When facing multiple enemies:

The impact of host development time and exposure to multiple parasites in shaping coevolutionary adaptations in host and parasites.

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Summary

Interactions between hosts and parasites represent a very important ecological relationship, occurring across ecosystems. Because of the tight nature of these interactions, hosts and parasites enter into coevolutionary dynamics which can be, to a certain extent, replicated and studied under laboratory conditions. In experimental coevolution of host and parasites, specific antagonistic selective pressures are employed to study population dynamics, genetics of adaptations, disease dynamics and species diversification. Though some studies have focused on host's interaction with multiple strains of the same parasite, a facet of host-parasite coevolution that has received little attention is the involvement of multiple species of parasites.

In nature, host interaction with multiple parasites either simultaneously or sequentially is more a norm than an exception and has been reviewed in chapter 1 *Introduction*. Therefore, in this thesis, I aimed to assess a multicellular host's interaction with multiple parasites in an experimental evolutionary context. For the first time, adaptations in both host and parasites in a simultaneous multiple parasite exposure has been explored using a tripartite (one host-two parasite system) experimental coevolution approach. Also for the first time, the consequence of sequential multiple parasite exposure has been investigated in a previously parasite coevolved host.

Tripartite experimental coevolution was performed using the red flour beetle *Tribolium castaneum*, and its two natural parasites *Beauveria bassiana* and *Bacillus thuringiensis*, for an equivalent of ten host generations, with all the treatments replicated in two host development regimes, FAST (generation time of 21 days) and NORMAL (generation time of 28 days). Host and parasites were sampled throughout the experiment, and host immune response (internal and external immune proxies) was measured. I observed that host development regime not only influenced host immune response but also led to adaptations in the parasites. Beetles from the coevolution experiment were resistant to non-evolved *B. thuringiensis* and

varied in their immune profile based on their development time. Neither parasites showed any significant trend regarding virulence but displayed very distinct protective adaptations as a consequence of coevolutionary interactions. *B. thuringiensis* isolated from the environments of FAST regime unanimously formed biofilms, reported for the first time for this strain. *B. bassiana* isolates from the NORMAL regime exhibited resistance to the beetle's external defensive secretions.

I also investigated emergence of cross-resistance as a consequence of sequential multiple parasite exposure. For this, I used *T. castaneum*, previously coevolved beetles with the fungus *B. bassiana*, and exposed it to the parasites, *B. thuringiensis* and *P. entomophila*. Using survival and gene expression experiments I was able to show evidence that the positive cross-resistance displayed by the *B. bassiana* coevolved beetles towards *B. thuringiensis* can be attributed to a similarity in the route and mechanism of infection of both parasites.

With my thesis, I have been able to highlight the different host and parasite responses to simultaneous and sequential interactions of multiple parasites and one host. While simultaneous exposure led to evolutionally adaptation in host immune response and parasites' protective features, the host exhibits an advantage in sequential exposure, when the routes of infection are similar between the parasites. Finally, these results draw the attention to the need for host-parasite evolution experiments involving multiple multicellular hosts and natural parasites, with longer evolutionary timescales, for better understanding of a ubiquitous natural interaction.

Zusammenfassung

Die Interaktion zwischen Wirt und Parasit stellt eine der fundamentalsten ökologische Beziehung da, welche allgegenwärtig in den unterschiedlichsten Ökosystemen auftritt. Aufgrund der engen Bindung zwischen Wirt und Parasit unterliegt diese Interaktion koevolutionären Dynamiken, welche unter Laborbedingungen nachgestellt und erforscht werden können. In der experimentellen Koevolution zwischen Wirt und Parasiten werden spezifische antagonistische selektive Drücke eingesetzt, um Populationsdynamik, genetische Anpassungen, Krankheitsdynamik und Artenvielfalt zu untersuchen. Obwohl bereits ein paar Studien die Interaktion zwischen einem Wirt und mehreren Stämmen des gleichen Parasiten untersucht haben, erlangte eine Facette der Wirts-Parasiten-Koevolution bis jetzt wenig Aufmerksamkeit, und zwar die Beteiligung mehrerer Arten von Parasiten.

In der Natur ist die Interaktion eines Wirts mit mehreren Parasiten, entweder simultan oder sequenziell, eher die Norm als die Ausnahme und wird in Kapitel 1 *Introduction* näher erläutert. Darauf begründet war das Ziel meiner Thesis die Interaktion eines multizellulären Wirts mit multiplen Parasiten in einem experimentellen evolutionären Kontext zu untersuchen. Erstmalig wurde die Anpassungsfähigkeit sowohl des Wirtes als auch der Parasiten in einer simultanen Exposition in einem dreigliedrigen (Ein-Wirt-Zwei-Parasiten-System) experimentellen Koevolutionsansatzes analysiert. Des Weiteren wurde auch zum ersten Mal die Folge einer sequenziellen Exposition in einem bereits mit Parasiten koevolvierten Wirt untersucht.

Der dreigliedrige experimentelle Koevolutionsansatz basierte auf dem roten Mehlkäfer *Tribolium castaneum* und seinen beiden natürlichen Parasiten *Beauveria bassiana* und *Bacillus thuringiensis*. Dieser wurde für ein Äquivalent von zehn Wirtsgenerationen durchgeführt, wobei alle Behandlungen in zwei Wirtsentwicklungsregimen repliziert wurden, FAST (Generationszeit von 21 Tagen) und NORMAL (Generationszeit von 28 Tagen). Die Immunantwort des Wirts (interne und externe Immunproxies) wurde gemessen und der Wirt und die Parasiten wurden während des gesamten Experimentes beprobt. Ich

beobachtete, dass das Wirtsentwicklungsregime nicht nur die Immunantwort des Wirts beeinflusste, sondern auch zu defensiven Anpassungen in den Parasiten führte. Die Käfer aus dem Koevolutionsexperiment waren resistent gegen nicht-evolvierte *B. thuringiensis* und ihr Immunprofil variierte in Abhängigkeit der Entwicklungszeit. Keiner der Parasiten zeigte einen signifikanten Trend zur Virulenz, dennoch wiesen sie unterschiedliche Anpassungen ihrer Schutzmechanismen als Konsequenz der koevolutionären Wechselwirkungen auf. Der aus dem FAST Regime isolierte *B. thuringiensis* formte ausnahmslos Biofilme, was zum ersten Mal für diesen Stamm beschrieben wurde. *B. bassiana* Isolate aus dem NORMAL Regime zeigten Resistenz gegen die externen Verteidigungssekrete des Käfers.

Ich habe auch die Entstehung von Kreuzresistenz als Folge der sequenziellen Exposition untersucht. Dazu verwendete ich den zuvor koevolvierten *T. castaneum* mit dem Pilz *B. bassiana* und exponierte ihn den Parasiten *B. thuringiensis* und *P. entomophila*. Unter Verwendung von Überlebens- und Genexpressionsexperimenten konnte ich zeigen, dass die positive Kreuzresistenz von den *B. bassiana*-koevolvierten Käfern gegen *B. thuringiensis*, auf einer Ähnlichkeit des Infektionsweges und -mechanismus beider Parasiten zurückzuführen ist.

Mit meiner Thesis konnte ich verschiedenen Anpassungsstrategien auf simultane und sequenzielle Interaktionen mehrerer Parasiten und eines Wirts hervorheben. Während die simultane Exposition zu evolutionären Anpassungen der Wirtsimmunantwort und der Schutzmechanismen der Parasiten führte, weist der Wirt einen Vorteil bei der sequenziellen Exposition auf, wenn die Infektionswege zwischen den Parasiten ähnlich sind. Letztlich zeugen die Ergebnisse meiner Thesis von der Notwendigkeit von Wirt-Parasiten-Evolutionsexperimenten mit mehreren multizellulären Wirten und natürlichen Parasiten über längere evolutionäre Zeitskalen, um das Verständnis der komplexen Interaktionen in unseren Ökosystemen verbessern zu können.

List of definitions

Coevolution: an ecological interaction between two or more organisms characterized by reciprocal adaptive changes [1]

Host: a living organism that provides resources to another living organism to complete a part or the entirety of its life-cycle.

Infection and Pathogenesis: while infection is the process of parasites colonizing and multiplying inside the host, pathogenesis is the negative effect on host fitness that occurs post infection [2].

Mechanism of infection: the process of by which parasites employ virulence factors (biomolecules that aid the parasite in establishing and causing disease in the host) to cause pathogenesis in hosts. E.g. *Cry* toxins of *Bacillus thuringiensis* that cause disruption of the enterocyte membrane integrity in the gut of insect hosts [3].

Multiple parasite infection: infection of one host species with more than one species of parasite (implying micro & macroparasites). Such an infection may occur simultaneously or sequentially.

Parasite: an organism that uses another organism (host) for shelter and resources, causing harm to it [4]. In this thesis, the use of the term 'parasite' always implies micro & macroparasites that can cause pathogenesis in the host.

Resistance: mechanism(s) that prevent infection by killing or stopping parasite growth upon attack in the host organism [5].

Route of infection: the path of parasite entry into the host's body, breaching the first line of host defence. In this thesis, route of infection primarily includes oral and systemic (via the cuticle as in the case of insects) route [6]. In this thesis, route of infection is to be seen only in the context of horizontal parasite transmission (unless otherwise specified).

Virulence: the rate at which a parasite induces host mortality [1], virulence implies the ability of the parasite to invade and colonize the host as well as the severity of the disease that it brings about in the host [7].

Sequential parasite exposure: exposure of the host to a different parasite after successfully defending one parasitic exposure

Simultaneous parasite exposure: when the host is exposed to two or more parasites at the same time point

“Nothing in biology makes sense except in the light of evolution.”

— Theodosius Dobzhansky, 1973

1

Introduction

I am never really satisfied that I understand anything; because understand well as I may, my comprehension can only be an infinitesimal fraction of all I want to understand.

|

Ada Lovelace, Mathematician (1815-1852)

- *Insects are the largest taxa spread across a wide variety of ecosystems and therefore are exposed to a variety of natural parasites. Evolutionary interaction of the host with multiple parasites and the outcomes thereof form the basis of this thesis. Close interactions between insect hosts and parasites are under permanent coevolutionary dynamics, where hosts and parasites exert negative selection pressure on each other, leading to reciprocal adaptive changes in both.*
- *Here, the impact of host interaction with multiple parasites has been addressed in the context of experimental host-parasite coevolution, which seems largely absent in existing literature. Also when it comes to experimental coevolution involving animal hosts, the impact of variability in host life-history trait is not fully understood. Using a one host-two parasite system my goal was to understand the outcome of coevolutionary interaction in the context of multiple parasites and variable host development time. It is important to note that, coevolution not only results in responses specific to the antagonist in question but can produce broad-scale effects. In this regard, the phenomenon of host cross-resistance towards different parasites has been discussed. Cross-resistance to different parasites and its basis was tested in beetles coevolved with a parasite.*
- *Through various experiments using the red flour beetle Tribolium castaneum as host and Beauveria bassiana, Bacillus thuringinesis bv. tenebrionis and Pseudomonas entomophila as parasites my thesis aims to understand the impact of such multipartite host-parasite interactions on both host and parasite.*

INTRODUCTION

1.1 Host-parasite interactions: bipartite evolution in a complex community

The omnipresence of host-parasite interactions makes it a well-studied antagonism in the fields of immunity, ecology and evolution [1,8,9]. This tight bipartite evolutionary interaction has helped researchers understand adaptive responses in both the host [8,10–12] and parasite [13–15]. The hosts and parasites can partake in complex ecological interactions [16–20], capable of shaping the life-histories of both host and parasite [21–23]. These ecological interactions are not limited to one host-parasite system, but also involve the interaction of one host with several parasites and vice versa, affecting the life-history traits of each another [22,24–26]. Complexity is one of the main reasons why this interaction is often studied in pairs, involving one host and one parasite species.

Within-host interactions in multiple parasite exposures, lead to adaptive changes in parasite virulence [27,28]. For instance, various experimental and theoretical studies have reported that parasite virulence increases due to competition for host exploitation, thereby selecting for the strongest parasite [29–31]. However, with increasing number of strains or different parasites infecting the host decrease relatedness and therefore, cooperation among them [32], consequently leads to a decrease in parasite virulence (reviewed in [33]). Such interactions are missing in single parasite infections.

In single parasite infection, the host often has an upper hand on the parasite, by employing various defence mechanisms [34–36]. In contrast, multiple parasite infections are mostly shown to be more detrimental towards the host. For example, in the rainbow trout *Oncorhynchus mykiss,* mortality was found to be significantly higher when simultaneously infected with the parasites *Argulus coregoni* and *Flavobacterium columnare* than in single infections [37]. Multiple parasite infections can also influence disease dynamics. When the English plantain, *Plantago lanceolata* was co-infected with two strains of *Podospora plantaginis*, it transmitted significantly higher numbers of parasite spores than plants infected with a single strain of *P. plantaginis*, thereby accelerating the rate of disease spread across different susceptible populations [38]. Multiple parasite exposures have been addressed by studies that investigate

the potential of hosts to defend against different parasites [39–41] by employing a variety of immune strategies.

Defence strategies employed in host-parasite interactions

Hosts and parasites have different strategies to counter each other's antagonism. In insect hosts, innate immunity forms the primary defence system. Most of our knowledge of insect innate immunity comes from the classic model of the fruit fly *Drosophila melanogaster* [42], the red flour beetle *Tribolium castaneum* [43] and the mosquito *Aedes aegypti* [44]. Upon recognition of parasite presence by pathogen-associated molecular patterns (PAMPs) through pathogen recognition receptors (PRRs) [35], broad and narrow range immune responses can be employed by the insect. Melanisation (encapsulating the parasite with melanin and inhibiting any contact with the insect body) is one such broad range response which is primarily mediated by the enzyme phenoloxidase (PO) that oxidizes the amino acid Tyrosine [45]. Specialized cells called hemocytes are also important in encapsulation as well as performing phagocytosis of parasites, thereby contributing to a broad response [42]

Of the narrow range of defence mechanisms, different classes of antimicrobial peptides (AMPs, synthesized in the fat body) are active against different types of microbial parasites [46]. The production of AMPs are regulated by the NF-ƙB pathways Toll and immune deficiency (IMD) that can be induced by different types of parasites [42,47]. While the AMPs of the Toll pathway are primarily active against fungal and Gram-positive bacterial parasites those of the IMD pathway are primarily active against Gram-negative bacterial parasites [35,48], with evidence of cross-talk between these two immune pathways [47]. Cytotoxic reactive oxygen species (ROS), as well as heat shock proteins (Hsp) in response to stress, are also employed as a defence against parasites [42].

For the parasites, entering the insect body and releasing various virulence factors[49], are primary for host exploitation. Among entomopathogens, the *Cry* and *Cyt* toxins of *Bacillus thuringiensis* are the best-studied virulence factors [3]. The route of parasite entry governs the progression of pathogenesis (the collective damage caused to the host) with generally systemic infections being faster at killing the host than oral infections [49]. Another important aspect of parasite virulence is its ability to persist, inside and outside of the host. Formation of an extracellular structure called biofilm protects the micro-organisms from their harsh environment, making it possible for them to persist and attack the host when conditions become favourable for them [50,51]. Together, these mechanisms of hosts and parasites, to counter the antagonistic pressures of each other often leads to an arms race between them, leading to them shaping each other's evolution.

Red queen dynamics at the heart of host-parasite evolutionary interactions

Rapid reciprocal changes in host-parasite interactions can be attributed to three kinds of selections; negative frequency-dependent [52], directional [53] and disruptive [54] selections. Theory, as well as experimental studies, often seek to understand host-parasite coevolutionary interactions with the point of view of negative frequency-dependent selection [52,55,56]. In this context, the negative frequency-dependent selection is popularly referred to as the 'Red Queen Dynamics' (RQD), where hosts and parasites are locked together in adaptive dynamics, without any party emerging as the sole winner [57–59]. This theory is inspired by the famous quote of the Red Queen in Lewis Carroll's *Through the looking glass*, "Now, here, you see, it takes all the running you can do, to keep in the same place. If you want to get somewhere else, you must run at least twice as fast as that!"

The Red Queen Hypothesis in evolutionary biology was first proposed by van Valen to explain the 'The law of Extinction'[60]. In the context of host-parasite interactions, RQD implies that parasites are better at

infecting sympatric hosts. An excellent example is the study of the New-Zealand freshwater snail *Potamopyrgus antipodarum* and its trematode parasite *Microphallus* sp. [61]. Naturally obtained snail hosts were infected in the lab with trematode parasites from two lakes after which the authors observed that parasites exhibited a significantly higher rate of infection to sympatric hosts as opposed to allopatric ones. Based on the RQD framework host-parasite coevolution studies often seek evidence of local adaptation in the coevolved host and parasite populations [62–64] and experimental coevolution is a useful tool to test this.

Host life history influences evolutionary dynamics with parasite

It is known that faster generation time and pace of development increase rate of adaptation, which in turn allows for rapid fixation of genetic changes [5,65]. In the context of evolutionary ecology, the 'Pace of life' hypothesis forms an interesting premise; investment of resources in rapid growth and development is traded-off against immune defence [66]. Agnew and Koella reported that in *A. aegypti*, populations selected for earlier pupation showed less mortality upon infection with the microsporidia *Edhazardia aedis* [67]. In *D. melanogaster,* populations selected for faster development, higher hemocyte density and PO hemolymph activity was found to be traded-off against larval competitive ability, which decreased [68]. Alternately, Boots and Begon showed that experimental evolution to a granulosis virus infection in the Indian meal moth *Plodia interpunctella* resulted in a longer development time for the moth [69].

Recently, Tate and Graham addressed the conundrum of the trade-off between immune defence and life history related constraints with a modelling approach whereby it was revealed that resource allocation to development is impaired if there is too much investment in immune defences [70]. Their modelling results also showed that the situation where the larvae incur developmental costs, resource allocation to faster development led to higher overall resistance. Therefore, manipulation of host development time in an

evolution experiment can drive evolutionary adaptations in different trajectories. In the community context, variation in host-development time can also help the host survive certain parasites that are virulent at earlier host life-stages [21,71]. In spite of the importance of life-history, there is a substantial lack of experimental work addressing the impact of the same in host-parasite coevolutionary dynamics, let alone in coevolution with multiple parasites.

1.2 Experimental approaches to study host-parasite evolutionary interactions

The dynamics of host-parasite coevolutionary interactions are important for the prevalence and maintenance of biodiversity [72,73] and sexual dimorphism [74–76]. Here, natural selection leads to hosts fighting parasites by selecting for host genes that contribute to resistance [77,78] which in turn contributes to the parasite's ability to fight host resistance [1,79–81]. In the laboratory, experimental evolution (Figure 1.1) is a powerful tool to investigate a number of evolutionary questions including those pertaining to antagonistic host-parasite [63,82–85] and predator-prey interactions [86]. Here, control populations and populations under imposed selection pressure, allow us to track adaptations in real-time by artificial manipulation [53,87–89]. Pioneering experimental evolution studies performed by Lenski et al. with the bacterium *E. coli* under controlled laboratory conditions [90,91] have provided valuable insights into processes driving evolution such as the dynamics of adaptation [92,93], selection of beneficial mutations [94], competition [95,96] and host-parasite antagonism [13,79,85,97]. Such studies laid the groundwork for experimental coevolution, which allowed for both the host and the parasite to adapt and counter-adapt simultaneously [98].

Experimental evolution allows for the performance of time-shift experiments, where host and parasite populations from different evolutionary time-points are tested against each other [63,99]. When Bérénos *et al.* after experimental coevolution, performed infection experiments on the beetle *Tribolium castaneum*

from current generation with the microsporidian parasite *Paranosema whitei* from eight different time points in coevolutionary history (across twelve host generations), they found parasite virulence to decrease over time [100]. Host survival increased over the time and was higher when current hosts were exposed to parasites from recent time points than when exposed to parasites from the past [100], providing evidence for local adaptation.

Artificial manipulation of the system in different ways can lead to conflicting outcomes. Rafaluk et al. observed that a number of antagonistic coevolution experiments, although involving the same host-parasite system, provides contrasting outcomes in terms of parasite's virulence based on methodology [14]. For instance, while Schulte et al. reported an increase in the virulence of *B. thuringiensis* post coevolution with *C. elegans* [63], Masri and co-workers observed no change in the virulence of *B. thuringiensis* upon coevolution with the same host [99]. For the host, while Bérénos *et al.* [101] reported an increase in resistance to *P. whitei* in *T. castaneum* as a result of experimental coevolution, Rafaluk et al., [102] reported a rapid decrease in resistance of *T. castaneum* to *P. whitei* in their coevolution experiment. In spite of its limitations, experimental coevolution is a compelling method to study the effects of reciprocal selective forces and their effects on the life history of the host and parasite [98].

Because the system can be manipulated, incorporating life-history traits of the host (e.g. developmental time, body size etc.) and parasite (e.g. growth rate, biofilm-forming ability etc.) can lead host-parasite dynamics in an interesting direction. In addition to traits that are directly under reciprocal selection pressure, traits related to host life-history often show correlated response upon evolutionary interactions with parasite [81]. For instance, experimental evolution of *Daphnia magna* with the microsporidia *Octosporea bayeri* led to increased competitive ability in the evolved populations [103]. Evolution of D. *melanogaster* with the parasite *Bacillus cereus* resulted in longer development time in the evolved fly populations [104]. However, owing to complexity of the system, such added factors have so far not been incorporated in the experimental coevolution of hosts and parasites.

In natural environments, one type of host (species or genotype) can potentially coevolve with more than one type of parasite (species or genotype) or *vice versa* [21,28] but studying the dynamics of such a system in the lab can be challenging [21,28]. There have been few theoretical attempts to study evolutionary interactions involving multiple players (table 1.1). Simulating a system with multiple hosts and multiple parasites, Rabajante et al. demonstrated that oscillatory RQD can occur in such system under the conditions of high basal growth rate (host), an intermediate death rate (parasite) and infection specificity (parasite) [105]. It was also shown that environmental effects, can lead to the replacement of a dominant host/parasite population by a rare host/parasite type, indicating that RQD helps in the maintenance of biodiversity across the evolutionary time-scale [105,106]. While there are experimental coevolution studies investigating the outcome of the bipartite interaction between an animal host and its parasites [81,98], there is a lack of experiments studying host coevolutionary dynamics with multiple parasites. I aim to fill this gap in the literature with my evolution experiment, additionally incorporating development time as a host life history variable.

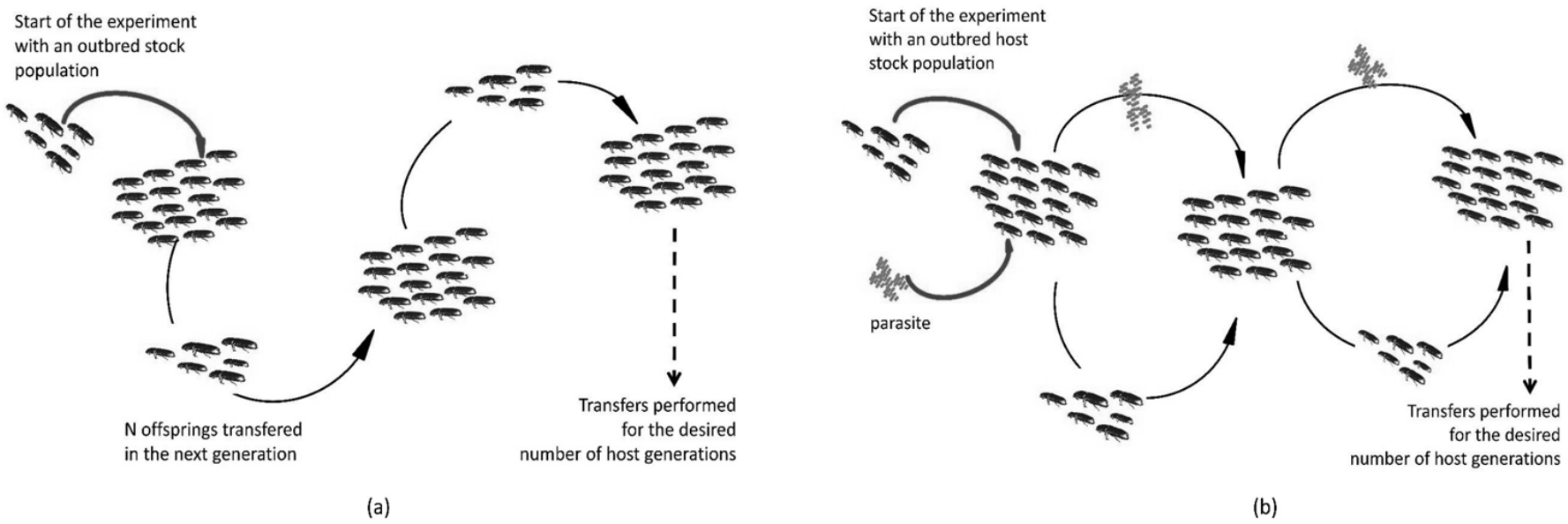


Figure 1.1 Schematic of experimental coevolution. (The thick arrows correspond to the addition of host/parasites from the stock population). Brockhurst & Koskella [98] proposed that a selection experiment can be termed as an experimental coevolution only if they meet the following criteria: (i) interacting species are co-cultured, (ii) the experiment investigates evolutionary responses in all the parties involved and (ii) evolutionary responses from the coevolutionary treatments in the co-cultured lines are compared to that of control lines (where coevolution is prevented) [98]. In this illustration depicting a simple coevolution experiment, panel (a) represents control populations devoid of any parasites in the environment and panel (b) represents coevolution treatment with both the hosts and the parasites transferred simultaneously at fixed time-points. Several studies have explored host-parasite interactions in accordance with such an experimental design. *C. elegans* and *Bacillus thuringiensis* observed in experimental coevolution studies exhibit greater molecular level changes as opposed to their control counterparts [11]. It has also been observed in *C. elegans* that after experimental coevolution with *B. thuringiensis* there is an elevated food avoidance behaviour [63].

Table 1.1 Reports of host-parasite coevolutionary studies involving multiple host and enemies (parasite or predator)

Characteristics of the host-parasite system	Type of study	Hypothesis evaluated	Key results	References
Multiple hosts and parasites (mammalian hosts and helminths)	Meta-analytical study using phylogenetic and statistical analysis	Whether parasite diversity is itself a part of 'parasitic pressure'	Long living hosts have increased immune investments and hosts facing a greater parasite diversity invest more in immunity.	[41]
Flavobacterium *Cellulophaga baltica* (host) and ϕS_T and ϕS_M (viral parasites)	Experimental	How do bacteriophages influence the diversity of bacterial strains at a population level?	Bacteriophages are potent drivers of bacterial strain diversity at a population level	[107]
One host, two parasites	Theoretical	Within host-parasite interactions	Cooperation between parasites can affect virulence dynamics	[28]
Multiple hosts and parasites	Theoretical	Investigating the impact of life host and parasite life-history factors on RQD	High host growth, intermediate parasite death and infection specificity contribute majorly to RQD	[105,106]
One host, one parasite and one predator	Theoretical	Does increasing defence against one enemy limit the host's defence against the other enemy	Investment of resources towards any type of enemy depends on the composition of the enemy populace and host growth rate.	[24]

1.3 Cross-resistance as a consequence of host-parasite interaction

Few experimental studies investigate the consequence of bipartite host-parasite interactions on host response upon exposure to other parasites [6,108–111]. Evolutionary interactions with a single parasite may not only have specific outcomes in response to that particular parasite but can also produce broader-scale effects such as cross-resistance, displayed towards different parasites the host may encounter [108].

Cross-resistance (Figure 1.2) is a host defence mechanism that incidentally develops as a response to parasite infection in which host evolutionary interactions with one parasite **(A)** leads to either host resistance or hyper-susceptibility to another parasite **(B or C)** [112]. When evolutionary interaction with a parasite **(A)** results in the host being resistant to previously un-encountered parasites **(B)** it is termed as positive cross-resistance [112]. In a study on *D. melanogaster*, fly populations that evolved to have increased resistance to the parasitic wasp *Leptopilina boulardi* were positively cross-resistant to the parasitoid wasp *Asobara tabida* [108]. Martins *et al.* [6] showed that *D. melanogaster* populations evolved to *Pseudomonas entomophila* was positively cross-resistant to the closely related *P. putida*. It was also shown by Martins et al. that experimental evolution to *Drosophila* C virus (DCV) led to *D. melanogaster* being positively cross-resistant to cricket paralysis virus (CrPV) and flock house virus (FHV) which relied just on few major genes [111]. It is imperative to mention here that immune priming i.e. protection from the type of parasite that the insect was previously and subsequently exposed to [113–115], should not be confused with positive cross-resistance.

While discussing costs associated with immune defence, McKean and Lazzaro highlight the concept of 'Multiple-fronts' costs which is displayed when the defence against parasite **(A)** results in higher susceptibility to the parasite **(B)** [116]. Since resources in nature are mostly limited, investments in defence against one parasite often lead to a trade-off in the investment of resources towards defending another parasite [116–118], leading to negative cross-resistance [112]. In an experimental evolution study performed by Martins et al. it was observed that the *Pseudomonas entomophila* treated *D. melanogaster* populations were more

susceptible to infection by the viruses FHV and DCV compared to control flies [6]. It was proposed that the higher survival of flies evolved to *P. entomophila* upon infection with *P. putida* comes at a cost which is manifested in the form of hypersusceptibility to viral infections [6]. Since different immune defence pathways are activated upon infection by different parasites, trade-offs between different constituents of immunity (e.g. [119,120]) can potentially explain negative cross-resistance.

Cross-resistance can be expressed at an evolutionary or ecological level [112]. At the evolutionary level, it has been proposed that cross-resistance to a parasite **(A)** is connected with resistance to a different parasite **(B, C)**, by means of shared defence mechanism [111]. For example, *D. melanogaster*, which had experimentally evolved with DCV, was reported to be positively cross-resistant to cricket paralysis virus (CPV) and FVH, mediated by similar underlying genetic elements [111]. At the ecological level, cross-resistance is the result of the activation of immune defence caused by the previous exposure to a different parasite **(A)** [112]. For instance, within the same generation, prior exposure of the mosquito *Anopheles gambiae* to the microsporidian parasite *Vavria culicis* **(A)** results in the mosquitoes being more resistant to *Plasmodium berghei* **(B)**, compared to control mosquitoes, due to an enhanced melanisation response [121]. Within the context of this thesis, I refer to the evolutionary definition of cross-resistance.

Some studies on cross-resistance (see Supplementary Table A.5) have tried to understand the underlying mechanism at play, such as specificity of the route of infection [6] or the genetic basis of resistance [111]. In cases which have reported positive cross-resistance, the first **(A)** and the subsequent parasite **(B)** the host was exposed to were closely related [6,108,111], resulting in the host employing similar immune mechanism against both. Furthermore, in studies which have reported negative cross-resistance or no difference in resistance, the first **(A)** and the subsequent parasite **(B, C)** to which the host was exposed to, belonged to different taxonomic groups [6,111,112,122]. These observations hint that relatedness of the parasite is of relevance for the occurrence of different types of cross-resistance. In *D. melanogaster* that evolved with *P. entomophila* by oral infection, Martins *et al.* [6] observed that positive cross-resistance to the closely related

P. putida was observed only upon oral infection and not when the flies were infected systemically (i.e. cuticular breaching); indicating that route of infection might be an important factor in cross-resistance. Adaptations to different routes have been shown to have different genetic underpinnings. In a study by Behrens et al. [123], it was shown that *T. castaneum* has different gene expression profiles upon oral and systemic infection by the same parasite. Keeping in mind the current trends in invertebrate immunology [124,125] and evolutionary ecology [81,98], it is compelling to investigate how coevolution with a parasite affects host survival upon infection by another parasite and what actually is the major governing factor driving cross-resistance.

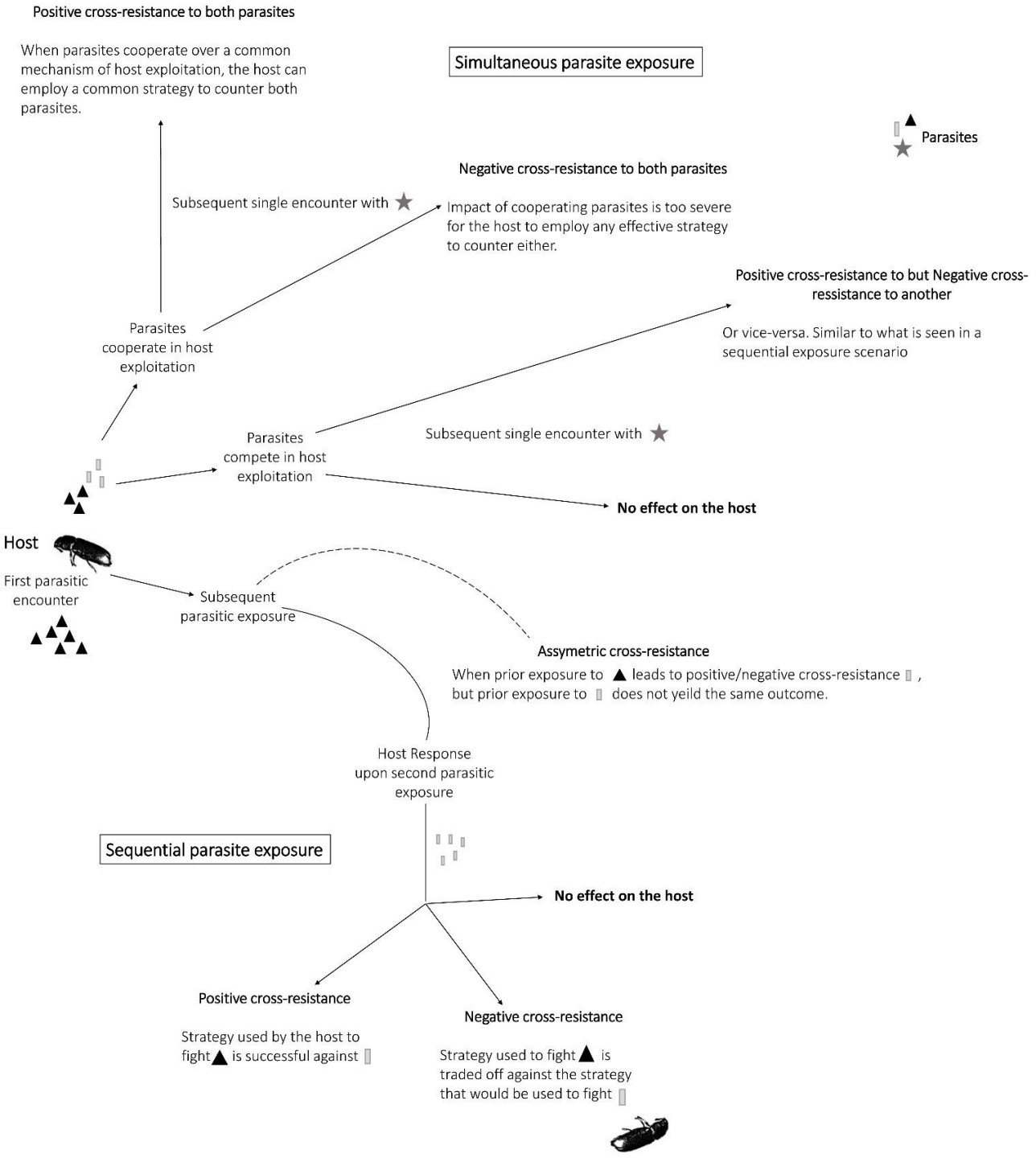


Figure 1.2 for hosts living in a complex community, multiple parasites and multiple encounters are inevitable. Development of cross-resistance upon exposure to multiple parasites: sequential exposure to different parasites can lead to cross-resistance while exposure to the same parasite genotype can lead to the expression of primed immunity (*Also see* Box 1.). Cross-resistance is a broader phenomenon and may confer either negative or positive effect when exposed to a previously un-encountered parasite. Note: the outcomes of cross-resistance illustrated here are to be viewed with respect to control populations that have not encountered parasites before.

1.4 Aims and hypotheses

In nature, evolutionary interactions of hosts and parasites are complex and multi-dimensional but have not been addressed in an experimental context in multicellular hosts, simply for the very same reasons. The broad goal of this thesis, therefore, is to assess adaptive changes in both host and parasite when the host is exposed to multiple parasites. This I approached via simultaneous and sequential exposure to multiple parasites in the purview of experimental coevolution. For this purpose, the model insect, *Tribolium castaneum*, commonly known as the red flour beetle was used as the host and *Bacillus thuringiensis* bv. *tenebrionis* (henceforth *Bacillus thuringiensis*), *Beauveria bassiana* and *Pseudomonas entomophila* were used as the parasites in the different experiments that constitute this thesis. The governing hypothesis addressed in this work is that aspects of host evolutionary interactions with multiple parasites shape the adaptive response of both host and parasites which have been subdivided into two testable hypothesis, whose results form the basis of this thesis:

Aim 1: *To assess the effect of host development time during tripartite experimental host-parasite evolution, on all protagonists.*

Hypothesis 1: *Adaptations of host and parasites under tripartite host-parasite coevolution is governed by host developmental time.*

To test this, coevolution to the parasites *B. bassiana* and *B. thuringiensis* bv. *tenebrionis*, present simultaneously, was performed on *T. castaneum*. Apart from being natural parasites of *T. castaneum, B. bassiana* and *B. thuringiensis* are both able to persist in dry environments as spores. In an effort to simulate natural conditions in experimental coevolution, the parasite spores persisting in the environment were transferred periodically for an equivalent of 10 host generations. The coevolution experiment was performed in two regimes (with identical treatments and replicates), one having host development at a NORMAL pace (28 days generation) and another where the hosts were selected to develop at a FAST (21

days generation) pace. At the end of the experiment, following questions were investigated, with either or more of the possible outcomes in Figure 1.4 which illustrates the evolutionary change in the host with response to either of the parasites or vice-versa.

(a) *Does host development time influence host immune parameters and ability to resist parasites post experimental coevolution?*

T. castaneum possess the ability to condition its immediate environment by secreting a mix of volatile chemical compounds, primarily consisting of quinones [126,127], which has been referred to as its external immune defence [89,128]. Also, the enzyme PO is classical representative of innate immunity [45]. To answer the question formulated here, PO (internal immune defence proxy) and quinone secretion (external immune defence proxy) were measured during the course of the experiment. Additionally, host resistance to non-evolved *B. thuringiensis* bv. *tenebrionis, B. bassiana* and *P. entomophila* were tested via survival assays.

(b) How do parasite virulence and other life-history traits (growth-rate and inhibition by quinone) change as a result of coevolution in a one-host two parasite system? Does host development time play a role?

Survival assays with non-evolved *T. castaneum* populations were conducted with coevolved parasites to test for change in parasite virulence. Furthermore, parasite growth rate was measured as a proxy of life-history costs associated with coevolution. Minimum inhibitory concentration assays were carried out to examine any changes occurring in the parasites' resistance to the quinone secretions of the beetle.

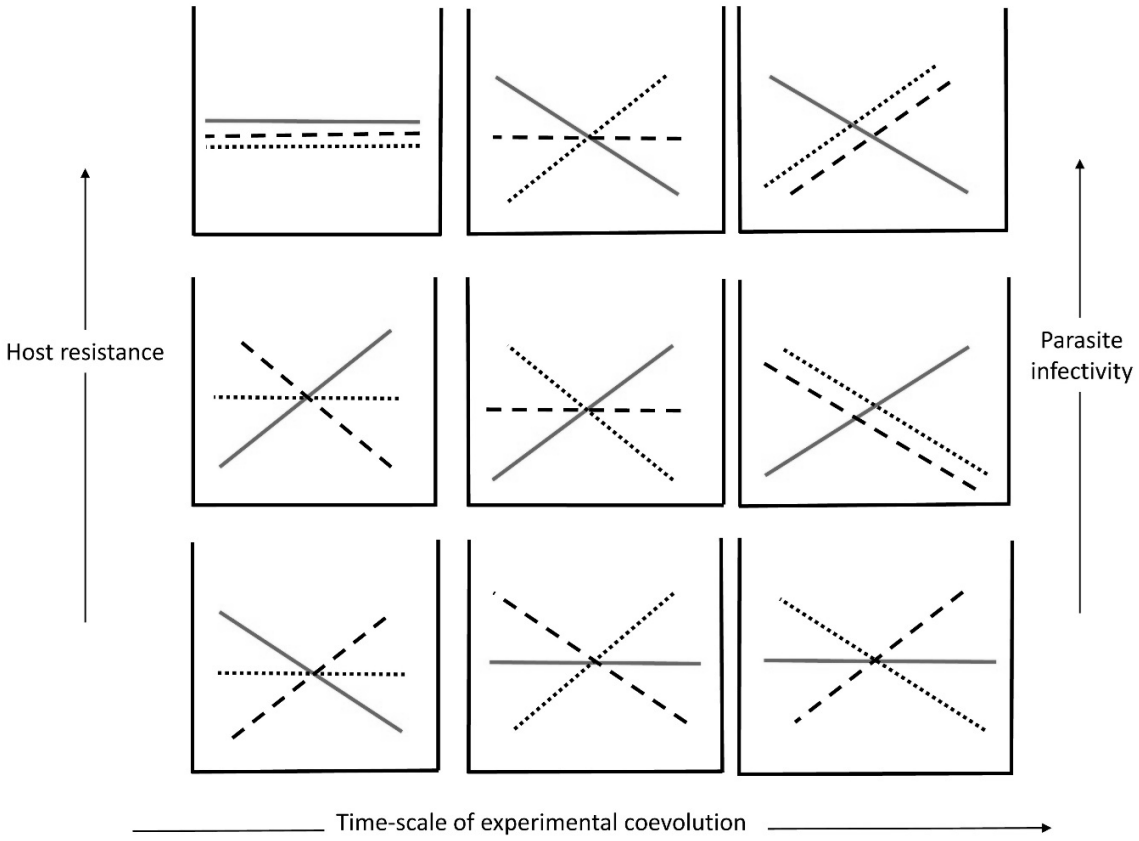


Figure 1.4 Experimental coevolution in a one host-two parasite system. In such a dynamic system host resistance and parasite virulence can change based on the strength of the selective forces. Tripartite evolutionary interactions can produce any of these outcomes or some unpredictable changes, only observed post experiment.

(*T. castaneum* ——— ; *B. thuringiensis* – – – , *B. bassiana* ·····

Aim 2: *To test the impact of host-parasite coevolution on host cross-resistance properties*

Hypothesis 2: *Cross-resistance of the host to different parasites is a consequence of bipartite host-parasite coevolution*

The following questions were addressed to test this hypothesis

(a) *Do coevolved hosts display cross-resistance to novel parasites? What is the nature of this cross-resistance?*

B. thuringiensis differs from *B. bassiana* not only taxonomically but also in terms of infection route; *B. bassiana* has been traditionally thought to infect hosts systemically, i.e. through the cuticle [129] although, evidence exists that it can infect the host orally [130,131]. Through *B. thuringiensis* exposure, I wanted to test whether beetles, coevolutionarily adapted to *B. bassiana*, displayed any cross-resistance based on the route of infection. I also used systemic exposure to *P. entomophila*, which can infect the host both orally and through cuticular breaching [39], to rule out a generally elevated immune response in coevolved beetles. Figure 1.3 illustrates different types of cross-resistance that the coevolved host can display.

(b) *What is the basis of this cross-resistance? Does the route of infection matter?*

For this, I tested the gene expression profiles of *T. castaneum* evolved to *B. bassiana* upon exposures with *B. thuringiensis*, *P. entomophila* and non-evolved *B. bassiana*. Candidate genes were selected based on a survey of existing gene expression studies which span RT-qPCR [132], transcriptomic [123] and functional analysis [133–135] approaches. The genes tested represent stress (Hsp90, p450), phenoloxidase (PO) (Laccase-2 (Lac-2; [133]) and Apolipophorin-III (Apo-III; [135,136])) and antimicrobial peptides (Attacin-2 (Atta-2) & Defensin-3 (Def-3) [132]). Additionally candidates for external immune defence (quinone-related; Gt39 [137]), fungal challenge (Thaumatin-like; Thaumatin [132]), for innate immunity (Lysozyme (Lyso-4; [136])) and chitin

metabolism (chitin deacetylase (TcDA6; [134])) were analyzed. Markers for oral (Apo-III & ObpC-12) and systemic (Hsp-90 & p450) routes of infection were used to test our hypothesis that cross-resistance is route dependent.

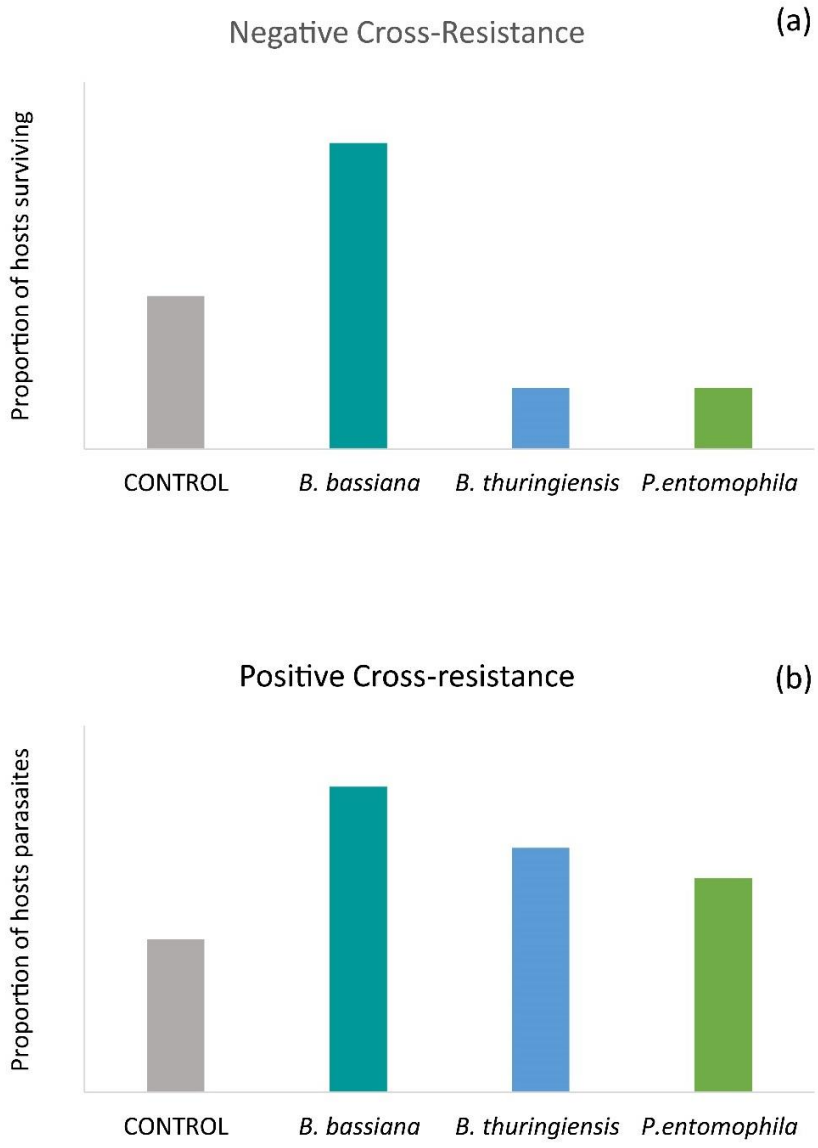


Figure 1.3 Cross-resistance to natural parasites. Predicted response of *B. bassiana* coevolved *T. castaneum* hosts upon exposure with non-evolved *B. bassiana*, *B. thuringiensis tenebrionis* and *P. entomophila.* Coevolved host is resistant to non-evolved *B. bassiana* but might be (a) hyper-susceptible (significantly lower survival compared to control treatment) or (b) resistant to parasites, previously not encountered. The colours corresponding to each parasite species are going to be followed throughout this thesis

Note: CONTROL *all bars in hypothetical results with respect to control treatment.*

1.5 The host: red flour beetle *Tribolium castaneum*

The red flour beetle *T. castaneum* (Figure 2.1) is a tenebrionid beetle which is widely used as a model organism in a variety of disciplines within biology for example, genetics [138–140], developmental [141–143], experimental ecology [144–147], eco-immunology [114,148,149] and evolutionary biology [89,102,150]. Globally, *T. castaneum* is a recognized pest insect of stored food grains [151,152], with evidence suggesting their association with humans since the time of ancient Egyptian [153]. In the lab, the growth optimum is at 30-32°C and 70% humidity. Under these conditions, the egg to adult development time is 28 days with each female laying up to 20 eggs/day. The lifespan of the beetle is approximately two years [154].

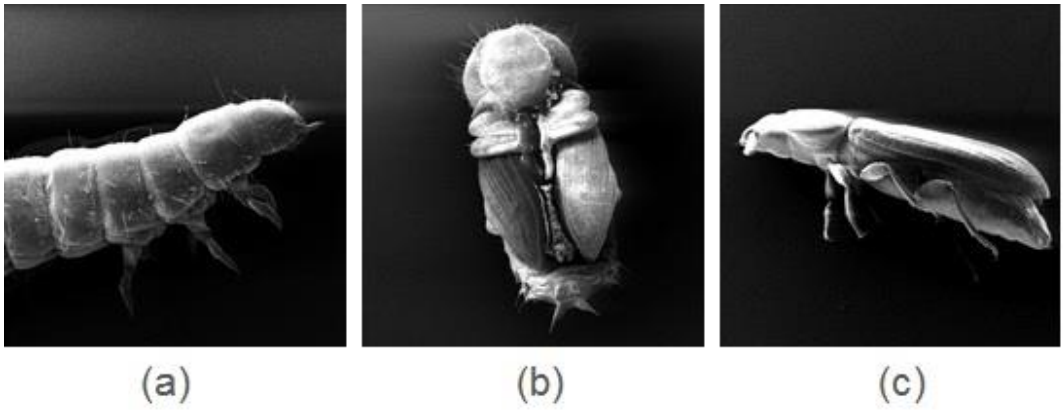


Figure 2.1 Different life stages of *T. castaneum*. As a holometabolous insect, the beetle goes through all the three major life stages post hatching, namely (a) larva, (b) pupa and (c) adult. (Pictures courtesy Dr. Gerrit Joop)

In addition to the ease of maintenance, the fast generation time and the applicability in high-throughput experiments, *T. castaneum* has a fully sequenced and annotated genome [155]. The *T. castaneum* genetic database (beetlebase) [156] together with established molecular tools like RNAi [157,158] make the beetle

an attractive model for different fields in biology including genetics, developmental biology, ecology and evolution.

T. castaneum lives and breeds in the same environment and therefore is subject to crowding and environmental contamination with dead individuals and faeces. To protect themselves, *T. castaneum* conditions their environment by secreting a mixture of volatile compounds [159], especially quinones, which possess broad antimicrobial properties [127]. *T. castaneum* beetles of the stock Cro1 [160] were used for all the experiments mentioned in this thesis.

1.6 The parasites

The word parasite is used in a broad context for the purpose of this thesis, collectively signifying both micro-and macro-parasites. The entomopathogens *Bacillus thuringiensis* bv*. tenebrionis* (*B. thuringiensis* henceforth), non-evolved *B. bassiana* and *P. entomophila* were used as parasites in the experiments discussed in this thesis (Figure 2.2). Non-evolved *B. bassiana* and *B. thuringiensis* used in the experiments throughout the thesis were kindly provided by Dr. C. Rafaluk.

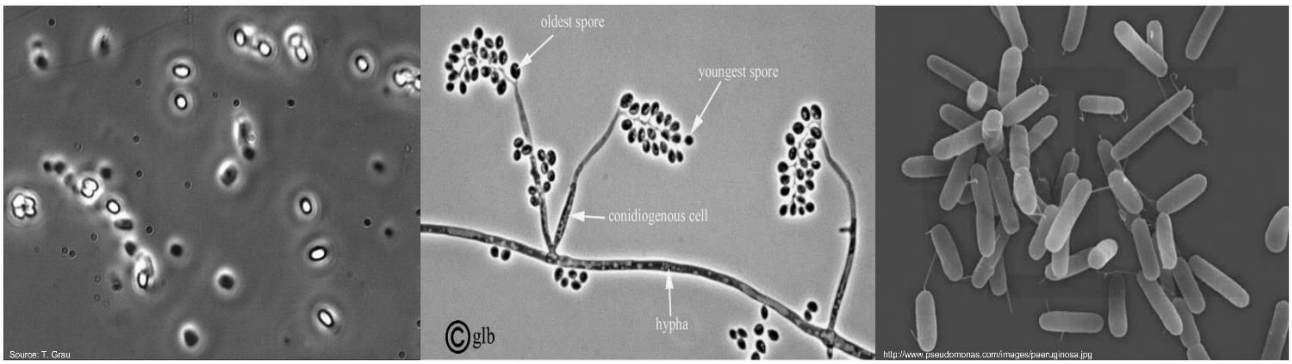


Figure 2.2 Parasites used in various experiments throughout the thesis

Bacillus thuringiensis

B. thuringiensis is an entomopathogenic Gram-positive bacterium found in the soil and in the intestine of many different insect larvae. *B. thuringiensis* displays specific oral toxicity towards various species of insects and nematodes [161] and brings about infection by producing crystalline (*Cry*) and cytolytic (*Cyt*) toxins during sporulation [3]. Once the larval host ingests the *Cry* toxins, the crystals solubilize in the insect gut where the pro-toxins are activated by midgut proteases [3]. The activated toxins then bind to the cadherin receptors in the midgut apical microvilli and subsequently produces pores in the host midgut, resulting in enterocyte cell death, ultimately leading to starvation and death of the host [162,163].

Due to its ease of culturing and availability of ample background knowledge, *B. thuringiensis* has been successfully used to address various questions in host-parasite interactions and eco-immunology in model organisms such as the beetle *T. castaneum* [114,123,164,165], the nematode *Caenorhabditis elegans* [63,99] and the moth *Galleria mellonella* [36,166].

Beauveria Bassiana

B. bassiana is a fungal parasite of insects belonging to the phylum Ascomycete. It was first discovered as the causative agent of the white muscardine disease that plagued silkworms (reviewed in [167]). The fungus infects the insect host via the cuticle where spores present in the environment germinate by producing proteases and chitinases in addition to a variety of structures such as penetration pegs and/or appressoria that help the developing fungal hyphae to enter the hemocoel [8,129]. Additionally, evidence of *B. bassiana* infecting the insect via oral route also exists [130,131].

To defend against the fungus, insects employ behavioural mechanisms such as grooming [168], cuticular defences increased melanisation and hardening [169], as well as humoral defences in the form of fungi specific anti-microbial peptides [170]. Additionally, the insect hosts also employ the so-called external

immune defence in form of a mixture of volatile compounds to defend themselves against fungal parasites such as *B. bassiana* [127,171,172].

Pseudomonas entomophila

Since it was first isolated from *D. melanogaster* [173], the Gram-negative *P. entomophila* (See Figure 2.2) is widely used to study host-parasite interactions [174–176]. Closely related to the soil inhabitant *P. putida*, genome sequencing of *P. entomophila* revealed the presence of many genes which are reported to be responsible for insect-specific virulence [177]. *P. entomophila* displays oral toxicity towards *D. melanogaster* [174] as well as the wax moth *Galleria melonella* [178] which is mediated by a variety of virulence factors.

A major enterotoxin which mediates the virulence of *P. entomophila* is the ß-pore-forming protein Monalysin which disrupts the gut membrane physiology of the host, aided by another virulence factor namely the metalloprotease AprR that cleaves the precursor of Monalysin [179]. Albeit mechanistically similar in the mode of action, monalysin does not share any sequence homology with *Cry* toxin [179].

2

Material and methods

It is not who I am underneath, but what I do that defines me.

|

Bruce Wayne, Batman Begins

2.1 Rearing *T. castaneum*

The beetles used in all experiments were of Cro1 strain, isolated from a granary in Croatia [160]. They were allowed to adapt to standard laboratory condition for beetle rearing (32°C and 70% relative humidity) for a period of four years prior to their use. For rearing, the beetles were provided with a mixture of organic whole wheat flour (type 550, Alnatura, Bickenbach Germany) enhanced with 5% brewer's yeast (Leiber, Kiel Germany). This flour mix was heat sterilized at 60°C overnight prior to the culturing of beetles. Since the medium consists of fine particles, all life stages can be separated with ease by using sieves of different mesh sizes. Colonies were maintained in 500ml glass jars containing 140g of flour mix sufficient to sustain 300 adult beetles and offspring for 2-2.5 months. The jars were covered with fine tissue paper and rubber bands.

2.2 Culturing parasites

B. thuringiensis

B. thuringiensis spores used to orally infect beetles were cultured as described by Milutinovic et al [160]. To prepare the Sporulation medium, 0.75% Bacto peptone w/V (Sigma-Aldrich, Munich Germany), 0.1% glucose w/V, 0.34% KH_2PO_4 w/V, 0.435% K_2HPO_4 w/V were added in 1L of dH_2O (all chemicals from Carl-Roth, Karlsruhe Germany) and the pH was adjusted to 7.2 using a pH meter (Mettler-Toledo, Giessen Germany). To prepare Phosphate buffer saline (PBS) 0.877% w/V NaCl, 0.224% w/V KCl, 0.069% w/V $NaH_2PO_4.H_2O$ and 0.089% w/V $Na_2HPO_4.2H_2O$ were added to 1L of dH_20 (all chemicals from Carl-Roth, Karlsruhe Germany), the pH was set to 7 and the solution was then autoclaved (at 121°C and 100kPa). For supplementing the culture during sporulation, salt solution (0.246% w/V $MgSO_4$, 0.04% w/V $MnSO_4$, 0.28% w/V $ZnSO_4$, and 0.40% w/V $FeSO_4$ in water (all chemicals from Carl-Roth, Karlsruhe Germany)) and 1M $CaCl_2.2H_2O$ was prepared. The salt and the 1M $CaCl_2.2H_2O$ was then filter sterilized using 0.22µm hydrophilic PVDF syringe filters (Carl-Roth, Karlsruhe Germany). The entire process of culturing *B. thuringiensis* spore production takes 10 days [160].

B. thuringiensis from glycerol (Carl-Roth, Karlsruhe Germany) stock (stored at -80°C), solution, was plated out on LB agar plates (10g/l Tryptone, 5g/l yeast extract, 10g/l NaCl (prepared recipe bought from Carl-Roth, Karlsruhe Germany) and 15g/l Agar-Agar (Carl-Roth, Karlsruhe Germany)) and allowed to grow overnight at 30°C in the dark. The following day, an overnight culture containing 5ml of sporulation medium enriched with 125µl of salt solution and 6.25µl of 1M $CaCl_2.2H_2O$ was set up in a 15ml culture tube (Greiner Bio-One, Kremsmünster, Austria), inoculated with several colonies of the bacteria from LB plates. The tube was then covered with aluminium foil and the bacterium was allowed to grow in a shaking incubator (Multitron Infors-HT, Bottmingen-Basel Switzerland) at 200RPM, 30°C for 14-16 hours.

The next day, *B. thuringiensis* culture was started in a 1L baffled conical glass flask (Schott-Duran, Wertheim Germany) containing 500ml sporulation medium, 2.5ml of salt solution and 125µl of 1M $CaCl_2.2H_2O$, inoculated with 2.5ml of overnight culture. The flask was then closed with a cotton stopper (Carl-Roth, Karlsruhe Germany), covered with aluminium foil (Carl-Roth, Karlsruhe Germany) and put in the shaking incubator under the same conditions as those for overnight culture. On day 4, the culture was supplemented with 2.5ml of salt solution and 125µl of 1M $CaCl_2.2H_2O$ and allowed to grow for three more days.

On day 7, the spore suspension was distributed over several 50ml culture tubes (Greiner Bio-One, Kremsmünster, Austria), centrifuged at 4000 RPM (Rotina 420R Hettich, Tuttlingen Germany) for 15 minutes and the supernatant was discarded. The spore pellet thus obtained was then washed and re-suspended in PBS and then centrifuged again. After two washes, all the spore pellets were fused and finally re-suspended in 100ml of PBS. The concentration of the spore suspension was determined using a Thoma counting chamber (depth = 0.01 mm & area of each square field = 0.0025mm^2, Marienfeld, Lauda-Königshofen Germany) viewed under a magnification of 400x (Stereomicroscope 102M Motic, Wetzlar Germany) and was then diluted accordingly as per the requirements of the experiment in PBS.

B. bassiana

B. bassiana was grown on Petri dishes containing Potato dextrose agar or PDA ((20g/l Dextrose, 4g/l potato extract and 17g/l Agar-agar (Carl-Roth, Karlsruhe Germany)) by inoculating from glycerol stock using a sterile loop. The fungal spores were then grown for three weeks at room temperature (24-26°C). This time period is optimum for generating enough spores from each plate, in addition to making spore collection an easier process since the plates get dry.

To extract the spores, a coloured piece of A4 paper folded laterally around the middle was used. Spores were gently teased out of the PDA plates onto the paper using a sterile plastic spreader (Greiner Bio-One,

Kremsmünster, Austria) and the spores were collected into a 50ml culture tube. The spores were weighed in a weighing balance (Mettler-Toledo, Giessen Germany). This process was always carried out inside a fume hood with the window pulled down as low as possible to prevent electrostatic spores from contaminating lab spaces.

Prior to their use, the spore concentration was always counted in a Thoma counting chamber (same specifications as above, Marienfeld, Lauda-Königshofen Germany) with a magnification of 400x (Stereomicroscope 102M, Motic, Wetzlar Germany). Since the spores of filamentous fungi are hydrophobic in nature due to the production of hydrophobins [180], 0.020g of extracted fungal spores were dissolved in 0.005% Tween® 20 (Carl-Roth, Karlsruhe Germany)

P. entomophila

P. entomophila from glycerol stock was grown overnight in LB medium (Roth chemicals, Germany) in a 250ml culture flask at 30°C and under shaking conditions of 200RPM (Multitron Infors-HT, Bottmingen-Basel Switzerland). The overnight culture of *P. entomophila* was centrifuged at 4000RPM (Rotina 420R Hettich, Tuttlingen Germany) to obtain bacterial pellets, discarding the supernatant. The pellets thus obtained were suspended in PBS (pH = 7) prior to use. Spore dilution prior to counting was done in the same way as for *B. thuringiensis*. Counting was performed using Thoma counting chamber (same specifications as above, Marienfeld, Lauda-Königshofen Germany) under a magnification of 400x (Stereomicroscope 102M, Motic, Wetzlar Germany).

2.3 Protocol for experimental coevolution

2.3.1 Experimental design and setup

T. castaneum was exposed to a mixture of *B. bassiana* and *B. thuringiensis* throughout the experiment. This allowed not only for the hosts and parasites to impose selection pressure on one another but also for the parasites to compete for host resources, thereby having the potential to apply selection pressures on each other. Additionally, the duration and the stage of development has an effect on the hosts' ability to fight parasites [181,182]. To observe the influence of host development time on host-parasite coevolution, the experiment was set up in two developmental regimes, FAST (fastest time period reported for *T. castaneum* to develop from egg to adult) and NORMAL (normal egg to adult development time for *T. castaneum*).

For the host, 12 jars were prepared with 140g of heat sterilized flour mix. 100 adult beetles (randomly selected from CRO1 stock populations) were placed in each for egg laying, to produce age synchronized beetles for the experiment. After three days of egg laying the adults were removed and offspring thus produced was allowed to develop for three weeks. The coevolution experiment was started in two developmental regimes. In NORMAL regime, the offspring were transferred every 28 days and in FAST development, the offspring were transferred every 21 days.

The parasites were cultured as per their culturing protocols (section 2.2). The concentration of *B. bassiana* spores adjusted as mentioned in section 2.2. *B. thuringiensis* cultured and the concentration adjusted as per section 2.2. Based on the results of preliminary experiments concentration of *B. bassiana* and *B. thuringiensis* which results in a 70% mortality in *T. castaneum* was used (T. Biswas, unpublished). A mixture of *B. bassiana* and *B. thuringiensis*, used for experimental coevolution, had a final collective spore concentration 10^9 spores/g of flour mix.

For each developmental regime, 7 replicate populations were set up. Adults were separated using a 710 µm mesh sieve (RETSCH, Haan Germany) and the offspring were put in falcon tubes.). 17g of flour or flour &

parasite spore mix was added to each falcon tube (Sarstedt, Nümbrecht Germany) for setting up the experiment, per replicate populations per treatment per developmental regime. To these, 100 pupae were added and the tubes were then closed with a double folded face tissue fastened by a rubber band.

The experiment consisted of the following four treatments, three of which contained both *B. bassiana* and *B. thuringiensis*:

- *Control*

  The host was allowed to adapt to the experimental evolution protocol without any parasite.

  The offspring (n = 100) were transferred at every designated time point based on whether belonging to normal or fast developmental regime to falcon tubes containing 17g freshly prepared flour mix.

- *One-sided Host adaptation*

  Here the host was allowed to adapt but not the parasites.

  The offspring (n = 100) were transferred at every designated time point based on the developmental regime to falcon tubes containing 17g of freshly prepared parasite flour mix. Here the parasites were not allowed to adapt but the host as freshly cultured parasites from the non-evolved strains were added every transfer.

- *One-sided parasite adaptation*

  Here the parasites were allowed to adapt but not the host.

  At every transfer, 12g of the environment from the previous transfer and 5g of fresh flour mix was added to a fresh falcon tube. To this 100 Cro1 pupae were added from stock that was maintained in parallel.

- *Coevolution*

  Both host and parasites were allowed to adapt.

For the coevolution treatment, at every transfer 12g of material from the previous environment and 5g of freshly prepared flour mix and to this 100 offspring from the previous transfer (pupae and fat larvae to complete the count) were added to falcon tubes.

Following these protocols, experimental coevolution was conducted for a period amounting to 10 host generation equivalents, starting December 2014 and ending in July 2015 for the FAST regime and September 2015 for the NORMAL regime. At the end of the experiment, F1 adults were maintained in sterile flour mix without the presence of any parasites in the environment and these populations were transferred every 21 days to keep the generations discreet. All the subsequent experiments were performed on F2 individuals generated from the transfers 5 and 10, of which F1 adults that were maintained in the lab from here on until August 2016.

2.3.2 Sampling protocol during the experiment

Sampling hosts

Starting transfer 2 onwards, adult hosts were sampled from both FAST and NORMAL regimes for assessing hemolymph PO and the quinone content of adults, per treatment per developmental regime. At transfer number 5, around 25-30 adults from each replicate populations were used to set up the F1 beetle stock for use in subsequent survival assays, these were stored as per the general laboratory requirements for *T. castaneum*.

At the end of the experimental coevolution, F1 adults belonging to transfer 10 were set up and maintained for phenotypic assays. The hosts generated from the coevolution experiment were assessed for changes in their resistance to non-evolved *B. thuringiensis* and *B. bassiana* via separate survival assays. Also, oral infection survival assay with *P. entomophila* was performed to check for a general change in resistance to an un-encountered parasite.

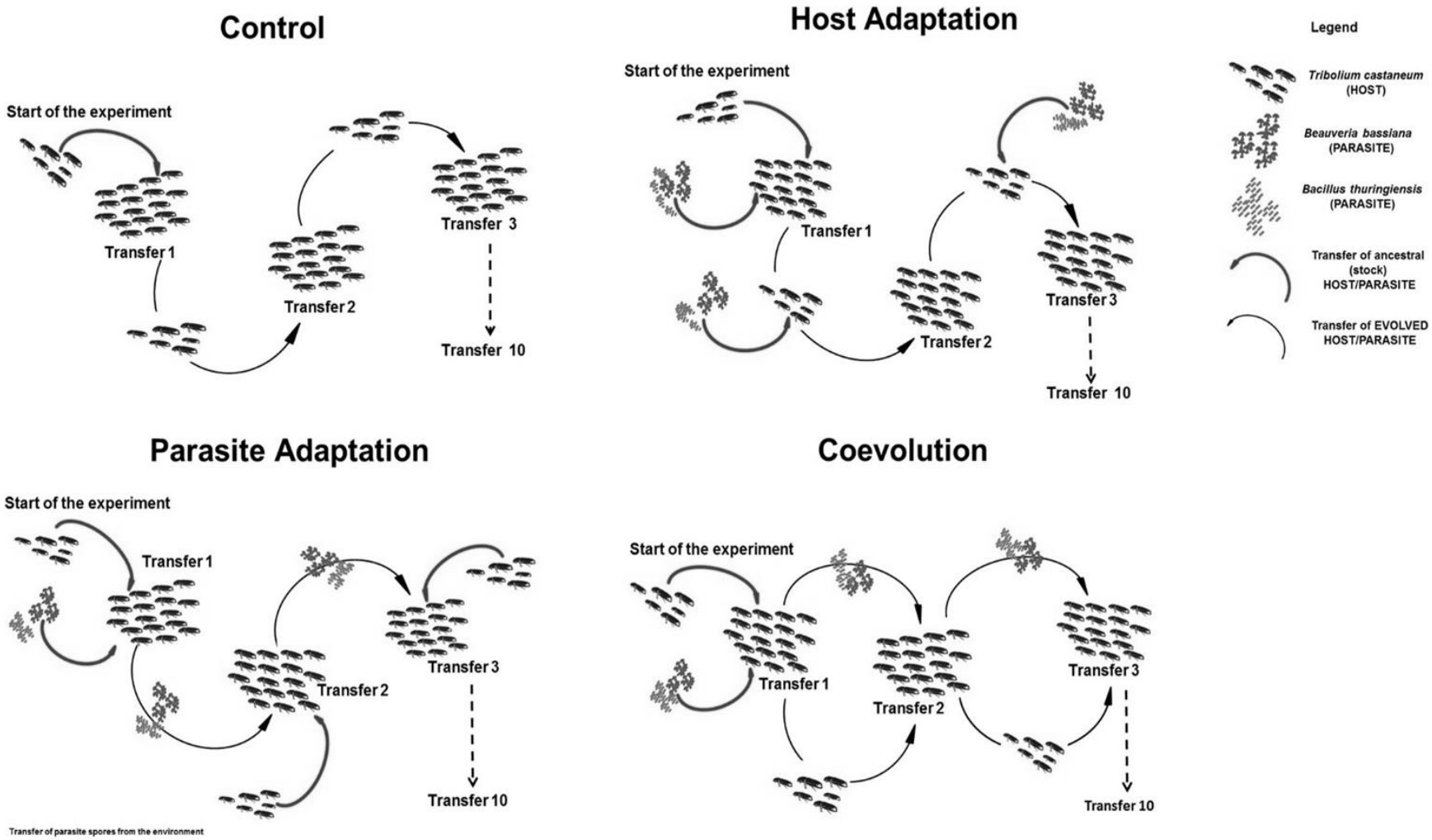


Figure 2.2 Schematic of different treatments during coevolution experiment

Sampling and isolation of parasites

Starting from transfer 4 onwards and every second transfer subsequently, 2g of the environment containing a mixture of parasites and flour was sampled from each replicate populations per treatment per developmental regime from transfers 4, 6, 8 and 10 (Table 2.1). The samples thus collected were stored in 2ml Eppendorf tubes (Eppendorf, Hamburg Germany) and then stored at 20°C. *B. bassiana* and *B. thuringiensis* were then isolated from these samples by dissolving 0.1g of the sample in 1ml of sterile PBS and vortexing vigorously. Following this, *B. bassiana* was isolated by serial plating on PD agar plates with the bacteriostatic chloramphenicol (Carl-Roth, Karlsruhe Germany) at a concentration of 250µl of stock (100mg/ml of 100% ethanol) per 500 ml of PD agar. *B. thuringiensis* was isolated by serial plating on LB agar plates containing anti-fungal Biofonazol ™ (Carl-Roth, Karlsruhe Germany) in a concentration of 250µl of stock (100mg/ml of 100mg/ml of dimethyl sulfoxide) per 500ml of LB agar. The parasite isolates thus obtained were assessed from changes in their growth rate, metabolic activity (*B. bassiana*) and virulence (via survival assays with ancestral Cro1 population). Additionally, the biofilm forming ability for *B. thuringiensis* was determined via the 96-well plate biofilm assay.

Table 2.1 List of all the parasites isolated from various transfer points during the coevolution experiment.

Generation in terms of Transfer number	*B. thuringiensis* isolates				*B. bassiana* isolates			
	FAST		NORMAL		FAST		NORMAL	
	PA	TWC	PA	TWC	PA	TWC	PA	TWC
4	From All 7	From All 7	From 4,5 and 6	From 1,2,3 and 7	X	X	X	X
6	From All 7	From All 7	From 1	From 2 and 6	X	X	X	From 2, 3, 6 and 7
8	From All 7	From All 7	From 1	From 4	X	X	X	X
10	From All 7	From All 7	From 2 and 6	None	X	X	From 2 and 6	X

PA = one sided parasite adaptation, TWC = coevolution

2.4 Origin of beetles for testing cross-resistance

To test for cross-resistance in the *B. bassiana* coevolved beetles arising out the experiment performed by Dr. C. Rafaluk was used [183] (Figure 2.1).

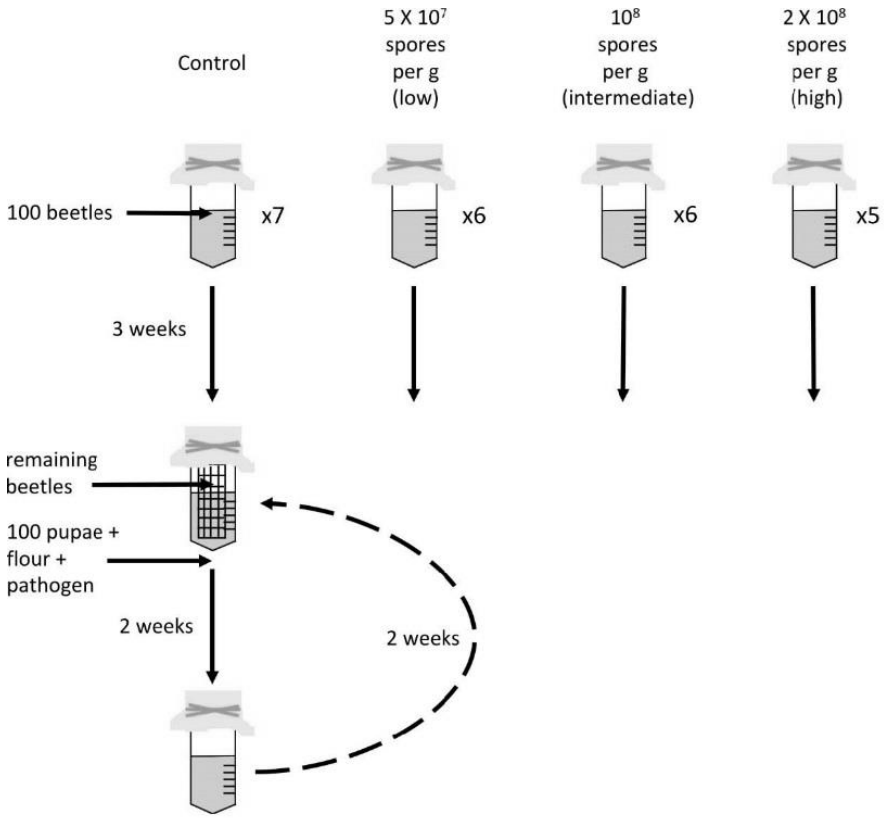


Figure 2.1 generation of B. bassiana coevolved beetles. The beetles used in the cross-infection experiments came from the experimental coevolution performed by C. Rafaluk [183]. For every new generation, 100 pupae were added to the area of the large tube outside of the cage. When there were less than 100 larvae, the largest of the larvae were added to make the count 100. After two weeks the cage and adults from the previous generation were removed. This procedure was then repeated every 4 weeks and is referred to as a 'transfer'. Experimental coevolution was carried out for 13 such transfers. For all the cross-infection experiments, F2 individuals, generated from F1 adults that were maintained in the lab, were used. Figure adapted from [183].

2.5 Phenotypic Assays

2.5.1 Survival assay

In survival assays, individuals which are given a particular treatment along with other individuals which are given the control treatment are monitored, in parallel, over a specific period of time until death. In the context of this thesis, all survival assay setups consisted of a 'control' and an 'infection' treatment and the assay was monitored for different time periods depending on the parasite with which the hosts were infected.

Before every survival assay, F1 adult beetles were allowed to oviposit on heat sterilized flour mix and removed after two days. Following this, F2 individuals were allowed to develop for 10 days, at the end of which they were used in survival assays. Prior to setting up of the experiment, the larvae were sieved out and collected to maintain equality in body size across treatments. For all survival assays, 40 individuals per replicate population per treatment were used.

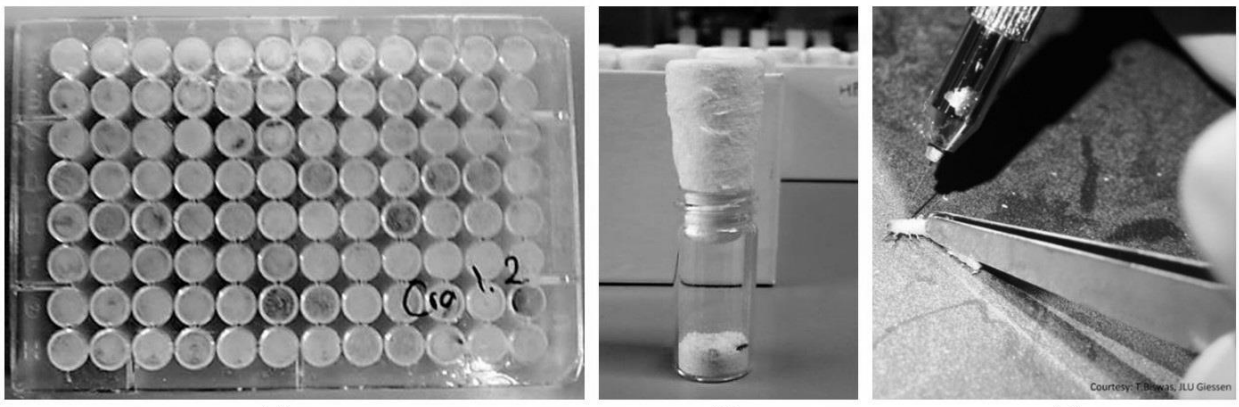


Figure 2.3. Experimental setup to test cross-resistance: Survival Assay. (a) *B. thuringiensis* survival assay where larvae are individualized and infected in each well. (b) *B. bassiana* survival assay where larvae are individualized in glass vials and allowed to dwell in a mixture of food and *B. bassiana* spores. (c) Infection with *P. entomophila* was done piercing right under the pronotum with a needle dipped in the bacterial cell suspension.

B. thuringiensis survival assay

The assay described in this section was modified after Milutinovic *et al.* [160]. The pure spore suspension was counted using a Thoma counting chamber (Marienfeld, Lauda-Königshofen Germany) and the spore concentration was adjusted to 5 x 10^9 spores/ml of food solution which was prepared by adding heat-sterilized flour mix in a ratio of 0.15g/ml of spore suspension.

For control food, a suspension of flour mix and PBS was prepared with a concentration of 0.15g/ml. 40µl of this liquid diet was pipetted into each well of a 96-well flat-bottom plate (Greiner Bio-One, Kremsmünster, Austria) under sterile conditions which were then covered with two layers of paper tissue bound (AG-052 Funny, Germany) with rubber bands and dried overnight in an oven at 50°C. This treatment does not harm *B. thuringiensis* spores as they can still germinate on LB agar plates [160].

The next day, single F2 larvae were placed into individual wells using forceps, after which the well was sealed with transparent adhesive tape (Tesa SE, Norderstedt Germany). 9 holes per well were punctured using a needle (0.3mm diameter) to allow air circulation (Figure 2.3a). To prevent larvae from flying away due to the static electricity the setup was prepared on a wooden platform. Always the control plates were set up first to avoid any chance of cross-contamination. The plates were put in plastic boxes (separate for control and infection) which were then placed under standard laboratory conditions for beetles and survival was monitored every day for a period of seven days [165].

B. bassiana survival assay

The assay described in this section was modified after Joop *et al*. [89]. Small glass vials (40x13mm, Roth Germany) were set up, where 10-day old larvae were infected individually with a mixture of flour-mix containing 10^8 of *B. bassiana* spores/g. Each vial was filled with 0.17g of flour mix (control or infected) with one

larva, which was then capped with cotton wool stoppers (Roth, Germany) and stored under standard laboratory conditions for beetles (Figure 2.3.b). Survival was monitored every alternate day for a period of 30 days and dead individuals were recorded.

P. entomophila survival assay

For survival assay with *B. bassiana* coevolved beetles, larvae were infected systemically with a 0.05mm diameter needle (Bioform, Nuremberg Germany) dipped in a 10^9 cells/ml suspension of *P. entomophila* or with sterile PBS solution for control treatments (Figure 2.3c). Post pricking, they were individualised in 96-well plates containing 40µl of flour-mix & PBS solution dried overnight at 30°C.

Survival was recorded every day for a period of 10 days. Beetles from the coevolution experiment with *B. thuringiensis* and *B. bassiana* were infected with *P. entomophila* via the oral route. $5x10^9$ cells/ml, in 96-well plates similar to *B. thuringiensis* survival assay. Plates for control treatments were prepared in a similar manner, without the presence of *P. entomophila* cells. Here again, survival was monitored for a period of 10 days (based on preliminary tests and on a daily basis.

2.5.2 Hemolymph extraction and phenoloxidase assay

The enzyme phenoloxidase (PO) is a major component of the innate immune system which contributes to a very important defence mechanism insects known as melanisation [45,184] and has been used as a proxy for innate immune defence in insects [185,186]. Prior to hemolymph extraction, BisTris buffer (0.1M, for 50ml = 1.05g BisTris (Carl-Roth, Karlsruhe Germany) in 50ml deionized water, pH 7.5, sterile filtered and stored at 4°C (Kirsch, Offenburg Germany)) was prepared and 20µl of this was pipetted into each well of a 96 well PCR plate (Sarstedt, Nuembrecht, Germany) using a multi-channel pipette.

Adult beetles were placed on ice for 5 minutes to anaesthetize them. Once anaesthetized, a beetle was picked up using forceps and pricked with a 0.3mm diameter needle (Bioform, Nuremberg, Germany) laterally on the pronotum, under a dissecting microscope. The drop of hemolymph which appeared from the wound was then extracted using a capillary tube (1µl capillary, Hirschmann end-to-end), previously chilled on ice. The amount of hemolymph thus collected was measured using digital Vernier callipers (150mm, VonHaus, United Kingdom).

Sample concentration was adjusted to 0.1µl in 20µl of BisTris buffer. After collecting the hemolymph samples (sample collection was performed on ice to prevent coagulation and evaporation), the plates were capped using 8-strip PCR lids and placed in a cryo box which was then stored at -80°C (GFL, Burgwedel, Germany) until PO measurements. A solution of L-DOPA (L-3, 4-dihydroxyphenylalanine, Sigma-Aldrich, Munich, Germany) was prepared at a concentration of 4mg/ml of BisTris buffer and filter sterilized and stored in the dark. L-Dopa acts as a substrate for PO which then leads to the production of dopachrome, measured using a spectrophotometer [187].

Prior to measuring hemolymph PO activity, a flat bottom 96-well plate with lid (Greiner-Bio, Germany) was prepared by pipetting 50µl of H_2O and 50µl BisTris Buffer, following which 20µl of the hemolymph sample was added to each well using a multi-channel pipette. Finally, 50µl of L-Dopa were added right before measuring the contents of a plate. An appropriate number of controls were used in every plate. The plates thus prepared were covered with lid, secured with Parafilm® (Sigma-Aldrich, Munich, Germany) and read at 490nm every 2 minutes for 90 minutes at 37^{o}C (Take3 BioTek-Eon plate reader, Friedrichshall, Germany). The V_{max} of the linear phase of the reaction was used as a measure of PO activity.

2.5.3 Quinone quantification assay

Since *T. castaneum* secretes a mixture of volatile compounds primarily consisting of methyl-1,4-benzoquinone (MBQ), ethyl-1,4-benzoquinone (EBQ), and 1-pentadecane [126,159,188] with anti-microbial properties into its

environment [127,128]. Quinone secretion of the adult beetles from the coevolution experiment was quantified using the protocol used by Joop et al. [89] as a proxy for external immune defence [171,183].

First, 150µl of acetonitrile (Carl-Roth, Karlsruhe, Germany) was pipetted into each well of a PCR plate using a multi-channel pipette (Mettler-Toledo, Giessen, Germany). In order to minimize the amount of acetonitrile lost to evaporation, each column of the plate was closed immediately with an 8 lid PCR strip. The plates thus prepared were stored in the refrigerator at 4°C (Kirsch, Offenburg Germany) until use.

Adult beetles were given cold shock by putting them in Petri plates (9cm diameter, Greiner Bio-One, Germany) on ice for 5 minutes, resulting in the relaxation of muscles around the quinone glands leading to the release of quinones [138]. Following this, each beetle was placed into one well of the PCR plate, again closing each column with an 8 lid PCR strip. Appropriate control wells were assigned per plate which contained no beetles, to be used as blanks. The plates were stored at 4°C (Kirsch, Offenburg Germany) for 24 hours to allow for the quinone to dissolve in the acetonitrile.

The next day, 120µl of the supernatant from each of the wells of PCR plate was pipetted into a 96 well quartz plate (Hellma-Analytics, Müllheim Germany), under the fume hood. The quinone mixture produced by each adult beetle was then measured plate in the plate reader (Take3 BioTek-Eon, Bad Friedrichshall Germany) over a spectrum of 220-300nm. The blank subtracted integral of the area under the curve for each well, read over 220-300nm is used as a proxy for the amount of quinone contained on the glands of each beetle. Once done, the contents of the plate were absorbed on paper towels and then washed using deionized H_2O followed by a wash with 70% ethanol and allowed to dry.

2.5.4 *B. thuringiensis* growth assay

The growth rate is an important microbial trait which is influenced by a variety of factors such as nutrients, competition and space [189]. Additionally, experimental evolution experiment with bacteria report that growth characteristics of the bacteria do change during the course of the evolution [190]. To assess the change in growth rate of the *B. thuringiensis* isolates from the coevolution experiment, ´they were first plated on LB agar plates from their respective glycerol stocks (stored at -80°C) and incubated overnight at 30°C. Following this, the isolates were cultured overnight at LB liquid medium in 15ml culture tubes (Greiner-Bio, Germany) post which the concentration of each isolate's culture was determined. The liquid cultures were then diluted in sterile PBS to a concentration of 10^5 cells/ml.

In a flat bottom lid containing clear 96-well plate (Greiner Bio-One, Kremsmünster, Austria), 200µl of the diluted culture was pipetted in each well, with three technical replicates per isolate as well as appropriate blanks containing just LB liquid were added. Non-evolved *B. thuringiensis* was also used as a reference strain as well a positive control. The plate was then closed using the lid and secured tightly with parafilm (BRAND® PARAFILM® M sealing film, Sigma-Aldrich, Munich Germany) to prevent evaporation. The plate thus prepared was read in the plate reader (Take3 BioTek-Eon, Bad Friedrichshall Germany) at 30°C with the absorbance measured at 600 nM every 10 minutes for a period of 16 hours. All the *B. thuringiensis* isolates enlisted in Table 2 were assessed for their change in growth rate

2.5.5 Biofilm quantification assay

Biofilm was first observed in the form of wrinkled colonies by B. thuringiensis isolates from the coevolution experiment on LB agar plates, post 36 hours of incubation at 30°C. This was not observed with non-evolved *B. thuringiensis*. Non-evolved *B. thuringiensis*, as well as isolates from the coevolution experiment belonging to both FAST and NORMAL developmental regimes, were cultured in LB medium overnight (30°C with shaking at

200RPM). The concentration of the overnight cultures was adjusted to 10^9 cells/ml, with LB liquid medium (supplemented with 1% Glucose).

After this, 150µl of the diluted *B. thuringiensis* isolates (6 technical replicates per isolate) were pipetted into a flat bottom 96-well plate (Greiner Bio-One, Kremsmünster, Austria) including appropriate number of wells for buffer, positive and negative control and empty wells for Crystal violet (Carl-Roth, Karlsruhe Germany), under the laminar airflow and then incubated at 30°C for 36 hours without shaking, with the lid secured using Parafilm. *Staphylococcus epidermidis* (strain: RP62A; American Type Culture Collection (ATCC) 35984) was used as the positive control.

Majority of the extracellular matrix that is the biofilm is constituted by polysaccharide [191]. Therefore, $NaIO_4$ (40mM in H_20, Sigma-Aldrich, Munich Germany) was used as a polysaccharide-degrading agent to determine the quality of the biofilm produced by the *B. thuringiensis* isolates. Post the 36 hour incubation period, the plates were pouted out on layers of tissues (paper towels over two layers of Korsolin extra (Carl-Roth, Karlsruhe Germany) in combination with Bode-X-wipes) and then washed 3 times with PBS (pH 7.4). 150µl of the test substance namely $NaIO_4$ was added to each well and then incubated for 24 hours at 30° C with lid, without shaking. After this, the plates were again poured out on layers of tissue and washed 3 times with PBS and dried at 55°C for up to 2 hours to fix the biofilm.

The fixed biofilm was then stained using 100µl of 0.01 % of Crystal violet (Carl-Roth, Karlsruhe Germany) for 10 minutes at room temperature. Post this, the plates were dried on layers of tissue and washed as described previously, washing until loose dye stops coming out. To each well, 150µl of a mixture of ethanol/acetone (70:30) was added to dissolve the bound for 10 minutes after which 100µl of the liquid in each well was pipetted onto a fresh flat bottom 96-well plate. Absorption was then read at 570nm and the average (n = 6) was calculated by eliminating the highest and the lowest values of absorption per isolate.

2.5.6 Fungal metabolic activity assay

A florescence-based assay using Fluorescein diacetate (FDA), modified from Troskie et al. [192] was carried out to measure fungal metabolic activity of the *B. bassiana* isolates from the coevolution experiment in the absence and presence of different concentrations of MBQ, compared to non-evolved *B. bassiana* isolates from the coevolution experiment or non-evolved strain were grown on PD agar plates for 2 weeks, while keeping them at room temperature.

After this time, spores were extracted from the plates as per mentioned in section 2.2 and the concentration of pure spore powder was determined by counting under a light microscope 400x magnification using a Thoma counting chamber. A suspension of 2000 spores/ml in 0.005% of Tween® 20 (Carl-Roth, Karlsruhe Germany) was prepared for each isolate. A stock solution of MBQ (11µg/ml of Methanol (Carl-Roth, Karlsruhe Germany) and FDA (2mg/ml of Acetone) were prepared and stored in black 1.5ml Eppendorf tubes (Eppendorf, Hamburg Germany).

The *B. bassiana* isolates were tested against '1' (1µl of MBQ stock in 999µl of PD broth), '5' (5µl of MBQ stock in 995µl of PD broth), '10' (10µl of MBQ stock in 990 µl PD broth) and '25' (25µl of MBQ stock in 975µl of PD broth) beetle equivalents (calculated from the reported amount of MBQ secreted by 100 adult beetles [193]) of secreted MBQ in 3 technical replicates. 70µl PD agar (6% w/V) was first pipetted into each well using a multi-channel pipette using the reverse pipetting method and allowed to solidify for 5 minutes. After this, 10µl of a spore suspension of interest was reverse pipetted into the plate and allowed to settle onto the PD agar for another 5 minutes following which 10µl of required MBQ solution diluted appropriate in was pipetted into the wells. Finally, 5µl of FDA was pipetted into each well followed by 5µl of PD broth. Following controls were added per plate:

- 0 MBQ containing spores, FDA and PD agar and broth
- Only FDA containing spores and MBQ

- No spores but only MBQ and FDA

All wells contained a final volume of 100µl and once prepared the plate was read in the plate reader (BioTek Synergy H4 microplate reader). Fluorescence (excitation 490nM and emission 520nM) was read at intervals of 30 minutes for 140 hours.

2.5.7 Minimum inhibitory concentration (MIC) of MBQ on *B. thuringiensis* isolate

MIC is used to determine the lowest concentration at which a compound is inhibitory to bacterial growth. Since during experimental coevolution, the beetles secreted quinone in the environment in which *B. thuringiensis* was also present, I decided to check for the inhibition of B. thuringiensis by MBQ. Different concentrations of MBQ (similar to that in section 2.5.6) were tested.

B. thuringiensis isolates were grown overnight (30°C, 200 RPM) and diluted to a concentration of 10^6 cells/ml in LB medium. 96-well plates (Greiner Bio-one, Germany) were prepared by adding 50µl of bacterial suspension and 50µl of MBQ solution and the assay was performed in the plate reader (Take3 BioTek-Eon, Bad Friedrichshall Germany) for 16 hours at 30°C and absorbance was measured at 600nm. All plates had appropriate blank wells.

2.6 Molecular methods

2.6.1 RNA isolation and cDNA preparation

For analysis of expression of candidate genes, RNA was isolated from pools of 15, 10-day old F2 larvae (coming from different treatments and time-points). Using 350µl of TRIzol (Sigma-Aldrich, Munich Germany) as the lysis buffer in a 2ml Eppendorf tube (Eppendorf, Hamburg Germany) containing 1 stainless steel bead

(manufacturer), samples were homogenised in the TissueLyser II (Qiagen, Hilden, Germany). This was followed by the manufacturer's protocol for isolating RNA as specified in the RNA extraction kit (Direct-zol™ RNA MiniPrep, Zymo Research, Freiburg Germany).

The RNA thus isolated was checked for concentration (>200 ng/µl), purity (A_{260}/A_{280} and A_{260}/A_{230} ~ 2) spectrophotometrically (Take3 BioTek, Bad Friedrichshall Germany) which was followed by quality (two sharp bands corresponding to 23S and 18S rRNA) check in agarose gel electrophoresis (1.5% agarose, Carl-Roth, Karlsruhe Germany)) with 1xTAE (Tris-acetate-EDTA) buffer. The RNA samples not upholding to these criteria were concentrated using 100% ethanol and 5M sodium acetate (Thermo-Fischer Scientific, Schwerte Germany). cDNA was synthesized using 2µg of total RNA, oligo $(dT)_{18}$ primers and the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Schwerte Germany) as per the protocol specified by the manufacturer. The resulting cDNA was diluted to a working concentration of 10ng/µl, and stored at –80°C for future use.

2.6.2 Gene expression using RT-qPCR

For gene expression, F2 larvae of *B. bassiana* coevolved *T. castaneum* were exposed to *B. thuringiensis*, *B. bassiana* and *P. entomophila*. Appropriate control treatments were performed in parallel. Larvae were sampled 12 and 24 hours post-exposure and RNA was extracted as per the protocol mentioned before. RT-qPCR was performed on the cDNA synthesized from the RNA thus produced.

Primer design Protocol

Specific primers were designed for genes of interest. FASTA files for coding nucleotide sequence for these genes were obtained from the online database of National Centre for Biotechnology Information (NCBI) [194]. Using the tool Primer3plus [195] which were validated for the parameters mentioned in table 1 using the

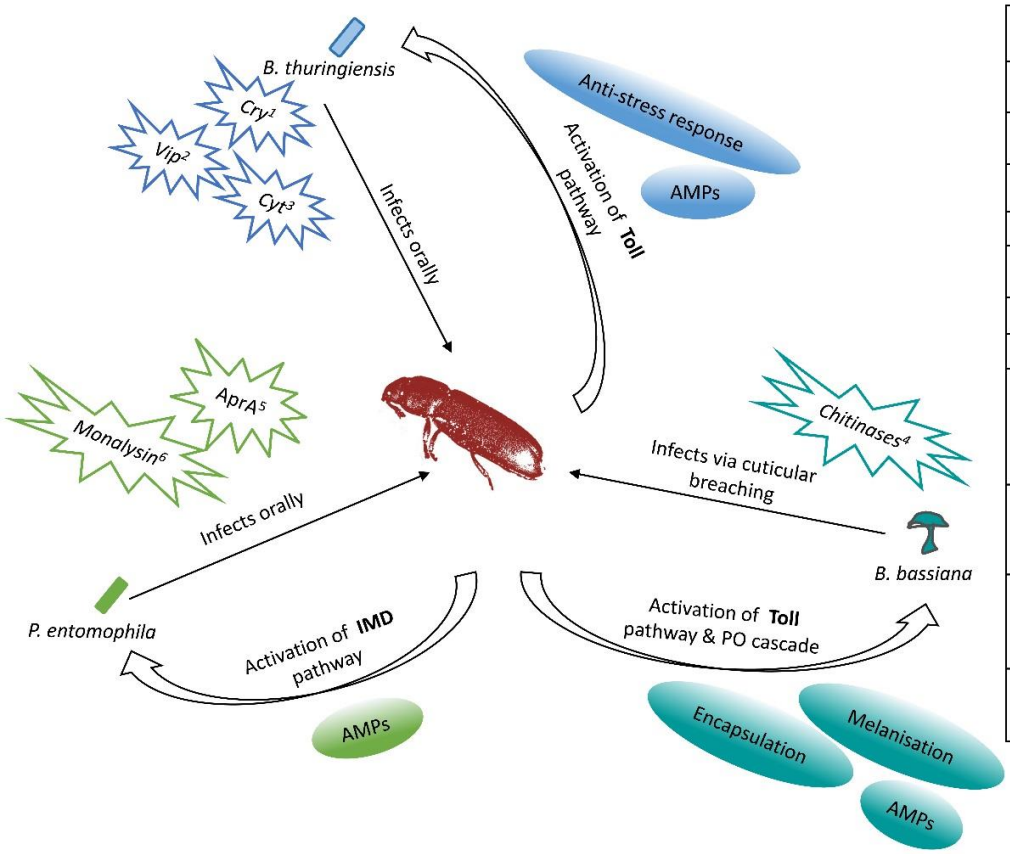
software Oligo Analyzer v3.1 from Integrated DNA technologies. As an additional control measure, the primers thus designed were checked using BLAST software [196] to ensure that they only amplified the gene of interest. Gene-specific primers (Biomers, Ulm Germany) were finally standardized using StepOnePlus Real-Time PCR machine (Applied Biosystems).

A literature survey of the prominent candidate genes (Figure 3.4 and Table 2.2), expressed in *T. castaneum* post infection, was performed and specific primers were designed A list of primers representative of different arms of immune response upon infection was prepared with candidates representing functional gene families such as stress (hsp90, p450), PO-related (Laccase-2 [197] and Apolipophorin-III [135]) and antimicrobial peptides (Attacin-2 & Defensin-3). Additionally, candidates for external immune defence (Gt39 [138]), expressed upon fungal challenge (Thaumatin-like [132]), innate immunity (Lysozyme) and chitin metabolism (TcDA6 [198]) were selected.

Primer validation

All primers were designed to have 19–23 nucleotides with a T_m ~60°C and amplification products of length 70–150 base-pairs (bp). Primers were tested in melt curve assay to determine optimum forward to reverse primer ratio (see appendix Table 1), followed by a standard curve assay using the StepOnePlus Real-Time PCR System (Applied Biosystems) to determine primer efficiencies (table 2.3).

Different cDNA concentration spanning 0.001ng to 100ng in 5-fold dilutions with the cycling conditions as described in the next section, was used for performing standard curve analyses. Primer efficiency was calculated using StepOne Software v2.3 and only primers with an efficiency of 85–110%, regression fit of $R^2 \geq 0.98$ and a single sharp melt curve peak corresponding to specific amplification were used for RT-qPCR experiments. Additionally, all primers were tested with water and RNA as a template to check for unspecific amplification.



Candidate genes	pathway
Attacin-2	IMD (Tanji et. al. 2007)
Defensin-3	Toll & IMD (Tanji et al. 2007)
Hsp90	Stress Response (Sørensen et. al. 2005)
P450	
Lysozyme-4	Toll (Tanji et al. 2007)
ApoIII	ProPO (Whitten et. al. 2004, Arakane et.al. 2005)
Laccase-2	
GT39	Possible function in the melanin synthesis pathway (Li et al 2013)
ObpC12	Chemoreception , anti-fungal (Contreras et. al., 2013)
TcDA6	Synthesized in peritrophic membrane (Arakane et. al. 2009)
Thaumatin	Stress Response, anti-fungal (Altincicek et. al. 2008)

[1]Crystalline protein toxin (PFT, enterotoxin); [2]Vegetative insecticidal protein; [3]Cytolytic toxin; [4]cuticular & peritrophic membrane chitin degradation; [5]AMP degrading Zinc metalloprotease; [5]PFT, enterotoxin; AMP: Anti Microbial Peptides; PFT: Pore Forming Toxin

Figure 3.4 a schematic representation of infection route and mechanisms naturally known to be taken by *B. bassiana*, *B. thuringiensis* and *P. entomophila* and the immune response they evoke from the host.

Reaction protocol

The same reaction protocol was used for primer validation as well as for gene expression studies. Hot-start PCR with denaturation at 95°C was run for 10 minutes followed by 40 cycles of extension at 95°C for 15 seconds and at 60°C for 60 seconds. Finally, melt curve analysis was run with a step-wise temperature increment from 60°C to 95°C in steps of 0.5°C.

Table 2.2 Checklist of criteria to be followed for primer design.

Parameters to check	Acceptable range
Amplicon length	70- 150 bp
Primer Length	19 – 22 nucleotides
GC Content	30-80% (ideally 40-60%)
Tm	63-67°C (ideally 64°C)
Hairpins3' end hairpin	-2 kcal/Mol (ΔG)
internal hairpin	-3 kcal/Mol (ΔG)
Self Dimer3' end self-dimer	-5 kcal/Mol (ΔG)
internal self-dimer	-6 kcal/Mol (ΔG)
Cross Dimer 3' end cross dimer	-5 kcal/Mol (ΔG)
internal cross dimer	-6 kcal/Mol(ΔG)

Note ΔG = quantity of energy needed to break a secondary DNA structure. The lower ΔG values (more negative values), the higher the quantity of energy needed to "separate" the DNA strands if a secondary structure (a primer dimer or hairpin, for instance) is formed.

3' End Stability: It is the maximum ΔG value of the five bases from the 3' end. An unstable 3' end (less negative ΔG) will result in less false priming

Table 2.3 List of primers for candidate genes used from RT-qPCR

Gene	Gene ID (sourced from NCBI)	Forward primer	Reverse primer	EFFICIENCY (85-110%)	R^2 (>=0.98)
Rps3	NM_001172392.1	ACCTCGATACACCATAGCAAGC	ACCGTCGTATTCGTGAATTGAC	96.67	0.994
Rps18	XM_968539.2	TGATGGCAAACGCAAAGTCA	TCGGCCGACACCTTTGA	93.809	0.985
Attacin-2 (Atta-2)	100141947	AGTCGGCGTTGAAGCATC	CCCGAACCTCTGACCATAG	89.595	0.995
Defensin 3	655548	TGTCACACTAGTGATGGGGC	ATCATTCTTTTGGTGTCCCG	85.154	0.997
Hsp90	656270	CCTCAAGTCCACGCATCCAG	TCGCCTCCTTGTGCATCTTC	110.7	0.989
Lysozyme4 (Lyzo-4)	NM_001166023.1	TGAGTTAGCCCGCGAATTGAA	TAGCCATTGCCAGGTGGTGA	85.419	0.997
p450	470011965	GGGGTTTGGTCACAGATGATG	CATTGCCGTGGATGATGTCTC	91.721	0.982
Apolipophorin-III (Apo-III)	655732	CCAAAACGCCGCTCAAAC	TTGCAAATTGTTGCTGACTTCA	107.682	0.993
Laccase2 (Lac-2)	641461	TCTGCGAAGGTGACAAGGTTG	GGGGCATTGGGTAACGAAAG	92.856	0.98
Tcas-ql VTGI(GT39)	JX569829.1	TACACTTCTCCACCTGACAATG	ACCCATACCTGGTTTTCGTAC	95.139	0.992
ObpC-12	656243	CAAGCCAAACAGCTAAGGAA	CCCGACACCGACTTGCA	95.622	0.992
TcCDA6 [199]	NM_001110435.1	CGGCAGAGTACTGGTTGAAAGC	CAATGGGAATGTTGGCAAAGTG	85.959	0.983
Thaumatin-like (Thauma)	663483	GGCAACGGGGTTATTGCTTG	ACGTGTCAGGTGTGCCGAAA	97.86	0.982

2.7 statistical analysis

For statistical analysis statistical analyses performed in R (for Windows), all packages were used in R version 3.3.2 [200]. Plots in R were made using the package ggplot2 [201]

Survival analysis

Kaplan-Meier estimator was used for analyzing and plotting the survival data using the 'survdiff' and 'survfit' functions built into the survival package in R [202]. Multiple pairwise comparisons were performed using an adaptation of a code by Terry Therneu (Dr. Gerrit Eichner, personal communications).

Additionally, cox-proportional hazard test was performed on each of the survival datasets using beetle origin i.e. Control/Coevolution and developmental time i.e. FAST/NORMAL based on the experiment and treatment (CONTROL/INFECTION) as factors, using the 'coxph' function in the survival package [202].

RT-qPCR analysis

Gene expression data from RT-qPCR were analyzed using the MCMC.qpcr R package [203,204] which implements a generalized linear mixed model analysis of qPCR data. I used the 'classic' mode, which normalizes the expression data of different candidate genes relative to 'control' genes (reference genes). I constructed a full factorial model with 'treatment' and 'timepoint' as interaction terms. P-values (significant at <0.05) were adjusted for multiple testing using the Benjamini & Hochberg correction method (incorporated in the package) implemented in the 'p.adjust' function implemented in R.

Analyzing immune parameters

PO and quinone were measured throughout the experiment every second generation, starting generation 2 onwards. The response variables PO and quinone were analyzed using the function 'lmer' in the lme4 package in R [205] function in a mixed-effects model (REML) with a logit link function, per developmental regime. 'Treatment' and 'transfer' were taken as fixed effects and individuals nested within 'line' (replicate populations) as random effects.

Residuals of all models were tested for Normality using the function 'shapiro.test' in Shapiro-Wilk Test. Pairwise comparison of response variables based on the effects of 'treatment' and 'transfer' were analyzed using Tukey contrasts, employing the function 'glht' in the package multcomp [206], where p-value was corrected using Holm method.

B. thuringiensis growth assay

Doubling time of *B. thuringiensis* isolates was calculated from the absorbance values (at 600 nm) from the exponential growth phase, using the following formula

Growth rate constant (μ) =

$$\frac{(lnA2 - lnA1)}{(t2 - t1)}$$

Doubling time =

$$\frac{ln2}{\mu}$$

A = absorbance, t = time in hours

[207]

Two-way ANOVA analysis was performed on the doubling time of *B. thuringiensis* isolates using 'isolate' and 'transfer' as fixed factors, post-hoc analysis on which was performed using Tukey HSD (honest significance difference) test, the p-value was corrected using Holm method.

B. bassiana metabolic activity

Fluorescence was measured over 140 hours and area under the curve was calculated using the trapezoid formula in Microsoft EXCEL in the following manner.

Area under the curve =

$$\sum (T2 - T2) \times [(RFU1 + RFU2) \div 2]$$

T = time in hour

RFU = relative fluorescence unit

Two-way ANOVA analysis was performed on the area data with MBQ concentration and isolate as fixed factors. A series of pairwise t-tests were performed where fungal metabolic activity under different concentrations of MBQ with p-value corrected using holm method.

Biofilm quantification

Mean absorbance values for different *B. thuringiensis* isolates were computed after which two-way ANOVA was performed with isolate and generation as fixed factors. Multiple comparisons using Tukey contrasts were performed and p-values were corrected using holm method.

METHODS

MIC assay

A series of pairwise t-tests were performed for the MIC data for different *B. thuringiensis* isolates which were compared to the non-evolved *B. thuringiensis* and the p-value was corrected using Holm method.

3

Results

The scientific human does not aim at an immediate result. He does not expect that his advanced ideas will be readily taken up. His work is that of a planner for the future. His duty is to lay the foundation for those who are to come and point the way.

|

Nikola Tesla, Inventor *(1856-1943)*

Cross-resistance: a consequence of bipartite host-parasite coevolution

- *Tribolium castaneum* previously coevolved with *B. bassiana* is positively cross-resistant to *Bacillus thuringiensis.*
- Markers representing the oral route of infection were up-regulated upon *B. bassiana* exposure indicative for cross-resistance.

Tripartite host-parasite experimental coevolution in the background of host development time

- *T. castaneum* from the evolution experiment show no clear pattern in resistance towards non-evolved *B. bassiana.*
- Coevolved beetles from FAST and NORMAL regimes are equally resistant to non-evolved *B. thuringiensis.*
- Trade-off observed between internal and external immunity in NORMAL developing beetles.
- No clear pattern in virulence displayed by either *B. bassiana* or *B. thuringiensis.*
- *B. bassiana* isolates show increased metabolic activity in the presence of quinones.
- *B. thuringiensis* isolates from FAST regime display biofilm forming ability, which increases during the course of evolution.
- Biofilm forming ability in *B. thuringiensis* isolates traded-off against slower growth rate, during evolution.

Schematic to for the abbreviations used in this chapter

<table>
<tr><td>Color code for parasites when used to infect T. castaneum</td><td colspan="2">Beauveria bassiana

Bacillus thuringiensis

Pseudomonas entomophila</td></tr>
<tr><td>Treatment</td><td colspan="2">CONTROL (no parasite)

INFECTION (with parasite)</td></tr>
<tr><td rowspan="2">Background</td><td rowspan="2">Tripartite coevolution</td><td>Host development time →

FAST (transfer every 21 days)

NORMAL (transfer every 28 days)</td></tr>
<tr><td>Code for individual beetle replicate population →

Control (no parasite)

HA (one-sided host adaptation)

PA (one-sided parasite adaptation)

TWC (coevolution between T. castaneum, B. bassiana and B. thuringiensis)

Code for parasite isolates →

Control (no parasite)

HostAdapt (one-sided host adaptation)

CoEvo (coevolution between T. castaneum, B. bassiana and B. thuringiensis)</td></tr>
<tr><td></td><td colspan="2">Cross-resistance as a consequence of coevolution

Coevolution* (with B. bassiana)

Control* (no B. bassiana)

*Always in italics</td></tr>
</table>

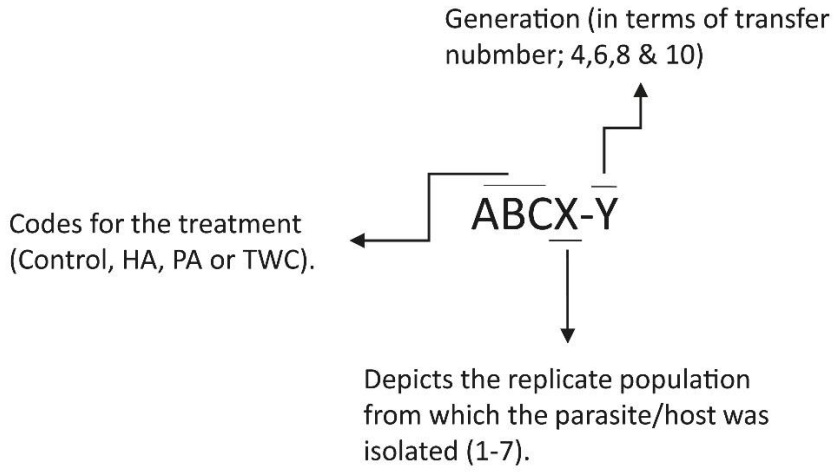


Figure 3.1 Host and parasite populations from the coevolution experiment are named as per the key. HA = one sided host adaptation; PA = one sided parasite adaptation; TWC = coevolution.

3.1 Tripartite host-parasite experimental coevolution in the background of host development time

3.1.1 Host development during evolution influences its response to non-evolved parasites

Susceptibility to *B. bassiana*

Kaplan-Meier survival analysis showed that *T. castaneum* larvae differ in their survival in CONTROL and INFECTION treatments ($\chi 2$ = 71.6, D.F. = 11, p <0.001) (Figure 3.2 and Table S1). Cox proportional hazard analysis (LrT = 58.28 on 4 DF, p < 0.001) revealed that *developmental time* (p = 0.037) and evolutionary *background* (p < 0.001) significantly influenced host survival to ancestral *B. bassiana*. Few groups were observed to differ from each other within treatments (Figure 3.2). The overall survival was observed to be circa 80%. In general, no resistance evolved to non-evolved *B. bassiana* during evolution but CoEvo beetles from FAST regime were most susceptible to infection.

Sceptibility to *B. thuringiensis*

Kaplan-Meier survival analysis revealed that the different groups of beetles are significantly different in treatment combinations (χ^2 = 501, DF = 11, p <0.001) (Figure 3.3 and Table S2). Upon Cox proportional

hazard analysis (LrT = 353.3 4 DF, p <0.001) it was revealed that *developmental time* (p <0.001) and evolutionary *background* (p <0.001) affect survival to ancestral *B. thuringiensis*. In CONTROL, only one group differed (HostAdapt (NORMAL) vs. CoEvo (FAST), p <0.001). Here, contrary to the observation upon *B. bassiana* INFECTION, FAST CoEvo beetles from both generations are highly resistant to *B. thuringiensis*.

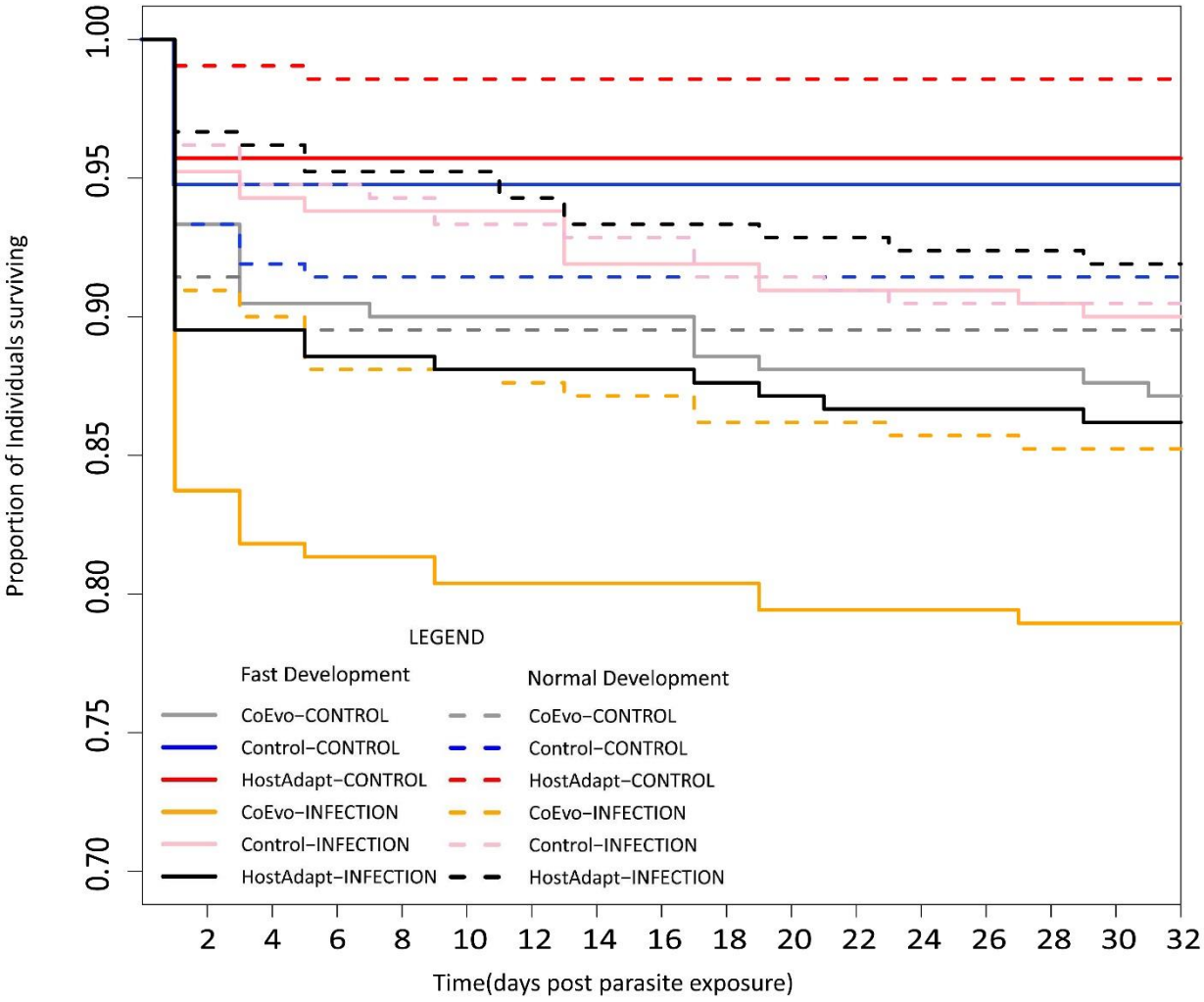
Table 3.1 summarizing the results of survival assay of F2 individuals of different beetle groups after exposure to *B. thuringiensis* and *B. bassiana*

	B. bassiana		*B. thuringiensis*	
	HA	TWC	HA	TWC
FAST				
NORMAL				

The colors here indicate higher or lower survival compared to control populations in a given treatment. Blue = CONTROL treatment (vs. control, significance); = lower than control; = higher than control. Pink = INFECTION treatment (vs. control, significance); = lower than control; = higher than control

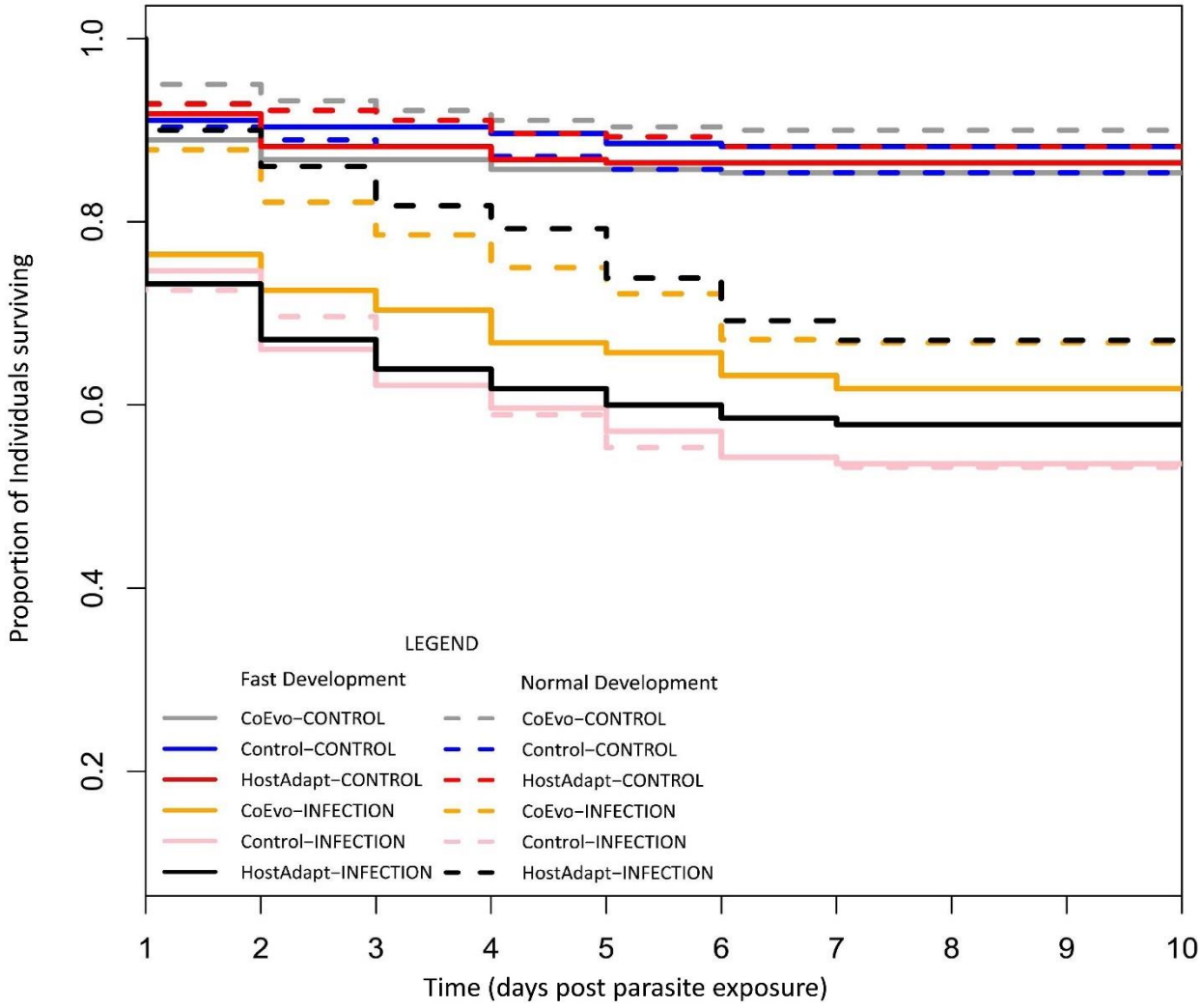
Evolved host is not resistant to a novel parasite

To check the evolved beetles had a generally high immune defence [208], survival to unrelated *P. entomophila* was tested. Kaplan-Meier survival analysis revealed a significant difference between beetle groups in CONTROL and INFECTION (χ^2 = 358, DF = 11, p <0.001) and survival dropped to a lowest of 57% (Figure 3.4 and Table S3). Cox proportional hazard analysis (LrT = 350 on 4 DF, p < 0.001) showed that *developmental time* (p = 0.42) had no influence on beetle response to *P. entomophila*, *T. castaneum* larvae from all background equally susceptible to *P. entomophila.*



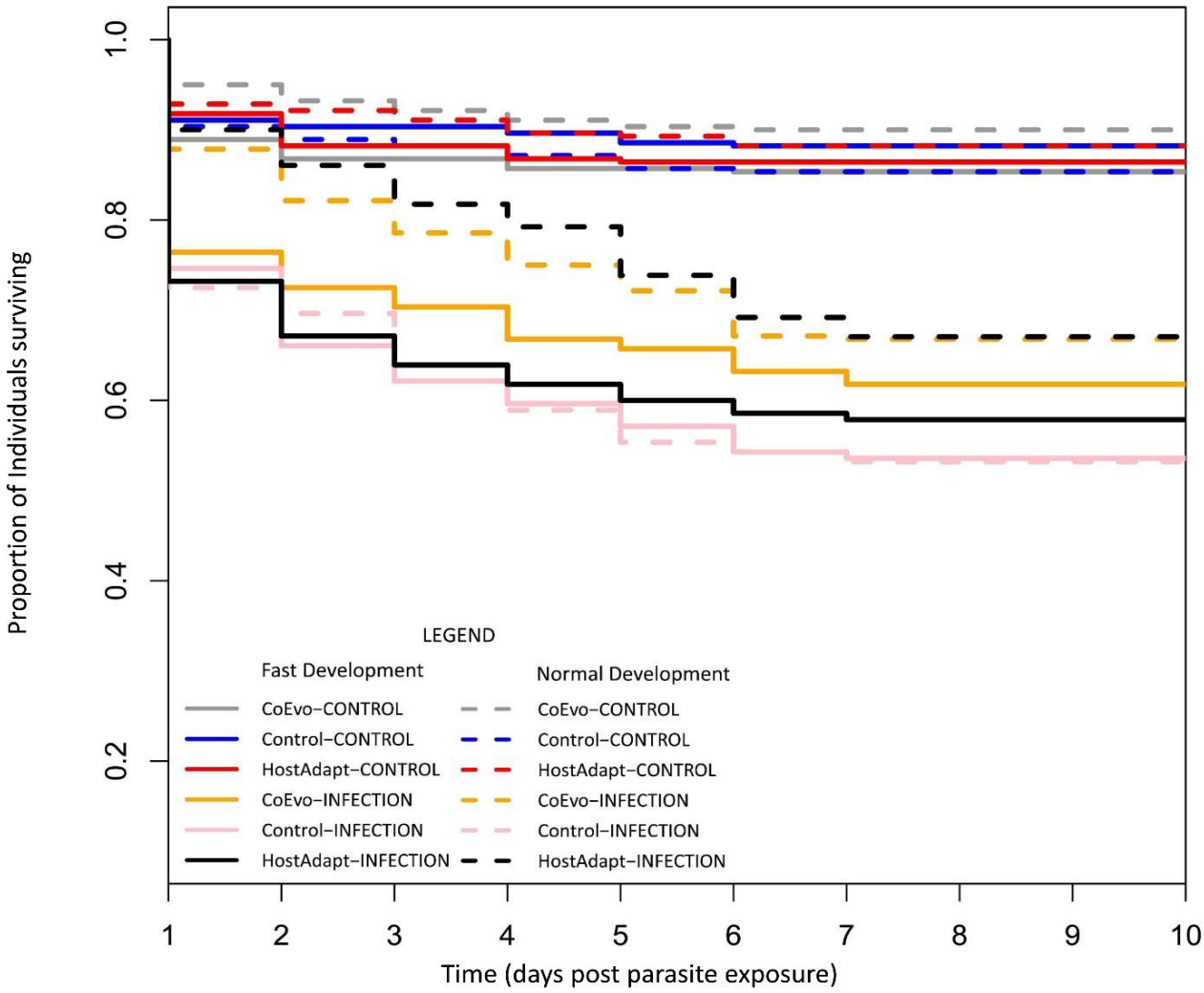
		CONTROL						INFECTION				
		CoEvo Fast	CoEvo Normal	Control Fast	Control Normal	HostAdapt Fast	HostAdapt Normal	CoEvo Fast	CoEvo Normal	Control Fast	Control Normal	HostAdapt Fast
CONTROL	CoEvo Normal											
	Control Fast	■										
	Control Normal											
	HostAdapt Fast											
	HostAdapt Normal	■	■		■							
INFECTION	CoEvo Fast			■	■	■	■					
	CoEvo Normal			■			■					
	Control Fast						■					
	Control Normal						■	■				
	HostAdapt Fast			■			■					
	HostAdapt Normal							■				

Figure 3.2 Survival of F2 of generation ten beetles upon infection with non-evolved *B. thuringiensis* (Kaplan Meier survival analysis). Highlighted fields indicate significant interactions in pairwise log-rank tests (p-value corrected using holm method). (For exact p values, see Table S4)



		CONTROL						INFECTION				
		CoEvo Fast	CoEvo Normal	Control Fast	Control Normal	HostAdapt Fast	HostAdapt Normal	CoEvo Fast	CoEvo Normal	Control Fast	Control Normal	HostAdapt Fast
CONTROL	CoEvo Normal											
	Control Fast											
	Control Normal											
	HostAdapt Fast											
	HostAdapt Normal											
INFECTION	CoEvo Fast											
	CoEvo Normal											
	Control Fast											
	Control Normal											
	HostAdapt Fast											
	HostAdapt Normal											

Figure 3.3 Survival of F2 from generation ten beetles upon infection with non-evolved *B. bassiana* (Kaplan-Meier survival analysis). Highlighted fields imply significant interaction in pairwise log-rank tests (p-values corrected using holm method. (For exact p values, see Table S2)



		CONTROL						INFECTION				
		CoEvo Fast	CoEvo Normal	Control Fast	Control Normal	HostAdapt Fast	HostAdapt Normal	CoEvo Fast	CoEvo Normal	Control Fast	Control Normal	HostAdapt Fast
CONTROL	CoEvo Normal											
	Control Fast											
	Control Normal											
	HostAdapt Fast											
	HostAdapt Normal											
INFECTION	CoEvo Fast											
	CoEvo Normal											
	Control Fast											
	Control Normal											
	HostAdapt Fast											
	HostAdapt Normal											

Figure 3.4 Survival of F2 of generation ten beetles upon infection with *P. entomophila* (Kaplan Meier survival analysis). Highlighted fields indicate significant interactions in pairwise log-rank tests (p-value corrected using holm method). (For exact p values, see Table S5)

3.1.2 Development time influences host immunity during evolution

Here, hemolymph PO and quinone secretions were used as proxies of external and internal immune defence respectively and measured every second generation during the course of experimental coevolution.

Using generalized linear mixed model analysis it was revealed that both *developmental time* (Sum of Square (SS) = 35799 on 4 DF, F = 216.006, p<0.001) and evolutionary *background* (SS = 3883 on 5 DF, F = 18.743, p<0.001) have an effect on the overall PO activity. CoEvo beetles from FAST and NORMAL development did not differ in their mean PO activity (Table 3.2).

Looking at the mean quinone production, again both *developmental time* (SS = 162383 on 4 DF, F = 50.370, p<0.001) and evolutionary *background* (SS = 69628 on 5 DF, F = 17.278, p<0.001) significantly affect the beetles' external defence secretions (Table 3.2).

Table 3.2 Post-hoc Tukey Contrasts (Multiple comparisons of means) of generalized linear mixed-model across experimental coevolution for PO and quinone production over all generations for FAST vs NORMAL developmental regimes

	Estimate	Standard error	|Z|	P value
	PO (NORMAL vs FAST, overall)			
Control	1.7077	0.4257	4.012	0.00148
HostAdapt	2.8127	0.4195	6.706	< 0.001
CoEvo	2.4672	0.4216	5.852	0.9461
	quinone (NORMAL vs. FAST, overall)			
Control	12.636	3.406	3.710	0.06347
HostAdapt	12.456	3.406	3.657	0.02703
CoEvo	22.001	3.406	6.459	< 0.001

Regime and evolutionary background as fixed effects and replicate beetle populations as random effect; P values corrected using Holm method

PO and quinone of beetles from the FAST development regime.

Analysis of linear mixed model for mean PO production revealed that PO activity varied across *generation* (ANOVA, SS = 62792 on 4 DF, F value = 430.92, $p < 0.001$) as well by the evolutionary background (ANOVA, SS = 1008 on 2 DF, F value = 13.84, $p < 0.001$). *Generation* (ANOVA, SS = 108938 on 4 DF, F value = 42.071, $p < 0.001$) and evolutionary *background* (ANOVA, SS = 8752 on 2 DF, F value = 6.760, $p < 0.001$) were significant factors in mean quinone production (Figure 3.5).

While the beetles do not show fluctuations in quinone production, fluctuations in PO activity is observed across the timeline of the coevolution experiment, providing no evidence for a trade-off between external and internal immune defence (Figure 3.5 & tables S4 and S5).

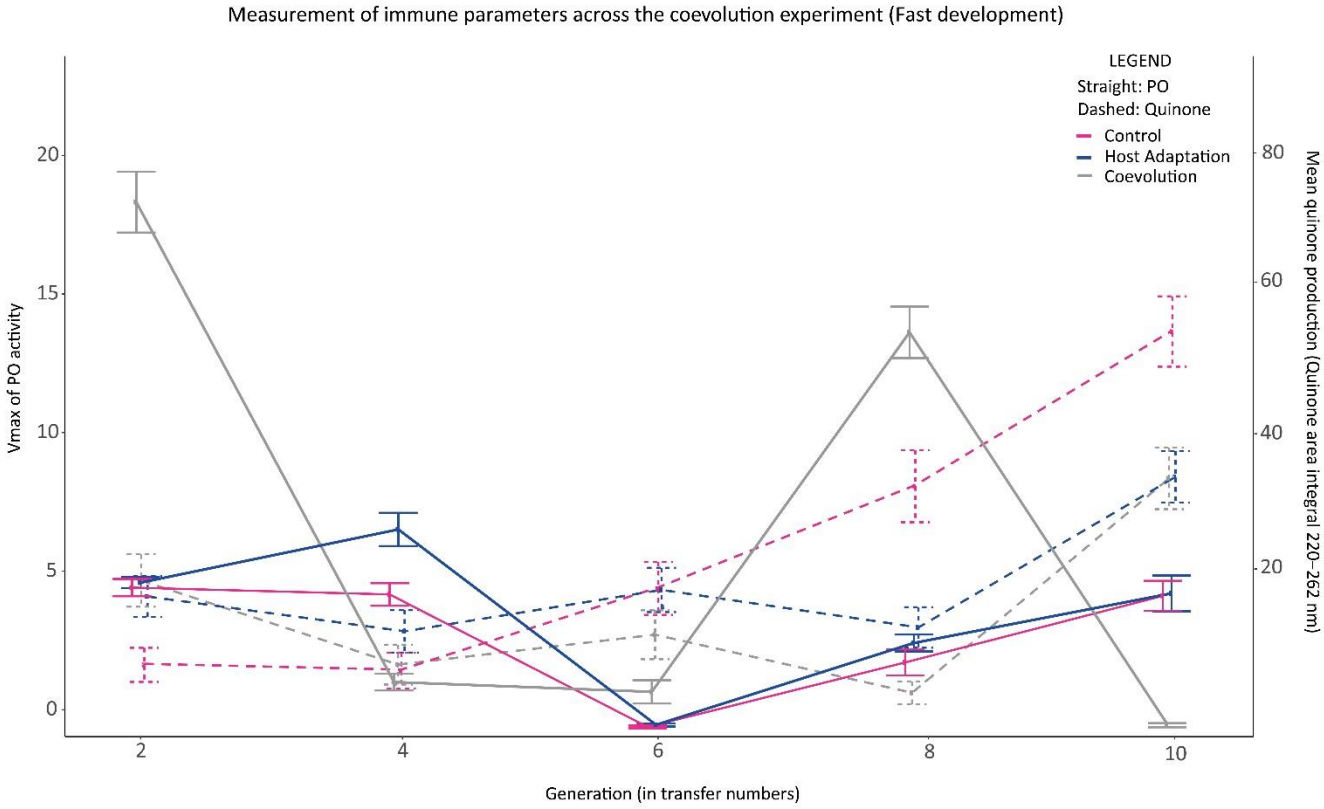


Figure 3.5 Immunity in FAST developing beetles. All measurements were performed on adult beetles. Error bars depict standard error of the mean. The Y-axis on the left represents Vmax of PO activity (µM/s) and on the right represents mean quinone production (given in terms of absorbance) (Tables S6 and S7).

PO and quinone measurements of beetles from the NORMAL development regime.

Analysis of mean PO activity for NORMAL beetles revealed that PO production varied across *generation* (ANOVA, SS = 4761.3 on 4 DF, F value = 77.375, $p < 0.001$) but the evolutionary *background* (ANOVA, SS = 37.6 on 2 DF, F value = 1.223, p = 0.3177) had no effect on it. While *generation* (ANOVA, SS = 272223 on 4 DF, F value = 75.491, $p < 0.001$) significantly affected mean quinone production of beetles, evolutionary *background* (ANOVA, SS = 1562 on 2 DF, F value = 0.866, p = 0.4373) was not (Figure 3.6). Here, as opposed to beetles belonging to FAST development regime, both PO and quinone production followed fluctuating trajectories during the course of the experiment (Figure 3.6 & tables S6 and S7). Therefore, when not faced with the stress of faster development, *T. castaneum* coevolving to *B. bassiana* and *B. thuringiensis* simultaneously, provides evidence for a trade-off between internal and external immune defence.

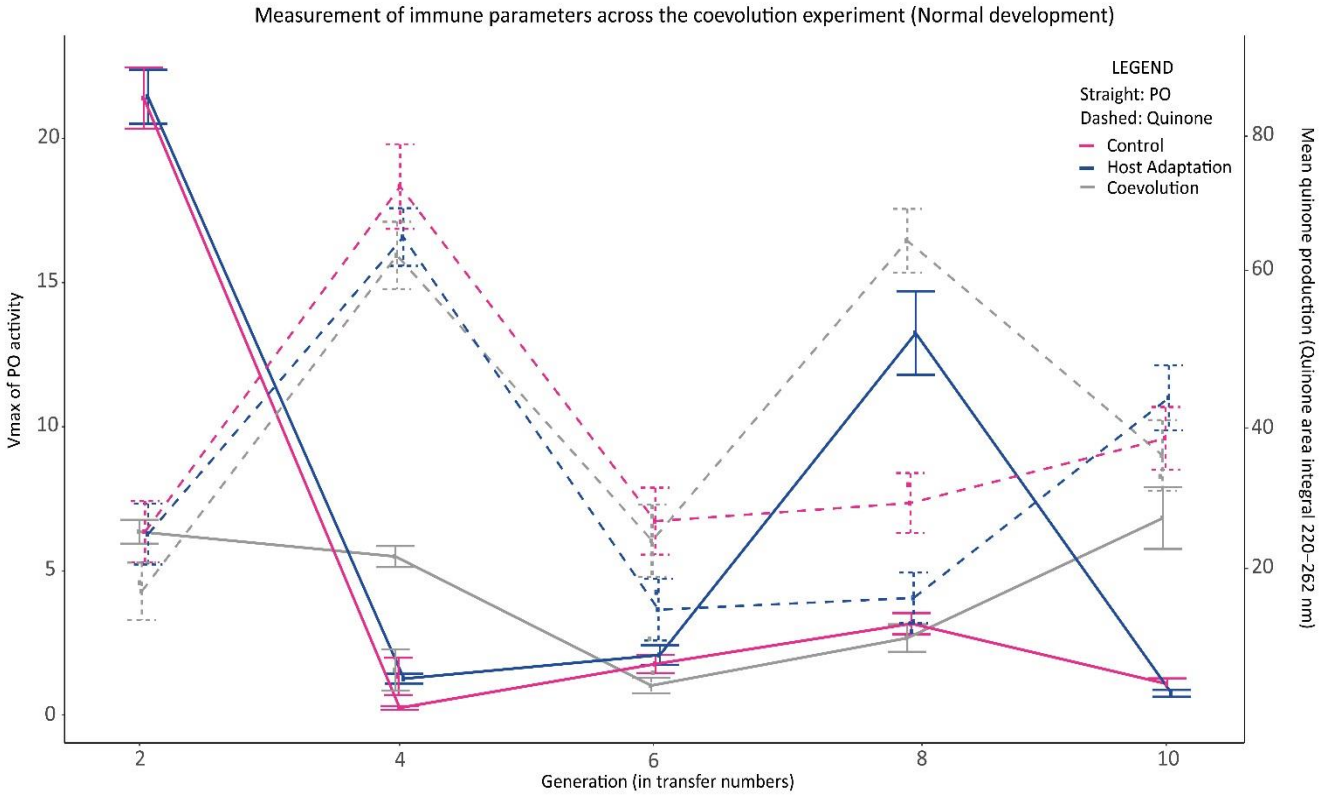


Figure 3.6 Immunity in NORMAL developing beetles. All measurements were performed in adult beetles. Error bars depict standard error of the mean. The Y-axis on the left represents Vmax of PO activity (µM/s) and on the right represents mean quinone production (given in terms of absorbance) (Tables S8 and S9).

3.1.3 Host development time drives parasite adaptation during tripartite evolution

Survival assays with *T. castaneum* belonging to the stock population were conducted with coevolved parasites to test for change in parasite virulence. Furthermore, parasite growth rate was measured as a proxy of life-history costs associated with coevolution. Minimum inhibitory concentration assays were carried out to examine any changes occurring in the parasites' resistance to the quinone secretions of the beetles.

A total of 56 *B. thuringiensis* isolates (treatments = CoEvo & ParasiteAdapt) belonging to FAST and 21 isolates belonging to NORMAL development regime, for the purpose of the experiments mentioned in this section I only used the isolates in Table 3.3. In contrast to *B. thuringiensis*, only five *B. bassiana* isolates (all belonging to the environment of NORMAL beetles) could be extracted from the environments of different replicate host populations (Table 3.3). *B. thuringiensis* isolates belonging the environment of FAST developing beetles started to developed biofilm. Therefore, the biofilm-forming ability of these isolates was quantified using a high throughput microtiter crystal violet assay [209].

Table 3.3 List of all parasites isolated from different treatments and generations

Generation (in transfer numbers)	Parasites			
	B. bassiana		*B. thuringiensis*	
	FAST	NORMAL	FAST	NORMAL
4	x	x	7 isolates from PA and TWC each	4 isolates from TWC
6	x	3 isolates from TWC[1]	7 isolates from PA and TWC each	1 isolate from PA and 1 isolate from TWC
8	x	x	7 isolates from PA and TWC each	1 isolate from TWC
10	x	2 isolates from PA[2]	7 isolates from PA and TWC each	

[1] TWC = coevolved, [2]PA = one-sided parasite adaptation

Virulence of B. bassiana isolates

Since *B. bassiana* could only be isolated from the environments of replicate populations belonging to NORMAL development regime, survival assays were also performed on beetles belonging to NORMAL development (F2 generations five and ten). In general, overall the survival assays, no statistical difference was observed among different groups (Figure 3.7).

When hosts were infected with PA3-10, Kaplan-Meier analysis (χ^2 = 17.2 on 13 DF, p= 0.19) show no overall significant difference between treatments (table S10). In survival assay with PA6-10 (χ^2 = 23.3 on 13 DF, p= 0.0383) overall difference was observed among different groups (tables S8 to S12).

None of the coevolved *B. bassiana* isolates (all from generation 6) showed any killing ability towards beetle hosts belonging to coevolution experiment (Kaplan-Meier analysis; TWC2: χ^2 = 14.4 on 13 DF, p = 0.347, TWC3: χ^2 = 18.7 on 13 degrees of freedom, p = 0.134 and TWC7: χ^2 = 9.6 on 13 DF, p= 0.724; (tables S8 to S12). Therefore, experimental coevolution did not lead to virulence in *B. bassiana*, irrespective of treatment.

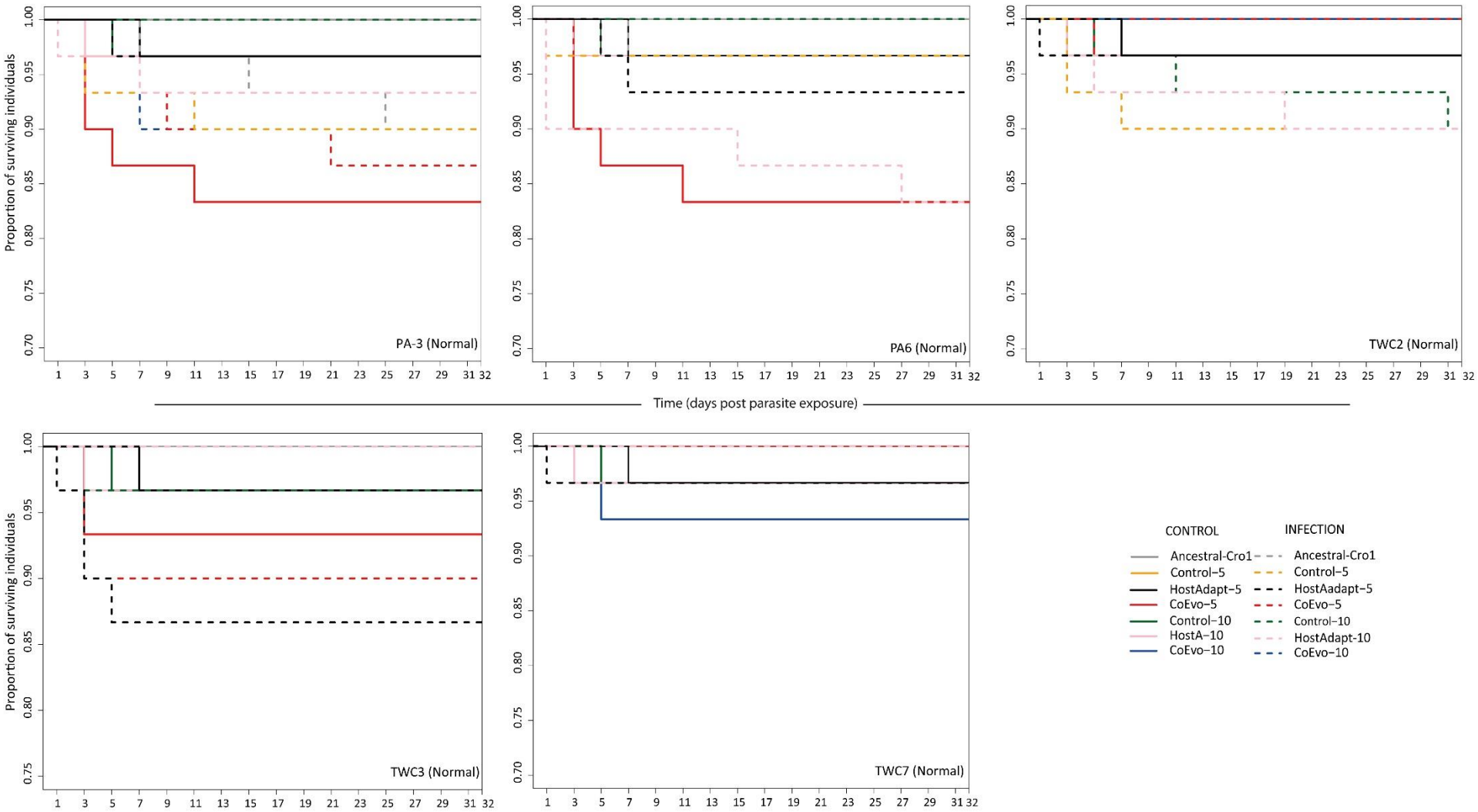


Figure 3.7 Virulence of coevolved *B. bassiana* isolates (represented in different panels) measured here as survival of hosts from experimental coevolution. None of the isolates show virulence towards the beetle hosts (different line represents different beetle populations) (table S8 to S12 for pairwise comparison of survival curves)

Virulence of B. thuringiensis isolates

Survival assays with representative *B. thuringiensis* isolates coming from FAST and NORMAL developing beetles did not reveal any general trend in virulence towards ancestral CRO1 beetles. From the FAST regime, the isolate PA7 (representing one-sided parasite adaptation) and TWC7 (representation coevolution) were chosen. Isolates belonging to generations 4, 6, 8 and 10 were used in the survival assays along with ancestral *B. thuringiensis.*

Infection with different isolates of PA7 (replicate) showed that PA7-10 had a highly increased virulence compared to isolates from previous generations (Kaplan-Meier analysis, χ^2 = 21.9 on 4 DF, $p < 0.001$, Cox proportional hazard analysis LrT = 18.41 on 4 DF, p = 0.001) (Figure 3.8 and Table S14). However, none of the other isolates (PA7-4, 6 & 8) showed any virulence on CRO1.

Upon infection with the coevolved isolate TWC7, CRO1 beetle hosts did not show any significant difference in their survival (Kaplan-Meier analysis χ^2 = 10.9 on 4 DF, p= 0.0272, Cox proportional hazard analysis LrT = 11.4 on 4 DF, p = 0.0224). Only the isolate TWC7-4 induces significant high mortality in CRO1, compared to the CONTROL (p = 0.022).

Some of the *B. thuringiensis* isolates from NORMAL developing beetles were virulent towards CRO1 (Kaplan-Meier analysis: χ^2 = 28.9 on 8 DF, p= 0.0039; Cox proportional hazard analysis: LrT = 29.4 on 8 DF, p = 0.0002702, figure 3.13 and table S14).

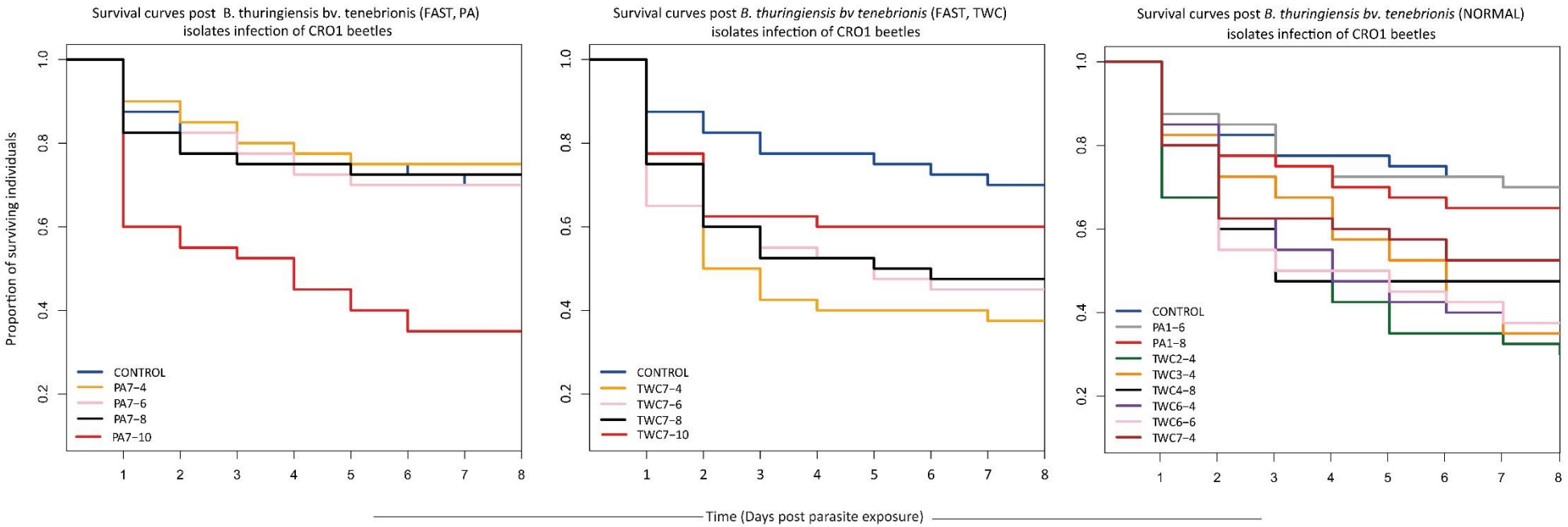


Figure 3.8 Virulence of *B. thuringiensis* isolates originating from experimental coevolution. Here virulence was measured as survival of Ancestral CRO1 beetle hosts to different parasite isolates (tables S13 and S14)

Note: PA = one-sided parasite adaptation; TWC = coevolution.

Metabolic activity of B. bassiana isolates

Metabolism of *B. bassiana* isolates from coevolution (coevolved isolates; TWC2 and TWC3 generation 6 and one-sided parasite adaptation; PA3 and PA6 isolates belonging to generation 10) was tested using fungal activity assay. Activity was measured in terms of fluorescence. Since methyl-1, 4-benzoquinone (MBQ) is the primary component of Beetle's external immune defence, different concentrations of MBQ were used to test *B. bassiana*'s growth against it.

In the absence of MBQ, coevolved isolates do not show any difference in their metabolic activity compared to ancestral *B. bassiana* (Figure 3.9 & 3.10 and Table 3.4). While growth and metabolic activity of ancestral *B. bassiana* is inhibited by the presence of MBQ in any concentration above that of one beetle equivalent, CoEvo and ParasiteAdapt *B. bassiana* isolates show metabolic activity at higher MBQ concentrations (table 3.3).

MBQ concentration equivalent to that produced by 25 beetles is inhibitory for both the isolates, TWC2 and TWC3. For ParasiteAdapt *B. bassiana* isolates, while PA3 was susceptible to MBQ beyond 1 beetle equivalent just as the ancestral, PA6 was highly resistant to MBQ. This is interesting because even within the same treatment, different isolates show very different responses to host defence.

Table 3.4 Pairwise t-tests comparing the activity of the ancestral and coevolved fungal strains at different MBQ concentrations (numbers indicate p-values upon comparison).

MBQ concentration (beetle equivalent)	Ancestral vs TWC2-6	Ancestral vs TWC3-6	Ancestral vs PA3-10	Ancestral vs PA6-10
0	0.1026	0.8056	1	<0.001
1	<0.0001	1	0.0104	<0.001
5	<0.0001	<0.0001	0.48328	<0.001
10	0.0411	0.0050	1	<0.001
25	0.1819	0.4731	1	<0.001

P values are holm adjusted.

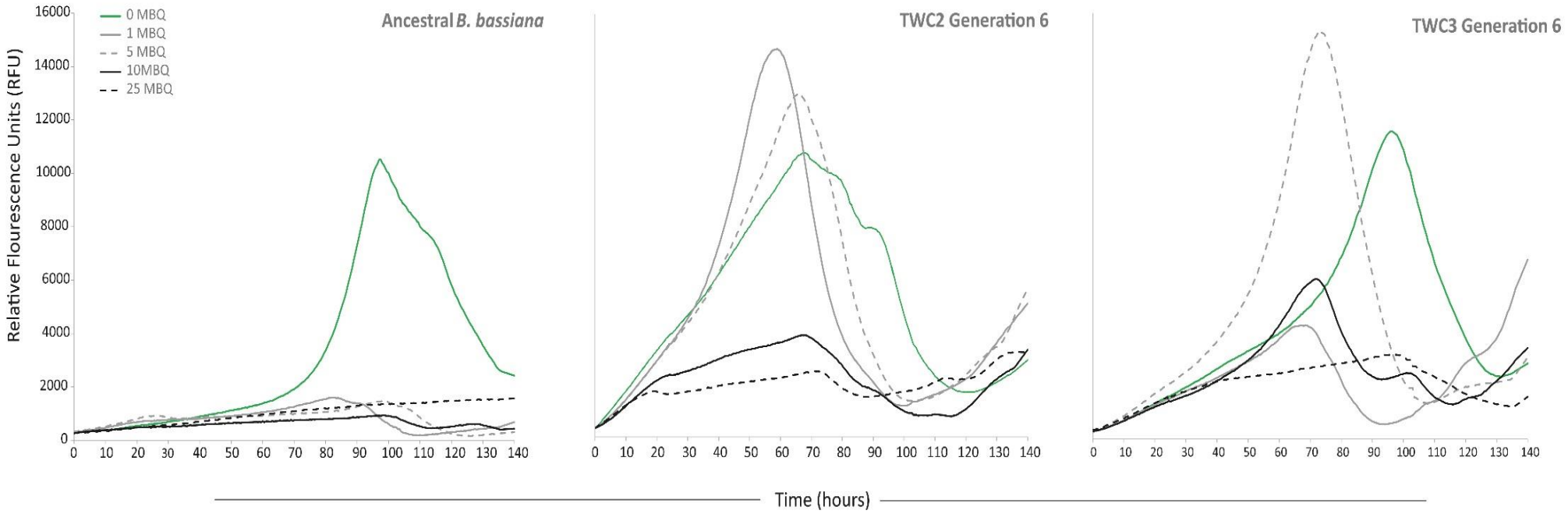


Figure 3.9 ***B. Bassiana*** **metabolic activity assay**: here activity is depicted in terms of relative fluorescence units (RFU) over time for ancestral and coevolved *B. bassiana* isolates TWC2 and TWC3 exposed to different concentrations of MBQ equivalent to the levels produced by 0, 1, 2, 5, 10 and 25 *T. castaneum* beetles [210]. The entire experiment was carried out for 140 hours. (Table S15)

Note: the y-axis runs from 0 to 16000 RFU

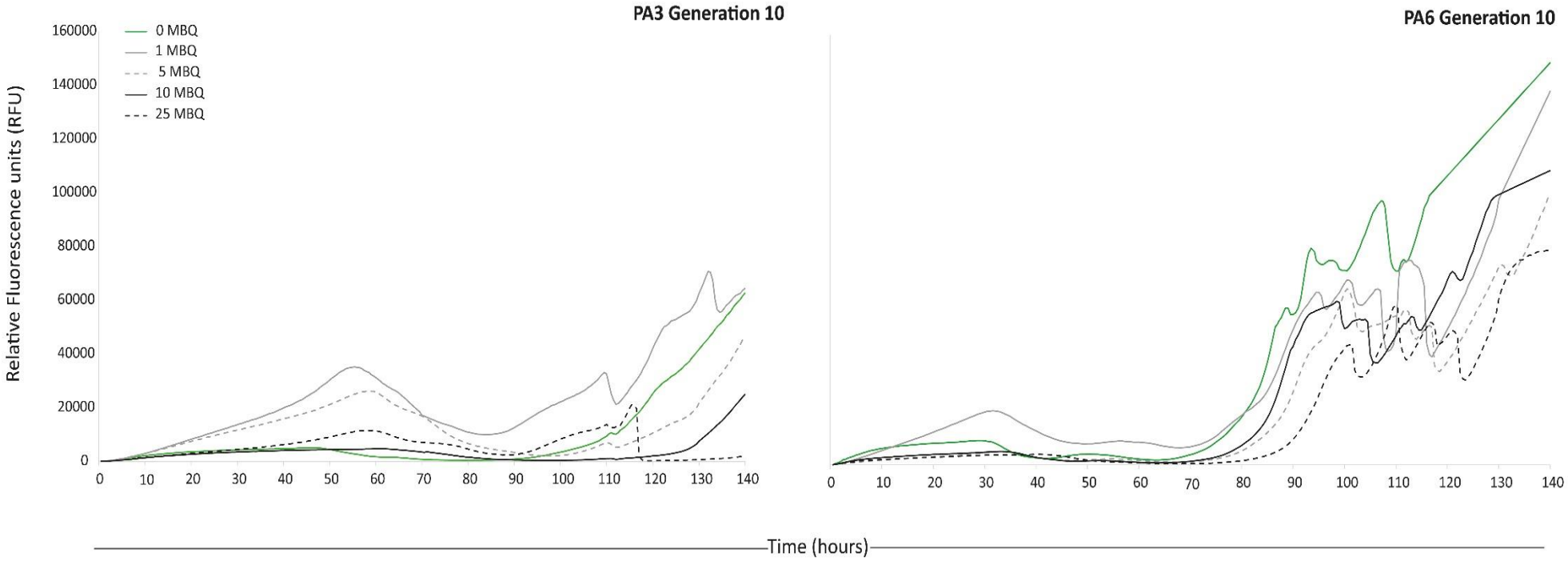


Figure 3.10 ***B. Bassiana*** **metabolic activity assay**: here activity is depicted in terms of relative fluorescence units (RFU) over time for *B. bassiana* isolates PA3 and PA6 belonging to one-sided parasite adaptation treatment, exposed to different concentrations of MBQ equivalent to the levels produced by 0, 1, 2, 5, 10 and 25 *T. castaneum* beetles [210]. The entire experiment was carried out for 140 hours. (Table S16)

Note: the y-axis runs from 0 to 160000 RFU

Growth analysis of B. thuringiensis

Growth is a major life-history trait of the parasite that contributes to its overall fitness, affecting both virulence and transmission [211]. For this purpose, I selected the isolates PA4 and PA7, and TWC4 and TWC7 from one-sided parasite adaptation and coevolution respectively.

In general, all isolates belonging to the FAST regime showed an increasing trend in growth rate during the course of the experiment (Figure 3.11 and table 3.6) and for all the isolates, the highest growth rate (longest doubling time) was always recorded at generation 10 (in terms of transfer), indicating that growth is an adaptive response to the coevolutionary interactions.

For the *B. thuringiensis* isolates belonging to the NORMAL regime, growth rate increased during the experiment compared to ancestral *B. thuringiensis* (Figure 3.12 and Table 3.6). ANOVA analysis revealed that *generation* and *isolate* were significant contributors in growth rate. This general increase in growth rate irrespective of FAST or NORMAL development regime, indicates that this trait was unanimously under selective pressure during the coevolution experiment.

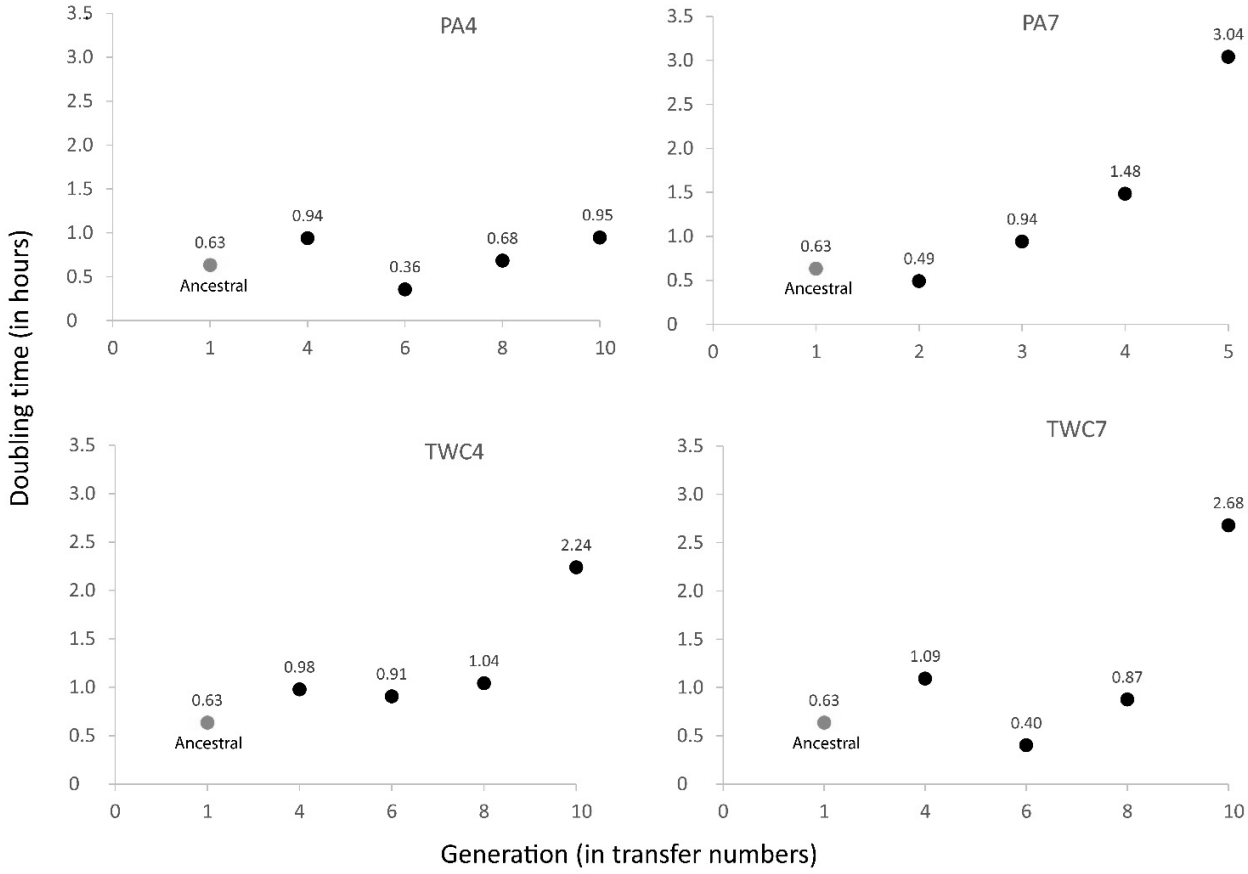


Figure 3.11 Measurements of changing growth rate of some representative *B. thuringiensis* isolates extracted at different time-points during experimental coevolution (Table S17)

Table 3.5 Two-way ANOVA table for doubling time for *B. thuringiensis* isolates belonging to FAST development regime (host)

	DF	Sum of Squares	Mean Sum of Squares	F-value	P-value
Generation	3	18.682	6.227	62275	<0.001
Isolate	4	3.902	0.976	9756	<0.001
Generation x Isolate	9	6.307	0.701	7008	<0.001
Residuals	34	0.003	0		

P values are holm adjusted.

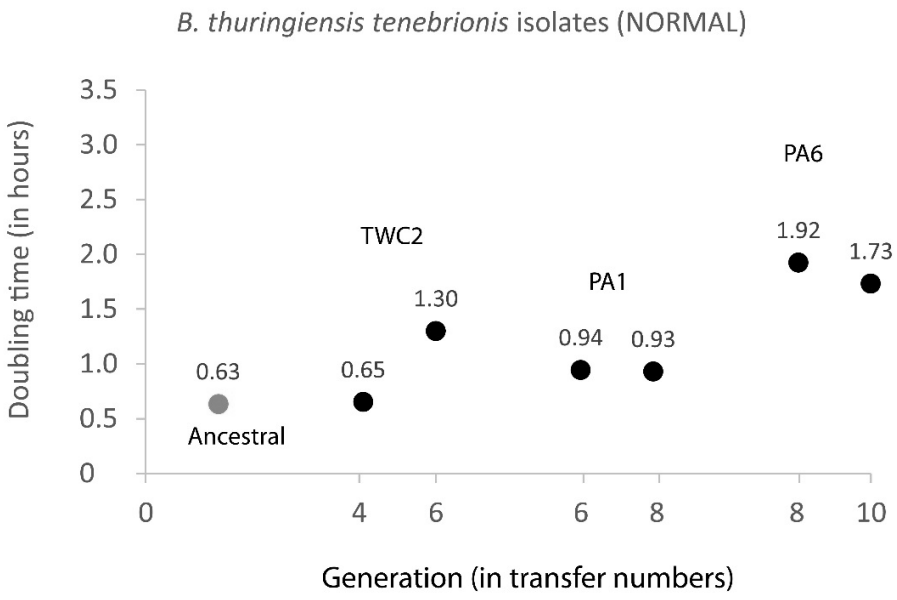


Figure 3.12 Growth rate of *B. thuringiensis* isolates (X-axis depicts generations (transfers)) from the environment hosts belonging to NORMAL development regime (Table S18)

Table 3.6 Two-way ANOVA table for doubling time for *B. thuringiensis* isolates belonging to NORMAL development regime (host)

	DF	Sum of Squares	Mean Sum of Squares	F-value	P-value
Generation	3	1.221	0.4071	4071	<0.001
Isolate	3	3.472	1.1573	11673	<0.001
Generation x Isolate	6	4.693	0.7822	7822	<0.001
Residuals	14	0.001			

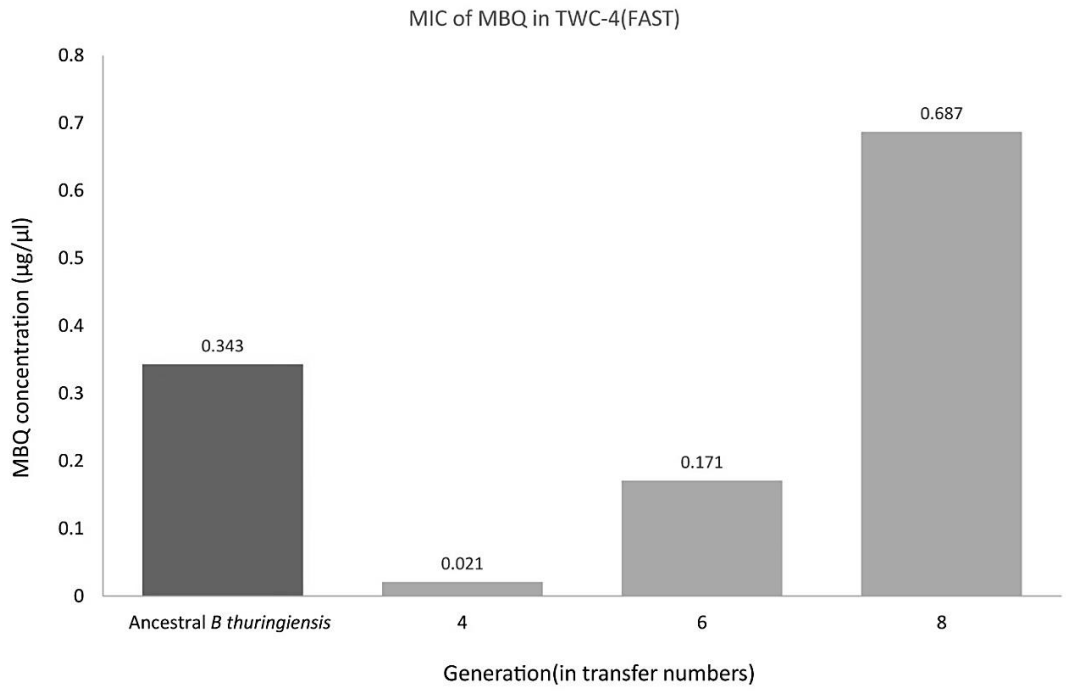
P values are holm adjusted.

MIC assay with B. thuringiensis

Minimum inhibitory concentration (MIC) assay was carried on a 96-well plate using different concentrations of MBQ. The biofilm forming isolate TWC4 belonging to FAST developing host was used as a representative to assess whether *B. thuringiensis* emerging from experimental coevolution show any inhibition towards the beetle host's external immune defence (Figure 3.13).

Since MBQ is a major component of *T. castaneum's* external immune secretions, different concentrations of MBQ, corresponding to 1, 5, 10 and 25 beetle equivalents were used. Ancestral *B. thuringiensis* was observed to be inhibited by MBQ at a concentration of 0.343 µg/µl.

The MIC value for MBQ kept increasing from generations 4 to 8 (Figure 3.13), implying gain in resistance to the beetle's external defence secretions.



MIC: Minimum inhibitory concentration
MBQ: methyl-1,4-benzoquinone

Figure 3.13 MIC assay for the coevolved *B. thuringiensis* strain TWC4 (from FAST development regime). The values mentioned in the figure correspond to the absolute MBQ concentration in µg/µl.

3.1.4 Emergence of novel phenotype: biofilm formation by coevolved *B. thuringiensis* isolates

While extracting *B. thuringiensis* isolates from different treatments and time-points, I noted wrinkled colonies formation of the isolates from the FAST regime, a phenotype associated with bacterial biofilm [212]. The ability to form biofilm emerged early in the experiment (from generation four in terms of host generation) and significantly differed between transfers (Two-way ANOVA, $p = 0.004$) (Table 3.6 for Two-way ANOVA results). The phenotype was quantified by a high throughput microtiter crystal violet assay [209].

For the crystal violet biofilm assay, PA1 and PA7 (PA = one sided parasite adaptation) and TWC4 and TWC7 isolates from generations 4, 6, 8 and 10 were chosen. Compared with the positive control i.e. *Staphylococcus epidermidis*, ancestral *B. thuringiensis* produced lower amounts of biofilm ($p < 0.001$). Overall, no specific trend was observed in the bacteria's ability to form biofilms across the timeline of the coevolution experiment (Figure 3.14). For one-sided parasite adaptation strains, the ability to form biofilm in comparison to the ancestor varied among different isolates.

While the isolate PA1 did not differ from the ancestor in its biofilm forming ability, PA7 belonging to transfer 10 differed significantly to the ancestral *B. thuringiensis* ($p = 0.02$). The coevolved *B. thuringiensis* isolates, TWC4 and TWC7, exhibited a high overall biofilm forming ability. TWC4 gained a significant increase in biofilm forming ability when compared to the ancestral isolate in transfers 6 ($p = 0.01$), 8 ($p = 0.015$) and 10 ($p < 0.001$). Similarly, TWC7 too showed a significant increase in its biofilm forming ability compared to the ancestor from transfer 6 onwards (results from TUKEY multiple comparisons of Two-way ANOVA see Table 3.7).

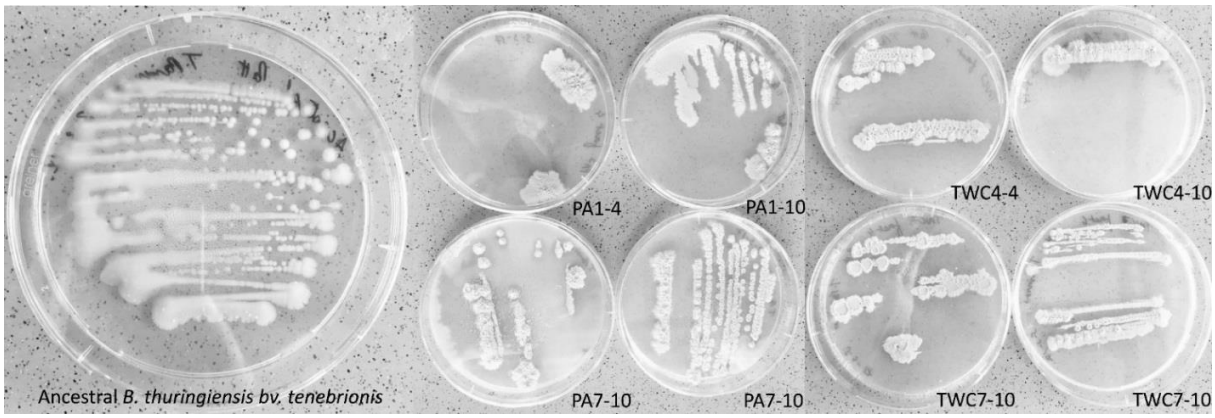


Figure 3.14 Biofilm formation on LB agar plates by one-sided adapted and coevolved *B. thuringiensis* isolates belonging to FAST host development regime. Note the typical wrinkled colonies associated with biofilm formation [213]. The ancestral *B. thuringiensis* does not form such colonies.

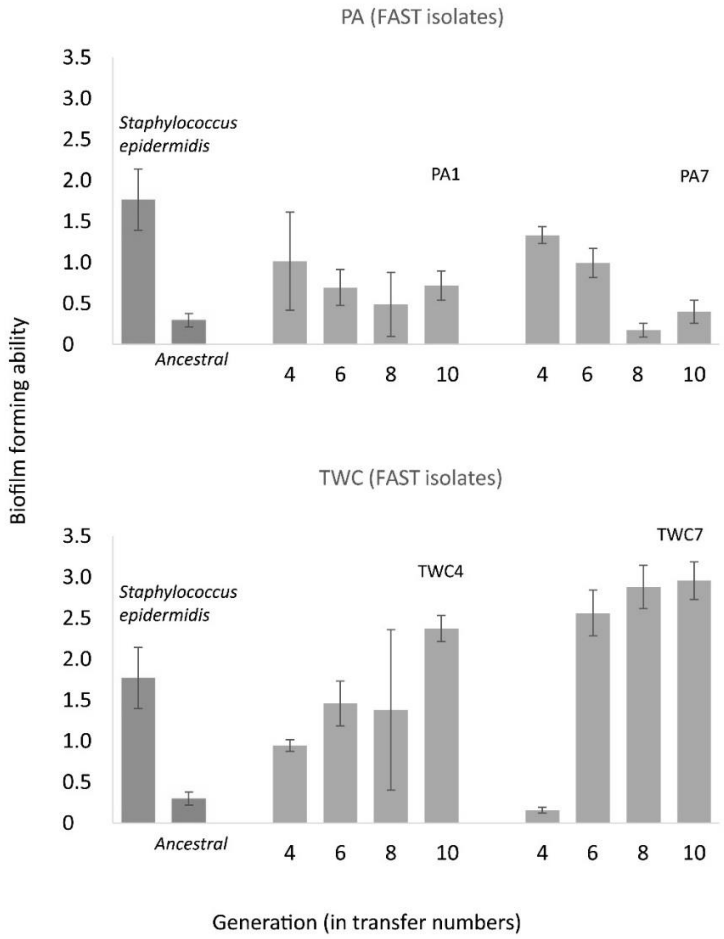


Figure 3.15 Relative biofilm forming ability of representative *B. thuringiensis* isolates from one-sided parasite adaptation and coevolved isolates. *Staphylococcus epidermis* is known to form biofilms and here used as a positive control. Ancestral *B. thuringiensis* did not produce any biofilm.

Table 3.7 Two-way ANOVA table for biofilm ability.

	DF	Sum of Squares	Mean Sum of Squares	F-value	P-value
Generation	3	2.53	0.844	4.834	0.004
Isolate	5	20.45	4.090	23.420	<0.001
Generation x Isolate	9	38.23	4.247	24.318	<0.001
Residuals	77	13.45	0.175		

The isolate and the generation (in transfer numbers) were used as fixed factors.

P values corrected using Holm method

Table 3.8 Tukey multiple comparisons of Two-way ANOVA output for differences in the ability to form biofilm of different *B. thuringiensis* isolates compared to Ancestral strain, across different time points (transfers) during the evolution experiment

Vs. Ancestral *B. thuringiensis*	*S. epidermidis*	PA1	PA7	TWC4	TWC7
	<0.001				
4		0.42	0.02	0.61	1
6		0.99	0.48	0.01	<0.001
8		0.99	1.00	0.015	<0.001
10		0.98	1.00	<0.001	<0.001

P values corrected using Holm method

3.2 Cross-resistance as consequence of host-parasite coevolutionary interactions

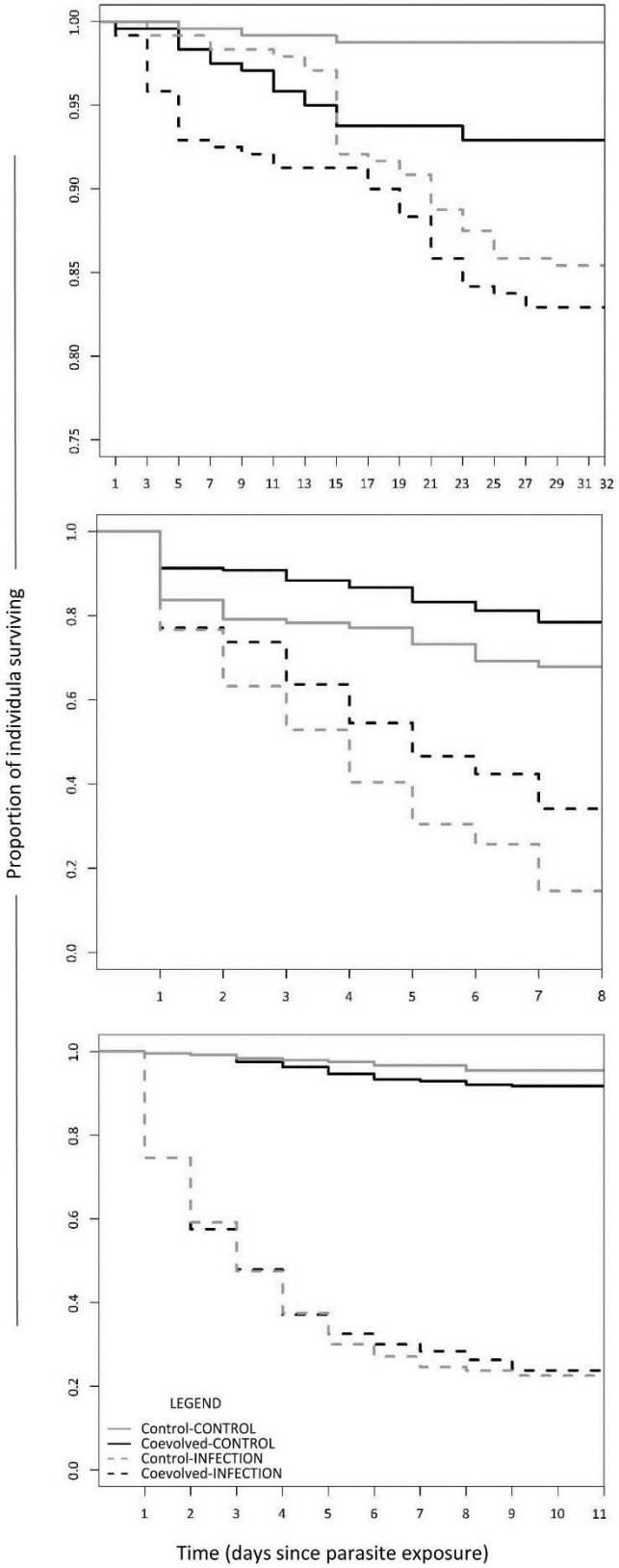
3.2.1 *T. castaneum* coevolved with *B. bassiana* is positively cross-resistant to *B. thuringiensis*

Survival of *B. bassiana* coevolved *T. castaneum* was monitored upon exposure to *B. bassiana, B. thuringiensis* and *P. entomophila* using survival assays.

When exposed to non-evolved *B. bassiana*, beetles differed between CONTROL and INFECTION treatments (χ^2 = 41.1 on 3 DF (degrees of freedom), p<0.001). Pairwise comparison of Kaplan-Meier curves (Figure 3.16(a)) revealed that *Control* and *Coevolution* groups differ from each other within CONTROL (p <0.001, with 7% drop in survival) but not within INFECTION treatment (p = 0.397). Therefore, coevolution to *B. bassiana* did not lead to increased resistance of beetles when infected with non-evolved *B. bassiana*.

When exposed to *B. thuringiensis* (figure 3.16 (b)), beetle groups differed significantly between INFECTION and CONTROL (χ^2 = 224 on 3 DF, p <0.001). Pairwise comparison of Kaplan-Meier curves showed that *Control* and *Coevolution* groups differed in CONTROL (p = 0.05) and in INFECTION treatment (p < 0.001). Here, higher survival of the *Coevolution* group upon INFECTION in comparison to the *Control* group indicates positive cross-resistance to *B. thuringiensis*, as a consequence of coevolution with *B. bassiana*.

In *P. entomophila* survival assay (Figure 3.16(c)), survival of groups in INFECTION and CONTROL treatments were significantly different from each other (χ^2 = 544 on 3 DF, p < 0.001). Pairwise comparison of Kaplan-Meier curves shows that *Control* and *Coevolution* groups do not differ from each other within CONTROL (p = 0.19) and INFECTION treatment (p = 0.80). Therefore, the response of *B. bassiana* coevolved beetles was not affected by their evolutionary background, with *Control* and *Coevolution* groups being equally susceptible to *P. entomophila*.



B. bassiana survival assay	Control-CONTROL	Coevolution-CONTROL	Control-INFECTION
Coevolution-CONTROL	0.004 *		
Control-INFECTION	<0.001 *	0.022 *	
Coevolution-INFECTION	<0.001 *	0.003 *	0.40

(a)

B. thuringiensis bv. *tenebrionis* survival assay	Control-CONTROL	Coevolution-CONTROL	Control-INFECTION
Coevolution-CONTROL	<0.001 *		
Control-INFECTION	0.05 *	<0.001 *	
Coevolution-INFECTION	<0.001 *	<0.001 *	<0.001*

(b)

P. entomophila survival assay	Control-CONTROL	Coevolution-CONTROL	Control-INFECTION
Coevolution-CONTROL	<0.001*		
Control-INFECTION	1	<0.001 *	
Coevolution-INFECTION	<0.001*	1	<0.001*

(c)

Figure 3.16 Survival of *B. bassiana* coevolved beetles upon infection by (a) ancestral *B. bassiana* (30 days), (b) *B thuringiensis* (7 days) and (c) *P. entomophila* (10 days). The assays lasted for different time periods based on the parasite in question. Pairwise comparisons of different background and treatment combinations are enlisted in the tables, next to their respective survival assays. Note: y-axis in (a) starts from 0.75 for better visualization of data.

3.2.2 Cross-resistance is mirrored in gene expression

RT-qPCR was employed to investigate differential expression of 11 candidate genes (post *B. thuringiensis, P. entomophila* and non-evolved *B. bassiana* exposures (Figure 3.17).

12 hours post *B. bassiana* infection Apo-III (8.5 fold) and ObpC-12 (~8 fold) were up-regulated (Figure 3.17(a)). After 24 hours of infection, the up-regulation of Apo-III (8.5 fold) and ObpC-12 (16.5 fold) was observed again. (Figure 3.17(a)). Both these genes are known to be expressed upon *Cry*-III (Coleopteran specific) toxin induction [123,135,214].

Most of the candidate genes were upregulated 12 hours post *B. thuringiensis* exposure with stress candidates Hsp90 (3 fold) and p450 (6.6 fold) being prominent (Figure 3.16 (b)). Attacins have been reported to be upregulated upon *B. thuringiensis* infection in *T. castaneum* and is also seen here. 24 hours post *B. thuringiensis* exposure revealed an up-regulation in all the candidate genes, although compared to 12 hours, stress genes Hsp90 (1.4 fold) and p450 (1.7 fold) were less abundant, candidates related to general immunity were all up-regulated (Figure 3.16 (b)).

Upon *P. entomophila* infection Attacin-2 (450 fold) and Thaumatin (34 fold) were highly upregulated 12 hours post-exposure. Although fold change in transcript levels of Attacin-2 (94 fold) and Thaumatin (15 fold) had decreased at the 24-hour time point (Figure 3.17(c)). Upregulation of Attacin-2 and Thaumatin are in consensus with previous findings which report the similar expression of these genes upon lipopolysaccharide induction [139,215,216] (abundant in Gram-negative bacteria).

Changes in gene expression were computed using the R package MCMC.qpcr upon *B. thuringiensis*, *P. entomophila* and *B. bassiana* exposure with respect to *time* (12 vs. 24 hours) and *treatment* (CONTROL vs. INFECTION) and are summarized in figure 3.5. A Greater number of candidate genes are differentially expressed upon *B. thuringiensis* exposure (Figure 3.18) than other infections, indicating that the host employs a more flexible approach towards this parasite, which can be attributed to its coevolution with *B. bassiana* [217].

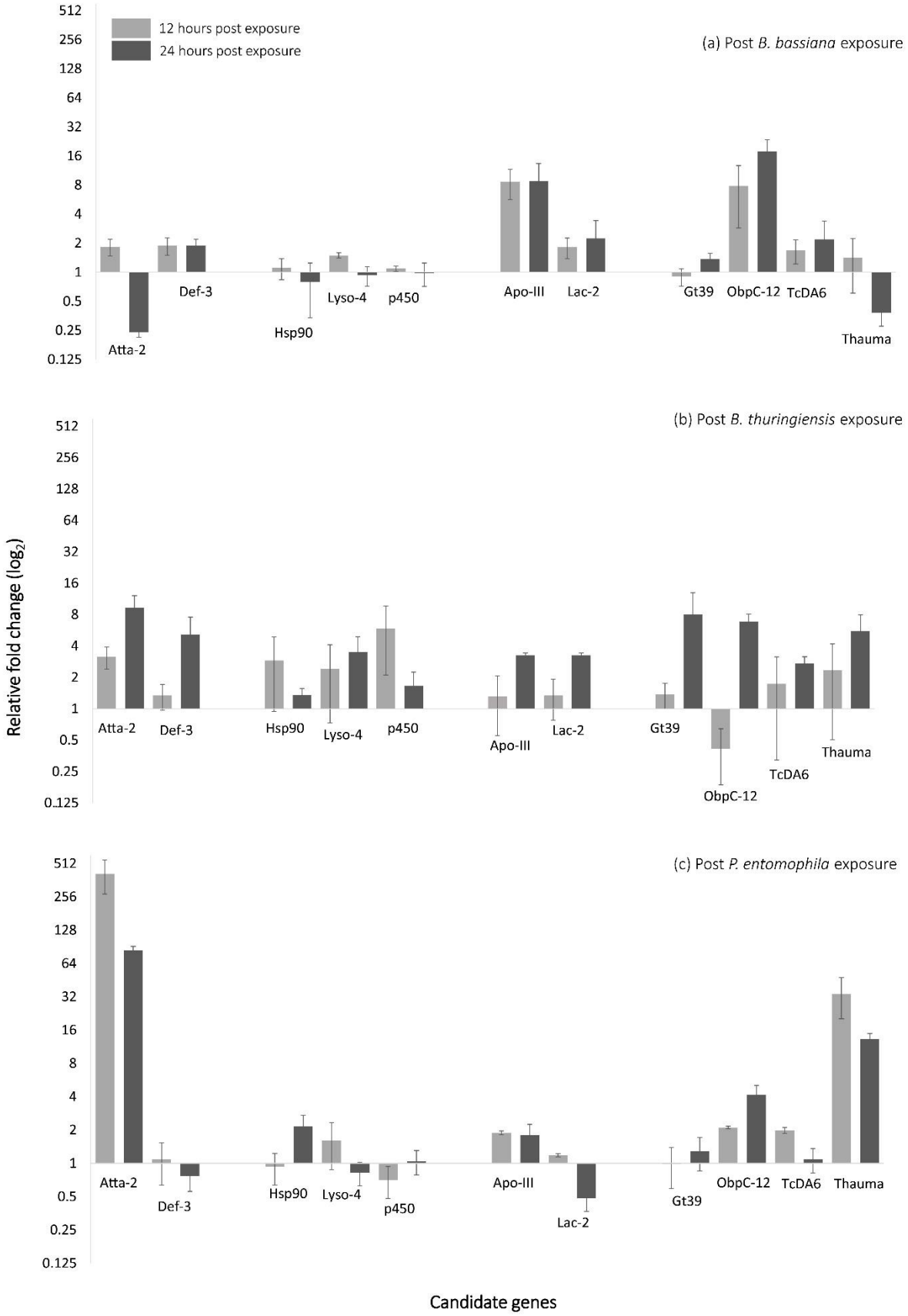


Figure 3.17 RT-qPCR results on the coevolved beetles upon infection with (a) ancestral *B. bassiana*, (b) *B. thuringiensis* and (c) *P. entomophila*, 12 and 24 hours post-exposure. The method of parasite exposure for the qPCR was kept the same as that used for the survival assay. *Note: Lyso-4 = lysozyme-4, Lac-2 = Laccase 2, Def-3 = Defensin-3 and Atta-2 = Attacin-2*

	B. bassiana			*B. thuringiensis* bv. *tenebrionis*			*P. entomophila*		
	Time	Treatment	Time & Treatment	Time	Treatment	Time & Treatment	Time	Treatment	Time & Treatment
Attacin-2									
Defensin-3									
Hsp90									
Lyzo-4									
P450									
Apo-III									
Laccase 2									
GT39 (quinone)									
Obpc12									
TcDA6 (chitin deacetylase)									
Thaumatin									

Highlighted fields depict changes in gene expression of candidate genes post normalization using reference genes. Significance of the factors are given as follows: *Time* is computed as change after 24 hours compared to 12 hours only, *Treatment* is computed as change upon infection only and the interaction term 'Time & Treatment' refers to changes after 24 hours compared to 12 hours upon INFECTION (vs CONTROL).

Note: Lyso-4 = lysozyme-4, Lac-2 = Laccase 2, Def-3 = Defensin-3 and Atta-2 = Attacin-2.

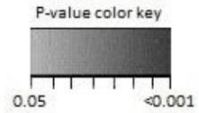


Figure 3.18 Results from the analysis of differential relative gene expression upon parasite exposure via generalized linear mixed models performed using the R package MCMC.qpcr

4

Discussion

He is a fool that practices the truth, without knowing the difference between truth and falsehood.

|

Krishna to Arjun (The Mahabharata)

With multiple parasites and hosts constantly interacting and shaping each other's adaptations, more experimental studies that tease apart the outcomes and causes of such interactions are needed [81,98]. Interaction with multiple parasites can be studied effectively in model insects such as the genetically curated *Tribolium castaneum* [155], which has the added benefit of ease of maintenance and the possibility of applying large sample sizes in concurrent experiments. In my doctoral thesis, I have aimed to understand host's exposure to multiple parasites, either sequentially or simultaneously, in the light of experimental evolution. Additionally, the impact of host development time in coevolutionary interactions was assessed.

Using tripartite (*T. castaneum*, *Beauveria bassiana* and *Bacillus thuringiensis*) experimental evolution with two host developmental time periods in parallel, I could show that host development time not only impacts host immunity but also influences changes in the tested parasite life-history traits. Additionally, I could also show that positive cross-resistance emerges as a consequence of coevolution in *B. bassiana*

coevolved *T. castaneum* towards *B. thuringiensis* but not to *Pseudomonas entomophila.* Here I propose that the route of infection forms the basis of positive cross-resistance.

4.1 Tripartite host-parasite evolution

Pace of development shapes adaptive changes in the host

A one host-two parasite experimental coevolution (with necessary controls [98]), using *T. castaneum* as the host and *B. thuringiensis* and *B. bassiana* as environmental parasites, was performed. Development time of the host was manipulated (grouping the experiment in two regimes with FAST (transferred every 21 days) and NORMAL (transferred every 28 days) beetle developmental time-periods) in addition to imposing evolutionary and coevolutionary pressures (Results summarized in Figure 4.1).

Coevolved beetles from both regimes gain resistance to non-evolved *B. thuringiensis*. The coevolved beetle populations belonging to NORMAL regime differ between CONTROL and INFECTION similar to the previous experiment by Bérénos *et al.,* with *T. castaneum* hosts coevolved to *Paranosema whitei* [101]. One-sided adapted hosts from the FAST regime do not show any difference in survival compared to control, similar to that observed in the experimental coevolution of Masri et al., where one-sided adapted *Caenorhabditis elegans* did not differ in survival to control when exposed to ancestral *B. thuringiensis* [99].

FAST coevolved *T. castaneum* display higher mortality even in the absence of *B. bassiana* (CONTROL treatment). This is in conjunction with the 'pace of life' hypothesis which states that animals with faster development time invest fewer resources in costly immune traits [218,219]. For instance, fast-evolving *D. melanogaster* populations are more susceptible to *E. coli* [220] and also have higher mortality compared to control populations [221]. Supporting this, it was shown that *D. melanogaster* experimentally evolved for higher resistance to *B. cereus* displays a significantly slower development rate [222]. Both these studies speculate that costs of immune defence are traded-off against the pace of development. In the context of tripartite experimental evolution, when under pressure to develop faster, the beetles invested

resources in development, thereby reducing allocation towards immunity. Indeed in FAST beetles, external and internal immune proxies measured do not display a fluctuating relationship, during the experiment, as per the trade-off hypothesis proposed in Joop et al. [89].

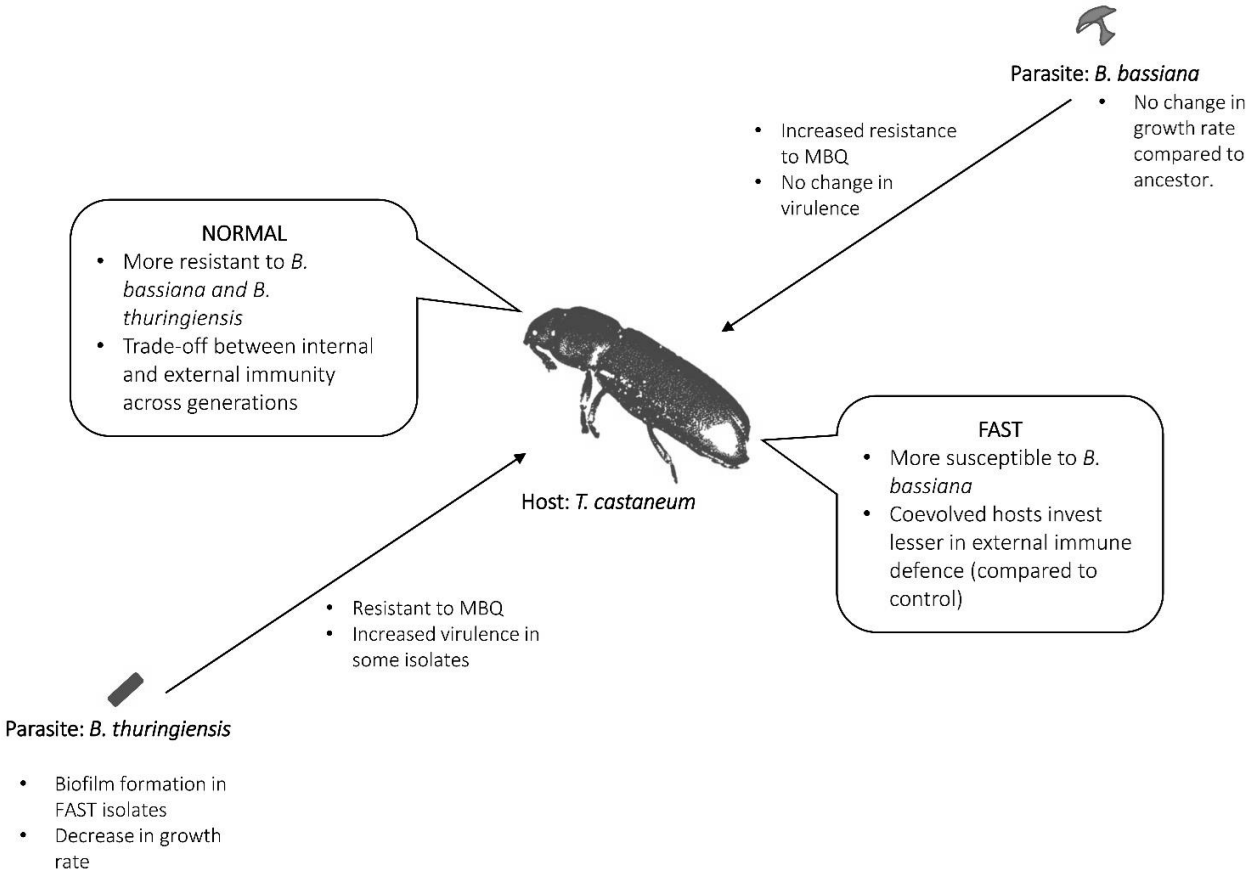


Figure 4.1 Primary outcomes of the one host-two parasite coevolution experiment. Host developmental time not only affected adaptive trajectories in the host itself but also in both the parasites. The phenotypic response for the parasite was more noticeable than that of the host. For comparison of the results present I thesis with other experimental coevolution involving *T. castaneum*, *B. bassiana* and *B. thuringiensis* (in separate studies) see supplementary tables A.4.1-3

One-sided adapted hosts were significantly more susceptible (vs Control and TWC hosts) when infected with the one-side adapted *B. bassiana* isolate. When infected with the evolved isolates hosts were

significantly more susceptible to infection in comparison to the ancestral (CRO1), control and coevolved beetles. The susceptibility of one-sided adapted beetle groups to some of the *B. bassiana* isolates is a likely outcome of the host and the parasite being at different points in the evolution, indicating a lack of local adaptation [10]. A plausible argument as to why not all one-side adapted and coevolved beetles are resistant to the parasite is that the parasites were extinct from the environment or went in a dormant stage, and therefore no selection pressure for the hosts to adapt to *B. bassiana.*

Every generation, pupae and big larvae were transferred to the next falcon tube. In holometabolous insects such as *T. castaneum*, the juveniles go through a number of molting stages prior to adulthood. Molting can significantly protect the insect from fungal infections by clearing the spores attached to the exterior of the host [129]. Components of the molting fluid can also render protection from parasites. Molting fluid of *Manduca sexta* produces Pr1, a protease highly inhibitory towards *Metarhizium anisopliae* [223]. *Bombyx mori* molting fluid was found to possess inhibitory functions towards bacteria [224]. Additionally, *B. bassiana* could not germinate on the newly formed cuticle of *B. mori* juveniles during ecdysis (the process of insect juveniles shedding off old cuticle during development) [224]. The molting associated protection might have prevented the adaptation of the beetles to *B. bassiana* infection in the conducted experiment since exposure was highly reduced and probably no infection occurred. Therefore, beetles forced to evolve with an additional selection pressure of extended development time directed their resources in defence against the second parasite being present in the environment, *B. thuringiensis*, a more lethal and faster-killing parasite.

In addition to biotic factors, abiotic factors such as resource availability (for instance dietary [225,226], space constraints [227–229]) and temperature [230,231] have been repeatedly shown to influence resource allocation and thereby the modulation of immune defence. For invertebrates like insects, the innate immune defence is the major player in fighting foreign agents within the body and PO is one such enzyme that provides protection against a number of parasites [45,184]. The external immune system is

an important barrier to parasitic attack and occurs among different animals [171,232–234]. In the case of *T. castaneum*, the external [89] and internal immune defence [87,185] have been shown to be heritable and therefore, can show responses under the right selection pressures. In *T. castaneum*, artificial selection for high and low levels of quinones (external immune defence) show that the effects of selection are retained in the populations even after 48 generations of no selection [89]. Schwazerbach and Ward also showed that yellow dung flies, *Scathophaga stercoraria* selected for low and high levels of PO also retained the effects after relaxed selection [185]. Additionally, it has been hypothesized that there is a trade-off between the primary components of internal (PO) and quinone since both require the amino acid Tyrosine as a precursor [89,138,234]. With regards to *T. castaneum* and other insects that secrete quinone compounds as an external defence [171], the potential for trade-off exists between internal and external immune defence.

Under conditions of constraint, increased investment in immunity is at the cost of important life-history traits [235–238]. FAST development coupled with coevolution in presence of parasites proved to be a very high selective force, directing higher investments in PO, albeit coupled with strong fluctuations. Quinone production in FAST beetles differed significantly between coevolved and control hosts, with an overall lower level of quinones compared to NORMAL beetles. In NORMAL beetles, quinone production did not vary significantly between different evolutionary backgrounds but fluctuated across the timeline of the experiment. It can be observed that the patterns of PO and quinone variation as the evolution experiment proceeds is opposite to that of each other. Here, in the absence of a strong pressure to develop faster, the beetle populations indicate a trade-off in the investment of resources in internal and external immune defence.

The dip seen in generation 6 in both PO and quinone production for both FAST and NORMAL developing beetles is plausibly due to a sampling of adults from generation 5 which resulted in lower number of individuals in the following populations. Decrease in population density potentially affected the beetle's

investment in immunity. Immune defence displays plasticity in response to population size as shown by the African Armyworm, *Spodoptera exempta* where two different density-dependent morphs exist; darker and light cuticular melanisation at higher and lower population densities, respectively [239]. Similarly, in lobster cockroaches, *Nauphoeta cinerea*, the activity of hemolymph PO increased with higher population densities [240]. Since population density in all generations except 6 was same, the PO and quinone response I see at other time-points are therefore correlated to the evolutionary background of *T. castaneum* and not to population density.

Host pace of development drives adaptive changes in the parasites

In my experiment*, B. thuringiensis* turned out to be the more successful parasite in terms of its ability to persist in the environment (all FAST and some NORMAL populations). Many of the isolates display a strong trend for the expression of biofilm formation. *B. thuringiensis* isolates from the environment of one-side parasite adaptations and coevolution treatments do not display a trend in terms of virulence and the ability to kill the host, instead of forming biofilms and protecting themselves from external factors.

While *B. thuringiensis* isolates could be extracted from all beetle populations (one-side parasite adaptation and coevolution) belonging to FAST development regime this was not the case for *B. thuringiensis* isolates from NORMAL beetles. During the experiment, NORMAL beetles not only produced higher quinone but here, the adults had a longer time to condition the environment, compared to FAST. The 'proof of concept' MIC assay with MBQ (representative of host external immune defence) revealed that the biofilm forming isolate TWC-4 from fast regime gained resistance against MBQ, with a significant trend displayed across the experiment. The susceptibility of *B. thuringiensis* to quinone coupled with NORMAL beetles conditioning their environment with higher levels of quinone for longer, potentially explain the extinction/dormancy of *B. thuringiensis* from the environment.

In total, only five *B. bassiana isolates* could be successfully extracted from the entire experiment and these did not show any significant increase in virulence when compared to the ancestor. The extinction of fungal isolates can be attributed to a combination of the following scenarios.

- ***Lack of standing genetic variation*** methodologies of evolution experiments influence their outcomes. Which in turn drives adaptations in different directions. In the evolution experiment of Rafaluk *et al.* new genetic material of *B. bassiana* was added during every transfer, increasing the standing genetic variation, creating greater adaptive potential. This was not the case in my experiment, where only the environmental parasites were transferred every generation.
- ***Dormancy of B. bassiana*** Fungi produce resilient dormant spores when faced with harsh environmental conditions [241]. Especially exogenous dormancy, which in the case of fungal spores is not broken even with the presence of moisture in the environment [241], making extraction of fungal spores from the environment impossible by simple microbiological approaches. Also, since environmental nutrient availability influences fungal sporulation and growth [241,242], the extremely dry external environment coupled with competition in infecting the host led to *B. bassiana* became dormant.
- ***Alternate infection route*** *B bassiana* possess enterotoxin (similar to *Cry*) producing coding regions in its genome creating a potential in the fungus to not only infect the host orally but also produce enterotoxins for killing [243]. Therefore, faster adaptation to *B. thuringiensis*, whose mechanism of infection is enterotoxin based, could have led to the hosts becoming resistant to environmental *B. bassiana*. This could have led to positive resistance of *T. castaneum* towards *B. bassiana*, leading to its extinction from the environment.

Contrary to the results reported here, Rafaluk et al. showed that under a strong selective force, *vis a vis* constant exposure to *T. castaneum* external immune defence, *B. bassiana* rapidly gains virulence and

resistance towards the beetle's defensive secretions [183]. However, metabolic activity patterns of *B. bassiana* isolates look promising. While coevolved *B. bassiana* did not show a significant change in activity compared to the non-evolved at higher quinone levels, one-side adapted *B. bassiana* were strikingly active, compared to both on-evolved and coevolved isolates. Here, my results are in similar to that reported in Rafaluk et al., [183]

The fact that none of the extracted *B. bassiana* or *B. thuringiensis* isolates could exhibit any general pattern of significance in terms of virulence towards either non-evolved, one-side adapted or coevolved beetles can be explained by in terms of ancestral population' diversity. Cro1 is a fairly new addition to the standard beetle laboratory conditions [160], and therefore possess enough genetic variation with regards to resistance to *B. bassiana*. Host genetic heterogeneity is an important criterion that affects parasite virulence [25,244–246]. Furthermore ample evidence point towards *B. bassiana*'s inability to efficiently kill beetles [247–249]. Together, they explain *T. castaneum*'s lack of susceptibility towards *B. bassiana*.

The two parasites used in the experiment belong to two different kingdoms of life and are generally known to employ different strategies for infecting the host. Their requirements not only differ in the mechanism of infection (toxins, enzymes etc. used to harm the host) [3,162,250,251] but also in the route of entry into the host body [129,252]. *B. thuringiensis* infects orally [160,252] and *B. bassiana* via breaching of the insect cuticle [167,253]. While *B. thuringiensis* produces enterotoxins (Cry most prominently), *B. bassiana* uses a barrage of enzymes (Chitinases most prominently [254]) to kill the host. Since both the parasites are natural antagonists of the beetle host and are present in the same environment (top soil), the parasites can potentially coevolve in nature, provided the presence of insect hosts and may cooperate or compete with each other for resources.

Adaptive strategies or outcomes are influenced by the way in which antagonistic species, like competing parasites, interact with each other. For instance, in the leaf-cutting ant *Acromyrmex echinatior*,

simultaneous infection with the obligatory parasitic *M. anisopliae* and opportunistically parasitic *Aspergillus flavus* resulted in the latter parasite outcompeting the former [255]. Bacteria and fungi are known to be competitors and when co-cultured, bacteria mostly outcompete fungi [256]. Competitive exclusion of a non-virulent parasite by a virulent one has been reported in theoretical studies of mixed infections [29,257]. Infection of *Daphnia magna* with different strains of *P. ramosa* resulted in the virulent strain outcompeting the non-virulent one, drastically decreasing the spore-producing ability of the non-virulent strain [30].

The two parasites used in my experiment vary in their virulence and their interactions with each other and with the host will result in different outcomes. Thomas et al. observed that when crickets were simultaneously infected with *Metarhizium anisopliae* and a non-virulent strain of *B. bassiana*, the non-virulent parasite majorly impacts host-parasite interactions [258]. A non-virulent parasite can just elicit an immune activation, which can persist in the population and be passed on across generations, and benefit the host in its evolutionary history if it ever encounters a parasite that has similarity in mechanism or route of infection with it [258]. Or parasites which are non-virulent can help in bringing about host death in the presence of another parasite. Broderick, Raffa and Handelsman showed that *B. thuringiensis* requires resident gut microbes (*Enterobacter sp.*) to kill the Gypsy moth [259]. Therefore, in spite of being eliminated in the host-parasite race, the effects that a non-virulent parasite has on the host population can persist and impact future host-parasite interactions.

An overall examination of all the outcomes of the experimental evolution points that development time drives evolutionary changes in different directions among different populations of host and parasites. The selective force imposed on the hosts to develop faster is a powerful driver of adaptations, majorly impacting changes in the host and in turn in the parasites. *B. thuringiensis* persistent in the environment of FAST hosts but neither increased in virulence (survival of ancestral host as a proxy) or transmission (reduction in growth rate across experimental time-scale). The parasite, however, started forming

biofilms, exhibited in all the isolates from the FAST regime. Indeed, the biofilm forming ability varied across different treatments with coevolved isolates showing a distinct increase across the experiment.

The time of transfer [260,261] and the way transfers [14] are performed have been shown to shape parasite traits. The NORMAL and FAST populations had different transfer time points which influenced adaptive changes in both the host and parasites, such as biofilm formation in *B. thuringiensis* isolates (from FAST regime) and decreasing susceptibility towards quinone in *B. bassiana* (from NORMAL regime). The lack of any clear reciprocal adaptations in my experiment can be explained by variable host development time and complex interactive environment involving three players. As such, any clear change would potentially require a longer evolutionary time-scale. Nonetheless, I do see changes in host immunity based on development time during evolution experiment. Also, both parasites show strong life-history changes in other as a response to selective pressures.

Staying strong together: bacterial resistance due to biofilms

In order to colonize new surfaces, bacteria produce biofilms, which are usually constituted of lipids, proteins, polysaccharides and extracellular DNA and such is the strength and integrity of the matrix that biofilm has been referred to as the 'most successful life form' [191]. Unfavorable environments are important triggers for the formation of biofilms. Under such conditions, bacteria signal each other to produce structural components required for biofilm formation [262,263]. Bacteria, mainly Gram-positive, form biofilms which are a protective extracellular matrix. For bacterial parasites, the host immune defence constitutes an unfavourable environment inducing biofilm formation. Biofilms are not only resistant to phagocytosis [264,265] but also possess reduced susceptibility to antibiotics [266,267]. Bacterial biofilms have been a major focus of study in microbiology and clinical research since they were first observed and reported [268].

DISCUSSION

B. thuringiensis strains have been reported to possess a biofilm forming ability although they are not strong biofilm formers [269,270]. To date, there has been no report of biofilm formation in *B. thuringiensis* bv *tenebrionis*. In my experiment, biofilm formation exhibits a dichotomous pattern based on the treatment, depending on the pace of development of the host. All isolates belonging to coevolution and one-sided parasite adaptation treatments from FAST development regime (from transfers 4, 6, 8 and 10) formed the typical wrinkled colony phenotype on LB agar plates [271]. However, only a couple of isolates from NORMAL regime exhibited any biofilm forming ability. In general, *T. castaneum* coevolving under FAST regime are more susceptible to environmental parasites (with over less quinone production), while *B. thuringiensis* from FAST regime gains more resistance to the beetle. The biofilm formation seen here could have also evolved as a mechanism against the harsh environmental conditions i.e. the dried state in which they had to persist. But this cannot explain the formation of biofilm in only FAST isolates and not the NORMAL isolates. In NORMAL beetles, devoid of any selection pressure to develop faster, resources were invested in developing resistance to *B. thuringiensis*. Indeed NORMAL beetles have higher levels of the external immune defence.

Biofilm formation is costly [191]. In the bacterial growth rate experiment, replication rate of *B. thuringiensis* appears to be under selection with growth rate slowing down during experimental coevolution. In accordance with a previous report on *B. thuringiensis* biofilms, the *B. thuringiensis* isolates tested for the purpose of this thesis, too, showed optimal biofilm formation post 96 hours of incubation [270]. In the experimental coevolution with *B. thuringiensis* and *C. elegans*, Masri et al. observed a correlation between biofilm formation and loss of pathogenicity [99]. Additionally, biofilm formation is traded-off against bacterial growth rate [272] and an increased growth rate (given in terms of doubling time) implies that the bacteria take longer time for transmission and also produce fewer spores in a given time period.

4.2 cross-resistance: a consequence of bipartite host-parasite coevolution

In nature, hosts live in complex communities interacting with multiple parasites [17,31]. Hence, close bipartite evolutionary interactions can impact on other ecological interactions within the community [18,25,41]. However, few experimental studies have reported the consequence of host-parasite evolutionary interaction, upon host exposure to other parasites [6,108,109,111,112]. To the best of my knowledge, the findings reported here are the first evidence for positive cross-resistance to a novel parasite, in an experimentally coevolved insect host. The increased survival of *Beauveria bassiana* coevolved *T. castaneum* upon *Bacillus thuringiensis* infection indicates positive cross-resistance towards this parasite. No change is observed between evolved and non-evolved populations of *T. castaneum* when infected with *Pseudomonas entomophila*. Cross-resistance is one of the broad-scale effects of close host-parasite interactions.

Here, I hypothesized that when a parasite infects a host with a similar 'route of infection' or 'mechanism of infection' to a previous parasite encountered by the host, positive cross-resistance occurs. Alternatively, physiological costs can be a consequence of mounting resistance to a parasite. When such a host is infected with the subsequent parasite, hypersensitivity and high mortality may occur, leading to negative cross-resistance [112]. A study on the experimental evolution of *D. melanogaster* as host to *B. bassiana* also reported that even after 15 host generations of selection regime, the evolved flies did not display higher fitness levels to ancestral *B. bassiana* as opposed to their control counterparts [273]. The coevolved beetles used in this study do not display increased survival upon infection with non-evolved *B. bassiana*, they exhibit increased phenoloxidase (PO) activity and a higher number of pupae during the course of coevolution (Rafaluk *et al.,* submitted). It has been reported that *T. castaneum* is particularly robust against *B. bassiana* infection [148,249] since in addition to possessing a highly melanized cuticle [198,274], the beetle also secretes defensive volatile compounds (methyl and ethyl-1,4-benzoquinones,

referred as quinones henceforth) as external immune defence into its immediate environment [89] which has been reported to be active against *B. bassiana* [128,172].

The *T. castaneum* larvae in all the experiments to assess cross-resistance were always exposed to *B. bassiana* spores via the environment which also serves as their food resource. As such, there is a high possibility of beetles ingesting spores and some of them germinating in the beetle gut and spreading infection in the host thereof. In the red imported ant, *Solenopsis invicta B. bassiana* was shown to successfully infect via oral ingestion of conidia [130]. Also in the *Aedes aegypti* gut germination and development of *B. bassiana* spores was observed [275] 12 hours post *B. thuringiensis* exposure, up-regulation of stress candidates Hsp90 (3 fold) and p450 (6.6 fold) was being prominent, in conjunction with studies that imply cross-talk between immune and stress response [132,139]. The high up-regulation of Attacin-2 upon *P. entomophila* exposure is in accordance with previous studies on *D. melanogaster* and *P. entomophila* [47,276].

It has been previously described that oral infection leads to the expression of several odorant-binding proteins [123,214] and therefore it is compelling to observe up-regulation of the candidate ObpC-12 upon *B. bassiana i*nfection since entomopathogenic fungi generally infect via cuticular breaching. Since Apo-III is known to upregulated upon oral intoxication with coleopteran specific *Cry*-III toxins [123,135], expression of this gene upon *B. bassiana* infection hints at the potential similarity in the infection dynamics between *B. thuringiensis* and *B. bassiana*. Additionally, Apo-III have been shown in *T. castaneum* to high anti-microbial activity against *B. bassiana* among other parasites [277].

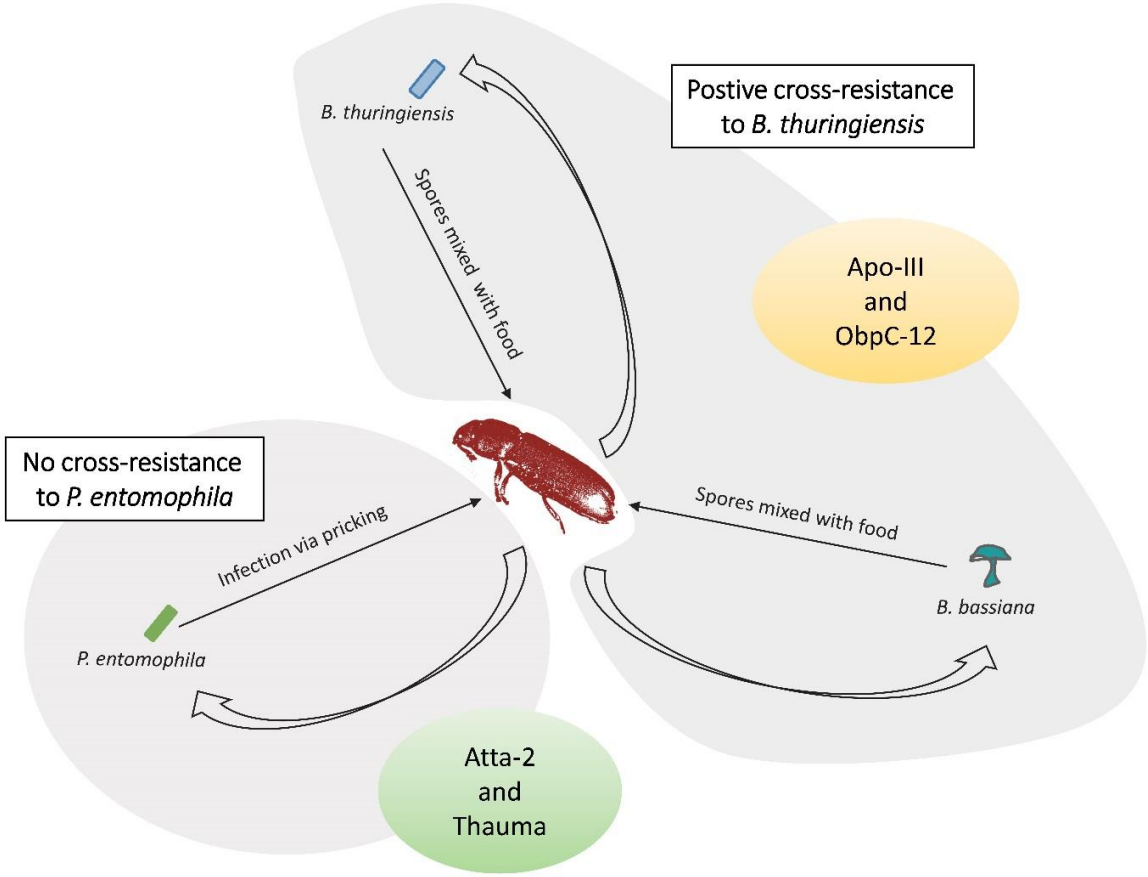


Figure 4.1. Summary of results after testing cross-resistance. Evidence for positive cross-resistance observed in *B. bassiana* coevolved beetles in survival assays with *B. thuringiensis* is supported in similar relative expression (up-regulation upon infection) of the two candidate genes previously reported to be upregulated up *B. thuringiensis* infection [123,135,214]. These are not only important candidates with respect to *B. thuringiensis* infection but also imply an oral infection route [123,214].

Studies have shown that adaptation to parasites in insect hosts can be route specific [6,110,123]. Experimental evolution of *D. melanogaster* to *P. entomophila* via oral and systemic routes revealed that adaptation to different routes was specific; flies adapted to one infection route were not resistant to *P. entomophila* infection via the route they had not evolved to [6]. Higher survival of *B. bassiana* coevolved beetles (compared to control beetles) upon oral infection with *B. thuringiensis* is compelling. *Bacillus thuringiensis* infects orally and kills the host via production of toxin proteins called *Cry* endotoxins [3,163]. *B. bassiana* is known to infect via cuticular breaching in the form of germination of spores and hyphal

growth thereby [278,279]. However, genomic investigation of *B. bassiana* revealed the presence of eight genes which are similar to *Cry* delta toxin genes of *B. thuringiensis* [243]. The potential for *B. bassiana* to express *Cry* toxins which show specificity and activity in insect gut need to be explored.

Infection routes matter in the host's response, even when it's the same parasite in question. For instance, *D. melanogaster* displays high levels of mortality when infected orally with the protozoan parasite *Crithidia fasciculata* [280]. However, when systemically infected with *C. fasciculate*, flies show no significant difference in mortality compared to uninfected individuals [280]. Similarly, the route of infection of *Serratia marcescens* also differentially affects mortality in *D. melanogaster* [281]. When *S. marcescens* infects *D. melanogaster* orally, the flies respond by local production of AMP in the gut to fight the bacteria. However, in systemic infections, *S. marcescens* kills the host at a faster pace, being resistant to AMPs [281]. The infection route can, therefore, generate different physiological responses in the host. This is nicely illustrated by Behrens and co-workers [123], using a transcriptomic approach, showing that the red flour beetle *T. castaneum* displays varying gene expression patterns upon oral vs. systemic infection with *B. thuringiensis.*

Expression of the candidate genes for oral infection (ObpC-12) and resistance to *B. thuringiensis* in *T. castaneum* (Apo-III) [123,135,214] upon *B. bassiana* infection coupled with the fact that genomic analyses of *B. bassiana* reveal the potential for oral toxicity [243], warranting further investigations. In the field of agricultural sciences, *B. bassiana* and *B. thuringiensis* are used as biological control agents, often in tandem [282–285]. However, several incidents of insects gaining resistance to them have been reported [286,287]. These observations can be explained by insects developing positive cross-resistance overtime and one way to combat this is to use these two control agents in rotation. Alternatively, the farmer can introduce a different biological control agent (to which the pest insect has been previously shown to negatively cross-resistant in such circumstances) in between rounds of *B. thuringiensis* and *B. bassiana*

usage, of course, both these solutions would require ample lab-based experimental proof prior to field application.

5

Outlook and limitations

Since host-parasite interactions in nature, more often than not, involve more than one host and one parasite, incorporating multiple hosts and parasites in studying the various aspects and basis of such evolutionary interactions are required. Experimental evolution has been employed in investigating host-parasite interactions, but these studies have so far focused on one host-one parasite systems in the case of multicellular hosts. Therefore, in this thesis, I have aimed to bridge this gap by investigating experimental host-parasite evolutionary interactions involving the red flour beetle, *Tribolium castaneum* and its natural parasites *Bacillus thuringiensis* bv. *tenebrionis, Beauveria bassiana* and *Pseudomonas entomophila*. Simultaneous and sequential exposures to different parasites were performed by conducting tripartite (one host-two parasite) experimental coevolution and cross-resistance experiment. An additional host life-history trait, i.e. host development time was incorporated in the tripartite experimental evolution which resulted in consequent responses in both host and parasites.

In the tripartite evolution experiment, development time emerges as a stronger selective force influencing external and internal immune expression, host resistance traits as well as changes in parasite life-history traits. After 10 generations, I found higher survival in the evolved beetles belonging to both FAST and NORMAL regimes when exposed to non-evolved *B. thuringiensis*. A fluctuating relation was observed between external and internal immunity in the NORMAL beetles, but this trend was not observed for FAST beetles. Perhaps the most striking outcome of the experimental coevolution was the formation of wrinkly

biofilm by *B. thuringiensis* isolates belonging to the FAST development regime. However, host local adaptation to parasites or vice versa was not observed at the end of the experiment. The primary outcomes and the potential future directions emerging out of the outcome tripartite evolution experiment are graphically depicted in Figure 5.1.

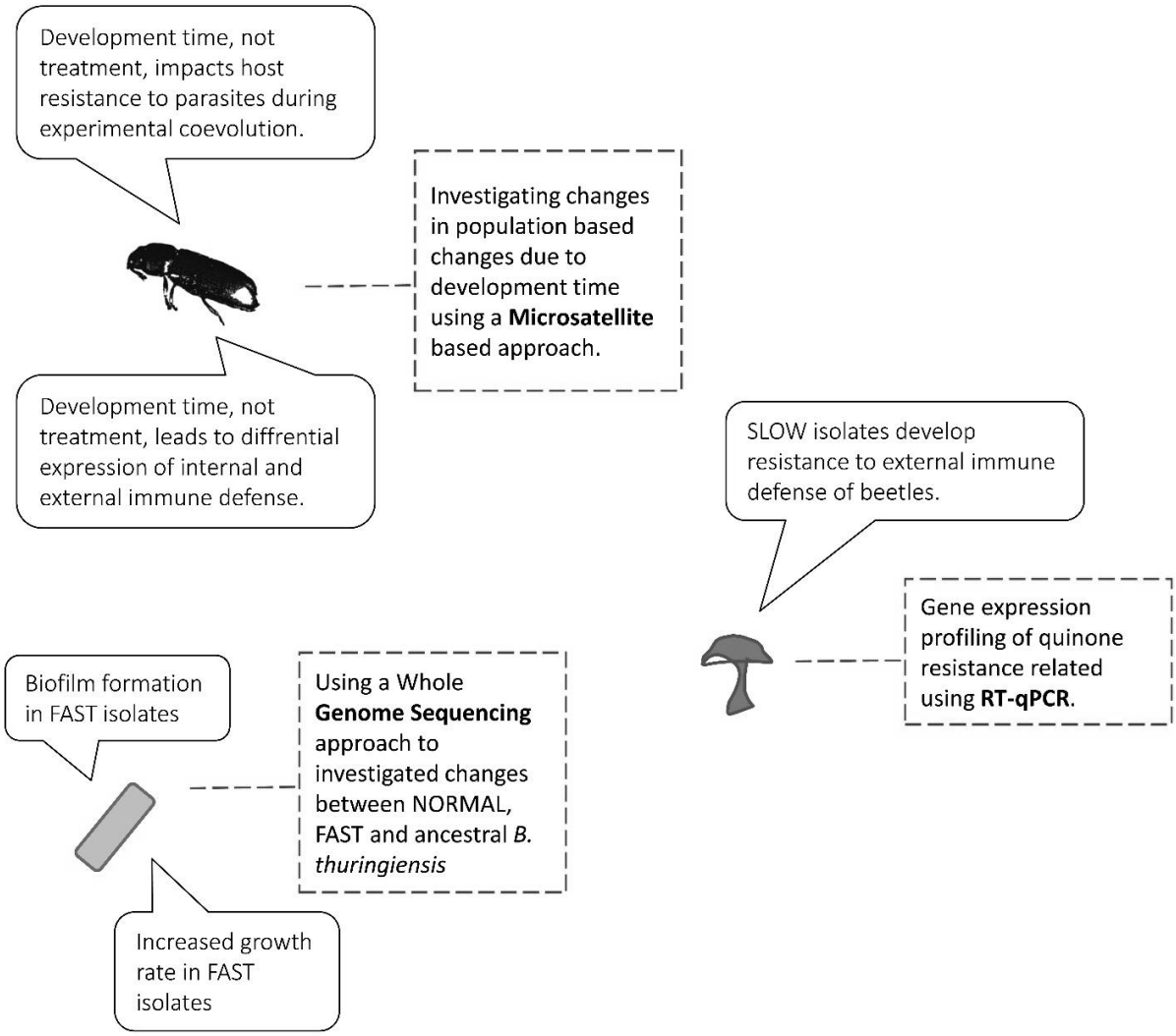


Figure 5.1. Outcome and future directions for the host and parasites arising out of evolution experiment. The boxes outlined in dashed lines propose potential future analysis.

OUTLOOK

Lande pointed out that while trying to adapt to very strong selection pressures, a population can undergo extinction, prior to adaptation and recovery [288]. Genetic diversity, therefore, allows for a parasite population to possess adaptive potential and successfully persist in an environment [289]. Not providing additional genetic material for *B. bassiana* during the experiment, turned out to be a limiting factor and the fungus was extinct/dormant from almost all treatments, unlike in Rafaluk *et al.*, [183]. The ones isolated, however, displayed resistance towards external immune secretion of the beetles, similar to the observations made in Rafaluk et al. [183]. If the dormant isolates can be extracted from the environment using strong chemical and mechanical methods [241] and would be pertinent to investigate the resistance of these isolates to quinones. In the case of lower resistance compared to ancestral *B. bassiana*, the dormancy can be partially explained. For spores which can stay dormant for long, investments in virulence increase are not necessary at a time when environmental conditions are unfavourable [290].

Limitations of the tripartite coevolution can be overcome by considering and controlling for more parameters. For instance, knowing the parasite spore load in the environment during the experiment can give a direct measure of which parasite dominates the host environment, under which treatment. Of course, the obvious conjecture, based on the mere number of parasite isolates per parasite type, is that *B. thuringiensis* was the dominant player among parasites. Whether the bacterium is able to form biofilm in the host gut, allowing persistence in a long-lived host which it can kill when the conditions turn unfavourable for the host, should be investigated. Another factor confounding factor is the time frame of the experiment. Experimental evolution must be conducted for a longer number of host generations in order to stabilize initial random fluctuations that might arise simply due to the novel environment.

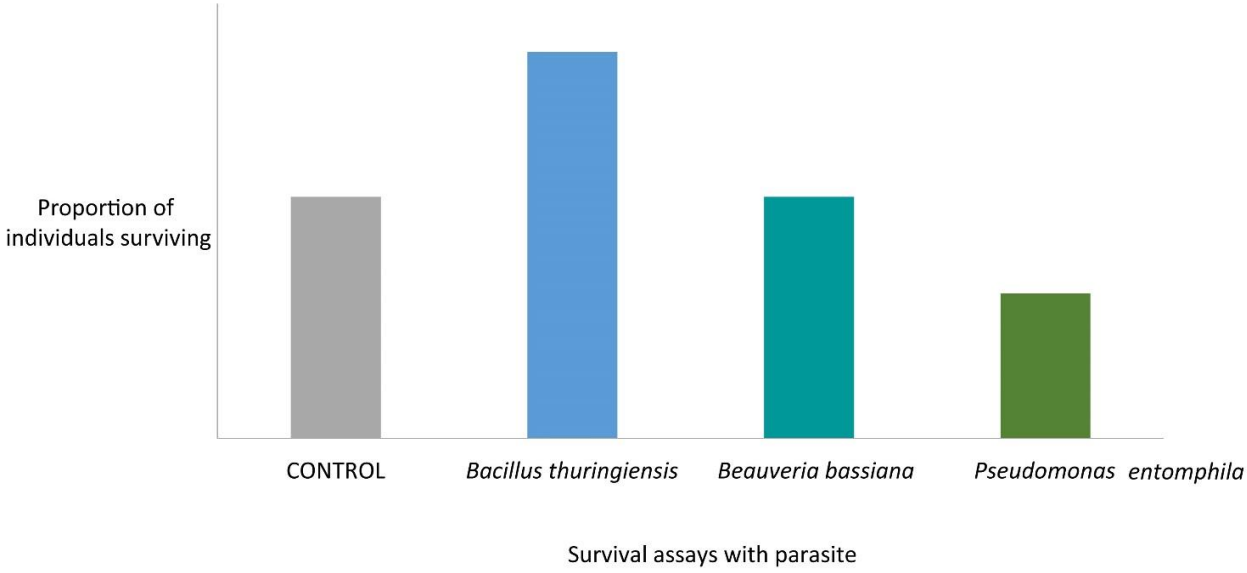


Figure 5.2 observed outcome of cross-resistance experiment. Enhanced resistance towards *B. bassiana* was not observed in *B. bassiana* coevolved beetles, similar to that observed in *Drosophila melanogaster* where experimental evolution to *B. bassiana* did not result in enhanced resistance.

Note: all bars here signify response with respect to control populations

In the case of sequential parasite exposure, *T. castaneum* was positively cross-resistant to *B. thuringiensis* but not to *P. entomophila,* as a consequence of coevolution with *B. Bassiana*. The route of infection has been shown to drive different adaptations in the same insect when infected with the same parasite [6,110,123], with specific genes being expressed based on the route of infection [123]. As *B. bassiana* has the potential for oral infection [130,243,278] and *B. thuringiensis* only infects orally, resistance to *B. thuringiensis* developed in the beetle as a consequence of coevolution, due to the similarity in the route of infection. I could also show similarity in the expression of the genes ObpC-12 and Apo-III upon *B. bassiana* and *B. thuringiensis* infections in *T. castaneum*, two candidate genes which are known to influence resistance to *B. thuringiensis* and *B. bassiana* [123,214,291]. Whether *B. bassiana* can indeed produce *Cry* like toxins and how similar is their chemistry and mode of action compared to the prototypical *Cry* toxins of *B. thuringiensis* needs to be studied and holds ample potential for field-based applications. For this, we need to employ experimental as well as an extensive -omics-based approach. In future studies aiming to

investigate the genetic basis of evolutionary cross-resistance, it will be pertinent to use the same parasite in different routes of infection for the second exposure.

In conclusion, experiments performed to address multiple parasite exposures in an experimental evolution setup, provide open questions with both ecological and commercial implications. The results of the experimental evolution are a snapshot of evolutionary interaction between *T. castaneum*, *B. bassiana* and *B. thuringiensis*. Although the complexity of the system did not produce a clear pattern in classic reciprocal traits namely resistance and virulence, phenotypic changes were seen in all the players. A longer evolutionary time-scale will help in streamlining the interactions and further studies are warranted in this direction. In an agricultural context, constant exposure to *B. thuringiensis* and *B. bassiana* is akin to an experimental evolution, albeit a less controlled one, occurring in the field. As such it is expected that insects gain resistance which beats the whole purpose of such a strategy. Therefore, judicious application of these two biological control agents is necessary and different strategies have been proposed to gain the maximum benefit of their application [282–285,292]. It is very important to keep in mind that positive cross-resistance can occur in the field, hampering attempts at optimizing pest management. Therefore, prior knowledge of the potential for positive cross-resistance is important to use a good strategy.

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Research aims

To study causes and effects of evolutionary processes leading to adaptations in insects resulting in phenotypes and in turn genotypes using a combination of molecular biology and –omics-based approach, and thereby better understand evolutionary trajectories of the most successful animal taxa.

Current Research

PhD student since **May 2013** at the Institute of Insect Biotechnology, Group of Applied Entomology, JLU Giessen, GERMANY

Principal investigator: Dr. Gerrit Joop and Prof. Dr. Andreas Vilcinskas

Cross-resistance to natural parasites: Using *Tribolium castaneum* (coevolved with *Beauveria bassiana*) as the host and two natural parasites, *Bacillus thuringiensis*, *B. bassiana* and *Pseudomonas entomophila,* I investigated phenotypic cross-resistance and its genetic basis in the host

One host-two parasite experimental coevolution: Experimental host-parasite coevolution using *T. castaneum* as host and *B. bassiana* & *B. thuringiensis* as parasites and investigating changes in host and parasites thereof.

Past Research Experience

- *Project student* (**August 2012 to February 2013**), Ecology and Evolution group at National Centre for Biological Sciences, Bangalore INDIA.

Title: To develop *T. castaneum* as a model system to measure adaptation in natural populations and its correlation with major life history related traits.

Principal investigator: Dr. Deepa Agashe

Responsibilities: building and maintaining beetle stocks and beetle lab organization, setting up resource choice and fitness assays across various resource types.

- *Practice School II Project/ Masters project* (**January to June 2012**) Ecology and Evolution group, National Centre for Biological Sciences, Bangalore INDIA.

Title: A Mycorrhizae and Endophytic fungal profile of some of the native wild grass species

Principal investigator: Dr. Mahesh Sankaran

Educational qualifications

MSc. (hons) Biological Sciences from Birla Institute of Technology and Science, Pilani INDIA **August 2008-June 2012** (Cumulative GPA: 7.18/10.00 and Core GPA: 8.90/10.00)

Skills

- RT-qPCR, molecular cloning, general microbiology, PCR and gel electrophoresis, survival & immune assays, light microscopy, bacterial biofilm assay and insect handling
- General statistics, R programming language, JMP software
- Training and supervising Bachelor and Master students

Recognition

- Recipient of Response Exchange Grant in collaboration with Prof. Dr. Klaus Fischer (**3rd-17th October 2016**)
- Won the *Best Oral Presentation* at the 20th Graduate meeting for evolutionary biology German Zoological Society (DZG**, 8th to 12th September, 2015**), Graz AUSTRIA.

Conferences and workshops:

- Oral Presentations
  - DZG meeting, **8th to 12th September, 2015**, Graz AUSTRIA.
  - German entomological society meeting, **2nd to 5th March 2015**, Frankfurt GERMANY
  - Spp 1399 DFG meeting, **29th August to 2nd September 2013**, Blossin GERMANY
- Poster presentations
  - Spp 1399 DFG meeting, Final Symposium **17th to 20th May, 2016,** Muenster GERMANY
  - DZG meeting, **8th to 12th September, 2015**, Graz AUSTRIA.

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"When you decide not be afraid, you can find friends in super unexpected places." – *Miss Marvel (Marvel comics)*

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Appendix 1. Supplementary result tables

Table S1. Results of pairwise comparison of Kaplan-Meier curves post-exposure of F2 individuals from generation ten beetles from the coevolution experiment to *ancestral B. bassiana* (p-value correction using Holm method)

	CoEvo-FAST	CoEvo-NORMAL	Control-FAST	Control-NORMAL	HostAdapt-FAST	HostAdapt-NORMAL	CoEvo-FAST	CoEvo-NORMAL	Control-FAST	Control-NORMAL	HostAdapt-FAST
CoEvo-NORMAL	1.0000										
Control-FAST	0.08967	0.68338									
Control-NORMAL	1.0000	1.0000	1.0000								
HostAdapt-FAST	0.31986	1.0000	1.0000	1.00000							
HostAdapt-NORMAL	0.00035	0.00537	1.0000	0.04173	1.0000						
CoEvo-FAST	0.92348	0.13457	1.5e-05	0.01681	1.0e-04	1.1e-08					
CoEvo-NORMAL	1.00000	1.00000	0.01571	1.00000	0.06291	3.6e-05	1.00000				
Control-FAST	1.00000	1.00000	1.00000	1.00000	1.00000	0.00936	0.06947	1.00000			
Control-NORMAL	1.00000	1.00000	1.00000	1.00000	1.00000	0.01571	0.04180	1.00000	1.00000		
HostAdapt-FAST	1.00000	1.00000	0.03741	1.00000	0.13457	0.00011	1.00000	1.00000	1.00000	1.00000	
HostAdapt-NORMAL	1.00000	1.00000	1.00000	1.00000	1.00000	0.06947	0.00768	1.00000	1.00000	1.00000	1.00000

Light grey fields = CONTROL treatment; Dark grey fields = INFECTION treatment

Table S2. Results of pairwise comparison of Kaplan-Meier curves post-exposure of F2 individuals from generation Ten beetles from the coevolution experiment to *ancestral B. thuringiensis* (p-value correction using Holm method)

	CoEvo-FAST	CoEvo-NORMAL	Control-FAST	Control-NORMAL	HostAdapt-FAST	HostAdapt-NORMAL	CoEvo-FAST	CoEvo-NORMAL	Control-FAST	Control-NORMAL	HostAdapt-FAST
CoEvo-NORMAL	1.0000										
Control-FAST	1.0000	1.0000									
Control-NORMAL	1.0000	1.0000	1.0000								
HostAdapt-FAST	1.0000	1.0000	1.0000	1.00000							
HostAdapt-NORMAL	0.18514	1.0000	1.0000	1.00000	0.53620						
CoEvo-FAST	0.00023	7.0e-08	3.1e-06	1.7e-06	3.5e-05	6.2e-11					
CoEvo-NORMAL	0.00831	1.1e-05	0.00028	0.00014	0.00181	2.7e-08	1.00000				
Control-FAST	1.7e-14	< 2e-16	< 2e-16	< 2e-16	< 2e-16	< 2e-16	0.00221	3.6e-05			
Control-NORMAL	1.6e-13	< 2e-16	< 2e-16	< 2e-16	5.7e-15	< 2e-16	0.00541	0.00012	1.00000		
HostAdapt-FAST	< 2e-16	< 2e-16	< 2e-16	< 2e-16	< 2e-16	< 2e-16	0.00101	1.2e-05	1.00000	1.00000	
HostAdapt-NORMAL	0.00043	1.6e-07	6.4e-06s	3.6e-06	7.0e-05	1.6e-10	1.00000	1.00000	0.00088	0.00181	0.00032

Light grey fields = CONTROL treatment; Dark grey fields = INFECTION treatment

Table S3. Results of pairwise comparison of Kaplan-Meier curves post-exposure of F2 individuals from generation 10 beetles from the coevolution experiment to *ancestral P. entomophila*(p-value correction using Holm method)

	CoEvo-FAST	CoEvo-NORMAL	Control-FAST	Control-NORMAL	HostAdapt-FAST	HostAdapt-NORMAL	CoEvo-FAST	CoEvo-NORMAL	Control-FAST	Control-NORMAL	HostAdapt-FAST
CoEvo-NORMAL	1.0000										
Control-FAST	1.0000	1.0000									
Control-NORMAL	1.0000	1.0000	1.0000								
HostAdapt-FAST	1.0000	1.0000	1.0000	1.0000							
HostAdapt-NORMAL	1.0000	1.0000	1.0000	1.0000	1.0000	-					
CoEvo-FAST	1.6e-08	2.4e-13	2.9e-11	1.2e-08	1.6e-09	2.2e-11					
CoEvo-NORMAL	2.7e-05	1.9e-09	1.2e-07	2.0e-05	4.0e-06	9.2e-08	1.0000				
Control-FAST	4.0e-14	< 2e-16	< 2e-16	1.9e-14	< 2e-16	< 2e-16	1.0000	0.0119			
Control-NORMAL	1.9e-14	< 2e-16	< 2e-16	1.3e-14	< 2e-16	< 2e-16	1.0000	0.0099	1.0000		
HostAdapt-FAST	3.4e-11	< 2e-16	2.9e-14	2.2e-11	2.2e-12	1.9e-14	1.0000	0.2651	1.0000	1.0000	
HostAdapt-NORMAL	4.8e-05	4.3e-09	2.3e-07	3.8e-05	8.1e-06	1.9e-07	1.0000	1.0000	0.0049	0.0039	0.1434

Light grey fields = CONTROL treatment; Dark grey fields = INFECTION treatment

Table S4. Tukey Contrasts of the output of generalized linear mixed model analysis (Multiple Comparisons of Means) of PO and Quinone secretion by FAST beetles, overall during experimental coevolution (p-values corrected using Holm method)

	Control		Host-adaptation	
	PO	quinone	PO	quinone
Host adaption	0.500	0.177		
Coevolution	0.289	<0.001	0.923	0.139

Table S5. Tukey Contrasts of the output of generalized linear mixed model analysis (Multiple Comparisons of Means) of PO and quinone secretion by FAST beetles with respect to generation (in transfer numbers) (p-values corrected using Holm method)

(Post-hoc test on linear mixed model with evolutionary background and generation as fixed factors and replicate beetle populations within different backgrounds as random factors)

	Eight		Six		Four		Two	
	PO	quinone	PO	quinone	PO	quinone	PO	quinone
Six	< 0.001	0.98306						
Four	< 0.001	0.00754	< 0.001	0.04137				
Two	< 0.001	0.99994	< 0.001	0.96302	0.954	0.00489		
Ten	< 0.001	< 0.001	< 0.001	< 0.001	0.901	< 0.001	1.000	< 0.001

Table S6. Tukey Contrasts of the output of generalized linear mixed model analysis (Multiple Comparisons of Means) of PO and quinone secretion by NORMAL beetles, overall during experimental coevolution (p-values corrected using Holm method)

	Control		Host-adaptation	
	PO	quinone	PO	quinone
Host adaption	< 0.001	0.393		
Coevolution	< 0.001	0.875	0.748	0.696

Table S7. Tukey Contrasts of the output of generalized linear mixed model analysis (Multiple Comparisons of Means) of PO and quinone secretion by NORMAL beetles with respect to generation (in transfer numbers) (p-values corrected using Holm method)

(Post-hoc test on generalized linear mixed model with evolutionary background and generation as fixed factors and replicate beetle populations within different backgrounds as random factors)

	Eight		Six		Four		Two	
	PO	quinone	PO	quinone	PO	quinone	PO	quinone
Six	<0.001	<0.001						
Four	<0.001	<0.001	0.792	<0.001				
Two	<0.001	<0.001	<0.001	0.953	<0.001	<0.001		
Ten	<0.001	0.930	0.370	<0.001	0.961	<0.001	<0.001	<0.001

Table S.8 Pairwise log-rank test on Kaplan-Meier survival curves upon infection with PA3 NORMAL generation 10 (p-values corrected using Holm method)

	CRO1	CoEvo10	CoEvo5	Control10	Control5	HA10	HA5	CRO1	CoEvo10	CoEvo5	Control10	Control5	HA10
CoEvo10	0.317												
CoEvo5	0.020	0.089											
Control10	0.317	0.99	0.086										
Control5	0.317	1.000	0.089	0.99									
HA10	0.317	1.000	0.089	0.99	1.000								
HA5	0.317	0.990	0.085	0.99	0.990	0.990							
CRO1	0.078	0.317	0.411	0.317	0.317	0.317	0.311						
CoEvo10	0.078	0.303	0.472	0.301	0.303	0.303	0.295	0.954					
CoEvo5	0.040	0.170	0.704	0.170	0.170	0.170	0.166	0.665	0.719				
Control10	1.000	0.317	0.020	0.317	0.317	0.317	0.317	0.078	0.078	0.040			
Control5	0.078	0.303	0.467	0.301	0.303	0.303	0.303	0.960	0.994	0.705	0.078		
HA10	0.154	0.557	0.241	0.557	0.557	0.557	0.557	0.670	0.641	0.407	0.154	0.648	
HA5	0.317	0.990	0.086	1.000	0.990	0.990	0.990	0.317	0.301	0.170	0.317	0.301	0.557

Light grey fields = CONTROL treatment; Dark grey fields = INFECTION treatment

Table S.9 Pairwise log-rank test on Kaplan-Meier survival curves upon infection with PA6 NORMAL generation 10 (p-values corrected using Holm method)

	CRO1	CoEvo10	CoEvo5	Control10	Control5	HA10	HA5	CRO1	CoEvo10	CoEvo5	Control10	Control5	HA10
CoEvo10	0.317												
CoEvo5	0.020	0.089											
Control10	0.317	0.990	0.086										
Control5	0.317	1.000	0.089	0.990									
HA10	0.317	1.000	0.089	0.990	1.000								
HA5	0.317	0.990	0.085	0.990	0.990	0.990							
CRO1	0.154	0.575	0.217	0.575	0.575	0.575	0.557						
CoEvo10	1.000	0.317	0.020	0.317	0.317	0.317	0.317	0.154					
CoEvo5	0.154	0.561	0.230	0.557	0.561	0.561	0.548	0.979	0.154				
Control10	1.000	0.317	0.020	0.317	0.317	0.317	0.317	0.154	1.000	0.154			
Control5	0.317	0.990	0.096	0.990	0.990	0.990	0.990	0.575	0.317	0.570	0.317		
HA10	0.020	0.088	0.979	0.088	0.88	0.88	0.088	0.227	0.020	0.231	0.020	0.089	
HA5	0.154	0.570	0.222	0.561	0.570	0.570	0.548	0.979	0.154	0.993	0.154	0.570	0.231

Light grey fields = CONTROL treatment; Dark grey fields = INFECTION treatment

Table S.10 Pairwise log-rank test on Kaplan-Meier survival curves upon infection with TWC2 NORMAL generation 6 (p-values corrected using Holm method)

	CRO1	CoEvo10	CoEvo5	Control10	Control5	HA10	HA5	CRO1	CoEvo10	CoEvo5	Control10	Control5	HA10
CoEvo10	0.317												
CoEvo5	1.000	0.317											
Control10	0.317	0.990	0.317										
Control5	0.317	0.990	0.317	0.990									
HA10	0.317	0.990	0.317	0.990	1.000								
HA5	0.317	1.000	0.317	0.990	0.990	0.990							
CRO1	1.000	0.310	1.000	0.317	0.317	0.317	0.317						
CoEvo10	1.000	0.317	1.000	0.317	0.317	0.317	0.317	1.000					
CoEvo5	0.317	0.990	0.317	1.000	0.990	0.990	0.990	0.317	0.317				
Control10	0.078	0.309	0.078	0.309	0.309	0.309	0.309	0.078	0.078	0.309			
Control5	0.078	0.297	0.078	0.303	0.307	0.307	0.297	0.078	0.078	0.303	0.988		
HA10	0.078	0.301	0.078	0.303	0.309	0.309	0.301	0.078	0.078	0.303	0.988	0.999	
HA5	0.317	0.990	0.317	0.990	0.990	0.990	0.990	0.317	0.317	0.990	0.988	0.319	0.311

Light grey fields = CONTROL treatment; Dark grey fields = INFECTION treatment

Table S11 Pairwise log-rank test on Kaplan-Meier survival curves upon infection with TWC3 NORMAL generation 6 (p-values corrected using Holm method)

	CRO1	CoEvo10	CoEvo5	Control10	Control5	HA10	HA5	CRO1	CoEvo10	CoEvo5	Control10	Control5	HA10
CoEvo10	0.317												
CoEvo5	0.154	0.557											
Control10	0.317	0.990	0.548										
Control5	0.317	1.000	0.557	0.990									
HA10	0.317	1.000	0.557	0.990	1.000								
HA5	0.317	0.990	0.548	0.990	0.990	0.990							
CRO1	1.000	0.317	0.154	0.317	0.317	0.317	0.317						
CoEvo10	1.000	0.317	0.154	0.317	0.317	0.317	0.317	1.000					
CoEvo5	0.078	0.299	0.626	0.295	0.299	0.299	0.295	0.078	0.078				
Control10	0.317	0.990	0.575	0.990	0.990	0.990	0.990	0.317	0.317	0.313			
Control5	1.000	0.317	0.154	0.317	0.317	0.317	0.317	1.000	1.000	0.078	0.317		
HA10	1.000	0.317	0.154	0.317	0.317	0.317	0.317	1.000	1.000	0.078	0.317	1.000	
HA5	0.040	0.163	0.386	0.317	0.163	0.163	0.156	0.040	0.040	0.700	0.172	0.040	0.040

Light grey fields = CONTROL treatment; Dark grey fields = INFECTION treatment

Table S12 Pairwise log-rank test on Kaplan-Meier survival curves upon infection with TWC7 NORMAL generation 6 (p-values corrected using Holm method)

	CRO1	CoEvo10	CoEvo5	Control10	Control5	HA10	HA5	CRO1	CoEvo10	CoEvo5	Control10	Control5	HA10
CoEvo10	0.15												
CoEvo5	1.00	0.15											
Control10	0.32	0.56	0.32										
Control5	0.32	0.57	0.32	0.99									
HA10	0.32	0.57	0.32	0.99	1.00								
HA5	0.32	0.55	0.32	0.99	0.99	0.99							
CRO1	1.00	0.15	1.00	0.32	0.32	0.32	0.32						
CoEvo10	1.00	0.15	1.00	0.32	0.32	0.32	0.32	1.00					
CoEvo5	1.00	0.15	1.00	0.32	0.32	0.32	0.32	1.00	1.00				
Control10	0.32	0.57	0.32	0.99	0.99	0.99	0.99	0.32	0.32	0.32			
Control5	1.00	0.15	1.00	0.32	0.32	0.32	0.32	1.00	1.00	1.00	0.32		
HA10	1.00	0.15	1.00	0.32	0.32	0.32	0.32	1.00	1.00	1.00	0.32	1.00	
HA5	0.32	0.57	0.32	0.99	0.99	0.99	0.99	0.32	0.32	0.32	1.00	0.32	0.32

Light grey fields = CONTROL treatment; Dark grey fields = INFECTION treatment

Table S13 **Virulence of *B. thuringiensis* isolates from FAST development regime.** Results of pairwise log-rank tests of Kaplan-Meier survival curves of PA7 (generations 4, 6, 8 & 10) and TWC7 (generations 4, 6, 8 & 10) (p-values corrected using Holm method)

	CONTROL	PA7-10	PA7-4	PA7-6
PA1-10	0.001			
PA1-4	1.000	0.003		
PA1-6	1.000	0.0129	1.000	
PA1-8	1.000	0.0092	1.000	1.000

	CONTROL	TWC7-10	TWC7-4	TWC7-6
TWC7-10	1.000			
TWC7-4	0.022	0.363		
TWC7-6	0.163	1.000	1.000	
TWC7-8	0.266	1.000	1.000	1.000

Light grey fields = CONTROL treatment; Dark grey fields = INFECTION treatment

Table S14 Virulence **of *B. thuringiensis* isolates from NORMAL development regime.** Results of pairwise log-rank tests of Kaplan-Meier survival curves of *B. thuringiensis* isolates (NORMAL)

	CONTROL	PA1-6	PA-8	TWC2-4	TWC3-4	TWC4-8	TWC6-4	TWC6-6
PA1-6	1.000							
PA1-8	1.000	1.000						
TWC2-4	0.011	0.012	0.072					
TWC3-4	0.115	0.123	0.523	1.000				
TWC4-8	0.823	0.778	1.000	1.000	1.000			
TWC6-4	0.132	0.137	0.571	1.000	1.000	1.000		
TWC6-6	0.123	0.123	0.532	1.000	1.000	1.000	1.000	
TWC7-4	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Light grey fields = CONTROL treatment; Dark grey fields = INFECTION treatment

Table S15 Metabolic activity test for coevolved *B. bassiana* isolates: Two-way ANOVA comparing area under the curve of fluorescence values over 140 hours of ancestral vs. coevolved *B. bassiana* isolates (TWC2 and TWC3, NORMAL generation 10)exposed to various concentrations of MBQ (p-values corrected using Holm method)

Effect	DF	Sum of squares	Mean sum of squares	F-value	P-value
Concentration	4	1.092e+12	2.184e+11	37.521	<0.0001
Isolate	2	1.132e+12	2.831e+11	91.255	<0.0001
Concentration x Isolate	8	3.474e+11	5.790e+10	9.749	<0.0001
Residuals	31	1.602e+11	5.340e+09		

Table S16. Metabolic activity test for one-side adapted. *B. bassiana* isolates: Two-way ANOVA comparing area under the curve of fluorescence values over 140 hours of ancestral vs. one-side adapted *B. bassiana* isolates (PA3 and PA6, NORMAL generation 10) exposed to various concentrations of MBQ (p-values corrected using Holm method)

Effect	DF	Sum of squares	Mean sum of squares	F-value	P-value
Concentration	4	1.067e+14	2.667e+13	24.923	<0.0001
Isolate	2	1.132e+12	1.216e+14	113.646	<0.0001
Concentration x Isolate	8	8.553e+13	1.069e+13	9.992	<0.0001
Residuals	27	2.889e+13	5.340e+09		

Table S17. The growth rate of representative *B. thuringiensis* isolates from the FAST regime. Tukey multiple comparisons of Two-way ANOVA to assess differences in s from one-side adapted and coevolved treatments and the ancestral strain Growth test of B. thuringiensis (p-values corrected using Holm method)

Vs. Ancestral *B. thuringiensis*	PA1	PA7	TWC4	TWC7
4	<0.001	<0.001	<0.001	<0.001
6	<0.001	<0.001	<0.001	<0.001
8	1	<0.001	<0.001	<0.001
10	<0.001	<0.001	<0.001	<0.001

Table S18. Tukey multiple comparisons of One-way ANOVA of *B. thuringiensis* isolates from NORMAL regime compared to the ancestral *B. thuringiensis.* (P-values corrected using Holm method)

Vs. Ancestral *B. thuringiensis*	P-value
TWC2 transfer-4	0.25
TWC2 transfer-6	<0.001
PA1 transfer-6	<0.001
PA1 transfer-8	<0.001
PA6 transfer-4	<0.001
PA6 transfer-10	<0.001

Appendix 2. List of consumables

Table A.1 list of disposables

Disposables	Supplier
Beetle rearing jars	Bardenhewer, Kiel
8-lid strips	Greiner-Bio
15ml tubes	Greiner-Bio
2ml tubes	Greiner-Bio
50ml tubes	Greiner-Bio
6-well plates	Greiner-Bio
96-well plates	Greiner-Bio
1 µl end to end capillaries	Eydam
Cotton wool fillers	Eydam
96-well qPCR plates	Applied Biosystems
96 well flat bottom plates	Greiner-Bio
Glass vials	Eydam
Petri dishes	Greiner-Bio
Needles	Bioform.de
Conical bottom PCR plates	Greiner-Bio
96-well Quartz plate	Greiner-Bio
Pasteur pipettes	Sarstedt

Table A.2 list of chemicals

Chemicals	Supplier
Flour, Alnatura type 405	DM-drogerie markt, Germany
Agar-Agar Kobe 1	Carl Roth
BisTris	Sigma-Aldrich
Chloramphenicol	Carl Roth
Amphotericin B	Sigma-Aldrich
Brewer's Yeast	Leiber, Germany
Tween 80	Carl Roth
Crystal violet	Carl Roth
L-DOPA	Sigma-Aldrich
MBQ	Sigma-Aldrich
Iron(II) Sulfate Heptahydrate	Carl Roth
Magnesium Sulfate Heptahydrate	Carl Roth
Glycerol	Carl Roth
Potassium Chloride	Carl Roth
Sodium Chloride	Carl Roth
Disodium Hydrogen Phosphate	Carl Roth
Dipotassium Phosphate	Carl Roth
Calcium Di-chloride	Carl Roth
Fluorescein diacetate	Sigma-Aldrich
TRIzol® reagent	Sigma-Aldrich

Table A.3 list of molecular biology kits used

Kits	Supplier
Direct-zol™ RNA MiniPrep	Zymo reaserach
SYBR® Green PCR Master Mix	ThermoFisher scientific
First Strand cDNA Synthesis Kit	ThermoFisher scientific

Table A.4.1 Coevolution experiments with *Tribolium castaneum* as the host against results from tripartite host-parasite coevolution

	Coevolving parasite	**Host adaptations**	**Parasite adaptations**	**References**	**Observed adaptations in the one host-two parasite coevolution from this thesis**	
***Tribolium castaneum* as host**	*Paranosema whitei*	Increased resistance towards non-evolved *P. whitei*	Decreased virulence towards non-evolved *T. castaneum*	[101]	**FAST**	**NORMAL**
		Effects of drift are countered by parasite-induced maintenance of genetic diversity		[293]	• Coevolved hosts showed the highest mortality upon exposure to non-evolved *B. bassiana* • Coevolved hosts are more resistant to *B. thuringiensis* • No resistance towards unrelated *Pseudomonas entomophila*. • No evidence for a trade-off between PO and quinone production during evolution.	• No evolution of resistance towards *B. bassiana* in any of the host background. • Variation in resistance to *B. thuringiensis* based on host evolutionary background. • No resistance towards unrelated *Pseudomonas entomophila*. • Evidence for trade-off between PO and quinone production
		Time-shift infections showed decreased mortality towards parasites from recent times than distant times		[12]		
		Lack of local adaptation towards parasite	Lack of local adaptation towards host	[217]		
		Red Queen dynamics leading to increased recombination frequency in coevolved hosts		[150]		
		Rapid decrease in host survival during the experiment	Rapid increase in parasite virulence	[102]		

Table A.4.2 coevolution experiments with *B. thuringiensis* as the parasite against results from tripartite host-parasite coevolution

	Coevolving host	Host adaptations	Parasite adaptations	References	Observed adaptations in the one host-two parasite coevolution from this thesis	
Bacillus thuringiensis as parasite	*Caenorhabditis elegans*	• Higher resistance to the non-evolved parasite than control hosts. • Lower fitness than control hosts. • More change in allele frequency of coevolved hosts compared to control, during coevolution.	• Higher virulence compared to control parasite. • Lower fitness control parasites. • More prevalence of parasite toxin gene in coevolved compared to control parasites, during coevolution.	[11]	FAST	NORMAL
		Some coevolved host populations display local adaptation to parasite.	Higher host mortality and reduced reproduction of hosts indicates parasite local adaptation in some populations.	[63]	• No evolution of virulence • Increased growth rate during the experiment. • Increased biofilm forming ability. • Increasing resistance to MBQ	• No evolution of virulence • Increased growth rate during the experiment. • No biofilm formation
		Coevolved hosts ingested less food containing pathogenic *B. thuringiensis* than non-pathogenic *B. thuringiensis*		[294]		
		Overall reduced male fitness and resistance in coevolved populations		[295]		
		• Evolution of increased host resistance to coevolving *B. thuringiensis.*	• Increased killing ability and major genomic changes observed. • Increased biofilm forming ability and loss of pathogenicity	[99]		

Table A.4.3 Coevolution with *B. bassiana* as the parasite against results from tripartite host-parasite coevolution

	Coevolving host	**Host adaptations**	**Parasite adaptations**	**References**	**Observed adaptations in the one host-two parasite coevolution from this thesis**	
***Beauveria bassiana* as parasite**	*Tribolium castaneum*	• Differential regulation of PO activity based on the route of infection of the parasite (Rafaluk *et al.,* submitted).	• Evolution of resistance towards beetle's quinones • Increased metabolic activity in the presence of quinones • Transcriptomic changes in coevolved parasites related to quinone resistance	[183]	**FAST**	**NORMAL**
					NO isolates	• No evolution of virulence towards host from any evolutionary background. • High metabolic activity of *B. bassiana* isolates in the presence of quinone.

Table A.5 Infection experiments with insect hosts and their natural parasites that investigate cross-resistance

Insect species	Cross-resistance to parasite							Reference
	Prior exposure to	Route of prior exposure	Cross-resistant to	Route of cross-infection	Assay for testing cross-resistance	Type of cross-resistance	Costs/Proposed mechanism	
Anopheles gambiae 	Exposure to microsporidia *Vavraia culicis*	Oral	*Plasmodium berghei*	Oral	Melanisation response	Positive	Enhanced melanisation response hampers malaria development	[296]
Culex quinquefasciatus 	Laboratory selection to *B. sphaericus*	Larvae exposed to *B. sphaericus* via water	*B. thuringiensis* bv. *israelensis*	Larvae exposed to *B. thuringiensis* via water	Survival	Negative	No mechanism proposed	[297]
Drosophila melanogaster (continued on the next page) 	*Drosophila* C virus (DCV) via experimental evolution	Systemic	Cricket paralysis virus and flock house virus (FVH)	Systemic	Survival	Positive	Candidate genes responsible for cross-resistance were identified by whole-genome sequencing and validated by RNAi	[111]
		Systemic	*Pseudomonas entomophila* and E*nterococcus faecalis*	Systemic		Negative	No mechanism proposed; *because the viruses and bacteria used in this study vary in their mechanism of infection*	

Table continued page 135

Insect species	Cross-resistance to parasite							References
	Prior exposure to	Route of prior exposure	Cross-resistant to	Route of cross-infection	Assay for testing cross-resistance	Type of cross-resistance	Costs/Proposed mechanism	
D. melanogaster (continued on the next page) 	Experimental evolution to *P. entomophila*	Systemic	*Pseudomonas putida*	Systemic	Survival	Positive	No mechanism proposed	[6]
			*E. faecalis, Serratia marcescens, Erwinia carovora, D*CV and FVH	Systemic		Negative		
			P. putida	Oral				
		Oral	*P. putida,*	Oral		Positive		
				Systemic		Negative		
	Experimental evolution to parasitoid *Asobara tabida*	Breaching of the puparium wall	*Beauveria bassiana*	Systemic (cuticular breaching)	survival	No change in resistance	NA; *difference in both the mechanism and route of infection between the parasites of prior and subsequent exposure.*	[112]
			Tubulinosema kingi	Oral				
	Experimental evolution to *B. bassiana*	Systemic (cuticular breaching)	*A. tabida*	Breaching of the puparium wall	encapsulation			
	Larvae infected with *T. kingi*	Oral						
			L. heterotoma			Positive		

Table continued page 136

Insect species	Cross-resistance to parasite							References
	Prior exposure to	Route of prior exposure	Cross-resistant to	Route of cross-infection	Assay for testing cross-resistance	Type of cross-resistance	Costs/Proposed mechanism	
D. melanogaster (continued on the next page) 	Experimental evolution to *A. tabida*	Breaching of the puparium wall	*L. boulardi*	Breaching of the puparium wall	encapsulation	Positive	No mechanism proposed	[108]
			L. heterotoma					
	Experimental evolution to *Leptopilinia boulardi*		*A. tabida*			No cross-resistance	*L. boulardi* is a specific parasite of *D. melanogaster*	
			L. heterotoma			Positive		
	Experimental evolution to *B. cereus*	Systemic wounding	*Drosophila sigma virus* (DMelSV)	Susceptibility of the evolved fly populations to DMelSV was tested via CO_2 sensitivity assay.	survival	No change observed across resistant populations but sex-specific significance in survival observed.	The results observed are a likely outcome of slower development time in resistant fly populations	[222]
Galleria mellonella 	selection to *B. bassiana*	Systemic (topical application of spore suspension)	*Metarhizium anisophilae*	Systemic (cuticular breaching)	survival	No change	No mechanism proposed	[298]

I have no special talents. I am only passionately curious – Albert Einstein