

Experimental Evolution of a Trypanosome Parasite of Bumblebees and its Implications for Infection Success and Host Immune Response

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Abstract Selection on basic growth properties of parasites may have many consequences for parasite traits, infection outcome, or host responses to infection. It is known that genotypes (strains) of the trypanosome parasite of bumblebees *Crithidia bombi* vary widely in their growth rates in their natural host, *Bombus terrestris*, as well as when cultured in medium. To test for changes in growth rates and their consequences, we here experimentally evolved six strains of *C. bombi* for fast and slow growth under controlled conditions in culture medium. Subsequently, we infected the evolved lines in live host and found that lines selected for slow growth attained higher infection intensity in the live bumblebee than those evolved for fast growth, whilst the immune response of the host was the same to both kinds of lines. These results fit the expectation that attenuation through rapid adaptation to a different environment, the culture medium, makes the parasite less successful in its next host. Selection for fast growth therefore does not necessarily lead to higher parasite success or more transmission. Hence, insect

trypanosome pathogens can be attenuated by experimental evolution in the culture; this could inform important aspects of host-parasite evolution and perhaps vaccine development.

Keywords Experimental evolution · Growth rate · Trade-off · Attenuation · *Bombus* · *Crithidia* · Trypanosome · Gene expression

Introduction

Inside their host, microparasites, such as viruses, bacteria, and protozoans, multiply in cell numbers to eventually gain transmission and be able to infect a next one. This multiplication is reflected in the growth rate of the infecting parasite population and can be measured with the infection intensity—the number of cells present in the host—at any one time. In turn, within-host parasite growth rate, for short, is of great importance in many ways. For example, a fast growing population of parasites extracts more resources from the host, thereby probably inflicting more damage. Yet, the host may respond more quickly or more strongly to such growth. Also, fast growing parasite populations may achieve higher transmission rates and thus have higher success. These considerations form the basic elements of the evolutionary theory of virulence, which stipulates that faster growth causes greater virulence, i.e. damage to the host, but yields higher transmission rates as more cells are produced that can infect a new host (Alizon et al. 2009; Anderson and May 1982; Doumayrou et al. 2012; Ewald 1983; Williams et al. 2014). Naturally, parasite population growth rate varies with host, with parasite species or strain, and depends on environmental conditions, but in every case is a key element that deserves study.

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Evidence to support assumptions of the evolutionary theory of virulence exist, e.g. (Mackinnon and Read 1999), but despite the rich theoretical efforts, the empirical knowledge on within-host parasite growth rates and how it responds to changing conditions is still limited. Addressing these questions using experimental evolution protocols, such as serial passaging, holds promise (Ebert 1998; Kawecki et al. 2012). In fact, serial passaging, a method borrowed from vaccine development, is a powerful method whereby parasites are propagated under defined and controlled conditions. This allows the parasite to evolve and adapt to a given environment either with living hosts or, alternatively, in culture. Experimental evolution has previously demonstrated that, for example, selection for horizontal transmission can lead to a higher within-host growth rate of the parasite and thus to higher virulence (Ebert 1998), as assumed with the evolutionary theory of virulence. In this study we aimed at testing whether trypanosomes—a group of important parasites for many organisms, including humans—can be evolved for different growth rates under a controlled setting in culture. Informed by the evolutionary theory of virulence, we further tested whether evolved parasite growth rates are reflected in differences of infection success in live hosts, and whether the host itself responds differently to these infections. We expect that parasites, which have been evolved towards fast growth, will also achieve higher infection intensities in live hosts. This is suggested by a simple numerical arguments, but it is actually not the case that fast parasite growth in culture always translates into higher infection intensities in the living host, because host defenses affect the parasite population and—in multiple infections—the co-infecting strains compete among each other (Ulrich and Schmid-Hempel 2012). Similarly, by numerical arguments and the obvious associated threat, a faster growing parasite population should elicit a higher defense response by the host; this is also assumed in more explicit models (Antia et al. 1994). Again, this expectation is somehow contradicted by studies of immune gene expression in our model system, where the generally most infective strains elicit the weakest rather than the strongest response (Barribeau et al. 2014). Hence, we here test whether expectations that are compatible with the evolutionary theory of virulence are observed or whether alternative processes are present.

In our model system the host, *B. terrestris*, the common European buff-tailed bumblebee, is naturally infected by the intestinal gut trypanosomatid *C. bombi* (Trypanosomatidae) (Schmid-Hempel 2001). The system has several practical advantages such as the fact that the parasites can be cloned and kept in culture, and that workers of the same colony represent a similar host genetic background. Moreover, bumblebees are important pollinators that are an important study model in the current pollinator crisis.

The parasite, *C. bombi*, infects when the bee ingests parasite cells, which then multiply in numbers in the host's hindgut. When infected, bumblebee queens have a seriously reduced colony success and thus lower fitness (Brown et al. 2003). In the field, transmission occurs via flowers that are visited by infected and uninfected bees (Durrer and Schmid-Hempel 1994). The parasite can infect all colony castes and is often present at high prevalence in natural populations (Schmid-Hempel 2001). Furthermore, a high degree of genotypic diversity among *C. bombi* infections in nature exists (Schmid-Hempel and Reber Funk 2004; Tognazzo et al. 2012) and it has been shown that growth rates within infected hosts vary among these different genotypes, which are here called 'strains' (Ulrich et al. 2011; Ulrich and Schmid-Hempel 2012). This is also the case when growing *C. bombi* in culture (see Fig. S1, Supplementary Material). Here, we evolved six different strains of *C. bombi* for either fast or slow growth in culture medium and tested their success as well as the host's response when infected into live workers of *B. terrestris*. We did not evolve the parasites in live hosts as previously done by (Yourth and Schmid-Hempel 2006) because the number of worker generations is limited. More importantly, the host background has a major influence on the infections and shows very strong host genotype background—parasite strain interaction that would confound the results in serious ways.

Materials and Methods

Insects and Parasites

The parasite, *C. bombi*, was obtained from isolates (faeces) of naturally infected queens collected in Northern Switzerland in the springs of 2008 and 2012. The parasite cells were sorted into single cells and then maintained as a range of clones (genotypes) in medium according to the method described by (Salathé and Schmid-Hempel 2012). Six different parasite genotypes ("strains") with similar and medium-range growth rates were arbitrarily chosen to be part of the experiments. The six strains used in this study are labeled with their corresponding project tag code as nos. 12.246, 12.444, 8.161, 8.261, 8.134, and 8.075.

For the later infection experiments (second part of study), we arbitrarily selected five healthy laboratory colonies of *B. terrestris* that had been started from queens collected in spring 2013 from a single population in Neunforn, Northern Switzerland. The bumblebee colonies were kept under red light at a temperature of 28 °C with pollen and sugar water (ApiInvert[®], Südzucker, Ochsenfurt, Germany) provided ad libitum.

Experimental Procedure

This study was performed in two parts: (1) experimental evolution for growth rate of *C. bombi* in culture, and (2) in vivo infection of bumblebees with the evolved *Crithidia* strains.

Experimental Evolution

We cultivated the six strains as clones of *C. bombi* in separate 96-well plates (Sarstedt). The wells of the marginal rows of the plate were filled with sterile dH₂O (in total, 40 wells with dH₂O), in order to avoid condensation of the plate while placed in the incubator. The remaining 56 wells were used for the cell cultures; each well contained 80 µl of medium (liquid “FP medium”, (Salathé and Schmid-Hempel 2012)) plus 20 µl of added cell suspension (inoculum). Plates were kept at 27 °C and 3 % CO₂. These standard conditions were kept for all cultures described below.

Before the actual start of the experiment, we first propagated the six different *C. bombi* strains in culture. Each strain was cultivated in its own plate; this plate was defined as the “ancestor strain plate” for this strain, containing many replicate clonal cell cultures. On this ancestor strain plate, we let the cells grow in their wells for 3 days and then measured the optical density (OD) of the cell suspensions in each well. OD measurements closely correlate with actual cell numbers (see Figs. S2, S3, Supplementary Material); wells with the highest ODs represent those cell populations that have the highest density of cells in the standardized volume. At the same time, ODs measured after the same time interval (3 days of growth) define the growth rates of the cell populations.

Based on their ODs we picked cell populations from the 56 wells containing cultures and assigned them to two treatment groups. Treatment ‘Fast’: we selected those four wells with the highest ODs in the plate; treatment ‘Slow’: we selected those four wells with the lowest ODs in the plate. In addition, for group ‘Control’: we randomly picked four wells from the remaining wells. Then, the cells from all wells selected for *Fast*, *Slow*, or *Control* were pooled together and the cell density of this mix standardized to 80'000 cells/ml (using a Cellometer Auto M10, Nexcelon Bioscience). This mix was large enough to be subsequently split into four (two) replicate inocula that were used to start the actual experimental evolution protocol. Hence, for every strain we thereby gained four replicates for each of the two treatment groups, *Fast* and *Slow*, and two replicates for the *Control* group. Each replicate was kept separated its own well plate for the duration of the experimental evolution procedure. Hence, barring the marginal wells, we kept 56 wells with the cell cultures for each replicate line,

group and strain. In other words, each of the six strains was present in three treatment groups, with four, four and two replicates each (a total of 10 replicate lines per strain), and each of this ten replicate lines was cultivated in 56 wells of a plate (Fig. 1).

We started the evolution experiment at time T₀, and used the same basic procedures as just described to transfer the parasite populations to new sets of plates at regular intervals. In particular, for every transfer (passage) we used inocula standardized to 80,000 cells/ml to seed the wells of a new plate. Subsequently, we let the cells grow for 3 days (72 h) under standard conditions in all 56 available wells. Then, in each of the four replicate plates seeded, for example, with cells from treatment *Fast* we selected the four wells (out of the 56) with the highest OD values to be propagated for this treatment, discarding all other wells. As before, the cell populations of these four wells were pooled, standardized, and then transferred to seed a next plate in the same manner. Correspondingly, we selected the four wells with the lowest OD values to be propagated in the four replicate plates of treatment *Slow*; four randomly picked wells in the two replicate plates of the *Control* group. This procedure was the same for the replicates of each of the six strains. In total, and for each strain, we therefore measured ODs for 224 wells (56 wells per plate, and 4 replicates), selecting 16 wells (4 wells per plate, and 4 replicates) to be propagated within the treatments *Fast* and *Slow*, respectively. For *Control* 112 wells (2 replicates used) were measured and 8 wells propagated. In each case, the replicate lines (four for *Fast*, *Slow*; two for *Control*) were kept separate for the duration of the experiment; hence, pooling only applied to the four selected wells from each plate/replicate line.

Before each cell population was transferred to a new plate, the cells were checked under the microscope for cell motility and cell size, as well as for contamination. After every second transfer, a sample of cells for each strain and treatment was stored for later use by putting them in 70 % glycerol and frozen at –80 °C using Nalgene Mr. Frosty Cryo freezing containers (Thermo Scientific) to be preserved for the second part of the experiment. This procedure was repeated until twelve transfers (time T₁₂) or 6 weeks of experimental selection had been reached; this amounts to somewhat over 120 generations of *C. bombi* (Salathé and Schmid-Hempel 2012).

Testing the Evolved Strains

In the second part of our study, the two evolved groups (*Fast*, *Slow*) within each of the six strains were used for infections into the live host. For this purpose, each sample freeze-stored at the end of the experimental evolution period was put in fresh culture medium to start its re-growth. Growth rates

(ODs) were measured again for each strain and replicate line to ensure that growth rates (fast/slow) were still in line with their assigned treatment. For the infection, the five bumblebee colonies were used as described above. Individual workers were age-controlled by selecting workers at the age between 3 and 4 days after eclosion. They were taken out from the colony and kept individually in clear Perspex boxes with sugar water and pollen provided ad libitum. After 3 days of isolation, the bees were starved for 2–3 h prior to infection. Then, each worker bee was individually infected with 10,000 parasite cells suspended in 10 μ l of 50 % sugar water. Each worker was checked that the full infection cocktail was taken up. Bees that did not imbibe the cocktail within 30 min were excluded from the experiment. Workers were then put back into their box. Nine days post-infection the bees were sacrificed by either freezing at -20°C (for infection quantification) or by flash-freezing in liquid nitrogen (for gene expression analysis). In total, 36 workers were used from each of the five colonies. For each strain, we used six workers for the test.

Infection Quantification

For the quantification of infections, workers were dissected, their hind gut removed and homogenized in 100 μ l Ringer solution. DNA was extracted from each individual worker using a QIAGEN protocol for purification of total DNA from animal tissues (DNeasy 96 protocol according to manufacturer's instructions). Infection intensity was then measured using quantitative real-time PCR (qPCR) according to the protocol by (Ulrich et al. 2011). All qPCRs were performed using Fast EvaGreen Master Mix (Biotium, Hayward, CA, USA) on an ABI 7500 Real-time PCR system (Applied Biosystems, Rotkreuz, Switzerland). The thermal profile used in these qPCRs were the same as described in the protocol of (Ulrich et al. 2011). Each biological sample was run in technical duplicates with distilled H_2O used as negative control and bumblebee DNA as the positive control. The number of cells present in workers (infection intensity) was estimated by first measuring qPCRs for a dilution series of DNA extractions from a known number of *C. bombi* cells. Then, we used this correlation to estimate cell numbers from the qPCR data (Figs. S2, S3, Supplementary Material). The same dilution series was included for each qPCR run in order to standardize our quantifications across plates.

RNA Preparation and Quantification of Gene Expression

Immune gene expression was measured by following an optimized version of the protocol suggested by (Brunner

et al. 2012). RNA was extracted from all abdomens of flash-frozen bees using an optimized Trizol-extraction protocol. The abdomen of each bee sample was first homogenized by putting the sample in 1 ml tubes containing small ceramic balls plus trizol, and homogenized using an Omni Bead Ruptor 24 homogenizer (OMNI international). Then, 450 μ l of the supernatant was transferred to an Eppendorf tube containing 40 μ l BCP (1-Bromo-3-chloropropane, a phase separation reagent). The contents were mixed well, incubated for 10 min at room temperature and centrifuged for 10 min at a speed of 11,500 rpm. Later, 180 μ l of the transparent phase was transferred to a next RNase-free Eppendorf tube containing 225 μ l isopropanol. The tube was carefully mixed and incubated for another 7 min at room temperature, followed by centrifuging 10 min at 14,000 rpm. To improve RNA quality this step was repeated twice. The supernatant was then completely removed and the pellet air-dried for at least 5 min. 50 μ l of DEPC RNase-free water was added to the tube with our extracted RNA. RNA integrity for all samples was confirmed using a 2100 Bioanalyzer (Agilent Technologies), and quantity as well as purity of the extracted RNA was evaluated using the Nanodrop 8000 (ThermoScientific). A ratio of between 1.6 and 2.05 at wavelengths 260/280 nm was accepted as pure RNA. Once the purity of the RNA was confirmed, we reverse-transcribed 0.4 μ g of RNA from each sample (quantitect reverse transcription kits, Qiagen) and amplified the cDNA product with intron-specific primers to confirm the absence of genomic DNA. Expression profiles were measured on a Fluidigm chip (96.96 Dynamic Array; Fluidigm Corp.) with EvaGreen as a DNA binding dye (Biotium). Three technical replicates were measured for each sample and the average of each triplicate was used as the raw expression value (Ct).

Based on previous studies and the genomic analysis of bumble bee immune defenses (Barribeau et al. 2015; Barribeau and Schmid-Hempel 2013), twenty-seven candidate genes were chosen that are representatives of the response by the insect immune system, including genes associated with the major canonical insect immune pathways: Toll, Imd, and JAK/STAT. The genes included in this study were (in alphabetical order): *abaecin*, *apidaecin*, *apolipophorin*, *basket*, *BGRP1*, *BGRP2*, *catsup*, *CYP4GII*, *defensin*, *domeless*, *ferritin*, *hemomucin*, *hopscotch*, *hymenoptaecin*, *jafrac1*, *lysozyme*, *myd88*, *PGRP-LC*, *PGRP-S3*, *PPO*, *PRX5*, *punch*, *relish*, *SPN27a*, *TEP-A*, *transferrin*, and *vitellogenin*. Our reference genes were *AK*, *PLA2*, *RPL13*, and *ef.1 fa2*. Previously published primers were used except for *vitellogenin* and *relish* (Brunner et al. 2012; Li et al. 2010; Schlüns et al. 2010).

Analyses and Statistics

Growth Rates During Experimental Evolution

All statistical analyses for this section were done with IBM SPSS Statistics V20 for Mac. A general linear mixed model was used to test for differences in growth rate between the start (T0) and the end (T12) of the experimental evolution period, with strain considered a random factor, transfer period (T0, T12) and treatment group (*Fast*, *Slow*) as fixed factors. The model was also used to test for each strain separately and all six strains together while looking for significant changes during the whole experiment. The post hoc tests used the Bonferroni method to correct for multiple testing.

Success in Live Hosts

For each sample we calculated the mean value of infection intensity (cells/ μl) and infection success (yes/no), as measured with qPCR. We then tested whether treatment group (*Fast*, *Slow*) had an effect on infection intensity and success (a binary variable) in the live host using a generalized linear mixed model. For the analysis of infection intensity, a general linear model (two-way ANOVA) was used. Data were log-transformed in order to reach normal distribution. Treatment group (*Fast*, *Slow*) and strain identity were both considered as fixed factors, colony as random factor.

Gene Expression Upon Infection

Gene expression of our candidate genes was evaluated with the Ct-values normalized against the geometric mean of our four reference genes, yielding the dCt-values, following the procedure of (Barribeau and Schmid-Hempel 2013). These values were further transformed to improve normality using the Yeo-Johnson transformation. Gene expression levels were then analyzed using a multivariate variance analysis (MANOVA). Colony, strain identities and treatment group were first level effects; in addition, treatment group was nested in strain as a secondary effect. All expression data were analyzed with R (2.15.2, R Core Team 2015).

Results

Experimental Evolution: Selection on Growth Rate

We successfully evolved different growth rates according to treatment for all six strains (which started as clones). In particular, there was a significant change in growth rate between T0 (start) and T12 (end) within the treatment

groups *Fast* ($F_{1,46} = 371.54$, $P < 0.001$), or *Slow* ($F_{1,46} = 20.21$, $P < 0.001$). By contrast, the *Control* group in all six strains showed no significant change in growth rates between start and end of experiment ($F_{1,22} = 0.096$, $P = 0.759$; Fig. 2; Table 1). Figure 3 shows the mean growth rate of all six *C. bombi* strains over the course of time (twelve transfers). The lines evolved significantly faster in the *Fast* group reaching a peak growth rate at T6 (21 days).

Infection Outcome

Infection Success

Evolved lines from the *Fast* and *Slow* treatment groups did not show any significant difference in their success (yes/no) at infecting live workers from various colonies ($F_{1,529} = 0.684$, $P = 0.409$) (Fig. 4). By contrast, the identity (genotype) of the strain, however, had a significant effect on infection success ($F_{5,529} = 12.788$, $P < 0.001$). That is, strains— independent of treatment group—differed in their infection success; there was no significant treatment \times strain interaction ($F_{5,524} = 1.886$, $P = 0.09$).

Infection Intensity

Infection intensity (cells/ μl in infected bees) differed among treatment groups ($F_{1,178} = 7.036$, $P = 0.009$) and according to strain ($F_{5,178} = 9.269$, $P < 0.001$). In addition, there was a significant interaction effect of strain with treatment group on infection intensity ($F_{4,178} = 3.387$, $P = 0.011$). Among all six strains, somewhat unexpectedly, lines from the *Slow* group reached higher infection intensity in the bees, except for strain no. 12.444. The lines from the *Fast* group clearly generated lower infection intensities, and one strain (no. 8.075) was not able to infect at all (Fig. 5).

Gene Expression in Hosts

Gene expression levels among bees showed a very strong colony effect (Table 2). We did not see any significant main effect of treatment group ($F_{29,90} = 0.867$, $P = 0.657$) or strain ($F_{28,94} = 1.232$, $P = 0.226$) on the gene expression profiles. When strain differences are ignored such that treatment group is instead considered a nested factor within strain, there is a significant effect of treatment ($F_{28,94} = 1.620$, $P = 0.045$) (Table 2). However, any effect seems to be marginal. The overriding strong colony effect can also be seen when each gene's expression is analyzed on its own. In nearly all genes, excluding *defensin*, *hymenoptaecin*, and *lysozyme*, there was a significant difference in

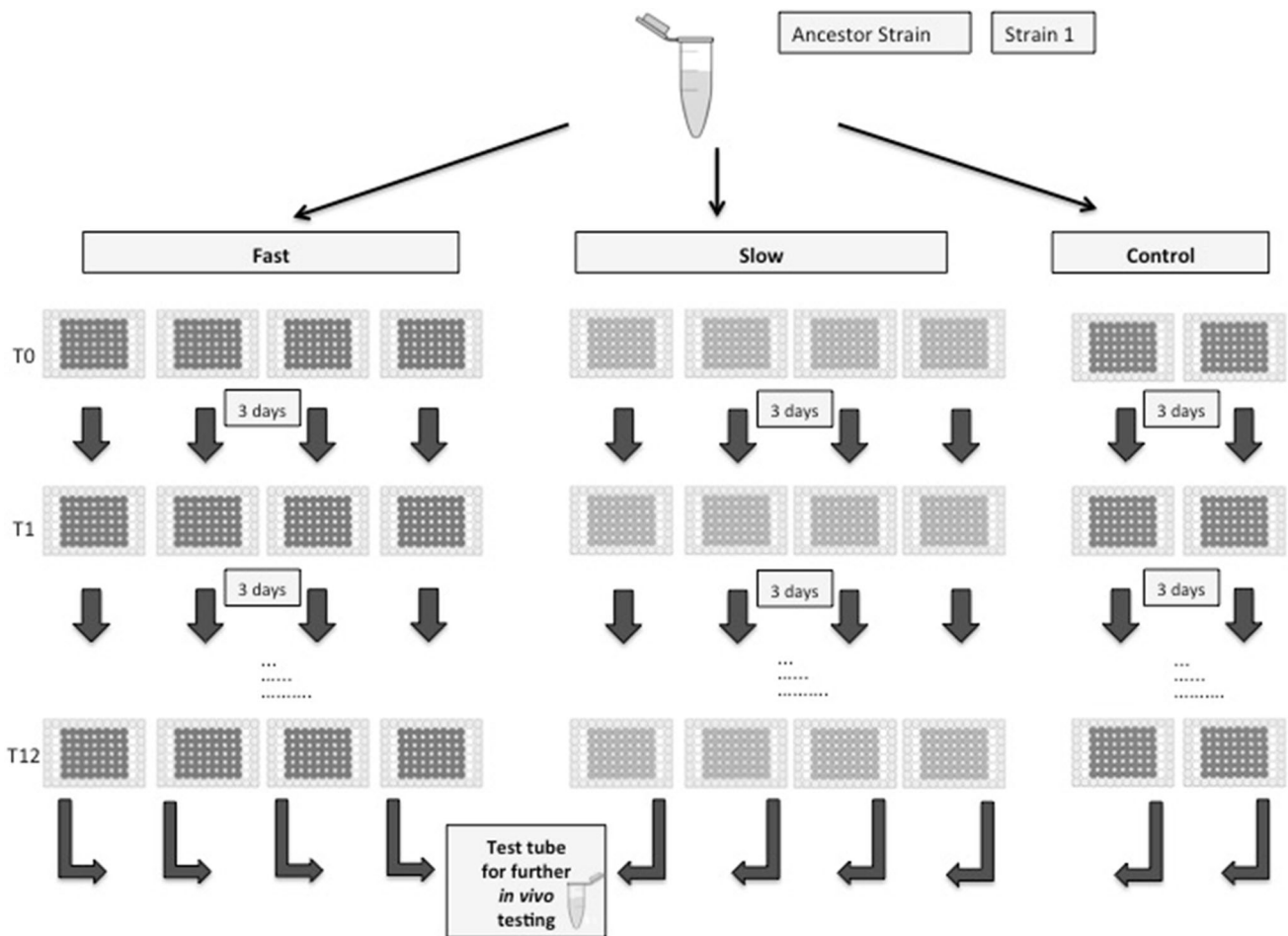


Fig. 1 Experimental design of the study. Six *C. bombi* strains were used throughout the experiment. Shown is the protocol for a given strain (“Strain 1”). First, before the start of the experiment, each strain was propagated in medium (shown as “Ancestor Strain”), such that four replicates for each of the two treatment groups (*Fast*, *Slow*), and two additional replicates for the *Control* group could be generated at time T0 (transfer to time 0), i.e. at the start of the experimental evolution process. Hence, the experiment subsequently was run for 10

replicate within each strain (a total of 60 replicates for all six strains of the experiment). Between transfers, the cells were grown in plates kept in the incubator at 27 °C. Every 3 days (72 h) the populations were selected and transferred to new plates as described in the text. The experimental evolution process was continued until twelve transfers were obtained (T12); after a further 3 days, the experiment was terminated. Probes of the evolved lines were stored for further use

the gene expression between colonies with fast and slow growing parasite infections (Table 3).

Discussion

Our results show, first of all, that experimental evolution of trypanosomes in culture is possible, using the bumblebee parasite *C. bombi*. This introduces a new experimental tool for the study of this important group of pathogens (Norman et al. 2015). In particular, we successfully selected for fast and slow growing lines after approximately 30 weeks of evolution in the culture (Fig. 3), whereas the control (randomly selected) group did not show any difference in growth rate during the experimental period. As is often the case for selection experiments, the effect was larger in one

treatment group (here, selecting for higher growth rate) than in the other (slower growth).

The evolved differences made it possible for us to test whether infection outcome and infection intensity in live hosts are affected by the evolved growth rate. On one hand, infection success (infection or no infection) did not differ between strains selected for fast or slow growth. On the other, however, infection intensity (parasite cell load) was affected by this treatment group. In particular, strains of the parasite selected to grow slower reached higher infection intensities in the host than those selected to grow fast. This was unexpected at first sight, as evolved higher growth rate should translate into higher infection intensities after a given period of time. It can be interpreted as showing that fast growth (corresponding to within-host replication of the parasite) in a given selection environment is associated

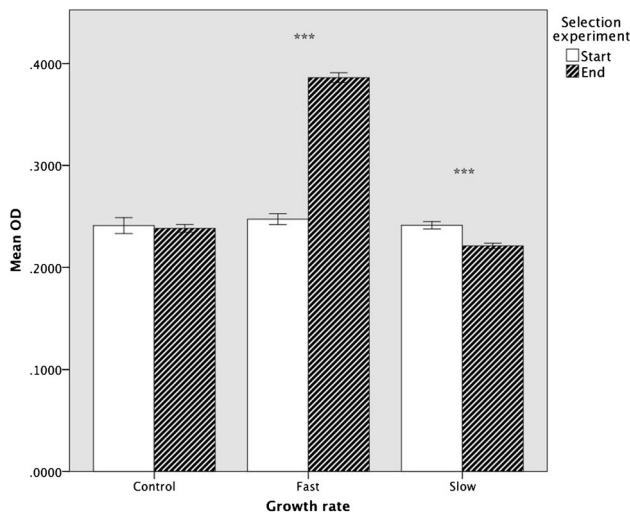


Fig. 2 Average difference in growth rate (optical density; mean OD ± SE) between the start (T0) and end of the experiment (T12; see Fig. 1) for all six *C. bombi* strains. The change is significant for strains under *Fast* and *Slow* treatment (***P* < 0.001), whereas *Control* did not show any significant change

with low success (measured by infection intensity) in a next host. This could mean a loss of virulence, which is supposed to correlate with parasite cell load. Hence, it

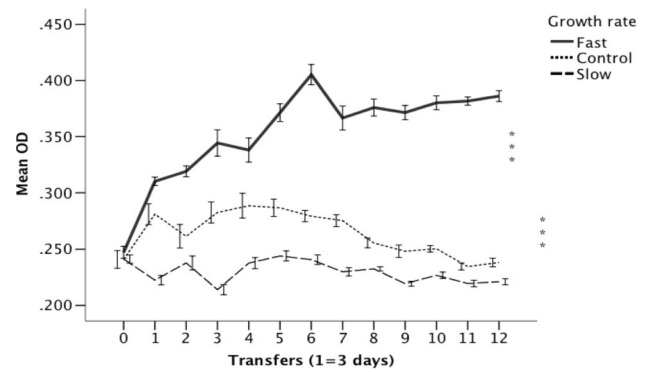


Fig. 3 Evolution of growth rates (optical density; mean OD ± SE) for all six *C. bombi* strains and different treatment groups during the experiment. Each time point is a transfer time T0–T12, separated by 3 days. Strains under *Fast* and *Slow* selection regimens grew significantly faster than the *Control* group and were different from their ancestors at time T0 (***P* < 0.001)

suggests a trade-off between growth rate in the current environment (i.e. a host) and success in the next. Moreover, better growth in the medium was associated with lower success in the next host, i.e. a phenomenon of attenuation.

It is yet unclear why such a trade-off occurs, but the most likely process is that selection for fast growth also implies adaptation of these lines to the conditions of the

Table 1 Summary table for mean growth rate of each of the six strains at the start (Transfer 0) and the end of experiment (Transfer 12), and for each selection regime (*Fast*, *Slow*, *Control*)

| Strain ID | Growth rate ^a | Transfer 0 Mean OD ± SE | Transfer 12 Mean OD ± SE | <i>P</i> value ^b |
|-----------------|--------------------------|----------------------------|-----------------------------|-----------------------------|
| 12.246 | Control | 0.25 ± 0.017 | 0.237 ± 0.017 | 0.615 |
| 12.246 | Fast | 0.261 ± 0.012 | 0.37 ± 0.012 | <0.01 |
| 12.246 | Slow | 0.248 ± 0.012 | 0.221 ± 0.012 | <0.05 |
| 12.444 | Control | 0.241 ± 0.012 | 0.226 ± 0.012 | 0.641 |
| 12.444 | Fast | 0.276 ± 0.009 | 0.383 ± 0.009 | <0.001 |
| 12.444 | Slow | 0.25 ± 0.009 | 0.213 ± 0.009 | <0.05 |
| 8.161 | Control | 0.256 ± 0.008 | 0.241 ± 0.008 | 0.514 |
| 8.161 | Fast | 0.256 ± 0.006 | 0.400 ± 0.006 | <0.001 |
| 8.161 | Slow | 0.252 ± 0.006 | 0.221 ± 0.006 | <0.01 |
| 8.261 | Control | 0.255 ± 0.014 | 0.246 ± 0.014 | 0.725 |
| 8.261 | Fast | 0.233 ± 0.01 | 0.398 ± 0.01 | <0.001 |
| 8.261 | Slow | 0.24 ± 0.01 | 0.232 ± 0.01 | 0.231 |
| 8.134 | Control | 0.223 ± 0.009 | 0.256 ± 0.009 | 0.328 |
| 8.134 | Fast | 0.225 ± 0.006 | 0.395 ± 0.006 | <0.001 |
| 8.134 | Slow | 0.222 ± 0.006 | 0.235 ± 0.006 | 0.092 |
| 8.075 | Control | 0.220 ± 0.015 | 0.223 ± 0.015 | 0.838 |
| 8.075 | Fast | 0.233 ± 0.011 | 0.372 ± 0.011 | <0.001 |
| 8.075 | Slow | 0.236 ± 0.011 | 0.203 ± 0.011 | <0.05 |
| All six strains | Control | 0.241 ± 0.007 | 0.238 ± 0.003 | 0.76 |
| All six strains | Fast | 0.247 ± 0.005 | 0.386 ± 0.004 | <0.001 |
| All six strains | Slow | 0.241 ± 0.003 | 0.221 ± 0.002 | <0.001 |

^a Growth rate is measured as optical density (OD) ± standard error (SE) of four replicates (for fast and slow group), respectively two replicates (control group)

^b Bold font indicates a statistical significant change in mean OD between start and end of experiment

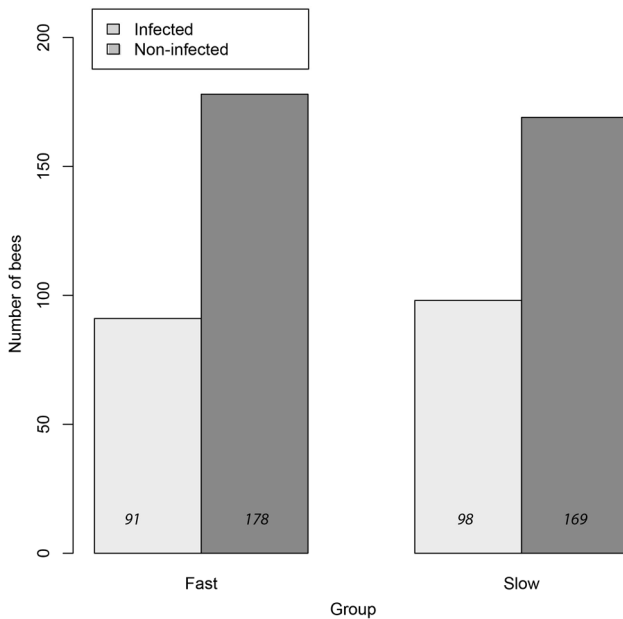


Fig. 4 Overall infection success (infected, non-infected) in live bees for all six *C. bombi* strains, selected for *Fast* or *Slow* growth. No significant difference in relative infection success (infected bees/total of bees) was found. Bars are number of individuals

selection experiment, i.e. the culture medium. As a result, these lines must have lost a capacity to successfully replicate in another environment such as a live host. Therefore, the effect of adaptation to the selection medium attenuated the lines in their natural host, and this effect seems to override the effect of generally faster growth rates. This fits the general outcome of serial passage

experiments (Ebert 1998). Moreover, the finding is similar to earlier findings in this system, where *C. bombi* was serially passed through workers within colonies and then infected into unrelated colonies (Yourth and Schmid-Hempel 2006). The serially passed parasites showed a decreased infection in the workers of unrelated colonies. The molecular changes responsible for the altered infectivity are not known here but have been scrutinized in other cases. For example, (Domenech and Reed 2009) demonstrated that the *Mycobacterium tuberculosis* bacteria showed an extensive loss of the cell wall lipid (PDIM) when cultured for several weeks in medium. The presence of this lipid is known to be essential for the virulence of *M. tuberculosis*.

An alternative explanation is that fast growing lines induce a faster and thus more effective immune response, as compared to the more slowly growing lines, and so suffer from reduced eventual infection intensity. However, our gene expression results suggested that selection regime had no effect on the response elicited in the host, as the gene expression levels did not vary between treatment groups or, at the very last, only had a very weak effect. Therefore, the results do not support this second hypothesis. A very strong colony effect, however, was observed, as there is a significant difference in immune responses among bumblebee colonies. This phenomenon has been also shown in previous works demonstrating strong variation in gene expression among insect populations, colonies and workers (Barribeau et al. 2014; Barribeau and Schmid-Hempel 2013; Brunner et al. 2012). Yet, the absence of effect of strain per se seems to contradict the results of

Fig. 5 Average infection intensity (mean ± S.E.) of six different *C. bombi* strains (labels on x axis), evolved as *Fast* and *Slow* growing lines. All strains, except strain no. 12.444, reached higher infection intensity when selected for *Slow* growth. The fast growing line from Strain no. 8.075 selected for *Fast* growth did not infect at all (* $P < 0.05$; *** $P < 0.001$)

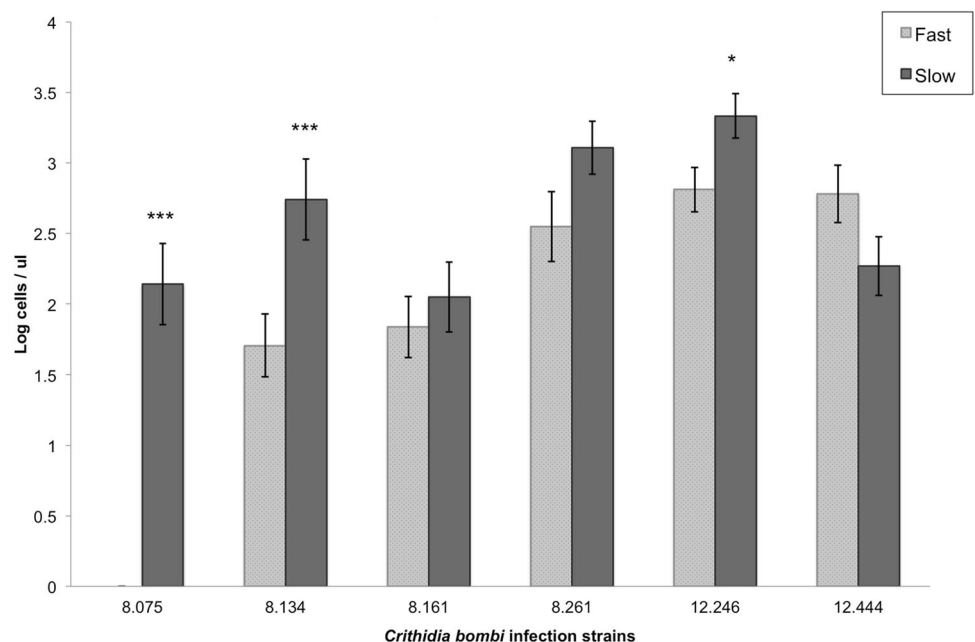


Table 2 MANOVA results for the expression of all selected genes

| Multivariate effects full data set | | | | | |
|------------------------------------|----|--------------------|---------|----------|------------------|
| Factor | Df | Roy's largest root | F value | Error DF | P value |
| Colony | 28 | 6.929 | 22.766 | 92 | <0.001 |
| Strain | 28 | 0.367 | 1.232 | 94 | 0.226 |
| Treatment | 28 | 0.270 | 0.867 | 90 | 0.657 |
| Strain (treatment) | 28 | 0.483 | 1.620 | 94 | 0.045 |

Treatment nested within strain

^a The full data set of the transformed dCT values was used to perform the MANOVA^b Effects that are statistically significant ($P < 0.05$) are highlighted in boldface**Table 3** ANOVA results for differential gene expression between colonies with fast and slow growing parasite infections

| Class | Gene | Putative gene function | Df | MS | F | P value ^a |
|--------------|-------------------|-------------------------------------|----|---------|--------|----------------------|
| Receptor | BGRP1 | Recognition (Toll) | 3 | 5.166 | 19.219 | <0.001 |
| | BGRP2 | Recognition (Toll) | 3 | 0.372 | 7.16 | <0.001 |
| | PGRP-LC | Recognition (Imd) | 3 | 1.219 | 19.057 | <0.001 |
| | PGRP-S3 | Recognition (Toll) | 3 | 8.416 | 32.135 | <0.001 |
| | Hemomucin | Potential recognition receptor | 3 | 2.021 | 26.396 | <0.001 |
| | Domeless | Potential cytokine receptor | 3 | 3.067 | 28.702 | <0.001 |
| Signalling | Relish | Signaling (Imd) | 3 | 0.774 | 33.154 | <0.001 |
| | Hopscotch | Signalling (Jak/stat) | 3 | 1.556 | 59.901 | <0.001 |
| | Myd88 | Adapter protein | 3 | 0.439 | 34.995 | <0.001 |
| | Basket | Signalling (JNK) | 3 | 1.75 | 32.846 | <0.001 |
| Effector | Abaecin | Antimicrobial peptide | 3 | 31.138 | 2.788 | 0.0440 |
| | Apidaecin | Antimicrobial peptide | 3 | 12.195 | 17.639 | <0.001 |
| | Defensin | Antimicrobial peptide | 3 | 23.947 | 1.971 | 0.1220 |
| | Hymenoptaecin | Antimicrobial peptide | 3 | 4.589 | 0.127 | 0.9440 |
| | TEP-A | Effector (Jak/stat) | 3 | 0.629 | 42.204 | <0.001 |
| | Lysozyme3 | Effector, bacteriolytic | 3 | 0.057 | 2.618 | 0.0540 |
| | Transferrin | Ironbinding, antibacterial | 3 | 32.416 | 10.973 | <0.001 |
| | Ferritin | Iron transportation | 3 | 33.801 | 17.492 | <0.001 |
| Melanisation | Punch | Enzyme, melanin synthesis | 3 | 0.991 | 44.181 | <0.001 |
| | Catsup | Enzyme, melanin synthesis | 3 | 28.775 | 18.234 | <0.001 |
| | SPN27a | Serin protease inhibitor (PPO) | 3 | 0.669 | 36.575 | <0.001 |
| | PP0 | Prophenoloxidase, melanin synthesis | 3 | 2.535 | 9.289 | <0.001 |
| ROS | PRX5 | Peroxiredoxin, ROS regulation | 3 | 13.392 | 74.951 | <0.001 |
| | Jafrac1 | Peroxiredoxin, ROS regulation | 3 | 43.19 | 16.625 | <0.001 |
| Metabolic | Apolipophorin III | Fat storage | 3 | 291.431 | 23.531 | <0.001 |
| | Cyp4gII | Cytochrome P450, stress response | 3 | 263.552 | 20.872 | <0.001 |
| | Vitellogenin | Endocrinological functions | 3 | 86.774 | 5.936 | <0.001 |

MANOVA results for differential gene expression between colonies with fast and slow growing parasite infections. Twenty-seven genes were tested which were known to be involved in three main immune pathways of the insect system: Toll, Imd, Jak/stat. Gene functions are associated with insect immunity, metabolism and stress response. All gene expression results were normalized against four common housekeeping genes of *B. terrestris*

^a Significant effects are in bold

(Barribeau et al. 2014), where an effect of strains, host background, and their interaction was found. We have no ready explanation for this divergence and can, for the time

being, only attribute it to the overriding differences among colonies in the current experiment.

We here show that it is possible to attenuate trypanosomatids infecting insects, too. Applied to the field

situation, strains of *C. bombi* should similarly adapt quickly to a given host colony environment and thus explain part of the observed genetic diversity found in natural populations (Salathé and Schmid-Hempel 2011; Schmid-Hempel and Reber Funk 2004; Tognazzo et al. 2012). The possibility of attenuation has many repercussions for controlling trypanosomes as, for example, attenuating the virulence of the parasite for use in vaccination and drug development. As it is widely known, many vaccines, such as Theiler's yellow fever vaccine, are composed of attenuated parasites (Fiuza et al. 2015; Keitany et al. 2014; Pompa-Mera et al. 2014), and the method is a general tool in vaccine development (Ebert 1998; Makela 2000). In fact, several studies report on the successful use of attenuated trypanosomes such as *Leishmania* for vaccine development (Daneshvar et al. 2003, 2014), or African trypanosomes for testing the host's response towards infection (Daneshvar et al. 2014).

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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