circles). Parameters (A)* $\beta = 0.08$, *(B,C)* $\tau = 100$, $\beta_{-1} = 10^{-3}$, $f = 10^{-2}$, $b_0 = 0.6$, $Y = 10$, *Grid size 256 x 256 in (A) and (B); 512 x 512 in (C).*

3.4.1.2 Plaque size assay

We measured plaque size after plating 10 µl of diluted phage lysate with 300 µl of ~10^7 cfu/ml mid-log bacterial suspension in 3 ml 0.8% top-agar. Individual plaques formed after 6h and were photographed. Plaques were counted and plaque size (in pixels) was measured by the shareware Java package ImageJ (http://rsb.info.nih.gov/ij/).

3.4.1.3 Adsorption assay

Adsorption measurements were done following the method of Schlesinger (Schlesinger 1960). Phage were added at MOI < 1:100 to a late log-phase bacterial culture (OD 1.0 at 600 nm) that was iced and physiologically blocked with 0.1M KCN to avoid the production of new phage particles. After 1 and 20 minutes the culture was centrifuged at 14k rpm and the supernatant was plated. Adsorption rate was determined as the decay of plaque forming units between the 1 min and 20 min samples. To account for the noise of the stochastic adsorption process, the adsorption assay was repeated 10 times independently for each evolved strain, and 20 times for the ancestor.

3.4.1.4 One-step growth curve assay

The one-step growth curve was quantified with an automated setup that is described elsewhere (chapter 2). Host cells of E.coli C122 mutT were kept in exponential growth for 3h at 33° C and were infected with the phage lysate of transfer 130 of evolution lines A-D at 10^6 pfu/ml. Infected host cells were applied to a filter-holder that carries a 0.2 μm PVDF membrane (impermeable to host cells). Infected cells were pre-rinsed by 20 times the volume of the filter chamber (0.2 ml) in order to remove free phage. Subsequently, infected cells were rinsed by a constant flow (8 volume changes per 10 min) of growth medium and the filtered fractions were collected in a 96-well block. The rate of phage production per time interval for each of the 8 growth curves was determined by the titer of all 96 obtained fractions independently, by plating in top-agar 12 h and incubation at 33 °C followed by plaque counting. We defined lysis time as the point at which the slope of the growth curve starts to decrease and burst size as the difference between initial pfu and maximal pfu after the first burst.

3.5 Results

3.5.1 Evolutionary changes in infectivity and viral plaque formation

After 130 serial transfers in a liquid environment all replicate lines showed a 2.2 to 4.5-fold increase in the adsorption rate to host cells (Figure 3). In the ancestral line (adapted to a Petri dish environment), 20% of the free phage particles infect bacterial cells within 20 min. The evolved strains are much more infectious and infect 44-88% of available hosts in the same time window (Figure 3). All evolved strains A-F differ significantly from the ancestor (Tukey-Kramer post-hoc comparison, in all comparisons $p < 0.001$ number of replicate assays $n = 19$ for ancestor and $n = 10,7,9,10,10,8$ for evolved strains A-F).

Increased infectivity was associated with a rapid reduction of mean plaque size by a factor 5 to 18 in all six evolved strains (Figure 4). Furthermore, increased infectivity leads to a change in the appearance of plaques that is strikingly similar to the effects of infectivity in our individual based simulation (Figure 2). Whereas strains with low infectivity show large plaques with a broad infection wave, high infectivity leads to small sharp-edged plaques. In the course of adaptation temporary relapses of plaque size did occur, which disappear towards the end of the experiment at transfer 130 (e.g. C25, D80 F15 in Figure 4).

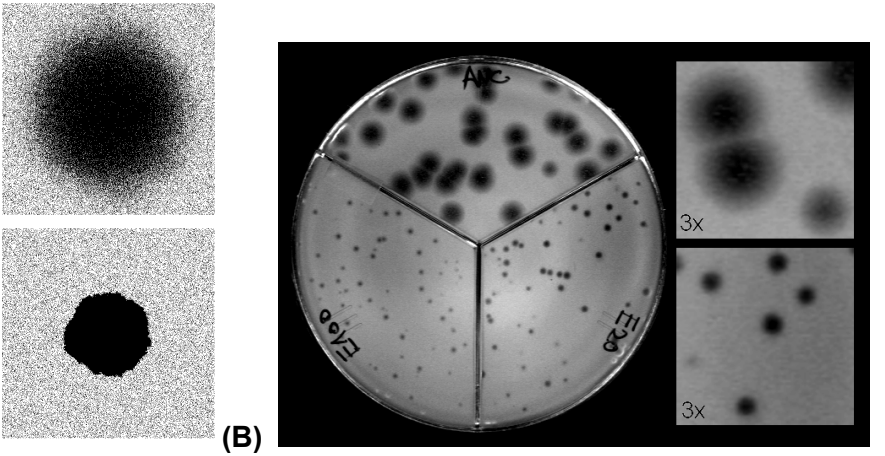

Figure 2: Spatial spread of viral epidemics. (A) Viral epidemics in the spatial simulation for a low adsorption rate (β=10-3, upper panel) and a high adsorption rate (β=0.5, lower panel). A high adsorption rate leads to small and slowly expanding plaques with sharp edges, while a low adsorption rate leads to large and more rapidly expanding plaques with fuzzy edges. (B) Plaques of ΦX174 in a lawn of host bacteria after 6h of growth. The ancestor (ANC, upper segment) produces large plaques with fuzzy edges, indicating that that the host population at the edge is only partially infected. After adaptation to a well-mixed liquid environment for 20 transfers (E20, lower right section) and 100 transfers (E100, lower left section) the evolved lines produce smaller plaques with sharper edges.

3.5.2 Evolution of the viral growth curve

All experimental results reported up to now are in line with our theoretical predictions: Evolution in a well-mixed environment led to the expected increase in infectivity (Figure 3) and this increase was associated with reduced plaque forming ability (Figure 4) and the predicted change in plaque morphology (Figure 2).

Two other results are, however, quite puzzling in the light of theoretical predictions. First, we tried to reverse the evolutionary trajectory by selecting for the ability of plaque formation in lines that had adapted to a liquid environment (lines A130-F130). Placing these lines into a spatially structured Petri-dish environment should lead to a reduction in infectivity and increased plaque sizes. This predicted response to reversed selection did, however, not occur (data not shown). Second, we quantified the one-step growth curve of the ancestral line and four of the evolved lines in a liquid medium (Figure 5). We expected the well-described trade-off between burst size and lysis time. Surprisingly, evolution of viral strains in liquid conditions led to a substantial fitness decrease in all evolved lines, since the evolved lines did not only exhibit a longer lysis time (30 min vs. 25 min for the ancestor), but also a substantially smaller burst size (between 100 and 200 pfu vs. 1000 pfu for the ancestor). Only line A has a comparable burst size, but still suffers from a delay in lysis time. Accordingly, the rate of offspring production was strongly reduced in all evolved lines, which is against all theoretical expectations.

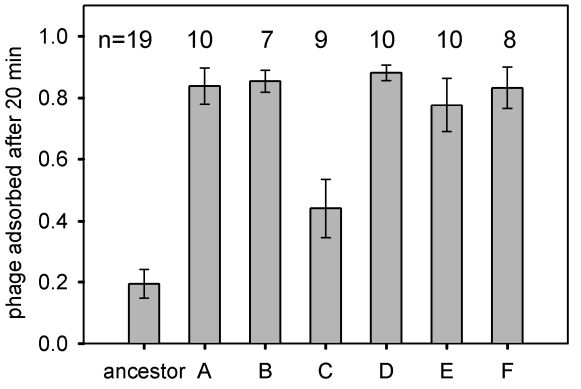

Figure 3: Adsorption rate of ancestor and evolved lines. Percentage of viral particles adsorbed to host cells within 20 minutes of the ancestor and evolved lines A-F. Error bars indicate 95% confidence intervals. The adsorption rate of the ancestor (20%) is considerably lower than that of all evolved lines (44% to 88%). Numbers above bars indicate the number of replicate assays performed per strain

3.6 Discussion

The spatial context of a pathogen-host interaction has a direct, short-term effect on the dynamics of epidemic spread (Grenfell & Harwood 1997; Wallinga et al. 1999; Keeling 1999). In addition, spatial structure has a long-term effect on the evolution of pathogen virulence and infectivity (Sato et al. 1994; van Baalen & Sabelis 1995; Rand et al. 1995; Boots & Sasaki 1999; Keeling 1999; Johnson & Boerlijst 2002; van Ballegooijen & Boerlijst 2004). We studied the effect of spatial structure on the evolution of viral infectivity in the context of bacteriophage plaque formation. By means of a spatially explicit stochastic model we show that in a spatially structured environment infectivity should evolve to an intermediate optimum, while a well-mixed environment should select for a maximal level of infectivity.

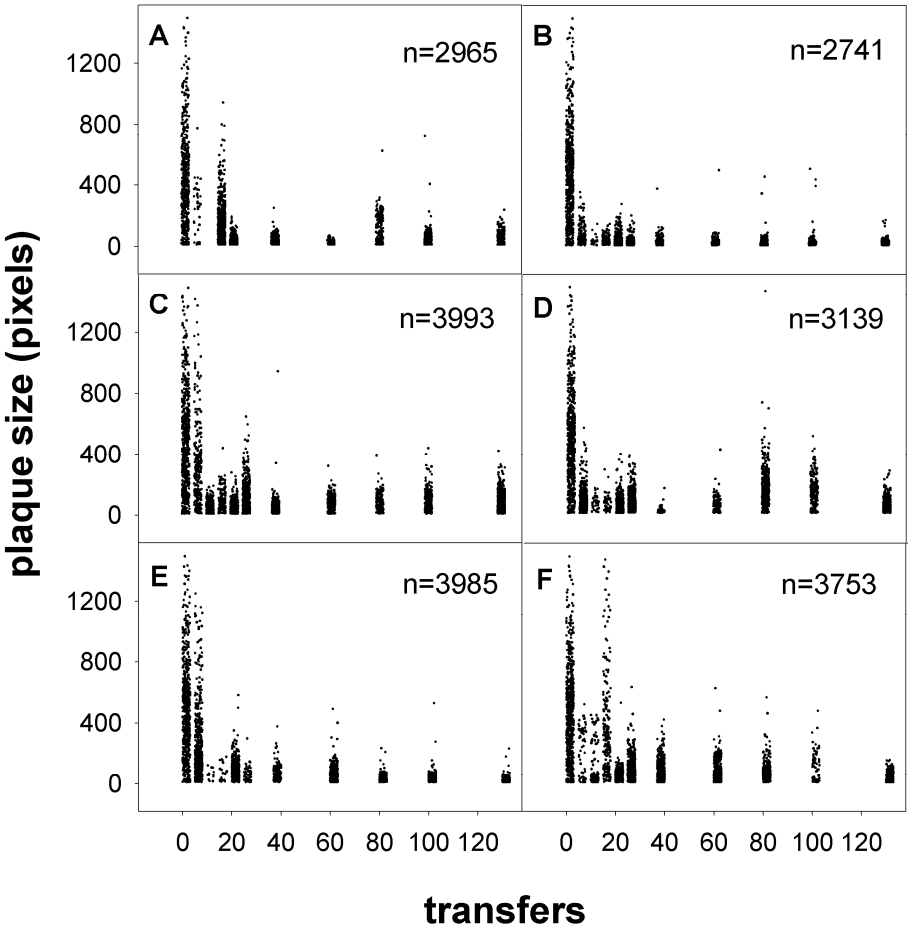

Figure 4: Evolution of plaque size during adaptation to a liquid environment. Each dot represents the size of an individual plaque. Plaque size was measured in each of the six replicate lines A to F at various time points of the evolutionary trajectory (transfer 0 = ancestor to transfer 130). n-values represent the total number of measurements per line.

The process of plaque formation is an ideal context to study the role of spatial structure on the evolution of pathogen life history. The biochemical properties that determine the phage life cycle and the process of plaque formation are well defined and can therefore directly be derived from first principles (Koch 1964; Yin & Mc Caskill 1992; Abedon & Culler 2007a; Abedon & Culler 2007b). As a conseuqence the effects of spatial structure can be related to micro-level mechanisms.

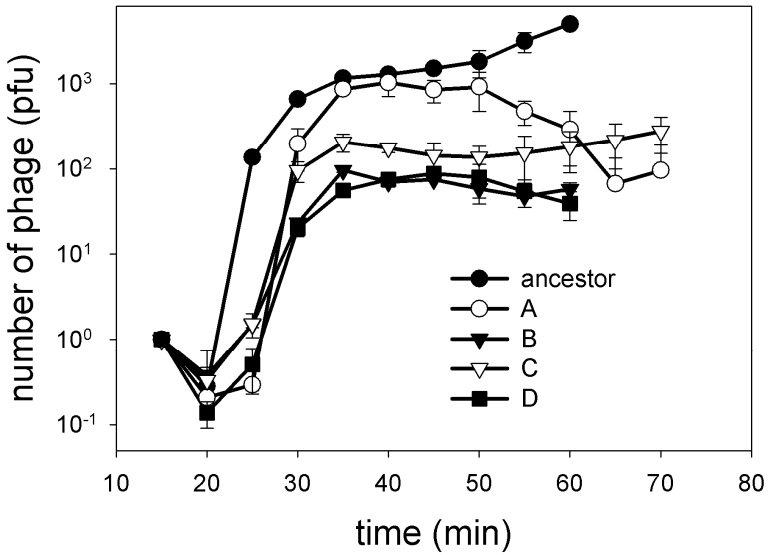

Figure 5: One-step growth curve of the ancestor and the evolved lines A to D. Time course of phage density in a liquid environment for the ancestor (that was adapted to a Petri-dish environment) and four lines that had an evolutionary history of 130 transfers in a liquid environment. Lysis time was about 25 min for the ancestor and 30 min for the evolved lines. Burst size was about 1000 for the ancestor and about 1000, 100, 200, and 100 for lines A to D, respectively.

In spatially structured host populations highly infectious pathogens may succumb to their own success when they locally exhaust the pool of susceptible hosts (Sato et al. 1994; Rand et al. 1995; Boots & Sasaki 1999). Pathogen impact on the host population during plaque formation is particularly severe, especially in lytic bacteriophages like ΦX174. In the center of the plaque the host population is driven close to extinction and any phage that fails to disperse to an area of uninfected hosts loses any future reproductive output (Koch 1964). These processes favor prudent predation of the host population for two reasons. First, high relatedness within one plaque can shift selection on the individual towards selection on the fecundity of the entire plaque. All phage individuals within one plaque are descendents of a single phage ancestor. Generally, relatedness of phage within one plaque should therefore be higher than the relatedness of phage originating from different plaques. When highly related individuals are clustered in space, long-term benefits for the cluster of relatives can outweigh the short-term benefits of increased virulence and infectivity for the individual (van Baalen & Sabelis 1995; van Baalen & Rand 1998; van Baalen 2002; Johnson & Boerlijst 2002; Lion & van Baalen 2008). By this mechanism long-term benefits for 'plaque fecundity' can overrule the fitness costs for the individual phage (Abedon & Culler 2007b). Second, higher infectivity does not necessarily lead to a higher fitness in a spatially structured population due to pathogen self-shading (Boots & Sasaki 2000; Boots et al. 2004). In the case of bacteriophage plaque formation, self-shading is mediated by a slightly different mechanism. During plaque formation the rim of the plaque contains a high concentration of infected host cells. Adsorption of phage particles to this barrier of infected hosts hampers the dispersal of phage particles in the center of the plaque towards an area of uninfected hosts. Reversible adsorption to infected hosts delays viral diffusion (Yin & McCaskill 1992). More importantly, the adsorption to infected hosts reduces viral fecundity. Even if a phage that adsorbs to an already infected host cell succeeds in co-infecting the host cell it has to share the resources of the host cell with the first infecting phage and will therefore produce at

best half the offspring it would gain from a single infection. Moreover, many viruses exhibit mechanisms that provide immunity against co- and superinfection (see chapter 4 of this thesis). Immunity to superinfection is well known in bacteriophages and has also been described in phage ΦX174 (Hutchison & Sinsheimer 1971). Adsorption to infected host cells that are immune to superinfection therefore creates a high fecundity cost which results in a strong effect of self-shading in the process of plaque formation.

So far we have considered the evolution of infectivity independent of changes in lysis timing and viral burstsize. Our observation that ΦX174 evolved towards reduced rates of reproduction (Figure 5) is puzzling since viral adaptation to a static environment generally leads to increased viral reproduction towards a fitness maximum (Bull et al. 2000). Nevertheless, evolution towards reduced rates of reproduction can occur when the environment of a virus changes in the time course of evolution. In our case the environment might have changed through three basic mechanisms: (1) increased host resistance caused by pathogen-host co-evolution (Buckling & Rainey 2002); (2) the accumulation of defective interfering particles (Dennehy & Turner 2004); or (3) the occurrence and accumulation of a viral competitor (Hattman & Hofschne 1967; Huppert et al. 1967; Dales & Silverbe 1968; Freda & Buck 1971; Goldman & Lodish 1975).

The possibility of host-pathogen coevolution was excluded on purpose by the setup of our experiment. At the end of each infection cycle all host bacteria were killed by chloroforming and replaced by host bacteria from a reference strain. Therefore the host bacterium could not evolve resistance to the prevailing virus population. Co-evolution of the host bacterium can therefore not explain the evolution towards reduced viral fecundity in our experiment.

In principle, the accumulation of defective interfering particles (DIPs) can be a factor that reduces viral fecundity (Dennehy & Turner 2004). Liquid conditions are prone to select for an increase of DIPs, since the

reproduction of DIPs requires their co-infection with a wildtype phage. In liquid conditions DIPs and wildtype are constantly mixed and co-infection readily occurs. In contrast, in a spatially structured Petri-dish environment DIPs can find themselves in a region that does not contain wildtype virus and therefore are not be able to reproduce. Furthermore, plaques that contain DIPs will have a lower fecundity than DIP-free plaques. Therefore selection in a spatially structured plaque will select against DIPs in the long run, whereas liquid conditions should allow for the invasion of DIPs. In principle at least, the reduction of viral reproduction during evolution in a liquid environment could therefore be explained by the accumulation of DIPs. Yet, we find this explanation improbable, since the formation of DIP's has never been described for ΦX174 under standard growth conditions (even though the formation of DIPs has been forced at low temperature (Espejo & Sinsheimer 1976)).

For this reason, we consider the occurrence and accumulation of another virus, which competes with the reproduction of ΦX174, as the most likely explanation for our puzzling findings. In the next chapter, we explore this possibility in more detail.

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Chapter 4
(Addition to chapter 3) Is the fitness loss of phage ΦX174 caused by competition with a pro-phage?

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4.1 Abstract

In a previous experiment several lines of phage ΦX174 that were adapted to a Petri-dish environment were transferred to a liquid environment were they could evolve for 130 transfers in batch culture. Unexpectedly viral fitness decreased (rather than increased) in the novel environment. This finding might be explained by competition of ΦX174 with a different species of phage. Here we show that the experimental lines did indeed contain a previously unknown phage. This phage has most likely entered our experiments from a pro-phage stage that is integrated in the genome of the host bacterium E.coli C122 mutT. We sequenced fragments of the genome of the unknown phage and compared them to known phage sequences. It turned out that the

unknown phage is a close relative to the lysogenic phages N15, HK22 and Φ80. We discuss possible mechanisms by which this unknown phage could reduce the reproduction of ΦX174.

4.2 Introduction

In a previous experiment several lines of phage ΦX174 that were adapted to a Petri-dish environment were transferred to a liquid environment were they could evolve for 130 transfers in batch culture. Unexpectedly viral fitness decreased (rather than increased) in the novel environment. This observation was puzzling since adaptation to a constant environment should generally lead to an improvement of reproductive output. After excluding alternative explanations we arrived at the conclusion that the fitness decrease of ΦX174 is caused by competition with a second species of phage. In order to detect and characterize this unknown phage we isolated viral DNA from a culture of E.coli C122 mutT, sequenced fragments of the viral DNA and compared them to known phage sequences.

4.3 Materials & Methods

E.coli C122 mutT was grown in LB (10 mM MgCl2 and 5 mM CaCl2) for 3h at 37°C until early stationary phase. To induce the unknown lysogenic phage, the culture was UV irradiated in a Petridish with 4.5 W, 233 nm UV (Mineralight UVS-54 handheld UV lamp) for 15 min and diluted 1:2 with LB medium and grown for one hour. The culture was filtered by 0.2 µm cellulose acetate filter (*Millipore*) and incubated with 200 U/ml DNAse (*Roche*) for 30 min at room temperature in order to digest remaining host genomic DNA. The DNAse activity was stopped by 50 mM EDTA, prior to destruction of the phage coat by 1% SDS and 5 vol% of 1:1 phenol/chloroform. The phage DNA was precipitated by 3 volumes of cold 100% Et-OH and 1 volume 3M Na-acetate. The isolated DNA was run on a 0.8% agarose electrophoresis gel. Phage DNA for cloning was digested by EcoRI and HindIII (*New England Biolabs*) overnight. The resulting restriction fragments were

ligated into EcoRI and HindIII digested plasmid puc18. Plasmids that carried an insert of phage DNA were sequenced by standad lacZ primers (*GATC Biotech AG*). The resulting sequences were queried against known sequences using BLAST (*NCBI*).

4.4 Results

DNA isolation and electrophoresis from the liquid fraction of an UV induced culture of E.coli C122 mutT showed a band of approximately 30kb which might represent the unknown phage (Figure 1). Sub-cloning and partial sequencing of 4 of the cloned DNA insertions revealed high sequence similarity with regions from the lysogenic phages Φ80, HK22 and N15 (see Table 1). The unknown phage is possibly a chimera of these lysogenic phages. Formation of chimeric variants between these lysogenic phages is common (Juhala et al. 2000). Yet, the presence of multiple lysogenic phages of similar genome size cannot be excluded.

Table 1: Sequence similarity of the unknown lysogenic phage with known phage sequences

Match	***Position***	***Nucleotide Similarity***
Accession number		
Bacteriophage Φ80	*2-126 (major coat*	*125 out of 125 (100%)*
emb|X13065.1|BP80ER	*protein)*	*560 out of 567 (98%)*
	5447-6013 (early region)	
Bacteriophage N15	*5479-6143*	*634 out of 665 (95%)*
gb|AF064539.1|AF064539		
Bacteriophage HK022	*17218-17490*	*250 out of 273 (91%)*
gb|AF069308.1|AF069308		

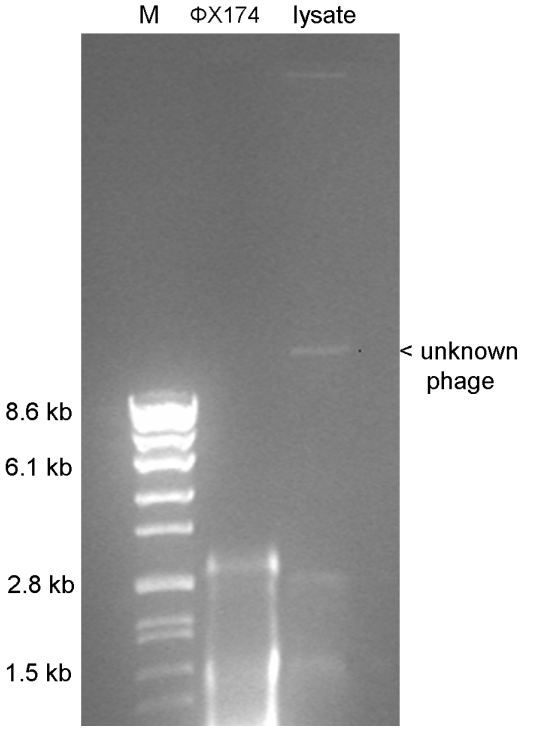

Figure 1: DNA isolation of phage ΦX174 and a UV induced lysate of host E.coli C122 mutT. First lane (M) contains marker DNA. Second lane (ΦX174) contains DNA from a ΦX174 isolate. Third lane (lysate) contains DNA of the unknown lysogenic phage that was extracted from the liquid fraction of an *E.coli C122 mutT* culture after UV induction. The genome size of the unknown phage is approximately 30 kb. [Note: The DNA band of ΦX174 appears smaller than its actual genome size (5.1 kb) as unrestricted ΦX174 DNA appears in its super-coiled form which runs faster in electrophoresis than linear DNA of the same size]

4.5 Discussion

We identified a previously unknown lysogenic phage which might be responsible for the decrease in reproductive efficiency of the lytic phage ΦX174 in our earlier experiments. This contaminant phage turned out to be notoriously difficult to detect and had therefore remained undetected for years, also in the work of several other labs. Lysogenic phages are known to stably integrate into the genome of the host bacterium and propagate to bacterial daughter cells through vertical transmission. We assume that the unknown lysogenic phage has entered our experiment from a pro-phage stage in the host bacterium *E.coli C122 mutT*, since the preperation of our host strain (loose colonies on an agar plate) exclude horizontal transmission.

Lysogenic phages are common. In a survey from the year 2003, 51 out of 82 full bacterial genome sequences were shown to contain at least one pro-phage, while some genomes contained up to 17 pro-phages accounting for approximately 10% of the bacterial genome (Casjens 2003). Even though lysogenic phages are common, they can easily go unnoticed. Lysogenic phage which are stably integrated into the host genome can have little effect on the host phenotype until a lytic cycle is artificially induced (e.g. by UV-mutagenesis or Mytomycin) (Oppenheim et al. 2005). Furthermore, classical plaque formation assays do often not detect lysogenic phage particles, since the original pro-phage carrying host strain is immune to infection of phage particles of the same species (Susskind et al. 1974; Kliem & Dreiseikelmann 1989). Phage particles therefore generally do not form plaques on their original carrier host. Our unknown lysogenic phage for example does not form plaques on its original host E.coli C122 mutT, but does form plaques on other pro-phage free E.coli strains. Detection of lysogenic phage therefore requires serendipity like the accidental plating on a pro-phage free host strain or a spontaneous lysis event. Alternative methods for the detection of lysogenic phages like electron microscopy or sequencing are quite involved. In our experiments the first indication

for the occurrence of the lysogenic phage was therefore the reduction of the fecundity of ΦX174 through competition with the lysogenic phage.

Even though the described interaction between a lysogenic and a lytic phage is accidental, it raises important question on (1) the mechanisms that trigged the activation of the lysogenic phage from the pro-phage state; (2) the mechanisms of competition by which the lysogenic phage reduces the reproduction of ΦX174; and (3) the selection on phage life-history that results from the competition of a lytic and a lysogenic phage in general. Competition between different species of viruses in mixed infections can result in significant loss of fecundity for both competitors (Huppert et al. 1967; Hattman & Hofschne 1967; Freda & Buck 1971). Yet, the mechanisms of competition are often unknown. In general the competition between two different species of viruses can occur on three levels: Competition for the availability of host cells, competition for resources during co-infection and the prevention of co-infection by interference mechanisms.

The ability to compete for available host cells is important for the success of a virus in our experimental treatment and is prone to select for altered life histories of both competitors. In every transfer we add a phage lysate that contains both competitors to a naïve host population (that carries the pro-phage). Rapid infection of the host population is therefore decisive for viral competition. A phage can increase its rate of infection by increasing its density in the lysate or by increasing its infectivity. Competition for available host cells therefore creates selective on two viral life-history parameters. First, competition for host cells selects for increased release of phage particles resulting in increased horizontal transmission. Second, competition for available host cells selects for increased rates of infection. Competition for host cells should therefore select for increased rates of infection in both phage and for an increased rate of horizontal transmission of the lysogenic phage. The selection for increased rates of infection might give an alternative explanation for the increase in adsorption rate of

ΦX174 that we observed in the experiments in chapter 3. Furthermore, the selection for increased horizontal transmission of the lysogenic phage can be a possible mechanism by which the lysogenic phage progressively increases its representation in the phage lysate and this way reduces the reproduction of ΦX174.

Alternatively, the competition for resources between phages that co-infect one host cell can be another mechanism by which the unknown lysogenic phage reduces the reproductive output of ΦX174. Within-host competition during co-infection acts though competition for the transcription and translation machinery of the host cell. Infection of the lytic DNA phage T4 , for example, can reduce the reproduction of RNA phage by competition for viral protein production (Hattman & Hofschne 1967) through competition of messenger RNAs (Goldman & Lodish 1975). Even though, intra-cellular competition for resources has not been studied in mixed infections with lysogenic phage, the competition for the production of proteins and messenger RNA are fundamental intra-cellular trade-offs that should also apply for competition of ΦX174 and the unknown lysogenic phage.

Next to competition for resources of lysogenic and lytic phage can involve interference mechanisms. In the lysogenic phage λ, for example, the exclusion proteins rexA and rexB prematurely lyse the host cell when a co-infecting lytic phage is detected and this way abolish the reproduction of the co-infecting competitor (Parma et al. 1992). The mechanism of premature cell lysis predicts a shortening in lysis timing. In contrast to this prediction we observed an increase in the burst time of all evolved strains (chapter 3, Figure 5). Premature lysis by the action of rexA and rexB is therefore not likely to be responsible for the reduced reproductive output of ΦX174.

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Chapter 5
Viral superinfection inhibition and the evolution of virulence

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5.1 Abstract

The competition of multiple pathogen strains within a single host is strongly affected by the replication rate and virulence of the competing strains. Strains with a higher replication rate and, hence, a higher virulence will typically have a competitive advantage. For this reason, many models for the evolution of virulence under superinfection assume that more virulent pathogens are better protected against superinfection. However this expectation is reversed in a broad range of benign viral systems, which deploy molecular superinfection inhibition mechanisms in order to win within-host competition. Superinfection inhibition mechanisms can lead to an increased rate of superinfection with increased virulence of the first infecting virus. By means of an evolutionary model that includes the molecular mechanisms of superinfection inhibition of bacteriophage λ we show that superinfection inhibition can lead to novel evolutionary dynamics like the evolutionary coexistence of virulent and non-virulent strains. Molecular mechanisms of superinfection inhibition also occur in other

benign viruses of different origins like phage M13, Hepatitis B Virus or Foamy Virus. Neglecting the mechanisms can lead to erroneous predictions on the outcome of viral evolution. In fact, these mechanisms might be crucial for the maintenance of viral benignity.

5.2 Introduction

The genomes of cellular organisms are interspersed by large numbers of viral genomes that reside in a dormant state. For example, 8% of the human genome consists of sequences of retroviral origin (Lander et al. 2001). Likewise, the average number of dormant pro-phage in all sequenced bacterial genomes is 2.6 and some bacterial genomes contain up to seventeen dormant viruses that constitute 10% of their total genome (Casjens 2003). It is still largely unknown why that many viruses remain dormant, while a more virulent strategy seems to provide obvious fitness benefits.

The evolution of virulence is affected by between-host and within-host competition. Between-host competition may reduce virulence when the reduction of host density, caused by virulence, hampers transmission (Anderson and May 1982; Ewald 1983). In contrast within-host competition is generally thought to favor increased virulence since rapidly replicating virulent strains have a competitive advantage over more slowly replicating variants (May and Nowak 1995; Nowak and May 1994; van Baalen and Sabelis 1995b; Frank 1996; Gandon et al. 2001; de Roode et al. 2005). The relative importance of within-host and between-host processes is still under debate. It has been argued (e.g. Ebert and Bull 2003) that direct within-host competition should typically dominate over between-host competition that is mediated by more indirect processes like the reduction of host density. If this is indeed the case an explanation has to be given how within-host competition can lead to reduced virulence and even viral dormancy.

The prediction that within-host processes should generally select for increased virulence are usually based on simple conceptual models that

do not consider mechanistic details of the competitive interactions of viral strains during co-infection. Yet, such details may be of crucial importance. An example are defective interfering particles (DIPs) that parasitize on the protein production of wildtype virus (Turner and Chao 1999; Chao et al. 2000; Dennehy and Turner 2004). By exploiting the wildtype, DIPs can spread, thereby reducing the overall production of viable viruses and, hence, virulence. However, DIPs can never spread to fixation, since they remain dependent on a co-infecting virulent wild-type strain. Accordingly, DIPs can partially explain a reduction of viral virulence, but not the widespread evolution of viral dormancy.

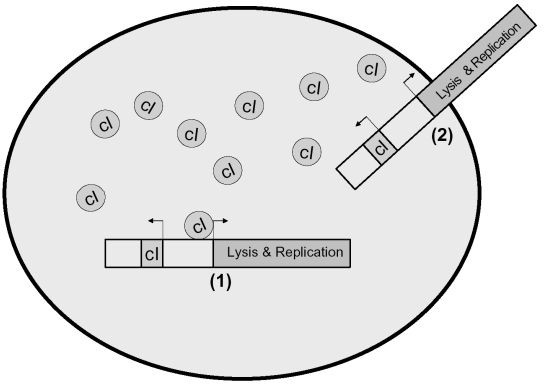
Here we discuss another mechanism that can yield such an explanation. In order to protect themselves from within-host competition, many RNA and DNA viruses of bacteria, plants and animals have evolved so-called superinfection inhibition that prevents the infection of already infected cells by other viral strains of the same viral species (Hutchison and Sinsheimer 1971; Susskind et al. 1974; Mcallister and Barrett 1977; Kliem and Dreiseikelmann 1989; Christen et al. 1990; Simon et al. 1990; Karpf et al. 1997; Ellenberg et al. 2004; Lee et al. 2005; Nethe et al. 2005; Huang et al. 2008). The mechanisms underlying viral superinfection inhibition are well studied. They often involve the repression of the replication machinery of the superinfecting virus. In many cases the molecular mechanisms that repress a co-infecting strain also limit self-replication of the resident virus and, accordingly, its virulence. Hence, reduced virulence might be a side effect of superinfection inhibition.

The mechanisms underlying the trade-off between superinfection inhibition and virulence are well studied in bacteriophage λ (Figure 1) (Bailone and Devoret 1978; Susskind and Youderain 1983; Oppenheim et al. 2005) and other benign viruses (Kliem and Dreiseikelmann 1989; Christen et al. 1990; Simon et al. 1990; Ellenberg et al. 2004; Lee et al. 2005; Nethe et al. 2005). Phage λ is a temperate bacterial virus that can propagate vertically, integrated as a pro-phage into the genome of its

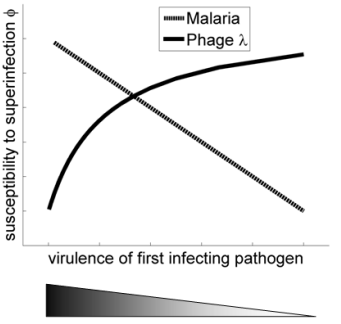
host, or horizontally by initiation of replication and host lysis. Since horizontal transmission requires host lysis it inevitably causes virulence. In contrast, during vertical transmission the pro-phage maintains a stable dormant state and virulence is low. The genetic mechanisms that repress the switch to the lytic cycle therefore control virulence. In phage λ, this switch to the lytic cycle is achieved by binding of the virulence repressor cI to the $p_L p_R$ promoter that controls the viral lysis and replication genes (Johnson et al. 1981; Ptashne 2004; Oppenheim et al. 2005). By the same mechanism the cI repressor also provides the host cell with superinfection inhibition against a second phage, when the cI repressor binds to the $p_L p_R$ promoter of the superinfecting phage (Figure 1A). Yet, this inhibition can be avoided by mutations in the the $p_L p_R$ promoter of the superinfecting phage (Figure 1A). These mutants however lose the ability to control their own virulence and are therefore termed ultra-virulent (Bailone and Devoret 1978).

Mechanisms as the one described above have not yet been incorporated into models of pathogen evolution. Generally superinfection models assume a decrease in the susceptibility to superinfection with increasing virulence of the resident pathogen (Mosquera and Adler 1998; Pugliese 2002; Boldin and Diekmann 2008), as has been demonstrated for malaria pathogens (de Roode et al. 2005) (Figure 1B). As the example of phage λ and other viral pathogens shows, it is by no means clear whether, and to what extent, this assumption is generally valid. It is therefore not self-evident that within-host competition should generally lead to higher virulence.

(A)



(B)



<u>Figure1: Virulence regulation and super-infection inhibition in phage λ</u> (A) A resident phage stays in the lysogenic state when the virulence repressor cI is bound to its pLpR promoter (1), thereby suppressing the resident's lysis and replication genes. By the same mechanism cI binds to the pLpR promoter of a superinfecting phage (2), thereby preventing its replication. This inhibits superinfection. Hence, viruses that produce a low level of repressor cI are more virulent and at the same time more susceptible to superinfection. (B) The mechanisms above create a positive association between virulence of the resident phage and the susceptibility to superinfection. This is in contrast to pathogens like malaria parasites where more virulent strains are less susceptible to superinfection.

In order to study the effect of these mechanisms on the evolution of viral virulence, we developed an evolutionary model that integrates biochemical mechanisms into a population model which considers the invasion of a rare viral mutant into an established ecological equilibrium of a resident virus. By this approach we aim to integrate the biochemical, ecological and evolutionary scales of superinfection and to provide a mechanistic view on the evolution of viral virulence under superinfection inhibition.

5.3 A model for the evolution of virulence under superinfection inhibition

We consider a virus that spreads by vertical and horizontal transmission between individual host cells. For simplicity we assume that the virus is highly infective and that, correspondingly, virtually all host cells are infected. Therefore a viral mutant can only increase in frequency when it is able to superinfect a host cell that is already infected by a resident virus. We will focus on the situation where co-infection eventually leads to take-over of the host cell by one of the competitors. The competition between the resident and a mutant strain of a virus can then be studied by following the dynamics of host cells that are infected by either type of virus (Nowak and May 1994). We represent this situation by two coupled differential equations that describe the density of hosts infected by the resident virus y_R and hosts infected by a mutant y_M, respectively:

$$\frac{dy_R}{dt} = ry_R\left(1-y_R-y_M\right)-\alpha_R y_R+\beta\left(\alpha_R\Phi_{MR}-\alpha_M\Phi_{RM}\right)y_R y_M \qquad (1a)$$

$$\frac{dy_M}{dt} = ry_M\left(1-y_R-y_M\right)-\alpha_M y_M+\beta\left(\alpha_M\Phi_{RM}-\alpha_R\Phi_{MR}\right)y_R y_M \qquad (1b)$$

The system has the following interpretation. In the absence of viral infection, the host grows logistically with intrinsic growth rate r and a carrying capacity that is normalized to 1. This part of the growth equation includes all mortality not induced by the viruses and it

determines the rate of vertical transmission of the viruses. The terms $\alpha_R y_R$ and $\alpha_M y_M$ correspond to virus induced mortality. Hence α_R and α_M characterize the virulence of resident and mutant, respectively. Virus-induced mortality is associated with cell lysis and viral reproduction. The number of newly produced resident virus is therefore proportional to $\alpha_R y_R$. The rate at which these viruses attempt to superinfect host cells incorporating the mutant virus is proportional to their abundance $\alpha_R y_R$ and to the abundance y_M of mutant-infected hosts. The constant of proportionality β includes the yield of virus production, diffusivity of the medium and the adsorption rate upon encounter with a host cell (which we assume to be the same for both types of virus). The crucial ingredient in our model is the superinfection term Φ_{MR}, which corresponds to the proportion of superinfection attempts that result in a 'take-over' of a mutant-infected host by the resident virus. Hence the rate of recruitment of new resident infected hosts due to successful superinfection by resident viruses is given by $\beta\alpha_R y_R y_M \Phi_{MR}$. Similarly, the recruitment of new mutant-infected hosts due to successful superinfection by mutant viruses is given by $\beta\alpha_M y_M y_R \Phi_{RM}$. Notice that the indices in the superinfection terms Φ_{RM} and Φ_{MR} reflect the order of arrival of the two types of viruses. Hence, Φ_{AB} corresponds to the probability that upon adsorption to a host infected by A a newly formed virus B takes over this host (Mosquera and Adler 1998).

When we consider the resident virus in the absence of a mutant (i.e. $y_M = 0$), the resident host population reaches the population dynamical equilibrium

$$y_R^* = \frac{r - \alpha_R}{r} \tag{2}$$

In this resident equilibrium a rare mutant can increase in frequency when its per capita growth rate is positive:

$$\frac{1}{y_M}\frac{dy_M}{dt}\bigg|_{y_R=y_R^*,\,y_M=0} = \alpha_R - \alpha_M + \beta\frac{r-\alpha_R}{r}\left(\alpha_M\Phi_{RM} - \alpha_R\Phi_{MR}\right) > 0\,. \qquad (3)$$

The per capita growth rate of a mutant in the resident equilibrium corresponds to the invasion fitness of the mutant (Geritz et al. 1998) that we will denote by W. Equation (3) shows how invasion fitness depends on virulence α and the susceptibility to superinfection Φ of both, the resident and the mutant virus. Although the rate of superinfection is often directly described as a function of virulence, e.g. $\Phi_{RM}(\alpha_R,\alpha_M)$, it is likely that virulence α and the rate of superinfection Φ are more indirectly related, e.g. via a correlation to some underlying trait or the concentration of some protein (like the virulence repressor protein cI of phage λ). In order to allow for both possibilities we choose a general approach in which α and Φ are functions of some trait x (that we will specify later). In other words we assume that

$$\alpha_R = \alpha(x_R),\ \Phi_{RM} = \phi(x_R, x_M) \qquad (4a)$$

and

$$\alpha_M = \alpha(x_M),\ \ \Phi_{MR} = \phi(x_M, x_R) \qquad (4b)$$

where x_R and x_M are the trait value of the resident and the mutant virus, respectively. Now, the invasion fitness can be rewritten as

$$W(x_M,x_R) = \alpha(x_R) - \alpha(x_M) + \beta\frac{r-\alpha(x_R)}{r}\left[\alpha(x_M)\phi(x_R,x_M) - \alpha(x_R)\phi(x_M,x_R)\right] \qquad (5)$$

Obviously $W(x_R, x_R) = 0$. This makes sense, since the resident should neither grow nor decline in a population of residents. A rare mutant with trait x_M will invade when $W(x_M, x_R) > 0$. The direction of selection (i.e. whether selection favors larger or smaller values of x) is therefore given by the selection gradient

$$\left.\frac{\partial W}{\partial x_M}\right|_{x_M=x_R} = -\alpha'(x_R) + \beta\frac{r-\alpha(x_R)}{r}\left(\alpha'(x_R)\phi(x_R,x_R) - \alpha(x_R)\left(\left.\frac{\partial\phi(x_M,x_R)}{\partial x_M} - \frac{\partial\phi(x_R,x_M)}{\partial x_R}\right|_{x_M=x_R}\right)\right) \quad (6)$$

Of particular importance are those resident strategies x_R^* where there is no directional selection any more (so-called evolutionarily singular strategies; Geritz et al. 1998), i.e. those resident strategies $x_R = x_R^*$ for which

$$\left.\frac{\partial W}{\partial x_M}\right|_{x_M = x_R^*} = 0 \quad (7)$$

The resident strategy x_R^* is an evolutionarily stable strategy (ESS) when the invasion fitness $W(x_M, x_R^*)$ has a maximum in the direction of the mutant strategy x_M or

$$\left.\frac{\partial W}{\partial x_M}\right|_{x_M = x_R^*} = 0 \quad \text{and} \quad \left.\frac{\partial^2 W}{\partial x_M^2}\right|_{x_M = x_R^*} < 0. \quad (8)$$

At an ESS all mutant traits x_M in the vicinity of x_R^* have a lower fitness than the resident. Accordingly, a resident population with strategy x_R^* is immune by invasion against mutants. An ESS is not necessarily reachable by a series of small gene substitution events

(Geritz et al. 1998). The resident strategy x_R^* is convergence stable (i.e. an evolutionary attractor) if

$$\left.\frac{\partial W}{\partial x_M}\right|_{x_M=x_R^*} = 0 \quad \text{and} \quad \left.\frac{\partial}{\partial x_R}\left[\left.\frac{\partial W}{\partial x_M}\right|_{x_M=x_R^*}\right]\right|_{x_M=x_R^*} < 0. \tag{9}$$

When x_R^* is convergence stable but not evolutionarily stable, evolution will converge to an evolutionarily unstable strategy; a so-called branching point. In this case, evolution a polymorphism of two (or more) coexisting strategies will result.

5.4 Simplified scenarios

5.4.1 Scenario 1: Superinfection directly related to virulence

We will now show that the relation between virulence and susceptibility to superinfection is decisive for the dynamics and outcome of evolution. To this end, we first consider the special case where virulence itself is the target of selection, i.e.

$$x_R = \alpha_R \text{ and } x_M = \alpha_M \tag{10}$$

Moreover we make the simplifying assumption that the rate of superinfection only depends on the virulence of the first infecting phage, which is related to its repressor concentration, or

$$\Phi_{RM} = \phi(\alpha_R),\ \Phi_{MR} = \phi(\alpha_M) \tag{11}$$

Thereby ϕ is a function taking on values between 0 and 1 and characterizes the nature of the trade-off between virulence of the first infecting phage and susceptibility to superinfection. (We will elucidate the biochemical link between repressor concentration, virulence and

superinfection in the next section.) Under these assumptions (10) and (11), the invasion fitness is given by

$$W(\alpha_M,\alpha_R) = \alpha_R - \alpha_M + \beta \frac{r-\alpha_R}{r} \left[\alpha_M \phi(\alpha_R) - \alpha_R \phi(\alpha_M)\right] \tag{12}$$

An evolutionarily singular strategy α_R^* is now given by the condition

$$\left.\frac{\partial W}{\partial \alpha_M}\right|_{\alpha_M=\alpha_R^*} = -1 + \beta \frac{r-\alpha_R^*}{r} \left[\phi(\alpha_R^*) - \alpha_R^* \phi'(\alpha_R^*)\right] = 0 \tag{13}$$

or equivalently

$$\beta \left[\phi(\alpha_R^*) - \alpha_R^* \phi'(\alpha_R^*)\right] = \frac{r}{r-\alpha_R^*} \tag{14}$$

In view of

$$\left.\frac{\partial^2 W}{\partial \alpha_M^{\,2}}\right|_{\alpha_M=\alpha_R^*} = -\beta \frac{r-\alpha_R^*}{r} \alpha_R^* \phi''(\alpha_R^*) \tag{15}$$

a positive solution $\alpha_R^* > 0$ of (14) is evolutionarily stable whenever $\phi''(\alpha_R^*) > 0$, or in other words whenever the superinfection function is convex in the vicinity of α_R^*. Making use of (14), the condition for convergence stability is given by

$$\left.\frac{\partial}{\partial \alpha_R}\left[\left.\frac{\partial W}{\partial \alpha_M}\right|_{\alpha_M=\alpha_R^*}\right]\right|_{\alpha_M=\alpha_R^*} = -\frac{r}{r-\alpha_R^*} - \beta \frac{r-\alpha_R^*}{r} \alpha_R^* \phi''(\alpha_R^*) < 0 \tag{16}$$

This is automatically satisfied whenever $\phi''(\alpha_R^*) > 0$. In other words, and evolutionarily stable strategy is always convergence stable.

However, an evolutionarily unstable strategy can also be convergence stable if $\phi''\left(\alpha_R^*\right)$ is not too negative. To be precise, a solution of (14) is a branching point whenever

$$-\frac{r}{\beta\alpha_R^*\left(r-\alpha_R^*\right)^2} < \phi''\left(\alpha_R^*\right) < 0 \tag{17}$$

Numerical analysis through a pairwise invasibility plot shows that in this case virulence α_R^* either evolves towards the branching point or towards the non-virulent strategy $\alpha_R^* = 0$, depending on the initial level of virulence (Figure 2).

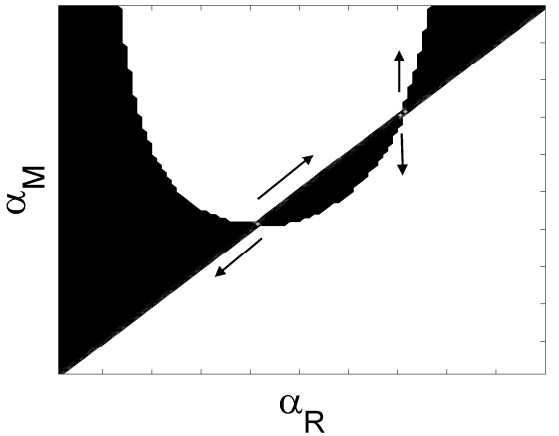


Figure 2: Pair wise invasibility plot for a concave superinfection function. A concave relation between the susceptibility to superinfection and virulence of the first infecting strain ($\phi''(\alpha) < 0$) can lead to three evolutionarily singular points: The ESS $\alpha^* = 0$, an evolutionary branching point for virulence and an evolutionary repellor which separates the two strategies (Superinfection function: $\phi(\alpha) = \alpha/(\alpha+1)$ Parameters: $\beta = 20$, $r = 1$).

5.4.2 Scenario 2: Virulence and susceptibility to superinfection are determined by repressor binding

In the previous section we showed that a concave positive relation between the rate of superinfection and the virulence of the first infecting virus can lead to evolutionary branching of virulence or the maintenance of a non-virulent population. Yet, we did not elucidate the origins of the relation between the susceptibility to superinfection and virulence. Therefore, in this section, we will derive this relation from the molecular mechanisms of viral virulence and superinfection control, along the example of bacteriophage λ. In λ the virulence α and the rate of superinfection ϕ are both determined by sigmiodal binding dynamics of the repressor cI to the pLpR promoter (Johnson et al. 1981; Hendrix et al. 1983; Ptashne 2004). Following these binding dynamics the virulence α_R of the resident phage is determined by the resident repressor concentration c_R and the resident promoter affinity k_R. The rate of superinfection ϕ_{RM} is in turn determined by the repressor concentration of the resident phage c_R and the promoter affinity of the super-infecting phage k_M (see Figure 1A). This way we can describe the proportion p of host cells, which have a repressor molecule bound to the $p_L p_R$ promoter, and remain in the lysogenic state, by second order receptor binding kinetics in the form

$$p = \frac{c^2}{k^2 + c^2} \tag{18}$$

In turn, the proportion of cells that switch to the lytic cycle is $1 - p$. Since the switching rate to the lytic cycle is equivalent to virulence α, we can write

$$\alpha_R\left(c_R, k_R\right) = 1 - p_R = \frac{{k_R}^2}{{k_R}^2 + {c_R}^2}, \tag{19a}$$

and

$$\alpha_M\left(c_M,k_M\right)=1-p_M=\frac{{k_M}^2}{{k_M}^2+{c_M}^2}. \qquad (19b)$$

On the other hand the susceptibility to superinfection is determined by the repressor concentration of the resident phage c and the promoter affinity of the super-infecting phage k, or

$$\phi_{RM}\left(c_R,k_M\right)=\gamma\frac{{k_M}^2}{{k_M}^2+{c_R}^2} \qquad (19c)$$

and

$$\phi_{MR}\left(c_M,k_R\right)=\gamma\frac{{k_R}^2}{{k_R}^2+{c_M}^2} \qquad (19d)$$

where γ is the relative binding efficiency between self and foreign promoter binding.

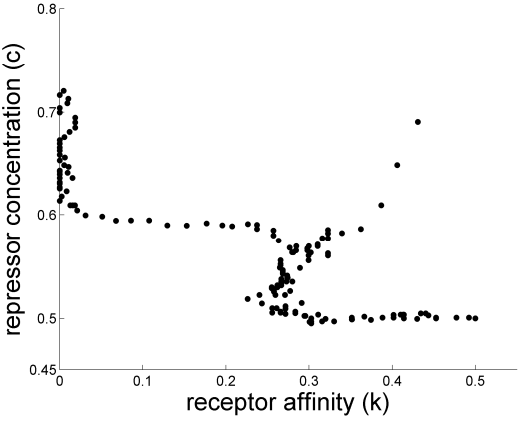
By the use of $\alpha_R\left(c_R,k_R\right)$, $\alpha_M\left(c_M,k_M\right)$, $\phi_{RM}\left(c_R,k_M\right)$ and $\phi_{MR}\left(c_M,k_R\right)$ we can now rewrite the system of two competing strains (1a, 1b) in terms of the underlying biochemical properties. This way we can represent the two trade-offs between virulence and superinfection inhibition, and the avoidance of superinfection inhibition and virulence, directly by the biochemical properties c and k. We can follow the evolution of the biochemical properties c and k by the numerical method of an evolutionary walk. Thereby, we introduce a rare mutant of c_M or k_M into a resident population c_R and k_R and integrate the system (1a, 1b) given the relations (8, 9). When a mutant increases in frequency and displaces the resident, the mutant becomes the new resident and the evolutionary path makes one step. By iteration of this process we can follow the correlated evolution of c and k and its effect on virulence α and the rate of superinfection ϕ.

The evolution of virulence for the biochemical model (1a, 1b, 8, 9) leads to evolutionary branching in virulence α, but evolution in the underlying parameters now proceeds in two dimensions (Figure 3). This leads to an adaptation process in several steps. At first virulence α decreases through directional selection towards lower receptor affinity k until it approaches the branching point. Close to the branching point the repressor concentration c increases until the population divides into two strategies: Low receptor affinity and low virulence and high receptor affinity and high virulence (Figure 3A). This way a single ancestor strategy can evolve into two coexisting strategies: A defense specialist that is analogous to the lysogenic phage λ wildtype and an attack specialist that is analogous to ultra-virulent mutants of phage λ. This example provides a mechanism by which virulent and non-virulent viruses can stably coexist in a natural virus population.

5.5 Disccussion

Within-host competition between parasites strains has fundamental consequences for the evolution of pathogen virulence (Pugliese 2002; Boldin and Diekmann 2008; de Roode et al. 2005; Nowak and May 1994; Gandon et al. 2001; Adler and Mosquera 2000). The competitive interactions of pathogen strains during co-infection are complex. A common approach to simplify these competitive interactions is the introduction of a superinfection function which describes the rate at which a first infecting pathogen is out competed and replaced by a second pathogen from its host cell. The superinfection function can be interpreted as a limiting case of co-infection with an instantaneous replacement of pathogens through competition.

(A)



(B)

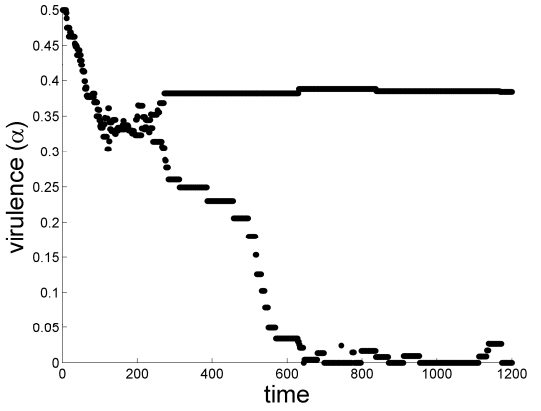


Figure 3: Evolutionary branching of virulence reflects the biochemical details of phage λ repressor binding. (A) Correlated evolution of repressor concentration and receptor affinity determine the evolution of virulence. Selection towards lower virulence decreases receptor affinity (= stronger repressor binding) until the branching point is reached. In the branching point repressor concentration continues to increase until a level is reached at which the population divides in into two strategies: Low receptor affinity and low virulence and high receptor affinity and high virulence. These strategies

are equivalent to lysogenic and ultra-virulent mutants of phage λ. (B) Accordingly, evolution decreases virulence until the branching point is reaches and disruptive selection leads to the co-existence of virulent and non-virulent population. (Parameters $\beta = 0.8$, $\gamma = 4$, $c_0 = k_0 = 0.5$*).*

Viral superinfection inhibition, as described in the case of phage λ has important consequences for the properties of the superinfection function and the evolution of virulence. First, in a host population which is fully infected by pathogen that deploys superinfection inhibition the relative benefit of horizontal transmission is reduced. Second, in phage λ, the molecular link between increased viral virulence and increased susceptibility to superinfection create a cost for virulence, since a more virulent virus has a higher probability to be displaced from its host cell by a superinfecting competitor. These two aspects of superinfection inhibition increase the relative benefits of vertical transmission and therefore enable the persistence of a fully vertical transmitting viral population or the co-existence of horizontal and vertical transmission strategies. The increase in the benefit of vertical transmission allows for the ecological specialization and co-existence of a non-virulent vertically transmitting 'defense' strategy and horizontally transmitting 'attack' strategy. Under these conditions a non-virulent virus can persist in the presence of a virulent counterpart.

The co-existence of virulence strategies has been described earlier. Two main properties of the superinfection function are decisive for the possible co-existence of virulence strategies. These are the slope of the superinfection function and the smoothness of the superinfection function, e.g. its behavior in the point of equal virulence of resident and superinfecting pathogen (Pugliese 2002; Boldin and Diekmann 2008). Most superinfection models assume a superinfection function that decreases with resident virulence and focus on the effect of non-smoothness around the point of equal virulence between resident and superinfecting pathogen. When the superinfection function has a strong non-smoothness in the origin, e.g. is a step function of virulence, a

large number of virulent strains can coexist around a virulence optimum (Nowak and May 1994). This is however an extreme case since a step-wise superinfection function enables mutants with infinitesimally small differences in virulence to enter and persist in the population (Pugliese 2002). A more realistic approach is the use of a piece-wise differentiable superinfection function $\phi_{RM}(\alpha_R, \alpha_M)$ that is $\phi_{RM} = 0$ zero for $\alpha_R > \alpha_M$ and steadily increasing for $\alpha_M > \alpha_R$. This less extreme non-smooth behavior of a piece-wise differentiable function can promote ecological and evolutionary and coexistence (Pugliese 2002; Boldin and Diekmann 2008).

The example of phage λ differs from these previous models in two important aspects. First, in contrast to previous models of superinfection the example of phage λ considers simultaneous horizontal *and* vertical transmission. Second, the mechanisms of superinfection inhibition introduce interference competition next to competition for host resources. Under these conditions the evolutionary coexistence of horizontal and vertical transmission strategies as well as the stable persistence of a fully vertically transmitting host population can occur even with a smooth superinfection function, under the premise that virulence and the susceptibility to superinfection show a positive (concave) relation.

Due to its stabilizing effect on vertical transmission strategies superinfection inhibition might play an important role in the maintenance of benignity in viral systems in general and therefore create an alternative explanation for the evolution towards lowered levels of virulence. Currently the major factor for the evolution of reduced virulence is seen in the ecological feedback of virulence on between-host transmission. Selection towards lowered virulence due to ecological feedback of virulence onto transmission might however be relatively weak in comparison to selection for increased virulence during within host competition during co-infection (Ebert and Bull 2003). In the absence of ecological feedback, other mechanisms are

required to enable the persistence of benign viruses. Superinfection inhibition might be an important mechanism for the maintenance of low virulence and should therefore be a common characteristic of benign viruses.

Indeed mechanisms that link viral virulence to superinfection inhibition which are similar to the mechanisms of phage λ can be pointed out in several other viral systems. Taking a closer look at other examples of benign DNA and RNA viruses, next to bacteriophage λ, we can see that mechanisms which relate of virulence to superinfection are indeed, common. Even though the genome organization of benign DNA and RNA can be vastly different these mechanisms show a certain 'core theme': Gene products that are involved in the limitation of viral self-replication are often used to suppress competing viruses. This regulatory core theme seems to have emerged multiple times. The single stranded DNA (ssDNA) phage M13, for example, causes a chronic infection of its host E.coli with a relatively mild effect on host mortality. In order to achieve this low level of virulence, M13 produces large amounts of protein P5 that on the one hand covers the single stranded form of M13 to prevent a conversion to the double stranded DNA (dsDNA) replicative form (RF) and on the other hand P5 inhibits the P2 gyrase that is required for the RF rolling circle replication. This way the replication repressor P5 limits the intra-host replication of M13 and, at the same time, blocks the replication initiation of a super-infecting M13 (Baas 1985). Therefore virulence and superinfection resistance are also tightly coupled in M13, although the mechanism is very different from virulence repression in phage λ. Another example is the retro-virus Hepatitis B (HBV), which causes a chronic liver infection. In order to escape immune suppression the HBV strictly limits its intra-cellular replication. It achieves this regulation by auto-repression of its reverse transcription protein P. This way reverse transcriptase P activity is high in the initial phase of infection but is repressed when protein P accumulates (Cao and Tavis 2006). When any competing HBV therefore enters an infected cell as a pre-genomic

RNA stage at a late stage of infection, its reverse transcription is also blocked by high levels of protein P that are produced by the residing virus, and superinfection is prevented. This way self-repression and superinfection inhibition are directly coupled in HBV.

Another class of retro-viruses, the Foamy Viruses use a system that is more similar to the one of phage λ. Foamy viruses cause benign infection. The genome of Foamy Virus contains next to the *gag*, *pol* and *env* proteins, the genes *tas* and *bet* that control the switch between latent, chronic viral infection and lytic viral replication. Thereby *tas* stimulates a switch to lytic replication, whereas *bet* represses the internal promoter and therefore suppresses a switch to the lytic stage. Strikingly, the expression of *bet* in Foamy Virus free cells has also been shown to provide resistance to superinfection by other Foamy Viruses (Nethe et al. 2005). The mechanisms of coupling between virulence and superinfection inhibition is therefore analogous to the cro and cI system in phage λ.

These examples suggests that genetic mechanisms that link virulence with increased susceptibility to superinfection, have evolved multiple times and could therefore be of major importance for the maintenance of viral benignity. The interpretation of the genetic coupling of increased virulence with increased susceptibility to superinfection might have analogies to the cost of 'cheater' strategies in kin selection theory that are required to maintain cooperation. Increased sensitivity to superinfection seems to be an intrinsic cost for high virulence. These associated costs of virulence are often required to stabilize the persistence of prudent host exploitation, i.e. low virulence against the invasion of virulent mutants (van Baalen and Sabelis 1995a; West et al. 2006). This way the genetic coupling of virulence and susceptibility to superinfection can be seen as a self applied limitation mechanism for the levels of virulence, which is 'hard-wired' in the viral gene regulation scheme. More attention should therefore be given to the evolution of mechanisms of viral superinfection inhibition as they have

the propensity to be at the core of the maintenance of low virulence in viruses.

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Chapter 6
Opposing selection pressures on receptor destroying enzymes of influenza virus limit viral adaptation and tissue specificity

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6.1 Abstract:

Opposing selection pressures on a viral protein can impose severe limits on viral evolution. The receptor destroying enzyme neuraminidase of influenza virus is an intriguing example. During late viral development destruction of the viral target receptor is required to release newly formed viral particles from the host cell. Early in the viral life cycle, however, receptor destruction can abort viral infection in the stage of host cell attachment. It is therefore not straightforward to predict the evolution of neuraminidase activity in different host and

tissue environments. By means of a viral life history model we demonstrate that optimal viral adaptation to an environment requires specialization in the rate of receptor destruction that can prohibit viral replication in other hosts or tissues. Adaptation of the receptor destroying activity to local conditions can therefore provide a plausible explanation for viral host and tissue specificity and, as a consequence, differences in viral transmissibility and virulence.

6.2 Introduction

Viral reproduction requires the balancing of many genetic and biochemical processes acting at various stages in the viral life cycle. Evolutionary adjustment of this balance is often constrained by conflicting selection pressures that arise when a change that is advantageous during one stage of the viral life cycle is disadvantageous at another stage. In lytic bacterial viruses, for example, reduced lysis time is coupled to a reduction in viral burst size (Wang et al. 1996; Bull et al. 2004); and in polio virus there is a trade-off between the rates of viral genome replication and viral genome encapsidation (Krakauer & Komarova 2003). When two fitness determining components of the life cycle are linked through a trade-off the outcome of evolution cannot be predicted on the basis of each component independently. Instead, an evolutionary analysis requires the integration of fitness effects over the whole viral life cycle (Stearns 1992; Caswell 2001).

Receptor destroying enzymes are an important class of viral proteins that exemplify such opposing selection pressures. These enzymes play an important role in the life cycle of a large family of human and animal pathogens including *Ortho-* and *Paramyxoviridae* (influenza, para-influenza, Newcastle disease virus, mumps and measles) and *Corona-* and *Toroviridae* (Smits et al. 2005; de Groot 2006). A well-studied representative of these families is influenza virus. Influenza virus contains two major surface proteins: the receptor binding protein

hemagglutinin (HA) and the receptor destroying enzyme neuraminidase (NA). HA and NA are essential for viral reproduction and determine important viral properties like host specificity, tissue tropism, virulence and transmissibility (Baigent & McCauley 2003). Yet, the HA and NA proteins counteract each other at several points of the viral life cycle. Whereas the binding of HA to the host sialic acid (SA) receptors establishes viral attachment to the host cell, NA destroys these SA receptors and therefore potentially hampers virus-host attachment in the early viral life cycle. Receptor destruction therefore 'does not seem like a good idea', was it not that the receptor destroying activity of NA is indispensable in at least three other steps of the life cycle: NA activity prevents the aggregation of viral particles in the mucus layer of epithelia in the lung and the intestine (Matrosovich & Klenk 2003; Matrosovich et al. 2004), it enhances the passage of viral particles through the endosome of the host cell (Suzuki et al. 2005) and it prevents the accumulation of newly formed viral particles on the host cell surface after viral budding from infected cells (Palese et al. 1974). Nevertheless, due to its detrimental effect on virus-host attachment, the receptor destroying activity of NA remains a double edged sword and needs to be carefully balanced in accordance with the strength of HA binding and the properties of the available binding receptors (Mitnaul et al. 2000; Wagner et al. 2000; Wagner et al. 2002; Bin et al. 2005).

The environment of a virus is determined by the availability and properties of viral binding receptors in a specific tissue and host or tissue. Accordingly, host and tissue characteristics markedly affect the optimal balance of receptor binding and receptor destruction. The occurrence of cell mucus is one of these characteristics. Mucus contains decoy SA receptors that act as surrogate binding targets for viral HA and immobilize and inactivate viral particles. Liberation from mucus attachment requires the destruction of mucus SA receptors by viral NA activity (Matrosovich & Klenk 2003). In addition to the occurrence and density of mucus, the variation in the binding efficiency of SA receptors also plays an important role. Hosts and tissues differ, for

example, in their concentration and relative frequency of 2,3-α-gal-SA and 2,6-α-gal-SA receptors, and avian and mammalian influenza virus differs markedly in the affinity of HA and NA with both types of receptor (Gambaryan et al. 2006).

Due to the multiple effects of receptor destroying enzymes on the viral life cycle, the evolution of NA activity is a complex process. A quantitative understanding of the evolution of NA activity requires the integration of costs and benefits of receptor destruction in a life history model for the viral life cycle (Stearns 1992; Caswell 2001). Such a model describes the transitions between the various stages of the life cycle by a life cycle graph that corresponds to a system of differential equations. It is then straightforward to derive viral fitness from the properties of the stage transition matrix. Obviously, viral fitness reflects the properties of host and tissues. Hence, a life history models not only allows to determine the optimal level of activity of receptor destroying enzymes, but also the degree to which a virus with an enzyme that is adapted to one particular host or tissue can survive in a different host or tissue. Here we study the evolution of NA activity and HA avidity of influenza virus in such a life history model. We derive the NA activity and HA avidity that maximize the overall growth rate of the viral population. Our emphasis is on the host and tissue dependence of these viral properties and the question whether, and to what extent, local adaptation can provide and explanation for viral host and tissue specificity.

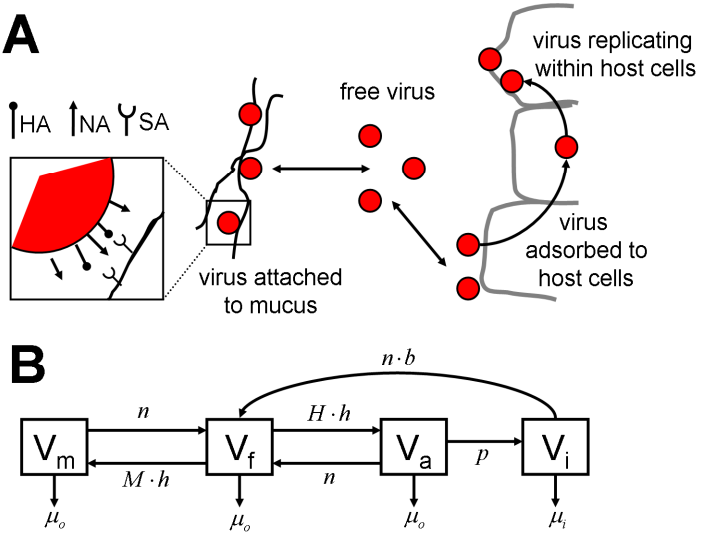

Figure1: The viral life cycle (A) Key processes in the life cycle of influenza virus. The virus can occur in four states: freely moving through the tissue, attached to mucus, attached to a host cell and internalized in a host cell, where it produces offspring virus. Viral attachment to mucus and host cells depends on the density and biochemical properties of sialic acid (SA) receptors and the avidity h of viral hemagglutinin (HA) to these receptors. Release from both, mucus and host cell attachment, to the free stage depends on the receptor destroying activity of the neuraminidase (NA) enzyme. (B) Life cycle graph summarizing the key assumptions of the life history model. The variables V_f, V_m, V_a and V_i denote the concentrations of virus in the four stages free, mucus adsorbed, host adsorbed and internalized. The transition rate from an absorbed to the free state is given by neuraminidase activity n. Adsorption rate to mucus and host cells is given by the product of hemagglutinin avidity h and the concentration H of mucus receptors and M of host cell receptors, respectively. Viruses adsorbed to a host cell are internalized with rate p and newly formed virus particles bud from infected cells at rate b and detach from the cell surface through neuraminidase activity n. Virus particles die at rate μ_o outside the host cell and at rate μ_i inside the host cell.

6.3 A model of HA-NA balance and its evolution

The structure of our model is motivated and summarized in Figure1. We consider four stages of the viral life cycle: free viral particles, mucus-adsorbed particles, particles that are adsorbed to the host cell, and particles that replicate within a host cell. The concentrations of the viral particles in these four stages are represented by the variables V_f, V_m, V_a and V_i, respectively. A virus from the pool of free particles can either enter the pool of mucus-adsorbed virus or host-cell adsorbed virus at a rate that is proportional to the receptor binding avidity h and the abundance of its preferred receptors in the mucus and on the host cell, which are denoted by M and H, respectively. Viral particles destroy receptors and detach from mucus and host cells at a rate determined by the receptor destroying activity n. Thereby the transition from the pool of host-adsorbed virus to free virus represents the abortion of infection, e.g. the negative side effect of receptor destruction. Viral particles that are adsorbed to the host cell penetrate the cell at rate p and enter the stage of replication within the host cell. Replicating particles in V_i produce new viral particles that bud from the host cell at rate b. Budded viral particles accumulate at the cell surface and require receptor destroying activity to detach and enter the pool of free viral particles, thereby finishing their life cycle. The rate of release from each infected cell is therefore proportional to the budding rate b and the detachment of viral particles n. All particles outside of the host cell decay at rate μ_o, while particles inside the host cell decay at rate μ_i. All these assumptions are represented by the life cycle graph in Figure 1B or, equivalently, by the following system of differential equations

$$\frac{dV_m}{dt} = -nV_m + hMV_f - \mu_o V_m \quad (2a)$$

$$\frac{dV_f}{dt} = +nV_m - hMV_f - hHV_f + nV_a + nbV_i - \mu_o V_f \quad (2b)$$

$$\frac{dV_a}{dt} = hHV_f - nV_a - pV_a - \mu_o V_a \quad (2c)$$

$$\frac{dV_i}{dt} = +pV_a - nbV_i - \mu_i V_i \quad (2d)$$

The properties of this linear system are determined by the 4 x 4 matrix of transition rates between states (Caswell 2001). In particular, the asymptotic growth rate λ of the viral population is given by the dominant eigenvalue of this matrix. Explicit calculation of λ, which we will later use as a measure of viral fitness, is intricate and inspires little insight. We therefore simplify the problem by making use of the different time scales of receptor binding and destruction and the process of viral replication. Receptor binding and destruction are spontaneous, fast, and energy-independent processes. In contrast, the processes of host cell penetration, replication within the cell and budding require complex interactions with the host cell and are therefore slow relative to receptor binding and destruction. Therefore, these two types of processes occur on different time scales. This means that on the fast time scale the particles redistribute very rapidly over the free, host-adsorbed and mucus-adsorbed stages, before the total number of viral particles noticeably changes due to the production of new particles. It is therefore reasonable to assume that the particles outside of the host cell reach a quasi-steady-state (Segel 1984) that is characterized by

$$\frac{dV_m}{dt} = \frac{dV_f}{dt} = \frac{dV_a}{dt} = 0 \ . \tag{3}$$

As a consequence the asymptotic growth rate λ of the viral population corresponds to the per capita rate of new infections, which according to (2d) is given by

$$\lambda = \frac{1}{V_i}\frac{dV_i}{dt} = p\frac{V_a}{V_i} - nb - \mu_i \tag{4}$$

In quasi-steady-state the viral particles distribute over the extra-cellular stages in the proportions

$$\tilde{V}_a = \frac{hH}{n + p + \mu_o}\tilde{V}_f = r_1\tilde{V}_f \tag{5a}$$

$$\tilde{V}_m = \frac{hM}{n + \mu_o}\tilde{V}_f = r_2\tilde{V}_f \tag{5b}$$

$$\tilde{V}_f = \frac{nbV_i}{h\left(M + H\right) - n\left(r_1 + r_2\right) + \mu_o} \tag{5c}$$

The ratios r_1 and r_2 represent the proportions of viral particles on the host cell and in the mucus relative to the amount of free virus

($r_1 = \tilde{V}_a / \tilde{V}_f$, $r_2 = \tilde{V}_m / \tilde{V}_f$). Substituting $\tilde{V}_a / V_i = r_1 \tilde{V}_f / V_i$ into (4), we get an explicit expression for the asymptotic growth rate of the virus:

$$\lambda = \frac{1}{V_i}\frac{dV_i}{dt} = \frac{p r_1 n b}{h(M+H) - n(r_1 + r_2) + \mu_0} - nb - \mu_i \qquad (6a)$$

In the appendix we show that under the assumption $\mu_0^{\,2} << H$ (that we will make from now on) this expression simplifies to

$$\lambda = nb\left[\frac{p}{p + \mu_0\left[1 + \frac{1}{H}\left(M + \frac{n+p}{h}\right)\right]} - 1\right] - \mu_i \qquad (6b)$$

The growth rate λ is a function of viral properties (n, h, p, b) and the conditions of the host tissue environment (M, H). We can therefore use λ to derive the optimal combination of the biochemical strategies, h and n, for a given host tissue that is characterized by M and H.

6.4 Optimal receptor destroying activity

The optimal receptor destroying activity n that maximizes the viral per-capita growth rate $\lambda(n,h)$ can be found by calculating the maximum of $\lambda(n,h)$ in the direction of n.

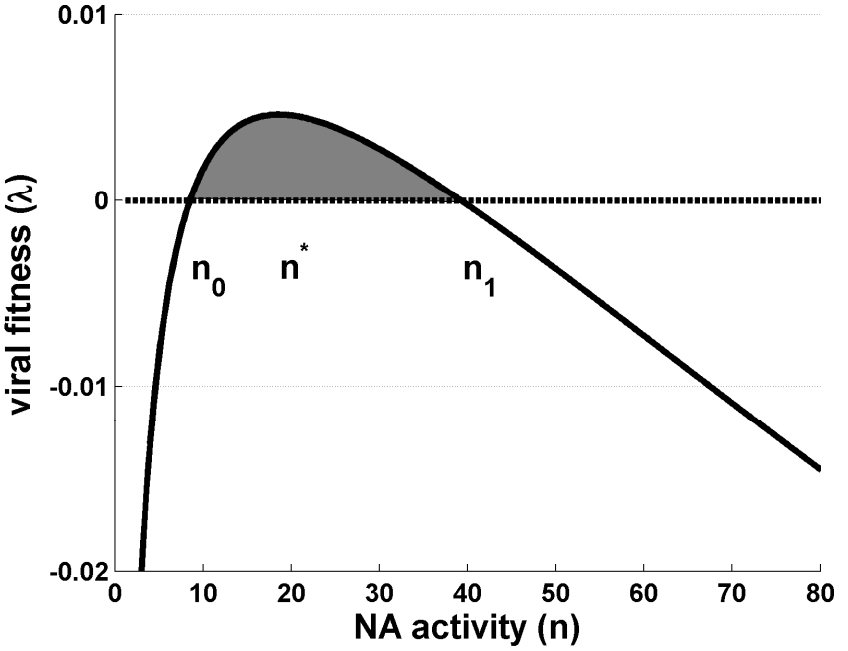

Figure 2: Viral fitness as a function of neuraminidase activity. A virus with a low NA activity $n \approx 0$ tends to accumulate within the host. Therefore it has a negative growth rate that is approximately given by $\lambda = -\mu_i$. A virus with a high NA activity tends to accumulate in the free state. Accordingly, it also has a negative growth rate that is approximated by $\lambda = -\mu_o$. Some host tissues do not allow viral growth irrespectively of the value of n. In the example presented here, there is a tissue specific interval $n_0 < n^* < n_1$ allowing viral growth. Viral fitness (= asymptotic growth rate) is maximized at an intermediate neuraminidase activity n^* (Parameter values: $h = 1$, $b = 3$, $p = 10^{-2}$, $\mu_o = \mu_i = 0.15$, $M = 1$, $H = 1$).

This optimum n^* is given by

$$\left.\frac{\partial\lambda(n,h)}{\partial n}\right|_{n=n^*} = 0 \qquad \text{and} \qquad \left.\frac{\partial^2\lambda(n,h)}{\partial n^2}\right|_{n=n^*} < 0\,. \qquad (7)$$

Let us first try to understand intuitively the effect of receptor destroying activity n on viral growth rate. It is easy to see that neither a very small n nor a very large n are beneficial. For $n=0$, all viral particles accumulate in infected hosts, where they decay at rate μ_i. Therefore the growth rate $\lambda=-\mu_i$ is negative for small n, implying viral extinction. On the other end, for large n, the ratios r_1 and r_2 become small and all viral particles accumulate in the free stage where they decay at rate μ_o. Large n therefore also leads to a negative growth rate $\lambda=-\mu_o$ and, accordingly, also to extinction. It is possible that the host tissue is too hostile to allow viral growth: $\lambda(n,h)<0$ for all n. For other tissues, however, λ is positive for intermediate values of n, i.e. for values from an interval of $n_0<n<n_1$, where n_0 and n_1 reflect the tissue properties M and H and the receptor binding avidity h (Figure 2). As shown in the appendix, the optimal receptor destroying activity of n^* is given by

$$n^* = \frac{h}{\mu_o}\left(\sqrt{pHu}-u\right) \qquad \text{with} \qquad u = pH+\mu_o\left(H+M+\frac{p}{h}\right) \qquad (8)$$

We can conclude that viral growth is negative for extreme values of n and optimal for an intermediate level of receptor destroying activity n^*.

The optimum n^* depends on the receptor binding avidity h and the properties of the target tissue H and M. For this reason differences in H and M between target tissues will lead to different optima in the receptor destroying activity n^* and different regions of positive viral growth $n_0 < n < n_1$. In the next section we will demonstrate how these differences in optimal receptor destroying activity n^* can prevent viral spread between tissues of different receptor availability H and M.

In our model the situation is markedly different for HA avidity h. As shown in the appendix, the asymptotic growth rate of the virus is positively related to the rate of receptor binding avidity h. Therefore h should always evolve towards the maximal attainable value.

6.5 Differences in optimal rates of receptor destruction determine tissue specificity

In order to determine if a virus can spread between different tissues we need to determine whether a virus that is adapted to a tissue of origin X has a positive growth rate in a certain target tissue Y. Within one host organism tissues differ in their relative abundance of receptors in the mucus M and on the host cell H. A virus that is adapted to a tissue X with an optimal strategy n_X^* will be able to spread to a target tissue Y when the growth rate $\lambda\left(n_X^*\right)$ of a virus with NA activity n_X^* is positive in tissue Y. The target tissue Y in turn only provides a positive viral growth-rate for a receptor destroying activity n within the interval $n_{0,Y} < n < n_{1,Y}$. A virus can therefore not spread from tissue X to Y when n_X^* falls outside the interval $n_{0,Y} < n < n_{1,Y}$ (see Figure 3).

Lets now consider which viruses that are adapted to a different environment can invade a given reference environment. To calculate whether a virus from an environment $X = \left(H, M\right)$ can invade the

reference environment Y we calculated the optimal strategy n_X^* and determined whether $n_{0,Y} < n_X^* < n_{1,Y}$. This procedure divides the parameter space into environments X that do not allow viral growth at all [i.e. $\lambda\left(n_X^*\right) < 0$; black region in Figure 4], regions that allow growth in the reference environment Y [i.e. $n_{0,Y} < n_X^* < n_{1,Y}$; white region in Figure 4] and in regions where viruses adapted X cannot grow in Y [gray regions in Figure 4]. For a given value of M viral growth does not occur below a threshold level for the concentration of host receptors H. This threshold increases with M. The minimal level of H that is required for viral growth can be approximated by a linear function of M (see Appendix). Combinations of M and H that allow for viral growth, or $\lambda\left(n_X^*\right) > 0$, fall into two categories: Environments which produce viruses that can invade the environment Y and those which cannot. Intuitively, environments X which are more similar to environment Y can produce viruses that can spread from X to Y. Distance in the direction of M and H has, however, a different effect on tissue specificity. Whereas viruses from an environment with a higher value of H can all invade the reference environment Y, viruses from environment of either very low or very high values of M are not able to invade the reference environment. Accordingly, the mucus concentration M is the primary factor that causes tissue specificity (Figure 4).

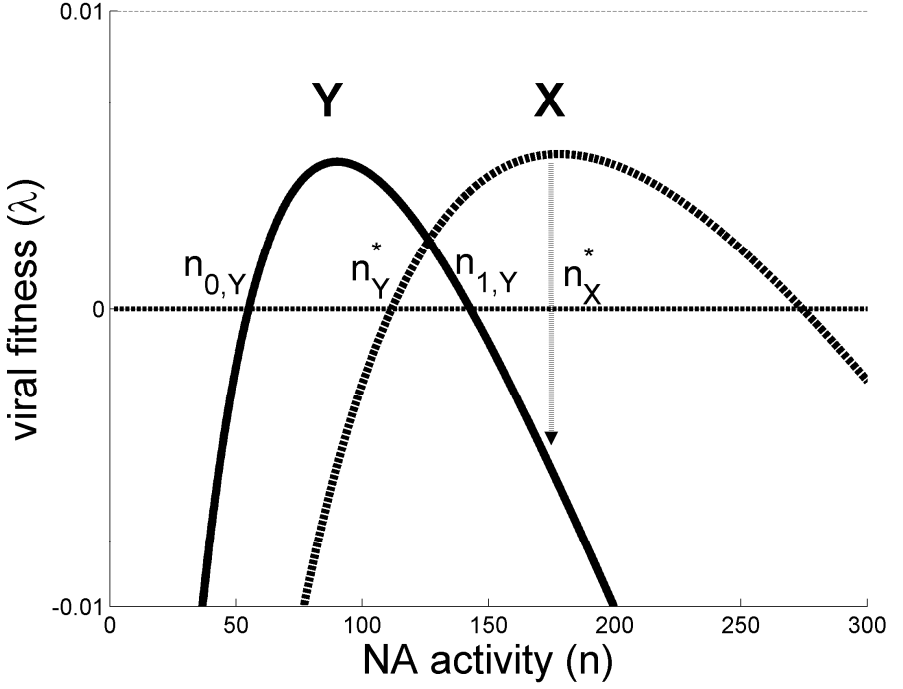

Figure 3: Tissue specificity of viral adaptation. Dependence of viral fitness (=asymptotic growth rate) on neuraminidase activity for two tissues X and Y. The tissues differ in the concentration of SA receptors on the host cell ($H_X = 3.7$, $H_Y = 2$) and in the surrounding mucus ($M_X = 200$, $M_Y = 50$). In environment Y, viral growth is possible for n-values between $n_{0,Y}$ and $n_{1,Y}$. The virus optimally adapted to environment X, does not satisfy this requirement (since $n_X^* > n_{1,Y}$) and hence has a negative growth rate in environment Y (see dotter arrow). Other parameters: $h = 1$, $b = 3$, $p = 10^{-2}$, $\mu_o = \mu_i = 0.15$.

6.6 Discussion

Conflicting selection forces can severely hamper viral adaptation. Here we studied the example of the receptor destroying enzyme neuraminidase (NA) of influenza virus to establish a framework that integrates conflicting enzymatic effects into the viral-life cycle. We furthermore use this model to predict viral adaptation to the environment. Our model demonstrates that viral adaptation to the prevailing tissue environment requires specialization of receptor destroying activity that can prohibit spread of the virus to other tissues. This specialization in receptor destroying activity could provide an additional mechanism for viral tissue and host specificity, next to other mechanisms, like the presence of suitable receptors and necessary proteases.

Viral fitness in the situation of an *in vivo* infection is affected by multiple factors like the viral replication rate, the ability to avoid the immune system and the rate of transmission between host organisms. In our model we focus on a single aspect of viral fitness which is its replication rate. Viral replication rate is generally strongly related to fitness. Maximal fitness can, however, deviate from the maximal replication rate, when viral replication has a negative side effect on other components of viral fitness. Viral replication can for example increase host mortality and therefore negatively affect viral transmission (Ewald 1994; van Baalen & Sabelis 1995); but see also (Ebert & Bull 2003). Furthermore, viral replication can have negative consequences for the avoidance of the specific immune system. Rapidly replicating viral mutants, for example, create a higher antigen dose and therefore a stronger specific immune response. In principle, this coupling between replication, antigen dose and immune response can select against strains with highest replication rate and create a rareness advantage of slow replicating mutants (Nowak et al. 1991). In some cases replication rate and immune avoidance can also be determined by a single mutation (Both et al. 1983). In our model, we do not address the action of a specific immune response. Instead, we

represent a non-specific immune response in terms of the decay parameters μ_o and μ_i. In principle the negative side effects of viral

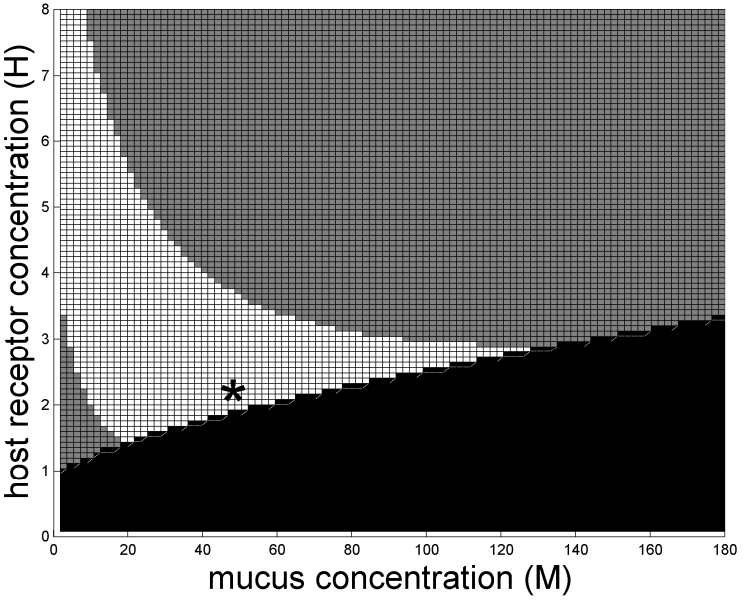

Figure 4: Parameter dependence of viral extinction and tissue specificity. Viral growth strongly depends on the tissue characteristics H (= concentration of SA receptors on host cells) and M (= receptor concentration on mucus). The black region corresponds to those tissues where viral growth is impossible irrespective of the value of neuraminidase activity n. The star marks a specific reference environment characterized by $M = 50$ and $H = 2$. The white region corresponds to those tissues where the virus optimally adapted to the given tissue is able to grow in the reference environment. Viruses derived from tissues in the grey parameter region cannot grow in the reference environment. Other parameters: $h = 1$, $b = 3$, $p = 10^{-2}$, $\mu_o = \mu_i = 0.15$.

replication onto future transmission and on immune avoidance can create other interesting trade-offs in addition to the here described conflicting selection on receptor destroying activity, which are

addressed elsewhere (Both et al. 1983; Nowak et al. 1991; Ewald 1994; van Baalen & Sabelis 1995). Assuming that the specific immune system is not affecting the early stages of viral infection we can nevertheless use the maximization of replication as a predictor for viral fitness as has been done by previous models for within-host viral growth [e.g. (Regoes et al. 2005)].

The intermediate optimum for receptor destroying activity has profound consequences for viral evolution. Most importantly, the adaptation to specific receptor destroying activity can prevent the spread of virus between different hosts and tissues. Yet, one has to consider the role of different optima in receptor destroying enzymes in relation to other mechanisms that determine viral tissue tropism. Viral spread between tissues is, for example also affected by the availability of cellular proteases that cleave the HA precursor HA0 to the active form of HA. For seasonal human influenza viruses and low pathogenic avian viruses the required proteases are expressed tissue-specifically. In contrast, highly pathogenic viruses, of avian origin (HPAI), contain a multibasic cleavage site which allows cleavage of HA_0 to its active form by ubiquitous proteases. Therefore, replication of HPAI does not require the presence of proteases that are specific for lung tissues. Consequently, avian HA_0 can be cleaved in tissues outside the lung which enables unrestricted viral spread and systemic infection. In avian hosts, the occurrence of a multibasic cleavage site shows a strong correlation with tissue tropism and virulence (Horimoto & Kawaoka 1994). In contrast, in mammalian hosts this correlation is weaker (Steinhauer 1999). In mammalian hosts, tissue differences in optimal receptor destroying activity could therefore be an important factor for tissue specificity, next to HA cleavability. Ultimately, the role of different optima of receptor destroying activity for tissue specificity remains, however, an empirical question.

Inhibitors of the receptor destroying activity of NA are currently the only possible pharmaceutical intervention for an influenza infection.

Obviously, NA inhibiting drugs disturb the balance of viral attachment and receptor destruction and therefore inhibit viral replication. Adaptation to NA-inhibitors can, however, readily occur through restoration of the attachment/detachment balance (Gubareva et al. 2001; Gubareva 2004; Reece 2007). Quantitative understanding of the evolutionary limitations of receptor destroying enzymes is an important tool to understand the limitations of viral adaptation to NA inhibitors. For example, different optima for receptor destroying activity between tissues predict situations in which the application of inhibitors of receptor destroying enzymes (NA-inhibitors) actually can promote the spread of virus between tissues. This undesired effect of NA-inhibitors can occur when a virus, that is adapted to an environment with a high optimum of receptor destroying activity, enters a tissue or host organism that requires a low rate of receptor destroying activity. In this case, reduction of NA activity by NA inhibitors moves the receptor destroying activity closer to the optimal level in the new environment and increase viral replication. Even worse, when the difference of optimal receptor destroying activity prevents viral spread between tissues, NA inhibitors could facilitate spread to new tissues and promote systemic infection. NA inhibitors have not yet been shown to increase viral replication directly. However, mutations that decrease the receptor destroying activity can indeed increase viral replication (Bin et al. 2005). It is therefore plausible that reduced receptor destroying activity, can increase viral replication in tissues with a low optimal receptor destroying activity and facilitate the spread from mucus to non-mucus tissues, regardless whether receptor destroying activity is reduced by NA-inhibition or directed mutations.

Tissue specific optima of receptor destroying activity imply that the adaptation to one environment worsens the success in another environment. When viral tissue specificity affects virulence and transmission, this effect can create a trade-off between viral virulence and transmission. As mucus-adapted viruses will be more successful in the mucus tissues which form the entrance route of infection, mucus

adaptation should promote transmission between host organisms. The virulence of a viral strain in turn depends on its ability to spread to mucus free tissues within one host orgamism, causing systemic infection. Even though viruses that are adapted to mucus tissues should transmit readily between host individuals, they are maladapted for growth in mucus free tissues and should therefore show low virulence. In turn, viruses that are adapted to mucus free tissues should be virulent but poorly transmitting. There is some evidence that highly virulent viral strains, indeed, transmit poorly in experimental transmission experiment even when the contact rate of animals is very high (Yen et al. 2007).

The trade-off between the specialization onto mucus containing and mucus free tissues, respectively, poses the question whether a generalist virus can evolve, which is able to reproduce in both types of environments and is therefore highly virulent and transmittable at the same time. Considering the evolutionary limitations on receptor destroying activity the occurrence of such a highly transmissible and highly virulent mutant might be less likely then previously thought. Evaluation of this possibility should focus on the measurement of HA avidity and NA activity as quantitative kinetic parameters in various tissues and host species.

The examples above demonstrate that biochemical constraints between different parts of the viral life cycle can be valuable to illuminate the limitations of viral evolution. Although biochemical conflicts in viral adaptation are likely to be ubiquitous, models that derive evolutionary limitations directly from the underlying biochemistry are scarce, probably because they require virus specific models. Current biochemical models of the viral life cycle focus on generic processes of the viral replication, like the dynamics of viral genome replication and protein production. For example, Krakauer & Komarova (2003) have focused on within cell processes like the encapsidation of viral genomes and its effect on viral genome replication. Regoes and

colleagues (2005) have investigated the optimal ratio of positive and negative RNA strands that maximizes the replication of polio virus. The example of receptor destroying enzymes demonstrates that mechanisms for conflicting selection lure also in other parts of the viral life cycle besides genome replication and packaging, like the attachment and detachment processes. Even though the mechanisms that constrain the evolution of receptor destroying activity are rather specific, these constraints have implications for a large group of animal pathogens.

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6.8 Appendix

6.8.1 Existence of a region of positive viral growth

In view of (6) and the definition of r_1 and r_2 in (5) the asymptotic growth rate of the virus is given by

$$\lambda = \frac{1}{V_i}\frac{dV_i}{dt} = nb\left[\frac{phH}{\left(n+p+\mu_0\right)\left[h\left(M+H\right)-n\left(\frac{hH}{n+p+\mu_o}+\frac{hM}{n+\mu_o}\right)+\mu_o\right]}-1\right]-\mu_i \tag{A1}$$

This can be rewritten as

$$\lambda = nb\left[\frac{p}{p+\mu_0+\frac{\mu_0}{H}\left[M+\frac{n+p}{h}\right]+\frac{\mu_0^2}{H}\left[\frac{M}{n+p}+\frac{1}{h}\right]}-1\right]-\mu_i \qquad \text{(A2)}$$

For large n the quotient in (A2) converges to zero. Accordingly, the term in brackets, and with it the per capita growth rate λ, becomes negative for large values of n and converges to $\lambda = -\mu_o$. On the other end $\lambda = -\mu_i$ for $n = 0$. Therefore there are two scenarios: either λ is negative for *all* values of *n* (implying that the virus cannot persist), or λ is positive for an interval $n_0 < n < n_1$. An optimal n^* that supports viral growth, exists in this interval, when $\lambda(n)$ is concave and $\lambda(n^*) > 0$. To simplify the analysis we assume that ${\mu_0}^2 << H$ allowing us to drop the small term $\frac{\mu_0^2}{H}\left[\frac{M}{n+p}+\frac{1}{h}\right]$ from (A2) to arrive at

$$\lambda = nb\left[\frac{p}{p+\mu_0\left[1+\frac{1}{H}\left(M+\frac{n+p}{h}\right)\right]}-1\right]-\mu_i \qquad \text{(A3)}$$

It is now easy to see that λ is positive when

$$\frac{1}{H}\left(M+\frac{n+p}{h}\right) > x_0 = \frac{p}{\mu_0}\left[\frac{nb}{\mu_i+1}-1\right]-1 \qquad \text{(A4)}$$

or, equivalently

$$H > \frac{1}{x_0}\left(M+\frac{n+p}{h}\right) \qquad \text{(A5)}$$

Correspondingly λ is positive when

$$h > \frac{n+p}{x_0 H - M} \qquad \text{(A6)}$$

We can conclude from (A5) that for $\mu_0^{\,2} << H$ the minimal H permitting viral growth is approximately linearly related to M (see also Figure 4A) and from (A6) we can see that the minimal h that enables viral growth asymptotically approaches infinity for $M \to x_0 H$ (see Figure 4B). Therefore at the critical mucus concentration $M_c = x_0 H$ no further increase in h can support viral growth.

6.8.2 Optimal NA activity and HA avidity

In order to calculate the optimal NA activity n^* we solve the equation $\partial\lambda / \partial n = 0$ which yields

$$n^* = \frac{h}{\mu_0}\left(\sqrt{pHu} - u\right) \quad \text{with} \quad u = pH + \mu_0\left(H + M + \frac{p}{h}\right) \tag{A7}$$

Furthermore, n^* is a local maximum when λ is concave in n or

$$\frac{\partial^2 \lambda}{\partial n^2} < 0 \qquad \Leftrightarrow \qquad \mu_0\left(H + M + p\right) + hpH > 0 \tag{A8}$$

This implies that n^* is always a local maximum for positive values of μ_0, h, p, H, M.

In contrast to the selection for intermediate for NA activity n^*, HA avidity h should evolve to its attainable maximum. This can be seen directly from (A3). When $h \to \infty$ the viral growth rate $\lambda(h)$ increases until it asymptotically reaches a maximum

$$\lambda_{\max} = nb\left[\frac{pH}{p + \mu_0\left(1 + \frac{M}{H}\right)} - 1\right] - \mu_i \tag{A9}$$

For high values of h the asymptotic growth rate is therefore insensitive to changes in h.

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Nederlandse samenvatting

Het reproductief succes van een virus is afhankelijk van een zorgvuldige balans tussen de temporele en kwantitatieve organisatie van reproductie in de levenscyclus van het virus. Deze balans wordt gewaarborgd door regulatie van de virale eiwitproductie en eiwitinteracties. De evolutie van virussen is afhankelijk van de mogelijkheden om deze eiwitinteracties optimaal op elkaar af te stemmen. In veel virale systemen zijn deze mogelijkheden sterk beperkt, vooral in situaties waarin het positieve effect van een eiwit inherent gekoppeld is aan nadelige neveneffecten – een zogenaamde moleculaire tradeoff.

Het doel van dit proefschrift is het identificeren van dergelijke moleculaire tradeoffs, het voorspellen van hun effect op de evolutie van virussen en het testen van deze voorspellingen in evolutionaire experimenten. Het speerpunt van onze aanpak is het reduceren van de biochemische complexiteit van een virus tot een gesimplificeerd model van de levenscyclus, om daarmee de cruciale biochemische stappen te identificeren die bepalend zijn voor de fitness van het virus. Door middel van deze aanpak worden de evolutionaire “zwaktepunten” van een virus geïdentificeerd. Kennis van deze evolutionaire zwaktepunten vormt een belangrijke basis voor de ontwikkeling van toekomstige antivirale strategieën die robuust zijn tegen virale adaptatie en resistentie.

De omgeving van een virus bepaalt de optimale afstemming van de eigenschappen van de levenscyclus van een virus. De optimale virulentie van een virus is bijvoorbeeld gerelateerd aan de dichtheid van de gastheer. In een lage gastheerdichtheid is het voor een virus nadelig om zijn gastheer teveel schade toe te brengen aangezien dit de

gastheer kan doden voordat het virus zich kan verspreiden naar een nieuwe gastheer. Theoretische modellen voorspellen daarom dat een virus zijn virulentie zou moeten aanpassen aan de gastheerdichtheid in zijn omgeving.

Aanpassingen van de levenscyclus van een virus zijn echter afhankelijk van de moleculaire mogelijkheden om deze levenscyclus te veranderen. Deze moleculaire mogelijkheden vormen de bron voor de mutaties die aanpassing aan de omgeving mogelijk maken. Beperkingen in deze moleculaire mechanismen zijn daarom van directe invloed op de aanpassing van een virus aan zijn omgeving. In theoretische modellen worden deze moleculaire mechanismen echter vaak buiten beschouwing gelaten. De introductie van expliciete moleculaire mechanismen in modellen van virale evolutie zou tot volledig nieuwe inzichten kunnen leiden.

In dit proefschrift ontwikkel ik methoden om moleculaire tradeoffs in de virale levenscyclus te identificeren, hun evolutionaire rol met behulp van wiskundige modellen te voorspellen, en deze voorspellingen door middel van experimentele evolutie van bacteriële virussen, zogenoemde bacteriofagen, te testen. Met deze aanpak laat ik in een viertal voorbeelden zien hoe moleculaire mechanismen de aanpassing van een virus aan zijn omgeving sterk kunnen beperken.

In hoofdstuk 2 onderzoek ik de consequenties van verstoorde gen expressie voor de fitness en adaptatie van het RNA virus MS2. Voor dit experiment werden de vier genen van MS2 één voor één in een productieplasmide gecloneerd. Met dit plasmide kunnen virale genen constitutief worden geproduceerd. Deze productie kan niet gecontroleerd worden door het virus en verstoort daardoor de natuurlijke balans van virale eiwitten tijdens de infectiecyclus. Dit heeft een sterk remmend effect op de groei van het virus, ook al produceert het plasmide viraal genproduct zonder dat het virus er “moeite voor hoeft te doen.” Het virus kan zich aan deze verstoring maar in beperkte mate aanpassen. Vooral de aanpassing aan een te hoog niveau van het

virale RNA replicatie enzym is lastig voor het virus. Overproductie van RNA replicatie enzymen blijkt zo even efficiënt als antivirale strategie, als de repressie van de productie van deze eiwitten – het doel van vele bestaande antivirale strategieën. Echter blijkt het voor het virus lastiger zich aan te passen aan de over-productie van RNA replicatie enzymen dan aan hun repressie. Dit biedt een perspectief voor een mogelijke antivirale strategie die bestand is tegen virale adaptatie.

Hoofdstuk 3 beschrijft de evolutie van de infectiviteit van bacteriofagen in enerzijds een vloeibare omgeving en anderzijds een ruimtelijk gestructureerde omgeving. Theoretische modellen voorspellen dat een te hoge mate van infectiviteit in een ruimtelijk gestructureerde omgeving nadelig is, omdat geïnfecteerde gastheercellen een barrière voor de verdere verspreiding van het virus vormen. Daarom kan een lage infectiviteit in een ruimtelijk gestructureerde omgeving voordelen hebben. In een vloeibare omgeving verdwijnen deze voordelen en zal een virus tot zijn maximale infectiviteit evolueren. Wij demonstreren dit effect door middel van experimentele evolutie van bacteriofaag ΦX174 in een ruimtelijk gestructureerde evenals in een vloeibare omgeving. Hoewel onze resultaten de theoretische verwachtingen bevesigen, hebben verdere controle-experimenten aangetoond dat deze resultaten beïnvloed zijn door de aanwezigheid van een tot nu toe onbekende (pro)faag in het genoom van de gastheerbacterie. De ontdekking van deze onbekende faag en zijn potentiële invloed op de experimentele evolutie van bacteriofaag ΦX174 is het onderwerp van hoofdstuk 4. Uiteindelijk identificeren wij in hoofdstuk 4 de onbekende faag door middel van sequentieanalyse als een virus uit de familie van lysogene virussen. Lysogene fagen kunnen zich via integratie in het gastheergenoom van moedercel tot dochtercel verspreiden. Deze manier van verspreiding wordt verticale verspreiding genoemd. Het bestuderen van deze vorm van virale verspreiding was de aanleiding voor het theoretische werk in hoofdstuk 5.

Hoofdstuk 5 beschrijft de evolutie van een virus dat zowel horizontale transmissie (tussen ongerelateerde gastheren) als ook verticale transmissie (van moeder tot dochtercel) vertoont. Een belangrijke voorwaarde voor verticale transmissie is het overleven van de gastheercel. Om overleven van de gastheercel te waarborgen moet een virus enerzijds zijn eigen virulentie beperken, maar anderzijds zijn gastheercel tegen de overname door een virulente virus-mutant beschermen. De mechanismen voor de beperking van virulentie en de bescherming tegen overname zijn in lysogene virussen nauw gerelateerd. Een virus dat zijn eigen virulentie beperkt is daarom automatisch immuun tegen overname door een virulent virus. Een virulent virus is daarentegen niet efficiënt in de bescherming van zijn gastheercel. Deze tradeoff leidt tot de mogelijkheid van coëxistentie van virulente (slecht beschermende virussen) en niet-virulente (goed beschermende virussen). Dit verklaart het voorbestaan van niet-virulente virussen in de aanwezigheid van virulente mutanten.

Hoofdstuk 6 beschrijft de tegenstrijdige effecten van het eiwit neuraminidase op de levenscyclus van het influenza virus en de gevolgen voor virale evolutie. Neuraminidase verbreekt de binding van het virus aan de gastheercel en heeft daarmee een nadelig effect op de vroege stadia in de virale levenscyclus. Anderzijds is de activiteit van neuraminidase noodzakelijk aan het einde van de virale levenscyclus wanneer nieuw gevormde virusdeeltjes de gastheercel verlaten. Neuraminidase heeft daarom tegelijkertijd voordelige en nadelige effecten op virale reproductie. De balans tussen deze relatieve voor- en nadelen is afhankelijk van de weefsels waarin een virus zich bevindt, en de aanwezigheid en kwaliteit van geschikte bindingsreceptoren. De optimale activiteit van neuraminidase verschilt tussen deze weefsels. Aanpassing van de neuraminidase-activiteit aan een bepaald type weefsel kan daarom de aanpassing aan een ander type weefsel verminderen. Een virus dat bijvoorbeeld aangepast is aan longweefsel heeft daardoor een neuraminidase-activiteit die groei op andere weefsels kan verhinderen. Aanpassing van het virus kan daarom tot

specialisatie voor een bepaald type weefsel leiden. Specialisatie in een bepaald type gastheerweefsel is op haar beurt een belangrijke factor voor virulentie en transmissie van het virus. De specialisatie van neuraminidase activiteit voor bepaalde typen weefsel is daarom een mogelijke verklaring voor de dichotomie tussen influenzastammen met hoge virulentie maar slechte transmissie en influenzastammen met lage virulentie maar goede transmissie.

Met deze vier voorbeelden wordt de rol van moleculaire beperkingen voor de optimale aanpassing van de virale levenscyclus aan de omgeving van een virus aangetoond. Onze aanpak vormt een algemene strategie om de evolutionaire zwaktepunten van specifieke virussen te identificeren en wijst de weg naar de ontwikkeling van antivirale strategieën die bestand zijn tegen de evolutie van virale resistentie.