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HOST GROWTH CONDITIONS INFLUENCE EXPERIMENTAL EVOLUTION OF LIFE HISTORY AND VIRULENCE OF A PARASITE WITH VERTICAL AND HORIZONTAL TRANSMISSION

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In parasites with mixed modes of transmission, ecological conditions may determine the relative importance of vertical and horizontal transmission for parasite fitness. This may lead to differential selection pressure on the efficiency of the two modes of transmission and on parasite virulence. In populations with high birth rates, increased opportunities for vertical transmission may select for higher vertical transmissibility and possibly lower virulence. We tested this idea in experimental populations of the protozoan *Paramecium caudatum* and its bacterial parasite *Holospora undulata*. Serial dilution produced constant host population growth and frequent vertical transmission. Consistent with predictions, evolved parasites from this “high-growth” treatment had higher fidelity of vertical transmission and lower virulence than parasites from host populations constantly kept near their carrying capacity (“low-growth treatment”). High-growth parasites also produced fewer, but more infectious horizontal transmission stages, suggesting the compensation of trade-offs between vertical and horizontal transmission components in this treatment. These results illustrate how environmentally driven changes in host demography can promote evolutionary divergence of parasite life history and transmission strategies.

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Parasites have numerous ways of exploiting their hosts to mediate their own growth and transmission (Combes 2001) and understanding the causes of this diversity is a central issue in evolutionary biology (Ewald 1994; Frank 2002). It has become increasingly clear that this requires the integration of epidemiological and co-evolutionary processes in an ecological framework, accounting

for spatial structure or environmental heterogeneity (Hochberg and van Baalen 1998; Koella and Restif 2001; Thompson 2006; Gandon and Nuismer 2009). Particularly, environmental conditions may have direct effects on the fitness functions of hosts and parasites, as well as indirect effects on epidemiological conditions, determining the availability of hosts and the force of infection. Thus spatial variation in environmental conditions may generate selection mosaics, with different evolutionary optima of attack and defense strategies (Thompson 1999).

Here, we will consider the impact of environmental heterogeneity on the evolution of parasites with horizontal and vertical transmission. The mode of transmission may be an important evolutionary driver of parasite virulence (= broadly defined as reduction in host fitness). Horizontal transmission, that is, the infectious spread from infected to uninfected hosts in the population, should be maximal for intermediate levels of virulence, balancing transmissibility against damage done to the host (Frank 1996). In contrast, vertical parent-to-offspring transmission depends on host reproduction and should therefore be maximal for low virulence (Fine 1975; Yamamura 1993; but see, van Baalen 2000; Day and Proulx 2004). Only few theoretical models have investigated epidemiology and evolution of parasites with mixed modes of transmission (Ewald 1987; Lipsitch et al. 1995b, 1996; Day and Proulx 2004; Ferdy and Godelle 2005). For such parasites, vertical and horizontal paths may not be optimized simultaneously because of the different virulence optima associated with them; selection may then operate on the component that contributes more to total transmission success. Hence, mixed-mode parasites may fall along a virulence continuum, ranging from more vertically transmitted and less virulent to more horizontally transmitted and more virulent (Ewald 1987; Herre 1993). This view has been criticized, mainly because epidemiological feedbacks can prevent selection for increased virulence (Lipsitch et al. 1995a, 1996). Nonetheless, optimal virulence is predicted to decrease with increasing rates of vertical transmission, for any level of horizontal transmission (Lipsitch et al. 1996). In other words, increased vertical transmission should select for lower mean virulence in the population, at least relative to a pure horizontal strategy (Altizer and Augustine 1997; Day and Proulx 2004; Ferdy and Godelle 2005).

Ecological conditions may strongly influence the relative contribution of the vertical or horizontal transmission pathway to total transmission (Lipsitch et al. 1995b; Kover et al. 1997; Kover and Clay 1998; Agnew and Koella 1999; Jaenike 2000). It is often argued that vertical transmission is a safer route of transmission when host density is low and finding a new host via horizontal transmission improbable (Levin and Lenski 1983; Lipsitch et al. 1996; Jaenike 2000). This may explain, for example, why temperate bacteriophages insert in the host DNA and become lysogenic (= vertically transmitted) when hosts grow

slowly (Chibani-Chennoufi et al. 2004). However, as pointed out by Jaenike (2000), periods of low host density may also occur when populations (re-)colonize a patch or habitat. In this case, host populations can grow very fast and high birth rates will be coupled with high rates of vertical transmission. Thus, in frequently disturbed populations with high intrinsic birth rates, vertical transmission may make an important contribution to total transmission (Altizer and Augustine 1997), and this may select for parasites with more efficient vertical transmission and possibly with lower virulence.

Experimental tests of these ideas are still rare. Several studies used serial passage or controlled culturing techniques to impose exclusive vertical or exclusive horizontal transmission on the parasite (Bull et al. 1991; Messenger et al. 1999; Jaenike 2000; Stewart et al. 2005; Sachs and Wilcox 2006). In most cases, lower virulence evolved with obligate vertical transmission and higher virulence and transmissibility with obligate horizontal transmission (Bull et al. 1991; Messenger et al. 1999; Stewart et al. 2005; Sachs and Wilcox 2006). To our knowledge, only one study (partly) relaxed the constraint of obligate mode of transmission to test the effect of host density on the evolution of a bacterial plasmid (Turner et al. 1998). Contrary to theoretical predictions, lower host density did not select for increased vertical transmissibility and lower virulence (Turner et al. 1998). Hence, it remains unclear how different ecological conditions influence the evolution of parasites allowed to freely transmit vertically or horizontally.

We used the protozoan *Paramecium caudatum* and its bacterial parasite *Holospora undulata* (Görtz and Dieckmann 1980; Fokin 2004) to test the above idea that host population growth conditions affect the evolution of vertical and horizontal transmission efficiency. Investment of this parasite in vertical and horizontal has shown to be condition dependent (Kaltz and Koella 2003). In rapidly dividing host individuals, the parasite remains mainly in the reproductive stage and is passed on vertically to the daughter nuclei of mitotically dividing paramecia. At low population growth rate, the parasite's reproductive forms differentiate into infectious forms, the agents of horizontal transmission (Kaltz and Koella 2003; Nidelet et al. 2009).

The goal of our study was to perpetuate these contrasting growth conditions for approximately 200 host generations and to investigate their long-term effects on parasite evolution. Following Kaltz and Koella (2003), two treatments were imposed. In the "high-growth" treatment, we kept infected host populations almost constantly under exponential growth, thereby allowing high rates of vertical transmission. In the "low-growth" treatment, populations were constantly kept near carrying capacity, with low rates of vertical transmission (Fig. 1). Thus, one key feature of these treatments is that they varied opportunities for vertical transmission, without, however, a priori excluding one or the other transmission mode. Responses to selection therefore

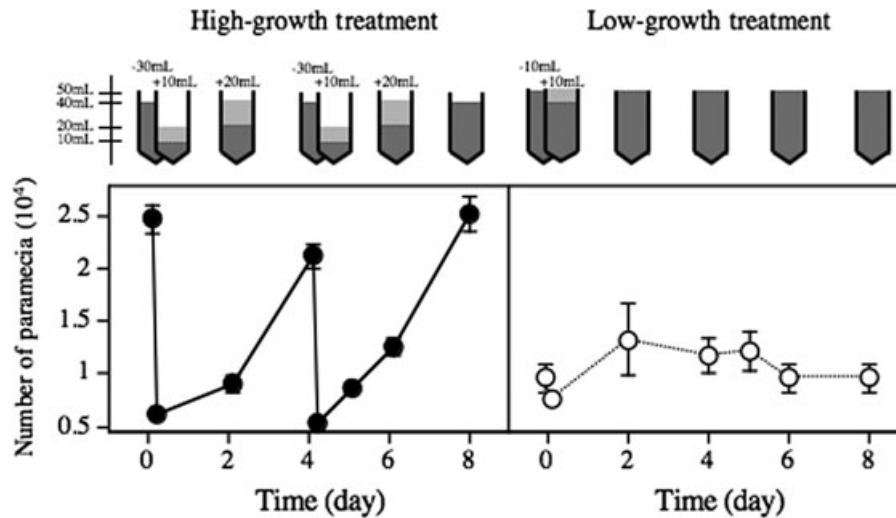


Figure 1. Experimental protocols in the high-growth (left) and low-growth (right) treatments. The tubes depict the changes of volume in the long-term cultures over the course of one 8-day cycle. Volume of culture was removed (–30 or –10 mL) and fresh medium added (+10 or +20 mL) to simulate episodes of non-selective mortality, followed by population regrowth. The corresponding changes in mean host population size are illustrated for cycle 33 (of a total of 45). High dilution rate kept high-growth populations under constant growth, whereas low-growth populations remained almost constantly near their carrying capacity.

depended on the relative contribution of each mode to total transmission. Because vertical transmission should make a relatively more important contribution in the high-growth treatment (Fig. 2), our main prediction was that parasites from this treatment evolved to become better at vertical transmission than those from the low-growth treatment. Increased vertical transmissibility may be achieved through higher fidelity of transmission (proportion of infected offspring) or lower virulence, resulting in a higher number of infected offspring. We further tested whether trade-offs evolved between vertical transmissibility and components of horizontal transmissibility, such as latency time, production of horizontal transmission stages or infectivity.

Material and Methods

STUDY ORGANISMS

Reproduction and population growth of the freshwater ciliate *P. caudatum* (Wichtermann 1986) is mainly achieved by asexual, mitotic division. Genes of the polyploid macronucleus are expressed during the asexual cycle, those of the diploid micronucleus during the sexual cycle (conjugation). Our cultures were maintained asexually (i.e., as single clones) on a medium made of organically grown lettuce, supplemented with the food bacterium *Serratia marcescens* (strain A173, Institut Pasteur, Paris, France), at 23°C (Nidelet and Kaltz 2007).

Infection with *H. undulata*, a gram-negative α -proteobacterium (Amann et al. 1991), starts with the ingestion of filamentous infectious forms (10–15 μ m) during food uptake.

Once transferred to the micronucleus, infectious forms differentiate in round-shaped reproductive forms (5 μ m). During initial stages of infection or when the host divides frequently, the reproductive forms achieve 1–2 doublings per day. About one-week post infection, accumulation of reproductive forms is followed by their differentiation into infectious forms, possibly induced by a density threshold in the micronucleus (Kaltz and Koella 2003), similar to density-dependent switches in trypanosomes (Vassella et al. 1997) or *Legionella* (Molofsky and Swanson 2004). From then on, provided host division is not too frequent, infected micronuclei usually carry both types of forms (Kaltz and Koella 2003). For vertical transmission, reproductive forms segregate into the new daughter nuclei, just like chromosomes, whereas infectious forms are released into the environment just after cell division is completed (Wiemann and Görtz 1989). Thus, horizontal transmission does not necessarily rely on host death. Individuals can be multiply infected, at least during early stages of infection (Fels et al. 2008). Therefore negative epidemiological feedbacks disfavoring horizontal transmission at high parasite prevalence (Lipsitch et al. 1995a, 1996) may be less strong.

Host population growth rates regulate the parasite's investment into vertical and horizontal transmission (Kaltz and Koella 2003; Restif and Kaltz 2006). In rapidly growing populations, parasite loads are small (50–100 bacteria) and predominantly at the reproductive stage. In static host populations, ongoing division of reproductive forms and their conversion into infectious forms leads to accumulation of bacterial loads and heavily swollen micronuclei (Kaltz and Koella 2003), causing reductions in host survival and division rate (Restif and Kaltz 2006).

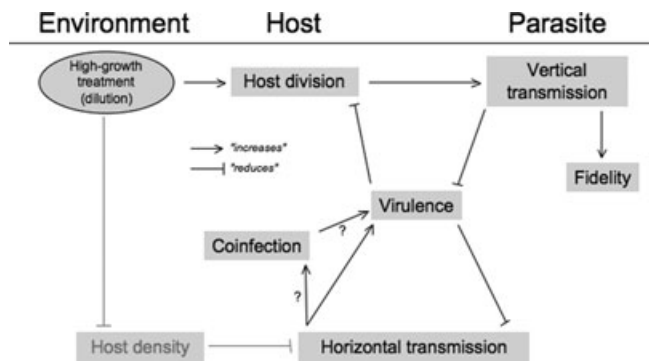


Figure 2. Potential epidemiological and evolutionary processes in the high-growth treatment. (1) Serial dilution (doubling of culture volume and regular nonselective replacement of a fraction of the population with fresh medium) increases rates of host division, and thereby vertical transmission. (2) Virulence-mediated feedback loops influence the relative rates of vertical or horizontal transmission. Lower virulence increases vertical transmission, but decreases horizontal transmission (because fewer horizontal transmission stages are produced). Higher virulence is the consequence of increased production of transmission stages, resulting in higher rates of transmission (i.e., higher parasite prevalence), but decreasing host division. Thus, there is an epidemiological trade-off between vertical and horizontal transmission. (3) Combined vertical and horizontal transmission can increase prevalence and thus the probability of coinfection. Frequent coinfection may reinforce within-host competition and thereby lead to higher virulence. Whether this is the case in our systems is unclear, hence the question marks on the arrows. If coinfection precluded, increased prevalence produces a negative feedback on horizontal transmission rates and on virulence. (4) Increased rates of vertical transmission can select for a higher fidelity (= fraction of infected daughter cells) of this transmission mode; it does not necessarily trade off with horizontal transmission. (5) Frequent dilution was expected to decrease host density and therefore reduce horizontal transmission. However, in our experiment, host density in high-growth populations was equal to or even larger than densities in the low-growth treatment.

DESIGN OF THE LONG-TERM EXPERIMENT

A clonal base population of paramecia (strain K8 (Restif and Kaltz 2006)) was infected with an unadjusted mix of inocula from three long-term parasite selection lines from another experiment (Lohse et al. 2006). These inocula did not significantly differ in infectivity or virulence on this host clone ($F_{2,6} < 1$, n.s.).

From the infected base population, two treatments were established (Fig. 1), each with 12 replicate lines. In the high-growth treatment, replicate lines (50 mL plastic tubes) were subjected to four-day growth cycles. To an initial volume of 10 mL of culture (ca. 2000 paramecia), we added 10 mL of fresh medium on day 0; on day 2, the volume was doubled by adding 20 mL of fresh medium. On day 4, 30 mL of the culture were discarded and a new growth cycle started. This removal/dilution protocol

keeps host populations under constant, near-exponential growth (ca. two doublings per four-day cycle; Fig. 1). In the low-growth treatment, replicate lines were kept at 50 mL, and 10 mL of the culture were replaced with fresh medium in eight-day intervals. This keeps populations at carrying capacity for most of the time, with moderate regrowth at the beginning of a new cycle (Table 1). We do not know the precise population turnover rate under these conditions, but given the generally high parasite loads, we assume that the average (infected) individual does not go through more than one to two divisions during a cycle.

SELECTION ASSAYS

Preparation of inocula

After 360 days (approximately 200 generations in the high-growth treatment), parasites from nine high-growth and nine low-growth replicate lines were arbitrarily chosen for the selection assay (the remaining six lines were not used because of logistic limitations). For the preparation of inocula, samples of each replicate line were grown to 100 mL, then concentrated in a volume of 5 mL by centrifugation; adding 1 mL of dimethyl sulfoxide (DMSO, Sigma, France) killed all paramecia. Additional centrifugation and replacement of supernatant with sterile Volvic washed out the DMSO. We then briefly vortexed the suspension together with several 2 mm glass beads (Sigma, France) to release infectious forms from dead paramecia. After determining the concentration of infectious forms, these freshly prepared inocula were stored at 5°C prior to use. The following experiments used hosts from the uninfected base population, maintained as a mass culture during the long-term experiment. Thus, only evolutionary responses in the parasite were considered in selection assay.

Infectivity

To measure the ability to establish an infection (= infectivity), we prepared two assay tubes (5 mL of medium with 2000 paramecia) per parasite selection line, to each of which we added about 3×10^4 infectious forms from the inoculum. Four control assay tubes received a mock inoculum, prepared from the uninfected base population (9 replicate lines \times 2 treatments \times 2 assay tubes + 4 controls = 40 assay tubes in total). Three days post inoculation, about 50 individuals from each assay tube were fixed with lacto-aceto-orcein (Görtz and Dieckmann 1980) and the proportion of infected individuals (= prevalence) determined at 1000 \times magnification (phase contrast).

Population measurements: High-growth assay

On day 3 post inoculation, each assay tube from the infectivity test was split into two new tubes. One subsample (4 mL) was placed in a 50 mL tube and the volume doubled with fresh medium (and split over several tubes) in 2-day intervals to obtain exponential growth. Because all assay tubes had started with the same density

Table 1. Overview of epidemiological and population dynamics in the high-growth and low-growth populations during first part of the experiment. Means and standard errors based on (occasional) routine measurements, and are therefore meant for illustrative purpose.

	High-growth treatment	Low-growth treatment	Comments
Parasite prevalence	Higher 83±4% (67–100%)	Lower 31±6% (10–50%)	Cycle 7 and cycles 21–23; initial: 25%
Host population size	Higher Control: 8331±723 Infected: 6435±617	Lower Control: 8033±578 Infected: 2538±408	Averaged over densities at end of each of first 10 growth cycles
Host population growth rate (per day)	Higher Control: 0.31±0.02 Infected: 0.30±0.01	Lower Control: 0.10±0.01 Infected: 0.04±0.01	($\log N_t - \log N_{t-1}$)/ Δt ; averaged over first 10 cycles
Investment in horizontal transmission	Higher 10±7%	Lower 67±11%	Proportion of individuals with massive production of infectious forms (nucleus size>10× of uninfected)
Concentration of infectious stages in the medium (×10 ⁴ /mL)	Higher 6.0±1.8	Lower 1.6±0.4	Cycle 24
Horizontal transmission	(Lower) 1.8±1.8%	(Higher) 5.3±2.2%	Proportion of newly (≤48 h) infected individuals; cycle 7

(2000 paramecia), we did not adjust densities in the 4-mL samples. On day 13 post inoculation, we combined all assay tubes from a given parasite selection line into a single flask and measured parasite prevalence, as described above, and host density, by counting the number of paramecia in 50 μ L samples under a dissecting microscope. (Note that the assay tubes were combined for use in another experiment. This precluded statistical comparison at the level of the selection line, but not at the treatment level).

Population measurements: Low-growth assay

On day 3, the other subsample (1 mL) from each assay tube was placed in a 5-mL tube and no medium added until day 9. This kept assay populations stationary, thereby allowing accumulation of bacterial loads. On days 3 and 9, host density was estimated in each tube. On day 9, we remeasured prevalence and recorded two parameters: parasite load [= length × width (μm^2) of the infected micronucleus] as an indicator of parasite within-host growth, as well as the proportion of individuals with micronuclei carrying newly produced infectious forms, as an indicator of parasite latency time (i.e., the timing of the onset of the production of infectious forms [Nidelet et al. 2009]). These assay populations were kept stationary during the following two weeks, with 500 μ L of fresh medium added on two occasions.

Singleton measurements

We used single individuals from the low-growth assay populations to measure virulence and the fidelity of vertical transmission. On

day 25 post inoculation, we selected up to 20 infected individuals from each of eight low-growth and six high-growth parasite selection lines under a dissecting microscope (for the remaining four selection lines, isolation of infected individuals was not possible because of low prevalence or total absence of infection); these individuals were then transferred to 500 μ L tubes, filled with 30 μ L of fresh medium. Half of the individuals were assigned to vertical transmissibility measurements, the other half to virulence measurements. Tubes were checked twice a day during the first three days, then daily for another six days. For estimates of vertical transmissibility, daughter cells were fixed after one cell division (in a few cases, a second division had already occurred) and their infection status was recorded. The proportion of infected daughter cells will be referred to as “fidelity of vertical transmission” or “vertical transmissibility” (i.e., the inverse of the curing rate). For estimates of virulence, we recorded the status of each tube (alive or dead) on day 9. For each group of tubes infected with parasites from a given selection line, virulence was calculated by subtracting the proportion of dead infected tubes from the proportion of dead uninfected control tubes. That is, negative values indicate a negative effect of the parasite.

STATISTICAL ANALYSES

Variation in proportions (infectivity, production of infectious nuclei, fidelity of vertical transmission, mortality of singleton lines) was analyzed by means of logistic regression and analysis of deviance (Schmid and Dolt 1994). Variation in (log-transformed)

host density was analyzed by mean of analysis of covariance (ANCOVA), with initial parasite prevalence in the assay tube as covariate; variation in (square-root transformed) estimates of parasite load by analysis of variance (ANOVA). Nested statistical models contained treatment (high- vs. low-growth) and parasite selection line (within treatment) as explanatory factors. To make treatment differences more comparable, effect sizes were calculated for each variable, as $[F/(F + df)]^{0.5}$ from F -values and denominator degrees of freedom (df) in the models (Cooper and Hedges 1994). Based on the means per parasite selection line, we examined pairwise correlations between traits across all selection lines pooled, as well as across selection lines within treatments (the latter complemented by ANCOVA). All analyses were carried out with the JMP (SAS 2003) and SAS (SAS 1996) statistical packages.

Results

Almost all analyses revealed significant differences between parasites from high- and low-growth treatments, with large effect sizes (0.60 ± 0.05 SE, range: 0.37–0.73; Figs. 3–5; Table 2). We found little differentiation among selection lines within treatments. Except for one trait (infectivity, $F_{1,17} = 6.24$, $P < 0.001$), the effect of “selection line” was nonsignificant in all analyses ($P > 0.1$) and explained little variance (variance components: $10 \pm 3\%$, range: 0–18%).

INFECTIVITY

For one low-growth parasite selection line, only half of the required dose had been available and both inoculated assay tubes remained uninfected. These two tubes were excluded from further analysis. Except for the two tubes from one high-growth line, all assay tubes became infected (range: 0–51%; mean: $23 \pm 2\%$).

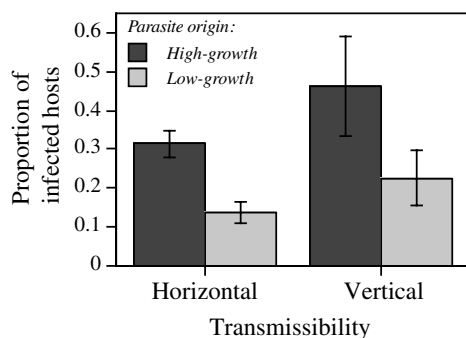


Figure 3. Means (\pm SE) of horizontal transmissibility (= infectivity after dose-controlled inoculation; left) and vertical transmissibility (= fidelity of vertical transmission; right), shown for parasites isolated from high-growth and low-growth treatments and tested on naive hosts. Means calculated from the averages per parasite selection line.

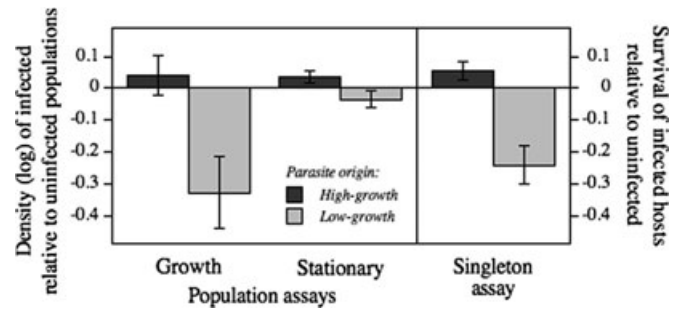


Figure 4. Effect of parasites from high-growth and low-growth treatments on assay population density and survival of single individuals. Left: Mean (\pm SE) density of infected assay populations after exponential growth; center: Mean (\pm SE) density of infected assay populations when kept at stationary phase; right: Mean (\pm SE) proportion of surviving infected hosts. For all three comparisons, values were standardized by subtracting density or survival of uninfected controls. Thus, negative values indicate a negative effect of the parasite. Means calculated from the averages per parasite selection line.

On average, parasites from the high-growth treatment were twice as infectious as those from the low-growth treatment ($F_{1,15} = 4.63$, $P = 0.048$; Fig. 3, left). All but one low-growth line produced fewer infections (5–18%) than all the other high-growth lines (19–47%, barring the extreme no-infection outlier; $F_{1,14} = 16.26$, $P = 0.001$).

HIGH-GROWTH ASSAY

At the end of 10-day period of population growth (day 13 post inoculation), the first secondary infections were observed, but parasite prevalences were still highly correlated with those measured on day 3 ($r = 0.72$, $n = 16$, $P = 0.001$). Prevalences in tubes infected with high-growth parasites were still higher than in those infected with low-growth parasites ($53 \pm 6\%$ vs.

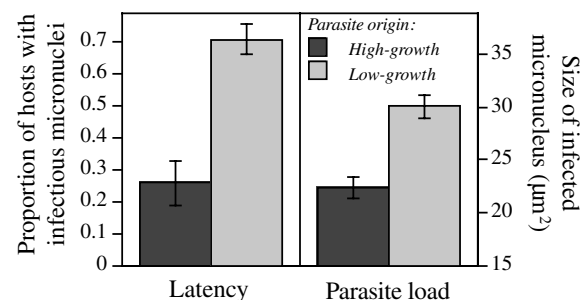


Figure 5. Mean (\pm SE) latency and parasite load of parasites isolated from high-growth and low-growth treatments and tested on naive hosts. Latency was taken as the proportion of hosts producing infectious forms on day 9 post inoculation (large values indicate short latency time). Parasite load was approximated by the size of the infected micronucleus on day 9 post inoculation. Means calculated from the averages per parasite selection line.

Table 2. Overview of characteristics of evolved parasites from high-growth and low-growth populations at the end of the long-term experiment (cycle 45).

	High-growth treatment	Low-growth treatment	Comments
Fidelity of vertical transmission	Higher 46.3±12.9%	Lower 22.6±7%	Figure 3; Percentage of vertically infected individuals
Virulence (host growth rate)	Lower 0.04±0.06	Higher −0.33±0.11	Figure 4; Log host population density relative to control
Virulence (host mortality)	Lower 4.5±2.8%	Higher 34.2±6%	Figure 4; Percentage of dead infected singleton tubes
Latency time	Longer 0.71±0.05 (≈9.72)	Shorter 0.26±0.07 (≈8.53)	Figure 5; Proportion of infectious individuals (≈time until 50% of population are infectious; days)
Parasite load (horizontal transmission stages)	Lower 522±51 (≈86)	Higher 948±66 (≈157)	Figure 5; Size of infected nucleus (μm ²) (≈number of infectious forms)
Horizontal per propagule transmissibility (infectivity)	Higher 8.4±1.4	Lower 4.2±0.9	Figure 3; Proportion infected individuals divided by inoculum dose (×10 ^{−6})

37 ± 5%; $F_{1,14} = 4.07$, $P = 0.063$). In both treatment groups, population densities tended to be negatively correlated with initial day-3 prevalences (ANCOVA: $F_{1,13} = 2.46$, $P = 0.141$), indicating a negative effect of infection on population growth. Despite the higher prevalences, densities in the populations infected with the high-growth parasites were significantly higher than in those infected with low-growth parasites (day 3: $F_{1,13} = 10.24$, $P = 0.007$; Fig. 4, left), suggesting lower virulence of high-growth parasites.

LOW-GROWTH ASSAY

In the low-growth assay populations, parasite prevalence on day 9 was highly correlated with that on day 3 ($r = 0.84$, $n = 32$, $P < 0.001$; treatment effect: $F_{1,14} = 9.05$, $P = 0.009$). Overall, 46% of the infected individuals had begun to produce infectious forms. The proportion of infectious micronuclei was nearly three times higher for low-growth parasites than for high-growth parasites ($F_{1,14} = 12.15$, $P = 0.004$; Fig. 5, left), indicating a substantially shorter latency time of low-growth parasites. Extrapolating from Nidelet et al. (2009), this corresponds to a difference of about 1 day until 50% of the individuals in an assay population begin to produce infectious forms (low-growth parasites: 8.53 days; high-growth parasites: 9.72 days). In addition, low-growth parasites also had larger parasite loads than high-growth parasites. This held for the comparison of the size of micronuclei carrying predominantly infectious forms ($F_{1,12} = 15.43$, $P = 0.002$), as well as for micronuclei carrying only reproductive or intermediate forms (both categories pooled: $F_{1,13} = 9.33$, $P = 0.009$; all three categories pooled: $F_{1,14} = 28.54$, $P < 0.001$; Fig. 5, right).

There was a relatively small, albeit significant, increase in host density between day 3 ($N = 539 \pm 9$) and day 9 (654 ± 19 ; $t_{37} = 5.21$, $P < 0.001$); densities on day 9 did not significantly differ between infected and uninfected assay tubes ($t_{36} = 0.14$, n.s.). Like in the high-growth assay, populations infected with high-growth parasites had a positive effect on population density and low-growth parasites a negative effect, although this difference was not statistically significant (after correcting for prevalence: $F_{1,14} = 2.23$, $P = 0.158$; Fig. 4, middle).

SINGLETONS

Virulence

Overall, 26% of the infected, but only 10% of the uninfected singleton tubes died during the nine days of the experiment. There also was a significant treatment effect ($F_{1,12} = 13.31$, $P = 0.003$) on host survival. More than a third of the individuals infected with low-growth parasites died, but only 4% of those infected with high-growth parasites (Fig. 4, right). Thus, although low-growth parasites increased mortality of their host, high-growth parasites were essentially avirulent or even slightly beneficial to their host.

Fidelity of vertical transmission

After discounting individuals that did not divide or where fixation failed, 53 replicates (from six high-growth and seven low-growth selection lines) remained for analysis. Of these, more than 70% showed incomplete vertical transmission, due to the large parasite loads interfering with the division of the nucleus. In several tubes, a second division had occurred before fixation, particularly

for individuals infected with high-growth parasites (37% vs. 9% for low-growth parasites: $\chi^2 = 6.53$, $P = 0.014$). Even when accounting for this additional division (by including division status as a cofactor in the analysis), we obtained a significant treatment effect on the fidelity of vertical transmission ($F_{1,11} = 8.36$, $P = 0.015$). Incomplete vertical transmission occurred in 91% of the individuals infected with the low-growth parasites, but only in 57% of those infected with high-growth parasites. On average, the fidelity of transmission (= proportion of infected offspring) was twice as high for high-growth parasites than for low-growth parasites (Fig. 3, right).

CORRELATIONS

Most of the 15 overall correlations, calculated across all selection lines (Table 3), showed medium (≥ 0.3) to large (≥ 0.5) effect sizes. Five of these correlations were significant and another three nearly so. Depending on the type of correction for multiple testing, only the two strongest correlations (Bonferroni method (Holm 1979)) or all five correlations (False Discovery Rate method (García

2004)) remained formally significant. Among the strongest correlations were those between latency time and parasite load, and between vertical and horizontal transmissibility (Table 3, Fig. 6). Furthermore, there was little evidence for consistent relationships between traits within treatments. Only five of the 30 within-treatment correlations were (marginally) significant. For the eight across-treatment correlations with the largest effect sizes, effects sizes of the corresponding within-treatment correlations were significantly smaller ($t_{22} = 2.53$, $P = 0.019$); only in two cases, the ANCOVAs detected significant within-treatment correlations of the same sign and similar magnitude as the overall correlation. In two other cases, the within-treatment and across-treatment relationships were of opposite sign (Table 3). Moreover, correlation coefficients were not significantly correlated between treatments ($r = 0.35$, $n = 15$, $P = 0.206$). These inconsistencies within treatments indicate that the overall correlations mainly reflect the differences in the treatment means. Figure 6 illustrates the across- and within-treatment relationships for four correlations with large effect sizes.

Table 3. Pearson correlation coefficients for six parasite traits, based on the means per parasite selection line. Correlations were calculated across selection lines within treatments (high-growth=HG, low-growth=LG) and for all selection lines pooled (overall=O). Within-treatment tests were complemented by analyses of covariance (ANCOVA), with treatment as a cofactor, one parasite trait as covariate (line), and another (column) as response variable. Significant correlations marked in bold (uncorrected for multiple testing). Note: ANCOVA can reveal significant relationships, while correlations within treatments are nonsignificant. $^+P < 0.1$; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.002$. ANCOVA: a main effect of covariate: $P < 0.05$; b covariate \times treatment interaction: $P < 0.1$. (1) Infectivity post dose-controlled inoculation; (2) Proportion of individuals producing infectious forms, day 9 post inoculation (large values indicate short latency time); (3) Size of the micronucleus of infected hosts, day 9; (4) Proportion of surviving single infected individuals, minus the survival of uninfected controls (i.e., negative values indicate negative effect of parasite); (5) Fidelity of vertical transmission (=proportion of infected daughter cells after one to two mitotic divisions of an infected host); (6) Population density after exponential growth (day 13), standardized by the density of uninfected populations (negative values indicate negative effect of parasite).

		Proportion of infectious nuclei (2)	Parasite Load (3)	Virulence (singletons) (4)	Vertical transmissibility (5)	Virulence (population) (6)
Horizontal transmissibility (1)	HG	+0.66 ^b	−0.11	−0.47	+0.92^{**a}	−0.57
	LG	−0.22 ^b	−0.55	−0.35	+0.22 ^a	−0.33
	O	−0.46 ⁺	−0.72^{***}	+0.50 ⁺	+0.72^{**}	+0.23
Proportion of infectious nuclei (2)	HG		+0.30 ^a	−0.35	+0.58 ^a	−0.32
	LG		+0.79^{**a}	−0.41	+0.76^{**a}	+0.50
	O		+0.83^{***}	−0.72^{**}	−0.03	−0.44 ⁺
Parasite load (3)	HG			+0.22	+0.14	+0.51 ^a
	LG			−0.24	+0.37	+0.54 ^a
	O			−0.70^{**}	−0.32	−0.26
Virulence (singletons) (4)	HG				−0.76	+0.90[*]
	LG				−0.49	−0.08
	O				+0.23	+0.38
Vertical transmissibility (5)	HG					−0.58 ^b
	LG					+0.55 ^b
	O					+0.25

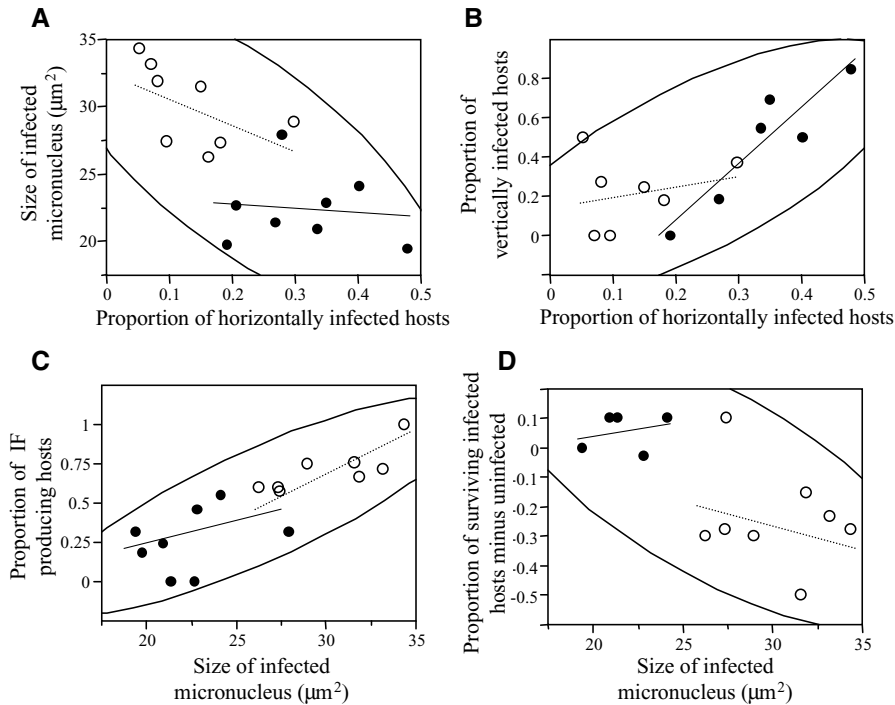


Figure 6. Relationships between life-history traits and virulence for parasites from the high-growth (closed circles) and the low-growth (open circles) treatments. (A) Horizontal transmissibility and parasite load; (B) Horizontal transmissibility and vertical transmissibility; (C) Parasite load and latency (= proportion of individuals producing infectious forms (IFs); higher values indicate shorter latency time); (D) Parasite load and virulence in the singleton assay (negative values indicate negative effect of the parasite). Each point is the mean for a single parasite selection line. Elliptical contours (95% probability) describe the correlation across all selection lines combined. Regression lines illustrate the relationships between the traits within treatments. In these four selected examples, overall correlations were significant (see Table 3). For precise definitions of traits, see Figures 3–5 and Table 3.

Discussion

After about 200 generations of culture, parasites from rapidly growing host populations (high-growth treatment) were more efficient at vertical transmission than parasites from stationary host populations (low-growth treatment). They had a higher fidelity of vertical transmission and interfered less with host reproduction (results overview in Table 2). This is consistent with the general prediction that increased opportunities for vertical transmission select for less-virulent parasites (Ewald 1987; Lipsitch et al. 1996; Day and Proulx 2004). Lower virulence was most likely due to lower within-host replication, which explains the longer latency time and lower number of transmission stages of the high-growth parasites. Similar trade-offs between components of vertical and horizontal transmissibility evolved in viral parasites (Bull et al. 1991; Messenger et al. 1999; Stewart et al. 2005). Here, the trade-off was alleviated by the higher per-propagule infectivity of the high-growth parasites.

RESPONSE TO SELECTION AND GENETIC CORRELATIONS

The substantial treatment differences indicate strong responses to selection. We believe that these result from *de novo* mutation

rather than preexisting variation in the ancestral parasite population. The three inocula founding the base population did not significantly differ in infectivity and virulence. Moreover, differences between treatment means for these traits in the final assay (≥ 2 standard deviation units) exceeded differences between the two most extreme ancestral inocula (< 1 SD units; see also Nidelet et al. 2009). We cannot, however, exclude the possibility that different genetic variants preexisted within the inocula.

Responses to selection in experimental evolution studies often result from few mutations of large effect (Bell 2008). If this also holds here, fixation of large-effect mutations may have caused the divergence of treatment means toward different local peaks in the adaptive landscape. Residual scatter around these means may then reflect experimental noise and/or standing genetic variation (from alleles of small effects) among replicate selection lines. Unlike in other studies (Ebert and Mangin 1997; Messenger et al. 1999), we found little evidence for correlations among selection lines within treatments and the significant overall correlations were mainly driven by the differences in treatment means (Table 3). This implies that phenotypic covariances were less constrained (by either selection or mutational correlations) in our experiment.

Because ancestral parasites were not available in the final assay, we cannot locate the starting point, from where populations diverged. However, it is conceivable that populations started out from (near) the low-growth treatment peak. This treatment is our standard long-term culture protocol, already experienced by the ancestral parasites for several years before this experiment. We therefore consider the low-growth environment as a control treatment that may not impose strong additional or new selection pressure on the parasite. Below, we discuss the selection pressures promoting the observed divergence of treatment means.

SELECTION PRESSURES ON VERTICAL AND HORIZONTAL TRANSMISSION COMPONENTS

With combined vertical and horizontal transmission, parasite prevalence in a population can reach 100%, if birth rates and vertical transmission are high relative to host mortality and virulence (Hochberg 1991; Lipsitch et al. 1996; Altizer and Augustine 1997). This is consistent with epidemiological characteristics of our high-growth treatment: high host division rates, reduced investment of the parasite in horizontal transmission, low virulence, and high prevalences (80–100%) (Fig. 2, Table 1; Kaltz and Koella 2003; Restif and Kaltz 2006). Under these conditions, theoretical models predict that a mutant strain can invade a resident population if it is less virulent (Lipsitch et al. 1996), or that a more vertically transmitted strain can invade a population of a more horizontally transmitted strain if its vertical transmission rate compensates the difference between the two horizontal transmission rates (Altizer and Augustine 1997). Parametrizing the model of Lipsitch et al. (1996) for our evolved lines, we find that a resident population of high-growth parasites cannot be invaded by low-growth parasites (see Supporting Information). Thus, higher fidelity of vertical transmission and lower virulence can be readily understood as adaptations to the high-growth treatment. Our findings also resemble those from a study on a meningitis virus in laboratory mice (Traub 1939, described in Lipsitch et al. (1996)).

However, caution should be taken when inferring past selection pressures from comparisons of the endpoint populations. The rationale of our treatments was to vary the rate of vertical transmission relative to the horizontal pathway. However, because we did not a priori impose constraints on either mode of transmission, we only have a limited picture of the epidemiology and the resulting selecting pressures in the two treatments (Table 1). Although it is safe to say that host division, and thus vertical transmission, was much more frequent in the high-growth treatment, the contribution of horizontal transmission is less clear. Indeed, measurements at earlier stages of the experiment indicated that horizontal transmission did occur in high-growth populations (Table 1): imperfect vertical transmission (Restif and Kaltz 2006) can constantly produce uninfected hosts, thereby allowing a baseline level of horizontal transmission, even at high parasite prevalence.

Furthermore, in our system multiple infection is possible, at least at early stages of infection (Fels et al. 2008). This violates a main assumption of Lipsitch et al.'s (1996) model, where multiple infection is precluded, thereby disfavoring investment in horizontal transmission at high parasite prevalence. If coinfection is possible, higher prevalences in the high-growth treatment may have increased rates of multiple infection. According to classical models on horizontal transmission, this may have increased within-host competition and thereby selected for higher virulence (Bull 1994; Frank 1996; Day and Proulx 2004). In principle, it is conceivable that coinfecting *Holospira* parasites compete for space and resources in the micronucleus and for access to horizontal transmission. More competitive and more virulent strains may replicate faster and thus bring forward conversion of reproductive into infectious forms (Nidelet et al. 2009; see also work on evolution of lysis time in phage (Bull et al. 2004)). However, we found that parasites from the high-growth treatment were less and not more virulent. This suggests that rates of multiple infection were not more elevated in this treatment, despite higher parasite prevalence, or that they were not sufficient to counter the strong selection acting on vertical transmissibility and concomitant lower virulence. Alternatively, multiple infection may have selected for more cooperative and less-virulent strains, sharing a common good (Brown et al. 2002): vertically infected host offspring. In this case, multiple infection would reinforce selection for lower virulence in the high-growth treatment.

BENDING THE TRADE-OFF: EVOLUTION OF A SUPER-GENERALIST?

Our results indicate that trade-offs between vertical and horizontal transmission are not invariable. Despite their higher vertical transmissibility, high-growth parasites also had a higher per-propagule horizontal transmissibility than low-growth parasites. This suggests that opposing selection pressures in the high-growth treatment were reconciled by improving the quality, rather than the quantity, of infectious forms. In fact, vertical and horizontal transmissibility were even positively correlated across high-growth parasite populations (Fig. 6B), and rough estimations suggest comparable total horizontal transmission capacities of high- and low-growth parasites (quantity \times quality of infectious forms; see Supporting Information). Thus, high-growth parasites seem to have reduced their virulence without compromising their capacity of horizontal transmission.

Why had such super-generalists not already evolved during the four years of culture (under low-growth conditions) prior to this one-year experiment? A simple explanation may be that shorter generation time and larger population size in the high-growth treatment speeded up evolution. It is also possible that selection on per-propagule infectivity requires concomitant selection on the vertical component (i.e., selection for reduced numbers

of infectious forms), which only occurs in the high-growth treatment. One may also speculate whether higher levels of multiple infection in this treatment facilitate bacterial recombination and thereby the assembly of novel advantageous mutations.

Recall, however, that our assay was based on whole-population samples. Populations showing a generalist strategy may in fact be composed of coexisting specialists (Barrett et al. 2005). Indeed, under certain conditions, theory predicts coexistence of vertical and horizontal transmission specialists (Lipsitch et al. 1996; Altizer and Augustine 1997; Ferdy and Godelle 2005). Testing for this possibility would have required sampling of individual parasite genotypes and is therefore beyond the scope of this study.

Finally, although host and parasite were allowed to coevolve, here we only considered parasite evolution, by doing the assays with naïve hosts. However, traits such as virulence may be determined by the genetic identity of both host and parasite. Such shared control can alter predictions about the evolution of virulence (Restif and Koella 2003). Thus, evolutionary changes in our experiment may not only reflect the relative importance of vertical versus horizontal transmission, but also (co)evolutionary change in life history or resistance in the host. Experiments are under way testing evolved parasites on evolved hosts from both treatments.

ADAPTATION THROUGH DEBILITATION?

With vertical transmission, within-host effective population size can be low and therefore genetic drift during transmission bottlenecks may cause accumulation of deleterious mutations. Thus, lower within-host growth and lower virulence of vertically transmitted parasites may result from stochastic loss of more virulent genotypes (Bergstrom et al. 1999; Elena et al. 2001). However, we believe that this is an unlikely explanation of our results, because vertical bottleneck size (≈ 50 – 100 bacteria per daughter cell) was large compared to predicted critical levels (Bergstrom et al. 1999). Moreover, some baseline horizontal transmission may already be sufficient to counter genetic drift within lineages.

GEOGRAPHIC MOSAIC

Central to the concept of the Geographic Mosaic of Coevolution (Thompson 1999, 2006) is the “selection mosaic”: spatial variation in fitness functions determining reciprocal selection (Gomulkiewicz et al. 2007). One possible driver of such mosaics is environmental heterogeneity (Hochberg and van Baalen 1998; Jessup and Bohannan 2008; Lopez-Pascua and Buckling 2008). Our treatments represent two classical ecological scenarios, characterizing disturbed versus constant environments that impose fundamentally different selection regimes on the host (r vs. K , Gadgil and Solbrig 1972). Here, the environmental variation experienced by the host promoted the evolution of distinct

life-history strategies in the parasite. However, this selection mosaic is not necessarily evolutionarily stable. If high-growth parasites are indeed both less virulent and equally infectious (see above), they may be able to invade populations of low-growth parasites. This would be an example of “roundabout selection” (MacLean and Bell 2002), where low-virulence (or even mutualist) genotypes do not evolve directly, but invade the population after originating in another environment (Michalakis et al. 1992). Ideally, hypotheses about invasibility should be tested in competition experiments (e.g., Bull et al. 1991).

Host evolution remains to be investigated. This concerns effects of the two treatments on the evolution of resistance or tolerance, but also the potential for coadaptation. High rates of vertical transmission may reinforce coevolution between host and parasite within a given clonal line of descent and lead to a higher degree of local adaptation or even mutual dependency in the high-growth populations.

Conclusions

We found that variation in host growth conditions affected the evolution of parasite life history and virulence. This highlights the potential role of ecological and epidemiological conditions as key determinants of host–parasite coevolution (Thompson 2006). Our results further confirmed basic theoretical predictions about the evolution of virulence of parasites with vertical and horizontal transmission (Ewald 1987; Lipsitch et al. 1996). It was one particular feature of our experiment that we did not a priori force the parasite into the vertical or horizontal mode. This seems to have created more complex responses to selection than anticipated by theoretical models. Thus, the experimental evolution approach allows us to give more degrees of freedom to our study systems, compared to, for example, artificial selection. Although this sometimes limits clear-cut interpretation of underlying selective forces (Ebert and Mangin 1997; Hochberg 1998), it makes this approach a valuable hypothesis-building tool.

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