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Experimental Host-Parasite Co-Evolution in a Changing Environment

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Abstract: Host-parasite co-evolution

Parasites with exclusive vertical transmission from host parent to offspring are an evolutionary puzzle. Any fitness costs for infected hosts risk the selective elimination of these parasites because their fitness is linked to host reproduction. One of the main evolutionary transitions from parasitism towards beneficial or mutualistic associations may therefore encompass a change from horizontal transmission to vertical transmission. In this thesis, the experimental evolution study on *Paramecium* and *Holospora* supports this hypothesis. The parasite nearly entirely lost horizontal transmission capacity in a treatment favouring vertical transmission and low virulence. However, many vertically transmitted parasites e.g. *Caedibacter taeniospiralis* impose detectable costs to their hosts. This endosymbiont imposes context-dependent costs to its host *Paramecium tetraurelia*. Fitness of infected paramecia was reduced in resource-limited conditions at all experimentally tested temperatures (16-32°C). These universal fitness costs along the temperature gradient necessitate universal cost compensation that can be the 'killer trait' that eliminates uninfected competitors. At acute heat stress the loss of infection indicates that cost compensation is impossible, thereby restricting conditions for parasite persistence. Surprisingly, the parasite persists in permanent stress and optimal temperature conditions. *Caedibacter* was able to adapt to high temperature conditions by increasing its number in the populations but without reducing virulence in high temperature conditions. Acute and intense stress harms the parasite and causes its extinction but the parasite was able to evolve and adapt to stress conditions. Moreover, the parasite reacts exactly in the opposite direction as it was expected. They do not suffer from stressful conditions, they benefit.

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GENERAL INTRODUCTION

Parasitism is one of, if not the most common lifestyle on earth (Leung & Poulin, 2008) and is expected to have strong influence on the ecology and evolution of organisms (Poulin, 2011). Parasites divert resources or energy from their host for their own growth and transmission. This parasite action harms the host and leads to a reduction in fitness, commonly defined as 'parasite virulence' (Read, 1994). Understanding the factors determining variation in the expression of virulence is central to concepts of parasite evolution or host-parasite co-evolution (Ewald, 1993; Read, 1994; Ebert, 1999). In classical models, virulence is considered as a parasite trait positively linked with transmission and optimised by natural selection to maximise parasite fitness (Anderson & May, 1982; Alizon *et al.*, 2009; Poulin, 2011). However, in more recent years, a number of studies have highlighted the context-dependency of virulence (Michalakis *et al.*, 1992; Brown *et al.*, 2003; Restif & Kaltz, 2006; Wolinska & King, 2009; Vale *et al.*, 2011). Genetic factors, parasite transmission mode and environmental conditions can all influence parasite-mediated effects on host fitness on both phenotypic and evolutionary levels (Ferguson & Read, 2002; Lambrechts *et al.*, 2006; Wolinska & King, 2009; Ebert, 2013). Therefore, the outcome of a symbiotic interaction might move along a continuum between antagonistic to neutral to even beneficial (Ewald, 1987; Leung & Poulin, 2008; Fellous & Salvaudon, 2009). Below, I highlight important aspects of virulence evolution linked to transmission mode, genetic factors and environmental variation, respectively. I review both theoretical and empirical/experimental literature.

Evolution of symbiotic transmission modes

Major transitions from parasitism to mutualism can be associated with a change in the transmission mode (Moran *et al.*, 2008; Sachs *et al.*, 2011). Vertical transmission is the transfer from parent-to-offspring and positively links parasite fitness to host reproduction (Fine, 1975). Any negative effects on host fitness will decrease parasite reproduction and transmission, which can consequently lead to the elimination of the parasite from the population (Fine, 1975; Régnière, 1984). Therefore the existence of purely vertically transmitted *and* virulent parasites runs against ecological theories (Lipsitch *et al.*, 1995b; Jones *et al.*, 2007). For spread and persistence of these parasites, lower levels of virulence or even mutualism would be an outcome favoured by selection (Fine, 1975; Ewald, 1987; Bull, 1994; Ewald, 1995). In contrast to vertical transmission, fitness of horizontally transmitted

parasites is not exclusively intertwined with that of its host. Parasite fitness can be improved by exploiting its host more aggressively, which allows the evolution of higher virulence levels (Alizon *et al.*, 2009). A simple model, the trade-off hypothesis, describes virulence evolution of horizontally transmitted symbionts as a trade-off between parasite transmission and parasite virulence (Anderson & May, 1982). Greater host exploitation allows a higher parasite reproduction and transmission rate, but is detrimental to the host because of increased host damage and mortality rate caused by infection. In contrast, a shorter life time of the host reduces overall parasite transmission success and thereby cost of infection (Anderson & May, 1982; Ewald, 1987; Ebert & Bull, 2003). Virulence is therefore supposed to evolve to an intermediate level along the virulence-transmission trade-off, because natural selection will balance the costs and benefits not only for hosts but also for the parasite (Anderson & May, 1982; Read, 1994; Frank, 1996). For parasites with both vertical and horizontal transmission, the level of expressed virulence may therefore depend on the ratio of vertical to horizontal transmission (transmission-mode hypothesis; Ewald, 1987; Herre, 1995; Lipsitch *et al.* 1995a; Kover & Clay, 1998, Ebert, 2013). In many systems, key factors manipulating the relative contribution of vertical and horizontal transmission to total parasite transmission are host density, host survival and its fecundity (Ebert & Herre, 1996; Lipsitch *et al.* 1996). While horizontal transmission will be favoured in conditions of high host population density because of high infectious contact rates, vertical transmission is just indirectly influenced by host density. The importance of vertical transmission will increase with the reproductive success of the host (Agnew & Koella, 1999; Kaltz & Koella, 2003; Refardt & Rainey, 2010; Ebert, 2013). Parasites with both horizontal and vertical transmission can combine the advantages of each transmission mode and will therefore switch between the two transmission modes depending on environmental conditions. Those symbionts with both vertical and horizontal transmission are most common in nature. However, parasites can also be strictly vertically or horizontally transmitted. The evolution of strictly horizontal or vertical transmission is, however, not yet sufficiently resolved (Ebert, 2013). Focussing on the origin of strictly vertical transmission, Ewald (1987) speculated that ancestral parasites in general had both vertical and horizontal transmission, and lost their infectivity because of mutations on genes involved in horizontal transmission pathway. In contrast, Sachs and colleagues (2011) hypothesized that the transitions towards strictly vertical transmission is exclusively host-driven. They argued that this transition has a higher impact on host fitness compared to parasite fitness because of the involved reduction in costs imposed on the host by the vertically transmitted symbiont. Contrary, theory predicts that strictly vertical transmission

will evolve as a consequence of epidemiological dynamics disfavouring horizontal transmission (Lipsitch *et al.*, 1995b). While genomic analyses of inheritable microorganisms revealed that the loss of infectivity evolved independently in many different host-symbiont systems (Moran *et al.*, 2008; Sachs *et al.*, 2011), experimental studies supporting one of the different hypotheses are missing. There is currently only one experimental study showing the loss of infectivity. In a bacteria-phage experiment, Bull and colleagues (1991) manipulated vertical or horizontal transmission. Here, a strictly vertical transmission treatment favoured 'benevolence'. Indeed, the inhibition of horizontal transmission in their 'partner fidelity' treatment caused not only a reduction in virulence but also the final loss of horizontal infectivity of the phage. However, the experimental design and their results are not supporting any of the hypotheses for the origin of strict vertical transmission.

Genetic factors influencing virulence evolution

Traditional models often described virulence as a characteristic of the parasite and an unavoidable by-product of host exploitation and parasite transmission (Anderson & May, 1982; Ewald, 1993; Bull, 1994; Ebert & Bull, 2003). In more recent studies, virulence is considered to be neither constant nor only a specific characteristic of the parasite (Restif & Koella, 2003; Alizon *et al.*, 2009). For example, virulence and transmission success of the parasitic protozoan *Ophrycystis elektroscirrha* varies not only between parasite genotypes, but also within different family lines of the host, the monarch butterfly (De Roode & Altizer, 2010). In a malaria rodent system, genetic variation of host and parasite influences the expression of virulence, resistance and the transmission rate (Grech *et al.*, 2006). Infection with one parasite genotype can be harmful for specific host genotypes, while other host genotypes do not suffer from infection and vice versa (Grech *et al.*, 2006; Salvaudon *et al.*, 2007; De Roode & Altizer, 2010). In conclusion, traits like transmissibility, infectivity, and therefore virulence depend not only on parasite genetic identity but also on host genotype, making virulence evolution a 'shared trait' (Carius *et al.*, 2001; Restif & Koella, 2003; Rauch *et al.*, 2006; Restif & Kaltz, 2006; Vale & Little, 2009).

Host resistance and tolerance are also not a characteristic of the host alone. These traits are influenced by the parasite genotype as well (Lambrechts *et al.*, 2005; Lazzaro & Little, 2009). However, especially in host-symbiont association with a strictly vertically transmitted symbiont, both the host and symbiont genotypes are jointly responsible for the evolutionary outcome (Wade, 2007; Feldhaar, 2011). Locked in a single host line, the symbiont creates an evolutionary unit with its host. Hence, both host and symbiont determine

the phenotype of this association (Feldhaar, 2011). This may lead to the accumulation of deleterious mutations or loss of function in the symbiont (Dale & Moran, 2006; Feldhaar, 2011), but can also facilitate host specialisation and co-evolution (Vavre & Kremer, 2014). Differences in the response to parasitism may therefore not only influence the population dynamics of the interacting species, but also produce variable co-evolutionary outcomes in different populations from distinct areas or habitats within the geographic range of the interacting species (Vale *et al.*, 2011).

Environmental factors driving co-evolution

"In host-parasite interaction, environment matters" (Thomas & Blanford, 2003). Environmental conditions such as temperature or nutrients are known to directly affect development, growth and reproduction of both host and parasite and this may feed back on parasite virulence, transmission and on co-evolutionary dynamics (Thomas & Blanford, 2003; Wolinska & King, 2009).

Host and parasite normally represent different species with potentially different environmental optima and stress responses (Thomas & Blanford, 2003). Changing environmental conditions may therefore promote either host or parasite fitness (Wolinska & King, 2009). For example, unfavourable temperatures for the host can be associated with increasing susceptibility to infection or parasite-induced mortality (Lafferty, 2009; Studer *et al.*, 2010). At that instant, the host has to handle the simultaneous impact of abiotic and biotic stress. It is further conceivable that abiotic stress weakens host defences, thereby aggravating the negative impact of parasite infection and consequently parasite virulence. This may or may not translate into increased parasite transmission, depending on how well the weakened hosts can still be exploited and, in particular, on how the parasite itself tolerates environmental stress (Moret & Schmid-Hempel, 2000; Lafferty & Holt, 2003; Jokela *et al.*, 2005). Increasing temperatures may further enhance parasite reproduction rate and host exploitation (reviewed in Thomas & Blanford, 2003). Consequently reduced host densities will negatively feed back on parasite transmission and therefore on parasite spread through a population or between host populations. These outcomes are more likely for parasites having at least some degree of horizontal transmission to evade the unfavourable feedback effects (Lipsitch *et al.*, 1995b; Lafferty & Holt, 2003). Any negative effect on the host has negative consequences on a vertically transmitted parasite because of the direct fitness link between host and parasite (Fine, 1975). Therefore, the operative range of a vertically transmitted parasite should be expected to be similar or narrow compared to that of the host. A

physiological mismatch between the environmental optima of host and parasite can therefore favour host fitness and/or disrupt parasite development or transmission, when parasites are less tolerant to a given environment (Hurst *et al.*, 2000; Rodriguez *et al.*, 2004; Anbutsu *et al.*, 2008; Weis, 2008). For example, reduced bacterial density of *Wolbachia* at 32°C suggests a decline in the negative effect of this symbiont imposed on its host (Van Opijnen & Breeuwer, 1999; Hurst *et al.*, 2000; Mouton *et al.*, 2006). Further, the cytoplasmic incompatibility or sex ratio distorting effect of *Wolbachia* infected hosts is reduced at higher temperature thereby favouring host fitness (Hurst *et al.*, 2000). Not only costs of infection might depend on environmental conditions, but also potential selective advantages or benefits of infection (Hurst *et al.*, 2000; Hurst *et al.*, 2001; Russell & Moran, 2006). On the other hand, parasites can also provide protection against abiotic or biotic stressors to the host. While the abundance of *Rickettsia* in insects is also decreasing at higher temperatures, this bacterial endosymbiont produces a protein at normal operating temperature which increase host heat tolerance at high temperatures (Brumin *et al.*, 2011). The bacterial endosymbiont *Hamiltonella defensa* provides protection against wasp parasitoids but also increases the heat tolerance of its aphid host (Russell & Moran, 2006). This is also true for *Serratia symbiotica*, another bacterial symbiont of aphids (Montllor *et al.* 2002; Russell & Moran, 2006). However, in the absence of the stressors these endosymbionts impose costs to their host by reducing fitness or longevity (Cayetano & Vorburger, 2013).

The precise impact of environmental changes may not only vary from system to system but also between different host and/or parasite genotypes (Mitchell *et al.*, 2005; Lambrechts *et al.*, 2006; Mouton *et al.*, 2007; Vale *et al.*, 2008). The differences in response to environmental changes may strongly impact both interacting species and thus traits involved in infection such as transmission, virulence, resistance or tolerance, but also possible beneficial traits. Therefore, environment-mediated changes in interactions are likely to feed back on population dynamics, epidemiology and on host-parasite co-evolution, with potentially important consequences for the geographic distribution of the interacting species (Thomas & Blanford, 2003; Wolinska & King, 2009).

In this thesis, I investigate the combined effects of temperature and genetic variation on the virulence and persistence of *Caedibacter taeniospiralis*, a vertically transmitted bacterial endosymbiont of the freshwater protozoan *Paramecium tetraurelia* (Preer & Preer, 1982; Beier *et al.*, 2002). The symbiont provides a selective advantage to its host, but also reduces host division rate, and thereby, its own vertical transmission (Kusch *et al.*, 2002). The

small size and short generation time of the host are convenient for studying population-level dynamics in experimental cultures in short and long-term experiments. Therefore, this system is ideal to address questions of permanent heat stress effects on the evolutionary outcome of a host-parasite system with strictly vertical transmission.

In a second experimental part, I focus on the question of the origin of strict vertical transmission and mutualism. Genomic analysis indicates that evolutionary transitions from parasitism to mutualism include a profound change in parasite transmission. Nevertheless, experimental studies are lacking to support this hypothesis. I will therefore use the *Paramecium caudatum – Holospora undulata* system to investigate the influence of environmental changes on the ratio of horizontal to vertical transmission and therefore on parasite evolution and host-parasite co-evolution.

Study system

Paramecium as model host organism

Paramecium is a widespread ciliate common in almost all water-related habitats (Sonneborn, 1957). As common to all ciliates *Paramecium* has two different kinds of nuclei, the generative micronucleus (MIC) and the somatic macronucleus (MAC). The polyploid macronucleus serves the vegetative functions and is responsible for gene expression during vegetative growth. The small diploid micronuclei represent the "germ-line" and the number of micronuclei per cell is a species-specific characteristic within the genus *Paramecium*. The micronucleus is active during the asexual process of binary fission and sexual processes such as conjugation and autogamy (Görtz, 1988). Sexual events take place every 18-25 generations (Görtz, 1988), otherwise *Paramecium* reproduces asexually. Under favourable conditions, *Paramecium* can reproduce up to 4 generations a day (Beale & Preer, 2008). *Paramecium* is a study organism for epigenetics and genomics research, but also for experimental evolution. *Paramecium* can carry several different endosymbionts (Görtz & Fokin, 2009). These endosymbionts are not only phylogenetic diverse but also in their host-and/or compartment-specificity and in their transmission mode (Fokin *et al.*, 2003).

1. *Paramecium tetraurelia* infected with *Caedibacter taeniospiralis*

Caedibacter taeniospiralis (Preer & Preer, 1982) is an obligate intracellular *Gammaproteobacterium* located in the cytoplasm of *Paramecium tetraurelia* (Fig. 1). It is exclusively vertically transmitted during asexual reproduction of the host (Preer *et al.*, 1974;

Kusch & Görtz, 2006). Though it reduces host fitness (Kusch *et al.*, 2002), the bacterium still provides a selective advantage to its host, the so-called 'killer trait' (Sonneborn, 1938; Pond *et al.*, 1989; Schrallhammer & Schweikert, 2009). This selective effect in the host population is comparable to manipulation of host reproduction by *Wolbachia* (Werren, 1997) or microsporidians (Dunn & Smith, 2001). The 'killer trait' in *Paramecium* acts against food competitors but not against predators (Pond *et al.*, 1989; Kusch *et al.*, 2002). Killing occurs when a *Caedibacter*-free and therefore sensitive Paramecium ingests a toxic particle released by a *P. tetraurelia* harbouring *Caedibacter*. These particles are *Caedibacter* cells containing an unusual structure termed R-body (refractile body). These R-bodies are proteinaceous ribbons, which are typically coiled within the bacterial cell (Preer & Stark, 1953; Pond *et al.*, 1989). The convoluted R-body represents a huge hollow cylinder, which in response to certain stimuli, can unroll in a telescopic fashion (Schrallhammer & Schweikert, 2009). Once an R-body carrying *Caedibacter* cell is ingested by a sensitive *Paramecium*, the acidification of the *Paramecium* phagosome leads to the unrolling of the R-body (Fig. 1, Schrallhammer & Schweikert, 2009). It penetrates the membranes of the bacterium and phagosome and involves the release of an unidentified toxin into the cytoplasm that ultimately kills the sensitive paramecia cell (Kusch & Görtz, 2006; Schrallhammer & Schweikert, 2009). The lethal symptoms occur almost immediately after the ingestion of an R-body containing *Caedibacter*. Therefore, the response time of the 'killer effect' depends only on the feeding rate of susceptible paramecia (Kusch *et al.*, 2002; Schrallhammer *et al.*, 2012).

Caedibacter taeniospiralis R-bodies are encoded by a small region of the plasmid pKAP298 (GenBank accession number AY422720.1, Jeblick & Kusch, 2005). These four genes are termed reb genes (Heruth *et al.*, 1994). Reb proteins are rather small (theoretical molecular weight 10-24 kDa) considering the enormous size of the overall R-body structure (completely unrolled up to 20 µm in length, Pond *et al.*, 1989) but these four *reb* genes are, however, sufficient to produce R-bodies (Heruth *et al.*, 1994; Schrallhammer *et al.*, 2012). This indicates that R-bodies consist of Reb multimers, which is in good congruence with the high transcription activity of these genes (Jeblick & Kusch, 2005).

The R-body expression is very costly for a *Caedibacter* bacterium: cells harbouring an R-body cannot divide anymore. Thus, only a proportion of *Caedibacter* within one *Paramecium* host cell actually expresses the reb genes. The ratio between R-body producing and reproductively active *Caedibacter* can vary between 10 up to 50 % (Kusch & Görtz, 2006) depending on host or parasite growth conditions and environmental factors.

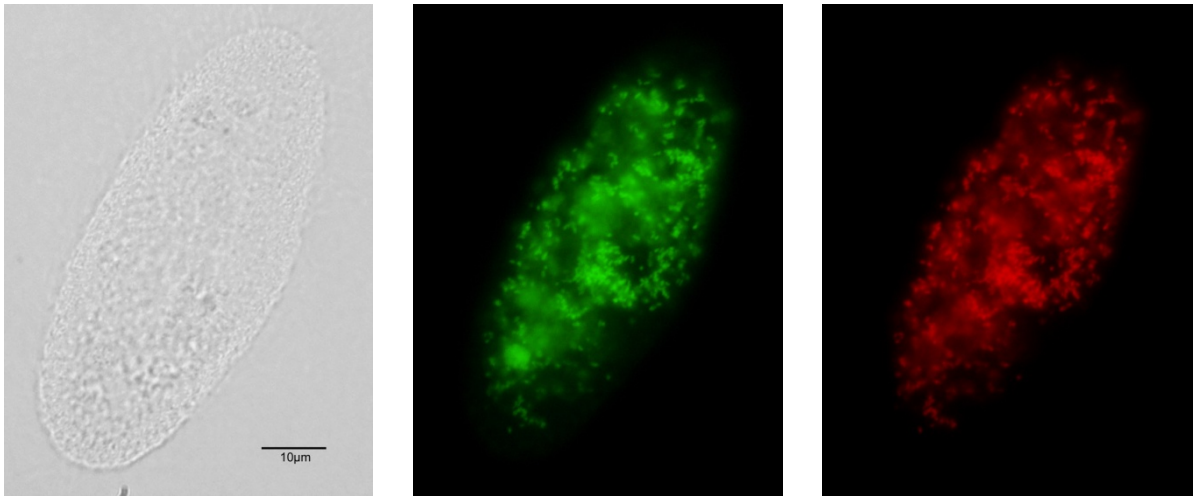

Fig. 1: *Paramecium* infected with *Caedibacter taeniospiralis*. (A) DIC of *P. tetraurelia* 298; (B) fluorescence *in situ* hybridisation performed with fluorescein-labelled general bacterial probe (EUB338 I; Amann *et al.*, 1991); (C) and with Cy3-labelled *Caedibacter taeniospiralis* specific probe (Ctaenio-998; Beier *et al.*, 2002). Positive probe signals allowed the determination of the parasite prevalence. Bar: 10 µm.

Caedibacter bearing hosts are not only emitting the 'killer trait', they are also resistant to pathogenicity of their own endosymbionts (Mueller, 1965; Preer *et al.*, 1974). This toxin resistance is provided by the harboured *Caedibacter* bacteria, as killer paramecia cured from their infection by antibiotic treatment lose the parasite's protection and become susceptible to the 'killer trait' (Kusch *et al.*, 2002). Thus, *C. taeniospiralis* infected *P. tetraurelia* cells are not killed by the uptake of their own parasite species from the medium, while parasite-free cells are killed (Pond *et al.*, 1989; Kusch & Görtz, 2006). It has, however, not been investigated so far, if massive exposure to toxic forms of *C. taeniospiralis* for any length of time still might be harmful but not lethal to *P. tetraurelia* hosting these bacteria. The toxin involved in the killing has not yet been identified (Kusch & Görtz, 2006; Schrallhammer *et al.*, 2012), but it was speculated that it should interact with the osmoregulation of the *Paramecium* cell (Jurand *et al.*, 1971). A membrane associated ATPase was identified as putative toxin (Jeblick & Kusch, 2005), but functional proof is missing.

Within this thesis, eight genetically different *Paramecium tetraurelia* strains with a different geographic origin (Table 1) were used to investigate the influence of genetic variation on the outcome of the host-parasite interaction. Five of these strains were naturally infected with their own *Caedibacter taeniospiralis* genotype.

Table 1. Origin of the *Paramecium tetraurelia* strains.

Strain	Origin	Infection level	Reference
51K (type strain)	Spencer, Indiana, USA	Infected	(Beale *et al.*, 1969)
47K	Berkeley, California, USA	Infected	(Dippell, 1950)
116K	Bloomington, USA	Infected	(Preer *et al.*, 1972)
298K	Empire Range Panama	Infected	(Preer *et al.*, 1972)
A30K	Littlehampton, Australia	Infected	(Stevenson, 1970)
51S	Spencer, Indiana, USA	Uninfected	(Preer *et al.*, 1974)
B	Kraków, Poland	Uninfected	(Przybos, pers. Comm.)

2. *Paramecium caudatum* infected with *Holospora undulata*

The gram-negative bacterium *Holospora undulata* belongs to the *Alphaproteobacteria* and infects the micronucleus of *Paramecium caudatum* (Gromov & Ossipov, 1981; Preer & Preer, 1982). This bacterium reduces host growth and survival rate (Restif & Kaltz, 2006) and is able to transmit horizontally and vertically because of a morphological dimorphism (Fokin & Skovorodkin, 1991; Fokin & Görtz, 2009). *Holospora undulata* occurs in infectious and reproductive forms. The immobile, spiral-shaped bacteria (≈ 15 µm) represent the infectious, horizontally transmitted forms that are ingested by new hosts during food uptake (Fokin & Görtz, 2009). They are able to escape the phagosome and are transferred to the micronucleus by the help of the host membrane and actin cytoskeleton system (Fujishima, 2009). After already 24 h, the infectious propagules will start to differentiate into shorter reproductive forms (≈ 2.5 µm) in the micronucleus. These reproductive forms will multiply and fill the entire nucleus. After 7 to 10 days a threshold bacterial density will be reached resulting in differentiation of a fraction of the reproductive forms into infectious forms (Görtz & Fokin, 2009). This step marks the end of the latent period and is strongly temperature-sensitive (Fels & Kaltz, 2006). While only reproductive forms are transmitted vertically to the daughter nuclei of the asexually dividing infected *Paramecium*, infectious forms are released into the environment during the host cell division or upon host death (Fokin & Görtz, 2009). This morphological and functional dimorphism of the parasite inflicts an inherent trade-off between the investment into vertical transmission (reproductive forms) and horizontal transmission (infectious forms; Kaltz & Koella, 2003).

After a new infection, infectious forms will occur in the nucleus within 7-10 days depending on environmental factors (Fels & Kaltz, 2006). The relatively long latency is different from the phenotypic switch from reproductive to infectious forms in already established infections (> 10 days), which can occur within 24-48 h. This phenotypic switch

might be regulated by bacterial density in the micronucleus and allows a different investment in the two transmission modes (Kaltz & Koella, 2003; Nidelet *et al.*, 2009). In slowly growing host populations, reproductive forms accumulate and differentiate into infectious forms within 24-48 h. Hundreds of infectious forms can fill the micronucleus causing its remarkable bloat. In contrast, rapid host division reduces bacterial density and the parasite will mainly remain in the reproductive form (Restif & Kaltz, 2006). Thus, this switch between vertical and horizontal transmission can be regulated by environmental conditions that alter host division rate (Kaltz & Koella, 2003). Host-growth conditions will therefore influence the proportion of vertical to horizontal transmission for the total transmission success (Restif & Kaltz, 2006) and therefore parasite virulence (Magalon *et al.*, 2010). Nidelet *et al.* (2009) demonstrated an evolutionary change in latency time, which suggests a genetic basis for this switch upon which selection can act.

During the first 48 h of the parasite's lifecycle, co-infections can occur after a first infection, although certain *Paramecium* genotypes become more resistant to co-infection, when newly infected (Fels *et al.*, 2008). At later stages of infection, with higher bacterial loads in the nucleus or when infectious forms are present, it is difficult to distinguish newly arrived infectious forms from those already present in infected host. However, there is evidence that co-infection with another *Holospora* species is not possible once a first species has established (Görtz & Fokin, 2009). This result suggests a mechanism prohibiting multiple infections after the onset of the production of infectious forms (reviewed in Görtz & Fokin, 2009).

Paramecium itself can inhibit a *Holospora* infection at several steps of the infection cycle, but resistance is commonly defined as host's ability to impede the invasion of infectious forms into the micronucleus or their differentiation into reproductive forms (Fujishima, 2009; Görtz & Fokin, 2009). Different *P. caudatum* genotypes have a natural variation in susceptibility to *H. undulata* infection (Görtz & Fokin, 2009), but resistance will also evolve in host populations co-evolving with the parasite (Lohse *et al.*, 2006). While this evolved resistance is costly due to a reduction in host fitness (Lohse *et al.*, 2006), host populations that have been completely recovered from infection can regain the fitness level of uninfected hosts without losing the resistance immediately (Duncan *et al.*, 2011). However, the underlying genetic or metabolic mechanisms of the resistance are still unknown.

THESES OUTLINE AND AIM OF EACH CHAPTER

In **chapter 1**, I investigate the role of genetic variability and acute environmental changes on the expression of virulence of a vertically transmitted symbiont. In order to identify the genetic and environmental influence on the host-parasite interaction, I tested different growth statuses, genotypes and temperatures including conditions stressful to the host.

- Hypothesis 1.1: The vertically transmitted symbiont *Caedibacter taeniospiralis* imposes context-dependent costs on its host.
- Hypothesis 1.2: Temperature variation renders the balance between cost and benefit of the parasite associated with a variable within-host density of *Caedibacter*.
- Hypothesis 1.3: Temperatures above host optimum are more stressful for the host being exposed to both parasite and thermal stress.

Central in **chapter 2** is the selective advantage of *Caedibacter* infected *Paramecium*. I describe the 'killer activity' of different *Caedibacter / Paramecium* interactions to investigate the influence of the genetic variability on the performance of the 'killer trait'.

- Hypothesis 2.1: The 'killer trait' compensates potential costs of the vertically transmitted symbiont.
- Hypothesis 2.2: The 'killer activity' depends on the genetic identity of the *Caedibacter / Paramecium* interaction.

In **chapter 3**, long-term virulence evolution of a vertically transmitted parasite was estimated at 32°C and 26°C. Prime focus here is to test whether this system can evolve in order to ensure symbiont maintenance at the stressful temperature (32 °C). Further, it was of interest if high-temperature adaptation trades off with the performance a permissive temperature (26°C). Therefore, microcosm populations of *Paramecium tetraurelia* infected with the vertically transmitted bacterium *Caedibacter taeniospiralis* were exposed to permanent high temperature stress, harmful for the parasite.

- Hypothesis 3.1: The parasite persists at and is able to adapt to high temperatures.
- Hypothesis 3.2: *Caedibacter* increases its within-host growth rate and thereby evolves to lower levels of virulence or even benevolence under continuous high-temperature conditions.

- Hypothesis 3.3: The direct response to selection trades off with the correlated response to selection, indicating costly heat adaptation.

In **chapter 4**, I describe the characteristics of 'heat-cured' paramecia that arose in the high temperature treatment of the previously explained long-term experiment. In theory, uninfected paramecia should be eliminated by means of the 'killer trait' of infected paramecia present in mixed populations.

- Hypothesis 4.1: The 'killer trait' is inefficient at high temperature.
- Hypothesis 4.2: 'Heat-cured' paramecia have a reduced sensitivity to the 'killer trait'.

In **chapter 5**, I used the parasite *Holospora undulata* with a mixed mode of transmission to study the evolution of strictly vertical transmission. In a long-term experiment, host growth conditions were manipulated to change the importance of vertical transmission in relation to total parasite transmission success. After ca. 800 host generations, I investigated host evolution and the horizontal transmissibility of the parasite to evaluate factors driving the transition from parasitism to mutualism. I compared the results with data of host generation 200 (Magalon *et al.* 2010).

- Hypothesis 5.1: Hosts of the vertical transmission treatment evolve a higher resistance due to the higher infectivity measured after 200 host generations.
- Hypothesis 5.2: The evolution of higher resistance reduces the importance of horizontal transmission.
- Hypothesis 5.3: Parasites of the alternating treatment, with the opportunity of horizontal and vertical transmission, evolve a more generalist transmission strategy.

CHAPTER 1

VIRULENCE REACTION NORM OF A VERTICALLY TRANSMITTED PARASITE ACROSS A TEMPERATURE GRADIENT

Vertical transmission and virulence

1.1 Abstract

Parasites with exclusive vertical transmission from host parent to offspring are an evolutionary puzzle. With parasite fitness entirely linked to host reproduction, any fitness cost for infected hosts risks their selective elimination. Environmental conditions likely influence parasite impact, and thereby the success of purely vertical transmission strategies. We tested for temperature-dependent virulence of *Caedibacter taeniospiralis,* a vertically transmitted bacterial symbiont of the protozoan *Paramecium tetraurelia*. We compared growth of infected and cured host populations at five temperatures (16-32 °C). Infection reduced host density at all temperatures, with a peak at 28 °C. These patterns were largely consistent across five infected *Paramecium* strains. Similar to *Wolbachia* symbionts, *C. taeniospiralis* may compensate fitness costs by conferring to the host a 'killer trait', targeting uninfected competitors. Considerable loss of infection at 32 °C suggests that 'killer activity' is not universal and that limited heat tolerance restricts the conditions for persistence of *C. taeniospiralis*.

Keywords: *Caedibacter*, genotype-by-environment interaction, *Paramecium*, temperature, vertical transmission, virulence

1.2 Introduction

The transmission mode of infection is a key factor for evolution in host-parasite interactions (Ewald, 1987; Lipsitch *et al.*, 1996; Day & Proulx, 2004). Horizontal transmission (infectious spread to uninfected hosts) can select for more harmful parasites, whereas vertical transmission (parent to offspring hosts) is intrinsically linked with host reproduction and should favour minimal host damage (Lipsitch *et al.*, 1996). Parasites with mixed modes of vertical and horizontal transmission are predicted to evolve intermediate level of virulence (i.e., reduction of host fitness), maximizing total transmission through both pathways (Lipsitch *et al.*, 1996; Day & Proulx, 2004).

Parasites with exclusive vertical transmission are an evolutionary puzzle (Ewald, 1987). Locked in a single line of host descent, their replication must be sufficiently high to ensure vertical transmission, but low enough to avoid damage to the host. In principle, any fitness cost imposed by the symbiont should lead to selective elimination of infected hosts from the population (Lipsitch *et al.*, 1996; Day & Proulx, 2004). Costs may be compensated by occasional horizontal transmission, manipulation of host reproductive system (e.g., sex-ratio distortion, biparental inheritance) or by conferring (novel) beneficial effects to the host, at least under some conditions (Ahlholm *et al.*, 2002; Fellous & Salvaudon, 2009).

Prominent examples include bacteria and microsporidia, which can strongly reduce host reproduction, but also manipulate the host reproductive system to increase their frequency in the population (Werren, 1997; Dunn & Smith, 2001). Endosymbionts of aphids compensate potential fitness costs by supplying hosts with nutrients or protection against parasitoid attack (Douglas, 1998; Vorburger & Gouskov, 2011). However, these host-endosymbiont interactions are also sensitive to genetic factors (Vorburger & Gouskov, 2011) and environmental conditions (Mouton *et al.*, 2007). Namely, temperature variation renders endosymbionts more or less harmful, or even beneficial (Thomas & Blanford, 2003); these effects are often associated with a change within-host density of the symbionts (Dunn *et al.*, 2006; Mouton *et al.*, 2007), which influences their fidelity of vertical transmission or capacity to manipulate the host reproductive system (Hurst *et al.*, 2000; Dunn *et al.*, 2006). The bottom line is that temperature can shift the balance between costs and benefits of the parasite and, consequently, the success of purely vertical transmission strategies. Experiments are lacking that investigate this issue at the population level over multiple generations.

We investigated combined effects of temperature and genetic variation on the virulence and persistence of *Caedibacter taeniospiralis*, a vertically transmitted bacterial

endosymbiont of the freshwater protozoan *Paramecium tetraurelia* (Preer *et al.*, 1974; Beier *et al.*, 2002). We hypothesized that *Caedibacter* imposes context-dependent costs to its host (hypothesis 1.1) and that temperatures above host optimum temperature are more stressful to the host because of a possible additive effect (infection and heat stress; hypothesis 1.3). Temperature will therefore render the balance between cost and benefit of the parasite (hypothesis 1.2). Analogous to *Wolbachia* (Werren, 1997), *Caedibacter* confers to its host a 'killer trait' that increases the frequency of infected carriers by causing death of uninfected competitors (Schrallhammer & Schweikert, 2009). However, the symbiont also reduces host division rate, and thereby its own vertical transmission (Kusch *et al.*, 2002). The small size and short generation time of the host are convenient for studying population-level dynamics in experimental cultures. We measured growth of infected and cured experimental populations from five infected *P. tetraurelia* strains along a temperature gradient (16-32 °C). We compared the impact of infection on population parameters (growth rate, carrying capacity) across temperatures and strains; we also assessed the persistence of the symbiont under these different conditions.

1.3 Material and Methods

The *Gammaproteobacterium Caedibacter taeniospiralis* (Preer *et al.*, 1974; Beier *et al.*, 2002) infects the cytoplasm of its host *Paramecium tetraurelia*. Vertical transmission occurs during mitotic division, from infected mother to daughter cells; free-living stages or horizontal transmission are unknown (Kusch *et al.*, 2002). Expression of the 'killer trait' requires release of killer-competent *Caedibacter* cells from an infected *Paramecium*. Once ingested by uninfected *Paramecium*, the bacteria burst and kill the *Paramecium,* presumably through a toxin (Preer *et al.*, 1974; Schrallhammer & Schweikert, 2009). We used one naïve and five *P. tetraurelia* strains (Table 1; Preer *et al.*, 1974), naturally infected with *C. taeniospiralis* and kept in the laboratory for several years at 22 °C in Cerophyl medium (Krenek *et al.*, 2011) with the food bacterium *Raoultella planticola* DMSZ 3069.

Caedibacter infection was verified with PCR and fluorescence *in situ* hybridisation (FISH). For PCR, total DNA was extracted from 20 *P. tetraurelia* cells using Chelex® 100 resin following the protocol of Barth and colleagues (Barth *et al.*, 2006). A 320 bp fragment of the 16S rRNA gene was amplified by PCR using the primer pair Ct_4F (5'-CTG TTG GTC CTG GTG TAA AAG GAT TA-3') and Ct_4R (5'-GCA GTC TCT CTA GAG TGC CCA ACT TA-3'). The PCR reaction mix contained 6 µl of Chelex extracted DNA, 3 pmol of each primer (Eurofins MWG Operon, Ebersberg, Germany), 0.375 U GoTaq Polymerase and

1xGoTaq Green buffer with 3 mM MgCl2 and 200 µM dNTPs (Promega, Mannheim, Germany) in a final volume of 15 µl. The PCR cycle program started with 3 min at 94 °C, followed by 35 cycles comprising 30 s at 94 °C, 30 s at 55 °C, 90 s at 72 °C and a final extension step of 10 min at 72 °C. FISH was used to confirm PCR results and to estimate the prevalence of *C. taeniospiralis* in the infected *P. tetraurelia* lines. Hybridization was performed following the protocol of Manz and colleagues (1992). Two fluorescently labelled oligonucleotides were used in parallel: Ctaenio-998 (specific for *C. taeniospiralis*, Beier *et al.*, 2002 and EUB338, Amann *et al.*, 1990; both probes obtained from Biomers, Ulm, Germany). The cells were mounted with Citiflour (Citifluor Ltd., London, UK) and observed with an Eclipse Ti epifluorescence microscope (Nikon, Tokio, Japan). Images were recorded using the Digital sight DS Fi1c camera and the NIS-Elements AR^{TM} Imaging Software (Nikon, Tokio, Japan).

For the temperature experiment, infected cells from each strain were cured with the antibiotic streptomycin. The streptomycin treatment was performed as follows (modified from Krenek *et al.*, 2011): approximately 30 cells of the stock culture were transferred to 500 µl of sterile Dryl's solution (1 mM $NaHPO_4$, 1 mM NaH_2PO_4, 1.5 mM $CaCl_2$, 2 mM Na-citrate; Dryl, 1959) with 100 µg ml^{-1} streptomycin (Sigma, St. Louis, USA) and incubated for 24 h at 22 °C. They were washed four times in sterile Dryl's solution with streptomycin. Single cells were incubated in 500 µl of sterile Dryl's solution with 100 µg ml^{-1} streptomycin at 22 °C for another 24 h. Single *Paramecium* cells were transferred to 500 µl of sterile Dryl's solution and then in 500 µl of Cerophyl medium inoculated with *R. planticola.* The volume was doubled every second day by adding bacterized medium. These monoclonal *P. tetraurelia* lines were incubated for eight days at 22 °C. Recovery was verified by PCR and FISH. For each strain, cured and infected (= untreated) precultures were established by pooling three monoclonal lines. Possible negative effect of the Streptomycin on paramecia growth was determined. For that purpose, uninfected control strains were antibiotic treated and their growth was measured. After four weeks post-treatment we found no influence of the antibiotic treatment on host growth (Fig. 2).

Established from precultures, 150 infected and cured replicate populations were tested at 16 °C, 20 °C, 24 °C, 28 °C and 32 °C (5 strains x 2 infection status x 5 temperatures x 3 replicates). Starting from 10 ml samples at 22 °C, populations were acclimated in steps of 2 K d^{-1}; then kept at final temperature for 3 d. We regularly doubled the culture volume to ensure exponential growth during acclimation.

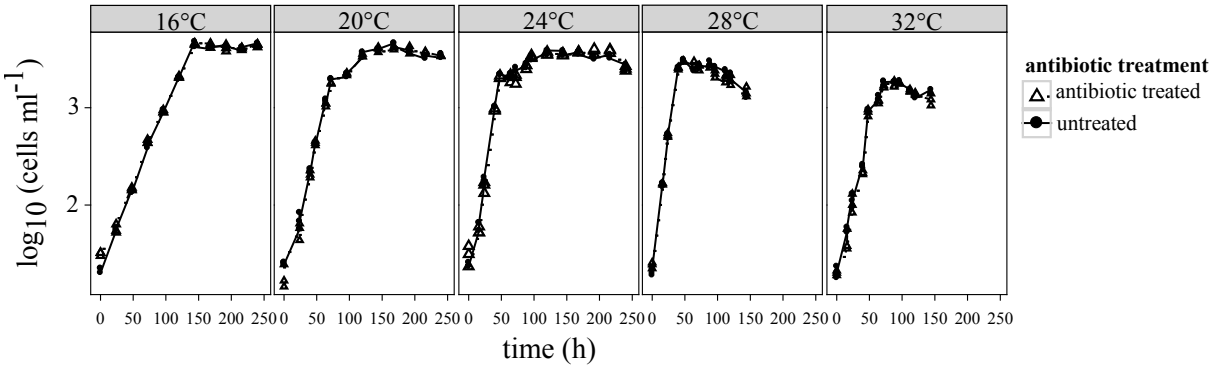

Fig. 2: Mean growth curves (± S.E.) of antibiotic treated and untreated, naive *Paramecium* populations at all experimental temperatures.

Replicate populations (60 ml flasks) were placed in temperature-controlled water tanks (2 tanks per temperature). Starting density was set to 25 cells ml^{-1} in 20 ml of culture (Krenek *et al.*, 2011); then measured every 8-24 h for up to 10 days, until growth curves were complete (ca. 15 generations). Density measurements consisted of counting paramecia in 25-300 µl samples under a dissecting microscope. Using FISH, we determined the proportion of infected cells (initially 100 %) at carrying capacity (4 or 9 days).

Using the R program (R Devleopment Core Team, 2012), we estimated intrinsic population growth rate r (d^{-1}) and carrying capacity K (cells ml^{-1}) for each replicate population, by fitting a logistic growth model (Eq. 1),

$$y_t = (k \times y_0)/(y_0 + (k - y_0) \times e^{-r \times t})$$

with the initial host concentration y_0 (cells/ml) and the host concentration y_t (cells ml^{-1}) after time t (d). As no additional food resources were provided during the experiment, many populations decreased in density after reaching carrying capacity. Decreases below 80 % of the maximum were not considered for model fitting. For parameter optimization, we used the Levenberg-Marquard method (R add-on package FME; Soetaert & Petzoldt, 2010). Estimates are based on real time as using physiological time (Mitchell *et al.*, 2005) gives nearly identical results (not shown). Effects of infection status, strain and temperature on K and log-transformed r were tested in factorial Analyses of Variance (ANOVA).

1.4 Results

Infection had no significant effect on intrinsic growth rate, but impacted host density carrying capacity (Fig. 3, Fig. 4, Table 2). Carrying capacity was always lower in infected populations than in cured populations (significant infection status effect).

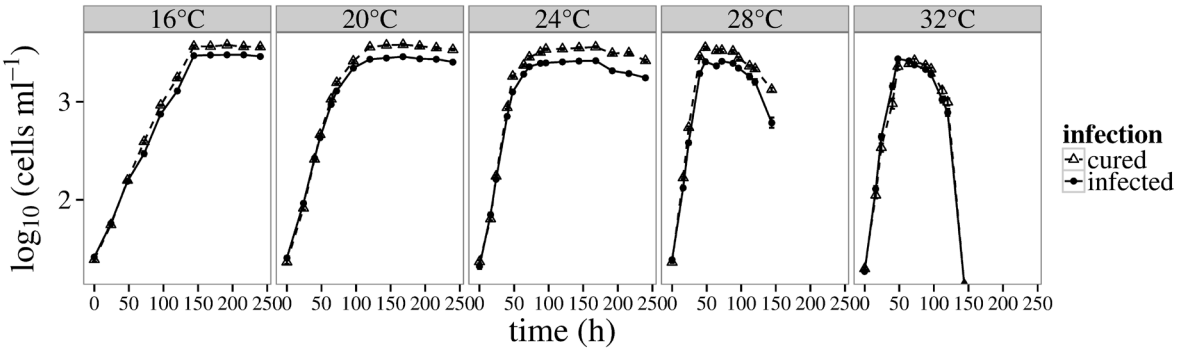

Fig. 3. Mean growth curves (± S.E.) of infected and cured populations at all experimental temperatures.

This negative effect of infection increased with temperature from a -16% density reduction (16 °C) to -30% (28 °C); then dropped back to -17% at 32 °C (Fig. 3; significant status x temperature interaction). At 32 °C, the drop of virulence coincided with reductions of infection prevalence, ranging from -10% (strain 298) to -80% (strain A30). Marginal loss of infection (-4%) occurred at 28 °C.

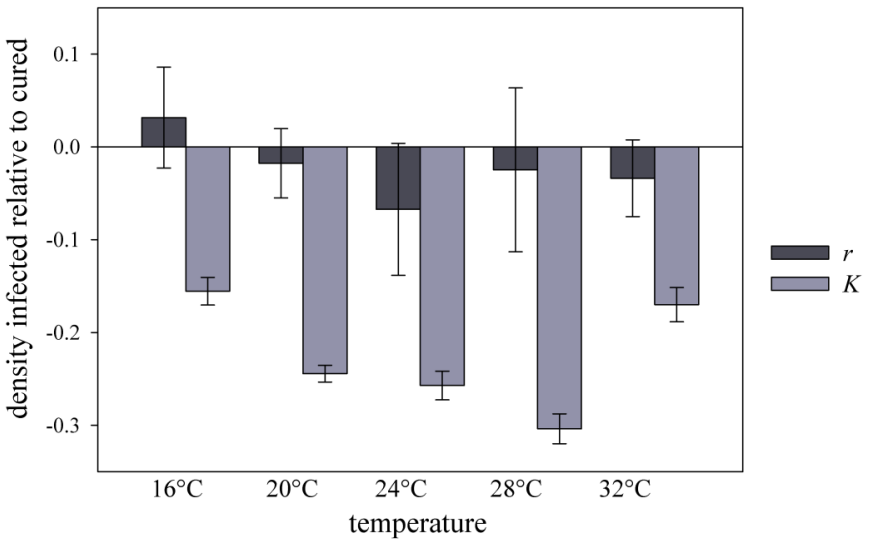

Fig. 4. Mean reduction (± S.E.) in host fitness by *Caedibacter taeniospiralis* at five temperatures, expressed as the growth rate (r) and density at carrying capacity (K) of infected relative to cured *Paramecium tetraurelia*. Reduction calculated as [(infected / cured) - 1]; negative values mean negative effect of infection

The temperature-dependent impact of infection varied with strain identity (significant infection status x temperature x strain identity interaction). However, *post hoc* tests (Tukey HSD) showed that the density reduction was significant for the majority of strains at all temperatures (22 out of 25 combinations; Fig. 5). Thus, the 3-way interaction explained only a small fraction of the total variance in carrying capacity (approximately 3%; Table 2), compared to the strong main effects of infection status (30%) and strain identity (50%).

Similarly, variation in growth rate (*r*) was mainly explained by temperature (86%), infection status and its interactions made only marginal contributions (ca. 5%; Table 2; Fig. 5).

Table 2. ANOVA testing effects of temperature, *Paramecium* strain and population infection status on host growth rate (*r*) and carrying capacity (*K*). The R^2 represents the fraction of the total phenotypic variability (total sums of squares) explained; MS is mean square.

		log *r*			*K*		
	d.f.	MS	p-value	R² (%)	MS	p-value	R² (%)
temperature	4	1.889	<0.001	85.8	0.023	0.075	5.7
strain	4	0.054	<0.001	2.5	0.195	<0.001	49.1
infection status	1	0.019	0.581	0.2	0.483	<0.001	30.3
temperature*strain	16	0.023	<0.001	4.1	0.009	<0.001	8.7
temperature*status	4	0.003	0.960	0.2	0.009	0.002	2.3
strain*status	4	0.053	<0.001	2.4	0.001	0.041	0.4
temperature*strain*status	16	0.014	<0.001	2.6	0.001	<0.001	1.3
residual	100	0.002			0.001		

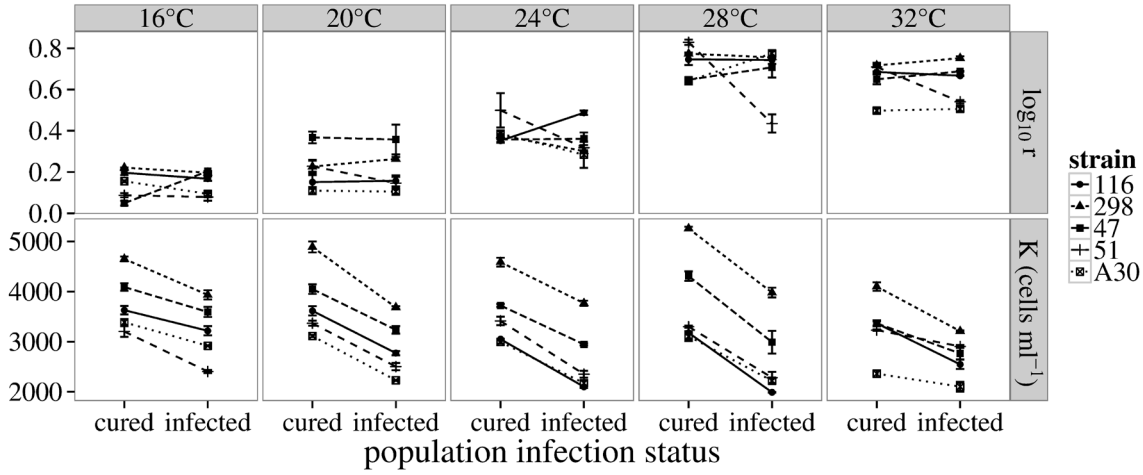

Fig. 5. Mean density (± S.E.) at carrying capacity (*K*) and maximal intrinsic growth rate (± S.E.) of infected and cured *Parameciumtetraurelia* genotypes at five temperatures

1.5 Discussion

Infection reduces host capacity

Infection reduced host carrying capacity (*K*), but had no detectable effect on growth rate (*r*). This suggests that infection becomes costly under resource limitation, characteristic of various host-parasite systems (Ahlholm *et al.*, 2002; Wolinska & King, 2009). During unlimited exponential growth, hosts may compensate the energy drain by the parasite and keep infection

in check. Possibly, rapid host division diluted the parasite within the host, thereby limiting parasite impact (e.g. Restif & Kaltz, 2006). This may also benefit the parasite, because having little impact on fast growing hosts increases vertical transmission rate (Magalon *et al.*, 2010). Nonetheless, when populations reach carrying capacity, our results indicate a substantial selective disadvantage of infected vs. uninfected *Paramecium*.

Temperature-dependent virulence

We observed a temperature-dependent increase in virulence with a peak at 28 °C. Higher temperature may favour bacterial within-host replication and lead to an accumulation of bacteria, when host division slows down at carrying capacity. The apparent drop in virulence at 32 °C can be explained by the observed loss of infection, such that only a fraction of the population may have actually suffered from infection. This result indicates that *C. taeniospiralis* is less temperature-stress tolerant than its host. Temperature-related changes in bacterial load as well as high-temperature sensitivity also exist in other endosymbionts (Van Opijnen & Breeuwer, 1999; Hurst *et al.*, 2000; Thomas & Blanford, 2003; Anbutsu *et al.*, 2008). Whether *C. taeniospiralis* can evolve heat-tolerance and how this affects its virulence, this question will be raised in Chapter 3.

Marginal role of genetic variation

Temperature effects on virulence varied with *Paramecium* strain identity. Such genotype-by-environment interactions are common in host-parasite systems (Thomas & Blanford, 2003; Wolinska & King, 2009) and may promote environment-specific virulence (Vale *et al.*, 2011). However, the strength of our strain x temperature x infection status interaction was marginal compared to the main effects. In fact, neither temperature nor infection caused strong rank reversal among the strains, as indicated by the parallel lines in Fig. 5. This lack of variation implies only weak selection potential for strains with lower virulence at any of these temperatures. In fact the patterns suggest that one single strain (298) is favoured at all temperatures, regardless of infection status. At this point it shall be emphasised that the experimental design did not allow assessing additional effects of *Caedibacter* genotype, as it was linked with *Paramecium* strain identity. This would require a technique of horizontal transfer of parasites for cross-infection experiments. In this experimental system with purely vertical transmission, host and parasite genomes are always selected jointly.

Compensation of the cost?

Similar to *Wolbachia* species inducing cytoplasmic incompatibility (Werren, 1997), *C. taeniospiralis* may compensate its fitness cost by conferring to the host the 'killer trait', which can eliminate uninfected competitors, despite their growth advantage (Kusch *et al.*, 2002; Schrallhammer & Schweikert, 2009). However, the expression of this trait is condition-dependent: the development of killer-competent cell types is induced by mild starvation (Kusch & Görtz, 2006; Schrallhammer & Schweikert, 2009). Here, loss of infection and persistence of uninfected cells suggests that this induction is abrogated at high temperature. The offset of the 'killer trait' is yet to be confirmed by competition experiments, but reduced cytoplasmic incompatibility at higher temperature is also known for *Wolbachia* (Hurst *et al.*, 2000).

1.6 Conclusion

Contrary to the common expectation that exclusively vertically transmitted symbionts should not harm their host (Ewald, 1987), *C. taeniospiralis* substantially reduced host density in resource-limited situations, across a range of temperatures and strains. These results verify the first hypothesis that *Caedibacter* imposes context-dependent costs on its host *P. tetraurelia* (hypothesis 1.1). Moreover, temperatures above host optimum are more stressful for the parasite, which falsifies the hypothesis, that temperature stress and parasitism have an additive negative effect on host fitness (hypothesis 1.3). High temperatures reduce the persistence of *Caedibacter* and thereby the negative effect of infection on host fitness. Successful spread and maintenance of this parasite therefore requires a universally efficient mechanism compensating this fitness cost, possibly the 'killer trait' (Schrallhammer & Schweikert, 2009). Our results suggest that this trait is inefficient at high temperatures, thereby limiting ecological conditions for persistence of *C. taeniospiralis.* Acute temperature variation therefore renders the balance between cost and benefit of infection (hypothesis 1.2). However, the question that remains is whether *C. taeniospiralis* can evolve heat-tolerance and how this affects cost and benefit for the host. One advantage of this model system is the possibility to test this question in experimental populations over larger ecological and evolutionary timescales.

CHAPTER 2

THE 'KILLER TRAIT' – A STRATEGY TO COMPENSATE HOST FITNESS COST?

The beneficial trait of *Caedibacter taeniospiralis*

2.1 Introduction

Vertical transmission is the infection spread from parents to offspring. Symbiont reproduction and transmission success is therefore positively linked with host fitness (Fine, 1975; Ewald, 1987). Vertically transmitted symbionts should minimize host damage because any costs imposed by the symbiont can be followed by the selective elimination of infected individuals from the population (Lipsitch *et al.*, 1996; Day & Proulx, 2004). Alternatively, costs can also be compensated by additional horizontal transmission, manipulation of host reproductive system or by beneficial effects to the host (Ahlholm *et al.*, 2002; Jones *et al.*, 2007; Fellous & Salvaudon, 2009).

The vertically transmitted symbiont *Caedibacter taeniospiralis* imposes costs to its host *Paramecium tetraurelia* by reducing host density at carrying capacity and therefore its own vertical transmission success. These costs are influenced by the genetic background and temperature changes (Chapter 1). However, *Caedibacter* confers a beneficial trait to its host, the so called 'killer trait' (Preer *et al.*, 1974; Pond *et al.*, 1989; Schrallhammer & Schweikert, 2009). Infected individuals have a selective advantage by killing their uninfected conspecifics through an unknown toxin carried in a special proteinous structure, the R-body (Pond *et al.*, 1989; Schrallhammer & Schweikert, 2009). This trait is expected to compensate costs imposed by the symbiont (hypothesis 2.1) and to depend on the genetic background of the host-parasite interaction (hypothesis 2.2). 'Killer tests' (Schrallhammer *et al.*, 2012) were conducted to test these hypotheses.

2.2 Methods

Experimental set-up

Five *P. tetraurelia* strains from different geographic origins (Table 1); each naturally infected with *C. taeniospiralis,* were tested for their 'killer activity' against different uninfected paramecia strains including antibiotic cured paramecia of the same genotype. Infected and

uninfected cells were set-up in a starvation treatment without adding food for 14 d. Afterwards, 8 'sensitive', uninfected paramecia cells were exposed to 8 infected paramecia cells in 200 µl exhausted cerophyl medium (Krenek *et al.*, 2011) for 5 h at 24 °C. 'Killer tests' were conducted according to the protocol of Schrallhammer *et al.* (2012) and replicated 16 times. Cell numbers were determined microscopically every 30 min to 1 h. Survival probability was estimated using the Kaplan-Meier estimate (Eq. 2),

$$\hat{S}(t) = \prod_{t(i)\leq t} \left(1 - \frac{d_i}{n_i}\right)$$

where d_i is the number of dead cells at the time point t_i and n_i is the number of cells at risk to die. Two parameters were used to describe the 'killer activity' of each *Caedibacter* genotype: the number of killed cells after 5 h and the 'survival time', which was calculated as the mean survival time of all uninfected cells of one replicate. The 'killer activity' as 'number of killed cells after 5 h' was correlated with virulence of the *Caedibacter*.

Electron microscopy

Cells of the infected *Paramecium* strain 116 and 298 were prepared for electron microscopy following the protocol of Sabaneyeva *et al.* (2009). Briefly, infected *Paramecium* cells were starved and concentrated by centrifugation. Samples were fixed in a mixture of 1.6 % paraformaldehyde and 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.2-7.4) for 1 h at room temperature, washed in the phosphate buffer containing 125 mg/ml sucrose and postfixed with 1.5 % Osmium tetroxide for 1 h at 4 °C. The cells were dehydrated through a graded series of ethanol and acetone and embedded in Epoxy embedding medium (Fluka, BioChemika). The resin was polymerized according to the manufacturer protocol. Ultrathin sections were prepared using a Reichert-Jung Ultracut E and stained with 1 % aqueous uranyl acetate and 1 % lead citrate. The samples were visualized using a Jeol JEM-1400 (Jeol, Ltd., Tokyo, Japan) electron microscope at 90 kV. The images were obtained with an inbuilt digital camera.

2.3 Results

The 'killer trait' was active for 4 out of 5 *Caedibacter* genotypes. The survival time differed from 3.30 h to 4.04 h (log-Rank $p < 0.001$; d.f. = 4) and the number of killed cells from 3.35 to 5.32 for the different genotypes (Table 3; Fig. 6). A pair wise comparison of the Kaplan-Meier estimates over time showed significant differences for all genotype combinations except for the pair 51K and A30K ($p = 0.14$; Fig. 6).

Table 3. 'Killer activity' estimated as mean killing time (h) and the mean number of killed paramecia after 5 h and mean virulence at 24 °C of the five different *Paramecium* strains harbouring their own *Caedibacter* genotype.

genotype	Virulence	Killing time (h)	Killed paramecia cells after 5 h
298	0.20 ± 0.01	3.301 ± 0.065	5.32 ± 0.12
51	0.31 ± 0.01	3.61 ± 0.060	4.71 ± 0.10
A30	0.28 ± 0.01	3.66 ± 0.061	4.21 ± 0.12
47	0.21 ± 0.004	4.04 ± 0.050	3.35 ± 0.10
116	0.31 ± 0.004	n.d.	0

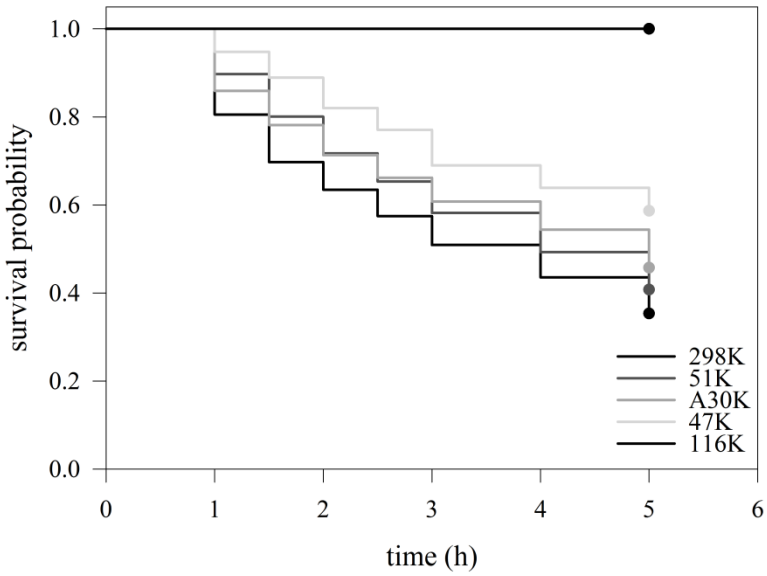

Fig. 6. Survival probability of uninfected host cells over time for five different *Paramecium* genotypes. Survival probability was estimated as the Kaplan-Meier estimate over time. Data are censored.

For 3 out of 5 *Caedibacter* genotypes, virulence was positively correlated with the number of killed cells (Fig. 7). The genotype 298 had the lowest virulence but the highest number of killed cells, while *Caedibacter* genotype 116, the most virulent one, was not able to kill any of the exposed sensitive cells.

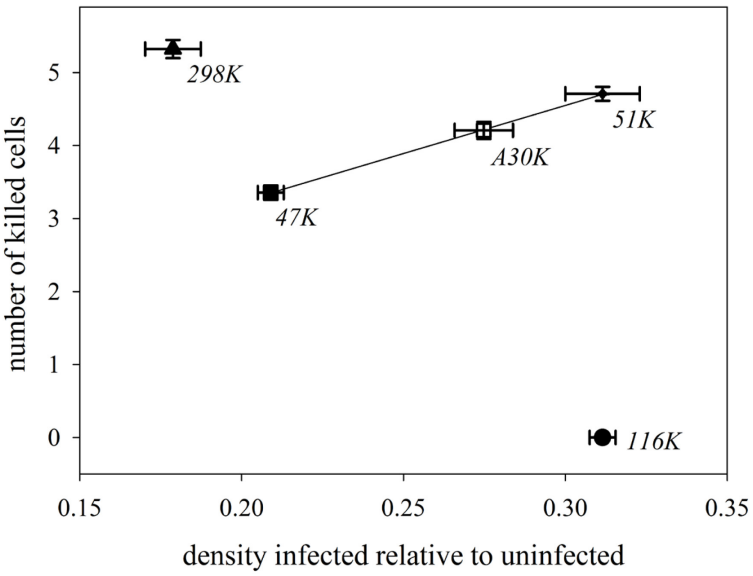

Fig. 7. Relationship between density of infected relative to uninfected hosts and the number of killed host cells. Benefit is defined as the *Paramecium* cell number killed by *Caedibacter* infected hosts during 5 h of exposure. Virulence is defined as density infected relative to uninfected at 24°C. Both virulence and benefit were estimated for five different *Caedibacter* genotypes. There is only a correlation between cost and benefit of *Caedibacter* for three out of five genotypes.

Electron microscopy was conducted to prove the presence of R-bodies within the *Paramecium* strain 298 and 116. The analysis revealed the presence of R-bodies not only in the strain 298 but also in the strain 116 (Fig. 8).

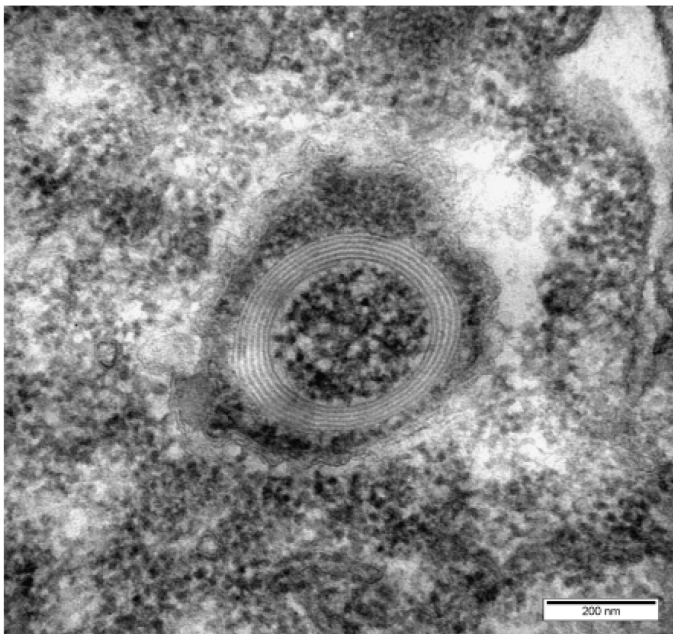

Fig. 8: Transmission electron micrographs of a *Caedibacter taeniospiralis* infecting *P. tetraurelia* strain 116K and harbouring a huge hollow cylinder, the coiled R-body. Bar, 200 nm.

2.4 Discussion

The 'killer trait' – a cost compensator?

Contrary to theory (Fine, 1975; Ewald, 1987; Lipsitch *et al.*, 1995b), *C. taeniospiralis* reduces host fitness, and hence its own transmission rate, in resource-limited situations (Chapter 1; Table 2). However, for successful spread and maintenance, the symbiont also shows an active 'killer trait' for 4 out of 5 *Caedibacter* genotypes under resource-limited situations. Analogous to *Wolbachia* species inducing cytoplasmatic incompatibility (Werren, 1997) or different microsporidian sex ratio distorting strategies (Dunn & Smith, 2001), *C. taeniospiralis* is able to increase the number of infected host individuals within mixed host populations by eliminating uninfected conspecifics (Preer *et al.*, 1974; Schrallhammer & Schweikert, 2009). Similar to genetic variation in virulence (Chapter 1), the 'killer activity' also varied between the different *Caedibacter* genotypes (Table 3) supporting hypothesis 2.2. For 3 out of 5 genotypes show a positive correlation between virulence and the number of killed cells (Fig. 7). These results support the hypothesis that this 'killer trait' compensates costs imposed by the symbiont (hypothesis 2.1). *C. taeniospiralis* genotype 298 probably has the best cost compensation strategy. It offers not only the most efficient 'killer trait' but also caused a minimal fitness reduction. The other extreme is *Caedibacter* genotype 116, which obviously lost the beneficial trait but imposes the strongest cost to its host. The loss of the beneficial trait might increase the risk of extinction for this *Caedibacter* genotype within mixed populations. However, the *Caedibacter* genotype 116 persists at temperatures below optimum (Chapter 1), even in resource-limited situations. This allows speculating about further mechanisms involved in symbiont maintenance, e.g. host tolerance or beneficial mechanisms of the parasite. All in all, these results demonstrate the genetic variability of the 'killer trait' (hypothesis 2.2).

Loss of the 'killer trait'

'Killer tests' with genotype 116 revealed a non-functional 'killer trait' but paramecia of this genotype are still resistant against the killer toxin (Hofmann 2014), the ultimate agent inferring the 'killer activity'. The loss of the 'killer trait' can have different genetic and mechanistic explanations: loss of the endosymbiont, loss of the plasmid, mutation in the reb genes, mutation in the promoter of the reb genes, mutations in the toxin, inactivation of the toxin. The infection level of the *Paramecium* strain 116 was confirmed by FISH and a PCR, amplifying the reb gene region (Kusch *et al.*, 2002), and revealed the presence of the plasmid.

The inactivity of the 'killer trait' caused by missing R-bodies, the toxin transmitter, could be neglected microscopically (Fig. 8). Furthermore, the functionality of the R-body should be similar to the R-bodies of the other *Caedibacter* genotypes because of 100 % similarity within the genes encoding the proteinous R-body structure (personal communication with M. Schrallhammer) and evidently their expression (Hofmann 2014). Therefore, the inactivity of the 'killer trait' might be related to a significantly reduced number of R-body containing *Caedibacter* cells per paramecia. This quantification of R-bodies within a cell or even a population is not feasible microscopically. The embedding of the samples and also the microscopically analysis is time consuming and not practicable for the number of replicates required for this analysis. A PCR based method would be more feasible for high-throughput analyses.

A possible explanation for the malfunctioning 'killer trait' might also be related to the toxin involved in the 'killer trait'. However, this hypothetical protein is still unknown and therefore its expression and activity within the different *Caedibacter* genotypes remains unclear.

Perspective

The 'killer activity' of the five *Caedibacter* genotypes was shown to be as different compared to their virulence. Therefore, the 'killer trait' can be an excellent candidate as cost compensator in the *Caedibacter taeniospiralis – Paramecium tetraurelia* system. However, 'killer tests' and microscopy are not efficient approaches to test the 'killer activity' for different genotypes probably sensitive to environmental changes. The established experimental approaches are time consuming because of the low number of replicates, which can be performed at once. The 'killer activity' might be correlated to the amount of R-bodies produced by a *Caedibacter* population so that the quantification of R-body gene expression would allow testing this hypothesis. The proportion of R-body producing *Caedibacter* within one population might be correlated to reb gene expression. The reb genes (rebA, rebB, rebC, rebD) encode the R-body structure (Heruth *et al.*, 1994; Schrallhammer *et al.*, 2012) and are expressed in R-body producing *Caedibacter* with high transcription rates (Jeblick & Kusch, 2005). Potential target region for a specific primer and probe design is the highly expressed plasmid region containing the rebB locus (Heruth *et al.*, 1994; Jeblick & Kusch, 2005). A specific primer pair (forward primer reb_B_F1 5'- ACA CGG CAG CTA ATG CTC AA-3' and reverse primer reb_B_R1 5'-CAG CAA GGG CAT CAG TTA AAC G-3'; Eurofins MWG Operon, Ebersberg, Germany) and Taqman hydrolysis probes (reb_B_pr 5'_TCA GTG

AAC CGT TCT AAT GAC ACA ACC C-3', labelled with the BHQ 2 quencher at 3'end and Texas Red as reporter dye at the 5' end; Eurofins MWG Operon, Ebersberg, Germany) were designed based on the rebB gene sequence (GenBank accession number AY422720.1) using the program Primer3 (Rozen & Skaletsky, 2000). The rebB gene as target gene and the *Caedibacter* 16S rRNA gene as reference gene, for which specific primer and Taqman probes were designed as well (Hofmann 2014), were used to perform relative quantification. By comparing rebB gene expression between different *Caedibacter* genotypes and at different temperatures, the differential expression of rebB can be quantified. To reduce the amount of PCR reactions and to increase assay sensitivity, we considered to perform a multiplex RT-qPCR approach by analysing 16S rRNA and rebB gene expression of each collected RNA sample within a single PCR reaction. However, first trials revealed a very low amount of rebB RNA making the establishment of the qPCR assay more challenging. Therefore, further work is needed to establish this method as a potential tool to study 'killer activity'.

CHAPTER 3

EXPERIMENTAL EVOLUTION OF HEAT-STRESS ADAPTATION IN A HOST-SYMBIONT ASSOCIATION WITH OBLIGATE VERTICAL TRANSMISSION

Heat stress evolution

3.1 Abstract

Strictly vertical (parent-to-offspring) transmission of symbionts is often associated with benevolence and mutualism, possibly because host reproduction and symbiont transmission are positively correlated. However, many such symbionts still impose costs on their host and the factors driving evolution in such systems are largely unclear. Using microcosm populations of *Caedibacter taeniospiralis*, a vertically transmitted bacterial symbiont of the protozoan *Paramecium tetraurelia*, we investigated evolutionary change under permissive conditions (26 °C) and under heat stress (32 °C). We demonstrate the capacity of the symbiont to adapt to heat stress and to persist in long term (150 host generations). Adaptation involved increased infection proliferation (= total number of infected offspring produced), but there was no sign of a reduction in symbiont virulence or protection of the host against heat stress, contrary to other systems. For certain genetic backgrounds, heat-adapted infected lines were also more prolific at the permissive temperature, suggesting cost-free evolution of super-generalists. This illustrates the idea of 'roundabout selection', where adaptations evolving in marginal habitats may invade back into central populations, stuck on an adaptive peak. Our results show that vertically transmitted symbionts can evolve adaptations that do not necessarily help their hosts. Spread and maintenance of infection requires additional mechanisms, namely the 'killer trait', allowing the selective elimination of uninfected host conspecifics in a given population. However, the genotype-specific outcomes suggest that it can be difficult to predict host-symbiont evolution along the geographic range of their interaction.

Keywords: costs of adaptation, *Paramecium*, parasite, specialist-generalist, temperature, virulence

3.2 Introduction

Symbiosis generally involves transfer of resources and services between the two interacting species, thereby generating reciprocal impacts on their fitness (Thrall *et al.*, 2007; Leung *et al.*, 2008). The outcome of an interaction, from negative/antagonistic (parasitism) to neutral (commensalism) to positive (mutualism), depends on the net effect of costs and benefits for the players involved (Leung & Poulin, 2008; Fellous & Salvaudon, 2009). Over the past years, an increasing number of studies have suggested that this balance between costs and benefits is strongly condition-dependent, with systems potentially shifting back and forth on a mutualism-parasitism continuum (Michalakis *et al.*, 1992; Brown *et al.*, 2003; Restif & Kaltz, 2006; Wolinska & King, 2009; Vale *et al.*, 2011). Factors determining sign and strength of an interaction are the symbiont transmission mode, environmental conditions, as well as the genetic background of symbiont and host (Ewald, 1987; Wolinska & King, 2009; Ebert, 2013). Most empirical and experimental work has highlighted the short-term consequences of variation in these factors over one or very few generations (Thomas & Blanford, 2003; Wolinska & King, 2009), but it is still unclear how they drive long-term evolutionary and co-evolutionary processes (Hatcher *et al.*, 2005).

Symbionts with exclusive vertical transmission are particularly interesting in this context. Vertical transmission occurs from infected parents to offspring, and therefore symbiont transmission success is directly linked with host reproduction (Fine, 1975). Unlike in systems with infectious horizontal transmission, allowing some degree of host exploitation and damage (Alizon *et al.*, 2009), symbiont and host fitness are positively aligned so that vertically transmitted symbionts should evolve to avoid any harm to their host or even become beneficial (Fine, 1975; Ewald, 1987; Jones *et al.*, 2007; Ebert, 2013). Moreover, under exclusive vertical transmission, genes of symbiont and host are locked up in a single line of descent. This may lead to the accumulation of deleterious mutations or loss of function in the symbiont (Dale & Moran, 2006; Feldhaar, 2011), but also facilitate specialisation and co-evolution (Brucker & Bordenstein, 2012). These ideas are consistent with the observation that some of the major evolutionary transitions from parasitic to mutualistic relationships are associated with a switch from horizontal to vertical transmission (Moran *et al.*, 2008; Sachs *et al.*, 2011).

Nonetheless, many vertically transmitted symbionts are known to be harmful to their host, qualifying them as parasites (Kelly *et al.*, 2003; Mouton *et al.*, 2004; Ebert, 2013; Dusi *et al.*, 2014). In some cases, this can be explained by the existence of residual horizontal

transmission, offsetting the negative effects on host fitness (e.g. virulence). In other cases, symbionts manipulate host's reproductive system in such a way that uninfected individuals are eliminated from the population (e.g., cytoplasmic incompatibility or male killing in Wolbachia; Werren, 1997), thereby increasing the frequency of the symbiont in the population despite its actual fitness costs (Werren, 1997; Dunn & Smith, 2001; Kusch *et al.*, 2002). Moreover, vertically transmitted symbionts provide benefits against natural enemies (Oliver *et al.*, 2005; Haine, 2008; Brownlie & Johnson, 2009; Jones *et al.*, 2011), competitors or adverse environmental conditions, such as pollutants or high-temperature stress (Douglas, 1998; Russell & Moran, 2006). The possibility of condition-dependent benefits may open the evolutionary avenue towards mutualism.

We still know very little about how environmental variation impacts the evolutionary dynamics of systems with obligate vertical transmission. For example, in *Buchnera*, a bacterial symbiont of aphids, a single point mutation in the symbiont genome seems to drive a balanced polymorphism of wild-type strains, which confer heat-stress protection, but impose fitness costs at lower temperature, and non-protecting mutants that bear no fitness costs at lower temperatures (Dunbar *et al.*, 2007). Variation in heat-stress protection also exists among genotypes (or species) of other aphid-symbionts, indicating a genetic basis on which selection can act (Russell & Moran, 2006; Cayetano & Vorburger, 2013). Similarly, genetic variation in temperature sensitivity is known for within-host density of *Wolbachia* spp., a widely distributed bacterial symbiont of arthropods (Mouton *et al.*, 2003), although it is less clear how this effect impacts host fitness and thus potential responses to selection (Mouton *et al.*, 2007).

Experimental work on vertically transmitted symbionts is typically based on single host individuals and their offspring, spanning little more than one host generation and limiting information on selective processes (but see Rouchet & Vorburger, 2014). In an attempt to fill this gap, we investigated evolution under heat stress conditions in experimental microcosm populations of the freshwater protozoan *Paramecium tetraurelia,* infected with the strictly vertically transmitted bacterium *Caedibacter taeniospiralis*. Vertical transmission occurs through the segregation of bacterial cells into the daughter cells of the asexually dividing host. Infection with the symbiont reduces host fitness (Dusi *et al.*, 2014), but it also confers a so-called 'killer trait' to the host, leading to the selective killing of uninfected conspecifics in the population (Pond *et al.*, 1989; Schrallhammer & Schweikert, 2009). This effect is comparable to the male-killing or cytoplasmatic incompatibility of *Wolbachia* or *Cardinium*, which also

promotes an increase in the frequency of symbiont carriers in the population by eliminating non-carriers (Werren, 1997; Gotoh *et al.*, 2006).

Dusi *et al.* (2014) showed that infection reduced density at or below temperatures optimal for *Paramecium* growth. At a more stressful temperature (32 °C), infection prevalence declined, indicating limited heat tolerance of the symbiont and thus limited capacity to persist under heat stress (Dusi *et al.*, 2014). Thus, the main objective of this study was to test whether this system can evolve in such a way that infection is maintained at the stressful temperature (32 °C). We were particularly interested in whether adaptation to high temperatures involved the evolution of reduced virulence or even beneficial effects of infection on its host. We hypothesized that the symbiont is able to adapt to high temperature (hypothesis 3.1). *Caedibacter* increases its within-host growth rate and thereby evolves to lower levels of virulence (hypothesis 3.2). In a long-term experiment, we exposed infected populations with 5 different genotype associations to a 32 °C high temperature treatment as well as to a 26 °C control treatment. After ca. 150 host generations, we performed fitness assays measuring the growth performance of evolved and ancestral host-parasite combinations at these two temperatures. We assume a cost free adaptation to heat stress (hypothesis 3.3). Therefore, we compared the direct responses to selection at each temperature, but also the correlated responses at the respective 'foreign' temperature. This allowed us to assess whether adaptation to the high-temperature environment traded off with the performance at the original permissive temperature.

3.3 Material and Methods

Study system

The *Gammaproteobacterium Caedibacter taeniospiralis* (Preer & Preer, 1982) lives exclusively in the cytoplasm of its host *Paramecium tetraurelia,* a cosmopolitan fresh-water ciliate (Ciliophora). Transmission occurs vertically during mitotic host cell division, from the infected mother cell to the two resulting daughter cells; free-living stages or horizontal transmission are unknown. Infection reduces host fitness (Dusi *et al.*, 2014), but the parasite also confers a so-called 'killer trait' to the host, leading to the selective elimination of uninfected *Paramecium* in the population (Pond *et al.*, 1989; Schrallhammer & Schweikert, 2009). Infection prevalence is stably maintained at temperatures ≤26 °C, but strongly declines within less than 10 asexual generations at 32°C (Dusi *et al.*, 2014). This indicates that high

temperature cures *Paramecium* from infection; it also suggests that the 'killer trait' is inefficient at this temperature.

Long-term experiment

The long-term experiment was initiated from five *Paramecium tetraurelia* strains, each associated with its own *Caedibacter taeniospiralis* symbiont (Preer *et al.*, 1974, Table 1.). Stock cultures of these strains were kept in 0.25% cerophyl medium (Krenek *et al.*, 2011), inoculated with *Raoultella planticola* (DMSZ 3069) at 22 °C. Bacterized medium was added to stock cultures in 1:2 ratio bi-weekly. Wheat Grass Powder used for the cerophyl medium was purchased from GSE Vertrieb GmbH.

Following the results from Dusi et al (2014), we established a high-temperature selection treatment at 32 °C and a control treatment at 26 °C, close to the optimum for *Paramecium* growth and allowing stable persistence of *Caedibacter*. For each strain, three long-term replicate populations (referred to as 'selection line', hereafter) were assigned to each selection treatment, giving a total of 30 populations (5 host strains x 3 replicates x 2 selection temperatures; Fig. 9). These selection lines were started with an initial host cell density of 500 cells ml^{-1} in 40 ml of culture medium (60 ml tissue culture flasks); initial infection prevalence was 100%. Every 2-3 days, 20 ml of culture were discarded and 20 ml freshly bacterized medium was added. This ensured constant population growth, with approximately three host generations per week. We measured host density and parasite prevalence (Dusi *et al.*, 2014) approximately every 25 host generations.

In an attempt to preserve as closely as possible the ancestral state of the founder populations, cultures of the ancestral paramecia were kept at 10 °C, with 25% of the population replaced with freshly bacterized medium every three weeks (Fig. 9). This protocol minimised cell division and therefore the number of host generations (≈ 1 complete population turnover in 12 weeks) over the duration of the long-term experiment (52 weeks; Lohse *et al.*, 2006).

The flasks with the long-term cultures were kept in computer-controlled water baths, with temperature regulated by a heating foil. A magnetic stirrer produced a homogenous temperature distribution in the water bath, reducing differences between single cell culture flasks to a maximum of 0.05 °C, on average. Several water baths were assigned to each of the two treatment temperatures, and flasks randomly assigned to a given water bath. During the experiment, flasks regularly changed water baths as well as position within water baths.

Growth assay

After approximately 150 host generations (52 weeks), we isolated single *Paramecium* cells from each selection line (Fig. 9). The cells were washed by transferring them three times to sterile Dryl's medium (Dryl, 1959), and then grown up individually for 8 days at their respective treatment temperature of the long-term experiment (26 °C or 32 °C). We also established monoclonal lines from ancestral population of each strain. To ensure host growth, we transferred a part of the ancestral population to 22 °C and isolated single *Paramecium* cells after 8 days of acclimatisation. The presence of infection in the resulting monoclonal cultures was verified using FISH and PCR techniques (Dusi *et al.*, 2014). In this way, three independent 100 % infected monoclonal lines were established for each long-term replicate population, and the same was done for the ancestral populations. In a next step, each monoclonal line was split and one half was cured from infection by adding the antibiotic streptomycin (Krenek *et al.*, 2011; Dusi *et al.*, 2014). The success of the antibiotic treatment was verified using FISH and PCR (Chapter 1).

Growth was assayed at both 26 °C and 32 °C (Fig. 9). To this end, each infected or antibiotic cured half of a monoclonal line was further split into two pre-cultures for the assay. One remained at its original treatment temperature; the other was acclimatized in steps of ±2 °C per day to the 'foreign' assay temperature and remained at this temperature for another two days prior to the assay. Ancestral monoclonal cultures were stepwise acclimatized to 26 °C or 32 °C (Fig. 9).

An individual assay replicate was started from the acclimatised pre-cultures with an initial host density of ca. 25 cells ml^{-1} in a flask containing 20 ml of culture medium. The flasks were then placed in water baths at 26 °C or 32 °C, as described above. Density was measured by counting paramecia in 25-300 µl samples under a dissecting microscope in 6-10 h intervals, for a total of 80 h. Using FISH (Beier *et al.*, 2002), we determined the proportion of infected cells (initially 100%) after 42-48 h, when replicates had reached carrying capacity (Dusi *et al.*, 2014).

Due to loss of infection in the 32 °C long-term treatment (see Results), all long-term replicate populations from the strain 47 and one from the strain 51 were omitted from the assay. Thus, in total, we assayed 23 long-term replicate populations and the four ancestral populations, with a total of 324 replicates (27 populations x 3 monoclonal lines x 2 infection statuses (infected / cured) x 2 assay temperatures).

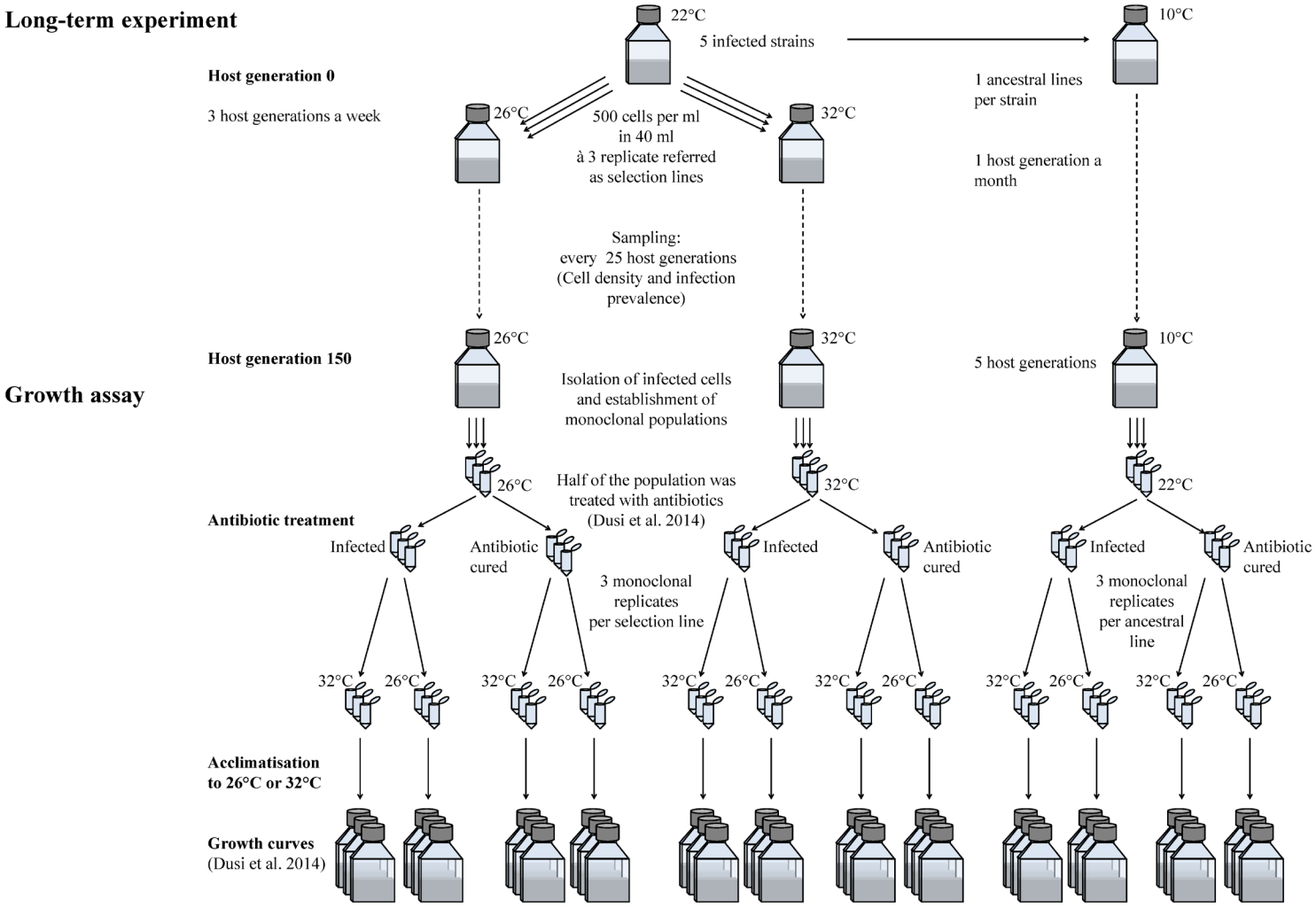

Fig. 9. Experimental set-up of the long-term experiment and the growth assays. In the long-term experiment, 5 *Paramecium* strains, infected with their own *Caedibacter* genotype, were set-up in 2 selection temperatures (26°C and 32°C) in 3 replicates, in total 30 selection lines. After 150 host generations, 3 monoclonal populations of each selection line were established for the following growth assays. Half of these populations were treated with antibiotics (Dusi *et al.* 2014). Each experimental population was acclimatized to one of the assay temperatures (26°C and 32°C) and then set-up for growth. One ancestral line per strain was kept at low growth and 10°C, and also included in the growth assay.

Statistical analysis

We analysed variation in three measures: (1) Infection prevalence, taken as the arcsine-transformed proportion of infected individuals after 2 days in the assay. This measure describes the capacity of symbiont persistence. (2) Infection proliferation, taken as the product of infection prevalence and host population size after 2 days in the assay. Integrating over host reproductive rate and the fidelity of vertical transmission, this measure describes the proliferation of infected cells and is therefore linked to the vertical reproductive rate (R_0) of the symbiont (Lipsitch *et al.*, 1995b). (3) Virulence, taken as the difference in growth performance between infected and cured assay replicates. Infection influenced both growth rate (*r*) and carrying capacity (*K*), but more strongly the latter (Fig. 14). To take into account both effects, we estimated growth performance of each replicate as the area under the curve (AUC) of *Paramecium* density. The AUC represents the cumulated density during the assay, weighted for the interval length between each time point the density was taken (Capaul & Ebert, 2003; Adiba *et al.*, 2010). The AUC is convenient, because it summarises performance in a single value per replicate. For each combination of monoclonal line and assay temperature, there was one infected and one cured assay replicate, and thus virulence was calculated as the difference between them: $\log_{10}(AUC_{infected}) - \log_{10}(AUC_{cured})$. Negative values indicate a negative effect of infection on growth performance.

Using linear mixed effect model (LME) approaches, we tested fully factorial models with selection temperature ('treatment'; long-term 26 °C, 32 °C or ancestor) and assay temperature ('assay'; 26 °C or 32 °C) as fixed factors. As random factors, we included strain and all its interaction with assay and selection temperature. Following Pinheiro and Bates (2000), we simplified full models to obtain models that minimised the Akaike information criterion (AIC). Random factors were omitted first and then fixed factors. For the final models, we employed log-likelihood ratio tests or quasi-F tests to test for the significance of model terms. Obtained significance levels have to be treated with caution as the determination of the degree of freedoms is insufficient (Bates *et al.,* 2014). Infection prevalence was arcsine-transformed and infection proliferation log-transformed to meet model assumptions; all final models showed approximately normally distributed and homogeneous residuals. We used the R software (R Development Core Team, 2014) with the add-on package lme4 (Bates *et al.*, 2014) and JMP (SAS, 2013) statistical packages for analysis.

3.4 Results

Demography and infection prevalence during the long-term experiment

Over the 52 weeks of the experiment, *Paramecium* cell density and infection prevalence were generally higher in the control treatment (26 °C) than in the high temperature treatment (32 °C; Fig. 10 a). While density at 26 °C reached levels of over 4000 cells ml^{-1}, it rarely exceeded 1000-2000 cells ml^{-1} at 32 °C (Fig. 10a). Infection prevalence in the controls persisted at nearly 100 %. In comparison, it declined in most populations at 32 °C, with three populations even losing infection completely (Fig. 10b).

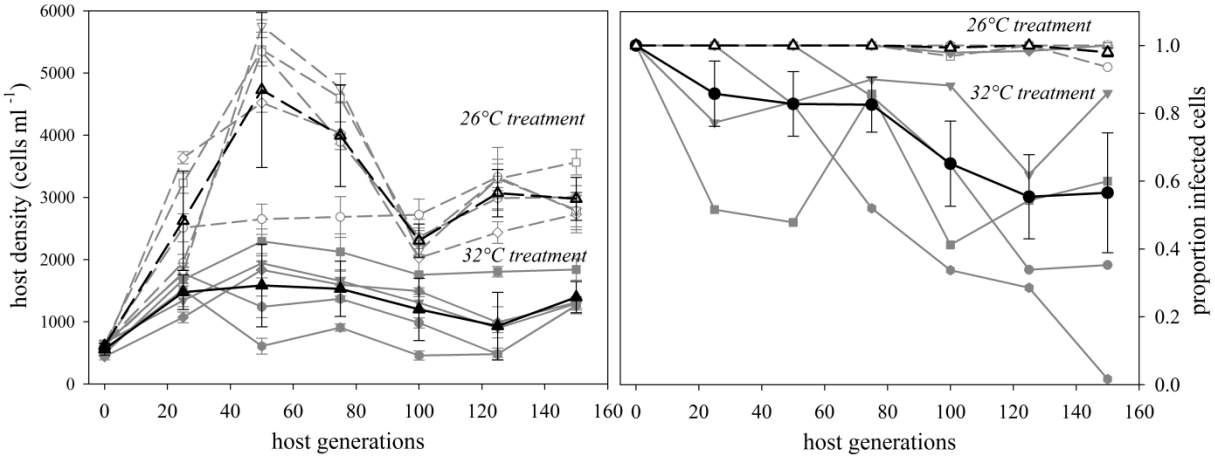

Fig. 10. (a) Mean host density (± S.E.) during the experiment for 5 different host-symbiont genotype association at 32 °C (black, straight line and filled triangle) and 26 °C (black, dashed line and open triangle). Density of every single host-symbiont genotype association is in grey. (b) Mean prevalence (± S.E.) during the experiment for 5 different host-symbiont genotype associations at 32 °C (black, straight line) and 26 °C (black, dashed line). Density of every single host-symbiont genotype association is in grey.

Growth assay

Infection prevalence. Analysis of infection prevalence revealed a significant interaction between long-term selection temperature and assay temperature ($F_{2,6} = 12.39$; $p < 0.001$; Table 4, Table 5). At 26 °C assay temperature, infection prevalence in all tested populations remained at approximately 100 % during the assay (99 ± 0.3 %; Fig. 11), whereas it decreased at 32 °C assay temperature. However, this decrease was much less pronounced for selection lines evolving at 32 °C (mean prevalence: 87 % ± 3.7 %) than for the lines from the 26 °C control treatment (56 % ± 9.3 %; Fig. 11) or the ancestral lines (57 % ± 11.3 %; Fig. 11).

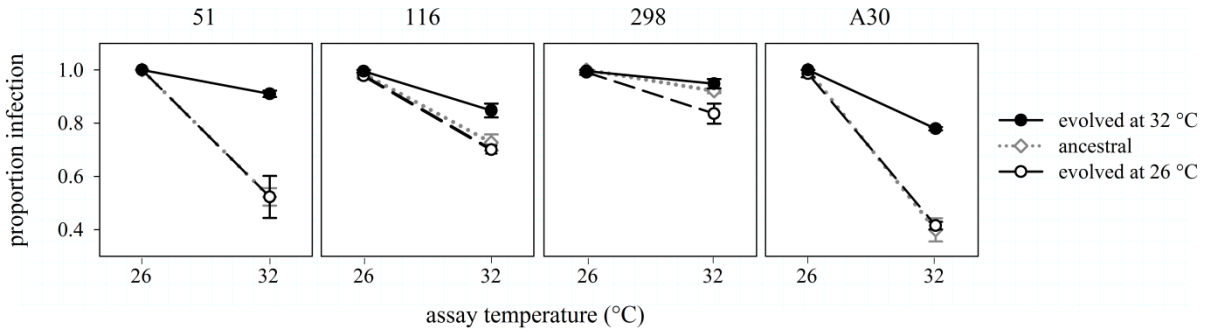

Fig. 11. Mean proportion of infected cells (± S.E.) for every single *Paramecium* strain. The proportion of infected cells was measured at day 2 of the assay for populations from the 32°C high temperature treatment (black, straight line and filled circles), the control treatment (black, dashed line and open triangle) and ancestral (grey dotted line and open rhomb) tested at 26 °C and 32 °C.

Infection proliferation. Results for the net proliferation of infection (= infection prevalence x host density) closely mirrored those for infection prevalence (Table 7). In addition, long-term lines selected at 32 °C also showed a twice as strong proliferation capacity at this temperature than did lines from the 26 °C treatment or the ancestor (Fig. 12). Additional patterns varied depending on the genetic background of the strains (significant strain x selection temperature x assay temperature interaction; Table 4). For strains 51 and A30, 32 °C selection lines also tended to be superior at the 26 °C assay temperature, indicating a positively correlated response to selection (Fig. 12). In contrast, for strain 298, high-temperature lines lost proliferation capacity and control lines tended to gain proliferation capacity at 26 °C (Fig. 12).

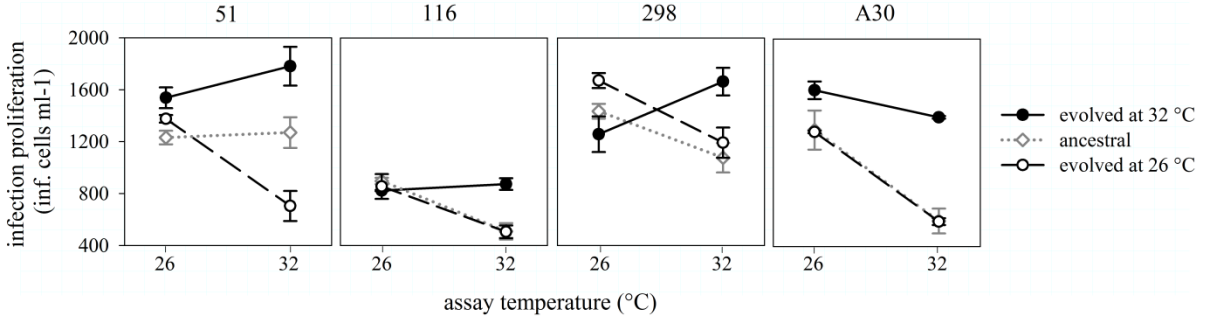

Fig. 12. Mean net performance (infected cells ml^{-1}; ± S.E.) for every *Paramecium* strain. Infection proliferation was calculated as the product of prevalence and host cell density at day 2 of the assay for populations evolved at 32 °C (black, straight line and filled circles), 26 °C (black, dashed line and open triangle) and from ancestral strains (grey dotted line and open rhomb) tested at 26 °C and 32 °C.

Table 4: LME testing effects of selection temperature, *Paramecium* strain and assay temperature on prevalence, infection proliferation and virulence. In LMM testing selection temperature ('treatment'; long-term 26°C, 32°C or ancestor) and assay temperature ('assay'; 26°C or 32°C) were included as fixed factors and strain and all its interaction with assay and selection temperature as random factors. Variance (± S.E.) was obtained for the random effects and quasi-F tests were performed for the fixed effects. The obtained significance levels have to be treated with caution as the determination of the degree of freedoms is insufficient (Bates *et al.*, 2014; d.f. =degrees of freedom, MS = mean square, S.E. = standard error. Terms separated with 'x' are interaction terms).

	Prevalence				**Infection proliferation**				**Virulence**		
Optimal model		Variance	S.E.			Variance	S.E.			Variance	S.E.
random effects											
strain		-	-			0.016	0.125			0.001	0.031
strain x assay		0.016	0.126			0.003	0.055			-	-
strain x treatment		-	-			0.003	0.050			0.001	0.033
strain x assay x treatment		0.002	0.047			-	-			0.003	0.053
Residual		0.005	0.070			0.004	0.064			0.002	0.043
	d.f	MS	F value		d.f.	MS	F value		d.f.	MS	F value
fixed effects											
assay	1	0.14	28.46	.	1	0.04	9.34		1	2.72E-05	0.015
treatment	2	0.08	16.54	*	2	0.03	6.51	.	2	7.55E-04	0.408
assay x treatment	2	0.06	12.39	**	2	0.14	34.02	***	2	1.52E-03	0.823

Virulence. We found substantial levels of virulence for all long-term selection lines. Overall, infected lines produced on average 40% lower densities than their cured, symbiont-free counterparts (t_{23} = 19.9, p< 0.0001). Virulence did not significantly differ between evolved and ancestral lines (t_{22} = 0.22, p > 0.6, all lines pooled). Unlike the analyses of infection prevalence, there was no clear general effect of the high temperature treatment on virulence (Table 4, Table 9). Instead, responses to selection varied with both strain identity and assay temperature (treatment x strain x assay temperature interaction; Table 4; Fig. 13). For strain 116, selection at 32 °C was associated with decreased virulence, and for strain 51 with increased virulence. Selection in the 26 °C control treatment had no significant consequences for virulence at this temperature.

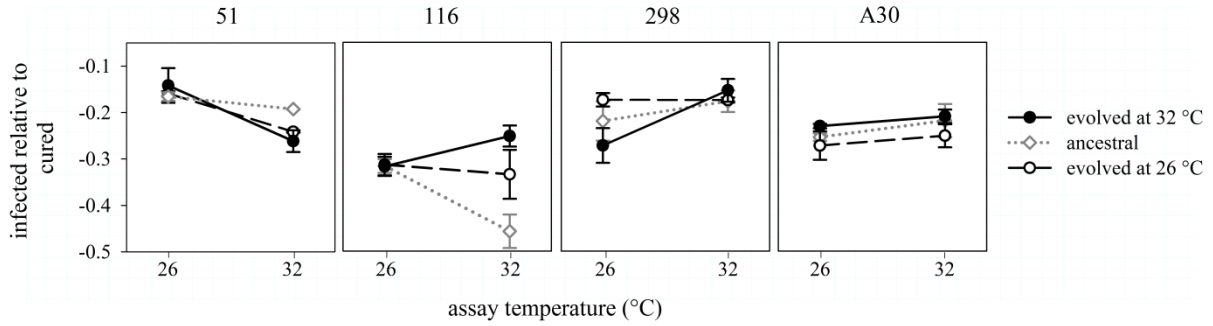

Fig. 13. Mean virulence (± S.E.) for each investigated *Paramecium* strain. Virulence was calculated as difference of Area under the curve between infected and uninfected populations for paramecia evolved at high temperature (black, straight line and filled circles), 26 °C treatment (black, dashed line and open triangle) and from ancestral strains (grey dotted line and open rhomb) tested at 26 °C and 32 °C.

Our virulence estimates did not account for the loss of infection at the 32 °C assay temperature, which may potentially lead to an underestimation of virulence (if freshly cured cells divide more than infected cells). However there was no significant relationship between infection prevalence and virulence in the assay, nor did relationships significantly vary with the identity of strain genetic background or temperature selection regime (ANCOVA of virulence, with infection prevalence as covariate plus strain and selection temperature as cofactors: prevalence, prevalence x cofactor interactions all p > 0.2). Thus, it is unlikely that loss of infection introduced a significant bias in our analysis of virulence.

3.5 Discussion

This study investigated how a host-symbiont system with obligate vertical transmission adapts to environmental stress. Because strictly vertical transmission links host and symbiont fitness, we had hypothesised that adaptation to stress in our experimental *Paramecium-Caedibacter* system may involve the evolution of lower levels of virulence or even

benevolence, similar to symbiont-mediated heat-stress protection in aphids (Douglas, 1998; Russell & Moran, 2006). We found that long-term exposure to high temperature indeed selected for an increased capacity to maintain infection at this stressful temperature. However, there was no evidence for the evolution of reduced costs of infection or heat-stress protection.

Parasite adaptation to high temperature conditions

We had previously demonstrated a limited capacity of *Caedibacter* to persist at 32 °C, such that population infection levels were quickly driven to very low levels (Dusi *et al.*, 2014). This temperature is stressful, but not lethal, to the host *Paramecium*. Our present experiment confirms the thermal limitation of the symbiont for both ancestral lines and lines from the long-term 26 °C control treatment. In contrast, lines from the long-term 32 °C treatment retained very high infection prevalence during the fitness assay, and they also showed an increased capacity of proliferation of infection at this temperature. These patterns were consistent across the four genetic backgrounds of strains, which had also shown stabilised infection prevalence over the course of the long-term experiment.

A straightforward explanation for these observations is adaptation of the *Caedibacter* symbiont to 32 °C, allowing it to maintain viable within-host densities. As *Paramecium* populations were kept under constant growth during the long-term experiment (and in the assay), sufficiently high within-host growth rates are required to track host division and to ensure vertical transmission. This life-history trait may respond to selection in our experiment, just like bacterial growth rate readily adapts to temperature stress in free-living bacteria (Bennett *et al.*, 1992). However, adaptation of the bacterial symbiont *Caedibacter* can also fail, as long-term lines from strain 47 almost all lost the symbiont during the long-term high temperature treatment.

Direct and correlated responses to selection

If adaptation to a novel environment involves specialisation, this can produce a negative correlated response to selection, i.e., a reduced performance in another environment (Bell, 1997; Nidelet & Kaltz, 2007). Such an evolutionary trade-off is illustrated for strain 298, where evolution of increased infection proliferation at 32 °C was accompanied by a correlated decrease at 26 °C. Conversely, long-term 26 °C lines increased their performance at this temperature, resulting in crossing reaction norms and local adaptation to the two respective temperatures. Costs of adaptation may be caused by antagonistic pleiotropy of genes advantageous at one temperature, but disadvantageous at the other, or by the accumulation of

mutations that are neutral at the temperature of selection, but deleterious at the other temperature (Kassen, 2002).

However, specialisation was not the rule in our experiment. Strains 51 and A30 showed positive correlated responses to selection, such that adaptation to 32 °C produced universally superior generalists, even better at infection proliferation at 26°C than the 'resident' lines selected at that temperature (Fig. 12). This is consistent with observations for free-living bacteria, where temperature stress did not only select for an increased temperature tolerance, but also for general improvement in fitness (Bennett & Lenski, 1993; 1996). All else being equal, our results suggest that infected cell lines from the 'marginal' 32 °C environments can spread back into infected populations in the permissive 26 °C 'mainland'. Indeed, theoretical papers have highlighted the importance of selection in marginal habitats for the evolution over the entire range of a species, even though these investigations are more focused on the question of how dispersal from the mainland affects local adaptation at the margins (Kirkpatrick & Barton, 1997; Sexton *et al.*, 2009). The bottom line in these theoretical publications is that evolutionary outcomes may critically depend on the genetic background, in which adaptations arise (Duputié *et al.*, 2012).

Virulence evolution

Basic theory predicts that virulent symbionts with exclusive vertical transmission can persist only under very limited conditions (Ewald, 1987; Jones *et al.*, 2007). One possibility is that fitness costs are compensated through protection against biotic or abiotic stress (Brownlie & Johnson, 2009; Jones *et al.*, 2011). However, we found no evidence that adaptation to 32 °C involved a benevolent action of the symbiont towards the host: *Caedibacter* still consistently reduced host growth in all selection lines. Nevertheless, some evolutionary change occurred. Replicate lines from the initially most virulent strain (116) evolved lower virulence at 32 °C, albeit to levels not particularly lower than those of the other strains. Conversely, the initially least virulent strain (298) did not significantly change its virulence levels. Hence, even though selection can act on virulence, there may be a limit beyond which virulence cannot be reduced. Indeed, unless symbionts 'invent' novel features conferring a benefit to the host, a minimal amount of host resources is required for their own reproduction and successful vertical transmission. Thus, just like for parasites with horizontal transmission (van Baalen & Sabelis, 1995), fitness of vertically transmitted parasites may be maximised for optimal levels of virulence, guaranteeing large enough within-host densities for successful vertical transmission (see Kover & Clay, 1998).

In the *Paramecium-Caedibacter* system with its strictly uniparental vertical transmission, host and symbiont are locked up within the same line of descendant. Consequently, traits like virulence may become a truly "shared trait" (Restif & Koella, 2003), with both partners contributing to its evolution. If adaptation of *Caedibacter* to 32 °C allows better within-host growth, decreased virulence may have resulted from concomitant host tolerance evolution, i.e. the capacity to maintain (or even increase) fitness despite increasing symbiont loads. Disentangling the precise relative contributions of host and symbiont will require controlled artificial infection techniques, which are currently not available for our system. More detailed investigations of tolerance are possible by the use of PCR techniques for accurate quantification of within-host symbiont density.

What maintains infection at high temperature?

As already said, the persistence of strictly vertically transmitted and virulent symbionts is restricted to very particular conditions (Ewald, 1987; Jones *et al.*, 2007). In the case of *Caedibacter*, fitness costs can be compensated through the 'killer trait', which brings infected hosts to eliminate uninfected *Paramecium* (Görtz & Fokin, 2009; Schrallhammer & Schweikert, 2009; Dusi *et al.*, 2014). This explains the stable maintenance of 100 % infection prevalence at 26 °C (control treatment) in our experiment. However, at 32 °C, uninfected individuals increased in frequency in many selection lines, indicating impaired 'killer activity' at this temperature (see also Dusi *et al.*, 2014). Similar temperature sensitivity is also known for comparable frequency distorter functions in the vertically transmitted *Wolbachia* bacteria (Hurst *et al.*, 2000).

But what maintained *Caedibacter* infection at 32 °C? Equilibrium levels of infection likely depend on the balance between three factors: (i) the rate at which cured, uninfected individuals arise in the population, (ii) the relative fitness of infected vs. uninfected individuals (virulence) and (iii) the rate at which uninfected individuals are removed from the population by the 'killer activity'. Our tests focused on the first two factors and showed that adaptation of *Caedibacter* to high temperatures reduces the input of uninfected individuals, but costs of infection continue to disfavour infected hosts. Thus, some degree of 'killer activity' is still required to prevent uninfected cells from taking over the population. Indeed, the strain with the *a priori* strongest 'killer activity' (298; Chapter 2) maintained nearly 100 % infection, whereas the two strains with the weakest 'killer activity' (47, 116) showed continuously declining infection levels, until even extinction. The intermediate 'killer activity' of strains A30 and 51 seem to allow coexistence of infected and uninfected cells.

However, we advise caution with these interpretations, as we took no measurements of 'killer activity' during the long-term experiment, nor do we know the 'killer activity' of the evolved lines. It would be particularly interesting to test whether this trait evolves in response to the selection temperature, and whether there are costs associated with such evolution, which could influence other traits, such as within-host growth. Even more complex (co-) evolutionary processes may be envisaged, for example, if uninfected hosts evolve resistance to the 'killer activity'. If this resistance is costly, the growth difference between infected and uninfected hosts may diminish, thereby facilitating coexistence.

3.6 Conclusions

Correlational studies suggest that strict vertical symbiont transmission is a perfect prerequisite for the evolution of mutualism (Kover & Clay, 1998; Ferdy & Godelle, 2005), in particular when the symbiont has protective functions in hostile environments (Oliver *et al.*, 2003). Here, using experimental evolution, we show that adaptation of a vertically transmitted symbiont to environmental change does not necessarily come to the benefit of the host. As we hypothesized (hypothesis 3.1), the parasite was able to adapt to high temperatures, but contrary to the hypothesis 3.2 without reducing virulence. We conclude that *Caedibacter* still qualifies as a parasite, imposing a growth cost on the host and conferring no protection to heat stress, contrary to our hypothesis 3.2 and what is known in other systems (Douglas, 1998; Vorburger & Gouskov, 2011). Furthermore, different genetic backgrounds produced different evolutionary trajectories in our experiment, with cost-free adaptation of the parasite (contrary to hypothesis 3.3). This suggests that more efficient parasites evolving in extreme or marginal habitats may spread back into the original populations. This raises interesting questions in times of global change, where host-parasite systems are experiencing profound modifications in their geographic range (Harvell *et al.*, 2002; Lafferty, 2009).

3.7 Appendix: Model selection

Statistical analysis

Data and statistical analysis was performed with the R system for statistical computing (R Development Core Team, 2014) and the add-on package lme4 (Bates *et al.*, 2014). The intrinsic population growth rate r (d^{-1}) and the carrying capacity K (cells ml^{-1}) of the host were estimated by fitting logistic growth models for each replicate population in dependency of infection state, selection temperature and assay temperature (Dusi *et al.*, 2014). We further estimated growth performance of each replicate as the area under the curve (AUC) of *Paramecium* density as infection affected both growth rate (r) and carrying capacity (K, Fig. 14).

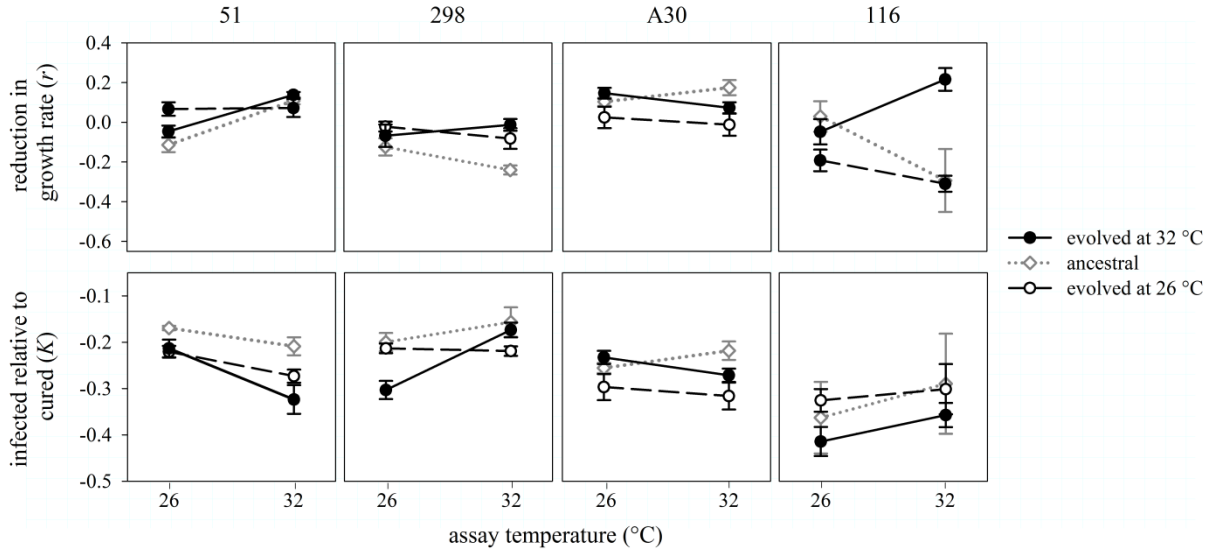

Fig. 14. Mean fitness reduction (± S.E.) in host growth rate (r) and carrying capacity (K) for paramecia evolved at 32°C high temperature treatment (black, straight line and filled circles), 26 °C control treatment (black, dashed line and open triangle) and ancestral paramecia (grey dotted line and open rhomb) tested at 26 °C and 32 °C. The reduction in growth rate (r) was estimated as the difference between the growth rates of infected and uninfected populations, and the cell density of infected relative to cured (K) as the difference between host cell densities at carrying capacity of infected and uninfected populations.

To appropriately address random and fixed effects of the experimental design, we have applied linear mixed models according to Pinheiro and Bates (2000). We started with a 'full' model, containing the experimental variables ('treatment' and 'assay') and their interactions as fixed effects, and the other independent variables (strain) and their interactions as random effects or with a 'beyond optimal model' (Zuur *et al.*, 2009) if the full model was not identifiable due to overparametrization. In a second step, random effects numerically close to zero were excluded. An optimal model with minimum AIC but still containing the experimental variables as fixed effects was derived by interactive model selection. This model

was then compared with further reduced models omitting single fixed effects by AIC and likelihood ratio tests (LRT) for verifying the experimental hypotheses. The model omitting the relevant fixed effects of our hypothesis was called null model. This procedure was carried out for indicators of prevalence, infection proliferation and virulence. The selected models fitted well without high correlations between the parameters of the fixed effects and showed approximately normally distributed and homogeneous residuals.

Model selection

Prevalence

We fitted mixed models with selection temperature ('treatment'), assay temperature ('assay') and their interaction as a fixed effect for prevalence and included strain and all its interactions as random effects. For model simplification, we excluded several terms of the random effects because of their variance close to zero (the main effect strain and the interaction between strain and selection temperature; Table 5). We further tested the influence of the fixed effects and their interaction on the model (Table 5). We found a strong increase of the AIC, when the fixed effects or their interaction were removed from the model fit.

Table 5. AIC based model selection (based on maximum likelihood method) for the dependency of prevalence on selection temperature ('treatment') and assay temperature ('assay'). From the full model the random effects strain and its interaction with selection temperature were removed. Further, the fixed effects selection temperature and assay as their interaction were individually removed from model fit 2 (d.f. = degrees of freedom, AIC = Akaike information criterion, BIC = Bayes' information criterion, t = Student's t. Terms separated with 'x' are interaction terms).

			AIC-based model selection			
	reduction in model	d.f.	AIC	BIC	log likelihood	deviance
1	full model	11	-82.63	-60.76	52.32	-104.63
2	treatment x strain; strain	9	-86.63	-68.73	52.32	-104.63
3	treatment x assay	7	-72.96	-59.03	43.48	-86.96
4	assay	6	-61.23	-49.30	36.62	-73.23
5	treatment	5	-67.09	-57.14	38.54	-77.09
6	null model	4	-55.45	-47.50	31.73	-63.45

The significance of the interaction term and the main effects was confirmed by the likelihood ratio test between the optimal model, the reduced models and null model (Table 6) without interaction. The pairwise comparison between the null model and the optimal model indicates a significant interaction between assay and treatment (Likelihood ratio test: χ^2 1df = 41.18; $p < 0.001$), and the lower AIC indicates that the optimum model (AIC = -86.63) is better than

the full model (AIC = -82.63). The effect strength of the optimal model (Table 4) revealed a strong influence of the selection temperature and its interaction with assay temperature as well as a minor influence of the assay temperature on infection prevalence.

Table 6. A pairwise comparison of the full model, null model and reduced models against the optimal model (model fit 2) were performed using the log likelihood ratio test. (d.f. =degrees of freedom).

		Loglikelihood ratio test vs. optimal model			
	reduction in model	d.f.	Chi²	p value	
1	full model	1	0	1	
2	treatment x strain; strain		optimal model		
3	treatment x assay	2	17.68	1.45E-04	***
4	assay	3	30.40	6.99E-07	***
5	treatment	4	27.55	1.54E-05	***
6	null model	5	41.18	8.62E-08	***

Infection proliferation

To test if infection proliferation was influenced by the selection temperature and assay temperature, we started with a 'beyond optimal model' (Zuur *et al.*, 2009) describing infection performance in dependency of selection temperature, assay temperature, and their interactions as fixed effects, and strain and all its interactions as random effects. We excluded the strain x selection temperature x assay temperature interaction of the random effects because of the variance close to zero (Table 7). As further model simplification did not decrease AIC (Table 7), the optimal model showed clear interaction between assay temperature and selection temperature.

Table 7. Linear mixed models for the dependency of infection proliferation on selection temperature ('treatment') and assay temperature ('assay'). From the full model the interaction of selection temperature, strain and assay was removed. The fixed effects selection temperature and assay as their interaction were individually removed from model fit 2. AIC value increased with further reduction. (d.f. = degrees of freedom, AIC = Akaike information criterion, BIC = Bayes' information criterion, t = Student's t. Terms separated with 'x' are interaction terms).

		AIC-based model selection				
	reduction in model	d.f.	AIC	BIC	log likelihood	deviance
1	full model	11	-93.026	-71.147	57.513	-115.03
2	treatment x assay x strain	10	-95.026	-75.136	57.513	-115.03
3	treatment x assay	8	-57.888	-41.977	36.944	-73.88
4	assay	7	-54.246	-40.323	34.123	-68.246
5	treatment	6	-53.451	-41.518	32.726	-65.451
6	null model	5	-49.811	-39.866	29.906	-59.811

The likelihood ratio test between the optimal model and null model without interaction (Table 8) confirmed the significance of the interaction between assay temperature and selection temperature (Likelihood ratio test: χ^2 1df = 55,215; $p < 0.001$), and the lower AIC indicates that the optimum model (AIC = -95.026) is better than the full model (AIC = -93.026). The effect strength of optimal model (Table 4) revealed a strong influence of the interaction between assay temperature and selection temperature and both fixed main effects on infection proliferation.

Table 8. The log likelihood ratio test between the optimal model and the full model, null model and reduced models were performed pairwise (d.f. = degrees of freedom).

		Loglikelihood ratio test vs. optimal model			
	reduction in model	d.f.	Chi²	p value	
1	full model	1	0	1	
2	treatment x assay x strain		optimal model		
3	treatment x assay	2	41.138	1.17E-10	***
4	assay	3	46.78	3.87E-10	***
5	treatment	4	49.575	4.43E-10	***
6	null model	5	55.215	1.18E-10	***

Virulence

For mixed modelling of virulence we started with a 'beyond optimal model' (Zuur *et al.*, 2009) describing virulence in dependency of selection temperature, assay temperature, and their interactions as fixed effects, and strain and all its interactions as random effects (Table 9). We could not exclude any random effect from the model fit for virulence (Table 9).

Table 9. Linear mixed models for the dependency of virulence on selection temperature ('treatment') and assay temperature ('assay'). Model selection based on the AIC (maximum likelihood method). Removal of the selection temperature x strain interaction and of fixed effects selection temperature, assay as their interaction increased AIC. (d.f. =degrees of freedom, AIC = Akaike information criterion, BIC = Bayes' information criterion, t = Student's t. Terms separated with 'x' are interaction terms).

		AIC-based model selection				
	reduction in model	d.f.	AIC	BIC	log likelihood	deviance
1	full model	11	-139.88	-118.00	80.937	-161.88
2	treatment x strain	10	-141.88	-121.98	80.937	-161.88
3	treatment x assay	8	-143.68	-127.77	79.841	-159.68
4	assay	7	-145.67	-131.75	79.834	-159.67
5	treatment	6	-146.78	-134.85	79.391	-158.78
6	treatment, assay	5	-148.77	-138.82	79.383	-158.77

The stepwise reductions of fixed effect increased the AIC value (Table 9) and the likelihood ratio test for the full model and our null model showed no significant difference ($p = 0.5246$, Table 10). Virulence is influenced by the interactions of strain x selection temperature x assay temperature, assay temperature x strain and the main effect strain, but was not systematically influenced by selection temperature and assay temperature (Table 4).

Table 10. Pairwise comparison of the full model and reduced models against the null model were performed using the log likelihood ratio test. (d.f. = degrees of freedom).

		Loglikelihood ratio test vs. null model		
	reduction in model	d.f.	Chi²	p value
1	full model	6	3.1085	0.7951
2	treatment x strain	5	3.1085	0.6833
3	treatment x assay	3	0.9157	0.8216
4	assay	2	0.9015	0.6371
5	treatment	1	0.0156	0.09005
6	null model		optimal model	

CHAPTER 4

EXPERIMENTAL ANALYSIS OF 'HEAT CURED' PARAMECIA – HOW DO THEY SURVIVE IN MIXED POPULATIONS?

Characteristics of 'heat cured' paramecia

4.1 Introduction

The vertically transmitted parasite *Caedibacter taeniospiralis* reduces host fitness, but also provides a selective advantage by killing uninfected host organisms (Study system; Chapter 1 and Chapter 2). Variation in costs (and benefit) exists between strains and temperatures (demonstrated in Chapter 1 and Chapter 2; temperature-sensitivity of the beneficial trait is unexplored). While cost of infection was increasing with rising temperatures until 28 °C, it was dramatically reduced at 32°C. This decline was positively correlated with a reduction in infection prevalence from 100 % to 50 % (genotype dependent; Chapter 1). Mixed populations of *Caedibacter*-harbouring and parasite-free *P. tetraurelia* not only arose in acute high-temperature stress but also in permanent high temperature conditions (described in Chapter 3). After 25 host generations of the long term high-temperature experiment (Chapter 3), mixed populations could be detected in 4 out of the 5 *Paramecium* strains. The number of 'heat cured' paramecia constantly increased for two host strains (47 and 116), while infection prevalence was fluctuating over time for two other paramecia strains (51 and A30). Further, one replicate of strain 51 and all replicates of strain 47 almost completely (98.3 % cured paramecia) lost the parasite between host generation 100 and 150. In contrast, 'heat curing' events were rarely occurring for host strain 298 (2.1 % cured paramecia at host generation 100 and 1.7 % at host generation 125); while they were more often detectable under acute heat conditions (10 %, Chapter 1). Rare 'heat-curing' events were also observed in the selection lines of the 26 °C control treatments on course of the long-term experiment.

Theory predicts the elimination of uninfected and cured paramecia by their infected conspecifics. Consistent with this theory, antibiotic cured paramecia lost the parasite's protection and became sensitive to the 'killer trait' (Chapter 2, Kusch *et al.*, 2002; Schrallhammer & Schweikert, 2009). 'Heat cured' hosts should also have lost this parasite's protection and consequently be killed in a mixed population with *Caedibacter* infected hosts, which are resistant to the released toxic form of the parasite. On the other hand, cured hosts have a fitness advantage in comparison to infected hosts (Chapter 1). There are two

hypotheses for the occurrence of 'heat-cured', parasite-free hosts contemporaneously with infected hosts: The 'killer trait' is inefficient at high temperature (hypothesis 4.1). 'Heat-cured' paramecia have a reduced sensitivity to the 'killer trait' (hypothesis 4.2). In this chapter, the focus is on the characterisation of 'heat cured' paramecia to provide a possible explanation for the observed population dynamics in the long-term high temperature treatment.

4.2 Methods

Growth assay

After 150 host generations, 'heat cured' paramecia were isolated from mixed populations. Infection level was verified using PCR and FISH (Chapter 1). Paramecia cultures were set up for fitness assays as described in Chapter 3. Briefly, host fitness was measured as carrying capacity at 32 °C. Per replicate population of the long-term experiment, three cured monoclonal populations were set up at 32 °C. Density was measured by counting paramecia in 25-300 µl samples microscopically. Carrying capacity K (cells ml^{-1}) was calculated for each replicate population in dependency of genotype and temperature assay by fitting a logistic growth model (Dusi *et al.*, 2014). Data were analyzed with the R system for statistical computing (Team, 2014) and compared with data of infected and antibiotic cured paramecia of the long term experiment (Chapter 3; grouped as 'statuses'). Effects of temperature, 'infection statuses' and genotype on K were tested in factorial analyses of variance (ANOVA).

Killer assay

In a second experiment, 'heat cured' hosts were tested for their resistance or sensitivity to the 'killer trait'. Infected host populations (100 % prevalence) were exposed to acute heat-stress temperature (32 °C) for 10 days and only recently 'heat cured' hosts were isolated for the following experiment to avoid potential adaptation or fixation processes occurred in the long term experiment. Infection level of isolated host cells was determined by FISH and PCR (Chapter 1) and 'heat cured' host cells were set up for proliferation. 'Killer tests' were performed following the protocol of Schrallhammer *et al.* (2012) with *Caedibacter* genotype 51, A30 and 298 (genotype 47 and 116 were excluded because of cultivation problems). Resistance of 'heat cured' and antibiotic cured paramecia (Chapter 2) was tested by exposing 8 cured cells to 8 infected paramecia of the same genotype. Experiments were conducted for

5 h at 24 °C in 24-well plates containing 200 µl exhausted cerophyl medium (Krenek *et al.*, 2011). Host cell number was determined microscopically every 30 min to 1 h. Survival probability was estimated using the Kaplan-Meier estimate (Eq. 2 in Chapter 2) and mean survival time of all cured paramecia per replicate was calculated. Kaplan-Meier estimates over time were used for pairwise comparison between resistance of 'heat cured' and antibiotic cured hosts to the 'killer trait'.

4.3 Results

Growth assay

The intrinsic growth rate of the host was neither significantly influenced by temperature ($F_{1,4} = 0.591$, $p = 0.485$), strain ($F_{2,4} = 2.152$, $p = 0.232$) nor 'infection statuses' ($F_{2,4} = 2.858$, $p = 0.169$). However, carrying capacity was always higher in 'antibiotic cured' host populations than in 'heat cured' and infected host populations (significant influence of 'statuses' $F_{2,4} = 127.6$, $p < 0.001$, Fig. 15). *Post hoc* tests (Tukey's HSD) revealed that carrying capacity between antibiotic cured and infected ($p < 0.001$) as well as 'heat cured' hosts ($p < 0.001$) were significantly different, while infected and 'heat cured' host populations did not significantly differ in their carrying capacity ($p = 0.432$). The genetic background also influenced carrying capacity (significant main effect: genotype $F_{2,4} = 10.9$, $p < 0.05$).

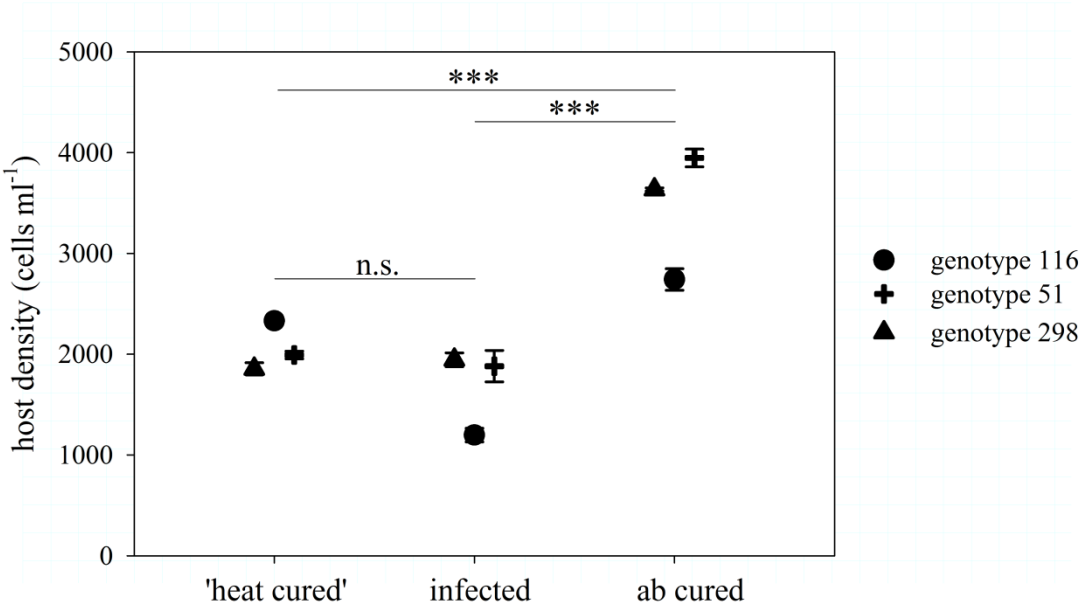

Fig. 15. Mean density (± S.E.) at carrying capacity of 'heat cured', infected and antibiotic cured (ab) *Paramecium tetraurelia* strains at 32 °C.

'Killer tests'

After 5 h exposure to infected paramecia, the number of killed cells per antibiotic cured replicate population was always higher than for 'heat cured' replicates (Table 11). Survival time of 'heat cured' individuals was higher than survival time of 'antibiotic cured' for *Paramecium* strain A30 and 298 (Table 11). However, the Kaplan Meier estimates over time were significantly different only for *Paramecium* strain 298 ($p < 0.001$; Fig. 16).

Table 11. Comparison of the characteristics (survival time (h) and number of killed cells after 5 h) of 'heat cured' and antibiotic cured paramecia.

genotype	curing event	survival time (h)	Number of killed cells after 5h
298	antibiotic	3.4 h ± 0.130 h	4.9 ± 0.3
298	'heat cured'	4.1 h ± 0.119 h	3.3 ± 0.2
51	antibiotic	4.2 h ± 0.129 h	4.7 ± 0.2
51	‚heat cured'	4.1 h ± 0.118 h	3.3 ± 0.1
A30	antibiotic	3.9 h ± 0.160	3.2 ± 0.2
A30	‚heat cured'	4.2 h ± 0.125	3.0 ± 0.3

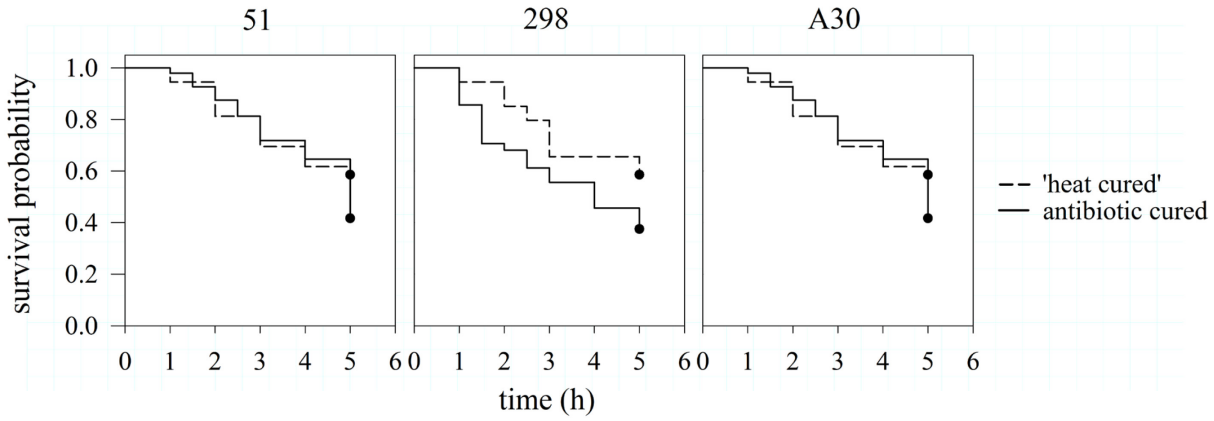

Fig. 16: Survival probability of antibiotic cured and 'heat cured' paramecia over time for three different *Paramecium* strains (298, 51, A30). The survival probability was estimated as the Kaplan-Meier estimate over time. Data are censored.

4.4 Discussion

In high temperature conditions, infected and uninfected hosts steadily co-existed in the same populations (see Chapter 3). Contrary to antibiotic-cured paramecia, fitness of the arising 'heat cured' hosts was not significantly different from infected hosts. Furthermore, 'killer tests' revealed that 'heat cured' paramecia might be less sensitive to the 'killer trait' than naive and antibiotic-cured hosts, supporting the hypothesis (4.2).

Population dynamics

In general, a fitness disadvantage caused by infection will select against infected hosts in mixed populations and consequently against their strictly vertically transmitted symbionts (Fine, 1975; Jones *et al.*, 2007; Chapter 1). *Caedibacter* can prevent its own elimination and the co-occurrence of cured and infected hosts in the same population by eliminating uninfected hosts via the 'killer trait'. Nevertheless, infection prevalence was reduced at 32 °C for all tested strains under acute heat stress conditions (Chapter 1) and for four out of five strains in the long-term high-temperature experiment (Chapter 3, Fig. 11). Therefore, the question arises what allows infected and uninfected hosts to co-exist. In Chapter 3 of this thesis, three factors were already postulated to manipulate the equilibrium level of infection: (i) the occurrence of uninfected, 'heat cured' individuals, (ii) the fitness difference between infected and uninfected individuals and (iii) the 'killer activity' of infected individuals. The balance between these three factors may not only be important for maintenance of infection but also for long-term survival of the 'heat cured' paramecia in mixed populations. At 32 °C, 'heat-cured' paramecia co-occur in host populations (Fig. 10). This result already supports the hypothesis (4.1) of an impaired 'killer trait' at higher temperatures. The rates, at which these 'heat cured' individuals are arising, obviously differ between the five strains (Chapter 3). These 'heat cured' hosts than have a general fitness disadvantage in comparison to antibiotic cured hosts, but not in comparison to infected hosts (Fig. 15). Therefore, infected hosts should not be outcompeted by the 'heat cured' hosts. Excluding the strain 298, 'heat cured' paramecia were not killed shortly after their emergence, which can have two different reasons: (i) inefficient 'killer trait' of infected hosts or (ii) insensitivity of 'heat-cured' paramecia to the 'killer trait'. Indeed, 'heat cured' paramecia seem to be more resistant to the 'killer trait' than antibiotic cured hosts, supporting hypothesis 4.2. This potential resistance might also explain the observed reduced fitness of the 'heat cured' paramecia. Nevertheless, there is also evidence for an impaired 'killer trait' at 32 °C (Fig. 10, hypothesis 4.1). The efficiency of the 'killer trait' at 32 °C might result from a balance between the costs for temperature adaptation and R-body production (dead end) of the parasite. Higher investment in heat adaptation may reduce R-body production and therefore the 'killer activity'. Vice versa, a stronger investment in R-body production may lower the ability for temperature adaptation, which might be the case for genotype 298 that is showing no generalist adaptation pattern (Chapter 3, Fig. 12), but probably the highest 'killer activity'.

Underlying mechanism

'Heat cured' host populations reached similar host densities as infected hosts contrary to antibiotic-cured paramecia that showed significantly higher carrying capacities. This is suggestive for a fitness-reducing cost associated with the previous infection. In most host-parasite systems with some degree of horizontal transmission, such costs are mostly associated with the evolution of resistance to infection (Simms & Rausher, 1987; Coustau *et al.*, 2000; Kraaijeveld *et al.*, 2002; Strauss *et al.*, 2002). However, *Caedibacter* is a strictly vertically transmitted symbiont, where no infectious stage is known (Kusch & Görtz, 2006; Schrallhammer & Schweikert, 2009; Dusi *et al.*, 2014). In this system, the potential costs causing similar fitness reduction in 'heat cured' compared to infected hosts might be associated with a heritable epigenetic change in the gene expression pattern of the host caused by *Caedibacter* infection. Since the genome of the parasite as well as the transcriptome of the infected host and parasite is still unknown, the parasite's influence on host gene expression is still theoretical. However, the parasite has a lower temperature tolerance than the host (Chapter 1) and thus high temperatures can reduce parasite growth and therefore cell density within a host. Hence, the parasite can be thinned out by the faster growing host (Kaltz & Koella 2006). This slow curing event (in comparison to antibiotics; Chapter 1) might leave a parasitic epigenetic influence ('parasite ghost effect'; Duncan *et al.*, 2011). This hypothetical parasitic effect on host's gene expression might be visible by the reduced fitness of infected and 'heat cured' hosts as well as by the reduced sensitivity of the 'heat cured' paramecia to the 'killer trait'. Theory and experimental analyses suggest a proteinous toxin involved in the 'killer trait' (Pond *et al.*, 1989; Schrallhammer *et al.*, 2012). This toxin has its target region in the host, which might be blocked by an antitoxin structure in infected paramecia, but not in uninfected hosts. The detected reduced sensitivity of 'heat-cured' paramecia to the 'killer trait' suggests a production of this antitoxin structure by the host, while the gene expression might be regulated by the parasite. In the absence of the parasite, the antitoxin expression would be stopped resulting in sensitive paramecia as shown for the antibiotic-cured hosts. The epigenetic influence of the parasite, however, might be preserved after a slow parasite loss for a short period of time and would allow the cured host to resist the 'killer trait'. However, these experimental results should be treated with caution as they are very preliminary but still remarkable data.

CHAPTER 5

LONG-TERM SELECTION EXPERIMENT PRODUCES BREAKDOWN OF HORIZONTAL TRANSMISSIBILITY IN PARASITE WITH MIXED-MORE TRANSMISSION

Loss of horizontal transmission

5.1 Abstract

Evolutionary transitions from parasitism towards beneficial or mutualistic associations may encompass a change from horizontal transmission to (strict) vertical transmission. Parasites with both vertical and horizontal transmission are amendable to study factors driving such transitions. Here we revisited a long-term experiment, exposing microcosm populations of the protozoan *Paramecium caudatum* and its bacterial parasite *Holospora undulata* to growth treatments and thus manipulating opportunities for vertical transmission over ca. 800 host generations. Inoculation tests revealed a near-complete loss of horizontal transmissibility of parasites from a 'high-growth' treatment, which generated high host birth rates and therefore high vertical transmission rates. This strongly reduced infectivity was consistent on paramecia from different treatments or with different genetic backgrounds. Parasites from a treatment alternating between high-growth and low-growth conditions showed a similarly reduced transmissibility. Our results complement previous findings of reduced investment in horizontal transmission and increased vertical transmissibility of parasites from the high-growth treatment. We explain loss of horizontal transmissibility by evolutionary and epidemiological feedbacks, reducing the availability of hosts for infection and thereby decreasing the selective advantage of horizontal transmission. Our study illustrates how environmental conditions may push parasites with a mixed transmission mode towards becoming vertically transmitted benevolent symbionts.

Keywords: Horizontal transmission, host density, infectivity, loss of function, resistance, vertical transmission

5.2 Introduction

The mode of transmission is a central trait in the life cycle of parasites, with important implications for the way hosts are exploited as a resource. One way to acquire new hosts is through horizontal transmission, the infectious spread from one host to another. Selection may enhance horizontal transmissibility by increasing the quality or quantity of transmission propagules, which is likely accompanied by increased exploitation of host resources, and therefore increased virulence (Ewald, 1987). A second common route of infection is vertical transmission from parent to offspring. Unlike horizontal transmission, vertical transmission aligns fitness of host and parasite, such that the evolution of high vertical transmission fidelity (e.g., the proportion of offspring infected) may be accompanied by low virulence (Fine, 1975; Ewald, 1987), resulting in a higher number of infected offspring. Because of their different virulence optima, the two modes of transmission are often considered as opposing strategies that can drive the evolution of symbionts towards parasitism or mutualism (Ewald, 1987).

It is not entirely clear from comparative study whether macroevolutionary transitions between parasitism and mutualism are necessarily accompanied by changes in transmission mode (Moran *et al.*, 2008; Sachs *et al.*, 2011). However, many contemporary parasites are capable of both vertical and horizontal transmission, and therefore provide an opportunity to study the selection pressures shaping their evolution (Ebert, 2013). Generally, selection should act on total parasite fitness, which is the sum of the contributions from each transmission pathway (Lipsitch *et al.*, 1995b). Total fitness may be maximized by specialist strategies investing predominantly in one of the two transmission modes (Lipsitch *et al.*, 1996) or by generalist strategies with some level of investment in both modes (van den Bosch *et al.*, 2010). Which strategy prevails will depend on trade-offs between the two modes, but also on how ecological and epidemiological factors affect transmission opportunities (Ebert, 2013). For instance, increased horizontal transmission can be expected at high population density, facilitating infectious contact. Conversely, at low density, or if co-infection is not possible, horizontal transmission can produce diminishing returns, due to depletion of susceptible hosts. Such epidemiological feedbacks may thus prohibit the evolution of highly virulent, horizontally transmitted specialists (Lipsitch *et al.*, 1996). Additional factors promoting one or the other transmission strategy are host survival and fecundity. Namely, vertical transmission should increase when hosts have high fecundity. Such conditions may occur when populations colonize a new patch and are still at low density, which may select for increased levels of

parasite vertical transmission, either by increasing vertical transmission fidelity or by reducing virulence (Lipsitch *et al.*, 1996).

Ultimately, however, evolutionary outcomes depend on how these environmental factors affect the relative, rather than the absolute, contributions of vertical vs. horizontal transmission. Obtaining this information for natural populations (see, e.g., Kover *et al.*, 1997) or tracking evolutionary change is very difficult. A well-known example is a comparative study on fig wasp parasites (Herre, 1993), demonstrating a negative relationship between the opportunity for vertical transmission and virulence, consistent with the idea that increased vertical transmission selects for less virulent parasites.

Alternatively, experimental evolution approaches can overcome some of these difficulties and investigate evolutionary change under controlled epidemiological conditions. In a seminal paper, Bull and colleagues (1991) experimentally forced a bacteriophage into either vertical or horizontal transmission over multiple generations. Consistent with theory, exclusively vertically transmitted phages evolved lower virulence. Moreover, the vertical treatment even led to the total loss of the capacity of the phage to transmit horizontally (Bull *et al.*, 1991). In similar experiments, exclusive vertical or horizontal transmission resulted in an improved efficiency of the selected transmission mode, as expected (Messenger *et al.*, 1999; Stewart *et al.*, 2005; Sachs & Wilcox, 2006). However, studies using less constrained and epidemiologically more realistic scenarios gave unexpected results. Contrary to theory, experimental manipulation of bacterial host density, and therefore the opportunity for horizontal transmission, had no detectable effect on phage evolution in a study by Turner *et al.* (1998). Magalon *et al*. (2010) manipulated growth conditions for populations of the protozoan *Paramecium caudatum* to change the relative importance of vertical to total transmission of the bacterial parasite *Holospora undulata*. This parasite is transmitted vertically during mitotic (asexual) division of its host, and horizontally by the release of infectious transmission stages (Görtz & Wiemann, 1989; Fokin & Görtz, 2009). In a high-growth selection treatment, host population growth was stimulated through frequent dilution, thereby keeping vertical transmission at a constantly high level and reducing investment in horizontal transmission (Kaltz & Koella, 2003). Consistent with theory, evolved parasites from this treatment were less virulent and had a higher fidelity of vertical transmission than parasites from a low-growth treatment, where host populations were less often diluted. However, evidence for an evolutionary trade-off with horizontal transmission was equivocal. Although producing fewer infectious forms, high-growth parasites had a higher per-capita

horizontal transmissibility, suggesting that the horizontal transmission pathway was nonetheless under selection in this treatment (Magalon *et al.*, 2010).

Here we revisited Magalon *et al.*'s (2010) long-term experiment, which had been continued for another 3 1/2 years (≈ 800 host generations in total). We re-measured horizontal transmissibility, for which Magalon *et al.* (2010) had obtained unexpected results. In addition to parasites from high- and low-growth treatments, we also tested parasites from a third treatment, alternating between high-growth and low-growth conditions. Parasites from the alternating treatment are expected to evolve a more generalist or plastic transmission strategy, as opportunities for vertical and horizontal transmission were fluctuating in time (hypothesis 5.3). This alternating treatment had not been included in the previous tests (Magalon *et al.*, 2010). Our new tests also investigated host evolution in this long-term experiment. We hypothesise that hosts of the vertical transmission treatment evolve a higher resistance due to the higher infectivity measured after 200 host generations (hypothesis 5.1). This evolution of higher resistance reduces hypothetically the importance of horizontal transmission (hypothesis 5.2). A factorial cross-infection assay tested all combinations of parasite and host from the three selection regimes, allowing us to disentangle the relative importance of parasite and host identity for horizontal transmissibility.

5.3 Material and Methods

Study system

Paramecium caudatum is a freshwater ciliate, filter-feeding on bacteria and detritus. Like all ciliates, it has two nuclei, a germline micronucleus and a somatic macronucleus. Reproduction and population growth is mainly achieved asexually, by mitotic division (Wichtermann, 1986). Our *Paramecium* cultures are maintained asexually, as single clones, on a lettuce medium bacterized with *Serratia marcescens* at 23 °C (Nidelet & Kaltz, 2007).

The *Alphaproteobacterium Holospora undulata* infects the micronucleus of *P. caudatum*. Two morphologically and functionally distinct forms exist (Fokin & Görtz, 2009). The S-shaped infectious forms (≈ 15 µm) are the horizontal transmission propagules. They are ingested by the host during food uptake and are subsequently transferred from the food vacuole to the micronucleus (Fokin & Görtz, 2009). Multiple infectious forms can start a new infection. During up to 48 h co-infections can occur after a first infection, although for certain host genotypes, a first infection increases resistance to secondary infection (Fels *et al.*, 2008).

Similarly, some *Holospora* species seem to be able to block co-infection with other species (Fokin & Görtz, 2009).

Ca. 24 h post infection, infectious forms differentiate into reproductive forms (≈ 5 µm), which multiply inside the micronucleus. Reproductive forms are transmitted vertically to the daughter nuclei of the mitotically dividing host. After 7-10 days post-infection, a fraction of the reproductive forms differentiate into infectious forms, marking the end of the latency period (Nidelet & Kaltz, 2007; Nidelet *et al.*, 2009). Infectious forms are released into the medium during host cell division or upon host death; they cannot divide and are not vertically transmitted. Hence, the morphological dimorphism imposes an inherent trade-off between investment into vertical transmission (reproductive forms) and horizontal transmission (infectious forms).

In established *Holospora* infections, a phenotypic switch exists for the investment in the two transmission modes, possibly regulated by intranuclear bacterial density (Kaltz & Koella, 2003; Nidelet *et al.*, 2009). When host division is slow, reproductive forms accumulate and differentiate into infectious forms within 24-48 h, eventually leading to a heavily swollen micronucleus with hundreds of infectious forms. In contrast, rapid host division reduces bacterial density (Restif & Kaltz, 2006) and the parasite remains in the reproductive state (Kaltz & Koella, 2003). This switch between vertical and horizontal transmission can be regulated by environmental conditions that increase or decrease host division rate (Kaltz & Koella, 2003). The phenotypic switch is also consistent with the idea of a trade-off between the two transmission modes, as a high production of infectious forms reduces host division and therefore rates of vertical transmission (Kaltz & Koella, 2003; Restif & Kaltz, 2006; Nidelet *et al.*, 2009).

Variation in susceptibility to infection among *P.* caudatum genotypes (Fels & Kaltz, 2006; Fels *et al.*, 2008; Fokin & Görtz, 2009) indicates a genetic basis of resistance. The underlying molecular and genetic details are largely unknown, but resistance is most likely determined by the host's capacity to obstruct invasion of infectious forms into the micronucleus or their differentiation into reproductive forms (Fokin & Görtz, 2009; Fujishima, 2009). Both host resistance and parasite infectivity can evolve in laboratory populations (Lohse *et al.*, 2006; Adiba *et al.*, 2010; Magalon *et al.*, 2010).

Selection regimes in the long-term experiment

We used selection lines from the long-term experiment described in detail in Magalon *et al.* (2010). The long-term experiment included three growth treatments, imposed on infected and

uninfected (control) selection lines (40-50 ml of culture, with up to 10^4 cells of the genotype K8; Duncan *et al.*, 2010). In the high-growth treatment, selection lines were diluted by 50% in 2-day intervals by the addition of fresh medium, thereby producing constant doubling of the *Paramecium* population (see Fig. 1 in Magalon *et al.*, 2010, and Fig. 17). This leads to frequent vertical transmission and decreases the production of infectious forms for horizontal transmission, as explained above (Kaltz & Koella, 2003). In the low-growth treatment, only 20% of the culture was replaced in 8-day intervals, resulting in low population turn-over rates and therefore lower rates of vertical transmission; because hosts divide less often, infectious forms accumulate in the micronucleus (Kaltz & Koella, 2003; Magalon *et al.*, 2010). In a third 'alternating' treatment, high-growth and low-growth conditions changed every eight days (Fig. 17).

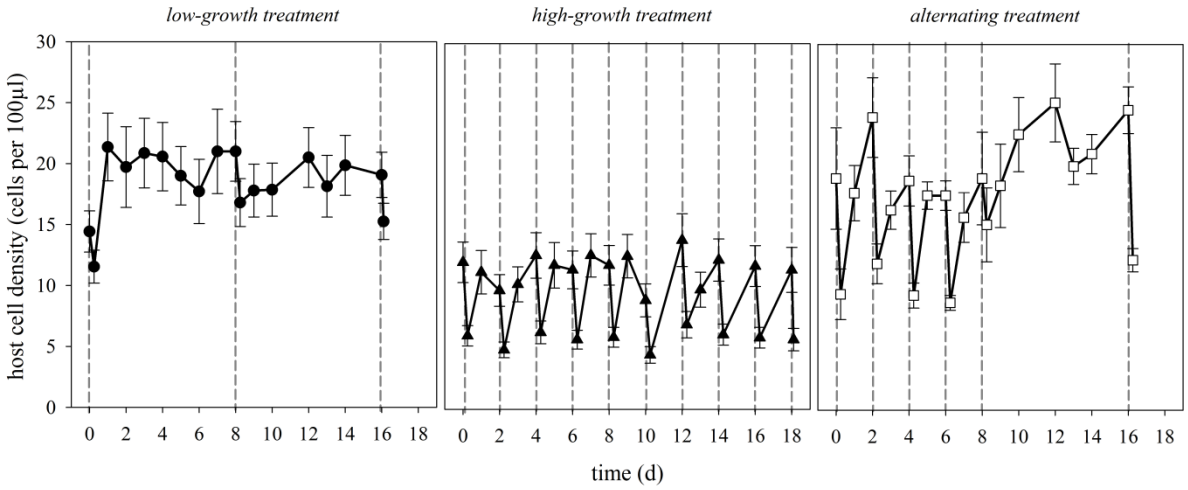

Fig. 17. Mean host density (± S.E.) per 100µl over the period of 18 days including two cycles for the high-growth, low-growth and alternating treatment.

Infection assays

For the present study, after 212 8-day cycles, we assayed horizontal transmissibility (= infectivity) and resistance for the three treatments. Planned assays of vertical transmissibility and virulence were not possible because parasites from the high-growth treatment failed to produce infected hosts (see Results), which would have been required for these additional tests.

Preparation of *Paramecium* and inocula for infection assays. Two weeks before the assay, we prepared uninfected monoclonal cultures ('monoculture', hereafter) of *Paramecium* from infected selection lines and from uninfected control selection lines. From each selection line, arbitrarily picked individuals were grown individually in 500 µl tubes, until monocultures of ca. 500 individuals were established. Monocultures from infected lines were

checked for infection, using the lacto-aceto-orcein stain (Görtz & Dieckmann, 1980) and phase contrast microscopy (1000x magnification). Infected monocultures were discarded, and only uninfected monocultures were used in the assays.

To prepare parasite inocula, we centrifuged 30 ml of culture from infected selection lines and then killed the *Paramecium* mechanically using a tissue lyzer (Qiagen^{TM}; samples placed in 2-ml plastic tubes with small glass beads, then agitated for 3 min at 3000 Hz). The inocula were stored at 5°C prior to use. In the infection assays, dose of infectious forms were adjusted by using a hemocytometer (100x magnification). In the infection assays, a replicate consisted of 40 cells from a given host monoculture that were placed in 300µl of medium containing inoculum. Three days after inoculation, the cells were fixed and checked for infection, as described above. Infectivity was taken as the proportion of infected individuals in the sample. The same measure also describes the degree of quantitative resistance of the *Paramecium*.

Infection assay 1. In pilot assays, inocula from high-growth lines did not produce infections, nor did natural horizontal transmission occur after introducing infected individuals into cultures of susceptible host genotypes (not shown). Further, for the infection assay, the quantities of infectious forms harvested from the high-growth parasite lines were not sufficient to inoculate all host lines, as planned. We therefore combined inocula from 5 high-growth selection lines to maximize the chance of infection success; accordingly, we also combined inocula from 10 low-growth selection lines and 4 alternating selection lines. The 3 combined inocula were tested against monocultures from an arbitrarily chosen set of 12 infected selection lines (4 high-growth, 4 low-growth, 4 alternating lines), and from 8 uninfected control selection lines (4 low-growth, 2 high-growth, 2 alternating lines). For each selection line, 6 monocultures were used, with 2 monocultures per combined inoculum and without replication. In total, 120 replicates were established (3 parasite treatments x 20 host selection lines x 2 monocultures), with inoculum dose adjusted to 200 infectious forms per µl.

Infection assay 2. Observed infection may result from a single active parasite line for combined inocula (assay 1). Therefore, to assess the variation in infectivity among parasite lines from the same treatment, a second infection assay tested individual inocula from 5 high-growth and 10 low-growth selection lines. High-growth parasites were tested against 3 naive host genotypes: K8 (used in the long-term experiment), as well as VEN and M3, two generally susceptible genotypes (Fellous *et al.*, 2012). Thirty replicates were set up (5 parasite selection lines x 3 host genotypes x 2 replicates). As inoculum sizes were again limited, we

varied the inoculum doses on each genotype, such that at least one of the two replicates received a very high dose of 130 – 420 (mean: 277) infectious forms μl^{-1}. The dose of the other replicate ranged from 65 to 210 (mean: 128) infectious forms μl^{-1}, which still represent substantial doses (Fels *et al.* 2008). All 3 host genotypes received the same mean dose (200 infectious forms μl^{-1}). Inocula from low-growth selection lines were tested against the genotypes K8 and M3, with a total of 20 replicates (10 parasite selection lines x 2 host genotypes; dose: 200 infectious forms μl^{-1}). No monocultures were established for this assay, replicates were established directly from the clonal mother culture of each genotype.

Infection assay 3. We further investigated specificity in resistance evolution by testing for local adaptation. This could only be done for the low-growth selection regime, as parasites from the other two treatments did not produce infections (see Results). *Paramecium* from 5 low-growth selection lines were confronted with parasites from their own (= sympatric) selection line and with parasites from foreign (= allopatric) low-growth selection lines. For this assay, we combined 4-6 monocultures from a given selection line, and confronted these mixes with the different inocula. We tested 3-5 replicates for sympatric combinations, and 2-6 replicates for allopatric combinations, depending on the availability of paramecia and/or inoculum, with a total of 37 replicates (dose: 200 infectious forms μl^{-1}).

Statistical analysis

We analyzed variation in infection success (= 'proportion infected individuals per replicate', or 'proportion of infected replicates' in some tests), by using logistic regression models with a binomial error structure to account for the binary data type ("infected / not infected"). For infection assay 1, we tested effects of parasite treatment origin, host treatment origin and whether hosts were taken from infected or from control selection lines. Host selection line identity was nested within treatment origin, as a random factor. In quasi-F tests, host treatment was tested over host line identity. Because fully factorial models failed to converge, we used simplified statistical models to validate the general trends in the data.

In assay 2, we compared infection success of high-growth vs. low-growth parasites separately for each host genotype (K8, M3). For assay 3, we combined the multiple replicates for each sympatric host-parasite combination to avoid pseudo-replication; we then fitted a model with parasite and host selection line, and 'sympatric vs. allopatric' as factors. We used the JMP statistical package (SAS, 2013) for all analyses.

5.4 Results

Long-term dynamics of infection prevalence

Infection prevalence in the high-growth selection lines initially reached very high levels (80-100%, Magalon *et al.*, 2010), but then steadily declined to average levels of 16% (range: 10-25%) at the end of the 4 1/2 years of the experiment (Fig. 18). Prevalence in the low-growth treatment never reached average levels over 50% and also showed a declining trend over time, with a final average of 17% (range: 12-48%). Patterns in the alternating treatment largely followed those in the high-growth treatment.

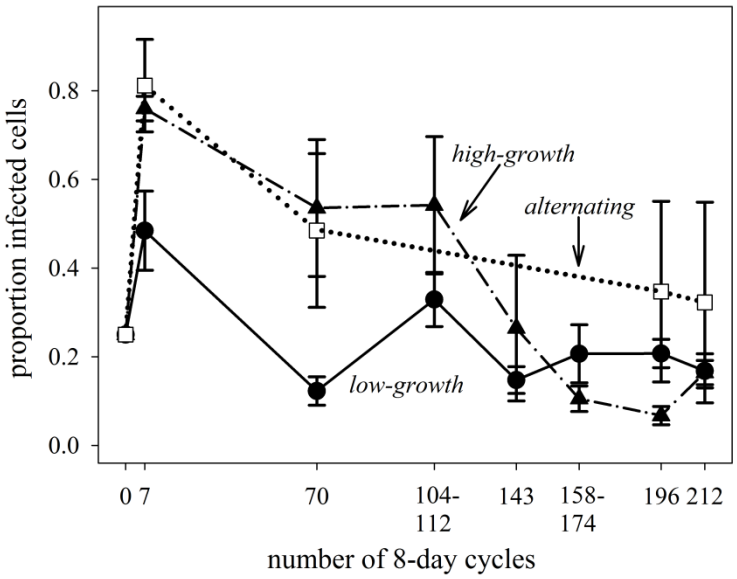

Fig. 18. Mean infection prevalence (± S.E.) in each of three growth treatments (high-growth, low-growth, alternating) over the 212 growth cycles (4 1/2 years) of the long-term experiment. One cycle refers to an 8-day period, during which high-growth populations were diluted twice by 50%, low-growth populations once by 20%; alternating populations changed weekly between high-growth and low-growth cycles. Means and standard errors based on occasional routine measurements, and therefore are meant for illustrative purpose. For two time periods, we combined estimates from different cycles because not all selection lines were measured at all time points.

Infection assay 1

No infection was detected for parasites from the high-growth selection lines in any of the 40 replicates (Fig. 19). This was significantly different from the infection success of parasites from the low-growth selection lines (17/40 = 42.5% infected) and from the alternating selection lines (8/40 = 20% infected; Fisher's exact tests: $p < 0.01$).

Only low-growth parasites produced infections on paramecia from infected selection lines (Fig. 19a), and these parasites also showed the highest infection success on paramecia from parasite-free control selection lines. Alternating parasites had significantly lower

infection success than low-growth parasites on these control paramecia (χ^2_1 = 32.5, $p < 0.0001$; Fig. 19b).

Overall, paramecia from parasite-free control selection lines were less resistant than those from infected selection lines ($F_{1, 16}$ = 20.61, p = 0.0004). There was also a significant general effect of growth treatment ($F_{2, 16}$ = 4.33, p = 0.0314). Namely, high-growth lines were more resistant than low-growth selection lines, a pattern that was characteristic for both infected and control lines. The resistance in alternating selection lines was generally more similar to that in high-growth lines (Fig. 19).

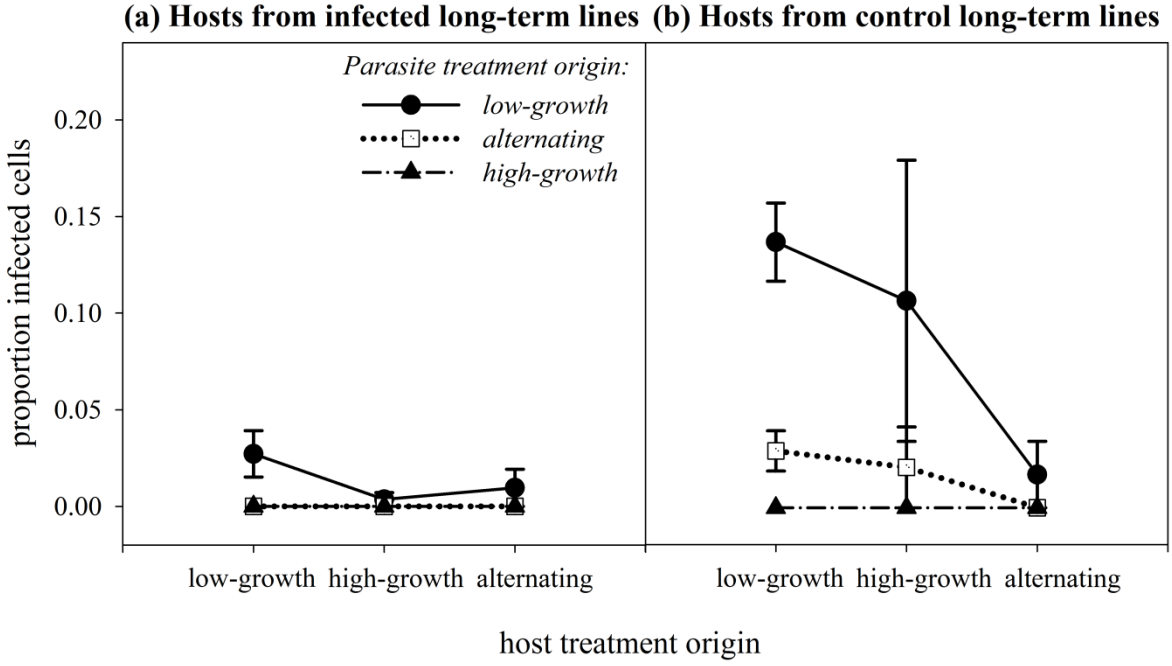

Fig. 19. Mean infection success (± S.E.) of parasites from three growth treatments (high-growth, low-growth, alternating), measured on hosts from these treatments. Hosts were taken (a) from infected long-term populations or (b) from uninfected control populations. In (a), lines for high-growth and alternating parasites are superimposed, with all values being 0.

Infection assay 2

Inoculum dose did not significantly affect infection success of parasites from the high-growth treatment (χ^2_1 = 0.02, $p > 0.8$) and was therefore omitted from subsequent analyses. The results from this assay confirmed the impaired infectivity of high-growth parasites detected in Assay 1. These parasites completely failed to infect paramecia from the K8 and VEN genotypes, and produced only low levels of infections on genotype M3 (Fig. 20). Low-growth parasites were significantly more infectious on both genotypes tested (K8: $F_{1, 13}$ = 33.16, $p < 0.0001$; M3: $F_{1, 13}$ = 27.01, $p < 0.0001$).

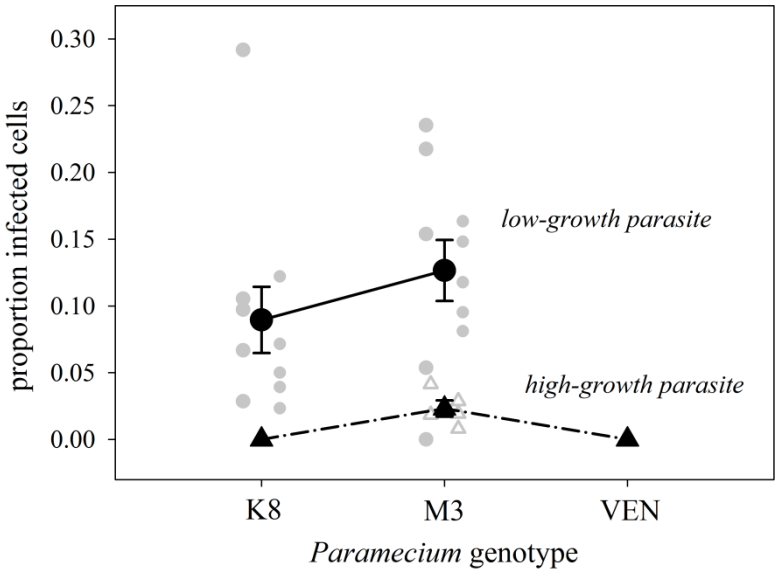

Fig. 20. Mean infection success (± S.E.) of parasites from high-growth and low-growth treatments, measured on three (naive) *Paramecium caudatum* genotypes, two of which (M3, VEN) were not used in the long-term experiment. Note that low-growth parasites were not tested on genotype VEN for this assay.

Infection assay 3

There was significant local parasite maladaptation in the low-growth treatment ($\chi^2_1 = 4.46$, $p = 0.0348$): overall, sympatric combinations of parasite and host showed lower infection success than allopatric combinations (Fig. 21).

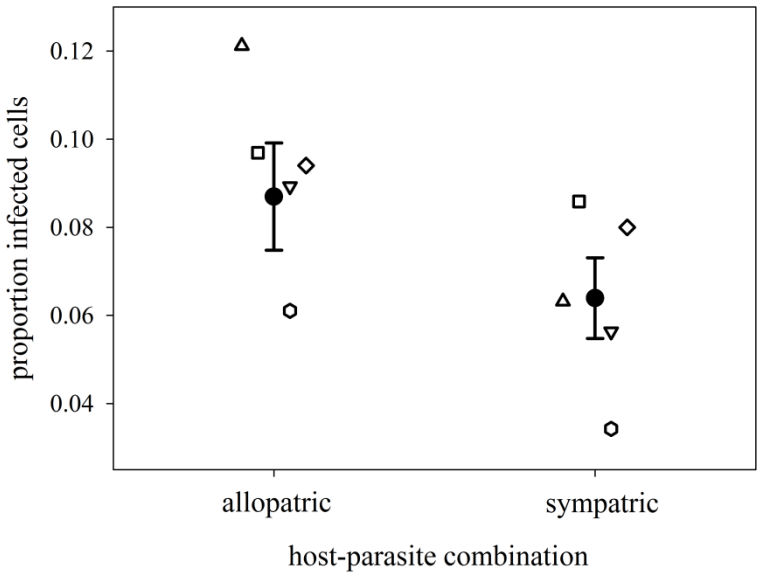

Fig. 21. Mean infection success (± S.E.) for sympatric and allopatric combinations of parasite and host from the low-growth treatment (filled circles). Host and parasite were taken from the same microcosm population in sympatric combinations, and from different populations in allopatric combinations. Smaller open symbols show the values for the five long-term replicate host replicate lines, from which sympatric and allopatric means were calculated.

5.5 Discussion

Loss of horizontal transmission has occurred in various natural host-symbiont systems and is considered a major evolutionary transition from parasitism towards beneficial or mutualistic associations with strict vertical transmission of the symbiont (Moran *et al.*, 2008; Sachs *et al.*, 2011). The main result in the present study was the near-complete loss of horizontal transmissibility of the parasite *Holospora undulata* after ca. 800 host generations in the high-growth treatment. This treatment allowed high host birth rates and therefore high rates of vertical transmission. An earlier study had already indicated that the high-growth treatment selected for increased vertical transmissibility and decreased virulence (Magalon *et al.*, 2010). Our new results complete this picture consistent with the idea that the transition from parasitism to benevolence is accompanied by a shift from mixed horizontal and vertical to mainly vertical transmission.

Loss of horizontal transmissibility in the high-growth treatment

Even if conditions favour high rates of vertical transmission, as such this does not automatically select against the horizontal transmission pathway. In fact, it should be abandoned only under very stringent conditions, if the contribution of horizontal transmission to total transmission is negligible and/or if horizontal transmission trades off strongly with vertical transmission. Several studies have addressed the evolutionary consequences of experimental manipulation of the relative contributions of vertical or horizontal transmission (Ebert, 2013). The most extreme result was obtained by Bull *et al*. (1991), who suppressed horizontal transmission of a bacteriophage and cultured it under exclusive vertical transmission. Similar to our result, the evolved phages did not only allow high host growth, but also lost the capacity to infect horizontally.

In our system, previous work had indicated that investment in the horizontal pathway trades off with vertical transmission capacity (Kaltz & Koella, 2003; Restif & Kaltz, 2006), but unlike in Bull *et al.* (1991), in our long-term experiment horizontal transmission was not a priori suppressed. In fact, although Magalon *et al*. (2010) already reported reduced investment in horizontal transmission (longer latency, reduced infectious parasite load), high-growth parasites had nonetheless shown a higher per-capita infectivity than parasites from the low-growth treatment after 45 experimental cycles (Magalon *et al.*, 2010). We offer the following tentative explanations for our new results of infectivity breakdown. Residual horizontal transmission is necessary to achieve high infection prevalence in a population (Lipsitch *et al.*,

1995a; Altizer & Augustine, 1997), and clearly, horizontal transmission has caused the massive infection outbreaks observed in the high-growth treatment during the initial period of the long-term experiment. Thus, the high per-capita infectivity observed after 45 cycles (Magalon *et al.*, 2010) may have reflected the utility of horizontal transmission, despite ongoing selection for lower virulence, better vertical transmission and reduced quantities of infectious forms. From these population-level assays it had not been possible to tell whether individual parasites were both better at vertical transmission and more infectious, or whether this reflected the coexistence of vertical and horizontal specialists (Altizer & Augustine, 1997). Regardless, in the long run, horizontal transmission may have become increasingly unprofitable for at least two reasons. First, with infection prevalences initially reaching very high levels and selection increasing the fidelity of vertical transmission in the high-growth treatment (Magalon *et al.*, 2010), fewer uninfected hosts were available for horizontal transmission. As such, this would render investment into horizontal transmission less profitable, in particular if access to already infected individuals in the population is limited (Lipsitch *et al.*, 1995a; Lipsitch *et al.*, 1996). Indeed, some studies suggest that already infected paramecia can be less susceptible to additional horizontal co-infection (Fels *et al.*, 2008; Fokin & Görtz, 2009), although we did not verify this for our present experiment. Second, our new results revealed increased levels of host resistance in the high-growth treatment. This may have further limited the availability of uninfected hosts, despite the observed decline in infection prevalence during later cycles (Fig. 18). However, higher host resistance was not only reducing the importance of horizontal transmission. Altogether, these epidemiological and evolutionary feedbacks may have disfavoured investment in horizontal transmission and ultimately caused the breakdown in infectivity. We acknowledge that these arguments hinge on several crucial parameters, namely the (im)possibility of co-infection or infection clearance, for which we have no strong data. We hope that our results can be helpful for future theoretical work, exploring the driving forces of transitions from mixed to vertical transmission modes.

The infectivity breakdown occurred independently across multiple high-growth selection lines. This suggests that it was the result of selection, rather than the random loss of (an unused) function. We can only speculate about the genes or functions involved. High-growth parasites still produce infectious transmission stages and release them into the environment. We also found a limited capacity to infect one of the three susceptible tester genotypes, which indicates that the transmission stages are still functional to some extent. As the infectious forms are still ingested by the paramecia (E. Dusi, personal observation), we

suspect that they fail to trigger the transfer from the food vacuole to the micronucleus (Fokin & Görtz, 2009). The transfer likely requires membrane contact and recognition between parasite and host, brought about by an unknown signal in the parasite. Only few genes are expressed in infectious forms at this stage (Görtz & Wiemann, 1989; Fujishima, 2009), and possibly expression levels are too weak to manipulate the host's within-cell trafficking system. In this sense, reduced infectivity may be the consequence of selection for reduced investment into provisioning of infectious forms for horizontal transmission.

The alternating treatment

Similar to an experiment by Messenger *et al.* (1999), the idea of the alternating treatment was used to generate fluctuations between conditions favouring vertical transmission (high growth) and horizontal transmission (low growth), thereby potentially facilitating the evolution of a more plastic transmission generalist. The long-term dynamics in this treatment resembled that of the high-growth treatment with infection prevalence quickly reaching ~100% and then decreasing over time (Fig. 18). Similar to the high-growth parasites, alternating parasites also failed to infect evolved paramecia in the infection assay (Fig. 18), falsifying our hypothesis (5.3). Because only few infections (or none) were produced by alternating and high-growth parasites, there was not enough material available to perform assays of vertical transmissibility or virulence. It is therefore difficult to interpret these results in a specialist-generalist context of transmission. From the data at hand, it would appear that alternating parasites followed the same evolutionary trajectories as the high-growth parasites, but at a slower rate, because they experienced high-growth conditions only half of the time. Furthermore, the high-growth treatment also resembled the alternating treatment, because host population growth tended to slow down during the second 24h of the 2-day dilution interval (Fig. 17). Consequently, infections may begin to switch from 'vertical' into 'horizontal' mode, before the new dilution causes a switch back into 'vertical' mode. Hence, the bottom-line is that two similar treatments, with more or less prolonged high-growth periods, produced congruent results: a strong reduction in horizontal transmissibility of the parasite.

Host evolution

In line with previous studies (Lohse *et al.*, 2006; Adiba *et al.*, 2010), paramecia from infected selection lines were more resistant to infection than naive paramecia from parasite-free control selection lines, indicating parasite-mediated selection for increased resistance. In addition, in the low-growth treatment, paramecia were more resistant to their own than to

foreign parasites. As all selection lines originated from the same founder host genotype, this pattern of local host adaptation illustrates how co-evolutionary interactions can drive population divergence (Buckling & Rainey, 2003).

Resistance in high-growth lines was generally higher than that in low-growth lines, independent of the presence or absence of parasite in the long-term experiment. This result supports our hypothesis (5.1) and suggests that growth conditions alone influenced resistance evolution. Possibly, this is an indirect result of selection on feeding behaviour or metabolic characteristics in this treatment (e.g., Meyer *et al.*, 2010). Importantly, however, our main finding regarding parasite evolution is largely unaffected by host identity: high-growth parasites, in general, had strongly reduced infectivity on hosts from different treatments or with different genetic backgrounds.

5.6 Conclusions

Horizontal transmission is implicated in parasite spread and virulence, and therefore a key parameter defining the nature of host-parasite interactions (Ebert, 2013). Our results indicate that environmental conditions can strongly impact the evolution of the horizontal transmission pathway and even lead to its near-complete breakdown in a parasite with an initially mixed mode of transmission. The concomitant changes in population infection prevalence and host resistance suggest that this breakdown was caused by evolutionary and epidemiological feedbacks, similar to those assumed in theoretical models for parasites with mixed transmission modes (Lipsitch *et al.*, 1996). Parameters such as host density and contact rates have also been invoked as drivers of co-evolution in experimental studies testing variable productivity regimes in bacteria-phage systems (Bohannan & Lenski, 2000; Forde *et al.*, 2004; Lopez-Pascua & Buckling, 2008).

In summary, our present study in addition to by Magalon *et al.* (2010) describe a cascade of profound changes in the life-history of this bacterial parasite, pushing it towards becoming a vertically transmitted benevolent symbiont. Whether there is the scope for the evolution of true mutualism remains to be shown.

SYNTHESIS

Parasites are generally harmful to their hosts by definition (Poulin, 2011). However, this is often an oversimplifying picture. In many systems, variation in the parasite's impact exists, and in some cases there are even potential benefits for the host being parasitized (Michalakis *et al.*, 1992; Leung & Poulin, 2008; Fellous & Salvaudon, 2009). For these reasons, the position of many given systems can shift back and forth on a continuum between parasitism and mutualism depending on a variety of abiotic and biotic factors (Michalakis *et al.*, 1992; Bronstein, 1994; Blanford & Thomas, 2000; Gomulkiewicz *et al.*, 2003; Fellous & Salvaudon, 2009).

Phylogenetic analyses indicate that evolutionary transitions from parasitism to commensalism to mutualism involve a radical change in parasite transmission (Moran *et al.*, 2008; Sachs *et al.*, 2011). The experimental evolution study on *Paramecium caudatum* and *Holospora undulata* described in Chapter 5 of this study supports this hypothesis. The parasite *H. undulata*, transmitting both vertically and horizontally, can switch to almost exclusive vertical transmission under conditions with high host growth (Kaltz & Koella, 2003). Based on this trait, microcosm populations of both infected and uninfected hosts were exposed to different host growth conditions for ca. 800 host generations, thereby manipulating the relative importance of the vertical transmission pathway to total transmission (Kaltz & Koella, 2003; Magalon *et al.*, 2010). Horizontal transmission was not explicitly restrained or favoured in this experiment. After 200 host generations, a first experimental analysis revealed the evolution of the parasite towards lower levels of virulence in the treatment favouring vertical transmission (Magalon *et al.*, 2010). Nevertheless, at this time point horizontal transmission efficiency of these parasites was similar or even higher compared to the control treatment with a lower vertical transmission rate (Magalon *et al.*, 2010). After 800 host generations of experimental evolution, host evolution and horizontal transmissibility were tested to evaluate factors driving the transition from parasitism to mutualism. Hosts from the treatment favouring vertical transmission were assumed to evolve higher resistance (hypothesis 5.1), which might reduce the importance of horizontal transmission (hypothesis 5.2). Indeed, the parasite nearly entirely lost its horizontal transmission capacity for all selection lines of the treatment favouring low levels of virulence and vertical transmission. Further, these hosts evolved higher resistance than hosts from the control treatment. Moreover, parasites of the alternating treatment, with the opportunity of horizontal and

vertical transmission, had also a reduced horizontal transmissibility. This falsifies the hypothesis (5.3) that these parasites might evolve a more generalist transmission strategy. Three potential factors were identified to influence the importance of horizontal transmission: (*i*) evolution of host resistance (Fig. 18), (*ii*) probability of co-infection (Fels *et al.*, 2008; Görtz & Fokin, 2009) and (*iii*) changes in the infection prevalence of host population during the long-term experiment (Fig. 17). These results and their explanation support the theoretical models by Lipsitch and colleagues (1996) on the evolutionary and epidemiological dynamics of parasites with mixed transmission modes, but not the loss of horizontal transmission as exclusively driven by the host or caused by mutation accumulation.

Horizontal transmission, however, is a key parameter defining the outcome of host-parasite interactions because of the strong implication in infection spread and virulence (Ebert, 2013). The loss of horizontal transmission creates a positive relationship between host and parasite fitness. Being locked-up in the vertical transmission pathway, parasite's reproduction must be sufficiently high to ensure vertical transmission, but low enough to avoid damage to the host because any host fitness reduction would also be detrimental to parasite fitness. This would suggest that vertically transmitted parasites must evolve to become avirulent or even beneficial, or they will go extinct (Fine, 1975; Ewald, 1987; Bull, 1994). Many strictly vertically transmitted symbionts are classified as mutualists and some of them are even essential for hosts' reproduction and survival. The loss of the endosymbiont *Polynucleobacter necessarius*, for example, is lethal for its host ciliate *Euplotes* because of the potential influence on host's glycogen metabolism (Vannini *et al.*, 2003). Another example is the bacterium *Buchnera,* well studied in aphids. This symbiont provides essential nutrients to the host and its removal decreases host fitness dramatically (Douglas, 1998). In other cases, vertically transmitted symbionts provide benefits against natural enemies (Oliver *et al.*, 2003; Haine, 2008; Brownlie & Johnson, 2009) or adverse environmental conditions, such as pollutants or high-temperature stress (Montllor *et al.*, 2002; Russell & Moran, 2006), but remain costly in the absence of stressors (Vorburger & Gouskov, 2011). Vertical transmission and mutualism are not depending on each other.

Vertically transmitted parasites are common in nature and known to be considerably virulent (Lipsitch *et al.*, 1996; Ebert, 2013). In some cases, this can be explained by the existence of some level of horizontal transmission. In other cases, parasites compensate virulence costs by conferring a benefit on the host (protection, nutrients, e.g.; Haine, 2008; Brownlie & Johnson, 2009) or by manipulating host's reproduction in such a way that uninfected conspecifics and thus potential resource competitors are eliminated from the

population (e.g., cytoplasmic incompatibility in *Wolbachia*; Werren, 1997; Dunn & Smith, 2001). The impact of a vertically transmitted parasite on its host and thereby its transmission success may vary with genetic background of host and/or parasite or with environmental conditions (Kelly *et al.*, 2003; Dunn *et al.*, 2006; Mouton *et al.*, 2006; Mouton *et al.*, 2007).

To investigate the role of genetic variability and environmental changes on the outcome of host-parasite interactions, virulence and persistence of *Caedibacter taeniospiralis*, a strictly vertically transmitted bacterial symbiont of the ciliate *Paramecium tetraurelia*, was determined. It was expected that the vertically transmitted symbiont *Caedibacter taeniospiralis* imposes context-dependent costs on its host (hypothesis 1.1). Further, temperatures above host optimum should be more stressful for the host being exposed to two stressors (parasitism and heat stress; hypothesis 1.3). To test these hypotheses, fitness of infected and antibiotic cured host populations was measured for five *P. tetraurelia* strains at five different temperatures (16-32 °C). Infection substantially reduced host density under resource-limited conditions at all temperatures, with the highest fitness reduction at 28 °C. The virulence profile was consistent for the five infected *P. tetraurelia* strains, whereas the magnitude of virulence differed between the strains. The endosymbiont *C. taeniospiralis* imposes remarkable but context-dependent costs and is therefore classified as parasite. While infection levels were 100 % for temperatures below host optimum, infection prevalence and also virulence strongly declined at a more stressful temperature (32 °C), indicating limited heat tolerance of *Caedibacter*. In contrast, growth of *P. tetraurelia* was reduced at 32 °C as well, but this temperature was not lethal to the host. Higher temperatures were more harmful for the parasite than for the host and may release the host from the negative impact of infection.

Still, very little empirical and experimental work exists on the evolutionary dynamics of systems with obligate vertical transmission and on the impact of stressful environmental conditions. A long-term experiment was set-up to study parasite evolution under heat stress conditions. Microcosm populations of *P. tetraurelia* and *Caedibacter* were exposed to a 32 °C high temperature treatment and a 26 °C control treatment for ca. 150 host generations. Central to this long-term experiment was the investigation of the evolutionary change in the host-parasite interaction under permissive conditions (26 °C) and under heat stress (32 °C). It was hypothesized that the parasite persists at and is able to adapt to high temperatures (hypothesis 3.1). *Caedibacter* should increase its within-host growth rate and thereby evolve to lower levels of virulence or even benevolence under continuous high-temperature conditions (hypothesis 3.2). Further, costly heat adaptation of *Caedibacter* was theorized (hypothesis

3.3). Fitness assays were performed to measure the potential evolutionary changes of the different host-parasite selection lines and their ancestral lines and to compare the direct and indirect responses to selection. The parasite *Caedibacter* was able to adapt and to persist under long-term heat stress conditions, which verifies the hypothesis 5.1. Only one parasite genotype (47) went extinct. The parasites of the other strains could increase rates of infection proliferation and had higher within-host densities, but they remained costly. Furthermore, the adaptation processes were genotype-dependent. Selection lines of certain strains evolved under high temperature conditions had not only a superior performance at the respective high temperature but also at the permissive temperature. These positive direct and indirect responses to selection indicate cost-free adaptation of the generalist strategy and are consistent with the idea of 'roundabout selection' (Bell, 1997) at least for some investigated genotypes. Contrary, heat-adapted lines of another strain (298) seem to have evolved towards a specialist with a higher performance at 32 °C, but reduced fitness at the permissive temperature. These genotype-specific outcomes suggest that it can be difficult to predict host-symbiont evolution along the genetic and geographic range of their interaction.

All in all, the vertically transmitted parasite *Caedibacter* can evolve in a way that does not necessarily facilitate its host. The parasite still induces costs for its own reproduction and transmission. Spread and persistence of the *Caedibacter* infection therefore require additional mechanisms. Thus, maintenance of *Caedibacter* infection may include some host contribution, e.g. a tolerance reaction defined as host's capacity to limit the harm caused by a given within-host parasite density. Investigations of such a potential tolerance reaction would be possible by the use of the quantitative real-time PCR (qRT-PCR) technique allowing the absolute quantification of within-host parasite densities. Nevertheless, separating host's and parasite's precise relative contribution to the evolutionary outcome would require the possibility of controlled artificial infections either by microinjection or by a hypothetical co-transmission with an infectious bacterium, such as *Holospora*. These techniques are, however, currently inefficient or not available for this system.

Universal costs of the *Caedibacter* infection for its host also necessitate a universal cost compensation to ensure successful spread and maintenance within a host population. In this thesis, the 'killer trait' of *C. taeniospiralis* influencing host population dynamics by eliminating uninfected conspecifics (Schrallhammer & Schweikert, 2009) is hypothesized as a cost compensator (hypothesis 2.1). This trait should prevent the co-occurrence of infected and uninfected hosts in one and the same population (Görtz & Fokin, 2009). The 'killer trait' is active in resource-limited situations (Kusch & Görtz, 2006; Schrallhammer & Schweikert,

2009); conditions where *Caedibacter* reduces host fitness (Dusi *et al.* 2014). This supports the hypothesis of cost compensation (hypothesis 2.1). However, only four out of five different *Caedibacter* genotypes showed a 'killer activity' thereby showing variation in the number of killed uninfected cells and in the survival time of these uninfected paramecia. This result supports the hypothesis of a genotype-dependent 'killer activity' (hypothesis 2.2). The 'killer activity' of the *Caedibacter* genotype 47, A30 and 51 was positively correlated with their virulence levels supporting a hypothetical relationship between these two traits. *Caedibacter* genotype '298', on the other hand, was the most potential killer but might also be the most efficient cost compensator, because of very low levels of virulence. In contrast, *Caedibacter* genotype 116 was not able to kill any of the exposed uninfected cells. Moreover, this parasite obviously was the most virulent genotype, but it persisted under optimal conditions for both host and parasite. This indicates that there might be further mechanisms or traits involved in parasite maintenance and cost compensation.

In the long-term high temperature treatment, 'heat cured', uninfected hosts steadily co-existed with infected hosts or even completely cured host populations arose indicating an inefficient or impaired 'killer trait' for all investigated *Caedibacter* genotypes at higher temperatures (hypothesis 4.1). This also supports the hypothesis that temperature variation renders the cost-benefit balance of the parasite (hypothesis 1.3). Similar temperature sensitivity was found for a comparable trait of the vertically transmitted parasite *Wolbachia*, the frequency distorter functions (CI or male killing; Hurst *et al.*, 2000). Here, the efficiency of the frequency distorter function is positively correlated with a strong decline of within-host parasite density at higher temperatures (Hurst *et al.*, 2000; Mouton *et al.*, 2006). A comparable correlation between within-host density and the 'killer trait' might exist for *Caedibacter* exposed to an acute temperature stress, but long-term heat adaptation processes allow the maintenance of occasionally high within-host densities. Here, 'killer activity' at 32 °C might result from the equilibrium between the costs for temperature adaptation and the costs for R-body production. These interpretations should, however, be treated with caution as 'killer activity' was not measured for the evolved lines and during the long-term experiment. One open question for a further study is how the 'killer trait' evolves under heat stress conditions. While 'killer tests' that are currently needed for estimating the 'killer activity' of the different genotypes, are very time consuming (see Chapter 2), the 'killer activity' might be positively correlated with R-body production. Testing this hypothesis by the use of qRT-PCR may provide the next step for further studies on the evolutionary response of this trait to different selection pressures.

However, the question that remains to be solved is what actually allows co-existence of infected and uninfected hosts. Experimental tests revealed that adaptation of *Caedibacter* to high temperatures reduces the occurrence of uninfected paramecia at 32 °C, but 'heat cured' hosts still arose. These uninfected hosts might have evolved a costly resistance to the 'killer activity' because they had no fitness advantage in comparison to infected hosts, but seemed to be less sensitive to the 'killer trait'. This possible resistance should be supported by more experimental 'killer tests' and analyses of host's and parasite's transcriptome.

Conclusion

This study advances the understanding of conditions for evolution and persistence of vertically transmitted parasites. While horizontal transmission can select for more harmful parasites, vertical transmission links parasite's reproduction and transmission to host's fitness. Any harm inflicted by the parasite will decrease its own reproduction success. Therefore, the loss of horizontal transmission is expected to push an interaction towards lower levels of virulence and mutualism as shown for the parasite *Holospora*. However, contrary to common expectations (Ewald, 1987), the strictly vertically transmitted parasite *C. taeniospiralis* causes substantial fitness costs for its host at all investigated temperatures. This finding indicates the necessity of universal cost compensation, probably through the 'killer trait'. However, the parasite is less temperature-tolerant then the host at 32°C. Therefore, the parasite should reduce cost to increase its persistence and reproduction success. However, adaptation of this vertically transmitted parasite to environmental change does not necessarily benefit its host. Heat adaptation of *Caedibacter* increases within-host density while still remaining costly. Further, the 'killer trait' seems to be offset at high temperature. Therefore, *Caedibacter* will still be qualified as parasite, imposing fitness costs on its host and conferring no additional benefit, contrary to observations in other systems. Furthermore, genetic variation strongly influences evolutionary trajectories; with sometimes costly or cost-free adaptation of the parasite. The evolution of super-generalist suggests that more efficient parasites evolving in marginal habitats may spread back into the original populations. On the one hand, this opens the question of competition between genetically different, infected host populations. On the other hand, in times of climate change where host-parasite systems are experiencing more often extreme conditions and therefore profound modifications in geographic distribution, this could be the starting for further research on species interaction.

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ABBREVIATIONS

%	percent
°C	degree Celsius
µg	microgramm
µl	microlitre
µl-1	per microlitre
µm	micrometer
µM	micromolar
AIC	Akaike information criterion
ANCOVA	analysis of covariance
ANOVA	analyses of variance
ATP	andeniosintriphosphate
AUC	area under curve
BHQ1	fluorescence dye
BIC	Bayes' information criterion
bp	base pair
ca.	circa
CI	cytoplasmic incompatibility
Ctaenio-998	*Caedibacter taeniospiralis* specific probe
Cy3	cyanine dye 3
d	day
d.f.	degree of freedom
d-1	per day
DIC	differential interference contrast
DNA	deoxyribonucleic acid
dNTPs	deoxynucleoside triphosphates
e.g.	exampli gratia (example given)
Eq.	equation
et al.	et alii or et alia - and others
EUB338 I	probe name of an eubacteria specific probe
Fig.	figure
FISH	fluorescence *in situ* hybridisation
h	hour
Hz	hertz
JMP	Jmp software of SAS®
K	killer *Paramecium* strain containing *Caedibacter*
K	carrying capacity
K d-1	kelvin per day
K8	*Paramecium caudatum* strain
kV	kilo volt
lme4	R package name
LMM	linear mixed effect model
log lik	log likelihood

LRT	likelihood ratio test
M	molar
M3	*Paramecium caudatum* strain
MAC	macronucleus of *Paramecium*
Mg	milligramm
MIC	micronucleus of *Paramecium*
Min	minute
Ml	millilitre
mM	millimol
MS	mean square
Nm	nanomol
PCR	polymerase chain reaction
pH	pondus Hydrogenii
pKAP	plasmid of kappa particle
pmol	picomol
qRT-PCR	quantitativ real time PCR
r	growth rate
R0	vertical reproductive rate of the symbiont
R^2	coefficient of determination
R-body	refractile body
Reb/ reb	R-body encoding DNA and produced protein in the subunits A, B, C, D
RNA	ribonuclein acid
ROX	fluorescence dye
rRNA	ribosomal ribonucleic acid
s	second
S.E.	standard error
SS	sum of square
t	time
Tukey's HSD	Tukey's Honestly Significant Difference
U	unit
VEN	*Paramecium caudatum* strain
yt	cell density at the specific time point t

ERKLÄRUNG ZUR ERÖFFNUNG DES PROMOTIONSVERFAHRENS

1. Hiermit versichere ich, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht.

2. Bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts habe ich Unterstützungsleistungen von folgenden Personen erhalten:

Thomas Berendonk

Oliver Kaltz

3. Weitere Personen waren an der geistigen Herstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich nicht die Hilfe eines kommerziellen Promotionsberaters in Anspruch genommen. Dritte haben von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.

4. Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt und ist – sofern es sich nicht um eine kumulative Dissertation handelt – auch noch nicht veröffentlicht worden.

5. Sofern es sich um eine kumulative Dissertation gemäß § 10 Abs. 2 handelt, versichere ich die Einhaltung der dort genannten Bedingungen.

6. Ich bestätige, dass ich die Promotionsordnung der Fakultät Umweltwissenschaften der Technischen Universität Dresden anerkenne.

Ort, Datum

Unterschrift des Doktoranden

REFERENCES TO OWN ORIGINAL PUBLICATIONS USED IN THIS THESIS

Chapter 1 has been published with minor alterations under the title:
Dusi, E., Krenek, S., Schrallhammer, M., Sachse, R., Rauch, G., Kaltz, O. & Berendonk, T.U. 2014. Vertically transmitted symbiont reduces host fitness along temperature gradient. *Journal of Evolutionary Biology* 27**:** 796-800)
The chapter differs from the original publication in the following respects: Title was changed. Material and method part were extended and the part presented in the Supplementary Information of the article were included in the text. The figure 2 of the manuscript was replaced by a figure combining Fig. 2 and Fig. S2 of the article. Due to the structure of the thesis, the conclusion was extended in comparison to original publication. Further, the numbering of figures and table was adapted to the structure of the thesis.

Chapter 5 has been published with minor alterations under the title:
Dusi E, Gougat-Barbera C, Berendonk T U and Kaltz O. Long-term selection experiment produces breakdown of horizontal transmissibility in parasite with mixed-mode transmission. EVOLUTION 69 (4): 1069-1076.
The chapter differs from the original publication in the following respects: The chapter contains an “Assay 3” in comparison to the publication. Therefore, the description, the results and a figure of “Assay 3” were included in the material and method part respectively in the results part. The conclusion section was extended to provide information on the hypothesis structure of the thesis. The numbering of figures was adapted to the structure of the thesis.

CURRICULUM VITAE

EDUCATION

2010 - present **PhD student of the TU Dresden, Institute of Hydrobiology**

Topic: „Experimental Host-parasite interaction in changing environment"

I am using common-garden experiments combined with molecular techniques to investigate the influence of temperature (constant or fluctuating), parasite competition or changed host growth conditions on the outcome of host-parasite interactions. (Supervisior: Prof. Dr. T.U. Berendonk, Dr. Oliver Klatz)

2004- 2010 **Diploma in Biology at the University of Leipzig, Institute of Biology II**

Thesis: *"Study of the ciliate community via molecular approaches"*

Annual differences of ciliate diversity within a constructed wetland were observed using denaturing gradient gel electrophoresis (DGGE) and 454 sequencing. (Supervisior: Dr D. Bernhardt and Prof. Dr. M. Schlegel)

2004 **School graduation**

EMPLOYMENT

2011- present **Institute of Hydrobiology, Technische Universität Dresden, Dresden**

Research associate

2010 (Feb – Dec) **Institute for Evolution and Biodiversity, University of Münster, Münster**

Research associate

2004 - 2010 **frunol Delicia®, Delitzsch, Germany**

Research assistant

2007 - 2010 **Institute of Biology II, University of Leipzig, Leipzig**

Student assistant

INTERNATIONAL INTERNSHIPS and SCHOLARSHIPS

2013 (Sep – Oct) **Research internship at the State University of St. Petersburg, Russia**

Institute of Animal Histology (Dr. Elena Sabaneyeva)

Project: "Morphological and molecular quantification of R-body production"

2011 (Apr – Oct) **Research internship at University 2 in Montpellier, France**

Evolutionary Community Ecology (Dr. Oliver Kaltz)

Project: „Experimental evolution of a parasite with vertical and horizontal transmission"

2014 (Mai- Aug) **Scholarship of the Graduate school, Technische Universität Dresden, Dresden**

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