Experimental test of the consequences of host-parasite coevolution of the nematode *Caenorhabditis elegans* and its microparasite *Bacillus thuringiensis*

Dissertation

in fulfillment of the requirements for the degree "Dr. rer. nat." of the
Faculty of Mathematics and Natural Sciences
at Kiel University

submitted by

LEILA EL MASRI

Kiel, 2012

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Second referee: Prof. Dr. Nico K. Michiels

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Experimental test of the consequences of host-parasite coevolution of the nematode *Caenorhabditis elegans* and its microparasite *Bacillus thuringiensis*

Leila El Masri

This thesis is based on the following manuscripts:

I **L Masri**, D Laehnemann, PS Günther, S Prahl, NK Michiels, H Schulenburg **An experimental test of the consequences of host-parasite coevolution,** unpublished manuscript

II **L Masri**, R Schulte, N Timmermeyer, S Thanisch, L Crummenerl, G Jansen, NK Michiels, H Schulenburg **A Stumbling Red Queen: Host-Parasite Coevolution handicapped by Feeble Males,** unpublished manuscript

III A Branca, A Sheppard, **L Masri**, M Saebelfeld, H Liesegang, E Brzuszkiewicz, R Daniel, J Kurtz, P Rosenstiel, E Bornberg-Bauer, H Schulenburg **Ecological functional genomics of the model pathogen *Bacillus thuringiensis* during experimental coevolution with its nematode host *Caenorhabditis elegans***, unpublished manuscript

Contribution of authors

L Masri: Development of original ideas, establishment of the study, the general data interpretation and providing material (**all chapters**), optimization of the experimental evolution protocol and development of new phenotypic measurements (**I and II**), performance of experiments (**I and II**), supervision of experiments (**I and II**), data analysis (**I and II**), and preparation of chapters for publications (**I and II**)

H Schulenburg: Original ideas, development of the original setup, supervision of experiments, data analysis and interpretation, and preparation of chapters for publication (**all chapters**).

NK Michiels: Scientific support and discussions (**I and II)**

D Laehnemann: Experimental help in the coevolution experiment and the related phenotypic measurements (**I**)

PS Günther: Experimental help in the coevolution experiment and the related phenotypic measurements (**I**), development of the infection load assay (**I**)

S Prahl: Performance of experiments and characterization of biofilm formation (**I**)

R Schulte: Development of ideas, data collection and analysis (**II**)

N Timmermeyer: Performance of experiments and development of the experimental set-up (**II**)

S Thanisch: Performance of experiments, data collection, data analysis and development of the experimental set-up (**II**)

G Jansen: Supervision of experiments (**II**)

L Crummenerl: Performance of experiments, data collection, data analysis and development of the experimental set-up (**II**)

A Branca: Conceiving the study, performance of the population genomics analysis, contribution to data interpretation and preparation of chapter for publication (**III**)

A Sheppard: Conceiving the study, performance of the screen of toxin genes and chromosomal codY gene, generation of genome sequence data used as reference of the analysis, contribution to data interpretation and preparation of chapter for publication (**III**)

M Saebelfeld: Performance of the screen of toxin genes and chromosomal codY gene, contribution to the data interpretation (**III**)

H Liesegang: Conceiving the study, generation of the genome sequence data used as reference for the analysis and contribution to the data interpretation (**III**)

E Brzuszkiewicz: Conceiving the study, generation of the genome sequence data used as reference for the analysis and contribution to the data interpretation (**III**)

R Daniel: Contribution to the design of particular aspects of the study and to the data interpretation (**III**)

J Kurtz: Contribution to the design of particular aspects of the study and to the data interpretation (**III**)

P Rosenstiel: Conceiving the study, generation of genome sequence data used as reference for the analysis and contribution to the data analysis and interpretation (**III**)

E Bornberg-Bauer: Conceiving the study and contribution to the data interpretation (**II**)

Summary

Host-parasite coevolution, or the evolution of host defence and parasite counter defence is predicted to associate with high selection dynamics that are crucial for the evolution of a number of biological processes, such as fitness traits related to both antagonists and the mechanisms generating fast genetic changes. The main objective of my PhD thesis is to enhance our understanding of host-parasite coevolution as a major selective force. Hence I addressed the complex set of the predicted evolutionary consequences that are unique to host-parasite coevolution, at both the phenotypic and molecular level, for both antagonists, and across time. I used an experimental evolution approach - under controlled laboratory conditions - using the nematode *Caenorhabditis elegans* as a model host, and the bacterium *Bacillus thuringiensis* as a model parasite. I optimized the selection protocol and multiple phenotypic measurements to compare the differences in the evolutionary outcomes between coevolution, one-sided evolution, and control conditions. After 28 host generations, I found that coevolution (i) causes reciprocal changes in both host resistance and pathogen virulence, (ii) affects their life history trade-offs, and (iii) produces patterns that are clearly different from one-sided adaptation and control conditions (Chapter I). In general the consequences of host-parasite coevolution were more pronounced in the parasite except for the patterns of temporal adaptations (Chapter I). Moreover, my results gave insights into the role of males and outcrossing in the evolution of the studied host-parasite interactions. I found an opposing interference of two selective forces that act either on the inter-species level (i.e., the high selective pressure that the antagonists exert on each other; Red Queen theory) or on the intra-species level (i.e., the differences in immunity among host genders; Bateman's principle of immunity). Males showed higher pathogen susceptibility than hermaphrodites, thus limiting but not abolishing the potential for outcrossing and recombination for fast host adaptation (Chapter II). Finally, for the parasite, we identified genetic changes in: (i) genotype frequency, (ii) the presence or combination of cry toxins, and (iii) the presence and frequency of single nucleotide polymorphisms (SNPs). The molecular analysis was done across three time points over all replicate populations. We found that the overall parasite evolution is dominated by clonal selection followed or combined with the spread of individual mutations (Chapter III).

Zusammenfassung

Wirt-Parasit-Koevolution ist die wechselseitige Evolution von Abwehrmechanismen des Wirtes und Pathogenitätsmechanismen seines Parasiten. Diese koevolutionären Interaktionen verursachen in der Regel starke Selektionsdynamiken, welche entscheidend für die Evolution biologischer Prozesse sind, inklusive derer, die evolutionäre Fitness der beiden Kontrahenten ausmachen oder auch zu schnellen genetischen Veränderungen führen. Das Hauptziel meiner Promotionsarbeit ist das bessere Verständnis von Wirt-Parasit Koevolution als zentrale einflussreiche Selektionskraft. Dementsprechend habe ich den Komplex der evolutionären Konsequenzen, die speziell für Wirt-Parasit Koevolution vorhergesagt werden, untersucht. Bei diesen Untersuchungen habe ich gezielt phänotypische als auch molekulare Veränderungen bei beiden Interaktionspartnern im Verlaufe der Zeit berücksichtigt. Ich habe den Ansatz der experimentellen Evolution unter kontrollierten Laborbedingungen eingesetzt und als Modelsystem die Nematoden *Caenorhabditis elegans* als Wirt und das Bakterium *Bacillus thuringiensis* als Parasit gewählt. Ich habe zunächst die Methoden für das Selektionsexperiment und mehrere phänotypische Messungen optimiert, um die Unterschiede der evolutionären Ergebnisse zwischen Koevolution, einseitiger Evolution und Kontrollbedingungen zu vergleichen. Nach 28 Wirtsgenerationen fand ich heraus, dass (i) wechselseitige Änderungen in Wirtresistenz und Parasitenvirulenz stattgefunden haben, welche (ii) die lebensgeschichtlichen Kompromisse beeinflussen und (iii) sich in ihrer Struktur klar von denen der einseitigen Adaptation und Kontrollbedingungen unterscheidet (Kapitel 1) . Generell waren die Konsequenzen der Wirt-Parasit-Koevolution deutlicher beim Parasiten,abgesehen von der zeitlichen Anpassung (Kapitel 1).

Des weiteren erlaubten meine Ergebnisse einen Einblick in die Rolle der Männchen und der Reproduktion durch Auskreuzung während der Wirt-Parasit Koevolution. Hierbei habe ich zwei gegensätzlich wirkende Selektionskräfte nachgewiesen, die zum einen zwischen Arten (z.B.reziproke Selektionskräfte zwischen den Antagonisten der Wirt-Parasit Interaktion; Rote-Königin-Theorie) und zum anderen innerhalb der Arten wirken (z. B. zwischen den Geschlechtern des Wirts; Bateman's Prinzip der Immunität). Männchen zeigten dabei eine höhere Anfälligkeit für Pathogene als Hermaphroditen, was die Möglichkeit für Auskreuzen und Rekombination zur

schnellen Anpassung beim Wirts einschränkt aber nicht verhindert (Kapitel 2). Schließlich haben wir bei den Parasiten genetische Veränderungen nachgewiesen, insbesondere im Hinblick auf (i) Genotypfrequenzen, (ii) das Vorhandensein oder die Kombination von *cry* Toxinen und (iii) die Existenz und Frequenz von *single nucleotide polymorphisms* (SNPs). Die molekulare Analyse wurde an drei Zeitpunkten für alle Populationsreplikate durchgeführt. (Kapitel 3). Die Ergebnisse zeigen, dass die Evolution der Parasiten durch klonale Selektion in Kombination mit der Ausbreitung von individuellen Mutationen geprägt ist.

Introduction

Consequences of host-parasite coevolution

Parasites are ubiquitous; they show high ability for rapid adaptation, reduce host fitness and impose selection for more resistant hosts, which in turn imposes selection for more virulent parasites. Thus parasites represent one of the strongest selective forces in evolution, and the long-term reciprocal evolution of host resistance and parasite virulence engage coevolving antagonists in an evolutionary arms race. The continuous counter adaptation of both antagonists is predicted to have manifold phenotypic and genetic consequences on both counterparts.

One of the direct responses of the long-term interaction is change in traits affecting host resistance and parasite pathogenicity. The continuous variation in value could result in two different coevolutionary dynamics (Woolhouse, Webster et al. 2002; Gandon, Buckling et al. 2008): (i) Fluctuating selection dynamics, where the host and parasite genotype frequencies oscillate through time and thus producing a pattern of temporally restricted adaptation (Buckling and Rainey 2002; Forde, Thompson et al. 2008) or (ii) Arms race dynamics where both counterparts continuously accumulate adaptive mutations and are hence expected to show a general increase in both host resistance and pathogen virulence (Decaestecker, Gaba et al. 2007; Gandon, Buckling et al. 2008; Gomez and Buckling 2011). The repeated cycles of parasite adaptation and host counter adaptation can result in patterns of local adaptation and the interaction between sympatric antagonists is expected to be more specific in comparison to allopatric locations. If local adaptation is found for only one of the antagonists, it is then expected to be ahead in the arms race (Buckling and Rainey 2002). Moreover, the continuous investment in resistance and virulence entails high costs due to the investment in effector molecules, leading to life-history tradeoffs (e.g. reproductive rate, competitive ability and change in body size (Forde, Thompson et al. 2008; Schmid-Hempel 2008; Schulenburg, Kurtz et al. 2009; Schulte, Makus et al. 2010). Host-parasite coevolution is also predicted to play a key role in increasing populations' genetic diversity, through the increase of recombination rates and mutations. Consequently, selection should favor diversity-generating mechanisms such as sexual reproduction and outcrossing. These mechanisms break up existing

linkage groups and generate new allele combination to which the antagonist is not or only poorly adapted.

What do evolution experiments tell us about host-parasite coevolution?

Recently, most experimental coevolution studies worked on microbial model organisms as a powerful system in the laboratory, profiting of their short generation time, large population sizes (favouring rapid evolutionary changes), cryopreservation, production of isogenic lines (thus the detection of changes due to mutations and selection), and the well-developed phenotypic and molecular methods (Buckling and Rainey 2002; Forde, Thompson et al. 2004; Brockhurst, Morgan et al. 2007; Forde, Beardmore et al. 2008; Kashiwagi and Yomo 2011; Meyer, Dobias et al. 2012). A well-investigated experimental system consist of bacterial hosts and their specific obligate killing viral phages, namely: *Escherichia coli* and various T-phages, (Bohannan 2000; Forde, Thompson et al. 2004; Forde, Thompson et al. 2008; Kashiwagi and Yomo 2011; Meyer, Dobias et al. 2012), and several Pseudomonas species and their specific phage parasites (Buckling and Rainey 2002; Brockhurst, Morgan et al. 2003; Brockhurst, Buckling et al. 2005; Brockhurst, Buckling et al. 2006; Buckling, Wei et al. 2006; Morgan and Buckling 2006; Brockhurst, Morgan et al. 2007; Gomez and Buckling 2011). The main results of these laboratory controlled coevolution experiments have shown:

(i) Patterns of coevolutionary dynamics and the related life history trade-offs: Bacterial resistance and phage infectivity was measured through time; in particular cross-infection experiments across time were conducted to detect the speed of coevolution between the interacting antagonists. Mainly it was found that the infectivity of phages from the past was lower than the infectivity of contemporaneous phages, and that the infectivity of phages from the future was higher than the contemporaneous ones. Coevolutinary selection has shown to be predominantly directional towards the evolution of generalists (Buckling and Rainey 2002; Lopes, Sucena et al. 2008; Gomez and Buckling 2011), and evidence of the related life-history trade-offs due to the increase in bacterial resistance was detected (Buckling, Wei et al. 2006).

(ii) Evolution of host diversity: In homogeneous environments, where diversity is

low, phage increased host diversity. The opposite was observed in heterogeneous environments. Phages reduced host diversity when diversity was high, because of a reduction in bacterial density, but increased diversity when it was low, because of coexistence and the competition of sensitive and resistant phenotypes (Buckling and Rainey 2002). Moreover, Pal et al demonstrated that coevolution with phages can drive the evolution of elevated mutation rates in bacterial populations (Pal, Macia et al. 2007).

(iii) Patterns of local adaptation: The analysis of the interaction between bacterial and phage clones across replicate populations, revealed patterns of local adaptation during which bacteria have shown to better resist phage from sympatric populations in comparison to allopatric ones (Brockhurst, Buckling et al. 2005; Morgan and Buckling 2006).

Gomez and Buckling, have taken the Bacteria-phage antagonistic coevolution to a different level. In their study, they developed a new experimental setup going from test tubes, to testing the relevance of natural environment on the dynamics of the coevolving interaction (Gomez and Buckling 2011). Their experimental approach of bacteria phage coevolution in a "controlled soil community" has shown that unlike in vitro, coevolution in the soil resulted in host more resistant to contemporary than past and future parasites. Hence a change from arms race dynamics in vitro, to negative frequency dependent selection in the soil, which can continue indefinitely. This observed negative frequency dependent selection is potentially due to the elevated fitness costs constraining the evolution of high levels of resistance in the soil (Gomez and Buckling 2011).

Similarly, the evolution of the antagonistic interaction was also studied in a variety of multicellular animal host systems, among others in the fruitlfly *Drosophila melanogaster* (Green, Kraaijeveld et al. 2000; Bangham, Obbard et al. 2007; Wilfert and Jiggins 2010), the crustacean waterflea D*aphnia magna* (Haag and Ebert 2004; Decaestecker, Gaba et al. 2007; Wolinska and Spaak 2009; Yin, Petrusek et al. 2012), the beetle *Tribolium castaneum* (Fischer and Schmid-Hempel 2005; Berenos, Schmid-Hempel et al. 2009), multiple snail species (Koskella and Lively 2007; Webster, Shrivastava et al. 2007; Berenos, Schmid-Hempel et al. 2009; Jokela, Dybdahl et al. 2009) and in the nematode *Caenorhabditis elegans* (Morran, Parmenter

et al. 2009; Schulte, Makus et al. 2010; Schulte, Makus et al. 2011). These studies provided an excellent empirical repertoire of the evolutionary consequences of the antagonistic host-parasite interaction in both the field and under laboratory controlled conditions. Nonetheless, none have been able to assess the processes that are unique to the coevolutionary interactions and their differences to "standard uni-directional selection" for both antagonists, at both phenotypic and molecular level, and across time. The lack of a complete experimental understanding of the consequences of this antagonistic interaction is likely due to two main reasons. First, most of the host-parasite systems benefit from analytical tools (i. e. phenotypic and molecular tools) for strictly one of the evolving antagonists. Second, it is difficult to disentangle in nature the consequences of host-parasite coevolution from other interfering factors since these additional interventions might select for different traits than those implicated in the evolutionary arms race.

In summary, the coevolutionary interactions between host and parasite have been predicted to be a fundamental selective force shaping the evolution of organisms and biological processes. However, complex empirical evidence for their importance is still scarce. Experimental coevolution under controlled laboratory conditions represents a promising approach to address this topic.

Enhancing the understanding of the role and consequences of these selective forces is the main focus of this PhD thesis. First, it requires the evaluation of the evolutionary consequences of host-parasite coevolution relative to "standard uni-directional" selection (where only one of the antagonists is able to adapt) and control evolution. For that purpose, a laboratory controlled evolution experiment should be performed to obtain adequate biological material for the subsequent phenotypic and molecular analysis. Second, it demands the performance of both broad and fine scale analysis of the biological material. The broad scale analysis done at the population level will serve to investigate the main differences between the coevolution and one-sided adaptation. The fine scale analysis done at the individual level genotype will identify genes and functional region responsible for the coevolutionary adaptation. These steps will evaluate in a single experiment, most of the predictions of the coevolutionary interaction, namely:

- Reciprocal change

- Changes in traits directly involved in the interaction: Host resistance and parasite pathogenicity
- Increased life-history trade-offs
- Patterns of temporal dynamics
- Increased diversity of molecular mechanisms
- Increased genetic diversity and diversity-generating mechanisms such as outcrossing rates.

The latter is predicted to be selectively favoured after host-parasite coevolution (i.e. sexual reproduction creates new genotype combinations, which help to escape from counter-adapting antagonist). Hence, since outcrossing depends on males, it should also selectively favour the presence of males, especially in species that can either reproduce by selfing or parthenogenetically.

The evolutionary importance of males in *Caenorhabditis elegans*

Caenorhabditis elegans has an androdioecious mating system composed of males and hermaphrodites (Brenner 1974; Cutter and Payseur 2003). Although *C. elegans* populations are primarily composed of hermaphrodites and reproduce via selfing, a low male frequency is maintained. In general, males are considered to be costly. To be produced, males require high resources and cannot produce offspring by themselves. Under equal conditions, if a selfed hermaphrodite produces the same number of offspring than a mated one and if all offspring have the same average fitness, the hermaphroditic population will produce twice as many hermaphrodites as the mated population. Hence the purely hermaphroditic population is expected to have a higher fitness relative to a population made of a mixture of males and hermaphrodites; and the population with males will have two folds of reproductive disadvantage compared to the purely hermaphroditic one (Bell 1982; Lively and Lloyd 1990; Anderson, Morran et al. 2010)

In addition to the costs of males, mating itself is associated with high costs, such as (i) the time and resources spent in mate search, (ii), direct physical damage, and (iii) potential transmission of sexual parasites (Lively and Lloyd 1990; Gems and Riddle 1996). When compared to their dioecious sister species *Ceanorhabditis remanei, C.*

elegans males perform worse. Opposite to *C. remanei*, *C. elegans* males are incapable of inducing female paralysis during mating (known as soporific effect) and hence have to mate with a mobile hermaphrodite. *C. elegans* hermaphrodites do not produce any sex pheromones making them even less attractive than *C. remanei* females. Finally, to make matters even worse, *C. elegans* hermaphrodites can eject male ejaculate and flee until their self-produced sperm is depleted (Barker 1994; Chasnov and Chow 2002; Chasnov, So et al. 2007; Garcia, LeBoeuf et al. 2007; Kleemann and Basolo 2007).

Therefore, we could expect natural selection to favour purely hermaphroditic populations and males to be eliminated from the androdioecious populations.

Then what role do males play in a hermaphroditic population?

In general, theories explaining male maintenance fall into two broad categories: mutation based models and ecological models. First, knowing that in *C. elegans* outcrossing is strictly possible between the two genders, males are thus considered as vehicles for outcrossing. Males prevent, in this mating system, the accumulation of mutations (both deleterious and advantageous) and their fixation in the population. They are considered to (i) reduce mutational load, (ii) limit the evolution towards homozygosity, and (iii) slow the consequences resulting from inbreeding depression. Second, from the ecological perspective, males or outcrossing enhance the adaptation to changing environmental conditions (Crow 1994; Hurst and Peck 1996). They increase the effectiveness of recombination; promote new adaptive mutations to arise and consequently breaking long-term linkage disequilibrium. Novel genotypes are then born through this process, which may help the populations to better adapt to environmentally changing conditions such as co-adapting parasites (Cutter and Payseur 2003; Cutter 2005; Morran, Parmenter et al. 2009; Anderson, Morran et al. 2010; Morran, Ohdera et al. 2010).

In the most common laboratory strain N2 (also known as Bristol strain), male frequency is generally maintained at non-disjunction meiosis rates, varying between 0.1 and 0.4% of the population (Hodgkin, Horvitz et al. 1979; Chasnov and Chow 2002; Cutter and Payseur 2003; Teotonio, Manoel et al. 2006). Males in this broadly used strain are known to mate less efficiently in comparison to other studied populations potentially due to their long term laboratory adaptation leading to genetic and behavioral changes. Male frequencies and outcrossing rates have been described

to vary between laboratory maintained strains and populations, likely due to the differences in their male mating related phenotypes including: (i) male sperm size, (ii) rate of male loss from the population, (iii) mating ability, (iv) rates of males in the population produced via nondisjunction (LaMunyon and Ward 2002; Teotonio, Manoel et al. 2006; Wegewitz, Schulenburg et al. 2008; Anderson, Morran et al. 2010)) A broader study of the natural ecology and natural variation of *C. elegans* populations was documented by Barrière and Félix (2005 and 2007). Barrière and Félix isolated thousands of *C. elegans* worms from 10 different locations throughout France and only found four males (Barriere and Felix 2005; Barriere and Felix 2007). Based on heterozygosity data, the outcrossing rates in these European populations were estimated to vary between 1.3 to 1.7% and thus outcrossing occurred rarely in contrast to the Californian populations where the outcrossing frequencies were estimated to be 22% (Barriere and Felix 2005; Sivasundar and Hey 2005; Barriere and Felix 2007). Surprisingly, Barriere and Felix (2007) found that in the largest and most stable isolated population, the genetic linkage between the same alleles persisted over 3 years despite a detectable rate of outcrossing. This suggests that selection acts against the progeny of a recombination event (Barriere and Felix 2007). The observed difference between degrees of heterozygocity and linkage disequilibrium could be explained by either (i) outbreeding depression from which *C. elegans* are known to suffer (Dolgin, Charlesworth et al. 2007) or (ii) the incompatibility between strains (Seidel, Rockman et al. 2008).

Several studies experimentally looked at the importance of males and outcrossing in this androdioecious species. Cutter (2005) tested the theory of maintenance of sex if the deleterious mutation rate is sufficiently high and thus the influence of mutation on the evolution of obligate outcrossing (Cutter 2005). In his experiment, he genetically transformed some hermaphrodites into functional females by a simple genetic manipulation and exposed these populations to high and low mutation rates. After 21 generations, he found that in all populations, regardless of the mutation loads hermaphrodites took over the populations; while outcrossing rates and female frequencies were higher in the high mutation treatment. Similarly Morran et al (Morran, Cappy et al. 2009; Morran, Parmenter et al. 2009; Morran, Ohdera et al. 2010; Morran, Schmidt et al. 2011) looked at the impact of stressful conditions on male production. First, they found that starvation elevated male frequencies and increased outcrossing rates and thus facultative outcrossing in *C. elegans* may

facilitate adaptation under stress. Second, they studied the changes in outcrossing rates under environmentally changing conditions, such as under high mutational loads and pathogen coevolution (Morran, Parmenter et al. 2009; Morran, Schmidt et al. 2011). In both studies, they found that under increased mutation rates and during adaptation to novel environments outcrossing is favoured. Changes in male frequencies to adapt to novel stressful environments such as pesticide resistance were also studied in Lopes et al (2008). In this study, no costs of resistance were found after 20 generations; however a reduction in outcrossing rate was observed in the adapted population due to lower male mobility and thus lower encounter rates (Lopes, Sucena et al. 2008).

Hence, the importance of males in the hermaphroditic *C. elegans* is mainly explained by one of the predictions of the Red-Queen hypothesis, proposing that coevolving parasites selectively favours outcrossing in the host.

Model organisms

Caenorhabditis elegans as a model organism

In nature, *C. elegans* is a worldwide distributed free living soil nematode inhabiting microbe-rich environments associated with compost and rotting fruits (Barriere and Felix 2007). During its lifetime, of approximately three weeks at 25 ºC, *C. elegans* has a high likelihood of encountering divers pathogenic microorganisms and, in turn, activating immune responses which seem to be mediated by the p38 MAPK, the JNK MAPK, and also the insulin-like cascades (Huffman, Abrami et al. 2004; Boehnisch, Wong et al. 2011; Kao, Los et al. 2011; Wang, Nakad et al. 2012).

In the laboratory the nematode can easily be maintained and manipulated. It bears many advantages as a model organism in multiple biological and medical fields. Its short generation time of 2-3 days at 20 ºC, its cryopreservation at -80 ºC in glycerol (Stiernagle 2006), the established behavioral and molecular assays, and the repertoire of phenotypic assays looking at the interaction between *C. elegans* and numerous micro-pathogens made it a suitable model organism for long term evolutionary experiments in general and for the study of the coevolutionary consequences of the host-parasite interaction in particular (Schulte, Makus et al. 2010; Schulte, Hasert et al. 2011; Schulte, Makus et al. 2011).

Moreover, *C. elegans* is a well-studied androdioecious model organism, having two genders: hermaphrodites and males (Brenner 1974) (Figure 1a and 1b). *C. elegans* hermaphrodites have two copies of the X chromosome and produce sperm, which is stored for self-fertilization. The males carry a single X chromosome as their only sex chromosome (Nigon and Dougherty 1949; Brenner 1974). Outcrossing is only possible between a male and a hermaphrodite, leading to the complete cross-fertilization of the eggs, because male sperm outcompete hermaphrodite sperm. Male production in *C. elegans* could either arise spontaneously after non-disjunction meiosis of the X chromosome in a self-fertilizing hermaphrodite (0.1% to 0.2% of male production) (Ward and Carrel 1979; Hodgkin 1987; Teotonio, Manoel et al. 2006) or after outcrossing events such as fertilization (30% to 50% of male production). Thus male frequency is believed to reflect the outcrossing rates (Teotonio, Manoel et al. 2006; Anderson, Morran et al. 2010; Morran, Schmidt et al. 2011) and *C. elegans* has proven to be a suitable model organism to look at the importance of males and outcrossing under environmentally fluctuating conditions such as host-parasite coevolution.

Bacillus thuringiensis as a model organism

Bacillus thuringiensis is a Gram-positive soil bacterium (Figure 1b), which is pathogenic to a wide spectrum of invertebrates, among them the nematode *C. elegans*. During sporulation, *B. thuringiensis* produces host specific crystal toxins, likely to represent a central virulence factor for the interaction with the *C. elegans* host. The latter are encoded by toxin genes present on their plasmids. Upon ingestion by the host, the toxin-spore mixture reaches the host gut; there the crystallized toxins are solubilized and proteolytically activated. They form cellular pores and cause the destruction of host intestinal cells. A spore germination step will follow during which bacterial cell will multiply in host tissue, causing host death (Borgonie, van Driessche et al. 1995; Borgonie, Claeys et al. 1996; Borgonie, Claeys et al. 1996; Borgonie, Claeys et al. 1996). Having these properties, Cry toxins have been employed to combat insect pests and disease vectors and thus transgenic plants incorporating the Cry toxin genes have been worldwide cultured (Forde, Beardmore et al. 2008). Such high selective pressure potentially led to the genetic adaptation of target hosts (Greeff

and Schmid-Hempel 2008; Wegner, Berenos et al. 2008; Wilfert and Schmid-Hempel 2008).

Like *C. elegans*, *B. thuringiensis* is a well-studied model organism. Particularly, the ease in the laboratory manipulation, its cryopreservation, its short generation time (16 hours to double the population size inside the nematode), its maintenance under similar conditions as the nematode, and the complex available molecular data made it a suitable model organism for our evolution experiment.

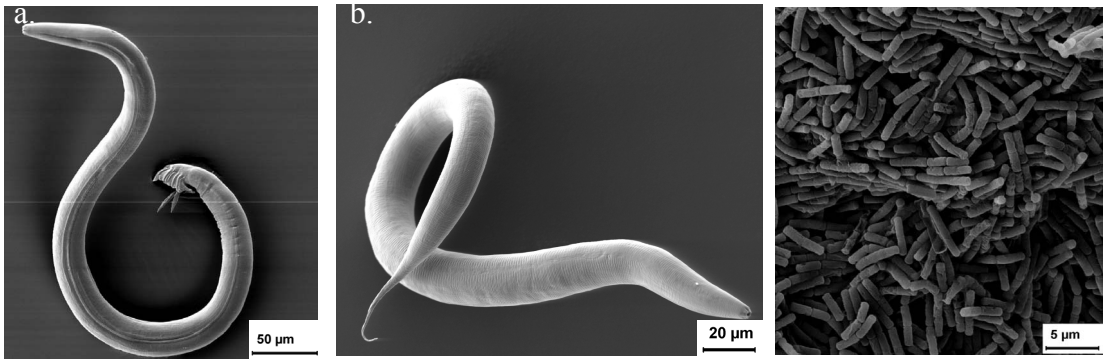

Figure 1. a. Electron microscopy picture (EM) of a male host nematode *Caenorhabditis elegans.* b. Electron microscopy picture (EM) of a hermaphrodite host nematode *Caenorhabiditis elegans. c.* Transmission Electron Microscopy picture (TEM) of the parasite *Bacillus thuringiensis.*

Main achievements and results

Main achievements

Optimization of the experimental evolution protocol

The previous evolution experiment performed by Schulte *et al* (2010) was able to demonstrate some of the particular selective consequences of host-parasite

coevolution, such as changes in host resistance, host feeding behaviour, parasite virulence, associated life-history trade-offs in both antagonists, increased local adaptation and patterns of increased genetic diversity (Schulte, Makus et al. 2010; Schulte, Makus et al. 2011; Schulte, Hasert et al. 2012).

However, in this previous study the phenotypic changes after host-parasite coevolution were lower for the host populations relative to the parasite populations. Moreover, the experimental setup did not include a one-sided adaptation regime, did not look at patterns of temporal adaptation, did not look at the role of males and outcrossing in the androdioecious species *C. elegans*, and the complete molecular understanding of the phenotypic changes was missing.

Hence, to increase the potential evolutionary responses, we decided to optimize the selection experiment by: (i) increasing the genetic diversity of the starting host population, (ii) elevating the host population size, and (iii) switching to a higher temperature of 19 ºC.

For that purpose we used a highly outbred host population (prepared by our collaborator Henrique Teotonio, Lisbon, Portugal). The host population derived from the consecutive crosses among 16 *C. elegans* natural isolates including: PB306, AB1, CB4858, CB4855, N2, JU400, MY16, JU319, PX174, MY1, PX179, JU345, CB4856, CB45507, RC301 and CB4852.

To avoid any artifacts in the results caused by environmental selection unrelated to the studied host-parasite interaction we maintained the host population for 10 generations at 19 ºC in the presence of the non-pathogenic *B. thuringiensis* (DSM-350). We then cryopreserved these populations at -80 ºC in 200 aliquots (containing each an average of 5000 worms).

Additionally we adjusted the protocol of the evolution experiments and of the related phenotypic assays to suit the elevated host population size of 500 worms (instead of 120 worms) per generation and the high temperature of 19 ºC. Such changes involved the optimization of the food (i.e *Escherichia coli*) and of parasites' concentrations and volumes, the number of transfer per week, the inoculation protocol, the growth durations of both hosts and parasites, and the phenotypic measurements of host resistance and parasite virulence and the related life-history trade-offs.

Development of new phenotypic measurements

- *I*

nfection load assay

To directly estimate parasite's fitness inside of the host and host resistance we needed to establish a new protocol for quantitative inference of parasite load. Hence we developed a method through which it is possible to extract the infecting bacteria and quantifying them. However extracting *B. thuringiensis* cells from their host and counting them, without destroying and losing them proved to be very challenging due to the *C. elegans* protective cuticle. The solution to overcome this obstacle, was to proceed as follows: (i) transfer three to five infected worms onto 12-well microscopic slides, (ii) measure their body size, (iii) repeatedly wash them with H_2O to remove the bacteria adhering to the cuticle, (iv) transfer the worms into new tubes and count the externally associated bacteria which could not be removed during the previous step, (v) extract the bacterial cells from the worms through sonication at 60 Hz (for 10 sec and 6 cycles) to break the cuticle, (vi) vortex the worms with 1mm zicrona beads form homogenization and (vii) count the extracted bacteria. Finally, the number of bacteria was calculated per size-adjusted nematode by subtracting the number of bacteria in the surrounding solution from the average number extracted from the worms. This method showed to be highly reproducible, and allowed us to have further valuable data on parasite virulence, its growth rates inside of the host, and on host resistance.

- *H*

ost escape behavior assay

This assay was essential to study the changes in host behavior known to be affected by the presence of the pathogen as well as the presence of conspecifics (Lipton, Kleemann et al. 2004; Schulenburg and Muller 2004; Chasnov, So et al. 2007). It is related to the previously performed pathogen avoidance assay (Hasshoff, Bohnisch et al. 2007), however a major limitation for such a protocol was the fact that the worms often stayed on the tested bacteria even if they were virulent as long as no other food sources were available. As an alternative, we developed new behavioral assay plates (Figure 2). The tested bacterium was mixed with *E. coli* and pipetted in the center of the plates to reduce the likelihood that the worms left the test spot due to the absence of food. Furthermore, we established an outer "food ring" by pipetting *E. coli* on the plate edges followed by the dispersion of the bacteria with an

"L shaped" sterile glass pipette (Figure 2). The escape rate was scored as the percentage of worms that left the central bacterial lawn after 24 hours.

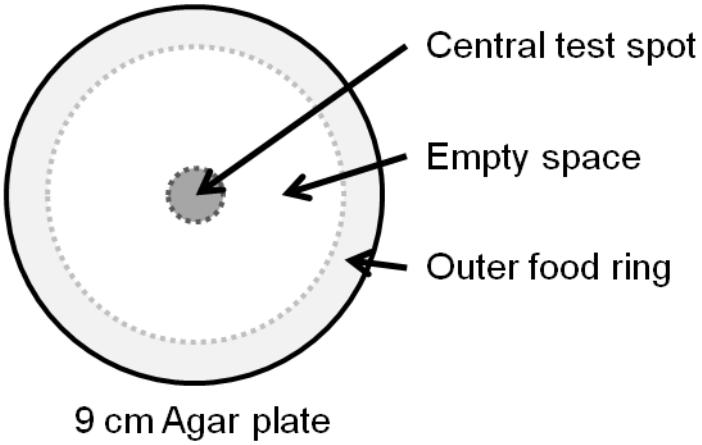

Figure 2. Drawing of the escape behavior plates.

- *Parasite characteristics in the absence of the host*

Quantifying the bacterial concentration was previously done using standard counting chambers (4.05 mm depth) (Schulte, Makus et al. 2010). However this method showed to be highly time consuming, and did not fit into our optimized selection regime during which an inoculation step was required twice per week (at every host generation). In addition, we needed to develop a more efficient counting method that eliminated the counting bias due the "clumps" formation of *B. thuringiensis*. For that reason, we developed a new bacterial counting method in which optical density was correlated to the cell number. For screens of large sample size, we used 96-well plates in combination with a plate reader.

Moreover, we have observed by the end of the evolution experiment changes in bacterial population characteristics, such as the ability of the bacteria to form biofilms. For this reason, we established new methods to study parasite fitness in the absence of the host, for example by measuring the bacterial colony size, transparency (Figure 3) and competitive ability under different nutrient conditions.

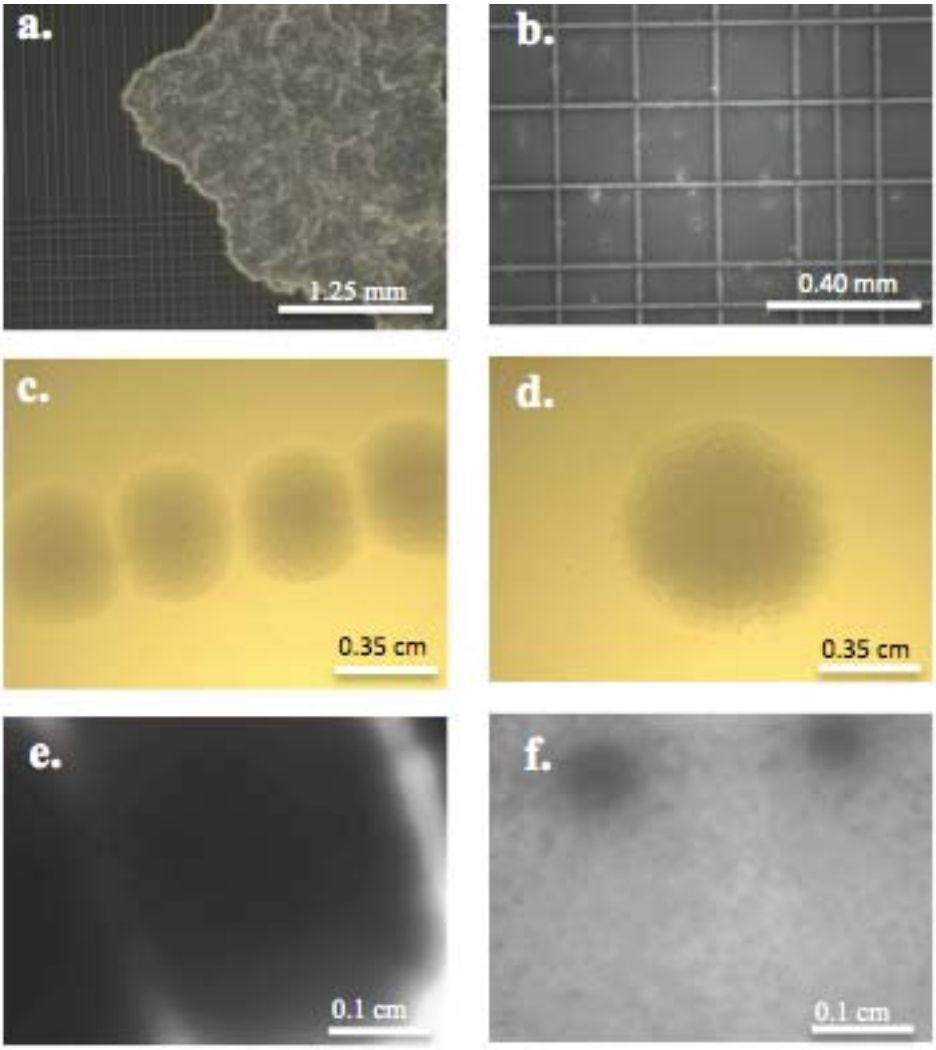

Figure 3. Pictures representing phenotypic differences between biofilm (on the left side) and non-biofilm forming bacteria (on the right side) after 72 hours of growth on enriched medium. a and b. DIG microscopy of the bacteria. c and d. different colony sizes. e and f. different transparencies of the colonies.

Establishment of a new selection experiment: Comparison between coevolution and one-sided adaptation through time

In our study, we focused on explicitly assessing the difference between reciprocal coevolution and one-sided adaptation, and thus understanding the particularity of coevolution relative to uni-directional selection, where only one antagonist is able to adapt, and to the control evolution. Moreover, for a complete evaluation of the dynamics of these interactions, we looked at the effects in various life-history characteristics across time since single traits and single time points may not be

necessarily representative of the exact dynamics (Thompson 2005; Gandon, Buckling et al. 2008).

Because under natural conditions, studying the evolutionary consequences of these interactions is constrained by the lack of control over environmental factors, the interference of other selection pressures and the limited ability of measuring the interaction over time, we simulated evolution under laboratory controlled conditions. We studied host-parasite coevolution and one-sided adaptation using, as model host, the nematode *C. elegans* and its microparasite the Gram-positive bacteria *B. thuringiensis* (Schulte, Makus et al. 2010). The starting material consisted of genetically diverse population. For the host we started with the Portuguese population deriving from the consecutive crosses among 16 *C. elegans* natural isolates. For the parasite the starting parasite population is similar to the population used by Schulte et al (2010). It consisted of a mixture of genotypes of nematicidal *B. thuringiensis*, including as the dominant genotypes at an abundance of at least 10% the strains MYBT246, MYBT18247 and MYBT18679 (provided by the Agricultural Research Service Patent Culture Collection, United States, Department of Agriculture). As a non-pathogenic *B. thuringiensis* strain we used DSM-350 (German Collection of Microorganisms and Cell Culture) for the host control treatment. Prior to the experiment, cultures of both antagonists were prepared, and frozen at either -80 ºC for the host, or at -20 ºC for the parasite for later use.

In our current selection experiment, both antagonists were allowed to evolve for 28 host generations under five selection regimes: (i) host control treatment in which the host evolved in the absence of the antagonist, (ii) host one-sided adaptation treatment in which the host adapted to a non-evolving antagonist, (iii) host-parasite coevolution treatment where both antagonists were forced to co-adapt to each other, (iv) parasite one-sided adaptation treatment in which the parasite adapted to a non-evolving host and (v) parasite control treatment in which the parasite evolved in the absence of the host (Figure 4).

In general, the host control was allowed to adapt to the non-evolving non-pathogenic *B. thuringiensis* which was taken from a frozen stock culture. In the host one-sided adaptation treatment, the host adapted to a non-evolving pathogenic *B. thuringiensis* taken from a frozen stock culture. Here, to enable evolution, surviving worms were selected and transferred to the next selection step. In the coevolution treatment, both

the host and the parasite were forced to coevolve with the coevolving antagonist. For that purpose, surviving worms and killing bacteria (collected from worm corpses) were selected, harvested and transferred to the next generation. Simultaneously, the parasite control was maintained in the absence of the nematodes, and the one-sided adapted parasite was continuously exposed to a non-evolving host taken from stock culture, where only the killing bacteria was taken to the next generation. All treatments were handled under exactly the same conditions. Each treatment was replicated ten times, and both host and parasite populations from every second generation were frozen for later phenotypic and genomic analysis.

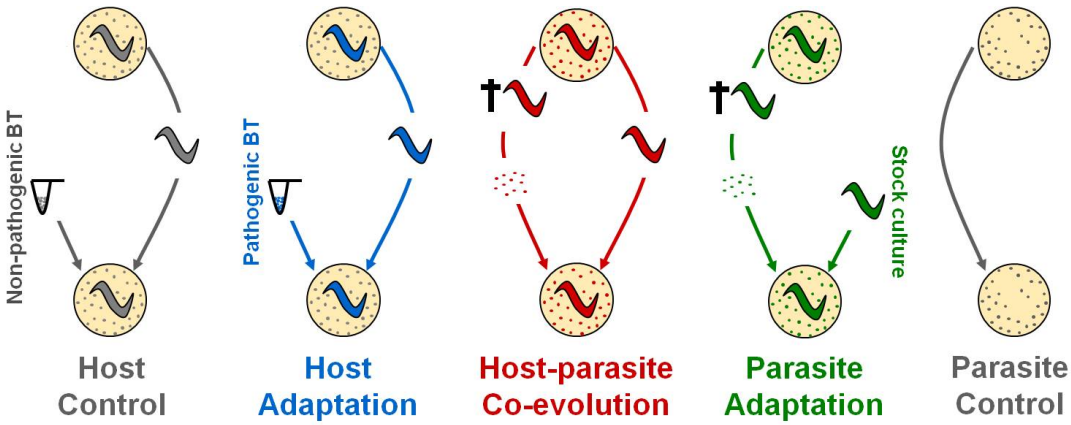

Figure 4. Experimental set-up of the selection experiment. In the control treatments, the host and parasite are maintained under the same laboratory conditions in the absence of the antagonist. During the host or parasite one-sided adaptation treatment, the host or the parasite were selected to evolve to a non-evolving antagonist taken from a frozen stock culture In the coevolution treatment, both host and parasite were forced to co-adapt to each other.

Results

Results on the evolutionary consequences for the host

We measured the evolution of host resistance through time, by looking at the changes in host survival rates, population growth, infection loads, body size and the related life-history tradeoffs. All phenotypic measurements were done respectively for host populations from generation 0, 12, 20 and 28.

On the one hand, coevolution led to a significant increase in host resistance at generation 12 and 20 in comparison to the ancestral host. Consistent with the evolutionary costs of resistance (Rolff and Siva-Jothy 2003; Schmid-Hempel 2003; Schulenburg, Kurtz et al. 2009; Schulte, Makus et al. 2010), the observed increase in resistance seemed to come at a fitness cost in the absence of the antagonist, expressed by the reduction of host body size ad-lib food conditions.

On the other hand, in the host one-sided adaptation we only found an increase in survival at host generation 12 relative to the ancestral population; however for all other comparisons we did not find any change in resistance. This result did not fit our expectation, since hosts from this treatment should have the advantage of a better adaptation to the non-evolving antagonist (on which they were tested) due to the uni-directional selection. This finding could either be explained by the insufficient selective constraints or generations to cause any observed effects under one-sided adaptation, or by a higher selective pressure on the coevolving host leading to the evolution of a generally higher resistance instead of a specific resistance. The latter is consistent with the lack of temporal adaptation observed for the coevolved host-parasite populations, tested by exposing coevolved hosts from a particular time point to coevolved parasites from the same time point, the past or the future.

Results on the evolutionary consequences for the parasite

Similarly, we measured the evolution of parasite virulence through time, by exposing the evolved parasite population to the ancestral host populations.

The control parasites, expressed a significant loss in their killing ability already after 12 host generations, while the coevolved parasite populations showed the highest killing rates followed by the one-sided adapted parasites. These results were confirmed by the additionally performed phenotypic measurements. The coevolved *B. thuringiensis* expressed the highest impact on host fitness and body size. It caused the highest reduction in host population growth and body size once compared to the control treatment. The one-side adapted parasite caused intermediate reduction of host population growth, but equal reduction in host body size in comparison to the coevolved parasite population. Thus we predict that virulence is costly and is only maintained at high levels if beneficial in the presence of a co-adapted antagonist.

The data on parasite infection load show that one-sided adapted parasites are the best at growing inside of the host, and thus having the highest fitness inside of the host. It

is important to note that: (i) the starting parasite population is a mixture of genotypes and a high parasite-parasite antagonistic competition is expected for host resources, favouring rapid parasite growth to achieve greater relative success within the host, (ii) the intra host replication could come at a cost such as a decrease of virulence (West and Buckling 2003), but see (Garbutt, Bonsall et al. 2011) and (iii) it is likely for the one-sided adapted parasite to easily overcome defences of the non-evolving host. Hence the combination of these factors, might explain the higher intra host replication of the one-sided adapted parasite in comparison to the two other parasite treatments.

On top of the differences in virulence, the evolved bacterial populations either formed biofilms or grew in planktonic forms. In detail, all control populations formed biofilms, while all the coevolved population grew in planktonic forms and the one-sided adapted parasite expressed both phenotypes depending on the replicate population. Further phenotypic measurements showed a competitive advantage of the biofilms under low nutrient conditions and a disadvantage under enrich food conditions. This observation comes in agreement with the fact, that biofilm is beneficial under stressful environmental conditions, such as the host-free (e.g, food-free) conditions present in the control treatment.

In summary, the measured phenotypical differences observed in the parasite populations are the outcome of three distinct selection pressures. First, coevolution selected for virulence, most probably to permit host entry and access to host resources. Second, one-sided adaptation favoured intra-host replication (see above). Third control conditions promoted biofilm production to overcome stressful conditions such as food deprivation, however came with costs of losing virulence.

Results on male maintenance during coevolution

Every second host generation, we looked at the male frequency in the evolving host populations. We observed maintenance of males at lower frequencies after host parasite coevolution and host one-sided adaptation in comparison to the host control population. Although unexpected, this result was confirmed by the data provided in the Schulte experiment (unpublished data).

Looking for possible explanations, we first measured males' susceptibility to the pathogenic *B. thuringiensis*. Indeed, the survival rate and infection load assay proved that males survive less and are more infected than the hermaphrodites.

Second, we assessed changes in the male mating behavior under pathogen exposure, since male abundance is influenced by the mating frequencies in such an androdioecious system. We found that the male sexual activity significantly dropped under pathogenic conditions. The change in the sexual activity was reflected in the F1 generation, where the male proportion was significantly lower under pathogenic conditions.

In summary, under pathogenic stressful conditions, males seemed to decrease in abundance because of their higher susceptibility and lower mating efficiency. Nevertheless, they are surprisingly still maintained at relatively stable frequencies that non-disjunction rates and migration cannot explain (see model Chapter II). We looked at possible explanations for such maintenance and tested possible indirect benefits of the males in the population. We found that F2 generation coming from outcrossed hermaphrodites is more parasite resistant than the one of selfed parents, which reflected potential indirect benefits of males and outcrossing.

Taken together, we found direct selective disadvantage of males but still indirect selective benefits, together explaining the observed low yet stable male frequencies in the parasite treatments.

Results on parasite genomics

Our selection experiment revealed pronounced differences among the three parasite treatments. First, the coevolved populations maintained the highest virulence levels, had low intra-host replication rates and never formed biofilm. Second, the one-sided adapted populations, showed intermediate levels of virulence, expressed the highest levels of intra-host replication and some of the replicates formed biofilm. Finally, the control populations manifested a complete loss of virulence, little intra-host replication and high ability for biofilm formation. This material provided the basis for a comprehensive understanding of the genomic consequences of coevolutionary adaptations. Our molecular analysis considered the genomic changes at three levels:

- Cry toxin gene frequencies were analyzed: as potential key factors of virulence against the nematode *C. elegans* (see above)
- Chromosomal gene *codY* and whole genome sequence frequencies: providing information on genotype frequencies and the main selective dynamics
- SNP variation, providing insight into the genes that are likely under selection

We found that MYBT18679 toxins to be present at high prevalence in the coevolving parasite populations and in some one-sided adapted populations. The whole genome sequencing approach confirmed this observation. Indeed, all replicate populations of the coevolved parasite treatment were fixed for the genotype MYBT18679 already at generation 12, as well as some of the one-sided adapted populations. This strain is known to express high levels of pathogenicity towards the nematodes, which explains its advantage during adaptation in the presence of the host. Under control conditions, the strain MYBT22 dominated all replicates. This strain might have spread in the control populations, where no host was available, because of its high competitiveness relative to other bacterial strains. In the one-sided adaptation treatment, several strains have gone to fixation depending on the replicate populations, namely: MYBT18679, MYBT22 and MYBT50. This correlates with the large phenotypic variation observed across replicate populations in this treatment. We argue that the constant and non-fluctuating selection has favoured two life-history strategies: either virulence and no biofilm formation or loss of virulence and biofilm formation.

Finally, SNP variation was identified for the populations dominated by the MYBT18679 genotype. Some of these variations refer to non-synonymous changes in genes potentially involved in the interaction with the host and bacterial cell surface characteristics and thus implicated in virulence and host manipulation.

In summary our three levels genomic approach, demonstrated that adaptation seems to be dominated by clonal selection followed or combined with the spread of individual mutations

General discussion

Parasites are spread worldwide; they show high ability for fast adaptation and impose high selective pressure on their host organisms. Parasite mediated selection is predicted to influence: (i) the mechanisms related to host resistance and parasite virulence, (ii) the related life-history tradeoffs, and (iii) the mechanisms generating fast genetical changes such as sexual reproduction and recombination. To date, numerous studies have looked at the consequences of the coevolutionary dynamics, using model organisms going from simple unicellulars (Forde, Thompson et al. 2004; Forde, Thompson et al. 2008; Kashiwagi and Yomo 2011; Meyer, Dobias et al. 2012)

to more complex organisms (Bangham, Obbard et al. 2007; Decaestecker, Gaba et al. 2007; Berenos, Schmid-Hempel et al. 2009; Wilfert and Jiggins 2010).

In this PhD thesis, I present to my knowledge, the first direct comparison of the selective consequences of host-parasite coevolution versus one-sided adaptation for both antagonists simultaneously across time. My findings show that the evolutionary history determines trait expression in both antagonists. For the host, an increase in resistance was observed across the coevolutionary interaction and to a lesser extent during one-sided adaptation, even though the treatments themselves did not vary significantly. For the parasite, coevolution seems to favour virulence, one-sided adaptation intra-host replication, and control evolution in the absence of a host the formation of a long-lasting stage. My study was able to test the key predictions of host-parasite coevolution simultaneously for both antagonists, and thus enhanced the understanding of the role of host-parasite coevolution as a potent selective force that impacts the evolution of organisms and biological systems (Chapter I).

Moreover, I was able to study the importance of males and outcrossing in the evolution of host-parasite interactions. Parasites show higher potential for rapid adaptations and therefore indirectly selects for outcrossing and recombination in the host (Ebert, Altermatt et al. 2007; Gandon and Otto 2007; Salathe, Kouyos et al. 2008; Lively 2010; Mostowy, Salathe et al. 2010; Drikas, Dixon et al. 2011; King, Jokela et al. 2011). However this prediction focuses on the selection constraints exerted between both antagonists and neglects the selective pressure present between the two host sexes. In our two independently performed evolution experiments, we observed maintenance of males at lower frequencies under pathogenic conditions in comparison the control conditions. The systematic comparison between the host two sexes showed that males showed a direct selective disadvantage by having lower immune defense against *B. thuringiensis* in comparison to the hermaphrodites. Nevertheless, our mathematical model predicted indirect selective advantages of the males and outcrossing explaining male maintenance at frequencies higher than non-disjunction. These selective advantages were found at the F2 generation during which offspring coming from mated grandparents showed higher pathogen survival in comparison to those produced by selfed grandparents. I argue that the reduced male frequencies are enough for the host to keep up with the coevolving parasite and lower male resistance limits fast adaptive responses (Chapter II). Thus my results demonstrate that intra-specific

variation in immunity interferes with parasite-mediated selection. As sex differences in immunity are widespread in animals, a complete understanding of host-parasite interactions demands the explicit consideration of such intra-specific differences (Chapter II).

Furthermore, the material generated during the selection experiment provides a unique opportunity to study the underlying molecular basis of evolutionary change. Our molecular analysis yielded one of the most comprehensive data sets on the genomic basis of coevolutionary adaptations (Chapter III). It gives unique insights in how mutational processes and clonal selection interact to determine the evolution of parasites in the presence of either a co-adapting host, a non-changing host, or no host (Chapter III).

Moreover, to ensure the continuity of this project, I prepared 25 isogenic family lines per replicate host populations, and approximately 25 clone cultures per replicate parasite population for different time points and cryopreserved them at -80ºC. This material in correlation with the complex phenotypic data set could permit the dissection of the genetics of coevolution in comparison to one-sided adaptation and control evolution, for the host as well as for the parasite. Further, future fine-scale analysis (whole genome sequence, SNP, etc.) may permit the mapping of candidate genes and/ or functional regions involved in the coevolutionary interactions. Theoretical predictions related to coevolution such as (i) high change in allele frequency over time, (ii) more changes in linkage disequilibrium, and (iii) increase of genetic diversity within replicate populations could be tested for both antagonists.

Conclusion

The main focus of my PhD thesis is to enhance the understanding of the consequences of host-parasite coevolution as a key selective force influencing multiple biological and ecological processes. For that purpose, I have optimized a selection experiment using the nematode *C. elegans* and its microparasite *B. thuringiensis* and divers phenotypic assays to highlight, for the first time in a single study, the difference between coevolution and one-sided adaptation for both antagonists. My assays, evaluated (i) the evolution of pathogenicity and resistance,

(ii) the related life history trade-offs, (iii) traits of temporal dynamics and temporal adaptation, (iv) the role of outcrossing and males in the evolution of these interactions, and (v) the underlying molecular genetics. My results provided one the most comprehensive insights on the evolutionary consequences of host-parasite coevolution. First my findings show that the evolutionary history determines trait expression in both antagonists. Second, they demonstrate that intra-specific variation in immunity interferes with parasite-mediated selection. Third, they give unique insights on how mutational processes and clonal selection interact to determine the evolution of parasites depending on their evolutionary history. Furthermore, I have provided material of both antagonists to continue future fine-scale analysis of the molecular consequences of these interactions.

Chapter I: An experimental test of the consequences of host-parasite coevolution

Leila Masri, David Laehnemann, Patrick S. Günther, Swantje Prahl, Nico K. Michiels, Hinrich Schulenburg

Abstract

Coevolution between host and parasite is believed to be associated with high evolutionary dynamics affecting various life-history characteristics. However, basic experimental information is as yet not available on the exact evolutionary importance of reciprocal co-evolution versus one-sided adaptation to a non-changing antagonist. Therefore, we specifically tested, for the first time, the relative impact of these two evolutionary scenarios for both antagonists. We additionally considered evolution in the absence of the antagonist as a control. The test was based on experimental evolution of the model nematode host *Ceanorhabditis elegans* and its microparasite *Bacillus thuringiensis*. Enforced coevolution did indeed produce results that differed from one-sided adaptation and control evolution. For the host, coevolution led to increased resistance**,** which however seems to come at a fitness cost in the absence of the antagonist. One-sided adaptation differed from coevolution, because it did not cause any change in resistance, contrary to expectations. The exact reason for this unexpected results is not yet clear. For the parasite, we uncovered pronounced differences among the three treatments. The coevolved populations showed highest virulence, followed by those from one-sided adaptation, whereas control bacteria lost their ability to kill. Intra-host replication was highest for the one-sided adapted bacteria. In the absence of the host, control-evolved bacteria form robust biofilms, which provides a competitive advantage under low food conditions. Such biofilm production is not found for the coevolved and only for some of the one-sided adapted bacteria. Taken together, these results demonstrate that the imposed evolutionary history determines differential expression of parasite life-history characteristics, whereby coevolution specifically enhances virulence and one-sided adaptation intra-host replication, suggesting a possible trade-off between these two infection-relevant traits. Absence of the antagonist then seems to favour production of a long-lasting stage, which should enhance persistence of the parasite in an unfavourable environment. Our study highlights the selective power of antagonistic host-parasite interactions in driving the evolution of life-history traits in both antagonists.

Introduction

Parasites are ubiquitous, they show high ability for adaptation and impose high selective pressure on their host organisms. As a consequence, host and parasite may engage in a coevolutionary arms race, consisting of the reciprocal evolution of host resistance and parasite virulence mechanisms (Woolhouse, Webster et al. 2002; Brockhurst, Morgan et al. 2007; Ebert 2008; Schulenburg, Kurtz et al. 2009). The resulting selective constraints can be extraordinarily high, resulting in fast evolutionary dynamics determined by negative frequency dependent selection, recurrent selective sweeps, or a combination thereof (Brockhurst, Morgan et al. 2007; Ebert 2008; Schulenburg, Kurtz et al. 2009). As a consequence, these interactions may influence the evolution of diverse biological systems, including host resistance and parasite virulence, the related life-history tradeoffs, and mechanisms generating fast genetical changes such as sexual reproduction and recombination (Hamilton, Axelrod et al. 1990; Woolhouse, Webster et al. 2002; Rolff and Siva-Jothy 2003; Schmid-Hempel 2003; Brockhurst, Morgan et al. 2007; Ebert 2008; Schulenburg, Kurtz et al. 2009; Lively 2010).

Intriguingly, direct empirical evidence of long-term host-parasite coevolution, especially proof of continuous reciprocal genetic changes in the antagonists is extremely scarce. The most convincing data sets have been collected for microbial systems studied under controlled laboratory conditions, including those involving *Escherichia coli* and its phages (e.g., (Forde, Thompson et al. 2004; Forde, Thompson et al. 2008; Kashiwagi and Yomo 2011; Meyer, Dobias et al. 2012)) or *Pseudomonas fluorescens* and phages (reviewed in (Brockhurst, Morgan et al. 2007)). Reciprocal changes were similarly analysed in a few animal host systems, like the waterflea *Daphnia magna* (e.g. (Decaestecker, Gaba et al. 2007)), the beetle *Tribolium castaneum* (e.g. (Berenos, Schmid-Hempel et al. 2009)), or the fruitfly *Drosophila melanogaster* coevolving with Sigma virus (Bangham, Obbard et al. 2007; Wilfert and Jiggins 2010). However, to date, only few studies attempted to assess the difference between reciprocal coevolution and one-sided adaptation, where only one of the antagonists is able to adapt. Such an assessment is pivotal for our understanding of the particular selective power of coevolutionary relative to "standard" uni-

directional selection. The few exceptions usually only included one-sided adaptation for one of the antagonists, for example for the phage in the *P. fluorescens* host system (Poullain, Gandon et al. 2008) or the host in a *Caenorhabditis elegans-Serratia marcescens* system (Morran, Schmidt et al. 2011).

Moreover, a detailed understanding of the consequences of coevolution needs to evaluate the effects in various life-history characteristics across time, since single traits and single time points may not be necessarily representative of the exact dynamics (e.g. (Thompson 2005; Gandon, Buckling et al. 2008)). Coevolutionary change across time is best understood for bacterial host systems (reviewed in (Brockhurst, Morgan et al. 2007), but has now also been addressed in several animal hosts like *Daphnia* waterfleas (Wolinska and Spaak 2009; Yin, Petrusek et al. 2012) or *C. elegans* (Morran, Schmidt et al. 2011). The complexity of coevolutionary effects has recently been dissected for the host of the *T. castaneum*-microsporidia system (Berenos, Schmid-Hempel et al. 2009).

In this study, we explicitly investigated the consequences that are unique to coevolution and differ from one-sided adaptation and also control evolution. The selective consequences were evaluated across time for several life-history characteristics simultaneously, including several traits of direct relevance to the interaction (resistance, virulence, intra-host replication) and also additional fitness-related traits. Our study is based on a laboratory controlled evolution experiment, which allowed us to precisely define alternative selection regimes. The experimental model consisted of the nematode *C. elegans* as host and the Gram-positive bacterium *Bacillus thuringiensis* as its micro-parasite. Both species are ideally suited for evolution experiments because of comparatively short generation times (for *C. elegans* 2-3 days at 20 ºC) and the accessibility of both antagonists to cryo-preservation. To infect its host, *B. thuringiensis* spore-toxin mixtures need to enter the host gut, where the crystallized toxins are solubilized, proteolytically activated and then destroy host intestinal cells through formation of cellular pores. Subsequently, spores will germinate and bacterial cells multiply in host tissue, ultimately causing host death (Borgonie, van Driessche et al. 1995; Borgonie, Claeys et al. 1996; Borgonie, Claeys et al. 1996; Borgonie, Claeys et al. 1996). The nematode host is able to activate an immune response upon infection, which seems to be mediated by the p38 MAPK, the JNK MAPK, and also the insulin-like cascades (Huffman, Abrami et

al. 2004; Boehnisch, Wong et al. 2011; Kao, Los et al. 2011; Wang, Nakad et al. 2012).

Our group previously established this model system for studying coevolution. We were able to demonstrate the particular selective power of these interactions, leading to fast reciprocal changes in host resistance, host responsiveness in feeding behavior, parasite virulence, associated life-history trade-offs in both antagonists, and also increased local adaptation (Schulte, Makus et al. 2010; Schulte, Makus et al. 2011; Schulte, Hasert et al. 2012). Moreover, we evidenced increased rates of genetic change and also increased genetic diversities, both within and between populations (Schulte, Makus et al. 2010). However, our previous study did not include a one-sided adaptation regime.

In the current experiment, both species were allowed to evolve for 28 host generations under five selection regimes: (i) host control treatment in which the host evolved in the absence of the antagonist, (ii) host one-sided adaptation treatment in which the host adapted to a non-evolving antagonist, (iii) host-parasite coevolution treatment where both antagonists were forced to co-adapt to each other, (iv) parasite one-sided adaptation treatment in which the parasite adapted to a non-evolving host and (v) parasite control treatment in which the parasite evolved in the absence of the host (Figure 1). Using the evolved material from this experiment, we tested for changes among treatments and time in host resistance, parasite virulence, various additional life-history traits like host population growth, host body size, parasite infection load, or bacterial growth rates. These traits were studied in either presence or absence of the antagonist, which permits assessment of the presence of life-history trade-offs. These characteristics were examined by exposing host and parasite from different time points and treatments to the ancestral population of the respective antagonist. To determine the selective dynamics during coevolution, we separately assessed the presence of temporal adaptation within the coevolving populations by exposing a particular host or parasite population to the coevolved antagonist from either the same time point (C, contemporaneous), the past (P), or the future (F).

Results

Evolutionary changes for the host

Host evolution of resistance was measured by looking at changes through time of host (i) survival rate, (ii) population growth, (iii) infection load, and (iv) body size. All host populations from generation 0, 12, 20 and 28 were exposed to the ancestral *B. thuringiensis* populations. Coevolved hosts increased significantly in resistance at host generation 12 and 20 if compared with the ancestral hosts (Table 1, Fig. 2A). A similar significant increase is found for one-sided adapted hosts at generation 12. For all other comparisons, there was no significant difference between the evolved populations and the ancestors (Table 1). In spite of the increased resistance for coevolved hosts, variation among the evolution treatments is insignificant (Table 2). None of the other host traits, which were measured in the presence of the parasite, produced significant variation (Table 2, Fig. 2B).

We then looked at potentially related life history trade-offs. We first examined host population growth under pathogen free conditions. Again no difference was found between the treatments (Table 2, Fig. 3A). Second, we measured host body size in the absence of the antagonist and ad-lib food conditions. Coevolved hosts showed significantly smaller body size when compared to hosts from the other two host treatments (Table 2, Fig. 3B).

Evolution of parasite virulence and related traits

As a direct measurement of parasite virulence, we quantified the parasite killing rate. All parasite populations from generations 0, 12, 20 and 28 were exposed to the ancestral host population. Control parasites lost significantly in virulence at host already at generation 12 compared with the ancestral parasite (Table 3).

Coevolved parasite populations showed the highest killing rate, followed by the one-sided adapted populations and last by the control parasite populations (Table 4, Fig. 4A).

We also examined the impact of the parasite on host fitness (i.e, host population growth) and host body size. Consistent with our previous results, coevolved *B. thuringiensis* caused a significantly higher reduction of host population growth and

host body size when compared to the control populations. The one sided-adapted parasites produced an intermediate reduction of host population growth when compared to the two other treatments but equal reduction in host body size as the coevolved parasites (Table 4, Fig. 4B,C). Taken together, coevolved parasites show the highest virulence followed by one-sided adapted bacteria, whereas the control-evolved parasites lost their virulence during the experiment.

We next asked whether parasite fitness inside of the host differed between treatments. We thus measured parasite infection load inside of the host after 48 hours of exposure. Our data shows that parasites adapting to the same non-evolving host (i.e., the bacteria from the one-sided adaptation treatment) had the highest infection load in comparison to the two other parasite treatments (Table 4, Fig. 4D).

Furthermore, we found that 60% of the one-sided adaptation parasite populations rapidly lost virulence, subsequently leading to their extinction; whereas virulence was maintained for all populations during coevolution (Table 5, Fig. 5).

Evolution of parasite population characteristics in absence of hosts

Additional to their differences in virulence, parasite populations expressed different phenotypes when cultured in the absence of the host on enriched nutrient medium like nematode growth medium (NGM) agar plates. The bacterial populations either grew in planktonic forms or formed biofilms, the latter characterized by the formation of “flakes” or “big particles” when washed-off the plates. In particular, 100 % of the control populations formed biofilm while this was the case for only 25-85 % of the one-sided adapted parasite and 0 % of the coevolved parasite populations (Table 5, Fig. 6A). Note that biofilm formation and the population’s loss of virulence strongly correlate; the same is true between non-biofilm formation and the maintenance of virulence.

We then quantified biofilm formation by measuring the average particle size per population. Indeed, control populations produced significantly larger p article sizes when compared to the two other treatments, followed by the one-sided adapted *B. thuringiensis* (Table 6, Fig. 6B).

Parasite clone characteristics in absence of hosts

For more detailed analyses of biofilm formation, we isolated four clones from the replicate populations with the following properties: (i) a coevolved clone with high virulence and lack of biofilm formation, (ii) a clone from one-sided adaptation with high virulence and lack of biofilm formation, (iii) a clone from one-sided adaptation with low virulence and biofilm-forming ability, and (iv) a clone from the control evolution treatment with low virulence and biofilm-forming ability. The selected clones were chosen out of 20 clones from a particular replicate population of the respective treatments, based on virulence measurements, as determined through parasite killing rate, the parasites' impact on host population growth, and a qualitative assessment of biofilm formation.

Variation among the biofilm-competent and incompetent clones was subsequently assayed for four phenotypes: colony size, optical density of colonies, average particle size within the clonal population across time (24 h intervals over 144 h), and competitive ability. All traits were measured on nutrient-rich nematode growth medium (NGM). The latter trait was additionally tested on peptone-free NGM (PF-NGM). The two biotypes consistently differed across these assays.

In particular, biofilm-forming clones showed significantly smaller colony size but higher optical density than non-biofilm forming clones (Table 7, Fig. 7A,B). Average particle size did not vary among the evolved clones during the first 48 - 72 h (Tables 8, 9; Fig. 7C). After 96 h of growth, the biofilm forming clones showed significantly larger particle sizes than the non-biofilm forming clones. In fact, the 96 h time point coincides with the maximum average particle size for the biofilm-competent clones. The non-biofilm forming clones did not vary between each other in particle size during their growth (Table 9, Fig. 7C). Particle size also varied among the three tested ancestral strains. MYBT246 and MY18247 produced significantly larger particles than MYBT18679 with a peak in particle size after 48 h of growth (Table 9, Fig. 7C). The strain MYBT18679 did not show any variation through time, but instead consistently produced very small particles (Fig. 7C).

Bacterial competitive ability varied among but not within the two biotypes. In particular, if two clones with the same biotype (i.e., both biofilm-competent or both biofilm-incompetent), then there was no difference in competitiveness (Fig. 8, Table 10). However, when two different types were combined, then success dependent on the medium. Under nutrient-rich conditions on NGM, non-biofilm forming clones outcompeted the biofilm-formers (Fig. 8A, Table 10). In contrast, under low nutrition

conditions, it was the biofilm producers that outcompeted the non-biofilm forming clones (Fig. 8B, Table 10).

Temporal adaptation of coevolved antagonists

To study the evolutionary dynamics during host parasite coevolution, we exposed hosts and parasites from the same contemporaneous replicate of the coevolution treatment to each other. We measured several phenotypic traits including (i) survival rate, (ii) host population growth, (iii) host body size, and (iv) host infection load. Our results show an increase of host survival over the studied time-scale of 28 host generations (Table 11, Fig. 9A). This particular change in survival did not affect any of the other measured traits even though population growth showed a trend of an increase for the last time point (Table 11, Fig. 9B,C,D).

Although these results suggest some form of temporal co-adaptation between the antagonists, the exact temporal dynamics remain unclear. To rectify this, we exposed coevolved hosts from a particular time point to coevolved parasites from either the same time point (C, contemporaneous), the past (P), or the future (F). An analogous exposure experiment was simultaneously performed for the parasites. The combined hosts and parasites were always taken from the same replicate population of the coevolution treatment. For the host, there was a significant time-shift effect between contemporaneous and future combinations in both survival rate and infection load, while it still showed a trend for body size (Table 12, Fig. 10). Significant effects were also observed between contemporaneous and past combinations in host body size and population growth, while a trend was found for survival rate (Table 12, Fig. 10). Thus, the coevolved hosts seems to perform better on future than contemporaneous antagonists and also better on contemporaneous than on past antagonists (Table 12 and Fig. 10).

For the parasite, we found no difference between contemporaneous combinations versus combinations with hosts from the preceding or subsequent time points. The only exception is host population growth where parasites in combination with future hosts reduce population growth to a larger extent than parasites in combination with a contemporaneous host (Table 12, Fig. 11).

Discussion

In this study, we present, to our knowledge, the first direct comparison of the selective consequences of host-parasite coevolution versus one-sided adaptation for both antagonists simultaneously. Previous studies focused on only one of the antagonists in similar comparisons, for example the phage of the *P. fluorescens* host system (Poullain, Gandon et al. 2008) or the host in a *C. elegans*-*S. marcescens* model (Morran, Schmidt et al. 2011). Our results now indeed highlight particular differences between these two selection conditions and also between these and control evolution in the absence of an antagonist. The consequences were more pronounced for the parasite than for the host, possibly due to their higher evolutionary potential because of a comparatively larger population size, faster replication rate, and their haploid genomes (Ebert 1998).

For the host, an increase in resistance was observed across the coevolutionary interaction and to a lesser extent during one-sided adaptation, even though the treatments themselves did not vary significantly. At the same time, coevolved hosts produced smaller body sizes in the absence of the antagonist than the other treatment groups. This result may indicate a cost of adaptation (i.e., resistance evolution), consistent with our previous results with the same model system (Schulte, Makus et al. 2010) and also in agreement with a large body of studies that inferred an evolutionary cost of resistance (reviewed in (Rolff and Siva-Jothy 2003; Schmid-Hempel 2003; Schulenburg, Kurtz et al. 2009)).

The results are still not entirely as expected, especially for the one-sided adaptation treatment. Hosts from this treatment were exposed to a constant uni-directional constraint, which should allow them to adapt quickly to the antagonist. Adaptation should have been particularly pronounced towards the ancestral parasites, against which they were tested in the final phenotypic assays and which they continuously encountered during evolution. Even though a small increase in resistance was observed, the increase was more pronounced for the coevolved hosts. This result may have two complementary explanations. On the one hand, selective constraints are likely larger for the host under coevolution conditions. A possible outcome may be the evolution of higher general resistance rather than specific resistance to only the coevolving parasites. This idea is consistent with the lack of high temporal adaptation

for the coevolved host-parasite populations (see below). On the other hand, the considered time and/or selective pressure may have been insufficient to cause stronger effects under one-sided adaptation.

For the parasite, significant variation was found among evolution treatments in several characteristics. Coevolution specifically favoured the maintenance of high virulence, which was lost completely under control evolution and to a lesser extent under one-sided adaptation. This result suggests that virulence comes at a cost and is only maintained if it provides an advantage such as in the presence of a co-adapting host. Interestingly, a non-changing host is apparently only to some extent sufficient to provide a benefit to high virulence. Under these one-sided adaptation conditions, parasites are favoured that are able to produce high infection loads, most likely as a consequence of intra-host replication and the efficient exploitation of host resources. Intra-host replication rates may be further accelerated in case of strong competition among bacterial clones. Since it is likely easier to overcome defences of a non-evolving host, the one-sided adaptation treatment may have led to increased individual numbers inside the host and thus increased competition and subsequently higher growth rates ((West and Buckling 2003), but see (Garbutt, Bonsall et al. 2011)).

In the absence of a host, a very distinct parasite phenotype is favoured that lacks virulence but instead is able to form robust biofilms. These biofilms provide a competitive advantage under low nutrition conditions, as used during the evolution experiment. In fact, biofilms allow the bacteria to persist under stressful environmental conditions. Thus, it is not surprising that the host-free condition favoured this particular bacterial biotype.

Taken together, the results for the parasite reveal that past evolutionary interactions with the host determine expression of virulence-associated fitness traits and that coevolution and one-sided adaptation favour different trait expressions. Firstly, coevolution specifically favoured virulence, most likely to ensure entry into the host and access to host resources. Secondly, one-sided adaptation specifically favoured increased intra-host replication. It is possible that under these conditions the bacteria may easily gain entry into the host, within which there is then high competition for host-resources and fast replication, possibly resulting in the observed higher infection load. Here, the ability to replicate quickly may come at the cost of losing virulence. Finally, control conditions favoured parasite traits like biofilm production that ensured

persistence in a supposedly hostile environment. The ability to persist by biofilm formation also seems to come at the cost of losing virulence.

The three favoured traits are representative of different steps of the life-cycle of a parasite, encompassing host entry, intra-host multiplication and persistence in the environment. Recent studies emphasized that consideration of the different parasite life-history steps is pivotal for in-depth understanding of host-parasite coevolutionary dynamics (Duneau, Luijckx et al. 2011; Scanlan, Hall et al. 2011). Our study now highlights the selective conditions that can specifically enhance expression of each one of these traits, which simultaneously appears to come at the cost of expressing one of the other traits.

The specific selection for virulence in combination with an apparent cost of virulence should lead to highly specific interactions during coevolution. For instance, an increase in host resistance should favour evolution of a novel virulence mechanism and a concomitant loss of the "old" virulence effect, since the latter has a cost. In contrast, our time shift analysis did not reveal the pattern of temporal adaptation or specificity, previously demonstrated for example for snail-trematode (e.g., (Dybdahl and Lively 1998)), *Daphnia magna – Pasteuria ramose* (Decaestecker, Gaba et al. 2007), and also bacteria-phage coevolutionary interactions (Hall, Scanlan et al. 2011). Instead, coevolving parasites were generally observed to lose virulence towards their contemporary and future coevolved hosts (Fig. 10). This loss in virulence may be enhanced by a concomitant increase in general resistance in the host, as indicated by our analysis of hosts exposed to the ancestral parasites (Fig. 2). A similar loss of parasite virulence was recently documented during an evolution experiment between *T. castaneum* beetle hosts and a microsporidian (Berenos, Schmid-Hempel et al. 2011). Loss was here proposed to be due to evolution for optimal levels of virulence. The same may apply to our system, especially when considering that initial virulence levels were particularly high and could easily decrease efficient transmission.

In conclusion, our approach of experimental evolution demonstrates an intricate pattern of selective constraints that determines evolution of host and especially parasite life-history traits dependent on the organism's evolutionary history and its current interaction with either a co-adapted or a non-co-adapted antagonist.

Material and method

Study organisms

The starting *Caenorhabditis elegans* host population derived from consecutive crosses among 16 natural isolates, namely: PB306, AB1, CB4858, CB4855, N2, JU400, MY16, JU319, PX174, MY1, PX179, JU345, CB4856, CB45507, RC301 and CB4852. It covers the entire worldwide spectrum of genotypes known for this model organism. The purpose of usage of this highly outbred host population (kindly provided by Henrique Teotonio, Lisbon, Portugal) is to increase the potential for evolutionary responses. We adapted this population to our experimental conditions by maintaining it for 10 generations at 19 ºC in 40 replicates in the presence of a non-pathogenic *Bacillus thuringiensis* (DSM-350). This adaptation step served to minimize potential artifacts in the results caused by environmental selection unrelated to the host-parasite interaction. These laboratory adapted strains were mixed and cryo-preserved in glycerol at -80 ºC (Stiernagle 2006) in 200 aliquots (containing each an average of approximately 5000 worms) and were later used during the selection experiment. For all phenotypic experiments, hermaphroditic fourth instar larvae (L4) were used.

The starting parasite population is similar to the population used by Schulte et al (Schulte, Makus et al. 2010). It consisted of a mixture of genotypes of the nematicidal Gram positive *Bacillus thuringiensis*, including as the dominant genotypes at an abundance of at least 10% the strains MYBT18246 and MYBT18247. Other strains like MYBT18679 were present at approx. 1%. As a non-pathogenic *B. thuringiensis* strain we used DSM-350 (German Collection of Microorganisms and Cell Culture) for the host control treatment. Prior to the evolution experiment, we prepared large amounts of *B. thuringiensis* cultures, aliquotted and conserved them at -20 ºC for later use. In all of the following experiments, *B. thuringiensis* and DSM-350 had a concentration of 1.2 x 10^9 particles/ml always mixed to a 1:10 ratio with the food source *Escherichia coli* OP.50 (concentration of 2 x 10^9 cells/ml).

Experimental evolution

The main experimental evolution protocol was similar to the evolution experiment described by Schulte et al (Schulte, Makus et al. 2010), but still differed in several aspects. First, we switched to an experimental temperature of 19 ºC to decrease host generation time, thus to increase the number of generations within a defined time period. Second, we increased the host population size to 500 individuals at each transfer step. Third, we used a higher genetically diver se starting population (see above). Fourth, 5 % of the original host genotypes (but not of the parasite) were added at every second transfer to simulate migration and to avoid random genetic diversity loss due to drift. And fifth, hosts and parasites were transferred to new wormballs twice per week (equivalent to every host generation) and two milliliters of a 10:1 mixture of *E .coli* and *B. thuringiensis* were applied to the worm balls on the same day of nematode addition.

In general, the evolution experiment consisted of five treatments (Figure 1): (i) host control, during which the host adapted to general laboratory conditions in the absence of pathogenic *B. thuringiensis*, (ii) host one-sided adaptation, where the host was allowed to adapted to a non-evolving pathogenic *B. thuringiensis* taken from a frozen stock culture at each transfer point, (iii) host-parasite coevolution, in which both antagonists were continuously forced to coevolve with each other, (iv) parasite one-sided adaptation , where the parasite was allowed to adapted to a non-evolving *C. elegans* taken from a frozen culture at each transfer point, and (v) parasite control, during which the parasite adapted to general laboratory conditions in the absence of the nematode host).

For the host control treatment, the host was allowed to adapt to the general laboratory conditions and to non-pathogenic *B. thuringiensis*. For that reason, the host populations were maintained on the mixture of non-pathogenic *B. thuringiensis* and food bacteria *E. coli*. The non-pathogenic *B. thuringiensis* was taken from a frozen stock, harvested for 3-5 days on NGM plates, pasteurized (at 80º C for 10 min) to avoid any contamination, adjusted to a concentration of 1.2 x 10^9 particles/ml, mixed to a 1:10 ratio with the food source *E. coli* (concentration of 2 x 10^9 cells/ml), and then applied to the worm balls. In parallel, at every second transfer step, the host populations were purified with alkaline hypochlorite: NaOH (Stiernagle 2006) resulting in survival of only the eggs of the nematodes and the death of bacterial cells and adults worms. The eggs were transferred onto NGM plates covered with *E.*coli, allowing them to hatch and to grow under optimal laboratory conditions. Once the

larvae reached the last stage before adulthood (fourth instar larvae L4), they were washed off the NGM plates, counted and a total of 500 worms (475 + 25 worms of the original host population) were transferred to the next round of the evolution experiment.

For the host one-sided adaptation treatment, surviving nematodes were exposed to the same ancestral pathogenic *B. thuringiensis* mixture. Surviving hosts were transferred every host generation and exposed to the same ancestral parasite population. Every second host generation, the host was treated with NaOH, counted, a 5 % of the original host population was added, and the worms were then transferred to the next round of the evolution experiment. In parallel, the pathogen as always taken from the frozen *B. thuringiensis* cultures, grown for 3-5 days on NGM plates, counted and mixed 1:10 with *E. coli.*

In the coevolution treatment, surviving nematodes and host-killing bacteria were transferred to the next evolution round. The host population was prepared using exactly the same protocol and as in the host-one sided adaptation. The host killing bacteria were isolated from dead worms, which were isolated from the experimental populations at each host generation, maintained for 2 days in liquid PBS buffer to extend the proliferation of the parasite inside of the host, pasteurized at 80 ºC for 10 min and later cultured on NGM plates for 3-5 days. The bacteria was then mixed with *E. coli* and transferred to the next selection round.

For the parasite one-sided adaptation treatment, parasites were continuously exposed to the same ancestral nematodes. Simultaneously, freshly thawed ancestral nematode population were prepared 10 days prior to the next exposure round, purified and synchronized using the bleaching protocol (Stiernagle 2006) and 500 L4 nematodes were exposed to the one-sided adapted parasite at each transfer step. In summary, the treatment followed the same protocol as in the host-parasite coevolution treatment, except that the host was always freshly prepared from frozen ancestral worm populations.

The parasite control treatment allowed the bacterial adaptation to the laboratory environment in the absence of the host. These bacterial populations went through exactly the same rhythm as the bacteria in the coevolution treatment; the bacteria were washed off the wormballs at the same time point when the bacteria were isolated from dead hosts. The washed off bacteria was similarly, maintained in PBS-buffer, pasteurized, grown on NGM plates, mixed with *E. coli* and added to the wormballs.

We replicated each treatment 10 times, and the selection experiment was run for a total of 28 host generations. Random sample from all replicate populations (both *C. elegans* and *B. thuringiensis*) were frozen at host generations 12, 20 and 28. The ancestral host and parasite population were also cryo-preserved prior to the evolution experiment.

Overview of final phenotypic measurements

Phenotypic changes across time and treatments were studied for the frozen host and parasite samples from host generations 0, 12, 20 and 28. We considered the following phenotypic traits: (i) nematode survival and parasite killing in the presence of the ancestral antagonist to assess respectively changes in host resistance and parasite virulence, (ii) nematode/parasite infection load in the presence of the ancestral antagonist to look at changes in host resistance and parasite pathogenicity, (iii) nematode body size and population growth rate on both pathogenic bacteria and non-pathogenic bacteria as an indication of host resistance and the related costs, (iv) ancestral host population growth and body size on different *B. thuringiensis* as an indirect measurement of parasite pathogenicity, (v) changes in parasite population characteristics and competitiveness.

The phenotypes were examined under the same experimental conditions as in the evolution experiment. All treatments respectively from generation 0, 12, 20 and 28 were studied simultaneously and in random order to avoid artifacts due to observer bias and/or random environmental or temporal fluctuations. Both nematodes and bacteria were raised and purified prior to the experiments (bleaching for worms, pasteurization for bacteria), the hermaphroditic worms were used once they reached the L4 stage and the final *B. thuringiensis* concentration was adjusted to 1.2×10^8.

Survival and killing assay

The evolution of host resistance was evaluated by measuring the proportion of surviving worms (Schulte, Makus et al. 2010). 50 worms per evolved replicate population were exposed to the ancestral *B. thuringiensis*. The percentage of surviving worms was counted 48 hours after exposure and thus determined the survival rate per studied population. Simultaneously, the inverse of this measure, namely 50 worms of the ancestral host population was exposed to all three evolved parasite populations,

the percentage of dead worms and thus the parasite killing rate, was used as a measure of parasite pathogenicity (Schulte, Makus et al. 2010).

Infection load assay

We developed a new protocol for quantitative inference of parasite load as a direct estimate of the parasite's fitness inside of the host. For this assay 9 cm PFM plates were covered with 500 µl of the *B. thuringiensis* – *E. coli* mixture (final concentrations respectively of 1.2 x 10^8 particles/ml and1.8 x 10^9 cells/ml). 35 L4 hermaphrodites of the evolved nematode population were exposed to the ancestral bacteria, 48 hours later three to six alive worms per combination were transferred onto a 12-well microscopic slide, and then photographed for subsequent body size measurements using ImageJ (http://rsb.info.nih.gov/ij/). To remove bacteria adhering to the cuticle, the worms were carefully washed with 5-20 µl sterile H_2O under a dissecting stereomicroscope. Washed worms were then transferred into 1.5 ml tubes containing 100 µl H_2O. The number of externally associated bacteria, which could not be removed, was estimated by counting cells in the surrounding solution using standard Thoma counting chambers (0.1 mm depth). For each replicate, bacteria were extracted for the group of nematodes by sonicating the worm solutions for 10 sec, 6 cycles at 60 Hz. Thereafter, three to four 1 mm Zirconia beads were added and the tubes were vortexed for 3 sec. As above, the number of bacteria was counted using the standard Thoma chambers. The infection load was then determined per replicate and per size-adjusted nematode as the number of extracted bacteria from worms of a particular replicate, and leaving out the number of bacteria in the enclosing solution. The infection load measurement was later correct by the average size per worm and the final number of worms per replicate used during the assay. In case of an insufficient number of transferrable animals (less than three worms per replicates) the replicate was excluded from the final statistical analysis. This method permitted the extraction of the ancestral *B. thuringiensis* cells from their evolving host without destroying them, and thus the determination of host resistance. The same method was used to study the parasite fitness inside of the host, however for that purpose evolved ancestral worm populations were confronted with the evolved *B. thuringiensis* populations.

Body size assay

Variation in host body size in the absence of the pathogen was used to either infer possible costs of host resistance (Schmid-Hempel 2003; Schulte, Makus et al. 2010) or to obtain an indirect measure of parasite virulence. Host body size is known to correlate positively with host reproductive rate (Hodgkin 1997; Houthoofd, Braeckman et al. 2005) and thus it represents a proxy for host fitness. 35 nematodes of a particular replicate population were maintained on non-pathogenic bacteria for 96 hours. Four to six nematodes were transferred onto a microscopic slide for differential interference contrast microscopy (DM5000B microscope; Leica), and the body size as whole worm area was determined using the program ImageJ 1.36b. We also used host body size as an indirect measure for parasite virulence. For this analysis, 35 hermaphrodites of the ancestral host population were exposed to replicate populations of the evolved bacteria. After 48 hours, the body size of four to six worms was measured as above. For later statistical analysis, we calculated the average body size per replicate population.

Population growth assay

Variation in host population growth in the absence of the pathogen was used to either infer possible costs of host resistance or to measure the impact of parasite on host fitness, thus its virulence. 35 L4 hermaphrodites per replicate of the evolved host population were maintained under non-pathogenic conditions. After five days, the number of nematodes per population was determined for each replicate by washing off all worms from the wormballs with 2 ml PBS-buffer, counting of animals in three 10 µl subsamples, and subsequent calculation of the total number of worms per sample. Similarly, 35 L4 hermaphrodites of the ancestral host population were exposed to the evolved parasite population, and population growth rate was measured five days later to determine the changes in parasite pathogenicity.

Parasite population characteristics and competition experiment

By the end of the evolution experiment, we also studied changes in bacterial population characteristics, including the ability of the bacteria to form biofilm.

First, we qualified the apparent phenotypes, such as the formation of flaks or not, thus biofilm formation or not and recorded the percentage of biofilm forming replicate populations per treatments.

Second, to quantify the extent of biofilm formation, we measured the size of particles formed within each of the replicate bacterial population. 20 µl per replicate bacterial population were plated onto 6 cm NGM agar plates and incubated at 19 °C for 48 hours. Thereafter, the plates were washed with 3 ml PBS buffer; the solution was transferred into 15 ml tubes and a five seconds vortexing step followed. Later, 20 µl of the collected solution per sample were transferred onto glass slides for differential interference contrast microscopy (DM5000B microscope; Leica) and the particles area (in mm^2) of the five biggest particles per replicate population was analyzed using the program Image J. To avoid random variation, each replicate bacterial population was measured four times and the mean value was taken for the subsequent statistical analysis.

Third, bacterial colony size and colony density were assessed on NGM plates. For this purpose, five 9 cm NGM plate were streaked with the studied bacteria and left to grow for 96 hours at 19 ºC. Thereafter, the plates were observed using contrast microscopy (DM5000B microscope; Leica) and the colony diameter (in mm^2) and density (absorbance) of 20 colonies per studied bacteria was analyzed using the program Image J.

Fourth, bacterial competitive ability was studied in the absence of the host, either under low nutrient conditions, using a peptone-free nematode growth medium (PF-NGM), or under nutrient enriched conditions, using the standard peptone-containing nematode growth medium (NGM). For this assay 9cm plates were used. Biofilm forming and non-biofilm forming bacteria from the selected evolved bacterial clones were streaked parallel to each other (5 mm distant to each other). The concentration of the studied bacteria was adjusted to $1.2*10^9$ particle/ml and the assay was performed at 19 ºC. Competitiveness was determined after 96 hours on NGM and after 21 days on PF-NGM (due to absence of nutrition, growth was substantially reduced under these conditions). In detail, the growth expansion of one bacterium in the direction of the other bacterium was measured as the distance from the original streak to the furthest area of the grown culture. An analogous measurement was taken for the competing bacterium. A competitiveness index was subsequently calculated for a particular strain by taking its growth expansion measurement and subtracting from it

the respective measurement of the competitor strain. Thus, if the competitiveness index is equal to 0, then none of the two strains is able to outcompete the other. If the value is positive, then the focal strain is more competitive than the other tested strain, and vice versa if the index is negative.

Statistics

JMP® 9 (SAS) was used for all statistical analyses. Variations between the treatments in all traits except of competitiveness was evaluated with a general linear model including generation and treatment as fixed factors, and replicate nested within treatment as random factor. A likelihood ratio test was used to assess the importance of every factor in the model. Variation in competitiveness was compared with the Mann-Whitney U test (MWU). Graphs were generated with SigmaPlot version 11.0 (Systat Software Inc.).

Figures

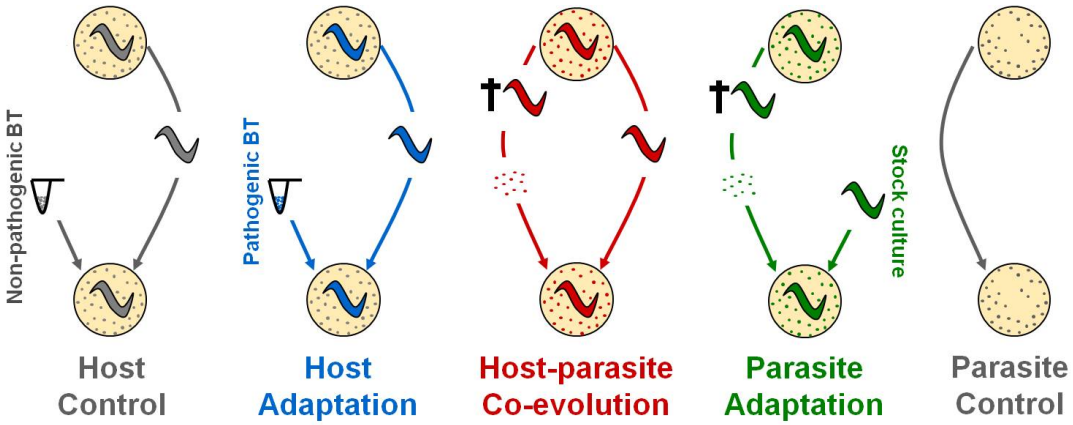

Figure 1. Design of the evolution experiment. The central treatment is coevolution, given in red, for which host and parasite were forced to co-adapt to each other. The control host and parasite treatments are indicated in gray and involved maintenance of either the host or the parasite under the same experimental conditions in the absence of the antagonist. During the host or parasite one-sided adaptation treatment, given respectively in blue and green, the host or the parasite were forced to adapt to a non-evolving antagonist, which was removed at each transfer step and immediately replaced with an antagonist from a frozen stock culture.

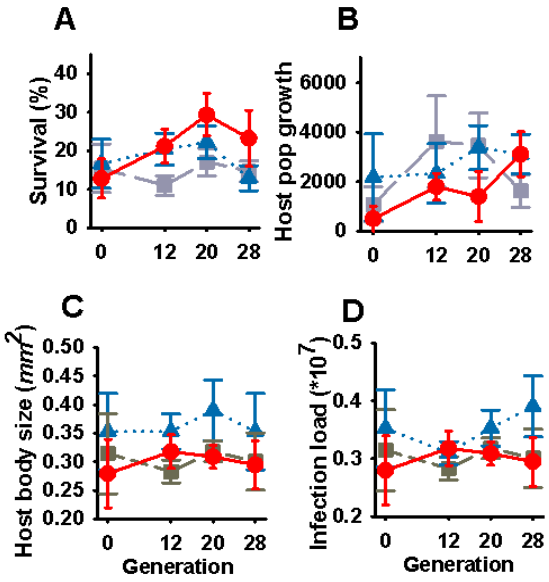

Figure 2. Changes in host resistance. (A) Survival rate, (B) host population growth, (C) host body size, and (D) infection load. Evolved hosts from different replicate population are always exposed to the ancestral pathogenic *B. thuringiensis*. Results for body size and infection load measures are only shown for alive worms. Infection load was adjusted by body size (see methods) Variation is shown across time (X-axis) for the treatments coevolution (red circle), one-sided adaptation (blue triangles), and control evolution (gray squares). Bars show 2* standard errors of the mean.

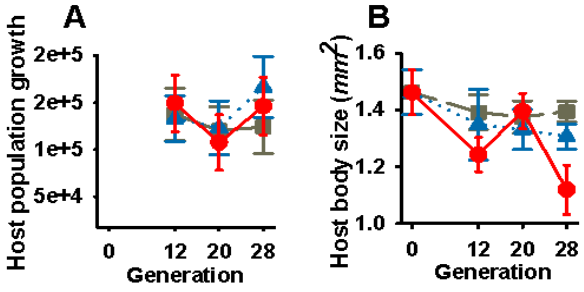

Figure 3. Indications of life history trade-offs in the host. (A) Host population, and (B) body size. Evolved hosts from different replicate population are always exposed to the non-pathogenic *B. thuringiensis*. Results for body size are shown for alive worms. Variation is shown across time (X-axis) for the treatments coevolution (red circle), one-sided adaptation (blue triangles), and control evolution (gray

squares). Bars show 2* standard errors of the mean.

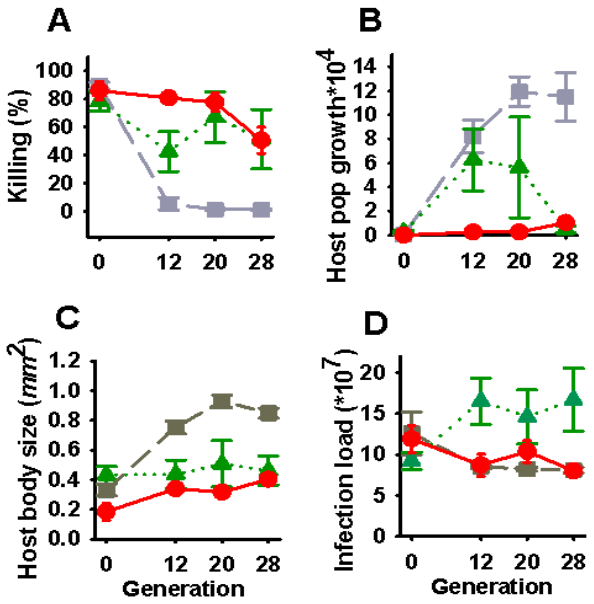

Figure 4. Changes in parasite virulence. (A) Killing rate, (B) host population growth, (C) host body size, and (D infection load. Evolved parasites from different replicate population are always exposed to the ancestral *C. elegans* population. Results for body size and infection load measures are only shown for alive worms. Infection load was adjusted by body size (see methods). Variation is shown across time (X axis) for the treatments coevolution (red circle), one-sided adaptation (green triangles), and control treatments (gray squares). Bars indicate 2* standard errors of the mean.

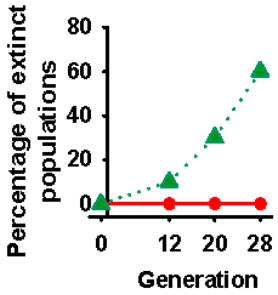

Figure 5. Parasite extinction rate. Percentage of extinct populations among the coevolved and one-sided adapted parasite population. Results are shown for treatments coevolution (red circle) and one-sided adaptation (green triangles).

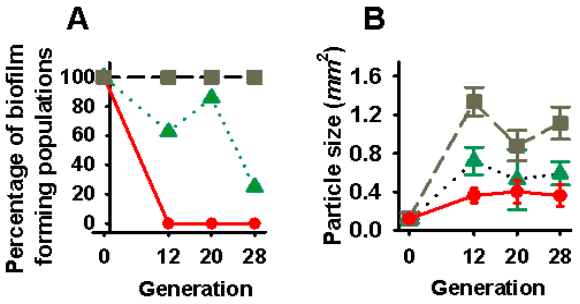

Figure 6. Changes in parasite population characteristics in the absence of the host. (A) Proportion of biofilm-formation among the evolved parasite populations. (B) Mean particle size of the evolved parasite populations. Results are shown for the treatments coevolution (red circle), one-sided adaptation (green triangles), and the control treatment (gray squares). The bars in (B) indicate 2* standard errors of the mean.

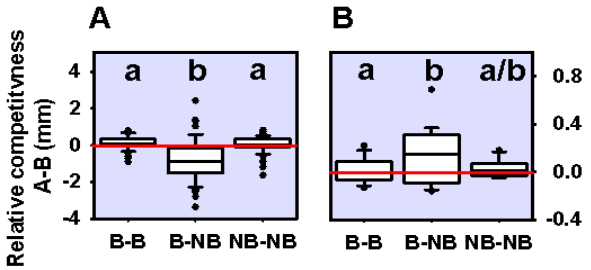

Figure 7. Parasite competitiveness. (A) Competitiveness on nutrient-rich NGM plates. (B) Competitiveness on nutrient-poor PF-NGM plates. Competitiveness is shown as the difference between alternative combinations of biofilm-forming (abbreviated B) and non-biofilm-forming (abbreviated NB) strains, as indicated on the X axis. Error bars show 2* standard errors of the mean, different letters show significant differences.

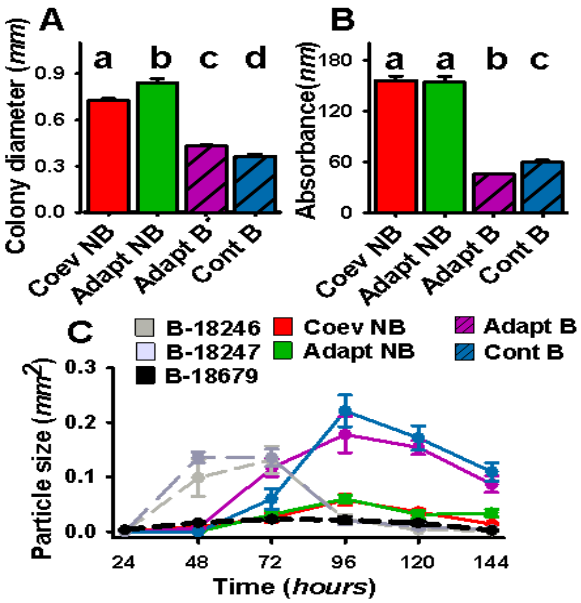

Figure 8. Changes in parasite clonal characteristics in the absence of the host. (A) Mean colony size of the evolved representative clones on NGM plates. (B) Mean absorbance per colony of the evolved representative clones on NGM plates. (C) Mean particle size of the evolved parasite clones and of the three original strains. Results are shown for non-biofilm and highly virulent coevolution clone (red), non-biofilm and highly virulent one-sided adaptation clone (green), biofilm and low virulent one-sided adaptation clone (purple), biofilm and low virulent control clone (blue), original parasite strain B-18246 (light shadow grey), original parasite strain B-18247 (dark shadow grey) and original parasite strain B-18679 (black). Error bars show 2* standard errors of the mean, different letters show significant differences.

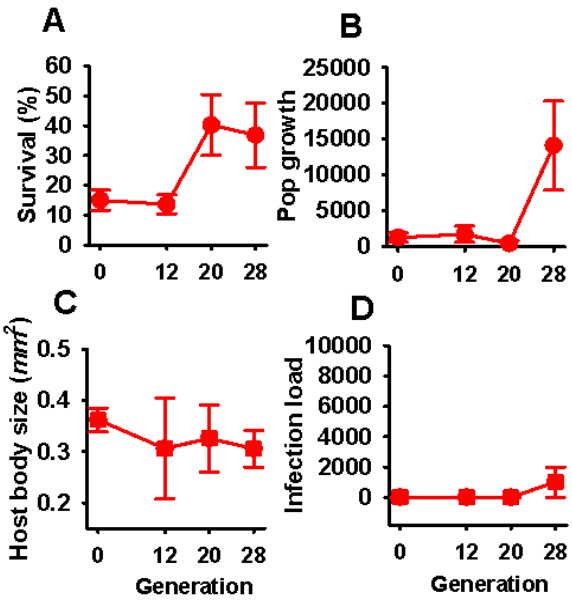

Figure 9. Trait variation for coevolved host-parasite combinations across time. (A) Host survival rate, (B) host population growth, (C) host body size, and (D) infection load. The combined hosts and parasites are always from the same contemporaneous replicate population of the coevolution treatment. Results for body size and infection load measures are only shown for alive worms. Infection load was adjusted by body size (see methods). The results for survival rate at host generation 20 and 28 differ significantly from the results for the ancestral combinations. Those for population growth at host generation 28 shows a trend of difference to the ancestral results (Table 7).

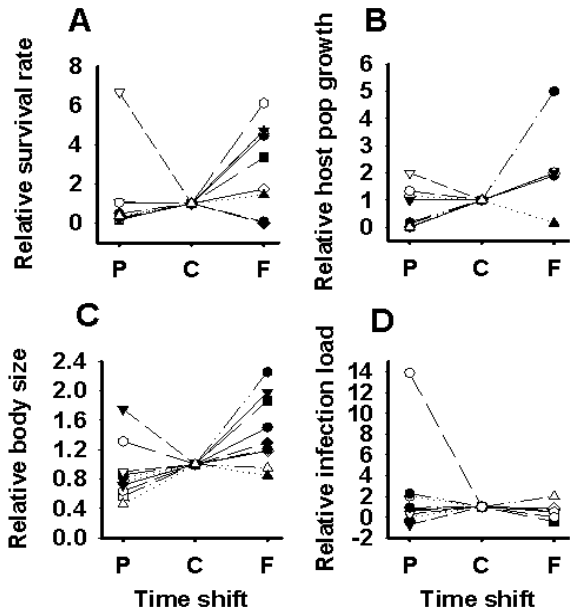

Figure 10. Time shift analysis for the host. (A) Relative survival rate, (B) relative population growth, (C) relative body size, and (D) relative infection load. In all cases, coevolved hosts from a particular time point were exposed to coevolved parasites from either the same time point (C, contemporaneous), the past (P), or the future (F). The combined hosts and parasites were always taken from the same replicate population of the coevolution treatment. The value for the contemporaneous combination was set to 1. Results for body size and infection load measures are only shown for alive worms. Infection load was adjusted by body size (see methods). The comparison between contemporaneous and future combinations differs significantly from chance for survival rate and infection load, while it shows a trend for body size (Table 8). The comparison between contemporaneous and past combinations varies significantly from a random distribution for population growth and body size, while it still shows a trend for survival rate (Table 8). Taken together, the results indicate that coevolved hosts perform better on parasites from the future than contemporaneous and also than those from the past.

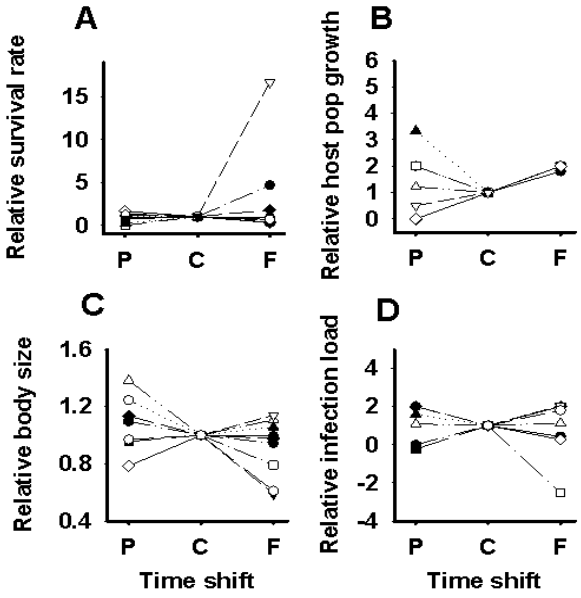

Figure 11. Time shift analysis for the parasite. (A) relative host survival rate, (B) relative host population growth, (C) relative host body size, and (D) relative infection load. In all cases, coevolved parasites from a particular time point were exposed to coevolved hosts from either the same time point (C, contemporaneous), the past (P), or the future (F). The combined parasites and hosts were always from the same replicate population of the coevolution treatment. The value for the contemporaneous combination was set to 1. Results for body size and infection load measures are only shown for alive worms. Infection load was adjusted by body size (see methods). For relative population growth, the comparison between contemporaneous and future combinations varies significantly from chance (see Table 8), indicating that parasites have a less severe effect on hosts from the future. All other comparisons between contemporaneous and either past or future combinations are insignificant.

Tables

Table 1. Comparison between evolved and ancestral hosts[1]

Trait	Treatment	Generation[2]	*F*	df	*P*
Survival rate	Coevolution	12	7.72	1,12	**0.0167**
		20	10.79	1,14	**0.0054**
		28	3.63	1,13	0.0788
	Adaptation	12	0.25	1,15	0.6210
		20	4.28	1,15	0.0562
		28	0.26	1,15	0.6118
	Control	12	0.19	1,14	0.6657
		20	2.52	1,16	0.1315
		28	2.08	1.14	0.1703
Host pop.	Coevolution	12	2.26	1,10	0.1633
growth		20	0.54	1,7	0.4841
		28	4.78	1,8	0.0602
	Adaptation	12	0.99	1,9	0.3445
		20	0.47	1.10	0.5047
		28	0.32	1,11	0.5782
	Control	12	0.00	1,9	0.9372
		20	1.49	1,10	0.2497
		28	0.25	1,10	0.6229
Host body.	Coevolution	12	0.29	1,11	0.5952
size		20	0.26	1,14	0.6142
		28	0.04	1,12	0.8380
	Adaptation	12	0.42	1,13	0.5242
		20	0.00	1,14	0.9963
		28	0.19	1,13	0.6638
	Control	12	0.18	1,12	0.6739
		20	0.00	1,15	0.9535
		28	0.02	1,12	0.8752
Host infection	Coevolution	12	1.12	1,12	0.3113

load		20	3.37	1,14	0.0877
		28	0.09	1,12	0.7733
	Adaptation	12	3.84	1,12	0.0737
		20	2.39	1,14	0.1447
		28	0.08	1,12	0.7801
	Control	12	1.06	1,11	0.3246
		20	2.23	1,15	0.1562
		28	0.03	1,12	0.8588

[1] Comparison between evolved and ancestral hosts both exposed to ancestral parasites using an analysis of variance. Degrees of freedom (df) are given for the comparison and the error (before and after comma, respectively). Significant values after FDR adjustment are given in bold.

[2] Time point is given as host generation number.

[3] Infection load is adjusted by body size.

Table 2. Regression analysis of the changes in host phenotypic measurements across time and treatments[1]

Experiment	***B. thuringiensis***	**Factor**	**df**	***F***	***P***
Host survival	Ancestral	Treatment	2	1.18	0.3155
	Ancestral	Generation	2	2.91	0.0641
	Ancestral	Treat.*Gene.	4	1.04	0.3941
Host pop. growth	Ancestral	Treatment	2	1.18	0.3191
	Ancestral	Generation	2	0.09	0.9096
	Ancestral	Treat.*Gene.	4	1.04	0.3635
Host body size	Ancestral	Treatment	2	0.58	0.5613
	Ancestral	Generation	2	0.48	0.6216
	Ancestral	Treat.*Gene.	4	1.85	0.1392
Host infection load	Ancestral	Treatment	2	0.05	0.9464
	Ancestral	Generation	2	2.86	0.0699
	Ancestral	Treat.*Gene.	4	0.37	0.8284
Host pop. growth	Non-pathogenic	Treatment	2	0.08	0.9186
	Non-pathogenic	Generation	2	0.80	0.4531
	Non-pathogenic	Treat.*Gene.	4	0.27	0.8901
Host body size	Non-pathogenic	Treatment	2	3.76	**0.0274**
	Non-pathogenic	Generation	2	0.37	0.6867
	Non-pathogenic	Treat.*Gene.	4	1.02	0.2904

[1] The defined models included evolution treatment, generation, the interaction between the two as fixed factors and replicate nested within treatment as a random factor. The specified models provide a better fit to the data than the corresponding minimal models ($P < 0.0001$). The table shows the results for the factor effect tests. For the host body size on non-pathogenic bacteria experiment, a posthoc test on treatments using Tukey HSD revealed a significant difference between host coevolution and the two other treatments (i.e., control and one-sided adaptation). Significant probabilities are given in bold.

Table 3. Comparison between evolved and ancestral parasites[1]

Trait	Treatment	Generation[2]	*F*	df	*P*
Killing rate	Coevolution	12	0.50	1,16	0.4858
		20	0.79	1,16	0.3869
		28	8.53	1,16	**0.0100**
	Adaptation	12	4.41	1,16	0.0517
		20	0.46	1,11	0.5086
		28	2.10	1,11	0.1748
	Control	12	215.88	1,15	**<0.0001**
		20	1715.68	1,15	**<0.0001**
		28	1542.75	1.14	**<0.0001**
Parasite impact on host pop. growth	Coevolution	12	1.02	1,14	0.3200
		20	1.26	1,14	0.2700
		28	2.94	1,14	0.1081
	Adaptation	12	3.20	1,14	0.0949
		20	1.64	1,10	0.2287
		28	0.79	1,8	0.3975
	Control	12	21.89	1,14	**0.0004**
		20	95.13	1,10	**<0.0001**
		28	18.70	1,14	**0.0007**
Parasite impact on host body size	Coevolution	12	4.89	1,14	0.0440
		20	4.19	1,13	0.0612
		28	11.84	1,15	**0.0036**
	Adaptation	12	0.00	1,14	0.9623
		20	0.27	1,10	0.6138
		28	0.08	1,10	0.7792
	Control	12	46.68	1,15	**<0.0001**
		20	111.18	1,11	**<0.0001**
		28	103.81	1,13	**<0.0001**
Parasite infection load	Coevolution	12	2.79	1,14	0.1168
		20	1.83	1,14	0.1967
		28	3.95	1,15	0.0653

Adaptation	12	4.10	1,13	0.0638
	20	3.40	1,8	0.1023
	28	5.18	1,8	0.0523
Control	12	3.42	1,15	0.0842
	20	2.26	1,11	0.1607
	28	3.2	1,13	0.0966

[1] Comparison between evolved and ancestral parasites both exposed to ancestral hosts using an analysis of variance. Degrees of freedom (df) are given for the comparison and the error (before and after comma, respectively). Significant values after FDR adjustment are given in bold.

[2] Time point is given as host generation number.

[3] Infection load is adjusted by body size.

Table 4. Regression analysis of the changes in parasite phenotypes across time and treatments[1]

Experiment	***C.elegans***	**Factor**	**df**	***F***	***P***
Parasite killing	Ancestral	Treatment	2	37.4	<0.0001
	Ancestral	Generation	2	8.07	0.0011
	Ancestral	Treat.*Gene.	4	1.77	0.1577
Parasite impact on	Ancestral	Treatment	2	17.9	<0.0001
host pop growth	Ancestral	Generation	2	2.46	0.0984
	Ancestral	Treat.*Gene.	4	0.27	0.5819
Parasite impact on	Ancestral	Treatment	2	17.3	<0.0001
host body size	Ancestral	Generation	2	6.08	0.0054
	Ancestral	Treat.*Gene.	4	1.64	0.1857
Parasite infection	Ancestral	Treatment	2	10.2	0.0003
load	Ancestral	Generation	2	1.05	0.3597
	Ancestral	Treat.*Gene.	4	1.10	0.3679

[1] The defined models included evolution treatment, generation, the interaction between the two as fixed factors and replicate nested within treatment as a random factor. The specified models provide a better fit to the data than the corresponding minimal models ($P < 0.0001$). The table shows the results for the factor effect tests.

Table 5. Fisher exact test of differences in bacterial biofilm formation and extinction rates[1]

Trait	**Treatment comparison**	**Generation**	df	N^2	*P*
Extinction rate	Coev. vs. Adapt.	12	1	10, 10	1.0000
	Coev. *vs.* Adapt.	20	1	10, 10	0.2105
	Coev. *vs.* Adapt.	28	1	10, 10	**0.0108**
Biofilm formation	Coev. *vs* Adapt	12	1	10, 9	**0.0031**
	Coev. *vs* Cont.	12	1	10, 10	**<0.0001**
	Cont. *vs.* Adapt.	12	1	10, 9	0.0867
	Coev. *vs.* Adapt	20	1	10, 7	**0.0034**
	Coev. vs. Cont.	20	1	10, 8	**<0.0001**
	Cont. *vs.* Adapt.	20	1	8, 7	0.2000
	Coev. *vs*. Adapt	28	1	10, 4	0.2857
	Coev. *vs*. Cont.	28	1	10, 8	**<0.0001**
	Cont. *vs.* Adapt.	28	1	8, 4	**<0.0182**

[1] Time point is given as host generation number. Significant values after FDR adjustment are given in bold.

[2] Sample sizes for first and second factor of the comparison, respectively.

Table 6. Regression analysis of the changes in parasite particle size across time and treatments[1]

Experiment	**Factor**	**df**	***F***	***P***
Parasite particle size	Treatment	2	19.68	**<0.0001**
	Generation	2	1.51	0.2297
	Treat.*Gene.	4	0.83	0.5103

[1] The defined models included evolution treatment, generation, the interaction between the two as fixed factors and replicate nested within treatment as a random factor. The specified models provide a better fit to the data than the corresponding minimal models ($P < 0.0001$). The table shows the results for the factor effect tests

Table 7. Mann-Whitney U test (MWU) of differences in bacterial colony size and density on NGM plates.

Trait	**Treatment comparison**		*U*	N[1]	*P*
Colony size	Coev. NB *vs.*	Adapt. NB	-3.36	23, 9	**0.0011**
	Coev. NB *vs.*	Adapt. B	6.52	23, 39	**<0.0001**
	Coev. NB *vs.*	Cont. B	5.92	23, 25	**<0.0001**
	Cont. B *vs.*	Adapt. NB	-4.37	25, 9	**<0.0001**
	Cont. B *vs.*	Adapt. B	-4.12	25, 39	**<0.0001**
	Adapt. NB *vs.*	Adapt. B	4.62	9, 39	**<0.0001**
Colony density	Coev. NB *vs.*	Adapt. NB	0.42	19, 13	0.6730
	Coev. NB *vs.*	Adapt. B	5.25	19, 19	**<0.0001**
	Coev. NB *vs.*	Cont. B	5.01	19, 16	**<0.0001**
	Cont. B *vs.*	Adapt. NB	-4.53	16, 13	**<0.0001**
	Cont. B *vs.*	Adapt. B	-3.75	16, 19	**<0.0001**
	Adapt. NB *vs.*	Adapt. B	4.71	13, 19	**<0.0001**

[1] Sample sizes for first and second factor of the comparison, respectively. Significant probabilities are given in bold.

Table 8. Statistical analysis of general differences in particle size among strains at different time points, using the non-parametric Kruskal-Wallis test

Time point	$\chi^2_{df=6}$	***P***
48h	31.25	**<0.0001**
72h	23.27	**0.0007**
96h	28.13	**<0.0001**
120h	29.73	**<0.0001**
144h	30.90	**<0.0001**

Probabilities, which are significant according to the false discovery rate, are given in bold

Table 9. Statistical analysis of differences in particle size between either coevolved non-biofilm or control biofilm forming clones and the remaining clones across time points, using the non-parametric Wilcoxon signed rank test

		Coevolved non-biofilm		**Control biofilm**	
Time	***B. thuringiensis***	$Z_{N=10}$	*P*	$Z_{N=10}$	*P*
48h	B-18246	2.09	0.0367	2.67	**0.0075**
	B-18247	2.51	**0.0122**	2.67	**0.0075**
	B-18679	2.09	0.0367	2.67	**0.0075**
	Coev. NB			2.67	**0.0075**
	Cont. B	-2.67	**0.0075**		
	Adapt. NB	-2.51	**0.0122**	2.24	**0.0254**
	Adapt. B	-1.67	0.0947	2.67	**0.0075**
72h	B-18246	2.30	**0.0216**	1.67	0.0947
	B-18247	2.51	**0.0122**	2.09	0.0367
	B-18679	-0.63	0.5309	-1.46	0.1437
	Coev. NB			-1.25	0.2101
	Cont. B	1.25	0.2101		
	Adapt. NB	0.63	0.5309	-1.04	0.2963
	Adapt. B	2.51	**0.0122**	1.88	0.0601
96h	B-18246	-2.09	0.0367	-2.51	**0.0122**
	B-18247	-2.09	0.0367	-2.51	**0.0122**
	B-18679	-2.30	**0.0216**	-2.51	**0.0122**
	Coev. NB			-2.51	**0.0122**
	Cont. B	2.51	**0.0122**		
	Adapt. NB	0.42	0.6761	-2.51	**0.0122**
	Adapt. B	2.51	**0.0122**	-1.04	0.2963
120h	B-18246	-2.51	**0.0122**	-2.51	**0.0122**
	B-18247	-2.30	**0.0216**	-2.51	**0.0122**
	B-18679	-2.30	**0.0216**	-2.51	**0.0122**
	Coev NB			-2.51	**0.0122**
	Cont. B	2.51	**0.0122**		
	Adapt. NB	-0.84	0.4034	-2.51	**0.0122**
	Adapt. B	2.51	**0.0122**	-0.42	0.6761

144h	B-18246	-2.51	**0.0122**	-2.51	**0.0122**
	B-18247	-2.51	**0.0122**	-2.51	**0.0122**
	B-18679	-2.51	**0.0122**	-2.51	**0.0122**
	Coev. NB			-2.51	**0.0122**
	Cont. B	2.51	**0.0122**		
	Adapt. NB	2.51	**0.0122**	-2.51	**0.0122**
	Adapt. B	2.51	**0.0122**	-1.04	0.2963

Probabilities, which are significant according to the false discovery rate, are given in bold

Table 10. Mann-Whitney U test (MWU) of differences in bacterial competition on NGM and on PF-NGM plates

Medium	**Treatment comparison**		*U*	N[1]	*P*
NGM	B/B	NB/NB	1.06	44, 45	0.2870
	B/B	NB/B	5.34	44, 59	**<0.0001**
	NB/B	NB/NB	4.86	59, 45	**<0.0001**
PF-NGM	B/B	NB/NB	1.02	14, 14	0.3071
	B/B	NB/B	-2.28	14, 23	**0.0221**
	NB/B	NB/NB	-1.48	23, 14	0.1333

[1] Sample sizes for first and second factor of the comparison, respectively. Significant probabilities are given in bold.

Table 11. Comparison between coevolved and ancestral host-parasite combinations[1]

Trait	**Generation**[2]	***F***	**df**	***P***
Survival rate	12	0.06	1,31	0.8142
	20	8.74	1,27	**0.0064**
	28	6.84	1,30	**0.0138**
Body size	12	0.21	1,28	0.6539
	20	0.12	1,24	0.7280
	28	1.43	1,26	0.2431
Population growth	12	0.14	1,19	0.7137
	20	0.70	1,15	0.4165
	28	6.37	1,18	0.0212
Infection load[3]	12	0.26	1.27	0.6137
	20	0.89	1,23	0.3541
	28	0.68	1,25	0.4164

[1] Comparison between coevolved host-parasite combination from a particular time point (second column) and ancestral host-parasite combinations using an analysis of variance. Degrees of freedom (df) are given for the comparison and the error (before and after comma, respectively). Significant values after FDR adjustment are given in bold.

[2] Time point is given as host generation number.

[3] Infection load is adjusted by body size.

Table 12. Statistical analysis of temporal specificity among coevolved hosts and parasites[1]

Focus	Trait	Comparison[2]	# smaller[3]	# larger[3]	$\chi^2_{df=1}$	*P*
Host	Survival rate	Past	9	3	3.14	0.0764
		Future	2	9	4.82	**0.0282**
	Infection	Past	5	4	0.11	0.7386
	load[4]	Future	6	1	3.96	**0.0465**
	Body size	Past	8	2	3.85	**0.0496**
		Future	2	7	2.94	0.0863
	Population	Past	1	7	5.06	**0.0245**
	growth	Future	6	4	0.40	0.5257
Parasite	Survival rate	Past	4	4	0	>0.99
		Future	6	3	1.02	0.3127
	Infection	Past	2	4	0.68	0.4097
	load[4]	Future	3	4	0.14	0.7050
	Body size	Past	3	4	0.14	0.7050
		Future	6	3	1.02	0.3127
	Population	Past	3	4	0.14	0.7050
	growth	Future	5	0	6.93	**0.0085**

[1] A likelihood ratio test was used to assess for each comparison whether the observed number of smaller and larger values is different from a random distribution.

[2] Comparison of contemporaneous combinations with those for which the focal interactor was combined with an antagonist from either the past (i.e., the preceding) or the future (i.e., the subsequent) time point.

[3] Number of comparisons where the combination with the past/future antagonist produced a value that was either smaller (first column) or larger (second column) than that for the contemporaneous combination.

[4] Infection load was adjusted by body size.

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Chapter II: A Stumbling Red Queen: Host-parasite coevolution handicapped by feeble males

Leila Masri, Rebecca Schulte, Nadine Timmermeyer, Stefanie Thanisch, Lena Crummenerl, Gunther Jansen, Nico K. Michiels, Hinrich Schulenburg

Abstract

The Red Queen hypothesis proposes that coevolving parasites select for outcrossing in the host. Outcrossing relies on males, which often show lower immune investment as a consequence of sexual selection. Our study revealed an interference of these two selective constraints. Two independent coevolution experiments with *Caenorhabditis elegans* and its microparasite *Bacillus thuringiensis* produced a decreased yet stable frequency of outcrossing male hosts. Subsequent tests verified that male *C. elegans* suffered from a direct selective disadvantage, as they were less resistant to pathogens. Yet males also offered an indirect selective advantage, because male-mediated outcrossing increased offspring resistance. As sex differences in immunity are widespread, the here reported interference of opposing selective forces is predicted to impose a fundamental limit to host adaptation during antagonistic coevolution.

Main text

Already Darwin pointed his finger at parasites as a potent selective force (Darwin 1859). They do not only decrease host fitness, but, relative to their antagonists, usually show higher potential for rapid adaptation (Woolhouse, Webster et al. 2002; Schulenburg, Kurtz et al. 2009). In the face of coevolving parasites, hosts should thus benefit from sexual reproduction and outcrossing, because these reproductive processes accelerate the combination of favorable alleles in spite of potential costs. This idea represents the central component of the Red Queen hypothesis (Hamilton 1980; Bell 1982; Lively 2010) and has found overwhelming theoretical and empirical support (Ebert, Altermatt et al. 2007; Gandon and Otto 2007; Salathe, Kouyos et al. 2008; Lively 2010; Mostowy, Salathe et al. 2010; Drikas, Dixon et al. 2011; King, Jokela et al. 2011). Current models focus on the high reciprocal selective pressures that the antagonists exert upon one another at the inter-species level. We here argue that intra-specific constraints are of additional importance, like those resulting from sexual selection in the host and predicted by Bateman's principle to cause sex differences in immunity (Rolff 2002). In particular, the sex with the higher reproductive potential, usually male, experiences increased fitness with increasing mating rates, whereas the sex with the lower reproductive potential, usually female, maximizes fitness through longevity, thus increasing the time available to produce the more costly gametes and find high quality mates. In response to these sex-specific constraints, females are predicted to invest more in immune function than males (Rolff 2002).

We studied the relevance of sex differences in immunity during the evolutionary interaction with parasites using the nematode *Caenorhabditis elegans* as host and the Gram-positive bacterium *Bacillus thuringiensis* as its microparasite (Schulte, Makus et al. 2010; Schulte, Makus et al. 2011; Schulte, Hasert et al. 2012). *C. elegans* has an androdioecious reproductive system defined by two genders, hermaphrodites and males (Brenner 1974). Hermaphrodites are modified females that first produce sperm, which are stored for self-fertilization of the subsequently produced eggs. Outcrossing is only possible between hermaphrodites and males. As male sperm outcompete hermaphrodite sperm (LaMunyon and Ward 1995), male-hermaphrodite matings result in complete cross-fertilization and subsequent 1:1 sex-ratios. Male

offspring may also be produced through spontaneous X chromosome non-disjunction during meiosis, usually at very low rates of no more than 0.005 (Hodgkin and Doniach 1997; Teotonio, Manoel et al. 2006). Thus, male frequency is often taken as a direct indicator for the outcrossing rate in *C. elegans* populations (Teotonio, Manoel et al. 2006). In the following, we will start with the observation of varying male frequencies during host-parasite coevolution and then continue with a detailed analysis of the constraints that either favor or disfavor males during their interaction with parasites.

Male frequencies were thus scored in nematode populations from two fully independent evolution experiments: a previously published experiment (Schulte, Makus et al. 2010), in which male abundance was recorded but not yet reported, and a new evolution experiment (supplementary online material, SOM). Both comprised a coevolution treatment, during which host and parasite were forced to co-adapt to each other, and a control treatment, during which hosts evolved in the absence of the antagonist (fig. S1, SOM). In apparent contrast to the Red Queen hypothesis, male frequencies were consistently lower under coevolution than control conditions across both evolution experiments ($F > 16$, $P < 0.0001$; Fig. 1A, 1B, table S1).

Following Bateman's principle, we then hypothesized that the lower male frequencies might be due to lower male pathogen resistance (Rolff 2002). To test this hypothesis, we assessed survival and infection load as proxies for pathogen resistance in a random nematode sample from the genetically diverse starting population of the second evolution experiment (SOM). Indeed, male survival was lower than hermaphrodite survival in the presence of pathogenic *B. thuringiensis* ($Z = 2.06$, $P = 0.039$; Fig. 2A, table S2), but not under non-pathogenic conditions ($Z = 0$, $P > 0.99$; Fig. 2A). Similarly, males were more heavily infected than hermaphrodites ($Z = 2.22$, $P = 0.026$; Fig. 2B).

We next asked whether pathogen exposure affects other traits known to influence male frequencies. One of these traits is male mating activity (SOM), which positively correlates with male abundance due to the androdioecious mating system and the competitive superiority of male sperm (LaMunyon and Ward 1995; Wegewitz, Schulenburg et al. 2008). Indeed, the average number of males that either mated or courted hermaphrodites was lower under pathogenic than control conditions ($Z = 4.43$, $P < 0.001$; Fig. 2C, table S2). Moreover, the decreased male

sexual activity translated into lower male numbers in the offspring generation (Z = 3.81, $P < 0.001$; Fig. 2D).

Another relevant trait is male-specific behavioral escape from pathogens (Schulenburg and Ewbank 2007), which might limit availability of males for outcrossing. Our analysis of this trait (fig. S2) revealed an intricate behavioral response contingent on gender type, presence of the pathogen, and potential mates (Fig. 3, table S3). In the treatments with only one of the genders, male nematodes fled from non-pathogenic bacteria more strongly than did hermaphrodites ($Z > 4.4$, $P < 0.0001$), whereas both avoided pathogens at similar rates ($Z < 0.9$, $P > 0.3$). This observation is consistent with previous reports of male roaming behavior aimed at finding mates (Lipton, Kleemann et al. 2004). Importantly, in mixed gender groups that reflected the conditions of the evolution experiment almost 100% of males escaped from pathogens, significantly more than observed for hermaphrodites (Z = 3.57, $P = 0.0003$; Fig. 3).

We conclude that reduced male resistance could have lowered male frequencies under pathogenic conditions. The observed decrease in male sexual activity might be a direct consequence of the lower male resistance, yet it may have still amplified the effect on male abundance through the reduced production of males in the offspring generation. Enhanced male escape behavior may have additionally biased sex ratio, even though its exact importance during the evolution experiment is unknown (SOM). Even in case of only one disadvantage (e.g., reduced male resistance), male abundance should have rapidly fallen to values close to zero. Yet males are present at decreased but relatively stable frequencies under coevolution conditions. We envisage two non-exclusive simple explanations for this: the spontaneous generation of males through X chromosome non-disjunction (Hodgkin, Horvitz et al. 1979) and male immigration. The latter was part of the design of both evolution experiments to prevent the random loss of genetic diversity (SOM).

To explore the relevance of these two factors in male maintenance we developed a simple mathematical model (SOM) directly following a previously established model on *C. elegans* sex ratio variation (Chasnov and Chow 2002). Male abundance is calculated as a function of male-hermaphrodite mating frequencies and the non-disjunction rate, taking into account the proportion of immigrant males and the relative male "performance" (SOM). We assume that the latter factor, q, is determined by male resistance, given as the average survival rate of males relative to

hermaphrodites (0.7 in the presence of pathogens; Fig. 2A). Based on this model, simulated male frequencies rapidly dropped to below 1% under pathogenic conditions (Fig. 1C). Even if we assumed less severe reductions of male performance (0.9 or 0.95), then the proportion of males still decreased to below 3% within twelve generations (Fig. 1C), thus clearly below the values observed during experimental coevolution (Fig. 1A, 1B). We consider the simulated results to be conservative because the model disregarded the observed reductions in male sexual activity under pathogenic conditions and higher male escape from pathogen, both of which may accelerate male loss under coevolution conditions.

Consequently, the stable maintenance of males at a frequency of more than 10% requires a different explanation. One of the likely alternatives is provided by the Red Queen hypothesis: males confer an indirect benefit if male-dependent outcrossing increases offspring resistance. We tested this explanation by comparing the pathogen resistance of offspring from outcrossed versus selfed nematodes. Offspring were assayed in the F2 generation after having gone through one generation of selfing to remove potential maternal effects associated with outcrossing. The comparison was performed twice by different experimenters in different locations using small differences in the exact protocol (SOM). Both comparisons consistently demonstrated that outcrossing leads to more pathogen resistant F2 offspring than selfing ($Z > 2.2$, $P < 0.025$; Fig. 4; table S4). Such differences were not observed in the presence of non-pathogenic bacteria ($Z < 0.4$, $P > 0.7$; Fig. 4; table S4).

We conclude that the direct selective disadvantage of males was coupled with an indirect selective benefit, which were jointly responsible for the observed lowered yet stable male frequencies during the coevolution experiment. What are the evolutionary consequences of the two opposing selective constraints? The published results of the first evolution experiment reveal that decreased male abundance coincides with a small decrease in recombination rates (Schulte, Makus et al. 2010). At the same time, the indirect male benefit correlates with elevated resistance of the coevolved hosts compared with control hosts (Schulte, Makus et al. 2010). Thus, the reduced frequencies of males and recombination events are sufficient for the host to keep up with a coevolving parasite, consistent with theoretical models (Agrawal and Lively 2001). Nevertheless, the increase in resistance appears moderate (Schulte, Makus et al. 2010). We speculate that this may have been caused by the lower male abundance and the resulting decreased generation of new favorable host genotypes.

How widespread is the interference of these selective forces? Our study demonstrates their combined action when host sexes differ in immunity. For *C. elegans* this may depend on the pathogen. A recent evolution experiment revealed increased male frequencies in the presence of *Serratia marcescens* pathogens compared to non-pathogenic controls, suggesting high male resistance even though this trait was not directly measured (Drikas, Dixon et al. 2011). In general, however, sex differences in immunity are predicted by Bateman's principle to be common among animal hosts (Rolff 2002), and are indeed observed across a large variety of vertebrate and insect taxa (Nunn, Lindenfors et al. 2009). In all of these cases, the higher susceptibility of males imposes a fundamental constraint on the potential for outcrossing and fast adaptive responses in the host. This constraint may have contributed to previous observations of limited change, not only in our *C. elegans* model (Schulte, Makus et al. 2010), but also in other taxa like coevolving beetle or fly hosts (Green, Kraaijeveld et al. 2000; Berenos, Schmid-Hempel et al. 2012). The explicit consideration of these intra-specific variations is thus predicted to be essential for full appreciation of host-parasite coevolutionary dynamics.

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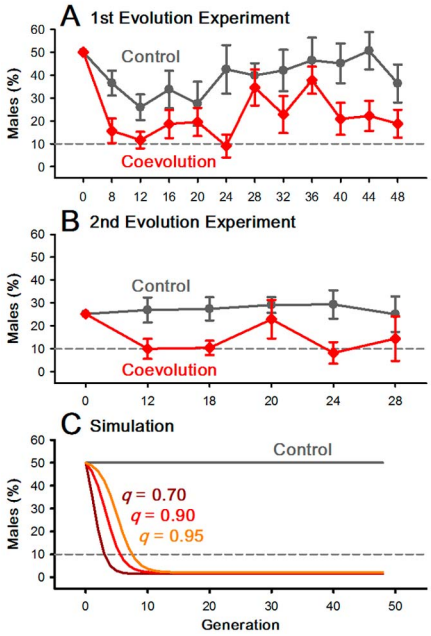


Figure 1. Male frequency over time. Frequencies are shown across (**A**) the first evolution experiment, (**B**) the second evolution experiment, and (**C**) simulations. Both evolution experiments (fig. S1) included a coevolution treatment, in which host and parasite were forced to co-adapt to each other (red), and a control, in which the host evolved in the absence of the antagonist (gray). Data in (A) and (B) are given as means with 2× standard errors of the mean (SEM). The simulations were based on a simple mathematical model that defines male frequency as a function of male-hermaphrodite matings, dependent on the male-hermaphrodite ratio, and relative male performance on pathogens (q). Values for q are varied from low to high, as indicated. The dashed gray line indicates a male frequency of 10% for comparisons across panels.

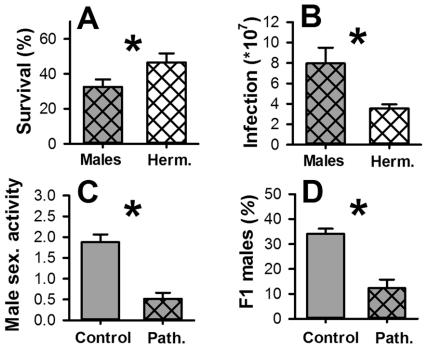


Figure 2. Pathogen resistance and male sexual activity. (**A**) Host survival. (**B**) Host infection load. (**C**) Male sexual activity, measured as the average number of males either courting or copulating with a hermaphrodite. (**D**) Resulting male frequency in the F1 generation. Bars show means with 2× SEM; asterisks indicate significant differences.

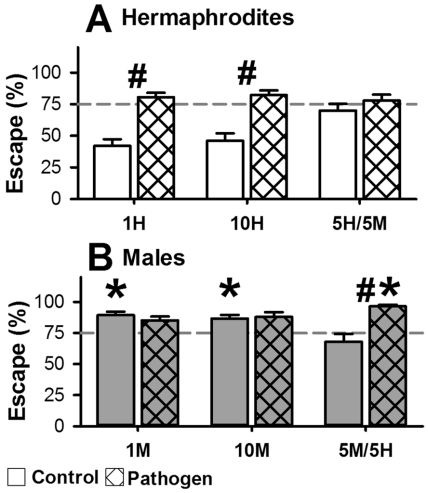


Figure 3. Escape behavior for **(A)** hermaphrodites and **(B)** males. Escape was evaluated in response to either pathogens (patterned boxes) or non-pathogens (solid boxes) for single worms, ten worms of the same gender, or ten worms of 50% hermaphrodites (H) and 50% males (M). Bars show means with 2× SEM. Asterisks in (B) indicate the treatments for which males and hermaphrodites show significant differences. Crosses in (A) and (B) point to significant differences between pathogen and control treatments for a particular sex and group composition. The dashed gray line indicates an escape of 75% for comparisons across panels.

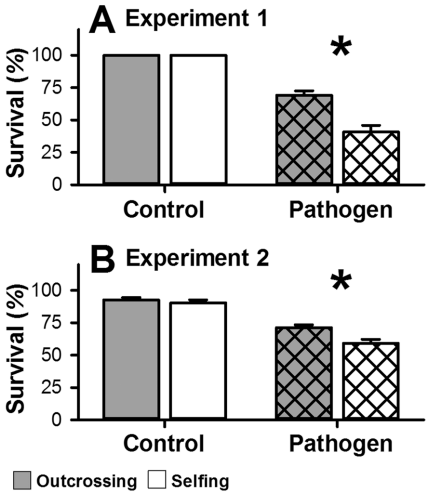


Figure 4. Survival of progeny from outcrossed versus selfed grand-parents. (**A**) First experiment. (**B**) Second experiment. Survival was measured under either pathogenic or control conditions for the F2 generation, derived from either outcrossed or selfed nematodes, including one generation of selfing in the F1 to remove potential parental effects caused by mating. Bars represent means with 2× SEM and asterisks indicate significant differences.

Supporting Online Material

Evolution experiments

- *First evolution experiment*

The methods for performance of the first evolution experiment were described previously (Schulte, Makus et al. 2010). Central aspects of its design differed from those of the second evolution experiment (see below), including: (i) the genetic diversity of the starting population was smaller; this starting population was derived from repeated reciprocal crosses of three natural isolates (MY8, MY15, and MY18), which covered a comparatively large, but still smaller proportion of the total worldwide genetic diversity than the starting population of the second experiment. (ii) The size of the host population was set to 120 individuals at each transfer step and was thus smaller than that of the second experiment. (iii) Males were added every fourth host generation to minimize the risk of random loss of genetic diversity; the male immigration rate was set to 5% of the total population size every fourth host generation. (iv) The temperature was lower for the first (16-18 °C) than for the second experiment (19 °C). As a consequence, the generation time was higher (first experiment: approx. 3-4 days; second experiment: approx. 2-3 days). (v) The two experiments were performed by different experimenters in different locations (first experiment: Institute of Evolution and Biodiversity at the University of Muenster, Germany; second experiment: Department of Animal Evolutionary Ecology at the University of Tuebingen, Germany), using the available equipment and climate chambers at the different departments. (vi) The first evolution experiment comprised 20 replicates and the second evolution experiment ten replicates per treatment.
In the first experiment, male frequencies were recorded at each second transfer step (every fourth host generation).

- *Second evolution experiment*

The starting *Caenorhabditis elegans* host population was obtained from Henrique Teotonio, Lisboa, Portugal, who prepared it for use in *C. elegans* evolution experiments. This population is genetically diverse and outbred. It was originally

derived from consecutive crosses among 16 natural isolates, which cover almost the entire worldwide spectrum of genotypes known for this species (strains PB306, AB1, CB4858, CB4855, N2, JU400, MY16, JU319, PX174, MY1, PX179, JU345, CB4856, CB45507, RC301, and CB4852). This population was adapted to our experimental conditions over ten generations in 40 replicates at 19 ºC in the presence of the non-pathogenic *Bacillus thuringiensis* strain DSM-350. This step aimed at minimizing the impact of selective constraints unrelated to the host-parasite interaction during the main evolution experiment. After the ten generations, the 40 populations of adapted worms were mixed and cryo-preserved in glycerol at -80 ºC (Stiernagle 2006) in 200 aliquots, each containing an average of approximately 5000 worms. The aliquots were later thawed for usage during the evolution experiment and the subsequent phenotypic assays.

For the microparasite *B. thuringiensis*, the starting population was identical to the one used for the first evolution experiment (Schulte, Makus et al. 2010). It consisted of a mixture of strains, including the three nematocidal strains B-18246, B18247, and B-18679 (provided by the Agricultural Research Service Patent Culture Collection, United States, Department of Agriculture) (Schulte, Makus et al. 2010). The non-pathogenic *B. thuringiensis* strain DSM-350 (German Collection of Microorganisms and Cell Culture) was used for the host control treatment. Prior to the evolution experiment, we also prepared *B. thuringiensis* cultures in large quantities following established protocols (Borgonie, van Driessche et al. 1995). These were aliquotted in phosphate-buffered saline (PBS) buffer and stored at -20 ºC for later use. For the evolution experiment, *B. thuringiensis* mixtures and the DSM-350 control were each used at a final concentration of 1.2×10^8 particles/ml. They were always supplemented with the standard nematode food bacterium, *Escherichia coli*, at a final concentration of 2×10^9 cells/ml.

For experimental evolution, we modified the previously published protocol (Schulte, Makus et al. 2010). The experimental temperature was increased from 16-18 ºC to 19 ºC to reduce host generation time (from 3-4 to 2-3 days). Host population size at each transfer step was increased from 120 to 500 individuals to reduce the risk of drift effects. For the same reason, we simulated migration of the host (but not the parasite) by adding 2.5% males and 2.5% hermaphrodites of original host genotype to the

worm populations at every second transfer step. Host and parasite were transferred to new wormballs twice per week (equivalent to one host generation per transfer step).
Both evolution experiments included the following two treatments (Schulte, Makus et al. 2010) (fig. S1): (i) host-parasite coevolution, during which both antagonists were continuously forced to coevolve with each other, and (ii) host control, during which the host adapted to the conditions of the experiment in the absence of pathogenic *B. thuringiensis*. The exact methods for these two treatments are provided in the previous publication (Schulte, Makus et al. 2010). Each treatment was replicated 10 times. The entire evolution experiment was run over 28 host generations.
The proportion of males was recorded for the replicate populations across the evolution experiment. A random sample of each population was collected at host generation 0, 12, 18, 20, 24, and 28, and subjected to alkaline hypochlorite:NaOH treatment, subsequently grown under pathogen-free conditions for approximately 2 generations, and then frozen in glycerol at -80 ºC (Stiernagle 2006). After completion of the evolution experiment, frozen samples were thawed and grown on peptone-free nematode growth medium (PFM) in the absence of pathogens. After approximately one generation, worms were washed off the plates with 2.5 ml H_2O. Three 10-µl subsamples were used to count the number of males versus hermaphrodites and thus to determine the average proportion of males per replicate.

Systematic analysis of male hosts

We compared the response to pathogens of males and hermaphrodites by studying, in independent experiments, their (i) survival rate, (ii) infection load, (iii) male sexual activity, and (iv) escape behavior. Survival rate, male sexual activity, and escape behavior were assessed in either the presence or absence of pathogenic *B. thuringiensis*. The experiments were performed with randomly chosen, fourth larval stage (L4) nematodes from the starting population of the second evolution experiment. Pathogenic *B. thuringiensis* always consisted of exactly the same mixture of genotypes used in the two evolution experiments. The *B. thuringiensis* strain DSM-350 served as a non-nematocidal control (Schulte, Makus et al. 2010).

- *Host survival rate assay*

Survival rate was measured as a proxy for resistance *sensu lato* (Schulte, Makus et al. 2010; Wang, Nakad et al. 2012). The assay was conducted on 6-cm PFM plates, covered with 100 µl of a 1:10 mixture of pathogenic *B. thuringiensis* (final concentration of 1.2 x 10^8 particles/ml) and *E. coli* OP50 (final concentration: 2 x 10^9 cells/ml). To assess a possible increase in male mortality due to male – male competition when maintained in single sex groups (Gems and Riddle 2000), a control treatment was established using a mixture of the non-pathogenic *B. thuringiensis* DSM-350 (final concentration of 1.2 x 10^8 particles/ml) and *E. coli* OP50 (final concentration: 2 x 10^9 cells/ml). *E. coli* OP50 was added to ensure that the worms had sufficient nutrition such that mortality results from the interaction with the pathogen but not starvation. About 10 days before the assay, the worms were thawed and grown for at least two generations, thus minimizing possible influences of freezing. Three days before the experiment started worms were bleached to age-synchronize them (Stiernagle 2006). Once the worms reached the fourth juvenile stage (L4), 10 individuals, either all males or all hermaphrodites, were transferred to specific assay plates and the survival rate was measured 48 h later. Worms were considered dead if they did not respond to touch with a worm-picker. In total, we examined 15 replicates per gender under pathogenic and five under non-pathogenic conditions.

- *H*

ost infection load assay

As an additional measure of resistance, we determined the pathogen load of exposed hosts (Boehnisch, Wong et al. 2011; Wang, Nakad et al. 2012). 6-cm PFM plates were covered with 100 µl of the *B. thuringiensis – E. coli* mixture (final concentrations as above). Either ten L4 males or ten L4 hermaphrodites were added to a given plate (total of 15 replicates per treatment). Three to five dead worms per plate were picked after 48 h, transferred onto 12-well microscopic slides, and then photographed for subsequent body size measurements using ImageJ (http://rsb.info.nih.gov/ij/). These nematodes were repeatedly washed with 15-20 µl sterile H_2O under a stereomicroscope to remove bacteria adhering to the cuticle. The worms from a given replicate were transferred into 1.5-ml tubes containing 100 µl H_2O. The number of externally associated bacteria that could not be removed was estimated by counting cells in

the surrounding solution. We took the average of 3 counts, using standard counting chambers (0.1 mm depth). For each replicate, bacteria were then extracted for the group of worms. The samples were sonicated for 10 sec, 6 cycles at 60 Hz to break the worm cuticle. 3-4 1 mm Zirconia beads were added and the samples were further homogenized by vortexing for 3 sec. The number of bacteria was determined using counting chambers as described above. The infection load was calculated per size-adjusted nematode and replicate by first subtracting the average number of bacteria in the surrounding H_2O from the average number of bacteria extracted from the worms of a particular replicate. This value was then divided by the number of worms in a replicate and by the average size of the worms included. Note that some of the replicates were not considered in the final analysis because of insufficient number of transferrable dead animals (less than three dead worms).

- *M*

ale sexual activity and resulting F1 male frequency

We tested whether pathogen exposure influenced the sexual activity of *C. elegans* males, resulting in a biased sex ratio in subsequent generations (Lopes, Sucena et al. 2008). The assay was performed on 3-cm PFM plates seeded with 50 µl of the *B. thuringiensis – E. coli* mixture, containing either the pathogenic or the non-pathogenic *B. thuringiensis* (final concentrations as above). Male sexual activity was assayed by adding three immature L4 males and three L4 hermaphrodites to a given plate. After 24 h, the number of scanning and mating adult males per plate was counted six times approximately every 45 min. The average of the six counts per assay plate and male was used as a proxy for male sexual activity and compared among treatments. A total of 20 replicates for the pathogen and 23 for the control treatment were included in the analysis. The consequences of male sexual activity on male frequency in the next generation were studied by scoring the sex of 100 randomly chosen F1 offspring grown to the adult stage. This analysis was based on 14 replicates of the pathogen and 13 of the control treatment.

- *H*

ost escape behavior assay

C. elegans expresses a strong pathogen-specific escape response, most likely to minimize infection risk (Schulenburg and Ewbank 2007) Sex-specific differences in this response might limit the availability of males for outcrossing and thus bias the offspring sex ratio. Note that the evolution experiment was done using so-called wormballs, which provide an environment that minimizes nematode escape responses against pathogens (Schulte, Makus et al. 2010). However, it cannot as yet be excluded that escape responses occur within this environment and thus influence mating rates. Variation in pathogen escape was studied using a modification of the previously described pathogen avoidance assay (Hasshoff, Bohnisch et al. 2007). A small spot of the tested bacteria (40 µl of the *B. thuringiensis* – *E. coli* mixture, final concentrations as above) was pipetted into the center of a 9-cm PFM plate and left to dry. The tested bacterium was mixed with *E .coli* to reduce the likelihood that worms left the test spot due to absence of food (Hasshoff, Bohnisch et al. 2007). Additionally, an outer "food ring" was established by pipetting 5 x 20 µl of *E. coli* on the plate's boundaries and dispersing it along the edges using an L-shaped sterile glass pipette. This outer food ring served to minimize the likelihood that escaped worms might return to the central spot because of food deprivation. One day later, individual L4 nematodes were placed into the middle of each central spot with a worm picker in the following combinations: 1 hermaphrodite, 1 male, 10 hermaphrodites, 10 males, or a mixture of 5 hermaphrodites and 5 males. These various combinations were considered to evaluate a possible bias in escape caused by presence or absence of conspecifics, which is known to influence nematode behavior, especially that of males (Gems and Riddle 2000; Lipton, Kleemann et al. 2004; Chasnov, So et al. 2007). The escape rate was determined in response to either pathogenic or non-pathogenic *B. thuringiensis*. It was scored as the percentage of worms that left the central bacterial lawn after 24 h and based on a total of 15 replicates per treatment.

Simple model on male frequencies under pathogenic or non-pathogenic conditions

To explain why males do not disappear from the experimental population, we adjusted a previously described mathematical model on *C. elegans* sex ratio

evolution (Chasnov and Chow 2002). The male proportion in the next generation, m', is here defined as: $m' = 0.5 \times \left(\frac{m+i}{h} \right) \times q + \left[1 - \left(\frac{m+i}{h} \right) \times q \right] \times u$.

The first term refers to the proportion of male offspring resulting from hermaphrodite-male matings. The occurrence of matings is dependent on the male ratio, which is the sum of the proportions of already present males, m, and immigrant males, i, divided by the proportion of hermaphrodites, h. The mating frequency is additionally influenced by the relative male "performance" in the presence of pathogens, q (i.e., a trait dependent on male pathogen resistance, male sexual activity, or male pathogen escape behavior). The entire term is set to a maximum of 0.5, which represents the maximum proportion of males produced through matings following Mendelian genetics. The second term refers to the proportion of male offspring produced through non-disjunction. It is determined by the non-disjunction rate, u, and restricted to the proportion of offspring that does not result from male-hermaphrodite mating. This latter term is set to a minimum of zero to exclude negative values for spontaneously produced males.

For simplicity, initial proportions of males and hermaphrodites (m_0 and h_0) are assumed to be 0.5. The male immigration rate, i, is set to 0.0125, which represents the average per generation used in both evolution experiments. We used a non-disjunction rate, u, of 0.005, which is above the value recorded for almost all natural isolates (Hodgkin and Doniach 1997; Teotonio, Manoel et al. 2006). We further assume that the relative male performance in the presence of pathogens, q, is exclusively determined by male resistance. Under pathogenic conditions, q is thus defined as the average survival rate of males relative to hermaphrodites (0.70; Fig. 2A of main text), while it is set to 1 under non-pathogenic conditions. As an alternative, we also assumed higher male performance under pathogenic conditions by setting q to either 0.90 or 0.95. Simulations were run across 50 host generations.

Resistance of offspring from self-fertilized or outcrossed hermaphrodites

The resistance of offspring derived from either selfed or outcrossed hermaphrodites was studied with the help of two independent experiments, performed by different experimenters in different locations: Experiment 1 at Tübingen University and experiment 2 at Kiel University. The experiments also differed in the degree of

pathogenicity expressed by the pathogenic *B. thuringiensis* (see below). In both cases, resistance was compared between the F2 offspring of selfed or outcrossed hermaphrodites that subsequently went through one generation of selfing to minimize potential effects on resistance due to mating, which may be caused by male-induced harm, as observed previously (Gems and Riddle 1996; Wegewitz, Schulenburg et al. 2008).

- *Experiment 1*

To generate selfed or outcrossed offspring, we added either only two hermaphrodites or a mixture of four males and two hermaphrodites to a given 3 cm PFM plate seeded with 50 µl of *E. coli* mixed with the non-pathogenic *B. thuringiensis* (concentrations as above). After 72 h, ten virgin L4 hermaphrodites of the F1 generation were transferred to 6-cm PFM plates containing the non-pathogenic *B. thuringiensis* – *E. coli* mixture and allowed to reproduce by selfing. Once the F2 generations reached the L4 stage, ten hermaphrodites per replicate were either exposed to the pathogenic or the non-pathogenic *B. thuringiensis* – *E. coli* mixtures seeded onto PFM plates (concentrations as above). Survival was scored after 48 h. Animals were considered dead if they did not respond to touch. Each treatment combination was replicated 25 times.

- *Experiment 2*

The parental generation was allowed to produce offspring through selfing or outcrossing by adding either only six hermaphrodites or a mixture of three males and three hermaphrodites, respectively, to a given 3-cm PFM plate containing 150 µl of the non-pathogenic *B. thuringiensis* – *E. coli* mixture (concentration as above). Virgin F1 L4 hermaphrodites were transferred to new plates and allowed to reproduce by selfing. Six L4 hermaphrodites per replicate of the resulting F2 generation were exposed to either the pathogenic or non-pathogenic bacterial mixtures, which were seeded in 300-µl volumes on 9-cm PFM plates. Prior to the assay, *B. thuringiensis* was maintained at room temperature, resulting in reduced pathogenicity of the pathogen mixture. Survival of the F2 individuals was scored

after 120-h exposure. A total of 40 replicates was included per treatment combination.

Statistical analysis

Variations in male frequency across each of the two evolution experiments were assessed with a general linear model including generation and treatment as fixed factors, and replicate nested within treatment as random factor. The Wilcoxon rank sum test was used to assess variations between males and hermaphrodites or between pathogen and control treatments in all other assays. In case of multiple testing and thus increased type I errors for analysis of the escape assay, the significance level was adjusted using the false discovery rate (Benjamini and Hochberg 1995). Statistical analyses were performed with the program JMP IN, version 9.0 (SAS Institute Inc.) and graphs were generated with SigmaPlot version 12.0 (Systat Software Inc.).

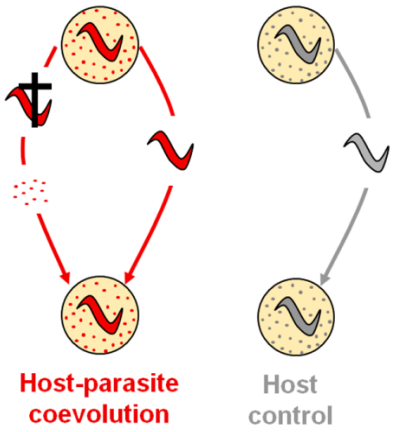


Figure S1. Design of the two evolution experiments. The central treatment is coevolution, given in red, for which host and parasite were forced to co-adapt to each other. The control treatment is indicated in gray and involved evolution of the host under experimental conditions in the absence of the antagonist.

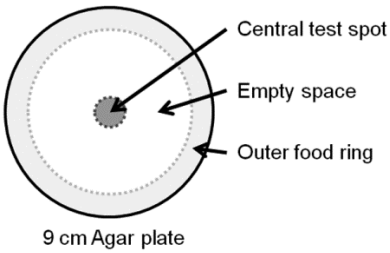


Figure S2. Schematic drawing of a test plate for the escape behavior assay. The center contains a spot of the tested bacteria (either pathogenic or non-pathogenic *B. thuringiensis*, in both cases mixed with *E. coli* OP50; dark shading), covering an area of up to 1.2 cm from the midpoint. The assayed worms are added to this bacterial spot at the beginning of the experiment. The plates also contain an outer "food ring" of *E. coli* OP50 at a distance of 3.5–4.5 cm from the center (light shading). This food ring serves to prevent escaped worms from returning to the central spot if they are hungry.

Table S1. Regression analysis of the changes in male frequency across time and treatments in the two evolution experiments[1]

Experiment	**Factor**	**df**	***F***	***P***
1st evolution experiment	Treatment	1	19.12	**< 0.0001**
	Generation	10	7.25	**< 0.0001**
	Treatment*Generation	10	2.82	**0.0021**
2nd evolution experiment	Treatment	1	16.56	**< 0.0001**
	Generation	4	2.11	0.0912
	Treatment*Generation	4	2.03	0.1021

[1] The defined models included evolution treatment, generation, the interaction between the two as fixed factors and replicate nested within treatment as a random factor. The specified models provide a better fit to the data than the corresponding minimal models ($P < 0.0001$). The table shows the results for the factor effect tests. Significant probabilities are given in bold.

Table S2. Wilcoxon rank sum test of the variation observed in resistance and male sexual activity.[1]

Trait	**Comparison**	***Z***	**N**[2]	***P***
Survival on control	Males – Hermaphrodite	0	5, 5	1.000
Survival on pathogen	Males – Hermaphrodite	2.06	15, 14	**0.0394**
Infection load in dead hosts	Males – Hermaphrodite	-2.22	15, 12	**0.0264**
Male sexual activity	Pathogen – Control	4.43	20, 23	**< 0.0001**
F1 male frequency	Pathogen – Control	3.81	14, 13	**0.0001**

[1] Resistance is measured as survival rate and infection load (top three rows). Male sexual activity (bottom two rows) considers the scanning and copulatory behavior of males (trait male sexual activity) and also the resulting frequency of males in the F1 generation (F1 male frequency).

[2] Sample sizes for first and second factor of the comparison.

Table S3. Wilcoxon rank sum test of the variation observed in avoidance behaviour.[1]

Considered groups	Comparison	*Z*	N[2]	*P*[3]
1 herm.	Pathogen – Control	4.22	15, 16	**< 0.0001**
10 herm.	Pathogen – Control	-4.03	15, 15	**< 0.0001**
5 herm. + 5 males	Pathogen – Control	-1.11	15, 14	0.2680
1 male	Pathogen – Control	1.12	15, 15	0.2629
10 males	Pathogen – Control	-0.45	16, 15	0.6520
5 males + 5 herm.	Pathogen – Control	-3.76	15, 14	**0.0002**
Path: 1 individual	Male - Hermaphrodite	0.64	15, 15	0.5219
Path: 10 individuals	Male – Hermaphrodite	-0.86	16, 15	0.3914
Path: Mixed groups	Male – Hermaphrodite	3.57	15, 15	**0.0003**
Control: 1 individual	Male – Hermaphrodite	4.58	15, 16	**< 0.0001**
Control: 10 individuals	Male – Hermaphrodite	4.44	15, 15	**< 0.0001**
Control: Mixed groups	Male – Hermaphrodite	-0.05	14, 14	0.9627

[1] Avoidance behavior was analyzed separately either for males and hermaphrodites (abbreviated herm.; top six rows) or for the different group sizes on pathogen or control (bottom six rows).

[2] Sample sizes for first and second factor of the comparison.

[3] Because groups were included in several comparisons, leading to an increased type I error, the significance was adjusted using the false-discovery rate. Significant probabilities after adjustment are given in bold.

Table S4. Wilcoxon rank sum test of the variation in the F2 survival rate between outcrossed and selfed parents.

Experiment	**Condition**	***Z***	**N**[1]	***P***[2]
Experiment 1	Control	0	20, 19	> 0.9999
	Pathogen	3.72	20, 19	**0.0002**
Experiment 2	Control	-0.32	24, 23	0.7484
	Pathogen	2.24	24, 27	**0.0248**

[1] Sample sizes for outcrossed and selfed offspring groups.

[2] Significant values are indicated in bold.

Chapter III: Ecological functional genomics of the model pathogen *Bacillus thuringiensis* during experimental coevolution with its nematode host *Caenorhabditis elegans*

Antoine Branca, Anna Sheppard, Leila Masri, Manja Saebelfeld, Heiko Liesegang, Elzbieta Brzuszkiewicz, Rolf Daniel, Joachim Kurtz, Philip Rosenstiel, Erich Bornberg-Bauer, Hinrich Schulenburg

Abstract

Bacillus thuringiensis is a pathogenic bacterium of invertebrates with a wide host spectrum, including the nematode *Caenorhabditis elegans*. We studied its ability to adapt to the nematode host with the help of an evolution experiment, during which a genotype mixture of *B. thuringiensis* was either coevolved with *C. elegans* (coevolution treatment), adapted to a non-changing host population (one-sided adaptation), or evolved in the absence of the host (control evolution), each replicated ten times. Three main outcomes were observed: (i) high virulence, low intra-host replication and lack of biofilm formation during coevolution; (ii) medium to high virulence, high intra-host replication and low levels of biofilm formation during one-sided adaptation, and (iii) avirulence, little intra-host replication and an increased ability for biofilm formation during control evolution. We explored the genetic basis of evolutionary change with whole-genome sequencing of replicate populations using Illumina HiSeq 2000 paired-end reads at more than 300X depth of coverage. We were able to identify which of the chromosomal genotypes from the starting populations were favoured during experimental evolution. While coevolution and control treatments were each dominated by two different chromosomal genotypes, an unexpected variability among replicate populations was observed in the one-sided adaptation treatment. The overall results suggest that evolution is driven by clonal selection, especially under coevolution and control conditions. Moreover, we also identified several single nucleotide polymorphisms (SNPs) which arose within the same chromosomal background and are specific to one of the treatments. Some of the variations were found in genes known to contribute to virulence, which could thus have been targets of selection during experimental evolution.

Introduction

The Red Queen metaphor illustrates the continuous need for adaptation of species that are surrounded by a constantly changing environment (Van Valen 1973). This is assumed to be generally the case for any organism within any environment. Detailed information on the type of adaptive responses and its molecular underpinnings are thus central for our general understanding of the biology of species. Antagonistic interactions such as host-parasite are one of the major forces determining the dynamics of environmental variation. They are ubiquitous and usually associated with high rates of evolution. The dynamics may be determined by negative frequency dependent selection or a succession of selective sweeps. Therefore, these types of interactions provide an ideal model to dissect the dynamics and consequences of environmental variations. In this context, details on the underlying molecular mechanisms are of particular value because they provide direct insights into the trait functions under selection. In addition, they permit inferences of the possible causes of the enormous complexity encountered in biological systems, including genome organisation. The recently emerging field of ecological functional genomics is specifically devoted to such a dissection of the genetics of adaptation, using the recent technological burst in genomics. It is particularly powerful if combined with experimental evolution, which yields material with a known evolutionary history and defined selection conditions.

Intriguingly, to date, information about the genomic basis of adaptive changes during continuous host-parasite interactions (i.e., host-parasite coevolution) remains scarce. Most of the few well-documented cases refer to host-parasite coevolution experiments. To our knowledge, only two of these cases dissected the genetic basis for both antagonists. In the first case, *Escherichia coli* and the bacteriophage Qβ seem to co-adapt to each other through mutations in mainly one bacteriophage gene (A1) and two bacterial genes *traQ* and *csdA* (Kashiwagi & Yomo 2011). In the second case, coevolution between *E. coli* and phage λ is primarily mediated through changes in the virus's host-recognition protein J and variation in expression level of the corresponding host receptor *LamB* (Meyer et al. 2012). In several other studies, putative coevolution genes have been characterised for one of the antagonists. For example, experimental coevolution of the bacteriophage Φ2 with its host

Pseudomonas fluorescens led to increased molecular evolution especially in the phage tail fibre gene and, to a lesser extent, in the three structural protein genes *gp40*, *gp47* and *gp48*, suggesting their involvement in co-adaptation (Paterson et al. 2010; Scanlan et al. 2010). For animal host systems, the most comprehensive data sets are available for *Drosophila melanogaster* coevolving with a sigma virus. Here, repeated mapping of resistance loci under both laboratory and field conditions revealed the importance of the major effect gene *ref(2)P* (Bangham et al. 2007, 2008; Wilfert & Jiggins 2010). Moreover, a recent study demonstrated an involvement of MHC receptor genes in the coevolutionary adaptation of sticklebacks to eukaryotic parasites (Eizaguirre et al. 2012).

In our study, we followed the genome evolution of a unique experimentally evolved host-parasite model system consisting of *Bacillus thuringiensis* and *Caenorhabditis elegans*. Indeed, both organisms are ideally suited for comprehensive experimental manipulation as well as detailed functional genomic analysis. *B. thuringiensis* is a pathogenic bacterium of arthropods and nematodes. It is characterised by the production of crystal toxins (i.e., cry toxins), which appear to mediate high specificity of individual *B. thuringiensis* strains to various host taxa, including the nematode *C. elegans*. For both species, several complete genome sequences, a versatile tool kit for functional genetic analysis and an enormous database on the species' biology are available. Moreover, the *B. thuringiensis – C. elegans* model system was recently shown to be a suitable for in-depth analysis of host-parasite coevolution based on a laboratory controlled evolution experiment (Schulte et al. 2010, 2011). Forty-eight host generations of enforced coevolution led to significant increases in pathogen virulence, host resistance, associated life-history trade-offs and local adaptation. Moreover, coevolution was also associated with increased rates of genetic change and increased genetic diversities, both within and between populations.

For this particular study, we used material from a new *C. elegans – B. thuringiensis* evolution experiment. The experiment had a total of five treatments: (i) host-parasite coevolution, for which both *C. elegans* and *B. thuringiensis* were allowed to co-adapt to each other; (ii) parasite one-sided adaptation, during which *B. thuringiensis* was allowed to adapt to a non-changing *C. elegans* population from a frozen stock culture; (iii) host one-sided adaptation, which was set up in analogy to above treatment, whereby the host could adapt to a non-changing *B. thuringiensis* population; (iv) parasite control evolution, during which *B. thuringiensis* was evolved in the absence

of the host; and (v) host control evolution, during which *C. elegans* was evolved in the absence of pathogenic bacteria. For our current work, we focused on the three treatments, in which *B. thuringiensis* was allowed to evolve (treatments (i), (ii), and (iv) as stated above). Indeed, these three treatments had a significant influence on phenotypic variation in *B. thuringiensis*. Coevolution produced higher virulence than one-sided adaptation, whereas complete loss of virulence was observed after control evolution. Moreover, infection load, as a measure of intra-host replication, was the highest for one-sided adaptation, followed by coevolution and control evolution. When studied in the absence of a host, all control evolved and some one-sided adapted populations showed strong biofilm formation, a phenomenon that was not observed in any of the coevolved populations (Table 1). These findings suggest that the evolutionary history determines trait expression in *B. thuringiensis*, whereby coevolution seems to favor virulence, one-sided adaptation intra-host replication, and control evolution in the absence of a host the formation of a long-lasting stage.

We now studied the genomic basis of the observed evolutionary changes for *B. thuringiensis*. We considered the relevant three treatments, three time points and all available replicates (up to ten per time point and treatment) to follow the dynamics of change across time. We specifically evaluated:

- Changes in genotype frequencies across time and treatments
- Changes in presence or combination of the cry toxin genes across time and treatments
- Variation in the presence and frequency of single nucleotide polymorphisms (SNPs) and structural variants among treatments and time points.

Material and methods

Material

The studied bacterial material was derived from three treatments of a new evolution experiment, as outlined above. These three treatments included *B. thuringiensis* populations, which were allowed to evolve in the presence of either a co-adapting host (coevolution treatment), a non-evolving host taken from a stock culture at each transfer step (one-sided adaptation treatment), or in the absence of the host (control evolution treatment). The treatments only differed as to the presence or absence of the host. All other parameters were identical to ensure high comparability of the material. The evolution experiment was run for 28 host generations, which is equivalent to approximately 14 weeks. The exact protocol generally followed the previously published procedures (Schulte et al. 2010) and will be described elsewhere (manuscript in preparation by Masri et al.). Important for the objectives of the current study is that the starting population consisted of a mixture of *B. thuringiensis* strains. Evolved *B. thuringiensis* were frozen every fourth host generation as either single clones in 15% glycerol at -80 °C or as entire populations at -20 °C.

Population genomic analysis (see below) was based on frozen population samples from all available replicates for host generations 0, 12, and 20. Four replicates, namely replicate 10 of coevolution treatment, 2 of control evolution), 3 and 10 of one-sided adaptation, were considered for additional time points, comprising host generations 12, 16, 20, and 24. A total of 65 samples were thus included in the population genomic analysis (Table 2). The toxin screen was based on individual clones from all available replicate populations from host generations 12 and 20. Note that some populations from the one-sided adaptation treatment went extinct during the experiment and thus were only considered for the earlier time points.

To validate sensitivity of our method to estimate the frequencies of each original strain in each sample (below sensitivity analysis), we extracted and sequenced aliquots of clones of the starting strains MYBT18246, MYBT18247, and MYBT18679 aliquoted at known frequency. We chose a panel of clone frequency from rare (1%) to high frequency (98%) (Table 3).

Sequencing of Reference Strains

We used five strains (MYBT246, MYBT247, MYBT679, MYBT22 and MYBT50) as references for the mapping of the population genomics' data. For each of these strains, genomic DNA was isolated using a DNeasy Blood and Tissue Kit (Qiagen). Whole genome sequencing was performed using the Roche 454 Genome Sequencer FLX platform. The resulting reads were assembled using GS *De Novo* Assembler (Roche) and a summary of the data and assemblies for each strain is shown in Table 4. For MYBT18679, PCR and Sanger sequencing was used to close gaps between contigs and the sequence used in this study was a partially closed reference consisting of 291 contigs.

Toxin screen

For each evolved population that was included in the toxin gene screen, 20 individual clones were isolated by plating the population on nematode growth medium (NGM) plates and picking single colonies. The clones were grown overnight at 28°C in LB medium, then frozen at 20°C. This frozen material was used directly in PCRs.

To test for the presence of the toxin genes, PCRs were carried out in 15.6 µL reactions with 0.39 units GoTaq DNA Polymerase (Promega), 1x Green GoTaq reaction buffer, 0.2mM each dNTP, 0.4 µM forward primer, 0.4 µM reverse primer, 0.4 µM CodY_F, 0.4 µM CodY_R and bacterial cells. Thermal cycling was performed with an initial denaturation step at 95°C for 2 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 57°C for 30 seconds and extension at 72°C for 90 seconds, then a final extension step at 72°C for 10 minutes. PCR products were analysed by agarose gel electrophoresis, with a positive result indicated by a band of the expected size. *CodY* primers were included in each reaction to ensure integrity of the template. Any reactions that did not give a *codY* product were repeated, in some cases adjusting the amount of template used, to obtain suitable conditions.

To analyse the composition of genes encoding crystal toxin proteins in the evolved *B. thuringiensis* populations, 20 clones were isolated from each coevolution and one-sided adaptation population at host generations 12 and 20, as well as four of the control populations at host generation 20. Each clone was then analysed individually for the presence of several toxin genes. The toxin genes that were investigated were

chosen on the basis of those that were known to be present in one of the strains making up the starting population (Table 5).

To determine the chromosomal background of each clone, PCRs were performed as above, but with only *codY* primers. The sequences of the PCR products were obtained by Sanger sequencing and compared to reference sequences for each strain.

Population genomics: DNA extraction

Prior to DNA extraction, 10 uL of the frozen bacterial populations were spread onto NGM plates, which was also used during the evolution experiment, and then grown for 14-16 h at 25 °C. Subsequently, NGM plates were washed twice with 1 mL of autoclaved water. DNA was isolated following Qiagen DNeasy® Blood and Tissue kit procedures for Gram-positive bacteria. For samples showing the biofilm phenotype, four replicates were extracted and pooled while three replicates were extracted for the other samples. Indeed, extraction was less efficient in biofilm-forming populations. Consideration of an additional replicate allowed us to obtain sufficient DNA for the Illumina Solexa sequencing platform. DNA quantity was measured using Qubit® Fluorometric Quantitation and ranged between 9.13 ng/µL to 55.1 ng/µL.

Population genomics: Sequencing

Genomic paired-end Illumina sequencing libraries were prepared for sequencing by synthesis according to standard methods (Bentley et al. 2008). Insert sizes (not including the adapters) ranged from ~200-450 nucleotides. Libraries were sequenced using GAII or GAIIx Illumina sequencing instruments to yield paired 100mer. The Illumina image analysis pipeline with default parameters was used for image analysis, base-calling and read filtering. Further filtering was done on later runs to remove adapter and PhiX contamination based on blast alignment (pairs with ≥ 14nt aligned at ≥ 98% were removed).

Population genomics: Mapping

First, we tested five short-read mapping software to determine the most suitable one for our dataset. The tested programs were chosen among the most commonly used: BWA (Li & Durbin 2009), BOWTIE (Langmead et al. 2009), MOSAIK (Strömberg & Lee 2009), SOAP (Li et al. 2008; Li, Yu, et al. 2009) and GSNAP (Wu & Nacu 2010). One hundred sixty five thousand reads from the draft genomes of MYBT18246, MYBT18247 and MYBT18679 were simulated using the tool dwgsim from DNAA's Whole-Genome Simulation software (http://sourceforge.net/apps/mediawiki/dnaa/index.php) and mapped onto the reference genome composed of the consensus sequence of the three genomes. Default parameters were used for all software except a common mismatches allowance parameter of two mismatches per reads. MOSAIK, GSNAP and BWA achieved the best mapping with respectively 88.4%, 89.03% and 68.95% of the reference genome covered by at least one read while BOWTIE and SOAP covered only 11.66% and 27.31% of the reference genome. Subsequently, only using MOSAIK, GSNAP and BWA, we tested the capacity to detect accurately variation in the genome by generating random SNPs across the genome at various frequencies. Variants were called using VarScan 2.7 (Koboldt et al. 2009) with neither mapping nor read quality filter. GSNAP provided the far best estimates of the known SNP frequencies. BWA estimates were biased toward an overestimation of invariable sites and MOSAIK estimates were biased toward high and low frequencies, underestimating intermediate frequencies. As a result of this testing step, GSNAP software was utilized for the subsequent analyses.

First, the raw read data were processed with SeqPrep software to remove adapter sequences from the data and to merge overlapping pairs of reads. The 100 nucleotide reads were mapped onto the backbone sequence with GSNAP software (Wu et al. 2010). Number of mismatches was set as the ultrafast level of

$$Nmismatches\ per\ read = ((readlength+2)/12 - 2)).$$

The insert size was set to 350bp, defined as the distance between the two ends of each reads of the pair.

To estimate the frequency of each clone (see below), a first mapping was done using a meta-reference comprising the complete genome sequence of five clones from the original starting populations. First, the contigs of the five draft genomes, namely MYBT18679, MYBT18246, MYBT18247, MYBT22 and MYBT50 were ordered with *B. thuringiensis* BMB171 (Genbank NC_014171) using the program MAUVE

(Wu et al. 2010). Thereafter, MAUVE was used to align them to each other. SNP information was added as supplementary information in GSNAP to perform a SNP-tolerant alignment. Therefore, presence of SNPs was not counted as a mismatch for calculating mapping quality as well as the edit distance between the reference genome and a read (Wu et al. 2010).

Population genomics: Frequency estimates of genotypes

For each sequence read, genotype assignment was based on comparison with the aligned five reference genomes, as described above. We then recorded the number of reads with a unique assignment to only one of the five reference genomes. The frequency of such unique assignments at each variable position in the alignment was considered to provide independent estimates of the reference genotype frequencies. However, the frequency distributions are often biased (as was the case in our study). Therefore, we chose the mode of the distribution as the final frequency estimate, because the mode was previously shown to be the most reliable estimate of the real frequencies, especially in comparison to the mean or the median (Figure1). The function mlv from package Modeest in R (Team & others 2010) was used to estimate the mode as the most frequent value of the distribution**.**

Population genomics: SNP and Indel discovery

For the discovery of new indels and SNPs, an independent mapping procedure was used. Reads were mapped independently on each of the five available genome references from the starting population. Reads were assigned to a particular reference genome (or genotype) if the comparison with this reference genome produced a smaller edit distance than those with the other references. If ties were observed, then the reads were removed to ensure high confidence in variant calling. Indeed, most of the ties contained a high number of mismatches, likely due to multiple sequencing error, contaminants or misalignment. SNPs and indels were assessed in MYBT18679 reference genome. SNVer software (Wei et al. 2011) was used to estimate SNP and short indel frequencies for each replicate population. Variations were filtered according to the partial conjunction test for multiple-pool data implemented in SNVer at a threshold p-value of 0.05. Particular attention was paid to SNPs in MYBT18679 and MYBT22 since these two original strains were found to be present in two

different treatments up to generation 20. SNPs frequency was compared between time points (Generation 12 vs Generation 20) and between treatments (Coevolution vs One-sided adaptation) using ANOVA. P-values were corrected for multiple testing using False Discovery Rate method and significant q-values were considered below 0.05. The localisation of the SNPs was determined by performing blastx (Tatusova & Madden 1999) on fragments flanking the SNPs by 100 bp on both side. Synonymous or non-synonymous changes on the 6 possible opening reading frames (ORF) were assessed using personal PERL script.

Longer insertion and deletion were detected using pairwise comparison of coverage between samples per contig where aberrant-looking patterns were identified by eyes.

Results

Toxin gene screen

In the control treatment, no toxin genes were found in any of the clones. In contrast, in the coevolution and one-sided adaptation treatments, many of the clones had one or more toxin genes from MYBT18679 (**Figure 3**). Of the other toxin genes, *cry13A*, derived from MYBT18246, was found in only one clone and *cry6B*, derived from MYBT18247, was completely absent in the analysed material. This indicates that either the MYBT18679 toxins themselves (or the plasmids that carry them) are advantageous in the presence of a host, or the MYBT18679 strain has a competitive advantage over the other strains under these conditions and the toxin genes are therefore found in high abundance due to their presence in this strain.

The one-sided adaptation treatment showed large variation in toxin gene frequencies between populations; some populations had a high proportion of clones with MYBT18679 toxin genes, while others had few or no clones with toxin genes (Table 6 and Figure 4). This is consistent with the phenotypic data from this treatment; many of the replicates lost virulence and these correlated with the replicates that lacked toxin genes (Table 6).

We also determined the chromosomal background of each clone by sequencing of the *codY* gene (Figure 5 and Figure 6). The results were largely consistent with those for the toxin genes. In both the coevolution and one-sided adaptation treatments,

MYBT18679 was the most prevalent genotype and the control populations, where no toxin genes were found, were composed entirely of MYBT22, a cry toxin-free strain. Seven of the tested populations showed evidence for horizontal gene transfer (HGT), where MYBT18679 toxin genes were found in a non-MYBT18679 chromosomal background (Tables 7-10). In one of these populations (replicate 3 of one-sided adaptation, generation 20), 14 out of 20 clones showed a signature of HGT. In the other six populations, however, HGT was observed for only one or two clones. This suggests that in most cases there has not been strong selection for the recombinant genotypes.

Interestingly, there was very little difference between generations 12 and 20 in terms of both toxin gene composition and chromosomal background (**Figure 3**, Figure 5 and Figure 6).

Population genomics: Frequency of ancestral strains in evolved populations

Short paired-end reads were mapped with a very high accuracy to the meta-reference, with 90 to 97 % of the reads mapping to the reference and 80 to 94% of total reads mapping properly (paired with good orientation of each mate of a pair and proper insert size).

The frequencies of the five considered ancestral strains in the evolved populations were estimated with little bias when present at very high abundance and higher bias when present at intermediate abundances (Figure 7). However, the bias was found to be unrelated to the inference method since estimates were very consistent across the three replicates of the different aliquots. Indeed, a bias in the estimates could be observed along the chromosome starting from the origin of replication (ORI), located using *dnaA* gene position (Figure 8).

In the coevolution treatment (Figure 9), strain MYBT18679 was always found at a frequency higher than 99% except in the replicate 3 at host generation 12. In the control treatment (Figure 9), at host generation 12, the genome of the strains MYBT50 and MYBT22 were recovered with MYBT22 being the most frequent strain in all replicates except replicates 5 and 6. At host generation 20, all samples were almost uniquely composed of the strain MYBT22. Three of the five considered strains were recovered in the one-sided adaptation treatment, only excluding strains

MYBT18246 and MYBT18247 (Figure 9). The replicate populations 1, 5, 7, and 8, which are the ones composed mostly of the strain MYBT22 at host generation 12, went extinct by host generation 20. Replicates 3, 4 and 6, which comprised the strain MYBT18679 at host generation 12, consisted of the same strain at generation 20. In contrary, the replicate 2 changed from a predominance of MYBT18679 at host generation 12 to MYBT22 at generation 20. Finally, replicates 9 and 10 stayed almost fully composed of strain MYBT50 at host generation 12 and 20.

For the four replicates, which were followed across all sampled generations (Figure 10), no change in frequencies of any strain was observed across time.

SNPs and Indels

The analysis of SNP and indel variation was restricted to populations dominated by the MYBT18679 genotype, in order to ensure comparability of the samples. Thus, this analysis focused on populations from the coevolution and the one-sided adaptation treatments. SNVer detected a total of 912 SNPs for the samples. Among these, 78 showed significant difference at least between the two considered treatments or generations (detailed data not shown). 5 out of the 78 SNPs showed significant frequency variation between treatments at host generation 12, while 31 were found at host generation 20. One of the SNPs differed between treatments at both time points (contig 217 position 15682, T-> C). Respectively 36 and 26 SNPs in the one-sided adaptation and the coevolution treatments varied in frequency between the two time points.

The majority of the blastx hits were found in hypothetical proteins of *Bacillus sp.* (N=21), followed by transposase-related proteins (N=17) and phage-related proteins (N=7). Poor-match hits (E-value >0.1, N=6) and non-significant hits (N=11) were found for 17 SNPs. Among the remaining hits, two were found in cell surface protein (Contig 217 position 15682, q-value(One-sided Adaptation vs Coevolution, Generation 20)<0.01, cell surface protein [GB ADY23698.1], E-value=7.00E-7 and Contig 100 position 11374, q-value(One-sided Adaptation vs Coevolution, Generation 20)<0.001, E-value=3.00E-29 LPXTG-motif cell wall anchor domain protein [ZP_04284995.1]) and, interestingly, two in FMRFamide neuropeptides (Contig 146 position 190 and 247, q-value(One-sided Adaptation vs Coevolution, Generation 20)<0.01, FMRFamide neuropeptide ([ZP_04263062.1], E-value=6.00E-

9 and 8.00E-7 repectively), which are also known as neuropeptides of *C. elegans* regulating digestion, feeding behaviour and reproduction (Li et al. 1999). These latter SNPs were all found to be non-synonymous.

From the coverage analysis, one region showed a pattern of deletion that is shared by four samples, three from the coevolution treatment and one from the one-sided adaptation treatment both from generation 20 (Figure 11). A blastx search shows that the 65 bp deletion is related to a putative teichoic acid/polysaccharide export protein associated with the mviN gene region (E-value=0.026, NZ_ACNC01000184.1). The deletion of the mviN virulence gene in *Salmonella enterica* has sown to reduce virulence in a mouse model of typhoid-like disease (Carsiotis et al. 1989). Mutants lacking homologs of mviN in the closer non-pathogenic related species *Bacillus subtilis* did not show any deficiency in growth even though the gene was previously linked to sporulation (Fay & Dworkin 2009).

Discussion

Our study provides one of the most comprehensive data sets on the genomic basis of coevolutionary adaptation. A bacterial parasite was allowed in a laboratory-controlled evolution experiment to adapt to a co-adapting host, a non-evolving host, or the general conditions of the experiment in the absence of a host. Based on this design, our approach allowed us to identify changes specific to reciprocal coevolution rather than unidirectional selection. We particularly considered genomic changes at three levels: (i) cry toxin gene frequencies were studied, because these toxins likely represent a central virulence factor for the interaction with the *C. elegans* host (Griffitts & Aroian 2005); (ii) chromosomal gene *codY* and whole genome sequence frequencies provided information on genotype frequency changes and the main selective dynamics in these populations; and (iii) SNP and indel variation yields insight into the genes that are likely under selection during the evolution experiment. Based on these three levels, we were able to generate an integrated view on the genomic consequences of fast coevolutionary adaptations.

Variation in toxin gene prevalence

We expected the spread of certain, highly effective toxin genes and HGT of toxins, especially under coevolution, where the resulting new toxin combination may permit more efficient infection of the co-adapting hosts. Indeed, our results suggest that the MYBT18679 toxins are favourable during host adaptation, because these showed extremely high prevalence in the coevolving and several of the one-sided adapted populations. Note that this strain was recently found to be the most pathogenic towards nematodes, most likely as a consequence of its toxins (Wang et al. 2011). Surprisingly, however, we were only able to identify a single clone (out of 800 tested) that had toxin genes from more than one of the starting strains. This result is not only in contrast to our expectations but also to the findings from a previous evolution experiment (Schulte et al, 2010), where a high level of toxin gene exchange between a similar mixture of *B. thuringiensis* strains was found. One important difference between the two experiments is that Schulte et al (2010) simulated migration by adding a small proportion of the original genotypes at every second transfer step. The current experiment did not include any immigration for the parasite. Therefore, a

possible explanation for the observed difference may be that in the current evolution experiment, some of the strains were lost early on in the experiment, thus precluding their contribution to populations from later time points.

Variation in whole genome genotype frequencies

Two possible outcomes were expected to result from coevolution: (i) the frequency of individual genotypes should vary across time due to negative frequency dependent selection and/or recurrent selective sweeps or, alternatively, (ii) single genotypes would dominate the populations as a consequence of clonal selection. During the first steps of our analysis, however, we encountered a bias during frequency estimation that may compromise subsequent inferences. Unexpectedly, strong variation in frequency estimates was observed along the bacterial chromosome. This bias is probably the consequence of the among strains' variation in the dynamics of DNA replication. In detail, when bacteria replicate DNA during cell division, the DNA polymerase starts from the ORI and goes in both directions along the DNA strand to form a so-called theta structure. If DNA replication proceeds at different speeds in the considered strains or if bacterial strains generally vary in growth rate, then the pooled DNA samples comprised bacteria at different stages of cell division possibly resulting in the observed variation of DNA quantities across the chromosome. Importantly, when the bias was found very strong in aliquots, it was negligible in the pooled population probably due to the difference in growth medium before sequencing (LB nutrient-rich liquid medium for aliquots and NGM nutrient-poor plates for the pooled populations).

The three evolution treatments were inferred to have very distinct strain compositions. In the coevolution treatment, the strain MYBT18679 was consistently favoured by selection across all replicates. Indeed, all replicates were fixed for this genotype already at host generation 12. Interestingly, this strain contained the toxin genes that were similarly dominated in the coevolved and some of the one-sided adapted populations. Moreover, as mentioned above, this strain was previously found to express a comparatively high level of pathogenicity towards nematodes, which was well above that recorded for MYBT18246 and MY18247 (Schulte et al. 2011; Wang et al. 2011), thus explaining its particular advantage in the presence of a host.

In the control treatment, the strain MYBT22 was similarly and consistently favoured in all replicates. Nevertheless, in this evolution treatment, the strain MYBT50 was still found to be quite frequent at generation 12. A possible explanation for this pattern is that selection in this treatment was less intense than under coevolution conditions or that both strains show high competitiveness in the conditions of this treatment. For the latter explanation, MYBT22 might have won the competition because of the production of specific bacteriocins, notably thuricin, which is well known for these bacteria to help them kill competitors (Favret & Yousten 1989).

In the one-sided adaptation treatment, a more complicated pattern was observed: The strains MYBT22, MYBT50 and MYBT18679 have each gone to fixation in different replicate populations. This result seems paradoxical since the treatment imposed a unidirectional selection pressure on the population across the experiment, which should have favoured only a single genotype across the replicates. However, the results are consistent with the observed variation in phenotype across these populations. In fact, some of the populations maintained a comparatively high level of virulence and all of these were dominated by MYBT18679. In contrast, the remaining populations lost virulence and these were composed of MYBT22 and/or MYBT50. Consequently, the constant and non-fluctuating selection conditions of this treatment may have favoured two alternative life-history strategies: either virulence and no biofilm or alternatively avirulence and biofilm formation. Chance may influence which strategy wins and/or which strategy spreads above a certain threshold frequency, thus favouring its fixation within the population.

As indicated, our study generally demonstrated a clear link between genotype and phenotype for the evolving populations, especially for the strains MYBT22 (avirulent and biofilm-producers) and MYBT18679 (virulent and no biofilm-producers). This pattern may suggest that clonal selection played an important role during evolution of these populations, allowing the spread to fixation of one of the genotypes present in the starting population – consistent with previous reports on microbial evolution in genotypically mixed populations (Levin 1981). Interestingly, the favoured genotypes were among those present at rather low frequency at the beginning (ca. 5% each for MYBT18679 and MYBT22), suggesting that initial abundance did not directly determine evolutionary success of the strain. These findings again contradict the increase in genetic diversity previously observed for the same model system (Schulte et al. 2010, 2011). As argued above, this discrepancy between experiments is most

likely due to the difference in immigration rates (previously with and in the current experiment without immigration). This observation is consistent with the previous notion that migration can play an important role in coevolutionary dynamics (Thompson 2005). In the *Pseudomanas fluorescens* – phage system, the lack of migration was indeed demonstrated to cause maladaptation of the parasite (Morgan et al. 2005).

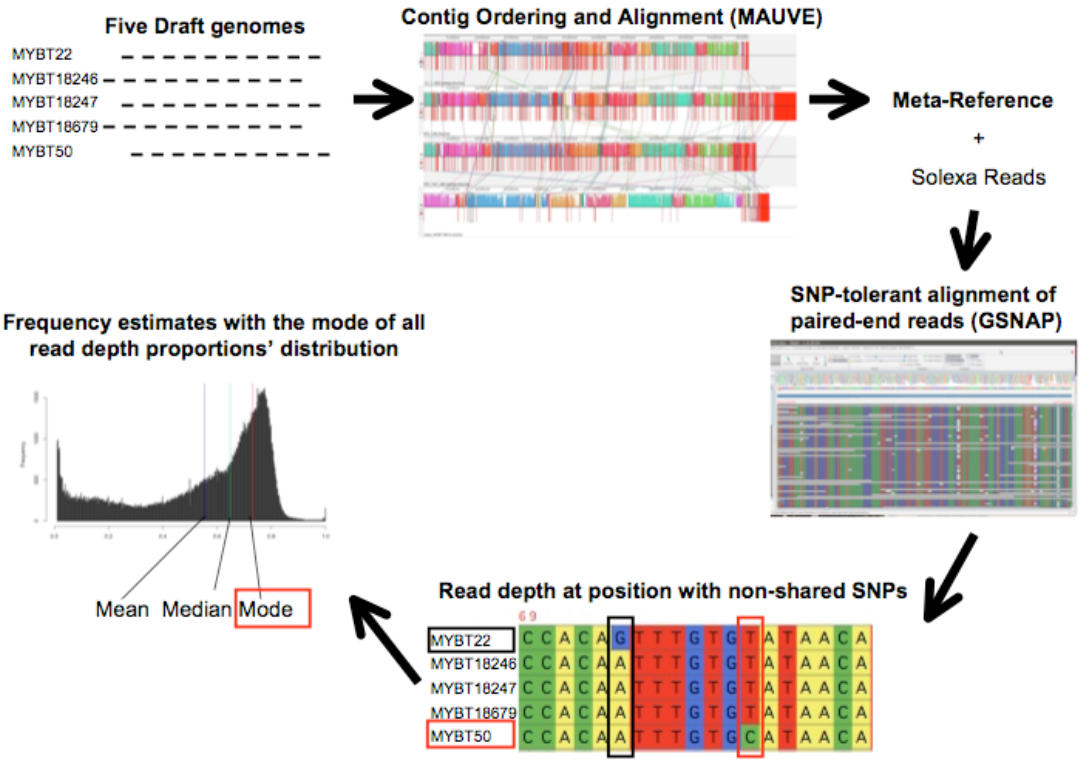
Candidate SNP and structural variation

In the populations dominated by the strain MYBT18679, we uncovered numerous SNPs and one major structural change (i.e., a deletion). Most importantly, some of these variations are treatment specific and also refer to non-synonymous changes in genes with a potential role in the interaction with the host, including: (i) A SNP in a surface protein, which may thus be located at the contact point of *B. thuringiensis* – *C. elegans* and possibly involved in virulence and/or host exploitation. (ii) A deletion in a protein that contributes to teichoic acid production, which represents another cell surface factor possibly involved in the interaction with the host (Bhavsar & Brown 2006). (iii) Two SNPs in close proximity in an FMRFamide neuropeptide. These neuropeptides are well known for their role in regulating gut activity, digestion, and reproduction in *C. elegans* (Li et al. 1999). Their presence in the bacterial genome and especially the treatment-specific change in variant frequency may suggest that they are used by *B. thuringiensis* for host manipulation, a trait universally expressed by parasites (Schmid-Hempel 2008). New neuropeptide variants may then have helped the bacteria to adapt to their hosts.

Conclusion

Our study uses comprehensive genomic analyses to dissect the molecular basis of coevolutionary adaptation in a bacterial parasite that was experimentally evolved with its nematode host. This approach demonstrated that adaptation is influenced by both clonal selection and the spread of novel variants. Our results suggest that cry toxin genes may play a role for clonal selection and thus the spread of a particular genotype associated with the nematocidal toxin genes. Within this genomic background,

selection subsequently favoured the spread of specific mutations and deletions, which most likely influenced cell surface characteristics and the interaction with the host. Our study provides unique insight in how mutational processes and selection interact to determine the evolution of parasites in the presence of either a co-adapting host, a non-changing host, or no host.



Figures

Figure 1. Pipeline for Read mapping and frequency estimates. First the five draft genomes are contig-ordered and aligned to produce a meta-reference. Then the Solexa reads are mapped onto this reference using the GSNAP software. Positions showing SNPs unique to one of the draft genome are recorded to estimate the frequency of each draft genome in the pool. Finally, the mode of the distribution of the estimates from all the positions unique to one draft genome provides the closest value from the real frequency of the draft genome in the pool.

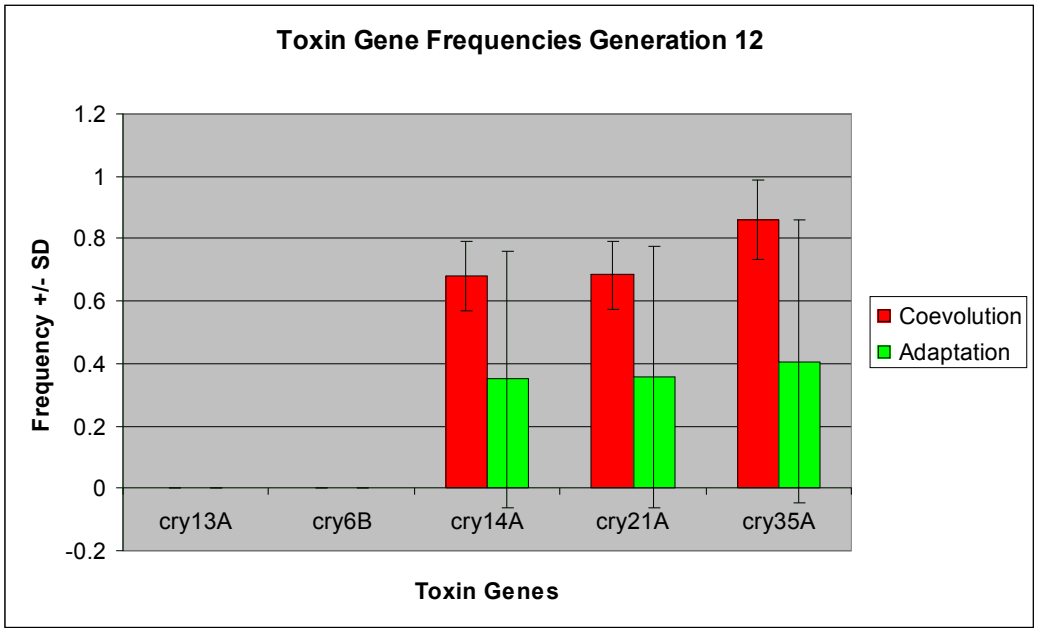


Figure 2. Toxin gene prevalence at host generation 12. Bars indicate the average frequency of clones from a population with each toxin gene. Error bars show standard deviation

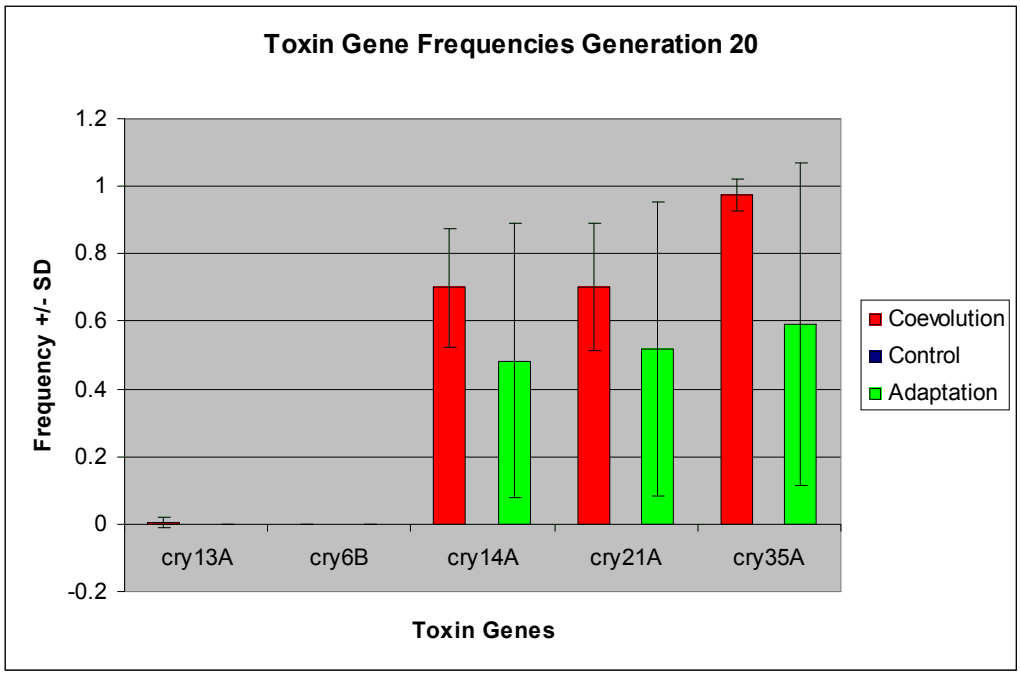


Figure 3. Toxin gene prevalence at host generation 20. Bars indicate the average frequency of clones from a population with each toxin gene. Error bars show standard deviation**.**

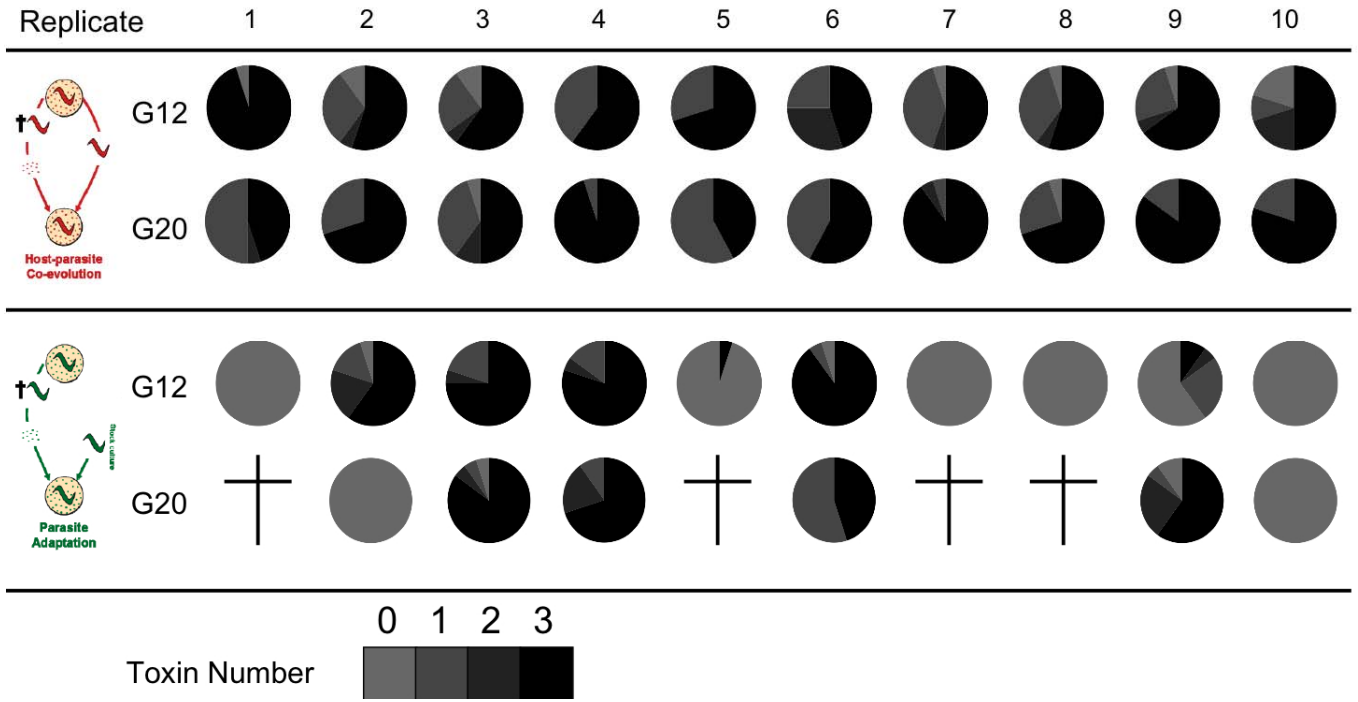


Figure 4. Number of toxins identified in the evolved populations at host generation 12 (G12) and host generation 20 (G20) for host-parasite coevolution (top panel), control (middle panel) and one-sided adaptation treatment (bottom panel). "+" represents samples that went extinct by a particular host generation (NB No toxins were found in the control treatment).

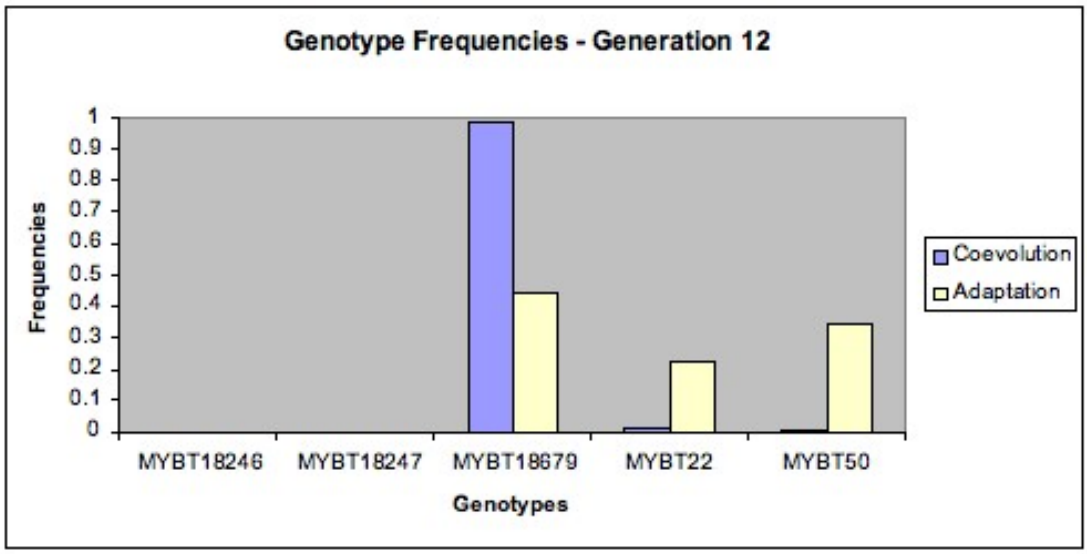


Figure 5. Frequency of clones with each chromosomal background at host generation 12.

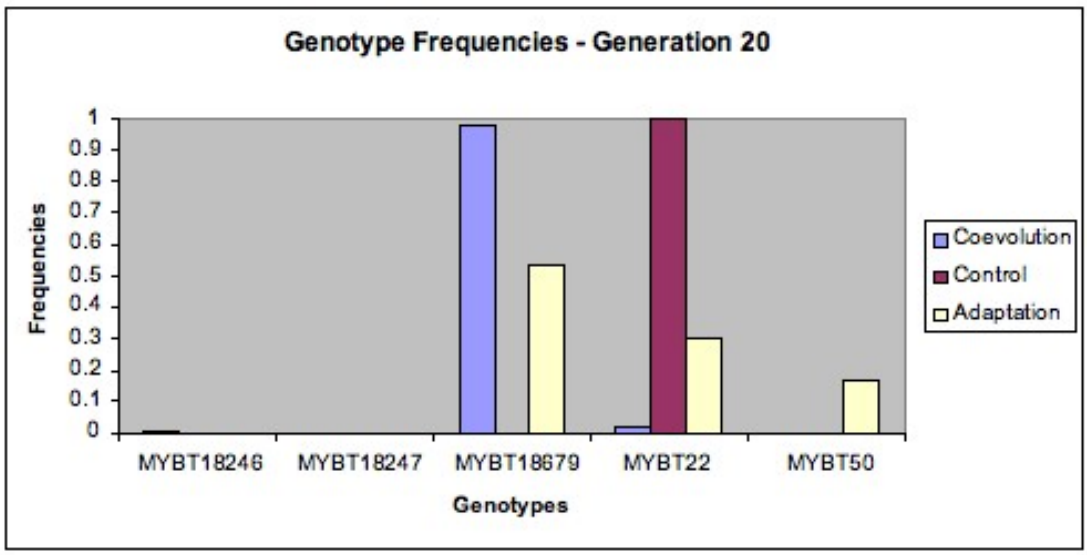


Figure 6. Frequency of clones with each chromosomal background at host generation 20.

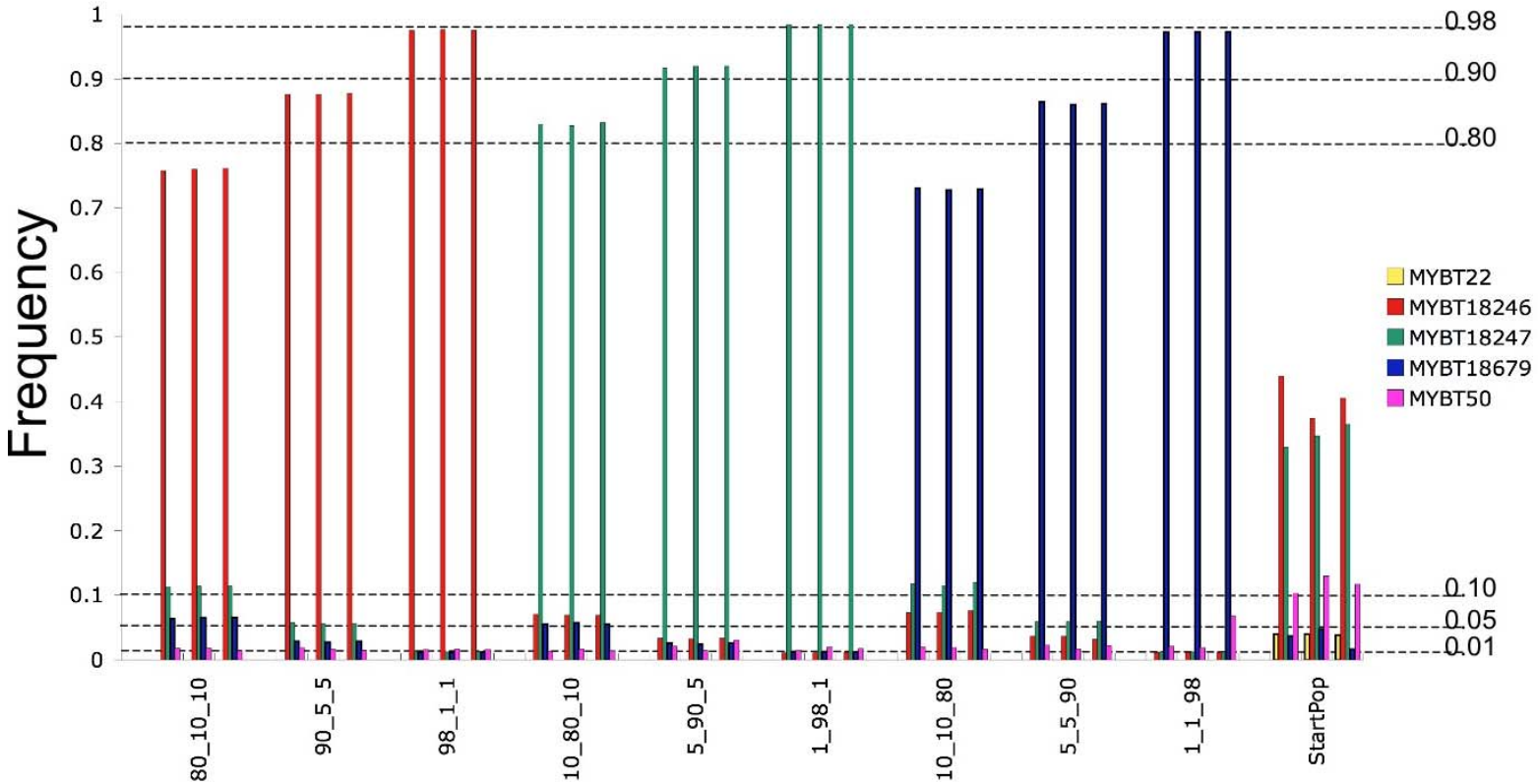


Figure 7. Frequency estimates of the different draft genomes in the aliquot test samples. Dashed lines represent the expected frequencies, as set by experimental combination of different quantities of the three clones for the sensitivity analysis, and as noted as categories along the X-axis, whereby the proportion of strains are given in the following order: MYBT18246, MYBT18247 and MYBT18679.

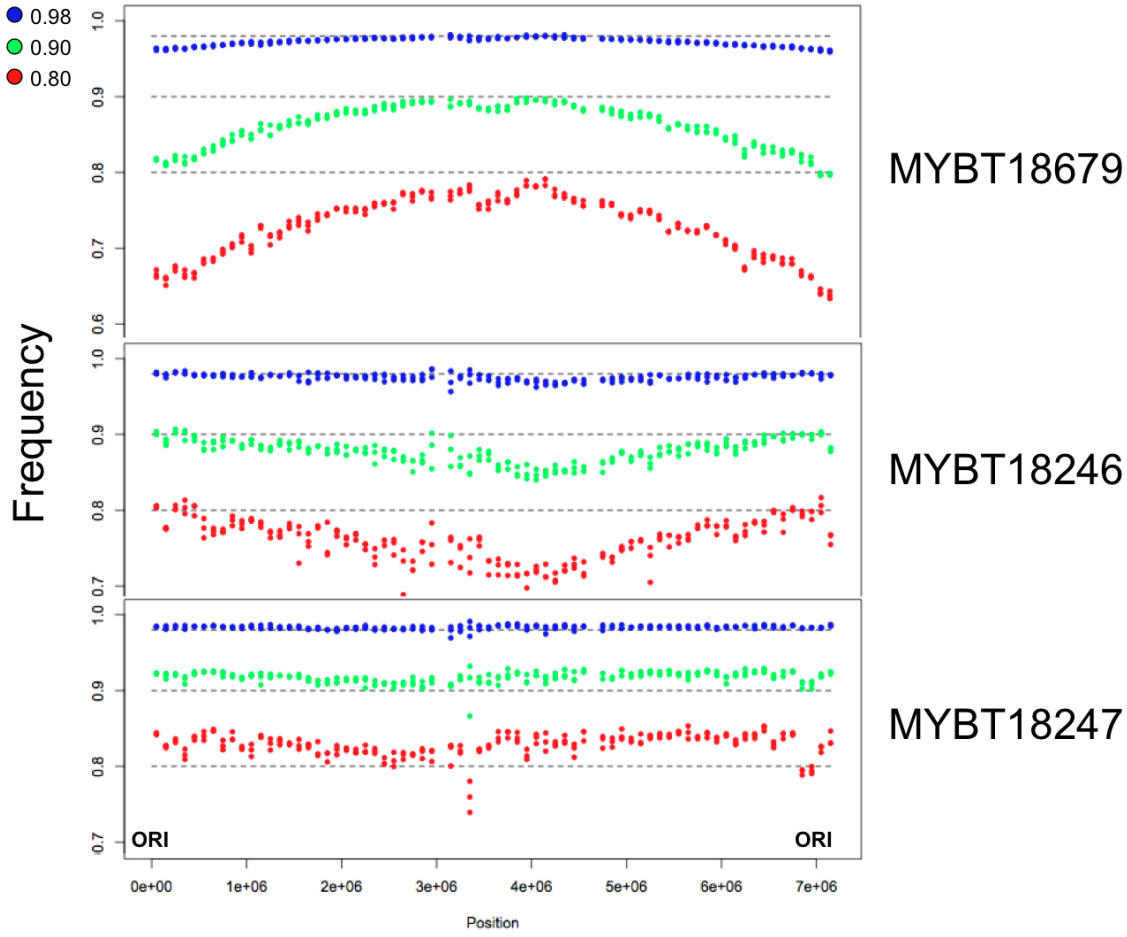


Figure 8. Biased observed in frequency estimates according to the position on the chromosome starting from ORI. Dashed lines represent the expected frequency at 0.8, 0.9 and 0.98, as set by experimental combination of strains for the sensitivity analysis.

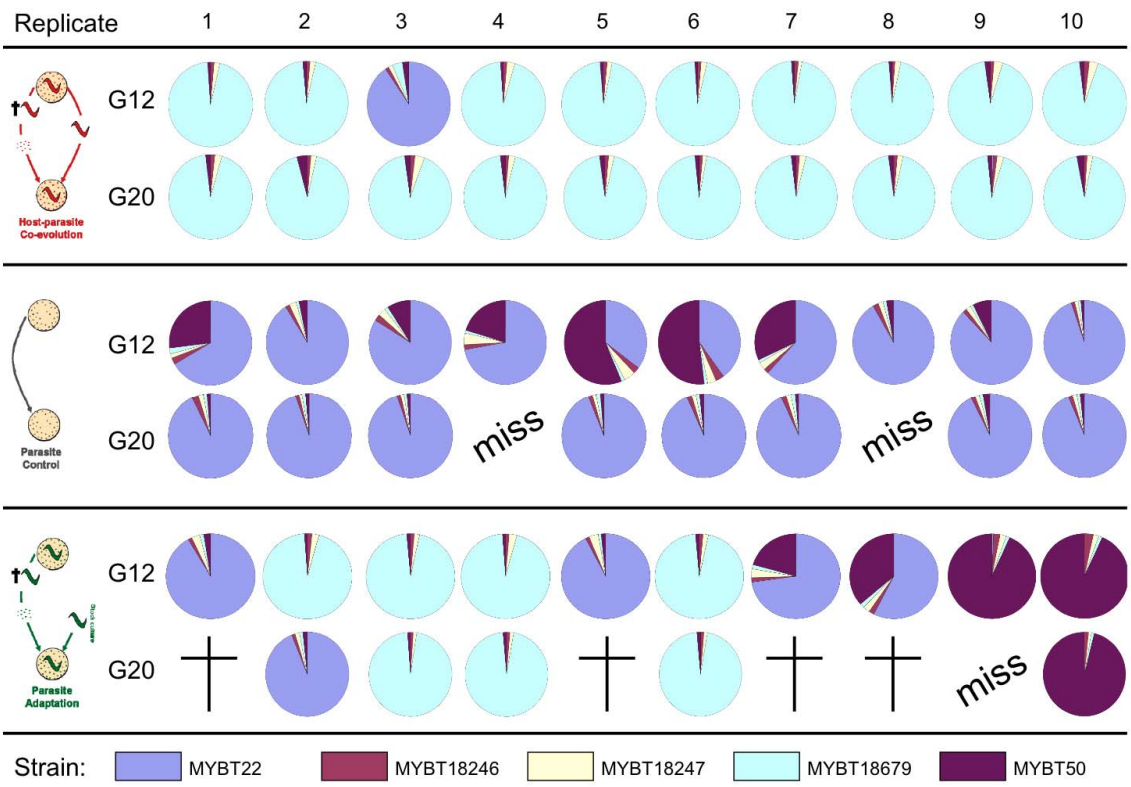


Figure 9. Proportion of the starting strains in the evolved populations at host generation 12 (G12) and host generation 20 (G20) for host-parasite coevolution (top panel), control (middle panel) and one-sided adaptation treatment (bottom panel). "miss" represents missing samples, "+" represents samples that went extinct by a particular host generation.

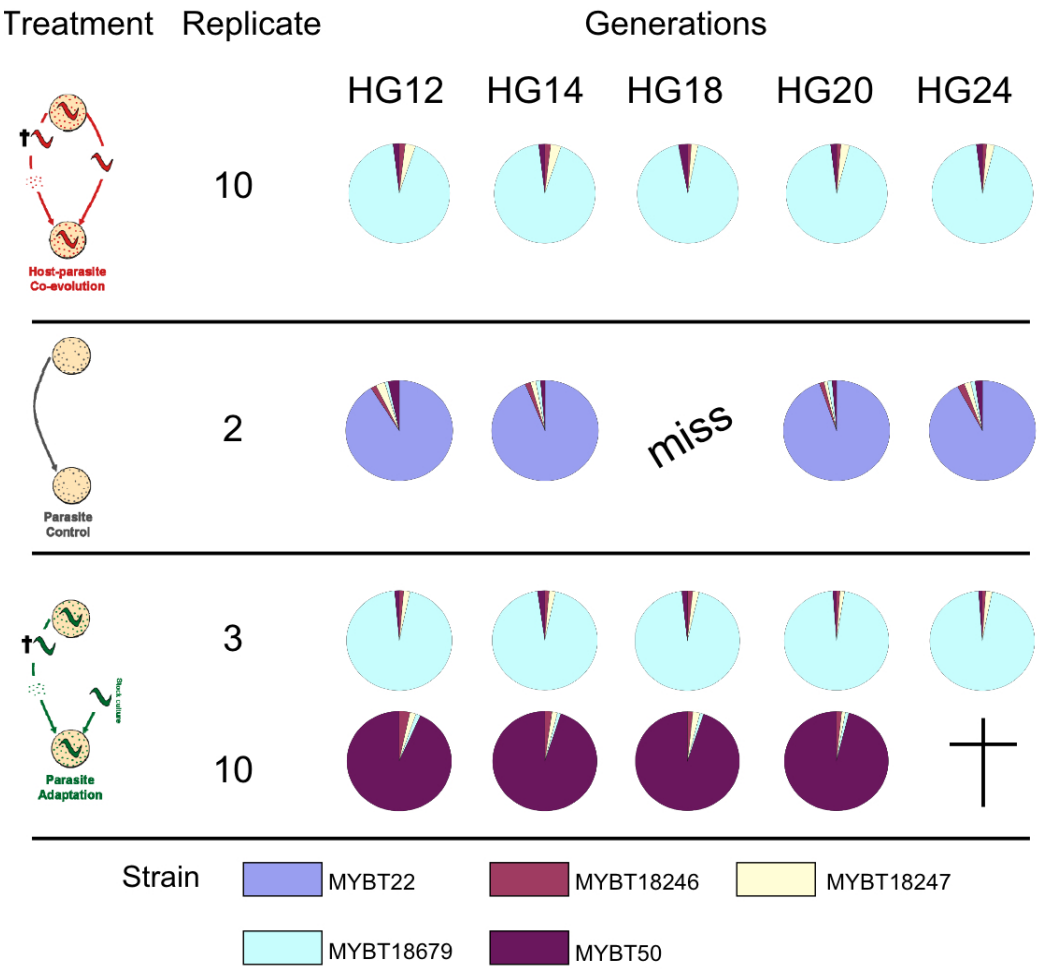


Figure 10. Proportion of the starting strains in the four replicates of evolved populations from host generation 12 (HG12) to host generation 24 (HG20) for host-parasite coevolution (top panel), control (middle panel) and one-sided adaptation treatment (bottom panel). "miss" represents missing samples, "+" represents samples that did not survived until a particular host generation.

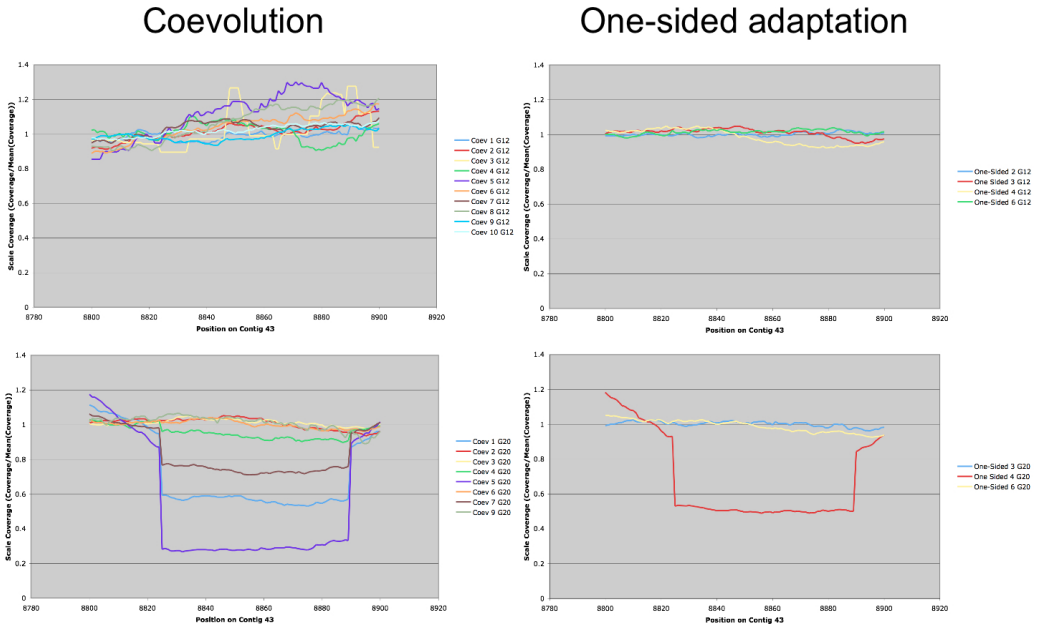


Figure 11. Example of a deletion that appeared independently at least four times on contig 43 of MYBT18679 genomes. Two top figures represent the replicates at generation 12 and the two bottom figures the replicates at generation 20. The replicates 1,5,9 and possibly 4 of Coevolution treatment (bottom-left) and 4 of One-sided adaptation treatment (bottom-right) at generation 20 show a drop of scaled read depth coverage of different intensity between position 8825 and 8889 while other samples do not show this pattern.

Tables

Table 1. Summary of the different phenotypes observed during experimental evolution for the three treatments + presence – absence

Phenotype Treatment	Biofilm	Virulence
Coevolution	-	+
Control Evolution	+	-
One-sided Adaptation	+/-	-/+

Table 2. Sample list

Treatment	Replicates	Generation	Biofilm
Coevolution	1	12	No
Coevolution	2	12	No
Coevolution	3	12	No
Coevolution	4	12	No
Coevolution	5	12	No
Coevolution	6	12	No
Coevolution	7	12	No
Coevolution	8	12	No
Coevolution	9	12	No
Coevolution	10	12	No
Control	1	12	Yes
Control	2	12	Yes
Control	3	12	Yes
Control	4	12	Yes
Control	5	12	Yes
Control	6	12	Yes
Control	7	12	Yes
Control	8	12	Yes
Control	9	12	Yes
Control	10	12	Yes
One-sided Adaptation	1	12	Yes
One-sided Adaptation	2	12	No
One-sided Adaptation	3	12	No
One-sided Adaptation	4	12	No
One-sided Adaptation	5	12	Yes
One-sided Adaptation	6	12	No
One-sided Adaptation	7	12	Yes
One-sided Adaptation	8	12	Yes
One-sided Adaptation	9	12	No
One-sided Adaptation	10	12	No
Coevolution	1	20	No
Coevolution	2	20	No
Coevolution	3	20	No
Coevolution	4	20	No
Coevolution	5	20	No
Coevolution	6	20	No
Coevolution	7	20	No
Coevolution	8	20	No
Coevolution	9	20	No
Coevolution	10	20	No
Control	1	20	Yes
Control	2	20	Yes
Control	3	20	Yes
Control	4	20	Yes
Control	5	20	Yes
Control	6	20	Yes
Control	7	20	Yes

Control	9	20	Yes
Control	10	20	Yes
One-sided Adaptation	2	20	Yes
One-sided Adaptation	3	20	No
One-sided Adaptation	4	20	No
One-sided Adaptation	6	20	No
One-sided Adaptation	9	20	No
One-sided Adaptation	10	20	Yes
Coevolution	10	14	No
Coevolution	10	18	No
Coevolution	10	24	No
Control	2	14	No
Control	2	24	No
One-sided Adaptation	3	14	No
One-sided Adaptation	3	18	No
One-sided Adaptation	3	24	No
One-sided Adaptation	10	14	Yes
One-sided Adaptation	10	18	Yes

Table 3. **Aliquots**

Number	Proportion 246	Proportion 247	Proportion 679
1	0.8	0.1	0.1
2	0.8	0.1	0.1
3	0.8	0.1	0.1
4	0.9	0.05	0.05
5	0.9	0.05	0.05
6	0.9	0.05	0.05
7	0.98	0.01	0.01
8	0.98	0.01	0.01
9	0.98	0.01	0.01
10	0.1	0.8	0.1
11	0.1	0.8	0.1
12	0.1	0.8	0.1
13	0.05	0.9	0.05
14	0.05	0.9	0.05
15	0.05	0.9	0.05
16	0.01	0.98	0.01
17	0.01	0.98	0.01
18	0.01	0.98	0.01
19	0.1	0.1	0.8
20	0.1	0.1	0.8
21	0.1	0.1	0.8
22	0.05	0.05	0.9
23	0.05	0.05	0.9
24	0.05	0.05	0.9
25	0.01	0.01	0.98
26	0.01	0.01	0.98
27	0.01	0.01	0.98

Table 4. Assembly summary of the five-draft reference genomes.

	MYBT22	MYBT18246	MYBT18247	MYBT50	MYBT18679
Average Contig length	17984.6	8941.49	13887.16	17671.93	20268.20
Median Contig length	10242.5	2334	6853	6815	7770
Min Contig length	102	102	110	102	107
Max Contig length	116305	194236	108816	300118	157625
Number of Contigs	310	715	411	357	291

Table 5. Genes analysed by PCR.

Gene	Strain	Forward Primer	Sequence (5'-3')	Reverse Primer	Sequence (5'-3')
cry13A	MYBT18246	Cry13A_F4	AATGTGCTGGGACAATCAGG	Cry13A_R4	TTGGGAATTTTCTGGAACACC
cry6B	MYBT18247	Cry6B_F2	CTGTTCAAGTACAACTAGCAC	Cry6B_R2	GGCTATCTCTTTCCATTGACC
cry14A	MYBT18679	Cry14A_F1	CTAATAATGCGCGACCTACTG	Cry14A_R1	GTACCAGCTATTGCACAACC
cry21A	MYBT18679	Cry21A_F1	CAACACCTTCAAATCGCATGG	Cry21A_R1	CATAAGTCCTGGTTGTTCTCC
cry35A	MYBT18679	CryF16	CCAGAAGTAGGAGGAGGTACA	CryR16	TTCATACCGAATGGTTTGTGAG
codY	all	CodY_F	TGAACACCAGCTTCAAGCAAT	CodY_R	GTTATTACAGAGCGCAGCAGG

Table 6. Toxin gene prevalence and virulence of evolved populations from the one-sided adaptation treatment from host generation 12. A total of 20 clones were tested for each population. High virulence indicates a minimum of 78% and low virulence indicates a maximum of 3% dead nematodes under standard assay conditions (Masri et al. in prep).

Replicate	Clones with at least one toxin gene	Virulence
1	0	Low
2	19	High
3	20	High
4	20	High
5	1	Low
6	19	High
7	0	Low
8	0	Low
9	8	High
10	0	Low

Table 7. Number of clones with different genotype compositions for each replicate from the coevolution treatment at host generation 12. + toxins: at least one toxin gene from MYBT18679 present. Shaded boxes indicate inferred cases of horizontal gene transfer.

Replicate	MYBT18679 + toxins	MYBT18679 - toxins	MYBT22 + toxins	MYBT22 - toxins	MYBT50 + toxins	MYBT50 - toxins	MYBT18246 + toxins
1	18	0	0	1	1	0	0
2	18	2	0	0	0	0	0
3	18	1	0	0	0	0	0
4	20	0	0	0	0	0	0
5	19	0	1	0	0	0	0
6	20	0	0	0	0	0	0
7	19	1	0	0	0	0	0
8	19	1	0	0	0	0	0
9	19	1	0	0	0	0	0
10	16	4	0	0	0	0	0

Table 8. Number of clones with different genotype compositions for each replicate from the one-sided adaptation treatment at host generation 12. + toxins: at least one toxin gene from MYBT18679 present. Shaded boxes indicate inferred cases of horizontal gene transfer.

Replicate	MYBT18679 + toxins	MYBT18679 - toxins	MYBT22 + toxins	MYBT22 - toxins	MYBT50 + toxins	MYBT50 - toxins	MYBT18246 + toxins
1	0	0	0	20	0	0	0
2	18	1	0	0	0	0	0
3	20	0	0	0	0	0	0
4	20	0	0	0	0	0	0
5	0	0	1	19	0	0	0
6	18	1	0	0	0	0	0
7	0	0	0	2	0	17	0
8	0	0	0	1	0	19	0
9	8	0	0	0	0	11	0
10	0	0	0	0	0	20	0

Table 9. Number of clones with different genotype compositions for each replicate from the coevolution treatment at host generation 20. + toxins: at least one toxin gene from MYBT18679 present. Shaded boxes indicate inferred cases of horizontal gene transfer.

Replicate	MYBT18679 + toxins	MYBT18679 - toxins	MYBT22 + toxins	MYBT22 - toxins	MYBT50 + toxins	MYBT50 - toxins	MYBT18246 + toxins
1	20	0	0	0	0	0	0
2	20	0	0	0	0	0	0
3	17	1	0	0	0	0	0
4	20	0	0	0	0	0	0
5	20	0	0	0	0	0	0
6	20	0	0	0	0	0	0
7	19	0	0	0	0	0	1[a]
8	17	0	2	1	0	0	0
9	19	0	0	0	0	0	0
10	20	0	0	0	0	0	0

[a] This clone also has *Cry13A*, which is derived from MYBT18246

Table 10. Number of clones with different genotype compositions for each replicate from the one-sided adaptation treatment at host generation 20. + toxins: at least one toxin gene from MYBT18679 present. Shaded boxes indicate inferred cases of horizontal gene transfer.

Replicate	MYBT18679 + toxins	MYBT18679 - toxins	MYBT22 + toxins	MYBT22 - toxins	MYBT50 + toxins	MYBT50 - toxins	MYBT18246 + toxins
2	0	0	0	20	0	0	0
3	5	0	14	1	0	0	0
4	19	0	1	0	0	0	0
6	20	0	0	0	0	0	0
9	18	2	0	0	0	0	0
10	0	0	0	0	0	20	0

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Scientific career

Personal information

Name	Leila El Masri
Born the	07.07.1984 in Beirut, Lebanon
Marital status	Married
Nationalities	German and Lebanese

School education

9/1988 – 7/1994	Mission laïque française, Tripolie, Libya
9/1994 – 7/1996	Ecole Saint Joseph de l'Apparition, Beirut, Lebanon
9/1996 – 7/2002	Mission laïque française, Verdun, Beirut, Lebanon

University education

9/2002 – 7/2006	Master I: Animal physiology. Lebanese University, Faculty of Science II, Fanar, Lebanon *(1st year: Biology & Chemistry; 2sd year: General Biology; 3rd year: Zoology, 4th year: Physiology).*
9/2006 – 7/2007	Master II: Structure and Interaction of Macromolecules and Functional Genomics. University of Saint Joseph, Faculty of Science, Mar Roukoz, Lebanon
1/2007 – 7/2007	Master Thesis: The study of the structure and genetic diversity of a mixed oak population using microsatellites. Institut National de Recherche Agronomique, Cestas, France
11/2007 – 10/2011	Doctoral thesis at the Department of Animal Evolutionary Ecology, Eberhard Karls, University of Tuebingen, Gemany. Thesis: "Experimental test of the consequences of host-parasite coevolution" (Supervisors: Prof. Dr. Hinrich Schulenburg and Prof. Dr. Nico K. Michiels).

11/2011 – present	Continuation of the doctoral thesis at the department of Evolutionary Ecology and Genetics, Zoological Institute, Christian-Albrechts-Universitaet of Kiel, Germany.

Publications

Arndt Hampe, **Leila El Masri**, Rémy J Petit (2010) Origin of spatial genetic structure in an expanding oak population., 459-71. In *Molecular ecology* 19 (3)

Leila Masri, David Laehnemann, Patrick S. Günther, Swantje Prahl, Nico K. Michiels, Hinrich Schulenburg (unpublished manuscript). An experimental test of the consequences of host-parasite coevolution

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Antoine Branca, Anna Sheppard, **Leila Masri**, Manja Saebelfeld, Heiko Liesegang, Elzbieta Brzuszkiewicz, Rolf Daniel, Joachim Kurtz, Philip Rosenstiel, Erich Bornberg-Bauer, Hinrich Schulenburg (unpublished manuscript). Ecological functional genomics of the model pathogen *Bacillus thuringiensis* during experimental coevolution with its nematode host *Caenorhabditis elegans*

Declaration

I, Leila El Masri, declare that:

- Apart from my supervisor's guidance the content and design of the thesis is all my own work;
- Specific aspects of my thesis were supported by colleagues; their contribution is specified in detail in the Chapter "Contribution of authors" at the beginning of the thesis;
- The thesis has not already been submitted neither partially nor wholly as part of a doctoral degree to another examining body and it has not been published nor submitted for publication;
- The thesis has been prepared subject to the Rules of Good Scientific Practice of the German Research Foundation.

Information about referent and second reviewer:

Referent: Prof. Dr. Hinrich Schulenburg

Scientific career

- Since 2008: Professor in Zoology, Kiel, Germany
- 2005-2008: Research scientist, Tuebingen, Germany
- 1999-2005: Research assistant, Muenster, Germany
- 1996-1999: PhD, Cambridge, UK

1991-1996: Studies in Biology, Bielefeld, Germany, and Cambridge, UK

Correspondence

Hinrich Schulenburg, Department of Evolutionary Ecology and Genetics, Zoological Institute, Christian-Albrechts-University of Kiel, Germany; Tel: +49-431-8804141; Fax: +49-431-8802403; Email: hschulenburg@zoologie.uni-kiel.de

Second Reviewer: Prof. Dr. Nico K. Michiels

Scientific career

- Since 2004: Professor in Zoology, Tuebingen, Germany
- 1999-2004: Professor in Zoology, Muenster, Germany
- 1993-1999: Research group leader (Associate Professor), Seewiesen, Germany
- 1992: Postdoctoral fellow, Sheffield, England
    - 1990-1991: Postdoctoral research fellow, Diepenbeek, Belgium
    - 1989: Ph.D. in Zoology at the University of Antwerp, Belgium
    - 1984: Graduation in Zoology at the University of Antwerp, Belgium

Correspondence

Nico K. Michiels, University Tuebingen, Faculty for Biology Institute for Evolution and Ecology, Animal Evolutionary Ecology Auf der Morgenstelle 28 D-72076 Tuebingen ; Tel: +49 7071 29 78876; Fax: +49 7071 29 5634; Email: nico.michiels@uni-tuebingen.de

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