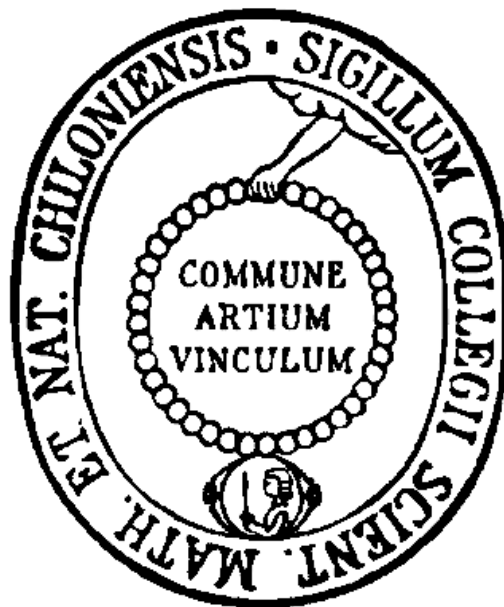


Extended interactions

- The influence of host external immunity and parasite transmissive stages on coevolutionary interactions



Dissertation

in fulfilment of the requirements for the degree
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Contents:

Declaration	4
Contribution of Authors	5
Summary	7
Zusammenfassung	8
General Introduction and Thesis Objectives	10
Chapter 1: Why selection for virulence leads to a loss of virulence	21
Chapter 2: Rapid evolution of virulence leading to host extinction under host-parasite coevolution	44
Chapter 3: Chemical warfare does not always win – rapid evolution of increased virulence in <i>Beauveria bassiana</i>	79
Chapter 4: Cryptic changes in immune response and fitness in <i>Tribolium castaneum</i> as a consequence of coevolution with <i>Beauveria bassiana</i>	101
Summary and outlook	135
Curriculum vitae	146
Acknowledgements	148
Appendix	150

Declaration

I, Charlotte Rafaluk, declare that:

Apart from my supervisor's guidance the content and design of the thesis is all my own work;

Specific aspects of my thesis were supported by colleagues; their contributions are specified in detail in the chapter "Contribution of authors" at the beginning of the thesis;

At the time of submission the thesis has neither been submitted partially nor wholly as part of a doctoral degree to another examining body nor has any part of the thesis been published or submitted for publishing;

The thesis has been prepared subject to the Rules of Good Scientific Practice of the German Research Foundation.

Contribution of authors

This thesis consists of four chapters, each represented by an unpublished manuscript. Charlotte Rafaluk developed original ideas and wrote the manuscripts with major contribution to all manuscripts. Gerrit Joop developed original ideas and supervised research in all manuscripts. Hinrich Schulenburg contributed to supervision of research on all manuscripts.

Chapter 1: Charlotte Rafaluk, Gunther Jansen, Hinrich Schulenburg and Gerrit Joop. **“Why selection for virulence leads to loss of virulence”**, unpublished review paper

Charlotte Rafaluk conceived the idea. Charlotte Rafaluk reviewed the relevant literature and wrote the manuscript. Gerrit Joop advised on writing, read and improved the manuscript. Hinrich Schulenburg and Gunther Jansen advised on writing and read and improved the manuscript but have not yet seen the final manuscript.

Chapter 2: Charlotte Rafaluk, Markus Gildenhard, Andreas Mitschke, Arndt Telschow, Hinrich Schulenburg and Gerrit Joop. **“Rapid evolution of virulence leading to host extinction under host-parasite coevolution”**, unpublished manuscript.

Charlotte Rafaluk conceived the project, performed the evolution experiment and phenotypical analysis, analysed data and wrote the manuscript. Markus Gildenhard developed the model and performed simulations. Andreas Mitschke established and assisted with the qPCR. Arndt Telschow assisted with the development of the model and advised the writing of the manuscript. Hinrich Schulenburg contributed to the supervision of the project and read and improved the manuscript. Gerrit Joop conceived and supervised the project, assisted with experimental work and read and improved the manuscript.

Chapter 3: Charlotte Rafaluk, Hinrich Schulenburg, Gerrit Joop. **“Chemical warfare does not always win - rapid evolution of increased virulence in *Beauveria bassiana*”**, unpublished manuscript

Charlotte Rafaluk conceived the project, performed the evolution experiment and phenotypical analysis, analysed data and wrote the manuscript. Hinrich Schulenburg assisted with the supervision of the project. Gerrit Joop conceived and supervised the project, assisted with experimental work and read and improved

the manuscript.

Chapter 4: Charlotte Rafaluk, Sophia Wagner, Hinrich Schulenburg and Gerrit Joop. **“Cryptic changes in immune response and fitness in *Tribolium castaneum* as a consequence of coevolution with *Beauveria bassiana*”**, unpublished manuscript

Charlotte Rafaluk conceived the project, performed the evolution experiment and phenotypical analysis, analysed data and wrote the manuscript. Sophia Wagner carried out the phenoloxidase activity assays on F2 beetles. Hinrich Schulenburg assisted with the supervision of the project. Gerrit Joop conceived and supervised the project, assisted with experimental work and read and improved the manuscript.

Hiermit bestätige ich als Betreuer der Arbeit die oben stehenden Angaben

Summary

Host-parasite coevolution is expected to present a strong force in nature, with interactions between hosts and parasites leading to rapid reciprocal adaptations. In recent years several coevolution experiments have been carried out, testing predictions on the dynamics and outcomes of coevolutionary processes. One aspect of host and parasite ecology that has received little attention in host-parasite coevolution experiments is the ability of both parties to shape their environment. A number of parasites are able to produce transmissive stages that persist in the environment across host generations and many host species are able to manipulate their environment through secretion of antimicrobial compounds or hygienic behaviours. The main objective of my PhD thesis is to extend our knowledge of how these traits may affect the outcome of coevolution. In *chapter 1* I review previous evolution experiments and discuss how transmission mechanisms may have been responsible for confounding results that have been seen in the past. The subsequent chapters relate to a host-parasite coevolution experiment where I allowed the red flour beetle, *Tribolium castaneum*, to coevolve separately with two parasites, the general entomopathogenic fungus, *Beauveria bassiana*, and its natural microsporidian parasite, *Paranosema whitei*. *T. castaneum* is able to secrete antimicrobial compounds into its environment and both parasites are able to produce persistent environmental stages. Within my experimental design I allowed for both the benefits of releasing transmissive stages into the environment for the parasites and secretion of antimicrobial compounds for the beetles to be realised. Coevolution resulted in both parasites increasing in virulence *chapters 2 and 3*. Fascinatingly, I was also able to show that in *B. bassiana* increased virulence was obtained via evolved resistance to the external immune compounds of the beetles *chapter 3*. Subtle reciprocal changes were also seen in hosts coevolved with *B. bassiana* *chapter 4*. This work highlights the importance of external immunity and transmissive parasite stages in coevolutionary interactions and the need for more studies where such effects are taken into account.

Zusammenfassung

Es wird angenommen, dass Wirt-Parasit-Koevolution eine starke Kraft in der Natur darstellt, mit der Interaktionen zwischen Wirt und Parasit zu schnellen reziproken Anpassungen führen. In den vergangenen Jahren wurden diverse Koevolutionsexperimente durchgeführt, um Vorhersagen über Dynamik und Folgen von Koevolutionsprozessen zu testen. Ein Aspekt der Wirt- und Parasit-Ökologie, dem in Wirts-Parasit Koevolutionsexperimenten bisher nur wenig Aufmerksamkeit geschenkt wurde, ist die Fähigkeit beider Seiten, ihre Umgebung zu formen. Viele Parasiten sind in der Lage, transmissive Stadien zu bilden, die über mehrere Wirtsgenerationen in der Umwelt überleben können, und viele Wirtarten können ihre Umgebung durch Sekretion von antimikrobiellen Substanzen oder hygienischen Verhalten manipulieren. Das Hauptziel meiner Doktorarbeit ist es, unser Wissen darüber wie diese Eigenschaften das Ergebnis von Koevolution beeinflussen können, zu erweitern. In *Kapitel 1* gebe ich eine Literaturübersicht über Koevolutionsexperimente und diskutiere, wie Transmissionsmechanismen die vergangenen Beobachtungen beeinflusst haben könnten. Die nachfolgenden Kapitel beziehen sich auf ein Wirts-Parasit-Koevolutionsexperiment, in dem ich den rotbraunen Reismehlkäfer, *Tribulium castaneum*, separat mit zwei seiner Parasiten koevolvieren ließ, dem entomopathogenen Pilz, *Beauveria bassiana*, und seinem natürlichen Mikrosporidium, *Paranosema whitei*. *T. Castaneum* kann antimikrobielle Substanzen in seine Umwelt abgeben und beide Parasiten sind imstande in der Umwelt persistente Stadien zu bilden. Durch mein experimentelles Design ermöglichte ich, dass die Vorteile der Parasiten, transmissive Stadien zu bilden, und die Vorteile des Wirts, antimikrobielle Substanzen in die Umwelt abzugeben, realisiert waren. Das Ergebnis dieser Koevolution war, dass beide Parasiten eine höher Virulenz entwickelten (*Kapitel 2 und 3*). Wir waren außerdem in der Lage zu zeigen, dass die höhere Virulenz in *B. Bassiana* auf eine erhöhte Resistenz gegenüber den externen Immunkomponenten der Käfer zurückzuführen war (*Kapitel 3*). Desweiteren konnten geringe Anpassungen des mit *B. Bassiana* coevolvierten Wirts (*Kapitel 4*)

nachgewiesen werden. Diese Arbeit zeigt die Wichtigkeit eines externen Immunsystems und transmissiver Stadien von Parasiten in coevolutionären Interaktionen, sowie die Notwendigkeit für weitere Studien, die diese Effekte betrachten.

General Introduction and Thesis Objectives

“One day, on tearing off some old bark, I saw two rare beetles, and seized one in each hand. Then I saw a third and new kind, which I could not bear to lose, so I popped the one which I held in my right hand into my mouth. Alas! it ejected some intensely acrid fluid, which burnt my tongue so that I was forced to spit the beetle out, which was lost, as was the third one.” — Charles Darwin, The Autobiography of Charles Darwin

The concept of coevolution was mentioned by Darwin (1859) in the *Origin of Species*, with the first data providing empirical support for the presence of coevolutionary processes coming mainly from observational studies (e.g. Ehrlich & Raven 1964). Recently, experimental evolution has emerged as a powerful method to test microevolutionary hypotheses (Kawecki *et al.*, 2012; Brockhurst and Koskella, 2013; Kerstes and Martin, 2013) and several host-parasite coevolution experiments have been carried out providing strong support for theoretical ideas relating to the dynamics, phenotypic and genotypic changes predicted to occur as a consequence of coevolution (Brockhurst and Koskella, 2013). Most of these experiments, however, have been carried out under extremely controlled conditions. Particularly in the case of animal systems, parasite (defined here in the broad sense incorporating both macro and micro parasites (Anderson and May, 1979)) propagules have been extracted predominantly from the bodies of dead or alive hosts and experiments carried out under strict environmental conditions (e.g. Koskella & Lively 2007; Béréanos *et al.* 2009; Schulte *et al.* 2010). Although these experiments have provided a valuable basis of knowledge and carefully tested coevolutionary ideas, such strict methods neither allow for natural parasite transmission nor for either of the interacting organisms to shape their environment. This reduces the chances of indirect mechanisms of attack or defence evolving and provides a quite different scenario from what is likely to be the case in nature.

When an individual is able to elicit change on, or directly affect the environment in which it lives, and there is heritable variation in this ability, the change elicited on

the environment can be thought of as an extension of the organism's phenotype (Dawkins 1982). This means that if, for example, a host organism alters their environment in a way that reduces their chance of infection by a parasite, or likewise a parasite adapts its environment in a way that increases infection success, it can be considered an extended phenotype and consequently can potentially be under selection as a result of host-parasite coevolution (Otti *et al.*, submitted). When the environment in which the organisms interact is frequently replaced, or tightly controlled, these extended phenotypic traits are excluded from the coevolutionary process.

One host trait which can be considered as an extended immune phenotype is the ability to secrete antimicrobial compounds into the environment (Otti *et al.*, submitted), or external immunity. This is a trait present in a number of insect species (e.g. Rosengaus *et al.*, 2004; Cotter *et al.*, 2010; Hamilton *et al.*, 2011; Kay *et al.*, 2014). For example, the burying beetle, *Nicrophorus vespilloides*, secretes antimicrobial compounds onto mammalian carcasses on which it lives (Cotter *et al.*, 2010). Similarly beetles of the genus *Tribolium* are able to secrete antimicrobial benzoquinone compounds into their environment, which have been shown to be able to control and manipulate the microbial community of the flour environment in which they live (Prendeville and Stevens, 2002; Yezerski *et al.*, 2007; Joop *et al.*, 2014). Furthermore, secretion of these antimicrobial compounds in *Tribolium castaneum* has been shown to be a highly heritable trait (Joop *et al.*, 2014), fulfilling all criteria for an extended phenotypic trait (*sensu* Dawkins 1982). When a host organism possesses such ability, it is likely that external immunity will play a role in coevolutionary processes with microbial parasites, as external immune defence will provide a first barrier of defence against infection. Consequently, coevolutionary processes will not be confined to occur solely within the body cavity of the host.

Many parasites are able to persist outside the body of their host, with some parasites producing long lived stages which are able to persist in the environment for extended periods of time (Dunn and Smith, 2001; Decaestecker *et al.*, 2007; King

et al., 2013). These parasites alter the environment for the host, posing a risk of infection, even when no infected hosts are present. Furthermore, the length of time these propagules are able to persist in the environment is theoretically predicted to be linked to their virulence and transmission success, with increased longevity outside of the host predicted to be correlated with increased virulence due to reduced costs of temporary or localised host extinction (Bonhoeffer *et al.*, 1996). Traits such as external immunity are likely to have a substantial effect on the evolutionary trajectory of these kinds of parasite by limiting the longevity of environmental stages. Furthermore, if these stages are ignored in evolution experiments, the dynamics of the host-parasite interaction are likely to be greatly altered from the natural state risking that the host and parasite simply adapt to the new state of the interaction rather than the selection pressure put upon them by the experiment.

The effect of extended phenotypes and long lived parasite transmissive stages on the dynamics and trajectory of coevolution is something that has received very little empirical attention. Parasite environmental stages are, however, predicted to have an effect on the evolution of fitness traits (Bonhoeffer *et al.*, 1996; Gandon, 1998; Kamo and Boots, 2004) and extended phenotypes are likely to be subject to selection and have a strong influence of host and parasite fitness (Dawkins 1982; Otti *et al.*, submitted). Consequently, studies allowing the costs and benefits of these traits to be realised are vital in creating a full understand of the evolutionary ecology of host-parasite interactions.

Objectives of the thesis:

The aim of this thesis is to test how the dynamics and outcomes of coevolution are affected by the inclusion of parasite transmissive stages and host external immune compounds in the coevolutionary interaction. To do this I carried out a large scale host-parasite coevolution experiment in which populations of the red flour beetle, *Tribolium castaneum* were allowed to coevolve separately with two parasites, it's natural, microsporidian parasite, *Paranosema whitei*, and the general entomopathogenic fungus *Beauveria bassiana*. At each transfer pupae of the beetle

population were taken to start the next generation along with a portion of the flour environment, including external immune compounds of the beetles and parasite stages present in the environment. This allowed interactions outside of the host, such as those between the external immune compounds of the beetles and the environmental parasite stages to be included in the evolutionary interaction (diagrams of the general experimental set up can be found in *chapters 2 and 3*). With this experiment I aimed to answer the following specific questions:

- i) **Does the presence of persisting parasite transmissive stages in the environment and allowance for natural horizontal transmission lead to a change in parasite virulence that differs from experiments where this is not the case?**

Both parasites produce long lived spore stages that can persist in the environment for longer than a single host generation. *P. whitei* can persist for over 6 months outside of a host (Milner, 1972) and *B. bassiana* is able to persist for approximately 80 days outside of a host under the temperature and humidity conditions used in my experiment (Hong *et al.*, 1997).

Consequently, the ability to shed spores into the environment and sit and wait for new hosts is likely to be an important aspect of the transmission strategy of the parasites. As my experimental design allows for the transfer of spores in the environment from generation to generation, the benefits of spore longevity and release of spores into the environment will be realised. Furthermore, my experimental design includes an overlap in host generations allowing for direct horizontal transmission from host to host across generations, which is a factor excluded in many host-parasite coevolution experiments (*chapter 1*). In previous coevolution experiments in the *T. castaneum* system contrary to theoretical predictions (e.g. Gandon *et al.*, 2002) a decrease in parasite virulence has been seen as a consequence of host-parasite coevolution (Béréños *et al.*, 2009; Béréños *et al.*, 2011). I hypothesise that the more natural method of transmission allowed for in my experiment will lead to increased virulence in comparison to experiments where spores

are extracted from a single time point (Figure 1). This question is further discussed in *chapter 1* and addressed in *chapters 2 and 3*.

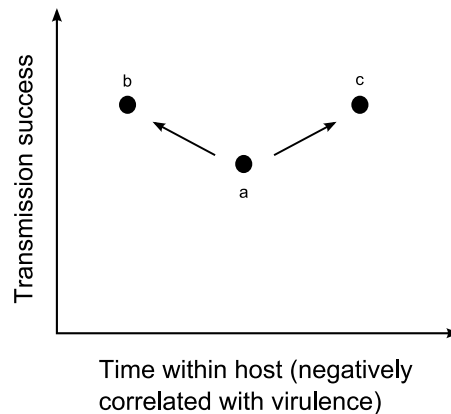


Figure 1. Predicted change in time the parasite spends replicating within the host from the ancestral state (a) as a consequence of coevolution under natural conditions (b) and under conditions where propagules are artificially extracted from a host at a specific (in this case late) time point (c). Extended time spent reproducing within the host is predicted to be linked to a slower rate of within host reproduction and therefore reduced virulence.

ii) Do host external immune compounds affect the trajectory of parasite evolution?

Host external immune compounds act as a first line of defence against parasites, coating the cuticle and disinfecting the environment (Cremer *et al.* 2007; Cotter & Kilner 2010; Otti *et al.*, submitted). They are likely to provide a direct, strong barrier to overcome for parasites such as *B. bassiana*, which infects via attachment to the cuticle (Leger *et al.*, 1986), and are likely to strongly affect the environmental stages of both parasites by reducing their longevity outside of the host. Consequently, external immune compounds

are likely to provide an important barrier to overcome for the parasites, potentially leading to selection for parasite genotypes resistant to the external immune compounds of the beetles (Figure 2). This question is addressed in *chapter 3*.

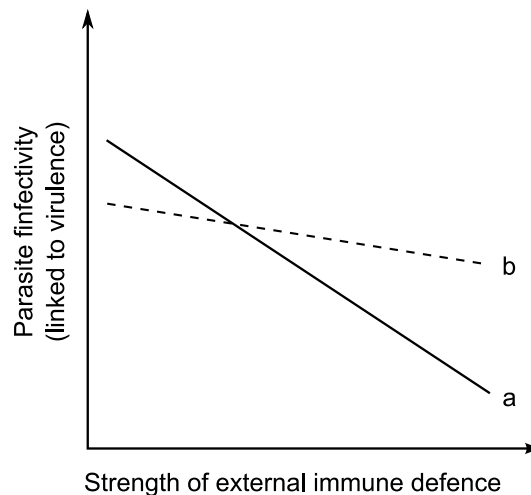


Figure 2. In the presence of increasingly strong external immune defences a susceptible parasite genotype (a) would be predicted to decrease in infectivity, which is likely to be linked to both virulence and parasite fitness, with increasing strength of external immunity. A resistant genotype (b) may have lower infectivity when external immunity is weak due to the costs of resistance but would decrease in fitness at a far slower rate.

iii) Is host external immunity traded off against host internal immunity?

Both external immunity, in the form of benzoquinones, and the phenoloxidase cascade, an important aspect of internal immunity, are likely to be tyrosine dependent (Cerenius *et al.*, 2008; Gorman and Arakane, 2010). Grain beetles are dependent on uptake of tyrosine from their diet (Taylor and Medici, 1966), meaning that tyrosine is likely to be a limited resource to the beetles. Consequently, when increased investment in external immunity is predicted as a consequence of coevolution I predict to see an inverse

response in phenoloxidase activity and vice versa (Figure 3). This question is addressed in *chapter 4*.

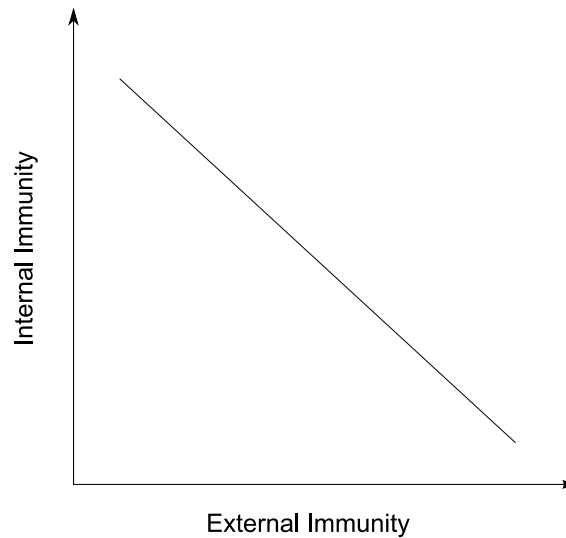


Figure 3. A negative correlation is predicted between increased investment in internal immunity, in the form of phenoloxidase activity, and investment in external immunity, in the form of benzoquinones and vice versa.

iv) Does the concentration of the parasite in the environment change the selective pressure on host immunity?

For both parasites there will be experimental evolution treatments with three different starting environmental concentrations of the parasite, representing differing levels of induced host mortality. My prediction is that low parasite concentrations, where infection probability is relatively low, are likely to select for increased levels of external immunity as the host will be able to fight off the parasite effectively without paying the cost of infection. As I predict that internal and external immunity will be traded-off against one-another, I predict that increased investment in external immunity will result in decreased investment in internal immunity. Conversely, when the parasite concentration is high, resulting in high infection probability, I predict that

increased investment in internal immunity will be selected for as external immunity is unlikely to be 100% effective at high parasite concentrations and the risk of having reduced internal immunity when infection occurs is likely to be, under such circumstances, too high. These predictions are illustrated in Figure 4. This question is addressed in *chapter 4*.

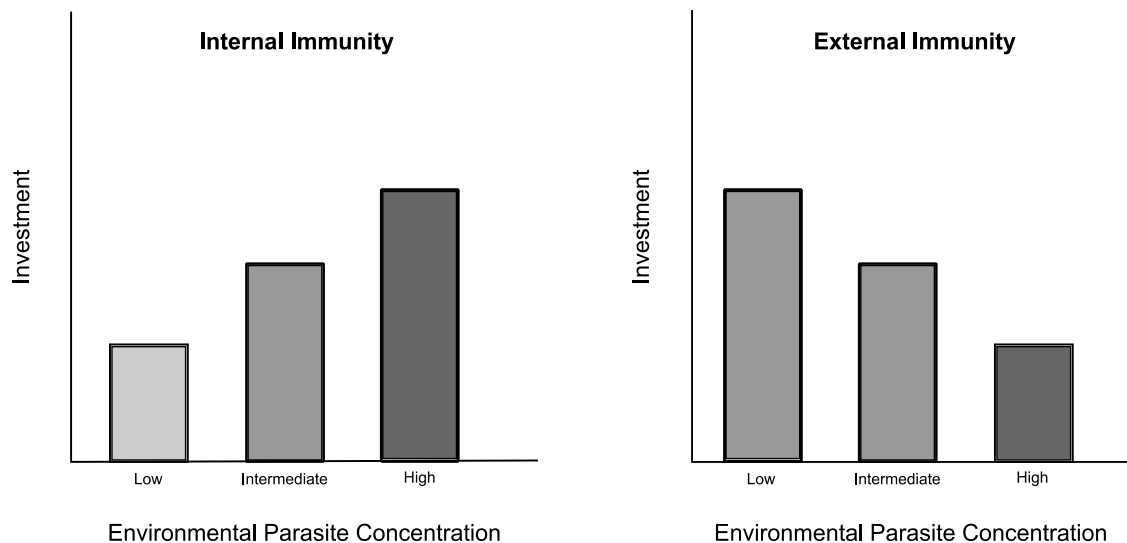


Figure 4. In the case of exposure to a horizontally transmitted parasite susceptible to external immunity, increased investment in internal immunity is predicted with increased pathogen concentration in the environment and the inverse predicted for external immunity.

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Why selection for virulence leads to a loss of virulence

1

"Virulence appears in a new light which cannot but be alarming to humanity; unless nature, in her evolution down the ages (an evolution which, as we now know, has been going on for millions, nay, hundreds of millions of years), has finally exhausted all the possibilities of producing virulent or contagious diseases -- which does not seem very likely." - Louis Pasteur in Cuny, Hilaire, 1965: Louis Pasteur: The man and his theories. Translated by P. Evans.

Charlotte Rafaluk, Gunther Jansen, Hinrich Schulenburg and Gerrit Joop

Abstract: There is a vast theoretical literature on virulence evolution largely based on the prediction that virulence levels should optimise at an intermediate level. Theory predicts that during certain periods of evolution virulence should increase, including during adaptation to a specific host and during host-parasite coevolution. Although these theoretical predictions are supported by natural observations, tests of these hypotheses in the form of evolution experiments have yielded confounding and contradictory results. Here we provide a critical commentary of experimental evolutionary tests of hypotheses regarding virulence evolution and provide potential explanations for the counterintuitive results highlighting transmission mechanisms in evolution experiments as a key factor that may explain the discrepancies that we see. Finally, we make suggestions for how evolution experiments could be carried out in the future to avoid some of the potentially confounding factors revealed in this paper.

Hypotheses of virulence evolution

The evolution of **virulence** (terms in bold defined in the glossary) has been a topic of great interest to biologists and epidemiologists for many years. The first inadvertent studies of virulence evolution can be traced back to the times of Pasteur and Koch, during which Pasteur and his colleagues noted the virulence attenuated when a **parasite** was left to grow in “atmospheric air” and regained its virulence when passage through a series of animal hosts (Mendelsohn, 2002). Initial hypotheses were largely related to what is now referred to as the “avirulence hypothesis” whereby it is assumed that parasites reduce virulence over time (Alexander, 1981; May and Anderson, 1983; Alizon *et al.*, 2009). This assumption was predominantly based on the observation that new host-parasite interactions, between partners which were not previously in ecological contact, are often highly virulent, whereas older interactions, between long associated partners, tend to be less virulent (May and Anderson, 1979; Read, 1994).

The avirulence hypothesis was superseded by the “trade-off hypothesis” (Anderson & May 1982), mathematically demonstrating that virulence, recovery and transmission are likely to be traded off against one another, resulting in heightened virulence emerging as a cost of increased transmission success. The trade-off hypothesis had a massive impact on the field and was followed by a huge amount of theoretical research and extensions to the basic model in the 1980s and 1990s (e.g. Frank, 1992; Nowak and May, 1994; May and Nowak, 1995; Frank, 1996; Bonhoeffer *et al.*, 1996; Mosquera and Adler, 1998). This has led to the general acceptance of the trade-off hypothesis, although it has been challenged more recently (Ebert and Bull, 2003; Alizon *et al.*, 2009), largely due to arguments that there is not a sufficient amount of evidence empirically supporting the hypothesis. Nevertheless, it still provides the basis for almost all current work on the topic. Interestingly, however, in spite of the wide ranging theoretical

Glossary:

Virulence – the damage and cost to host fitness inflicted on a host by a parasite (Read, 1994)

Parasite – any organism that depends on another larger (host) organism for survival and in doing so causes damage to the host.

literature on virulence evolution, only very few controlled experimental studies have as yet tried to evaluate the predictions proposed.

In the last few years evolution experiments have emerged as a powerful tool for testing predictions of theoretical models and disentangling cause-effect relationships (Kawecki *et al.*, 2012; Brockhurst and Koskella, 2013; Kerstes and Martin, 2013). These kinds of experiments may thus yield the critical litmus test for the vast theoretical literature on virulence evolution. In some of the experiments that have tackled this so far, however, predicted effects have not been seen or the effects have differed from what is observed in nature. Here we will briefly discuss the theoretical literature on virulence evolution and shortly review the different kinds of evolution experiments that have been used to test predictions made by this body of literature, moving up in complexity from serial passage experiments to one-sided evolution experiments and finally to coevolution experiments. We will end by discussing some reasons that may potentially be responsible for this discrepancies seen between theoretical predictions and the results of evolution experiments and make suggestions of how evolution experiments could be designed in the future to more precisely test theoretical predictions.

Predictions and models of virulence evolution

Virulence is not predicted to increase or decrease indefinitely. In order to maximise its fitness a parasite needs to achieve an optimal level of virulence, where it maximises the amount of resources extracted from an individual infected host while ensuring transmission to the largest number of new hosts (Anderson & May 1982; Jensen *et al.* 2006). The trade-off hypothesis (Anderson and May, 1982) explicitly predicts a link between virulence itself (i.e., the number of dead hosts caused by infection), transmission rate to new hosts and host recovery rates (Box 1). This hypothesis is supported by evidence from a number of field studies and experiments based on natural parasite isolates. For example, Roode *et al.* (2008) showed that in the protozoan parasite *Orphryocystis elektroschirrha* infecting the monarch butterfly *Danus plexippuss* that within host replication, virulence and transmission were genetically linked and that high parasite loads, resulting in

increased virulence were necessary to increase transmission rate. Similarly, a study of disease emergence of the bacterium *Mycoplasma gallisepticum* in house finches showed that both virulence and parasite load, which is linked to transmission, were highly correlated and furthermore that increased virulence emerged during adaptation to the finch hosts in two populations as a consequence of selection for heightened transmission success (Hawley *et al.*, 2013).

Many models have added factors and extensions to the original trade-off model providing more complex predictions for how virulence is expected to evolve. The mechanism of transmission is predicted to have an enormous effect on virulence evolution. A fact that was noted in the 1980s by Ewald (1983; 1987) and has since been mathematically formalised in a number of models. Frank (1992) included transmission in his co-infection model and demonstrated that the mechanism of transmission was important in determining the outcome of competition among parasites. Further models have focussed entirely on the mode of transmission. For example, parasites that are primarily vertically transmitted are predicted to evolve lower virulence than those that are horizontally transmitted as their reproduction is more aligned with that of their hosts (reviewed in Bull 1994). A phenomenon that is supported by numerous empirical observations (Ebert and Herre, 1996), for example in a comparison two strains of *Trypanosoma cruzi* with different rates of vertical transmission, the strain with the higher rate of vertical transmission showed a lower level of virulence (Hall *et al.*, 2010).

The precise mode of horizontal transmission also influences virulence evolution. Vector transmitted diseases are predicted to have higher levels of virulence due to their increased dispersal ability (Day, 2002), consistent with natural observations (Ewald, 1983). Furthermore it has been predicted that pathogens with transmissive stages that are able to persist for long periods in the environment may be able to evolve extreme virulence, as the costs of causing local extinction of the host population are phenomenally reduced (Bonhoeffer *et al.*, 1996). Consequently, the specific mechanism of transmission, perhaps more than any other factor, is of vital importance for the exact dynamics and outcomes of virulence evolution.

In addition to transmission, there are a number of further factors that can influence the trajectory of virulence evolution. Significantly, competition among parasites infecting the same host individual (i.e., multiple infection or co-infection) is predicted to have a substantial effect. In these cases although, overall transmission may be maximised by keeping the host alive for a prolonged period of time, fitness of individual parasite genotypes is likely to be maximised by producing more propagules than all other unrelated genotypes, thereby favouring more rapid reproduction and increased virulence (Frank, 1992; Nowak and May, 1994; May and Nowak, 1995; Mosquera and Adler, 1998). There are, however exceptions to this including when parasites are closely related, in which case kin selection plays a role (Frank, 1992; Lively, 2005). The effect of multiple infections on optimal virulence has been empirically demonstrated by Ben-Ami et al. (2008) who showed that when *Daphnia magna* were infected with multiple strains of their bacterial parasite *Pasteuria ramosa*, parasite induced mortality represented that of the most virulent bacterial strain, indicating that the most virulent genotype outcompeted the others.

In addition to the examples described above a multitude of further mathematical examples exist predicting additional factors likely to influence the trajectory of coevolution including the type of interaction between parasite propagules (Lively, 2001), host density (Lively, 2009) and the effects of host and parasite life history (Gandon *et al.*, 2002) (Box 1). Although, natural observations and field studies provide support for many of these predictions, evolution experiments in the lab provide the potential to carefully control specific parameters and control for confounding factors which are not possible in field studies, such as ensuring single infection when transmission mechanism alone is being tested or manipulation of the mechanism of transmission. Evolution experiments like these have the potential to provide critical evaluation of the extensive range of theoretical hypotheses.

Serial passage experiments

The first experiments to test (or accidentally yield information on) the dynamics of virulence evolution were serial passage experiments. These kinds of evolution experiment have been used extensively in vaccine development and currently constitute the most comprehensive empirical basis for our understanding of how virulence evolves (Ebert 1998). In these experiments a parasite is usually passaged through a series of naive, often genetically similar or identical hosts which are in many cases still living at the time of pathogen extraction (Box 2). The initial aim of these studies was to adapt a pathogen to a novel host, usually a taxon that can be easily maintained in the lab, in order to permit in-depth experimental analysis of the pathogen. Nevertheless, they have indirectly tested some of the hypotheses mentioned above (Ebert 1998).

A representative example of a serial passage experiment is that of Dortmans *et al.* (2011) who passaged an initially low virulent isolate of the Newcastle disease virus PPMV-1 intracerebrally through one-day-old chick hosts. Virulence, determined by a standard intercerebral pathogenicity (ICPI) test, was seen to rapidly increase in this case. Although the majority of serial passage experiments have been carried out in vertebrate models there are examples from invertebrate systems. For example, Kolodny-Hirsch & Van Beek (1997) passaged the multiply embedded nuclear polyhedrosis virus of the alfalfa looper, *Autographa californica* (AcMNPV), through the diamondback moth, *Plutella xylostella*. Viral particles for each passage were extracted from dead individuals from the previous passage. Again, in this case virulence, defined at LC_{50} , the lethal dose at which 50% of the population are killed, significantly increased as a result of serial passage. Interestingly the authors were able to show that a specific genotypic variant had been selected for from a genetically diverse starting population, indicating that among genotype competition was responsible for the increase in virulence (Frank, 1992; May and Nowak, 1995; Nowak and May, 1994; Lively, 2005).

Importantly, in the vast majority of serial passage experiments virulence in the novel hosts almost exclusively increased rapidly and to high levels (Table 1). The

most likely reason for this is simply that the cost of virulence is dramatically reduced under the experimental conditions. In many cases, particularly in vertebrate models, animals are euthanized and parasites extracted as soon as symptoms of infections occur, or if no symptoms occur after a set period of time (e.g. Dortmans et al. 2011; Hu et al. 2014). This puts a high selection pressure on rapid reproduction within the host as the time available for the production of propagules is reduced. Furthermore, in these experiments parasite transmission is almost guaranteed as propagules will be extracted from hosts by the experimenter. This means that inducing rapid mortality will not come at the cost of reducing the time window in which transmission can occur. Consequently, the best way to maximise fitness is likely to reproduce as rapidly as possible in the given time frame, in order to maximise chances of propagules being successfully extracted and transferred to the next generation. Although serial passage experiments have provided us with some vital information on virulence evolution (Ebert 1998), more complex experiments are required in order to test the specific hypotheses put forward in the theoretical literature.

One-sided or unidirectional adaptation experiments

One-sided or unidirectional selection experiments are similar to serial passage experiments, in that adaptation is to a non-evolving host, but add a level of complexity in that they attempt to answer more precise questions by specifically manipulating factors of interest. They further differ from serial passage experiments in that the host is not necessarily novel (Box 2). Many of these experiments are carried out in parallel to coevolution experiments to control for differences in adaption to the host alone and those resulting from reciprocal coevolutionary interactions. Other experiments specifically manipulate host life history characteristics in order to test their influence on parasite evolution.

For example, Nidelet et al. (2009) manipulated the life-span of *Paramecium caudatum* and allowed its parasite *Holospora undulata* to adapt to either long-lived or short-lived hosts. With this experimental design they varied the shape of the virulence-transmission trade-off (Anderson & May 1982) by either prolonging or reducing the

time available for within host reproduction and transmission, respectively. As predicted by theory, short host life-spans indeed favoured higher virulence than long life-spans. Contrary to this, however, *Daphnia magna* life span manipulation has yielded opposite results (Ebert & Mangin 1997). In this experiment parasites from the longer lifespan treatment showed higher virulence than those from the shorter lifespan treatment in apparent contrast to the predictions of the trade-off hypothesis (Anderson & May 1982). Here, however, the mode of transmission was quite different, with spores being able to naturally transmit from host to host. Furthermore, parasites from the low mortality treatment produced higher spore loads within their hosts which could be linked to an increased incidence of multiple infection, potentially selecting for increased virulence (Nowak and May 1994; May and Nowak 1995).

There are further examples of one-sided evolution experiments yielding counter intuitive results (Table 1) and in summary, both increases and decreases in virulence have been observed, often contradicting initial predictions even in cases where specific predictions were being tested. These selection experiments can provide one of the best methods for testing precise problems, however, all potentially confounding factors need to be considered and, ideally, controlled precisely. Although one-sided adaptation experiments can and do simulate conditions that occur in nature, they assume a static host which is not always the case under natural conditions, where hosts may also be reciprocally adapting to changes in the parasite.

Coevolution experiments

A further level of complexity can be added to evolution experiments by allowing the host species to coevolve with the parasite of interest (Box 2). This can provide a more comprehensive view of how virulence evolves under natural conditions by producing information on how predictions made in the theoretical literature hold in more dynamic natural systems. Due to their added complexity, however, they also present the biggest challenges for developing a system which truly tests the desired hypothesis.

Again these kinds of experiment have produced contradictory results. Schulte *et al.* (2010) found, for example that virulence increased in *Bacillus thuringiensis* as a consequence of coevolution with its host *Caenorhabditis elegans*. In this case the starting parasite population was genetically diverse, potentially providing opportunity for within host competition (Nowak and May, 1994; May and Nowak, 1995; Lively, 2005) and parasite propagules were collected from dead hosts every second nematode generation potentially allowing for natural parasite transmission within the two nematode generations. In contrast, in a coevolution experiment between *Tribolium castaneum* and its microsporidian parasite *Paranosema whitei* (formerly *Nosema whitei*) a decrease in virulence was observed (Bérénos *et al.*, 2009; Bérénos *et al.*, 2011). Interestingly, the parasite here is an obligate killer, depending on host death for transmission, a trait which is theoretically predicted to be associated with high virulence (Dieter Ebert and Weisser, 1997). In this case transmission also occurred through collection of dead hosts, however, in contrast to the methods of Schulte *et al.* (2010), parasite propagules were extracted every generation from hosts which died within a specific time frame.

Particularly here, decreases in virulence or lack of change have been observed (Table 1), with the decreases being inconsistent with theoretical predictions. Gandon (2002) predicts in his model of coevolution that virulence should increase during coevolution as hosts increase their reproductive output often to the cost of their own survival, reducing the costs of virulence and increasing the benefits of more rapid transmission. Even if hosts evolve tolerance rather than resistance the prediction of increased virulence holds due to reduced costs of increased reproductive rate within the coevolved hosts (Little *et al.*, 2010). These predictions are supported by field studies which have shown more virulence associations to be found in coevolved host species (Ebert, 1994; Sloan *et al.*, 2008). Raising the question of why this is not the pattern we see with virulence in many coevolution experiments (Table 1).

Why do the experimental results differ from theoretical predictions?

The results that we see across the types of experiment vary in their consistency with theoretical predictions raising the question of what might be responsible for the inconsistency of results that we see. In studies of natural systems that have aimed to test theoretical predictions, consistent results have been seen (Roode *et al.*, 2008; Ben-Ami *et al.*, 2008; Hawley *et al.*, 2013). Indeed, in addition to the examples already discussed, the few studies that have examined the trajectory of virulence evolution as a consequence of coevolution in nature have also seen increases in virulence and effects consistent with the theoretical predictions. For example (Sloan *et al.*, 2008) found that isolates of the anther smut fungus, *Microbotryum spp.*, showed the highest levels of virulence in species of host-plant that they had coevolved with as opposed to non-coevolved species. Similarly, (Ebert, 1994) found the highest spore loads and virulence levels in sympatric *Pleistophora intestinalis* and *Daphnia magna* associations, even on incredibly localised scales. So why is this not consistently the case for tests of theoretical predictions by experimental evolution?

Methodology may play an important role in the discrepancies we see between results and predictions, especially if it inadvertently manipulates the costs of increased virulence. For example, when host extrinsic mortality is effectively increased to 100%, as in the case of many serial passage experiments, killing a host carries virtually no costs. This affects the trade-off balance (Anderson and May, 1982): by eliminating the potential risk of reduced transmission when the host is killed too early, transmission is no longer traded-off against virulence.

Perhaps the most important factor that may influence the trajectory of along which virulence changes during evolution experiments is the precise mechanism of transmission. This, arguably above all other factors, may explain much of the contradiction in results that we see. Whenever the sole mechanism of transmission involves extraction of parasite propagules from either dead or living hosts by the experimenter we introduce artificial changes and biases in transmission and transmission probability of individual propagules. The effects of this may be tremendous. In burst killers where parasite transmission is dependent upon release

of propagules from cells or cadavers, such as the phi phage (Buckling and Rainey, 2002; Brockhurst *et al.*, 2007; Hall *et al.*, 2011) or *Paranosema whitei* (Béréños *et al.*, 2009; *chapter 2*), the cost of killing the host too slowly and not causing it to burst are effectively limited when propagules are extracted from entire dead hosts. In fact in such cases an extra cost is introduced on inducing early death as cadavers/cells that burst too early would not be collected, even if parasites had optimised the resources extracted from the host. Even for non-burst killers extracting propagules only from entire hosts can introduce a bias. The most virulent parasite may exploit the host they are in to the full before producing infective stages (Lohse *et al.*, 2006; Nidelet *et al.*, 2009) or in other cases may transmit more effectively from host to host than from environment to host or vice versa. Changes in any of these aspects of transmission would affect the predicted evolutionary outcome (Anderson and May, 1982; Bremermann and Pickering, 1983; Massad, 1987).

A significant issue with transmission mechanisms in evolution experiments is the fact that, particularly in animal systems, parasite propagules are almost always extracted from hosts at a specific time point (Kolodny-Hirsch and Van Beek, 1997; Béréños *et al.*, 2009; Schulte *et al.*, 2010; Garbutt *et al.*, 2011; Béréños *et al.*, 2011). In the case of Béréños *et al.* (2009; 2011), where a decrease in virulence was seen in *P. whitei* coevolving with *T. castaneum*, for example, this is likely to be responsible for the observed decrease in virulence. In this case spores were extracted from entire dead hosts at a relatively late time point for the parasite (Milner, 1972), which is likely to have selected against early killers, which may well have already caused the disintegration of their host by the time point of parasite extraction. This is particularly likely to be the case when results are compared to an experiment in the same system where environmental transmission was allowed for (*chapter 2*), in which case increased virulence evolved, potentially as in this case killing and bursting over a wide timeframe was allowed for, allowing selective advantages of early bursting to be realised. In any system, where a parasite is extracted at a specific time point it introduces a huge risk that the parasite will adapt to the new balance of the virulence-transmission trade-off inflicted upon it by the controlled

time point of extraction rather than the conditions intended to be imposed by the experiment itself.

There are of course additional factors that may have played a role in the inconsistency of results that we see. The variation in whether single or multiple infections are allowed for in experiments and the extent to which infections are allowed to diverge may play a role. In the case of the inconsistent results between Ebert and Mangin (1997) and Nidelet *et al.* (2009) this may well have been the case. In coevolution experiments we most often see an unexpected decrease in virulence (Table 1). An additional potentially confounding factor in coevolution experiments is that parasites may be best adapted to contemporary hosts (Dieter Ebert, 1994; Decaestecker *et al.*, 2007; Laine and Barrès, 2013). Consequently, if virulence is tested against the ancestral genotype alone, as is sometimes the case (e.g. Schulte *et al.*, 2010) changes in virulence may be overlooked. Indeed, experiments between *Pseudomonas fluorescens* and the phi phage have shown that the host genotype phage infectivity is tested against has an effect (Buckling and Rainey, 2002; Brockhurst *et al.*, 2007). Finally, inconsistencies in the definition of virulence may have an effect on the discrepancies we see. In Evolution experiments in general the range of measures of virulence reflect the variety of definitions (Table 1) (Read, 1994). Although, in the examples we report here (Table 1) the measure of virulence does not seem to correlate with whether an increase or decrease in virulence was seen, problems of definition could potentially cause false or inconsistent assumptions regarding change in virulence to be made.

Perspectives

The previously discussed contrasting results may highlight the pivotal influence of transmission on virulence evolution and our current inability to predict this in a precise way. Both theoretical predictions and studies of natural populations show us that direct selection on increased transmission success leads to increased virulence (Anderson and May, 1982; Bremermann and Pickering, 1983; Massad, 1987; Roode *et al.*, 2008; Hawley *et al.*, 2013). One key way that this may be achieved is by manipulating the time spent reproducing within the host and

consequently the time point of host death. In light of this, allowing for transmission to be flexible and change within an experiment could help to allow all potential trajectories of evolution to be realisable. A key point to consider in order to allow for flexibility in evolutionary responses is to, wherever possible, avoid extraction of parasite propagules from a defined time point

Furthermore, avoidance of artificial manipulation of transmission would help to prevent a counter pressure being put on transmission by the experimental design, which may otherwise lead to confounding results. In order to achieve this, it is necessary to consider how the parasite in question is transmitted in nature, how alteration of the method might shift the parasite from its current balance and how the natural mode of transmission affects the theoretical predictions of virulence evolution. For example, in the case of burst killers who are able to sit and wait for future hosts to appear, collecting spores from individuals burst across an experimental generation would help to remove biases. Similarly, if a parasite transmits most effectively via direct contact from host-host it would be beneficial to include an overlap in generations in order to allow for transmission to occur through contact and if a parasite is able to transmit both horizontally and vertically both should be allowed for in the evolution experiment.

Such considerations are likely to reduce the risk of inadvertently selecting for parasite strategies that reach their maximum exploitation level of their host at the experimental time point of propagule extraction or the experimentally imposed transmission mechanism. In the simplest situation this can be achieved by setting up conditions for initial infection and then allowing populations to persist with minimal interference for a number of generations. This of course introduces problems of its own and compromises will inevitably have to be made. It may nevertheless be possible to transfer a proportion of the environment from generation to generation, including parasite environmental stages, or to transfer a mixture of living and dead infected hosts. We indeed see that in several experiments where more natural transmission has been allowed for, such as the *Pseudomonas*-phage system (Buckling and Rainey, 2002; Brockhurst *et al.*, 2007; Hall

et al., 2011) and in experiments between both *P. whitei* and *Beauveria bassiana* and *T. castaneum* (chapters 2 and 3) (Table 1), that increases in virulence, as predicted, have been observed.

Of course other factors may be at play and starting experiments from well-defined parasite populations, carefully considering whether to use a single parasite genotype or a mixed parasite population depending on the question being asked would help to reduce confounding effects of having, or not having, multiple infections. Single infections only occur under rare circumstances, for example in hosts who have been treated with high doses of antibiotics. Thus, when the aim of an experiment is to test predictions of how virulence will evolve in natural epidemics, consideration of competing genotypes is essential. Moreover, in coevolution experiments, overlooking increases in virulence could be avoided by testing parasite strains against both coevolved and ancestral pathogen genotypes. Similarly, confounding effects of definition could be overcome by measuring virulence as we, as evolutionary biologists generally define it, i.e. the damage and cost to host fitness inflicted on a host by a parasite (Read, 1994). This would involve, in addition to mortality, taking a fitness measure, where possible lifetime reproductive success or number of grand offspring, and where not, a measure that most closely reflects the factor that would have most closely correlated to fitness in the experimental design.

Conclusion

Experimental evolution provides us with a powerful tool to test the complex set of predictions put forward by the theoretical literature. It gives us a unique opportunity to disentangle causal relationships and carefully examine very specific predictions. It is, however, an extremely sensitive method where even slight unintended selection pressures can have strong effects on the trajectory of evolution. Theoretical models and studies of naturally evolving populations show us that transmission, including the time point that transmission occurs, is likely to be under direct selection (Anderson and May, 1982; Bremermann and Pickering, 1983; Massad, 1987; Roode *et al.*, 2008; Hawley *et al.*, 2013). The fact that many

evolution experiments influence transmission as part of the selection protocol may explain some of the discrepancies that we see between theoretical predictions of virulence evolution and experimental evolution results. In this paper we have provided a critical commentary and put forward suggestions of how future evolution experiments could be carried out in order to reduce the risk of confounding factors and allow directional selection on traits associated with transmission to occur. Such experiments would likely greatly extend our abilities to carefully evaluate the hypotheses put forward by the extensive theoretical literature.

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Table 1. Examples of evolution experiments in which changes in parasite virulence have been measured.

Type of adaptation	Host	Parasite	Increase in virulence?	Transmission mechanism	Virulence measure	Reference
Serial passage	Mus musculus	Cryptococcus neoformans	Increase	From living host	Mortality	Hu et al., 2014
Serial passage	Gallus gallus domesticus	New castle disease virus Avulavirus spp.	Increase	From living hosts	Intracerebral pathogenicity test	Dortmans et al., 2011
Serial passage	Mus musculus	Friend retrovirus complex	Increase	From living hosts	Splenomegaly	Kubinak et al., 2013
Serial passage	Mus musculus	Murine rotavirus EB	Increase	Oral inoculation from living hosts	Presence of diarrhoea	Tsugawa et al., 2014
Serial passage	Plutella xylostella	AcMNPV	Increase	Oral infection from dead larvae	Mortality	Kolodny-Hirsch and Van Beek, 1997
One-sided adaptation	Paramecium caudatum	Holospira undulata	Increase	Horizontal (potentially some vertical)Collection of infectious particles	Host fitness	Nidelet et al., 2009
One-sided adaptation	Daphnia magna	Glugoides intestinalis	Decrease	Horizontal/environmental	Mortality	Ebert and Mangin, 1997
One-sided adaptation	Acyrtosiphon pisum + symbiont Hamiltonella defensa	Aphidius ervi	Increase	All hatched parasitoids to next generation	Rate of parasitism	Dion et al., 2011

One-sided adaptation	Capsicum annuum	PVY virus	No change	From leaves of living infected plants	Quantity of parasite induced damage caused to the host	Montarry et al., 2012
One sided adaptation to host, coevolution between parasite subspecies	<i>Plutella xylostella</i>	<i>Bacillus thuringiensis</i> subspecies <i>kurtstaki</i> (pathogen) and <i>tenebrionis</i> (non-pathogenic)	Decrease	From dead hosts	Mortality	Garbutt et al., 2011
Coevolution/ one-sided adaptation	<i>Pseudomonas fluorescens</i> SBW25	Phage SBW25F2	Increase	Environmental/ semi-natural	Reduction in growth due to infection	Hall et al., 2011
Coevolution	<i>Paramecium caudatum</i>	<i>Holospira undulata</i>	No clear change	Environmental/ semi-natural	Infectivity	Lohse et al., 2006
Coevolution	<i>Pseudomonas fluorescens</i> SBW25	Phage SBW25F2	Increase	Environmental/ semi-natural	Increased phage infectivity	Buckling and Rainey, 2002
Coevolution	<i>Tribolium castaneum</i>	<i>Paranosema whitei</i>	Decrease	From dead hosts	Mortality	Bérenos et al., 2009; 2011
Coevolution	<i>Escherichia coli</i>	Phage Q β	Decrease	Environmental/ semi-natural	Host fitness	Kashiwagi and Yomo, 2011
Coevolution	<i>Caenorhabditis elegans</i>	<i>Bacillus thuringiensis</i>	Increase	From dead hosts/ natural	Mortality	(Schulte et al., 2010)
Coevolution	<i>Tribolium castaneum</i>	<i>Paranosema whitei</i>	Increase	Environmental/ semi-natural	Mortality	Chapter 2
Coevolution	<i>Tribolium castaneum</i>	<i>Beauveria bassiana</i>	Increase	Environmental/ natural	Mortality	Chapter 3

Box 1. Mathematical models of virulence evolution.

Most models designed to examine predictions of virulence evolution are derived from a set of equations put forward by Anderson and May (1979) known as the SIR model. These equations consider a host population N to be constituted of susceptible individuals X , infected individuals Y and immune individuals Z . They include the rate of introduction of new hosts A and the background mortality of the population b . The population is said to be at equilibrium when $N^*=A/b$ i.e. birth rate is equal to death rate. A transmission coefficient β is introduced, with the rate of infection being defined as βXY . Disease induced mortality is defined as a , meaning that the mortality rate of infections is $b+a$. A recovery rate is considered, defined as v and immunity is predicted to be lost at a rate of γ , leading to equations 1-4.

$$\frac{dX}{dt} = A - bX - \beta XY + \gamma Z \quad (1)$$

$$\frac{dY}{dt} = \beta XY - (b + a + v)Y \quad (2)$$

$$\frac{dZ}{dt} = vY - (\gamma + b)Z \quad (3)$$

Which in turn lead to the following equation for the whole population:

$$\frac{dN}{dt} = A - bN - aY \quad (4)$$

These equations assume, however, that the parameters affecting parasite reproductive rate, β , a , b and v are not linked, which is contrast to what is the likely case in natural systems. Anderson and May (1982) adapted these equations to link the three parameters. Here they refer to parasite reproductive rate as R_0 , defined it as is shown in equation 5.

$$R_0 = \frac{\beta N}{(a+b+v)} \quad (5)$$

If the parameters are not linked a is optimised at 0, as predicted by the avirulence hypothesis. However, when parameters are linked increase in the parameters linked to virulence necessarily causes increases in virulence itself, dramatically changing predicted outcomes. R_0 has become one of the most discussed parameters in epidemiology, an R_0 value greater than 1 represents an outbreak, however, its dependence on all other parameters in the equation means that change in any of the parameters has an impact on whether an epidemic occurs.

These models create the backbone for numerous subsequent models designed for testing specific predictions. Day (2001), for example, further breaks down the transmission constant β into two components, the rate of contact between hosts and probability of transmission per contact, in order to examine the effect that different transmission mechanisms have on virulence evolution. Similarly, Bremermann and Pickering (1983) extended the model to include within host growth rate in order to examine the functional relationship between within host growth rate and parasite induced host mortality. There are an enormous amount of additional examples extending the model in various ways. More recent extensions have included consideration of the special case of sterilising parasites (O'Keefe and Antonovics, 2002), the interactions among propagule types (Lively, 2001) and the effect of coevolving hosts (Gandon, 2002) among countless others. Consequently, these models provide the basis of our understanding of how virulence evolves.

Box 2. Types of evolution experiment

Serial passage experiments

In serial passage experiments a parasite is transferred through several host organisms of the same species (Ebert 1998), usually a taxon that can be easily reared in the lab and often a host that is novel to the parasite. The mechanism of transmission is usually determined by the researcher, for example through injection or oral infection of parasite propagules extracted from previously infected hosts, which in most cases were not allowed to die from the infection. The initial aim of serial passage experiments was to adapt a parasite to a novel host causing an attenuation in virulence towards the ancestral host. The attenuated parasite could then be developed into a vaccine for use against the original parasite. A typical design of a serial passage experiment is detailed in figure i.

One-sided evolution experiments

One-sided or unidirectional evolution experiments are similar to serial passage experiments but differ in that adaptation is usually not to a novel host. Pathogens are still, however, adapting to a non-evolving host, usually through transfer from host population to host population after a set period of time. In these experiments specific aspects of the hosts biology, such as background mortality rate or

immunocompetence may be manipulated in order to test hypothesis relating to parasite evolution. One-sided evolution experiments are also often run in parallel to coevolution experiments to control for parasite adaptation to the host alone as opposed to reciprocal adaptations. An example of a typical one-sided evolution experiment is detailed in figure ii (C). In these experiments transmission is usually not natural but carefully controlled by the experimenter, often extracting parasite propagules from whole dead or alive individuals and often infecting the next host artificially in order to carefully control time of infection.

Coevolution experiments

In host-parasite coevolution experiments parasites are allowed to adapt to reciprocally adapting hosts. The main difference between these and the other two types of experiment is that hosts are also able to evolve. The design of a typical coevolution treatment is detailed in figure ii(B). Traits associated with parasite fitness are predicted to either steadily increase in value (as a consequence of recurrent selective sweeps, often termed arms race dynamics) or fluctuate over time (for example as a consequence of sustained cycles of negative frequency-dependent selection, often termed Red Queen dynamics). This creates a more complex and dynamic system which can be better related to certain natural situations. Again in these experiments transmission is carried out an artificial way, with propagules being

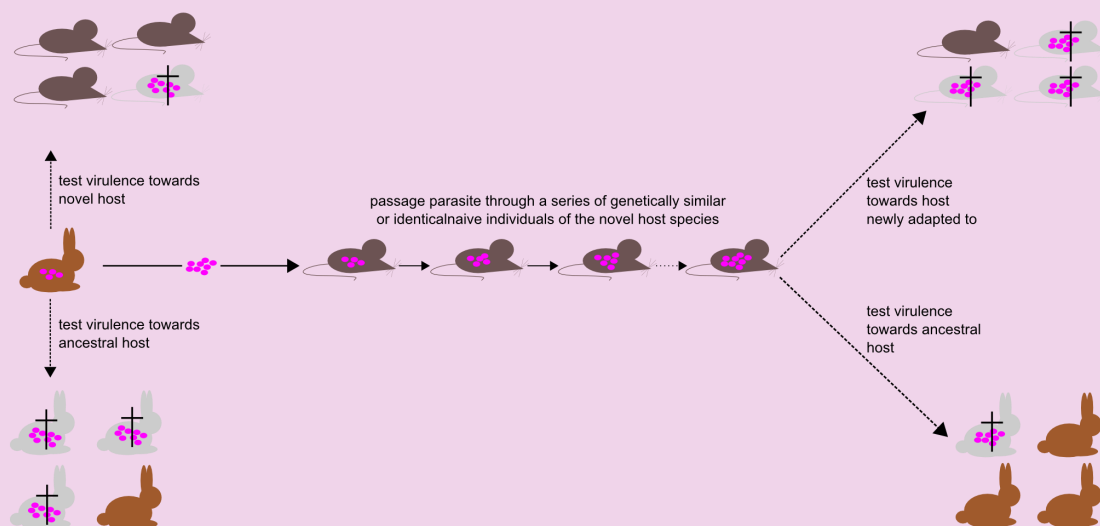


Figure i. A typical example of a serial passage experiment

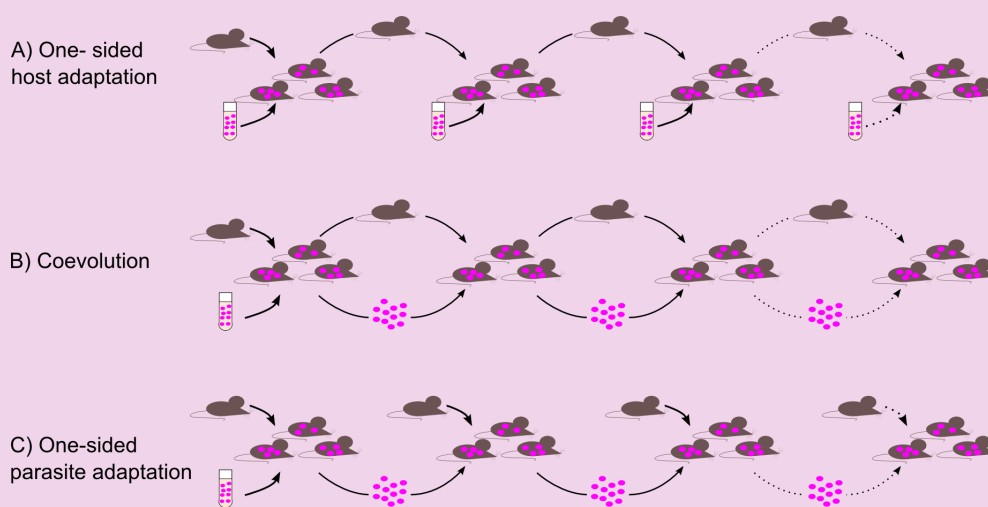


Figure ii. An example of a typical coevolution experiment (B) along with one-sided adaptation of both the host (A) and the parasite (C). Example (C) is also the basis of most one-sided evolution experiment designs.

Rapid evolution of virulence leading to host extinction under host-parasite coevolution

2

In the previous chapter I discussed how transmission, specifically the precise mechanism of transmission, is likely to effect the trajectory of parasite virulence evolution in evolution experiments. In the following chapter I present an empirical study where I carried out an evolution experiment with a relatively natural mechanism of parasite transmission. I indeed saw an increase in virulence as predicted.

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Abstract: Host-parasite coevolution is predicted to result in changes in the virulence of the parasite in order to maximise its reproductive success and transmission potential. The majority of coevolution experiments, however, use discrete parasite generations, discarding any spores or longer lasting parasite stages that made their way into the environment. For spore forming parasites, this differs from the likely natural scenario, where spores can survive for long periods of time in the environment and therefore may obtain the greatest evolutionary benefit by getting as many spores into the environment as possible. We carried out a coevolution experiment using the red flour beetle, *Tribolium castaneum*, and its natural microsporidian parasite, *Paranosema whitei*. Beetles and their environment, inclusive of spores released into it, were transferred from generation to generation. We additionally took a modelling approach to further asses the importance of transmissive parasite stages on virulence evolution. In all parasite treatments of the experiment coevolution resulted in extinction of the host population, with a pronounced increase in virulence being seen, contrary to previous results from the same system. This, in combination with results obtained from the model, highlights the importance of the inclusion of transmissive parasite stages when drawing conclusions about virulence evolution.

Introduction

During the course of host-parasite coevolution, reciprocal genetic changes are predicted to occur between the host and parasite (Hamilton, 1980). A key parasite trait often expected to change as a consequence of these dynamics is virulence (Anderson and May, 1982; Jensen *et al.*, 2006). Optimal virulence is reached by an evolutionary compromise between reproducing too rapidly within the host and reproducing at a suboptimal lower rate. Too rapid reproduction may result in reduction of their level of transmission, whereas too low reproduction would risk not extracting the maximum amount of possible resources from the host (Jensen *et al.*, 2006; Bérénos *et al.*, 2009). The outcome is likely to be highly dependent on the genetic diversity in the parasite population (May and Anderson, 1983; Barrett *et al.*, 2011) and the transmission mechanism of the parasite (Dunn and Smith, 2001; Kisdi and Boldin, 2013). Additionally, in experimental evolution the methods and parameters of the experiment may also play a role. For example, starting a coevolution experiment from a single parasite genotype might result in a less virulent parasite evolving in comparison to an experiment starting from a mixed stock, as competition pressure between differing genotypes would increase with the level of genetic diversity present (Read and Taylor, 2001). Furthermore, taking parasite propagules at a very specific time point may cause the parasite to evolve the optimal virulence for transmission at that time point rather than for the system as a whole.

The prediction of a trade-off between transmission and virulence is based on the idea that an increase in transmission rate should come at a cost in terms of duration of infection (Anderson and May, 1982; Bremermann and Pickering, 1983; Massad, 1987). Obligatory killing parasites are particularly interesting to consider in this respect as in their case transmission is itself dependent on death. Consequently, a reduction in virulence potentially comes at an extremely high cost, as transmission success is reduced to zero if the host survives (Ebert and Weisser, 1997; Bérénos *et al.*, 2009). In spite of this prediction, several studies have in fact provided evidence of a trade-off in obligatory killing parasites. Jensen *et al.* (2006) showed an

intermediate level of virulence to be optimal in the obligately killing parasite of *Daphnia magna*, *Pasteuria ramosa*. Furthermore, Bérénos et al (2009) showed that following co-evolution between *Tribolium castaneum* and *Paranosema whitei* virulence was reduced with the apparent benefit of increased spore production. However, in this system transmissive stages of the parasite entering the environment upon host death were not taken into account.

The majority of host-parasite co-evolution experiments using animal systems do not allow for transmissive stages of parasites to persist in the environment. Mechanisms of transmission involving prolonged environmental stages are, however, wide spread for intracellular parasites, including many of the horizontally transmitted microsporidia (Dunn and Smith, 2001) and therefore warrant consideration. Additionally, the presence of these stages has been hypothesised and theoretically predicted to have a substantial impact on virulence evolution, especially when they are long lived (Ewald, 1987). Known as “the curse of the pharaoh hypothesis”, mathematically formalised by Bonhoeffer et al. (Bonhoeffer *et al.*, 1996), the cost of virulence is hypothesised to be reduced for long lived parasites as they are able to endure periods of low host density or even complete host absence (Ewald, 1987; Bonhoeffer *et al.*, 1996). Furthermore, the presence of transmissive stages in the environment can increase the genetic diversity of the parasite population, potentially increasing competition between parasite genotypes, resulting in higher virulence due to reduced returns in terms of inclusive fitness (Frank, 1996).

In addition, the inclusion of transmissive stages in evolution experiments may also be critical for the effects of a trade-off, or lack thereof, to be realised. If spores are extracted from dead host at a specifically defined time point, as in most experimental evolution protocols (e.g. Kolodny-Hirsch and Van Beek, 1997; Raymond *et al.*, 2009; Bérénos *et al.*, 2009; Schulte *et al.*, 2010; Bérénos *et al.*, 2011), then spore numbers should be maximised for this exact time point and the virulence-transmission trade-off should be adjusted accordingly. If, however, transmissive stages are allowed to persist in the environment, as realistic under

most natural conditions, then selection may also favour higher virulence levels, as the earlier produced spores will not be lost from the experiment. We carried out a host-parasite coevolution experiment using the red flour beetle, *Tribolium castaneum* and its natural, obligately killing microsporidian parasite *Paranosema whitei* (Milner, 1972b; Blaser and Paul Schmid-Hempel, 2005) in order to test the consequences of environmental spore persistence on the dynamics of the interaction. *P. whitei* infects early stage larvae and usually kills in the late larval or early pupal stage (Milner 1972b; Blaser & Schmid-Hempel 2005). Spores are released upon host death and can persist for extended periods of time without losing infectivity (Milner, 1972a). In our experiment, parasite stages were given the opportunity to enter and remain in the environment, providing potential benefits of increased parasite longevity, and host generations were allowed to partially overlap. We additionally took a modelling approach in order to explain our results in the context of both the presence of environmental parasite stages in general and in the specific case of the curse of the pharaoh hypothesis.

Methods

Host

Tribolium castaneum is a tenebrioid beetle, that inhabits human grain stores, with the earliest recorded association with humans being in Egyptian times (Sokoloff, 1972). The *Tribolium castaneum* stock Cro1 (Milutinović *et al.*, 2013a; Milutinović *et al.*, 2013b), was used to start the evolution experiment. The line had undergone around 20 generations of adaptation to standard lab rearing conditions prior to use in the experiment.

In order to produce adults of a similar age with which to start the experiment, 20 oviposition jars (Bardenhewer, Kiel, Germany) were set up five weeks prior to the experiment start date. Each jar contained 140g of flour (Alnatura type 550) plus 5% brewer's yeast (Leiber, Bramsche, Germany). Approximately 200 adult beetles, of the strain Cro1, were transferred to each jar. The beetles were then left to oviposit for 14 days, following which they were removed. Offspring were left to

develop for a further three weeks, at the end of which there were a large number of adult beetles. 12 random jars were sieved and the adults taken off then mixed and used to start the experiment. Throughout the experiment beetles were stored at 32°C with 70% humidity and in complete darkness.

Parasite

Microsporidia are single celled eukaryotes that obligately parasitize cells of other eukaryotic organisms (Keeling and Fast, 2002). *Paranosema whitei*, formally *Nosema whitei* (Sokolova *et al.*, 2005; Sokolova *et al.*, 2003), is a specific microsporidian parasite of scarabid beetles, particularly *Tribolium* spp. (Milner, 1972a). The *P. whitei* isolate used to start the experiment was kindly provided by the lab of G. Bucher (University of Göttingen). In order to generate enough microsporidian material to start the evolution experiment, the material from Göttingen was taken split evenly between 5 standard 50ml tubes (Sarstedt, Nuembrecht, Germany), the 50ml tubes filled up to the 15ml line with a mixture of flour (Alnatura type 550) plus 5% brewer's yeast and the tubes vortexed thoroughly at multiple angles. 20 beetles from an outbred population generated from 4 different lab stock lines, GA2, 50, 51 and 61 which are of varied geographical origin, excluding the Cro1 line, were allowed to oviposit on the inoculated flour for 3 days, following which the flour was sieved and the adults removed. The tubes were subsequently sieved weekly and dead larvae collected until all larvae had died or become adults. This process was repeated twice on the same flour. The collected larvae were finally ground with a pestle and mortar and used to start the experiment. Spores were stored in the fridge at 4°C, under which conditions they have previously been shown to remain infective for extended periods of time (Milner, 1972a).

Experimental set-up

The evolution experiment consisted of four treatments, three different environmental concentrations of *P. whitei*, representing substantially differing levels of beetle mortality over a single generation, and a control treatment consisting of standard flour with 5% yeast mixture. The *P. whitei* concentrations used were 10²

(low), 10^3 (intermediate) and 10^4 (high) spores per g flour. Concentrations were chosen to be in the range of those used in previous experiments (Milner, 1972b; Blaser and Paul Schmid-Hempel, 2005; Béréños *et al.*, 2009) and to show strong differences in survival with the ancestral population based on preliminary assays (Rafaluk unpublished). Each treatment group consisted of 7 replicate populations. To start the experiment the spore concentration of the ground larvae mixture was counted, using a “Fast Read 102” disposable counting chamber (Biosigma, EU), and a portion diluted to 10^4 in a mixture of flour (Alnatura type 405) containing 5% yeast. This flour spore mixture was vigorously mixed in a large jar to produce an even distribution of spores. The lower concentrations were diluted following the same method, but with the relevant amount of the spore flour mixture from the next highest treatment. Each population was started with 17g of the relevant flour or flour spore mixture in a 50ml tube. 100 age synchronised adults of the line Cro1, synchronised as described above but with a slightly longer oviposition period of 7 days, were then added to each population and left for three weeks to oviposit and their offspring to become pupae.

Following this initial 3 week period all populations were sieved. 11g of the flour from the previous generation was then put back into the tube and 5g of new flour as food, mixed from the collected stock of spores where relevant and following the methods described above, was also added. A cage made from a standard 15ml tube (Sarstedt, Nuembrecht, Germany) with two 4cm long sections removed and replaced by 1 X 0.5mm plastic mesh. The remaining beetles from the parental generation were placed into this cage and the cage sealed. The aim of the cage system was to produce semi-natural conditions with overlapping generations present, whilst still allowing for generations to be distinguished. In addition the cage allowed for contact and chemical communication between parents and offspring. To start the new generation 100 pupae were counted and added to the area of the large tube outside of the cage. If there were less than 100 pupae, the largest larvae were added to make the number up to 100, when possible. As populations approached extinction this became no longer possible and as many

larvae and pupae as were present were added. After two weeks, at the point when the pupae had become fully matured adults, the cage and adults from the previous generation were removed. This procedure was then repeated every 4 weeks until population extinction. The setup of the evolution experiment is schematically represented in figure S1.

End concentration

As microsporidia spores are difficult to differentiate from flour grains via microscopy, the end spore concentration in the flour environment was determined by qPCR. A dilution series of spores, counted using the counting chamber method of spore quantification described above, was run in parallel for calibration. The 50ml tube containing the final spore flour mixture was kept and transferred to the fridge for storage. Following extinction of all populations, 1g of flour was sampled from each tube and total DNA extracted from the mixture using a standard Phenol-Chloroform extraction method. A subsample of the extracted DNA was then further purified (Machery & Nagel blood and Tissue DNA purification kit) for qPCR use and the quality and concentration checked using a Nanodrop® (ND-1000). qPCR was carried out using an Applied Biosystems 7300 qPCR machine in 15µl reaction volumes with 20ng of genomic DNA, pre-prepared qPCR mastermix with ROX (Eurogentec, Brussels, Belgium), the primers NW_16s_forw: 5'-GCT AGT CTT GGG GAC TTA GCC-3' and NW_16s_rev : 5'-GGCCCT TAC CCT ACC TAC CA-3' and a double labelled probe 5'-FAM-TAA GAC ATG GAC AGG CTG CA-6-BlackHoleQuencher1-3', following the methods and conditions of Wegner *et al.* (2008). A dilution series of *P.whitei* DNA of known concentration was run in parallel for standardisation.

Survival assay

For the survival assay dead larvae were taken from the experimental populations at the point of extinction, ground with a pestle and mortar and the spore concentration counted as described above. Spores were then diluted in flour to a concentration of 10^3 spores per gram, the initial intermediate concentration. The assay was carried out in small glass vials (40 x 13mm) (Assistant, Sondheim/Rhoen,

Germany) sealed with cotton wool filters (Herenz, Hamburg, Germany), following the methods of Blaser and Schmid-Hempel (2005). 0.17g of flour, where appropriate inoculated with the relevant spores, was weighed into each vial and a single young larva added. Thirty vials, each containing an individual larva were set up for each isolate. The ancestral isolate and spore free flour were tested in parallel. Tubes were checked every other day to identify whether each individual was alive or dead, and this process continued until no new dead individuals were recorded at two successive time points.

Spore load of individual dead larvae

Spore load was calculated for individuals infected with each microsporidian strain isolated from a population with a 10^2 (low) starting concentration of microsporidia, hereafter referred to as “low isolate”, used in the survival assay, as well as the ancestral parasite. 10 adults of the Cro1 stock line were allowed to oviposit on 5g flour containing 10^5 spores per gram of the relevant isolate for 7 days, following which they were removed and discarded. Offspring were allowed to mature for a further 5 weeks, following which 3 dead larvae from each isolate were taken and ground individually in 1ml water plus a small amount of Tween® 80 (Carl Roth, Karlsruhe, Germany) in a 2ml tube (Sarstedt, Nuembrecht, Germany) using a 2000 Geno/Grinder ball mill homogeniser for 15 seconds at a speed of 500rpm with 2mm diameter steel balls. Spores present in the homogenised solution were subsequently counted using a “Fast Read 102” disposable counting chamber (Biosigma, EU). This method was used in order to most accurately obtain all the spores contained in a single larva.

Statistical analysis of experimental data

All statistics were carried out in R version 2.15.1 (R core team 2013). Survival data were analysed using a series of pairwise binomial tests and corrected for multiple testing using the false discovery rate method, in order to compare each evolved line to the ancestral line at every time point. A method used previously by Henry et al. (Henry *et al.*, 2012) to assess homing probability in bees over time. The relationship

between spore load and parasite induced mortality was analysed using a spearman's rank test. All graphs were created using the R package ggplot2.

The model

We took a modelling approach in order to determine whether the observed increase in virulence could potentially be explained by the environmental transmissible propagules of the parasite. We augmented the model of Anderson and May (1981), which incorporates environmental parasites stages to include a larval and adult life-stage, in order to include both the structure of the host population and the environmental parasite propagules. Additionally, following Bonhoeffer *et al.* (1996), we incorporated the assumption that larvae are infected through spore uptake from the environment in order to better represent our system. Details of the model can be found in the supplementary information. The model and simulations were run in MatLab version R2010b.

Results and Discussion

All populations experimentally evolving with *P. whitei* went extinct between transfers 2 and 5 of the evolution experiment. By the third transfer there were no adults present in any population in the high treatment to start the next generation, by the fourth in the intermediate treatment and by the fifth in the low treatment (Figure 1.).

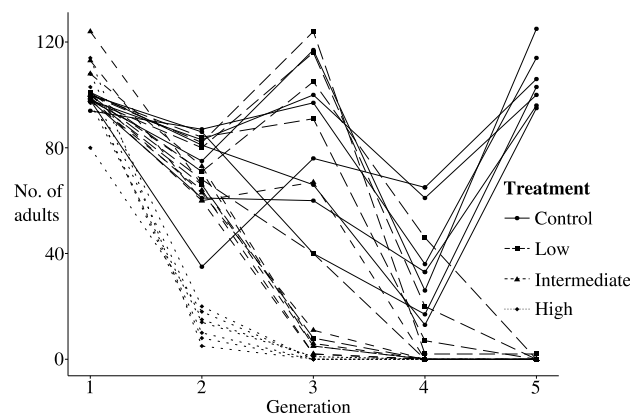


Figure 1. Number of adults alive in each population at each generation of the evolution experiment.

In order to establish whether an accumulation of parasite particles in the environment may have played a role, the end concentration of spores in the environment was measured by qPCR. Interestingly, regardless of the starting concentration of the population, or of the time until extinction, qPCR showed that the end concentrations of spores in the flour were all in the range of 10^6 spores per g flour and did not differ significantly from one another (ANOVA: $F=0.42$, $d.f.=2,15$, $p=0.6$) (Table S1.). This is consistent with the findings of (Milner, 1972b), who showed 10^6 to be a critical dose of *P. whitei*, above which the majority of individuals die as larvae. Our results therefore indicate that spore accumulation in the environment may have contributed to host extinction.

In spite of the observed increase in spore concentration it is possible that this alone was not responsible for host extinction and that an increase in virulence also contributed to host extinction. To test this we carried out a survival assay. Spores from dead larvae were collected from five low and five intermediate treatments for use in the assay. All spore isolates from the initial low treatment populations showed higher mortality than the ancestral isolate. In the case of the isolates from the intermediate treatment populations the result was more varied with some showing increased mortality in comparison to the ancestral isolate and some showing a slightly lower mortality than the ancestral isolate (Figure 2). Pairwise binomial tests for each time point showed that towards the end of the assay the 4 most virulent isolates differed significantly in virulence from the ancestral population, with no lines being significantly less virulent than the ancestral line (Figure 2, Table S2).

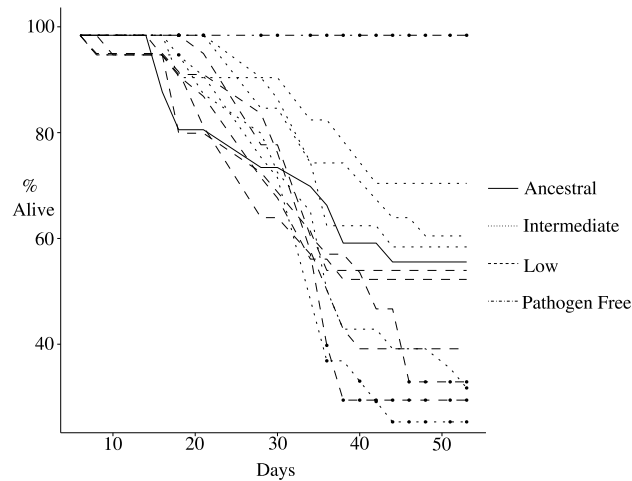


Figure 2. Percentage of individuals alive for each treatment at each time point. Percentage calculated in relation to the number of individuals recovered at the first time point. Diamonds indicate time points at which each evolved line differed significantly from the ancestral line (pairwise binomial tests).

There are several mutually non-exclusive reasons that could explain why only some of the intermediate treatment populations evolved higher virulence. One is that the opportunity for parasite evolution was constrained because the host killing rate, caused by higher starting concentration, was already at the upper limit of optimal virulence for this experimental setting. As a consequence of the higher starting concentration, infection probability would have been higher. This could have resulted in semi-random infection of beetles by various spore genotypes each infecting so many beetles that when the individuals from the first round of infection burst, transmitting the microsporidia, the maximum tolerable microsporidian concentration for the beetles was already reached. A second potential explanatory factor is that in the low population any allele with heightened reproductive success would instantly contribute to a higher proportion of the total environmental spore composition than in the intermediate concentrate, making its spread more efficient. The increase in virulence observed here is in stark contrast to the findings of other evolution experiments using the same host-parasite system, where a significant decrease in virulence was seen to occur (Bérénos *et al.*, 2009; Bérénos *et al.*, 2011).

The experiments aimed at approaching slightly different questions and therefore differed in some aspects of the methodology, potentially explaining this contrast. A major difference was the management of the parasite including the way in which the parasites were transferred from one generation to the next and the control of parasite concentration. In the experiments of Béréños *et al.* (2009; 2011) transmission of spores to the next generation was more controlled than in our experiments, with spores being collected purely from dead larvae, with any spores that were released into the environment being discarded. In their case dead larvae were collected within a time window of 35-50 days post egg laying, whereas in our experiment dead larvae were removed after 28 days, meaning that larvae releasing spores way before the time window used by Béréños *et al.* (2009; 2011) would have had a selective advantage. Furthermore, as spore concentration in the environment was carefully controlled by Béréños *et al.* (2009; 2011), the level of competition for infection between spore genotypes may have been reduced.

In the experiments of Béréños *et al.* (2009; 2011) a trade-off was observed between spore load of dead larvae, in the case of obligate killers a proxy for transmission, and virulence, which was the most parsimonious explanation for the decrease in virulence observed in their experiments (Béréños *et al.* 2009). We, however, found no correlation between the level of virulence of each of the lines and the average spore load per larvae (Spearman's rank test: $S=46$, $p=0.56$) (Figure 3), meaning that in our case an increase in virulence did not appear to come at a cost of decreased transmission potential.

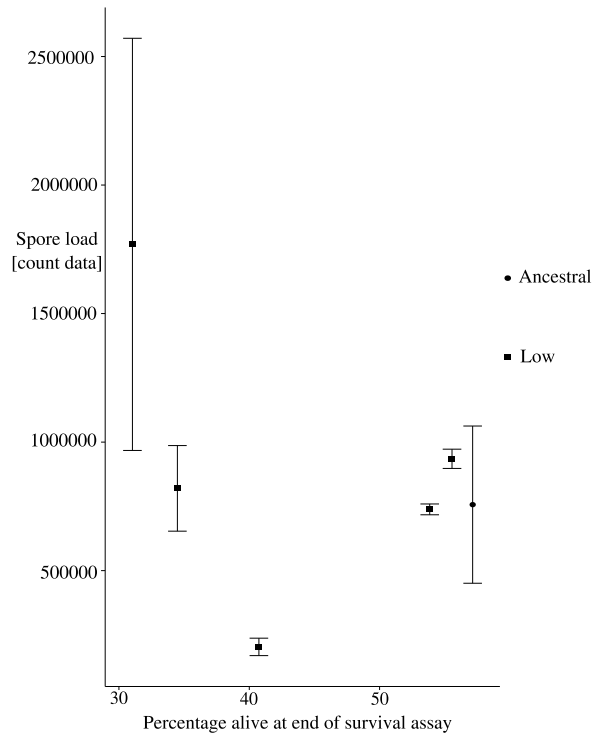


Figure 3. Correlation between percentage of individuals alive at the end of the survival assay and average spore load of individual larvae [count data] from 3 individuals from each line.

The lack of virulence/ transmission trade-off we see here, although consistent with some theoretical predictions (Gandon et al 2013), is inconsistent with previous empirical evidence that spore production/ virulence trade-offs do occur in obligately killing parasites (Jensen *et al.* 2006; Bérénos *et al.* 2009). In contrast to previous studies with *Paranosema whitei*, we did not observe obvious gigantism occurring in infected larvae (Blaser and Schmid-Hempel, 2005), perhaps the potential for spore production was less to start within our parasite population as a result of lack of induction of gigantism.

In order to further investigate the role of transmissive stages in the evolution of virulence we created a modified version of the “curse of the pharaoh” model (Bonhoeffer *et al.*, 1996) to fit a simplified version of the *T. castaneum*-*P. whitei* system. This approach allowed us to further explore the question of whether transmissive stages of the parasite being allowed to persist in the environment

plays a role in the evolutionary dynamics that we would expect to see in this system.

A mathematical simulation of the survival assay we carried out produced similar simulated mortality rates at differing levels of virulence to those seen with the evolved strains from the evolution experiment (Figure S3). This suggests that increased virulence levels could potentially account for the increased mortality seen in the evolved lines. Additionally, in the model low host density can only be achieved with increased virulence, with higher spore concentrations alone not leading to host extinction. This provides support to our hypothesis that virulence, rather than concentration alone, played a role in population extinction (Figure S4).

Evolutionary invasion analysis (Otto and Day, 2007) carried out with the model produced two possible explanations for host extinction. The first is that both reduced spore mortality and virulence evolved in parallel, reducing the cost of increased virulence as longer living spores are able to survive periods of temporary host absence or low population size under these circumstances, consistent with results based on the original model (Bonhoeffer et al., 1996). Under these circumstances high virulence in environmentally transmitted parasites should evolve if invasive mutants have higher survival outside of the host and the resident population was not at an evolutionary stable strategy (ESS) (Figure 4).

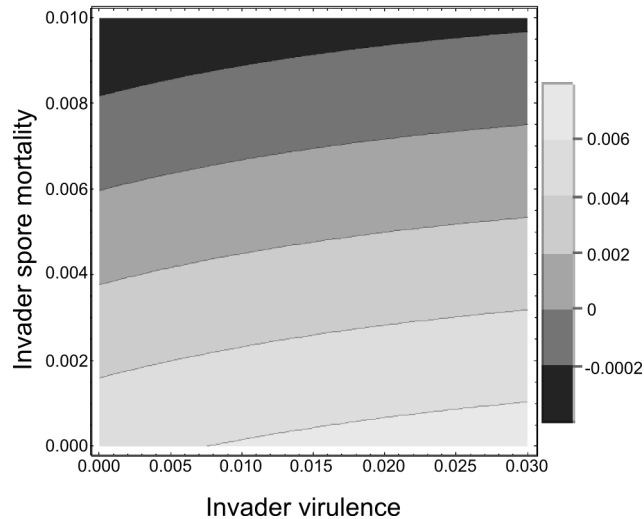


Figure 4. ‘Pharaoh Dynamics’ if the resident allele is not at an ESS decreasing spore mortality in the environment will always select for higher virulence. Greyscale represents values of resident R_0 . The following parameter values were used in this simulation propagule transmission (b) = 0.00083; Larval maturation (m) = 0.03; Adult removal (u) = 0.07; Carrying capacity (C) = 100; Larval birth (l) = 0.33; Larval background mortality (d) = 0.03; Resident spore mortality (k) = 0.0075; Resident virulence (v) = 0.03, Spore shedding (B_2) = $750000 / (v^2 + 0.5)$ and (B) = $750000 / (v + 0.5)$.

Interestingly however, when the trade-off between virulence and spore load was considered to be low or non-existent in the model, runaway virulence was predicted to evolve. Evolutionary invasion analysis showed that virulence mutants could invade a population of resident parasites at equilibrium if their reproductive factor was > 0 (Figure 5). Given our finding that in our evolved material there did not appear to be a trade-off between virulence and spore load (Figure 3) this would provide a more simple explanation than decreased spore mortality being the driving factor in the observed evolution of virulence.

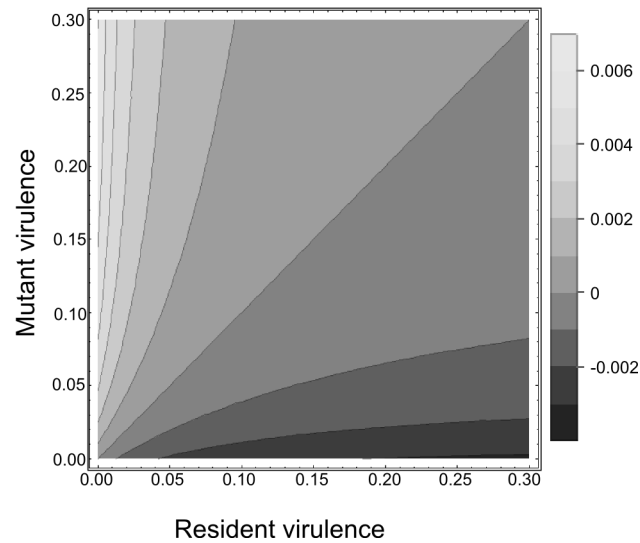


Figure 5. ‘Runaway virulence’, pairwise invisibility of virulence mutants with differing resident alleles in the absence of a virulence/spore production trade-off. Greyscale represents values of R_0 . The following parameter values were used in this simulation propagule transmission (b) = 0.00083; Larval maturation (m) = 0.03; Adult removal (u) = 0.07; Carrying capacity (C) = 100; Larval birth (l) = 0.33; Larval background mortality (d) = 0.03; Spore mortality (k) = 0.0075; Spore shedding (B) = 750000 and (B_2) = 750000.

Of the possible explanations for the increase in virulence observed here, we consider the hypothesis of a lacking trade-off between virulence and spore production is the most likely driver of the evolved increase in virulence. Despite the general presence of this trade-off being somewhat controversial (Ebert and Bull, 2003; Alizon *et al.*, 2009; Froissart *et al.*, 2010), most published studies showing a change in virulence demonstrate that trade-offs of some kind provide a limitation on its evolution. Even in bacteria-bacteriophage systems where transmissive stages have been included, decreases in virulence of evolutionary time have been observed. For example, Heineman and Brown (2012) observed in an *Escherichia coli*- phage system that increased phage longevity came at a cost to reproductive rate, a proxy for virulence. In this system however, although phages were allowed to burst kill and persist in the environment, the persistence phase in harsh conditions was a separate experimental step from the replication in *E. coli*, potentially eliminating the possibility for alternative strategies to evolve and compete (Heineman and Brown, 2012). Examples of increased virulence do also exist, for example, (Little *et al.*, 2006)

demonstrated in the *D.magna* – *P. ramosa* system that *P. ramosa*, a burst killer, increased in virulence upon adaptation to a specific host genotype without transmissive stages being present, even though King *et al.* (2013) have demonstrated empirically that long lived stage are formed. This was, however, a serial passage experiment, where the host could evolve in response and it has been shown empirically in the same system that an optimal level of virulence exists (Jensen *et al.*, 2006). Our study is a rare example alluding to its potential presence of apparent run-away virulence in an empirical system.

It is important to note that in our experiment the parasite is not extinct, only the host, when considering the results of this experiment in relation to how virulence may evolve in nature. The ecology of the parasite is critical to its evolutionary success. If host migration would have been present in our experimental design, the parasite would not have been at a disadvantage at all as a result of making the contemporary population extinct. As the spores are long lasting, even if a new susceptible host migrated into the environment a year in the future the parasite would be able to still infect it (Milner 1972; Blaser & Schmid-Hempel 2005). Invasion by new host individuals or even a population is likely the case in nature and may explain why an obligately killing parasite was able to evolve in this system in the first place. Resultantly, the parasite would not necessarily lose any fitness advantage under natural conditions by displaying this trait (Bonhoeffer et al 1996; Gandon 1998).

Conclusion:

When considering the evolutionary trajectories of traits under host-parasite co-evolution many ecological factors have to be considered and slight changes in these may have dramatic effects on the outcome. Here we have found evidence indicating that the presence of transmissive stages of a spore forming parasite may have a strong influence on the outcome of evolutionary dynamics. We have also demonstrated that even in experiments with the same host-parasite system, differences in methodology and handling can lead to opposite results. All in all we

feel that this highlights the need to carefully consider all aspects of an organisms' ecology when planning experiments and drawing conclusions. Furthermore, we feel that the consideration of transmissive parasite stages deserve more attention in empirical work on host-parasite co-evolution.

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S.1. The model

The model detailed below aimed to investigate the effect of transmissive parasite stages on virulence evolution in the *Tribolium castaneum* – *Paranosema whitei* system. The model is based on a modified version of the Anderson and May (1981) model for invertebrate hosts, which incorporates environmental parasite stages. We modified the model to include a larval and adult life-stage, importantly in our system only susceptible larvae can become infected after spore uptake from the environment. In our model we investigate a special case of Anderson and May's model, incorporating the assumption that a total number of spores are released into the environment when an infected larva dies. For comparability with Bonhoeffer *et al.* (1996) who created a general model to test assumptions of the Pharaoh hypothesis, we assumed that parasite density is not influenced by host uptake.

Stable equilibria for spores and host population were calculated in relation to the observed levels of virulence (Figure S4). The model was then extended further to include a virulence mutant genotype and a virulence spore-production trade-off in order to investigate the conditions under which virulence evolved. In simulations of the evolution experiment, adult mortality was increased to simulate the effect of their artificial removal during the experiment.

Susceptible larvae S are infected by pathogenic spore W uptake through the environment at the rate $\hat{a} * W * S$ through which they become infected individuals I . Susceptible larvae are produced by adults at the rate $l * A$. Background mortality given by $d * S$ or $d * I$ respectively. Larvae mature at the rate $m * S$ or $m * I$. However, due to cannibalism the carrying capacity C acts on maturation. If the carrying capacity is reached, maturing larvae are lost. Adults are only lost at their mortality rate $u * A$. Pathogen induced mortality $v * I$ kills infected larvae. Once an infected individual dies after $1/(v+d)$, B number of spores are shed into the environment. The spores in the environment W decay at the rate $k * W$.

To investigate the evolutionary dynamics, we extended the model to include two pathogen genotypes W_2 and I_2 differing in virulence v_2 . We assumed a trade-off between spore production and virulence, meaning that with increased virulence the number of spores produced per dead larva B is reduced. This was incorporated by assuming that $B = Z/(v+x)$ where Z is the maximum number of spores that can be generated from one corpse and x is some trade-off constant. Full details of parameter values can be found in table S3.

Epidemiological model:

$$\frac{dA}{dt} = m(S + I + I_2) \left(1 - \left(\frac{A}{C}\right)\right) - uA, \quad (1)$$

$$\frac{dS}{dt} = -\beta S(W + W_2) + lA - (d + m)S, \quad (2)$$

$$\frac{dI}{dt} = \beta WS - (v + d + m)I, \quad (3)$$

$$\frac{dW}{dt} = B(v + d)I - kW, \quad (5)$$

$$\frac{dI_2}{dt} = \beta W_2 S - (v_2 + m + d)I_2, \quad (6)$$

$$\frac{dW_2}{dt} = B_2(v_2 + d)I_2 - kW_2. \quad (7)$$

$$\begin{aligned} B &= Z/(v + x) \\ B_2 &= Z/(v_2 + x) \end{aligned} \quad (8)$$

Table S1. Start and end *P. whitei* concentrations of evolved populations.

Starting Concentration	Replicate	End Concentration
10 ² (low)	1	3.7X10 ⁶
	2	5X10 ⁶
	3	2.2X10 ⁶
	5	4.5X10 ⁶
	7	5.6X10 ⁶
10 ³ (intermediate)	2	2.5X10 ⁵
	3	2.3X10 ⁶
	4	1.4X10 ⁶
	5	3X10 ⁶
	6	2.1X10 ⁶
	7	6.1X10 ⁶
	10 ⁴ (high)	2
3		3.7X10 ⁶
4		2.4X10 ⁶
5		3.3X10 ⁶
6		2.6X10 ⁶
7		4.6X10 ⁶

Table S2. Results of binomial tests comparing proportions of alive and dead beetes following exposure to each of the evolved *P. whitei* isolates in comparision to the ancestral isolate. In all cases comparison is to the proportion of individuals alive in the ancestral treatment on each day of the survival assay.

Days	Treatment	Replicate	Successes	Trials	p value
6, 8, 10 and 12	Intermediate	1	29	29	n/a
		2	25	25	n/a
		4	26	26	n/a
		5	25	25	n/a
		7	27	27	n/a
	Low	3	27	27	n/a
		4	26	26	n/a
		5	29	29	n/a
		6	29	29	n/a
		7	27	27	n/a
16	Intermediate	1	29	29	0.0677
		2	25	25	0.1036
		5	25	25	0.1036
		4	26	26	0.1056
		7	26	26	0.1056
	Low	6	28	28	0.06714
		5	29	29	0.0677
		3	26	26	0.1056
		7	26	26	0.1056
		4	25	25	0.5182

18	Intermediate	2	23	25	0.295
		1	29	29	0.005976
		7	26	26	0.009223
		5	25	25	0.01516
		4	25	26	0.07107
	Low	7	22	26	1
		5	29	29	0.005976
		6	27	28	0.04759
		3	25	26	0.07107
		4	24	26	0.3009
21	Intermediate	2	23	25	0.295
		1	29	29	0.005976
		5	25	25	0.01516
		4	24	26	0.3009
		7	24	26	0.3009
	Low	7	22	26	1
		5	28	29	0.0486
		3	25	26	0.07107
		4	23	26	0.6074
		6	24	28	0.8065
28	Intermediate	4	20	26	1
		2	23	25	0.06178
		5	23	25	0.06178
		1	25	29	0.2009
		7	22	26	0.3648
	Low	7	20	26	1
		3	23	26	0.171
		6	27	28	0.3852
		5	23	28	0.5136
		4	19	26	0.8218

30	Intermediate	7	20	26	1
		5	23	25	0.06178
		2	22	25	0.1675
		1	25	29	0.2009
		4	19	26	0.8218
	Low	3	21	26	0.652
		6	19	28	0.3852
		4	18	26	0.4992
		5	23	28	0.5136
		7	19	26	0.8218
34	Intermediate	2	19	25	0.825
		4	13	26	0.02704
		5	21	25	0.1902
		1	22	29	0.6854
		7	18	26	0.8289
	Low	6	17	28	0.2134
		4	15	25	0.2663
		3	16	26	0.2798
		5	18	28	0.4063
		7	17	26	0.5167
36	Intermediate	4	10	26	0.002499
		5	21	25	0.09031
		7	14	26	0.1424
		1	22	29	0.43
		2	16	25	0.6725
	Low	6	12	28	0.00741
		3	14	26	0.1424
		7	15	26	0.2951
		5	17	29	0.3208
		4	15	25	0.3979

38	Intermediate	1	22	29	0.127
		4	10	26	0.02607
		5	20	25	0.0635
		7	12	26	0.1593
		2	16	25	0.8393
	Low	4	14	25	0.684
		6	9	28	0.002997
		3	12	26	0.1593
		7	15	26	0.8414
		5	17	29	0.8504
40	Intermediate	4	9	26	0.008353
		5	19	25	0.1516
		7	12	26	0.1593
		1	21	29	0.2543
		2	16	25	0.8393
	Low	4	14	25	0.684
		6	9	28	0.002997
		3	11	26	0.06955
		5	16	29	0.5714
		7	15	26	0.8414
42	Intermediate	4	8	26	0.002288
		7	12	26	0.1593
		5	18	25	0.3081
		1	20	29	0.4486
		2	16	25	0.8393
	Low	4	14	25	0.684
		6	9	28	0.002997
		3	11	26	0.06955
		5	14	29	0.1858
		7	15	26	0.8414

44	Intermediate	2	15	25	1
		4	7	26	0.000855
		7	11	26	0.06955
		5	18	25	0.3081
		1	19	29	0.7052
	Low	4	14	25	0.684
		6	9	28	0.002997
		3	11	26	0.06955
		5	14	29	0.1858
		7	15	26	0.8414
46	Intermediate	2	15	25	1
		4	7	26	0.000855
		7	11	26	0.06955
		5	18	25	0.3081
		1	19	29	0.7052
	Low	4	14	25	0.684
		6	9	28	0.002997
		5	10	28	0.01051
		3	11	26	0.06955
		7	15	26	0.8414
48	Intermediate	1	18	29	1
		2	15	25	1
		4	7	26	0.000855
		7	11	26	0.06955
		5	18	25	0.3081
	Low	4	14	25	0.684
		6	9	28	0.002997
		5	10	28	0.01051
		3	11	26	0.06955
		7	15	26	0.8414

51	Intermediate	1	18	29	1
		2	15	25	1
		4	7	26	0.000855
		7	10	26	0.02607
		5	18	25	0.3081
Low	4	14	25	0.684	
	6	9	28	0.002997	
	5	10	28	0.01051	
	3	11	26	0.06955	
	7	15	26	0.8414	
53	Intermediate	1	18	29	1
		2	15	25	1
		4	7	26	0.000855
		7	9	26	0.008353
		5	18	25	0.3081
Low	4	14	25	0.684	
	6	9	28	0.002997	
	5	10	28	0.01051	
	3	11	26	0.06955	
	7	15	26	0.8414	

Table S3. Parameter values included in the model. These parameters were used to run simulations for all figures.

Variable	Description	Value	Reference
A	Density of adult beetles	Was set to 100 individuals at beginning of	Number of beetles experiment started from
S	Density of susceptible larvae		
I	Density of infected larvae		
W	Density of pathogen propagules in environment		
J2	Density of larvae infected with pathogen genotype 2		
W2	Density of pathogen genotype 2 propagules in environment		
m	Maturation constant	0.03	Calculated from life tables in (Sokoloff, 1972)
\hat{a}	transmission constant	0.00083	
k	propagule decay constant	0.0075	Calculated from spore longevity values (Milner, 1972)
u	adult removal constant	0.003 (in evolution experiment 0.07)	Based on artificial removal of adults in the experiment
C	Carrying capacity	100	Imposed by the experiment
l	birth of susceptible larvae	0.33	Rafaluk (Unpublished)

d	background larval mortality	0.03	Calculated from life tables in (Sokoloff, 1972)
v	virulence	0.3	Calculated from average time point of death
B	Number of propagules shed into the environment shed	With trade off $750000 / (v + 0.5)$, without trade-off:	Calculated based on spore counts (Rafaluk unpublished)
Z	Maximum number of propagules in the absence of trade-off shed into the	With trade off: $750000 / (v^2 + 0.5)$, without trade-off: 750000	Calculated based on spore counts (Rafaluk unpublished)
x	some trade-off constant	0.6	

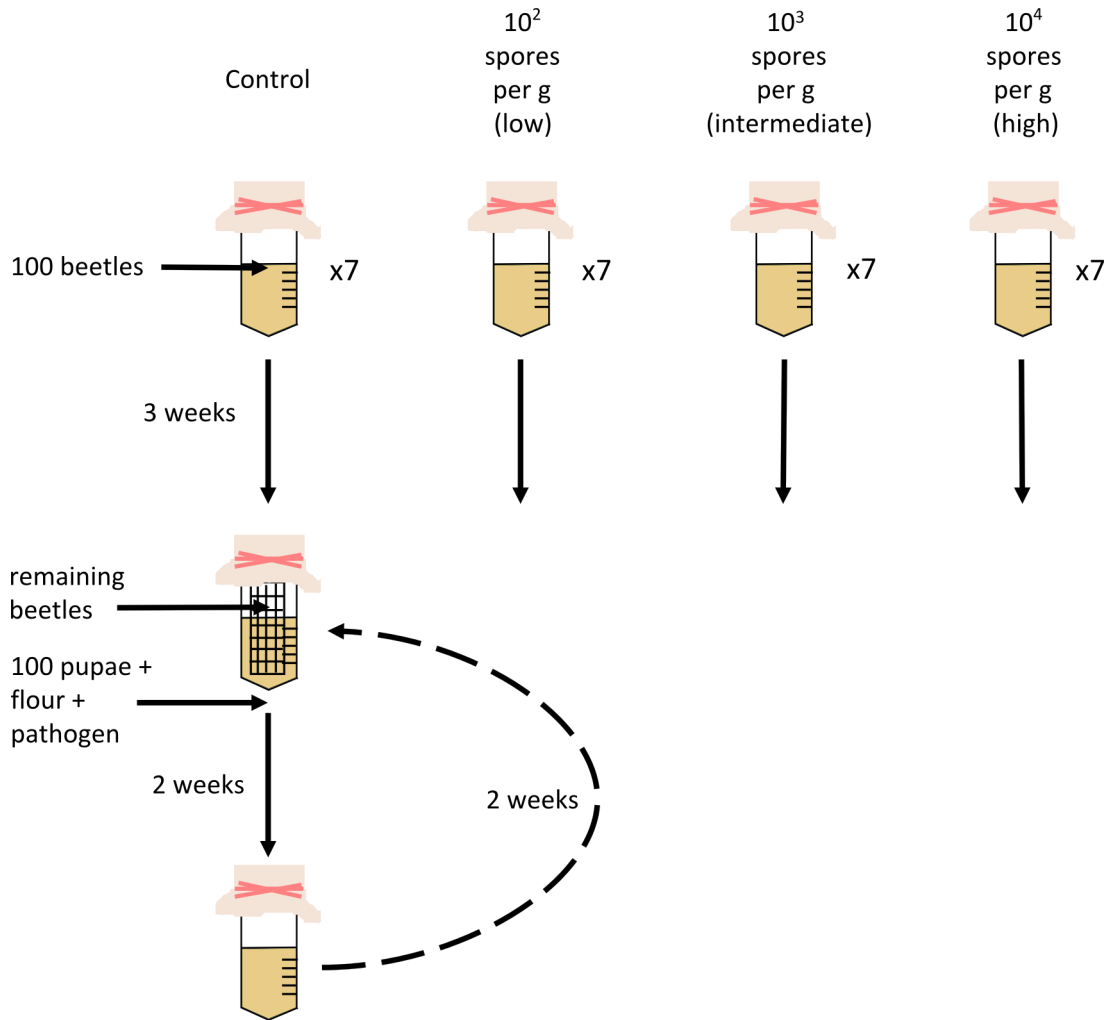


Figure S1. Diagram of experimental set up of the evolution experiment.

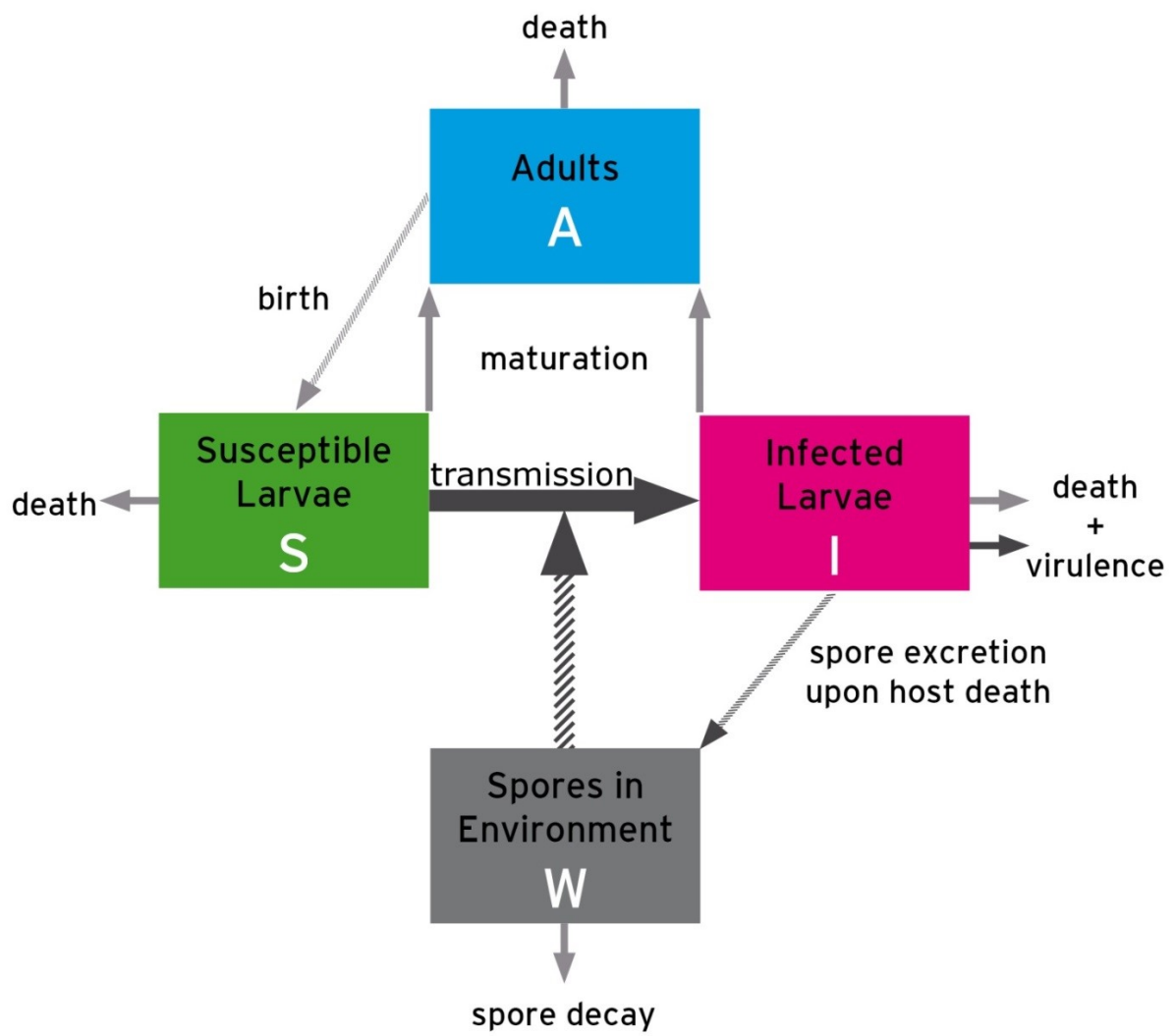


Figure S2. Schematic representation of the epidemiological model.

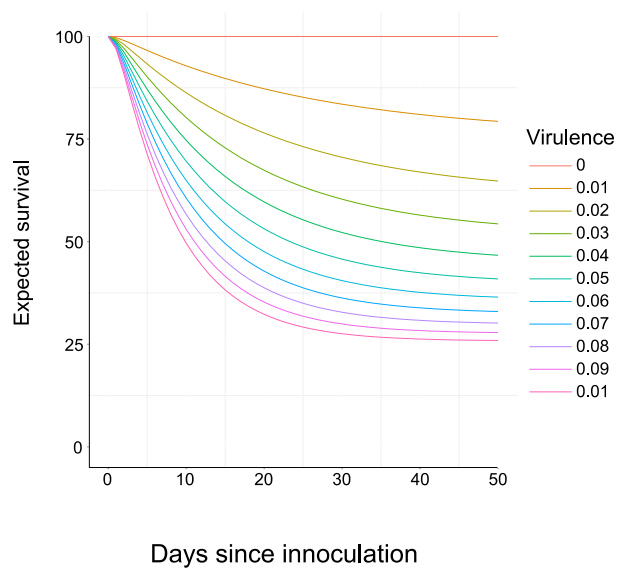


Figure S3. Simulation of survival from the model in the absence of reproduction with differing levels of virulence. The following parameter values were used in this simulation propagule transmission (b) = 0.00083; Larval maturation (m) = 0.03; Adult removal (u) = 0.003; Carrying capacity (C) = 100; Larval background mortality (d) = 0.03; Spore mortality (k) = 0.0075; Spore shedding (B) = 750000 and (B_2) = 750000.

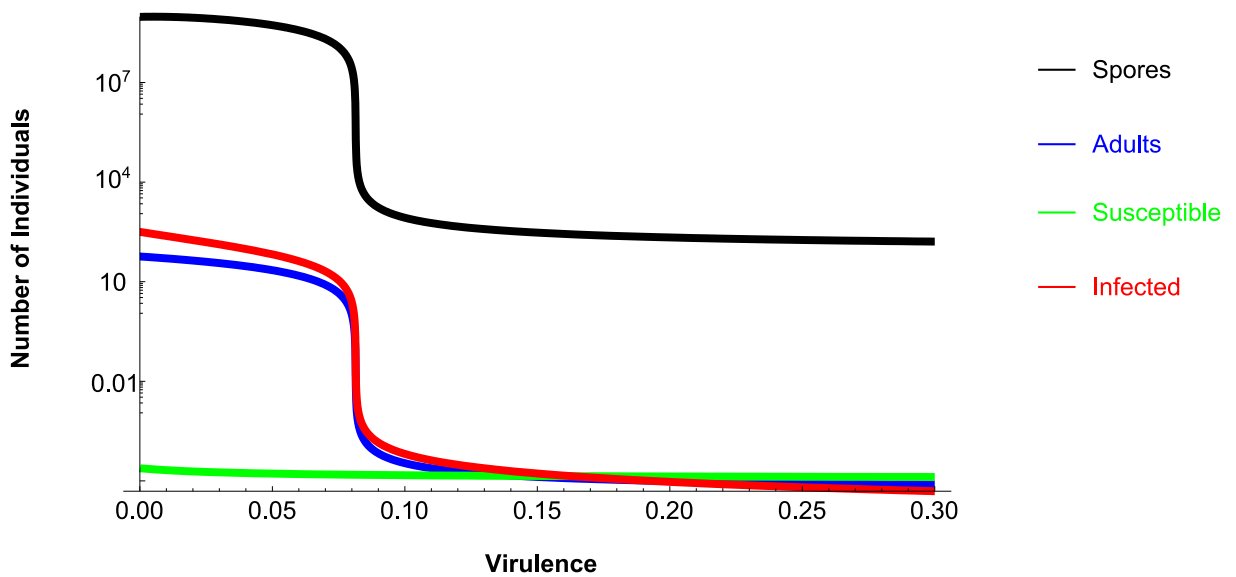


Figure S4. Here we show equilibria calculated from the simple model, with just one genotype, with all possible levels of virulence under experimental conditions (increased removal of adults). For high levels of virulence host populations are maintained below one individual, this is virtually extinction. The following parameter values were used in this simulation propagule transmission (b) = 0.00083; Larval maturation (m) = 0.03; Adult removal (u) = 0.07; Carrying capacity (C) = 100; Larval birth (l) = 0.33; Larval background mortality (d) = 0.03; Spore mortality (k) = 0.0075; Spore shedding (B) = 750000 and (B_2) = 750000.

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Chemical warfare does not always win – rapid evolution of increased virulence in *Beauveria bassiana*

3

In the previous chapter I demonstrated an increase in virulence as a result of host-parasite coevolution in an obligate intracellular parasite. However, in such a parasite that can only be cultured in vivo it is difficult to elucidate the mechanisms responsible for increased virulence. In the following chapter I present results of coevolution with Tribolium castaneum in a general entomopathogenic fungus that can be readily cultured outside of the host and is therefore amenable to further analysis. .



Charlotte Rafaluk, Hinrich Schulenburg and Gerrit Joop

Abstract: An increase in virulence is a trait predicted to evolve as a result of host-parasite coevolution. However, this is not an effect that is consistently seen in coevolution experiments and where changes are observed, specific immune responses overcome in order to achieve increased virulence can be difficult to elucidate. We carried out a coevolution experiment with the red flour beetle, *Tribolium castaneum*, and the general entomopathogenic fungus, *Beauveria bassiana*, allowing for both environmental manipulation by the host and persistence of fungal spores in the environment. After just seven host generations of evolution we saw a substantial increase in virulence in all evolved isolates of *B. bassiana*. Fascinatingly, we were able to show that this increase in virulence was a result of the *B. bassiana* isolates evolving resistance to the external immune defences of the *T. castaneum* beetles, who are able to secrete antimicrobial compounds into their environment. This presents a rare example of an experimentally coevolved increase in virulence, where the exact barrier of host immune defence overcome by the parasite in order to achieve the increase in virulence has been described.

Introduction

Antagonistic coevolution between hosts and parasites is expected to present a strong evolutionary force in nature (Woolhouse *et al.*, 2002). Parasites, here defined as both macro- and micro-parasites (Anderson and May, 1979), are ubiquitous, often having short generation times and rapidly adapting to their hosts, who in turn must reciprocally adapt to resist their parasites (Ebert, 1998). A body of literature obtained from coevolution experiments is emerging, whereby host-parasite interactions are allowed to evolve under carefully controlled laboratory conditions, empirically supporting ideas on the processes, phenotypic and genotypic outcomes of these antagonistic interactions (reviewed in Brockhurst and Koskella, 2013). These experiments have resulted in a number of interesting findings such as evidence of negative frequency dependent selection (Koskella and Lively, 2009), rapid evolution of host resistance (Schulte *et al.*, 2010) and both increases (Schulte *et al.*, 2010; Hall *et al.*, 2011) and decreases in virulence (e.g. Béréños *et al.*, 2009).

Virulence is generally predicted to increase as a result of host-parasite coevolution as parasites evolve to better infect their hosts and overcome increasingly strong host defences driven by reciprocal evolution of resistance in the host as part of a coevolutionary arms race (Dawkins and Krebs, 1979; Ebert, 1994; Gandon *et al.*, 2002). This is not, however, a pattern that is consistently seen in coevolution experiments, with either decreases in virulence (Béréños *et al.*, 2009; Béréños *et al.*, 2011; Kashiwagi and Yomo, 2011) or no change in virulence (Lohse *et al.*, 2006) frequently being seen. In many of these cases the transmission mechanism of the parasites is somewhat artificial, with parasite transmissive stages often being extracted from entire hosts at specific time points (*chapter 1*), potentially explaining this discrepancy. Experiments where more natural transmission mechanisms have been used, allowing for parasite transmissive stages to persist in the environment, have been shown to result in increased virulence (Hall *et al.*, 2011 *chapters 1 and 2*), consistent with theoretical predictions (e.g. Gandon *et al.*, 2002).

An increase in virulence as a consequence of host-parasite coevolution indicates that parasites have either increased in infectivity or increased in reproductive rate within the host. In order to achieve such an increase parasites have to either overcome, circumvent or manipulate the host's immune response. In the majority of insects, the barriers the parasite needs to overcome in order to infect consist mainly of the humeral and cellular defences of the innate immune system (Hoffmann and Reichhart, 2002; Cerenius *et al.*, 2008), physical barriers to infection and indirect defences such as avoidance behaviours (Schmid-Hempel, 2011). Particularly interesting are cases where the host organism has an additional line of defence in the form of external immunity, whereby antimicrobial substances are secreted or administered into the living environment of the animal (Cremer *et al.*, 2007; Cotter and Kilner, 2010). These barriers are likely to be highly important to the host, providing both a defence at a relatively low cost, by killing parasites before infection occurs thereby not paying the cost of infection and also, in many cases, additionally providing protection to kin (Cremer *et al.*, 2007; Cotter and Kilner, 2010; Schmid-Hempel, 2011).

External immunity has been shown to present a tough barrier to fungal infection in a number of insect species. Hamilton *et al.* (2011), for example, found the secretions of the termites *Reticulitermes flavipes* and *Reticulitermes virginicus* to show high levels of B-1,3 glucase activity and act strongly against the entomopathogenic fungus, *Metarhizium anisopliae*. Similarly Kay *et al.* (2014) showed metapleural gland secretions in the ant *Ectatmma ruidum* to be important in resistance to *M. anisopliae* and Tragust *et al.* (2013) found that formic acid secretions of the invasive garden ant, *Lasius neglectus* had inhibitory effects against *Metarhizium brunem*. Furthermore, it has been show in a number of earwig species that hydrocarbon and quinone containing secretions play a role in anti-fungal defence (Gasch *et al.*, 2013; Boos *et al.*, 2014). These examples suggest that external immune secretions in insects are likely to present an important barrier of defence against entomopathogenic fungi, potentially presenting a critical barrier to overcome in order to increase infectivity.

A further insect species able to secrete antimicrobial compounds into its environment is the red flour beetle, *Tribolium castaneum* (Chapman, 1926; Sokoloff, 1972), which secretes a mixture of methyl-1,4-benzoquinone (MBQ), ethyl-1,4-benzoquinone (EBQ), and 1-pentadecene into its environment (Loconti and Roth, 1953; Unruh *et al.*, 1998; Villaverde *et al.*, 2007). Quinone secretions such as these have a broad antimicrobial range, having been shown to have inhibitory effects against yeast (Prendeville and Stevens, 2002; Yezerski *et al.*, 2007), fungi (Ruther *et al.*, 2001), bacteria (Prendeville and Stevens, 2002; Yezerski *et al.*, 2007) and microsporidia (Joop *et al.*, 2014). These antimicrobial compounds are secreted into the environment by adult beetles, both disinfecting the grain environment in which they live and providing an antimicrobial coating to the cuticle of the beetle (Yezerski *et al.*, 2000), acting as a first line of defence for beetles against parasite infection. These defences may well be particularly important against entomopathogenic fungi, which infect via cuticular attachment.

One such fungal parasite is the general entomopathogenic fungus *Beauveria bassiana*, which infects a wide range of insects via enzymatic breakdown of the cuticle, followed by cuticular penetration (Leger *et al.* 1986). It has further been shown that infection by *B. bassiana* results in a decrease in quinone secretion by *T. castaneum* (Pedrini *et al.*, 2010), which could potentially be a mechanism of *B. bassiana* to minimise contact with the potent secretions of the beetle. Furthermore, a related species, *Beauveria brongniartii*, has been shown to be inhibited by quinone secretions of cockchafer (Ruther *et al.*, 2001). Together these factors make *B. bassiana* an interesting candidate for further investigation in host-parasite interactions involving external antimicrobial compounds.

We carried out a co-evolution experiment with *T. castaneum* and *B. bassiana* where both the antimicrobial quinone secretions of the beetle and fungal spores were able to persist in the environment across generations, allowing for external immunity to play a part in the coevolutionary process and for transmission to occur in an as natural as possible way. This allowed for the benefits of *T. castaneum*'s antimicrobial

secretions and any effects of evolved resistance to these secretions in *B. bassiana* to be realised.

Methods

Host

Tribolium castaneum is a tenebrioid beetle that inhabits human grain stores (Sokoloff, 1972). The *T. castaneum* stock Cro1 (Milutinović *et al.*, 2013a; Milutinović *et al.*, 2013b), was used to start the evolution experiment. The line had undergone around 20 generations of adaptation to standard lab rearing conditions prior to use in the experiment. A detailed description of how the beetles were reared in preparation for the evolution experiment can be found in *chapter 2*.

Parasite

The *Beauveria bassiana* strain used to start the experiment was obtained from the University of Giessen (Knorr *et al.*, 2009). *B. bassiana* was grown on potato dextrose agar (PDA) made from potato dextrose broth (PDB) (Carl Roth, Karlsruhe, Germany) with the addition of 15g agar (Carl Roth, Karlsruhe, Germany) and incubated at 25°C for 5 days in order to generate enough spores to start the experiment. Sporulating plates were subsequently kept at room temperature until use in experiments in order to reduce condensation. Stocks were maintained on PDA plates, incubated at 25°C for five days and then stored at room temperature throughout the experiment with transfers to new plates around once every two weeks.

Coevolution experiment

The coevolution experiment consisted of four treatments, three different environmental concentrations of *B. bassiana* in a flour-yeast mixture (Alnatura type 405 flour with 5% brewer's yeast (Leiber, Bramsche, Germany) and a control treatment containing only the flour-yeast mixture. The *B. bassiana* concentrations

used were 5×10^7 (low), 10^8 (intermediate) and 2×10^8 (high) spores per g flour-yeast mixture. Concentrations were chosen to show strong differences in survival with the ancestral population based on preliminary assays (Rafaluk, unpublished). The control group contained 7 replicate populations, the low and intermediate groups 6 and the high group 5. The differences in numbers of replicate populations in each group were due to difficulties in generating enough *B. bassiana* material at the beginning of the experiment.

A fungal spore powder was generated from which to start the experiment by banging spore filled plates of *B. bassiana* upside down onto a piece of coloured cardboard, in order to release spores. Spores were then transferred into a 50ml tube (Sarstedt, Nuembrecht, Germany), weighed, a subsample transferred to a 2ml tube (Sarstedt, Nuembrecht, Germany) and weighed again, 1 ml of water with Tween® 80 (Carl Roth, Karlsruhe, Germany) added and the spore concentration counted using a 0.02mm deep Neubauer counting chamber (Marienfeld, Koenigshofen, Germany). A portion of these collected spores were diluted in a mixture of flour and 5% yeast. This flour spore mixture was vigorously mixed in a large jar to produce an even distribution of spores. The lower concentrations were diluted following the same method, but with the relevant amount of the spore flour mixture from the next highest treatment. Each population was started with 17g of the relevant flour or flour spore mixture in a 50ml tube and 100 age synchronised adults of the line Cro1, synchronised as described in *chapter 2*. These adults were left for three weeks to oviposit and their offspring to become pupae.

Following the initial 3 week oviposition period, all populations were sieved and beetle stages separated. 11g of the flour from the previous generation was then put back into the tube and 5g of new flour as food inoculated where relevant with the starting concentration of ancestral spores, was added. A cage made from a standard 15ml tube (Sarstedt, Nuembrecht, Germany) with two 4cm long sections removed and replaced by 1 X 0.5mm plastic mesh was inserted into the 50ml tube. The remaining beetles from the parental generation were placed into this cage and the cage sealed. The aim of the cage system was to produce semi-natural conditions

with overlapping generations present, whilst still allowing for generations to be distinguished. In addition the cage allowed for contact and chemical communication between parents and offspring. To start the new generation 100 pupae were counted and added to the area of the large tube outside of the cage. If there were less than 100 pupae, the largest larvae were added to make the number up to 100 when possible. After two weeks, at the point when the pupae had become fully matured adults and where able to secrete quinones themselves, the cage and adults from the previous generation were removed (Figure 1). This procedure was then repeated every 4 weeks. We refer to each of these time periods as a transfer. The experiment was carried out for 13 transfers.

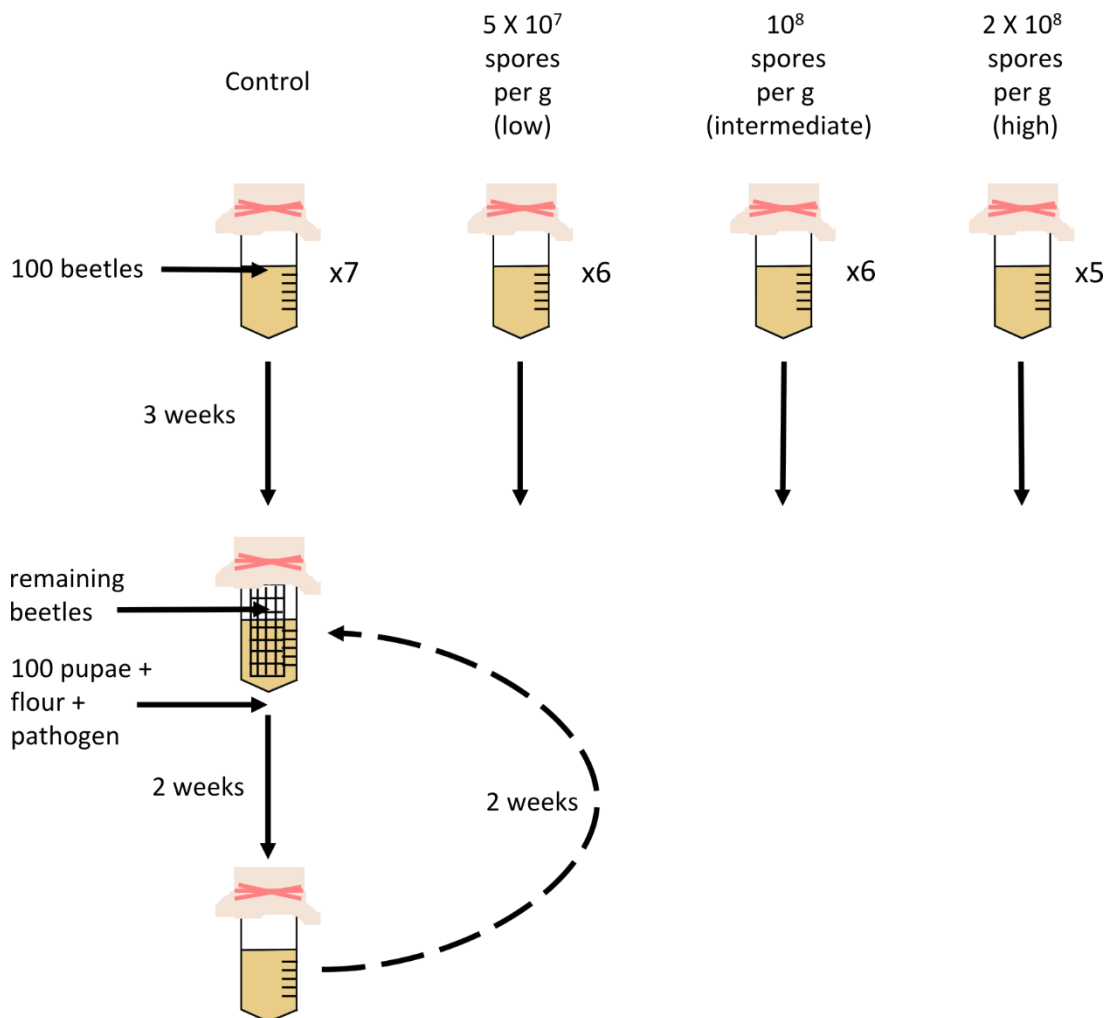


Figure 1 Diagram illustrating the set up and experimental design of the evolution experiment. Each treatment was initiated with between 5 and 7 replicate population each with 100 adult beetles and the relevant concentration of spores. Each transfer there was a two week overlap in generations, where adults of the previous generation were confined within a cage.

F2 control beetles were generated in order to produce naive individuals for use in assays with *B. bassiana*. The adults from the last generation of the evolution experiment were allowed to oviposit for 7 days on clean flour (Alnatura type 405) with 5% yeast. Following which adults were removed and the F1 offspring allowed to develop. A mixed control population was produced by mixing F1 from all control lines for oviposition, producing a mixed F2 population. The time the F2 individuals were allowed to develop depended on what life stage was required for the subsequent experiment.

Dead beetles were frozen from transfers 7, 12 and 13 in a mixture of 200µl glycerol (Carl Roth, Karlsruhe, Germany) and 300µl PDB. *B. bassiana* was re-grown from these dead beetles for use in phenotypic assays. For extraction of *B. bassiana* dead beetles were crushed with a spatula and a portion of the crushed beetle-glycerol/PDB mixture pipetted into a 15ml tube containing 3ml PDB with 50µg per ml chloramphenicol (Carl Roth, Karlsruhe, Germany) to prevent bacterial growth. This mixture was then pipetted onto plates for a further growth step and a portion frozen at -20° C, again in 200µl glycerol plus 300µl PDB. It was difficult to extract *B. bassiana* from later time points, transfers 12 and 13, with *B. bassiana* being successfully extracted from far less samples than at the earlier time point. Consequently, in order to generate enough material for comparison, both transfers were considered to be the same time point, representing the end point of the experiment in further analyses. Extraction from both transfer 7 and either 12 or 13 was possible for a total of 8 experimental populations, 2 from the high treatment, 4 from the intermediate treatment and 2 from the low treatment. Only these isolates were used in subsequent assays.

Increase in virulence of B. bassiana towards T. castaneum

To test if the coevolved strains of *B. bassiana* had changed in their virulence towards *T. castaneum* a survival assay was carried out. The survival assay was started from 7 day old F2 larvae of the mixed control population. Flour was inoculated at a concentration of 10⁸ spores per gram with *B. bassiana* isolates from generation 7 and

either generation 12 or 13 from each population. Only fungal strains from populations where extraction from both time points was possible were considered in the analysis, several test populations were lost due to contamination for one of the intermediate isolates, which was then excluded from further analysis. Test populations were set up with 50 larvae in 8.5g flour inoculated with the relevant *B. bassiana* isolate in a standard 50ml tube and closed with tissue paper and an elastic band. 3 replicates per treatment combination were prepared. Numbers of alive and dead individuals were counted after 14, 21 and 28 days. Tubes were kept at 32°C and 70% humidity throughout the experiment.

B. bassiana Resistance to beetle secretions – Zone of inhibition on plates

A zone of inhibition assay was carried out in order to establish whether the evolved strains of *B. bassiana* had changed in their susceptibility to the quinone secretions of the beetles in comparison to the ancestral strain, following the methods of Prendeville and Stevens (2002) and Li *et al.* (2013). PDA was prepared with an agar concentration of 6g agar per litre media. Individual bottles of media were inoculated with 10^7 *B. bassiana* spores per ml media. Plates were poured using Pasteur pipettes (Sarstedt, Nuembrecht, Germany) with 12 ml media per plate and kept in the fridge overnight. Following this, adult F2 beetles of the mixed control population from generation 12 of the evolution experiment were cold shocked on ice for 5 minutes then placed at set points on the plates, left there until they began to move and were then discarded. This process had been shown to cause beetles to release their quinones (Joop *et al.*, 2014). The mixed control population was used here in order to provide a naive population which had not encountered *B. bassiana* before. A positive control of the fungicide Amphotericin B (Sigma-Aldrich, Steinheim, Germany) was run on each plate in parallel. One fungal isolate from the high treatment was excluded from the analysis due to unsuccessful growth. Plates were incubated at 25°C for 96 hours then scanned using an Epson 2480 photo scanner and the inhibition zones measured using image J (version 1.46). Means of inhibition zones were taken from each plate for use in statistical analysis.

B. bassiana Resistance to a pure quinone compound - Inhibition in liquid media

In order to test whether it was indeed the quinone component of the beetles' secretion that the *B. bassiana* had evolved resistance to, an inhibition assay was carried out in PDB, following an adapted version of the methods of Du Toit and Rautenbach (2000), with a pure quinone product as the inhibitory agent. PDB was inoculated with 2×10^6 spores per ml of each of the evolved strains isolated from the intermediate treatment at generation 12/13. 90 μ l of broth was pipetted into each well of a 96 well flat bottom plate (Sarstedt, Nuembrecht, Germany), using a reverse pipetting technique to avoid cross-contamination. Subsequently, 10 μ l of Methyl-para-benzoquinone (MBQ) (Sigma-Aldrich, Steinheim, Germany) at a concentration of 0.11 μ g per ml diluted in methanol (Carl Roth, Karlsruhe, Germany), representing approximately the equivalent of the secretion of 100 beetles (Yezerki *et al.*, 2000), or 10 μ l of pure methanol as a control, was added to each well. OD was then measured at 595nm at 24, 72 and 96 hours post inoculation on a BioTek EON plate reader. In order to calculate the difference in growth of strains when exposed to MBQ or the methanol control the mean growth on each line in the presence of MBQ was subtracted from the growth values in the presence of methanol alone.

Statistical analysis

All statistics were carried out using R version 3.0.2 (R core team 2013). Overall survival curves were analysed using a series of pairwise ordinal log-rank tests. Data for each time point of the survival data were analysed using nested ANOVAs followed by pairwise t-tests on the proportion of individuals alive. The results of the zone of inhibition test on solid media were compared using a generalized linear model (GLM) with a Quasi-Poisson error distribution, followed by Tukey multiple comparison tests using the "lsmeans" package in R. Data from the zone of inhibition test in liquid media were analysed using Kruskal Wallis tests followed by pairwise Wilcoxon tests. Wherever appropriate, p-values were corrected using the

false discovery rate method. Graphs were created using the R package “ggplot2” and edited in Inkscape (version 0.48).+

Results

Increase in virulence of B. bassiana towards T. castaneum

Overall survival kinetics showed that all coevolved *B. bassiana* isolates differed significantly from the ancestral isolate (pairwise ordinal log rank tests Figure 2, Table 1). Examining the data for each time point individually we found that the proportion of individuals alive at days 14 and 21 of the survival assay did not differ significantly between lines (Nested ANOVAs). At day 28, however, significant differences emerged between parasite generations (Nested ANOVA: $F=16.9$, $d.f.=3,12$ $p<0.001$), the nested effect of parasite isolate within generation was not significant. Pairwise t-tests showed that all evolved lines differed significantly from the ancestral line in mortality at the end of the assay and that none of the evolved lines differed significantly from one another.

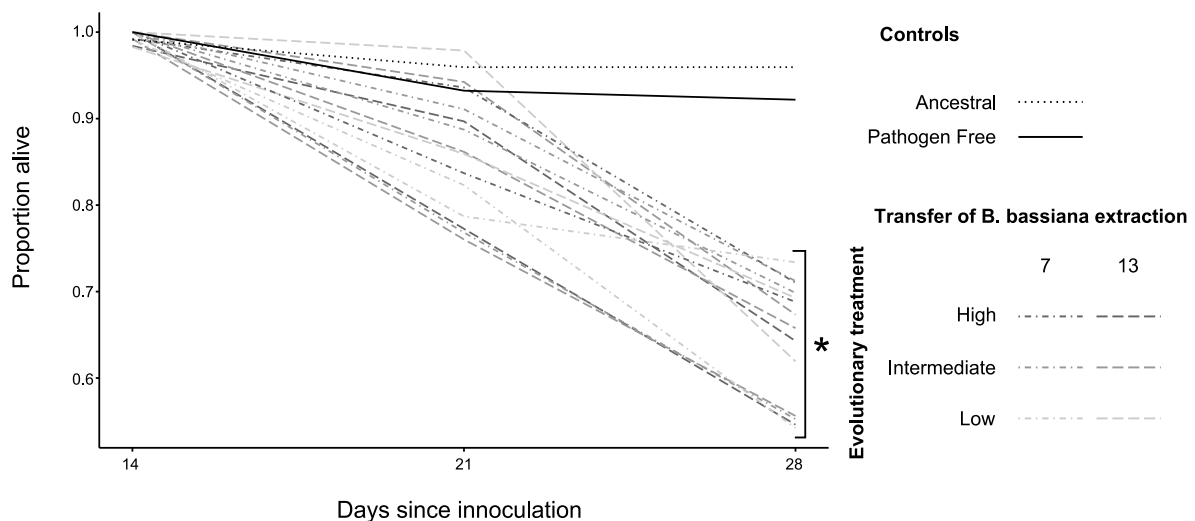


Figure 2. Proportion of beetles alive following exposure to each of the coevolved *B. bassiana* isolates, the ancestral isolate or pathogen free control flour over the course of the survival assay. Isolates within the bracket denoted by * differ significantly from the ancestral isolate (pairwise ordinal log rank test with fdr p-value correction).

Table 1. Statistical results of pairwise ordinal log rank tests comparing survival over time for all coevolved *B. bassiana* isolates and the pathogen free control to the ancestral isolate. P values are corrected using the *fdr* method.

Evolutionary treatment	Time point of <i>B. bassiana</i> extraction	<i>B. bassiana</i> isolate	χ^2 value	d.f.¹	p value
Intermediate	7	Intermediate, replicate 3	24.20	1	<0.0001
	13	Intermediate, replicate 3	49.50	1	<0.0001
	7	Intermediate, replicate 5	51.50	1	<0.0001
	13	Intermediate, replicate 5	31.90	1	<0.0001
	7	Intermediate, replicate 2	22.90	1	<0.0001
	13	Intermediate, replicate 2	29.20	1	<0.0001
Low	7	Low, replicate 3	28.90	1	<0.0001
	13	Low, replicate 3	39.30	1	<0.0001
	7	Low, replicate 6	52.90	1	<0.0001
	13	Low, replicate 6	23.20	1	<0.0001
High	7	High, replicate 1	30.10	1	<0.0001
	13	High, replicate 1	48.50	1	<0.0001
	7	High, replicate 2	24.20	1	<0.0001
	13	High, replicate 2	35.10	1	<0.0001
Pathogen	n/a	Pathogen Free	1.20	1	0.274

¹ Degrees of freedom

B. bassiana Resistance to beetle secretions – Zone of inhibition on plates

The results from the zone of inhibition assay on plates showed strong differences in inhibition between the ancestral and evolved *B. bassiana* isolates (GLM, Table 2) with all evolved isolates being significantly less inhibited by the quinone secretions of the beetles than the ancestral isolate (pairwise multiple comparison tests with *fdr* correction, Table 3, Figure 3).

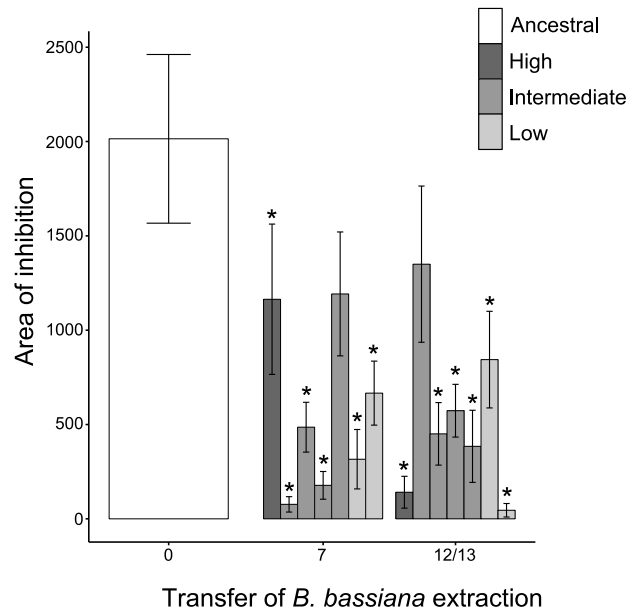


Figure 3. Area of inhibition zones of ancestral and coevolved *B. bassiana* isolates exposed to the quinone secretions of naive control line beetles. * denotes isolates differing significantly from the ancestral strain (Tukey multiple comparison tests).

Table 2. ANOVA Results of the generalized linear model comparing area of inhibition zones among *B. bassiana* isolates following exposure to secretions of naïve control line beetles across transfers.¹

Effect	d.f. ²	Deviance	Residual d.f.	Residual Deviance	p value
NULL			109	100622	
<i>B. bassiana</i> isolate	7	24155.8	102	76466	<0.0001
Transfer	1	105.6	101	76360	0.6582
<i>B. bassiana</i> isolate: Transfer	6	23169.5	95	53191	<0.0001

¹ In the model we used the mean inhibition zone induced by the secretions of all control beetles on each plate as a response variable. *B. bassiana* isolate and Transfer were considered as fixed effects.

² degrees of freedom

Table 3. Pairwise multiple comparison tests comparing area of inhibition zones of isolates of *B. bassiana* extracted at different transfers of the experiment exposed to quinone secretion of naïve control line beetles. P values are corrected using the *fdr* method.

Population isolate extracted from, transfer extracted	z ratio	p value
<i>Comparison to ancestral parasite strain</i>		
High replicate 2, 7	2.191	0.04264
Intermediate replicate 2, 7	3.047	0.00606
Intermediate replicate 3, 7	3.614	0.00158
Intermediate replicate 5, 7	4.016	0.00111
Intermediate replicate 6, 7	1.686	0.10704
Low replicate 3, 7	3.319	0.00316
Low replicate 6, 7	2.744	0.01415
High replicate 2, 12/13	3.26	0.00335
Intermediate replicate 2, 12/13	1.318	0.1968
Intermediate replicate 3, 12/13	3.777	0.00111
Intermediate replicate 5, 12/13	3.486	0.00206
Intermediate replicate 6, 12/13	3.861	0.00111
Low replicate 3, 12/13	2.482	0.02111
Low replicate 6, 12/13	2.539	0.01943
<i>Comparison with replicate population between transfers</i>		
High replicate 2, 7 - High replicate 2, 12/13	-2.073	0.0534
Intermediate replicate 2, 7 - Intermediate replicate 2, 12/13	2.611	0.01725
Intermediate replicate 3, 7 - Intermediate replicate 3, 12/13	-0.316	0.75233
Intermediate replicate 5, 7 - Intermediate replicate 5, 12/13	1.764	0.09606
Intermediate replicate 6, 7 - Intermediate replicate 6, 12/13	-2.65	0.01691
Low replicate 3, 7 - Low replicate 3, 12/13	1.454	0.1613
Low replicate 6, 7 - Low replicate 6, 12/13	-1.804	0.09354

B. bassiana Resistance to a pure quinone compound - Inhibition in liquid media

The inhibition assay in liquid media demonstrated that the evolved lines were significantly less inhibited by methyl-para-benzoquinone than the ancestral strain at both 24 (Kruskal Wallis Test: $\chi^2=22.1$, d.f.= 4, $p<0.001$) (Figure 4a) and 72

(Kruskal Wallis Test: $\chi^2=31.8$, d.f.= 4, $p<0.001$) (Figure 4b) hours post inoculation.

After 96 hours, however, a significant difference was no longer seen (Kruskal Wallis Test: $\chi^2=6.2$, d.f.= 4, $p=0.18$) (Figure 4c).

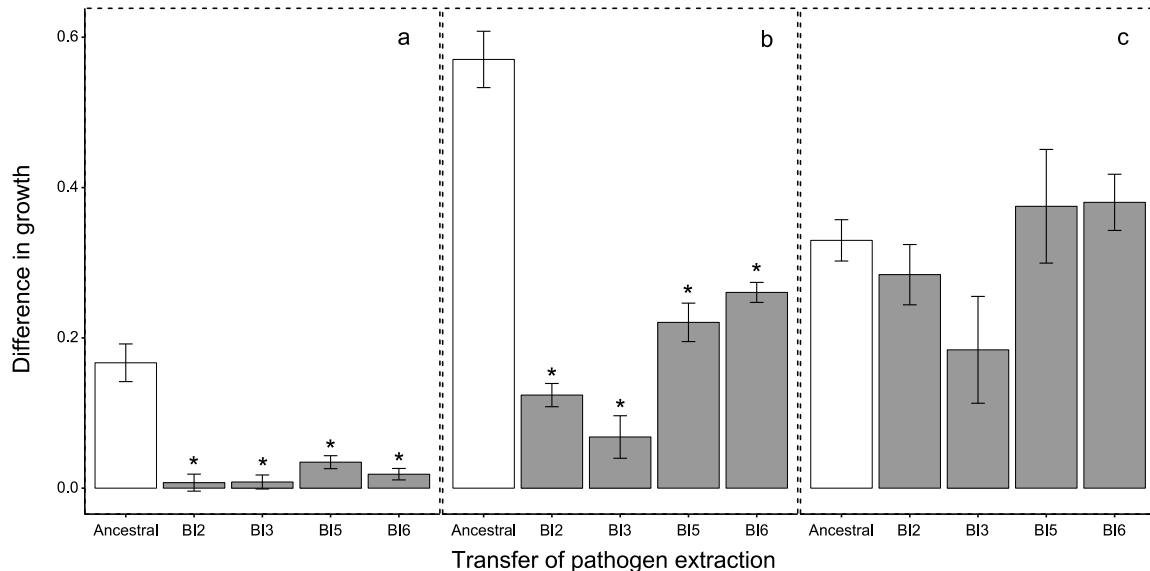


Figure 4. Difference in *B. bassiana* growth in liquid media containing methyl-para-benzoquinone diluted in methanol and a methanol only control for each isolate. Measured as optical density (OD) at 24 (a), 72 (b) and 96 (c) hours post inoculation. * denotes means differing significantly from the ancestral strain at the same time point (pairwise Wilcoxon tests with FDR p-value correction). A=the ancestral strain, PF=pathogen free control, BI2, 3, 5 & 6 = evolved isolates.

Discussion

Our data show both a profound and consistent increase in virulence in all evolved strains, providing clear evidence that virulence did seem to increase as a result of coevolution in our experiment. Interestingly virulence did not show a further clear increase between generations 7 and 12/13 indicating that *B. bassiana* may have reached its optimal virulence by generation 7 and that the populations then stayed around this optimum level for the rest of the experiment. The increase in virulence observed here is consistent with our previous experiment using the same set up but a different parasite, where we also saw an increase in virulence over evolutionary time (*chapter 2*). In addition, however, here we were also able to demonstrate a completely consistent increase in resistance to the antimicrobial quinone secretions of the beetles. We were not only able to demonstrate this effect with the entire

secretions of the control line beetles but also with a specific, representative component of the secretions, methyl-para-benzoquinone (MBQ), providing incredibly strong evidence that virulence increased as a consequence of *B. bassiana* evolving resistance to the quinone component of the beetles' antimicrobial secretions.

It is fascinating that we were able to not only demonstrate an increase in virulence but also show the aspect of the beetle immune system overcome in order for the increase in virulence to be achieved. Through showing that *B. bassiana* evolved to overcome the external immune defence of the beetles we have provided compelling evidence that external immune secretions are an important defence barrier against entomopathogenic fungi, consistent with previous studies showing the effectiveness of external immunity against fungal pathogens in other species (Hamilton *et al.*, 2011; Gasch *et al.*, 2013; Tragust *et al.*, 2013; Boos *et al.*, 2014).

Increased quinone resistance may benefit *B. bassiana* in two ways. It may facilitate more efficient cuticle attachment, which is important in *B. bassiana* infection (Leger, *et al.*, 1986), by allowing spores to attach and germinate in spite of the quinone coating on the *T. castaneum* cuticle (Yezerksi *et al.*, 2000). An additional benefit of increased resistance to the quinone secretions of the beetles is that it may allow *B. Bassiana* to persist for longer periods of time in the environment. Persistence was favoured by our experimental design (*chapter 2*) and the presence of quinones in the environment likely greatly heightened mortality of environmental spores, putting a strong selection pressure on environmental persistence in the presence of quinones. Persisting in spite of the quinones in the environment could therefore have greatly improved infection probability.

Environmental persistence of long lived parasite stages and transmission from environment to host is something that is often ignored in evolution experiments (*chapters 1 and 2*). In many cases, with parasite transmissive stages being extracted from entire hosts at a specific time point (Kolodny-Hirsch and Van Beek, 1997; Bérénois *et al.*, 2009; Garbutt *et al.*, 2011). As pathogen spores *per se* were not directly collected and re-introduced to the environment at each transfer, rather the

environment itself and a subset of individual beetles were transferred, only *B. bassiana* within a living beetle, pupa or older larva or persisting within the environment would make it to the next generation. When pathogens have the capability to outlive their host, increased virulence is predicted to be selected for under certain circumstances (Ewald, 1987; Bonhoeffer *et al.*, 1996; Gandon, 1998; Kamo and Boots, 2004). *B. bassiana* spores survive for approximately 80 days outside of a host under the temperature and humidity conditions used in our experiment, when quinones are not present (Hong *et al.*, 1997), meaning that without quinones the lifespan of *B. bassiana* spores would be greater than a single experimental host generation. Quinones likely greatly reduced this survival time, however, an increase in longevity in the presence of quinones, moving back towards the quinone free level, may have provided the conditions predicted by Bonhoeffer *et al.* (1996) to select for increased virulence.

Even without highly persistent spores, coevolution is predicted to select for increased virulence (Ebert, 1994; Gandon *et al.*, 2002). This is, however, not what is generally seen in coevolution experiments (*chapter 1*; Lohse *et al.*, 2006; Béréanos *et al.*, 2009; Kashiwagi and Yomo, 2011). The inconsistency of our results in comparison to previous coevolution experiments is likely to be linked to the mechanism of transmission. Our findings are consistent with results from the same experiment with a different pathogen (*chapter 2*) and with experiments in non-animal systems where more natural transmission was allowed for (e.g. Hall *et al.*, 2011). Our experiment allowed for horizontal transmission from generation to generation, by having a two week period of overlap where beetles from subsequent generations could come into contact with each other through the cage system. Additionally, through transfer of the environment from generation to generation, spore stages released into the environment were transferred. This provided a more natural mechanism of transmission and is likely to have been responsible for our results being more consistent with theoretical predictions (*chapter 1*).

Here we have not only described a clear example of an increase in virulence as a consequence of host-parasite co-evolution but also phenotypically shown the

precise barrier of the immune system that was overcome to achieve this increase in virulence. We have provided rare evidence of a pathogen overcoming a defined, complex and broadly antimicrobial barrier of host defence, which has implications for pest control, where *T. castaneum* has shown to be more resistant than other pest insect species to biological control agents such as *B. bassiana* (Padin *et al* 2002.). Furthermore, with microparasites rapidly evolving resistance to antibiotics people are beginning to consider antimicrobial peptides and other antimicrobial compounds produced by animals as alternatives, as these are thought to be more difficult to evolve resistance to (Hancock and Sahl, 2006; Perron *et al.*, 2006; Haine *et al.*, 2008). We have shown here that resistance can evolve even to the most broadly antimicrobial agents. Furthermore, we have again demonstrated that a more natural mode of transmission can lead to results consistent with predictions from the theoretical literature on how virulence should evolve under host-parasite coevolution (*chapters 1 and 2*; Gandon *et al.*, 2002). All in all we have shown that although external immunity provides a powerful first line of immune defence, it can be overcome in a relatively short period of evolutionary time.

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Cryptic changes in immune response and fitness in *Tribolium castaneum* as a consequence of coevolution with *Beauveria bassiana*

4

In the previous chapter I described changes that occurred in B. bassiana as a consequence of coevolution with T. castaneum where I observed substantial increased in virulence and resistance to host external immune compounds. Host parasite coevolution theory predicts that reciprocal changes should be seen in the host as it evolves to resist or tolerate the increasingly virulent parasite. In the following chapter I describe the phenotypic changes seen in the host.

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Abstract: Immunity is a key trait in host defence against parasites. Particularly interesting are organisms who possess an additional or extended line of defence in the form of an external immune system. One such organism is the red flour beetle *Tribolium castaneum* which is able to secrete antimicrobial quinone compounds into its environment. We carried out a host-parasite co-evolution experiment between *T. castaneum* and the general entomopathogenic fungus *Beauveria bassiana*. We measured levels of external immunity and an internal immune trait, phenoloxidase (PO) activity throughout the experiment and with F2 beetles at the end of the experiment, along with survival, as a proxy for resistance, of F2 coevolved and control beetles exposed to the fungus. No change in external immunity or survival was observed as a consequence of host-parasite coevolution, however, PO responses in evolved beetles showed increased flexibility dependent on infection method of the parasite. This more flexible PO response appeared to result in beetle populations being better able to cope with the parasite during the course of the coevolution experiment.

Introduction

In recent years several host-parasite coevolution experiments have been carried out with the aim of empirically testing theoretical ideas of how species interactions evolve (Béréños *et al.*, 2009; Schulte *et al.*, 2010; Hall *et al.*, 2011). Most of these experiments have resulted in phenotypic changes in both the host and parasite (here we use the term parasite to include both macro and micro parasites (Anderson and May, 1979) (reviewed in Brockhurst and Koskella, 2013). Reciprocal changes are often seen with, for example, hosts becoming more resistant to the parasite that they are evolving with (Béréños *et al.*, 2009; Schulte *et al.*, 2010) and the parasite in turn either increasing (e.g. Schulte *et al.*, 2010) or decreasing (e.g. Béréños *et al.*, 2009) in virulence. In some cases, however, change can be more difficult to see. The outcome for the host may be tolerance rather than resistance (Kraaijeveld and Godfray, 2008) or changes may only be seen when looking at phenotypes under very specific conditions.

Tolerance and resistance are distinctly different concepts (Raberg *et al.*, 2009; Little *et al.*, 2010). Raberg *et al.* (2009) define resistance as “the ability of a host to limit parasite burden” and tolerance as “the ability to limit the damage caused by a parasite burden” and whether a host evolves resistance or tolerance can affect the trajectory of coevolution. Nevertheless mechanisms of tolerance and resistance can be similar (Raberg *et al.*, 2009) and when specifically examining host change, it can be interesting to more closely examine mechanisms in order to better understand the specific trajectory along which evolution occurred and more comprehensively understand how immune systems evolve.

One way that an increase in host resistance or tolerance can be achieved is via increased investment in host immune traits (Hoffmann and Reichhart, 2002; Miller *et al.*, 2006). In insects the immune system consists of humoral defences such as antimicrobial peptide (AMP) responses (Hoffmann and Reichhart, 2002) and the prophenoloxidase (PO) cascade, which as well as being linked to cellular defences, results in antimicrobial substances such as melanin, phenols and quinones being secreted into the haemolymph (Cerenius *et al.*, 2008). Also present are cellular

responses, including phagocytosis and encapsulation (Lemaitre and Hoffmann, 2007) and physical barriers to infection such as the cuticle (Armitage and Siva-Jothy, 2005).

In addition to these standard barriers of immune defence, some organisms possess an additional or extended line of defence in the form of external immunity (Cotter and Kilner, 2010). The most common example of this is the ability to secrete antimicrobial compounds into or onto the environment in which the organism lives (Cotter and Kilner, 2010). For example the burying beetle, *Nicrophorus vespilloides*, secretes antimicrobial compounds onto vertebrate corpses where they live and raise their offspring (Cotter *et al.*, 2010). Similarly, sternal gland secretions of the dampwood termite, *Zootermopsis angusticollis*, show anti-fungal activity (Rosengaus *et al.*, 2004), as do the metapleural gland secretions of the leaf cutter ant, *Acromyrmex octospinosus*, which are also effective against bacteria (Poulsen *et al.*, 2002). All immune responses are predicted to come at a cost (Sheldon and Verhulst, 1996), and external immune reactions have been shown to be costly in several systems (Poulsen *et al.*, 2002; Cotter *et al.*, 2010). Furthermore, Cotter *et al.* (2013) demonstrated a direct link between internal and external immunity in *N. vespilloides* indicating that, at least in some cases, external immune defences come at a direct cost to internal immunity (but see Steiger *et al.* 2011). When there is a direct physiological link between two aspects of immune defence, it is likely, particularly when both defences derive from a shared resource, host organisms involved in antagonistic coevolutionary interactions would be unable to increase investment in all aspects of immune defence.

The mode of immune defence under selection is strongly influenced by the taxon and infection method of the parasite. Parasitoids, for example, are targeted by the cellular arm of the immune defence (Salazar-Jaramillo *et al.*, 2014), whereas antimicrobial peptide responses exist to directly target bacterial infections (Zaslhoff, 2002). Similarly, infections that involve breakage of the cuticle, such as those by parasitoids or entomopathogenic fungi often result in an up-regulation of PO activity resulting in melanin deposition and wound sealing (Kraaijeveld *et al.*, 2012;

Yassine *et al.*, 2012; Dubovskiy *et al.*, 2013a), whereas oral infections tend to elicit more specific anti-microbial peptide responses (Limmer *et al.*, 2011; Zhang *et al.*, 2014). When a pathogen has multiple mechanisms of infection the situation is complicated further as increased investment into multiple barriers of immune defence is likely to come at extreme fitness costs (Sheldon and Verhulst, 1996). It is, therefore, likely that these types of parasite are more difficult for hosts to evolve resistance to and that coevolution with a parasite with multiple infection routes will lead to evolutionary compromises within the host immune system.

One insect species that possesses an external as well as internal immune defences is the red flour beetle, *Tribolium castaneum*, which is able to secrete volatile, antimicrobial compounds into its environment (Chapman, 1926; Sokoloff, 1972). These secretions consist of methyl-1,4-benzoquinone (MBQ), ethyl-1,4-benzoquinone (EBQ), and 1-pentadecene (Loconti *et al.*, 1953; Unruh *et al.*, 1998; Villaverde *et al.*, 2007). Quinone secretions have a broad antimicrobial range, having been shown to have inhibitory effects against yeast (Prendeville and Stevens, 2002; Yezerksi *et al.*, 2007), fungi (Ruther *et al.*, 2001), bacteria (Prendeville and Stevens, 2002; Yezerksi *et al.*, 2007), microsporidia (Joop *et al.*, 2014) and even tapeworms (Yan and Phillips, 1996). These antimicrobial compounds are secreted into the environment by adult beetles, both disinfecting the grain environment in which they live and providing an antimicrobial coating to the cuticle of the beetle (Yezerksi *et al.*, 2000). The antimicrobial coating of the cuticle is likely to provide a particularly strong barrier of defence against parasites such as entomopathogenic fungi, which attach to the cuticle as the first step of infection. Interestingly, similarly to the case of burying beetles (Cotter *et al.*, 2013), we predict there to be a direct physiological trade-off between these secretions and the PO cascade as both require tyrosine derived compounds (Cerenius *et al.*, 2008; Gorman and Arakane, 2010) and have been shown in previous studies to be interlinked (Li *et al.*, 2013; Joop *et al.*, 2014). Although, clear evidence of a trade-off in this case is yet to be seen (Joop *et al.*, 2014).

A particularly interesting parasite able to infect *T. castaneum* is the general entomopathogenic fungus, *Beauveria bassiana*. *B. bassiana* has traditionally been thought to infect insects via attachment to and enzymatic breakdown of the cuticle, followed by cuticular penetration and proliferation within the haemocoel (Leger et al. 1986). Recent evidence from the sequenced genome of *B. bassiana* indicates, however, that it may also be capable of oral infection as it possess genes resembling the cry-toxin genes of the orally infecting bacterium *Bacillus thuringiensis* (Xiao et al., 2012). Interestingly, previous studies have demonstrated the *B. bassiana* infection elicits immune responses from both cellular (Hung and Boucias, 1992) and humoral (Wojda et al., 2009) arms of the insect immune system, including the PO cascade (Yassine et al., 2012; Dubovskiy et al., 2013a). It has further been shown that *T. castaneum* beetles' external immune defence is down-regulated upon infection by *B. bassiana* (Pedrini et al., 2010). Consequently, selection pressure by *B. bassiana* is likely to lead to evolutionary change in multiple arms of the insect immune system, presenting a potential evolutionary challenge for the host.

We carried out a host-parasite co-evolution experiment with the red flour beetle *Tribolium castaneum* and the general entomopathogenic fungus *Beauveria bassiana*. This experiment has resulted in a number of phenotypic changes in the parasite (*chapter 3*). Coevolved isolates of *B. bassiana* became more virulent after just 7 generations of coevolution and also became resistant to the external immune compounds secreted by the *T. castaneum* beetles (*chapter 3*). Based on the theoretical predictions of antagonistic coevolution (Hamilton, 1980), we would expect to see reciprocal adaptation in the host. Here we examine change in several host fitness traits and immune parameters, during and at the end of the coevolution experiment.

Methods

Host

Beetles from the Cro1 stock were used in this experiment (Milutinović et al., 2013a; Milutinović et al., 2013b). This is a natural isolate that was kept in the lab for

approximately 20 generations prior to use in this experiment and has also been used in previous coevolution experiments (B. Milutinović personal communication). Full details of the maintenance of the stock beetles can be found in *chapters 2 and 3*.

Pathogen

A *Beauveria bassiana* strain obtained from the University of Giessen was used to start the experiment (Knorr et al 2009). Full details of the maintenance of the fungus prior to and during the experiment can be found in *chapter 3*.

Evolution experiment

Detailed methods for the evolution experiment can be found in *chapter 3*. Briefly, a coevolution experiment was carried out with 3 different environmental concentrations of *B. bassiana*, 5×10^7 spores per gram flour (Alnatura type 405, Bickenbach, Germany) (low), 10^8 spores per gram flour (intermediate), 2×10^8 spores per gram flour (high), each with 5% brewers' yeast (Lieber, Bramsche, Germany) as food in all treatments. Beetle populations were started with 100 age synchronised adult beetles. The evolution experiment was carried out in 50 ml tubes with 7 populations per treatment. Every 4 weeks populations were sieved, 30 adults and 20 larvae removed, where possible, for phenotypic assays and the remaining adults placed into a cage. We refer to each of these time periods as a transfer. The cage was constructed from a standard 15 ml tube (Sarstedt, Nuembrecht, Germany) with holes covered in mesh (details can be found in *chapter 2*). The purpose of the cage was to allow for semi overlapping generations and chemical communication between adults and larvae while experimentally being able to differentiate between beetle generations. 100 pupae were placed around the outside of the cage. Where 100 pupae were not present numbers were made up to 100 by adding the largest larvae.

F2 beetles were generated for phenotypic assays at the end of the experiment. The adults of the last transfer of the evolution experiment were allowed to oviposit for 7 days on clean flour with 5% yeast, following which adults were removed and the

F1 offspring allowed to develop. This procedure was repeated with the F1 adults in order to generate F2 individuals. The time the F2 were allowed to develop depended on what life stage was required for the subsequent experiment. All evolved lines were kept separately, however, a mixed control and a mixed intermediate population were produced in parallel by mixing F1 from all control or intermediate lines for oviposition, producing a mixed F2 population. These mixed populations were used whenever the testing of all lines was not possible.

Benzoquinone content of quinone glands throughout the experiment:

The benzoquinone content of the beetle's quinone glands was assayed for ten adult beetles of each treatment every transfer at the time point that the adult beetles were transferred to the cage. Extraction of quinones from the glands was carried out by placing beetles in acetonitrile (HPLC Grade, Carl Roth, Karlsruhe, Germany) overnight. Quinones are soluble in acetonitrile (Peover and Davies, 1964) and when dissolved in acetonitrile can be quantified by reading of optical density on a plate reader (Joop *et al.*, 2014).

Three days prior to quinone extraction 150µl of acetonitrile was pipetted into each well of the relevant number of 96-well PCR plates (Sarstedt, Nuembrecht, Germany). Each 8-well column of the plate was sealed with an 8-lid strip (Sarstedt, Nuembrecht, Germany) immediately after being pipetted and prepared plates were stored in the fridge at 4°C prior to use.

After sieving of each population, 10 adult beetles were taken at random and cold shocked in a petri dish (Sarstedt, Nuembrecht, Germany) on ice for 5 minutes, causing them to release the contents of their quinone glands (Unruh *et al.*, 1998; Joop *et al.*, 2014). Subsequently, each beetle was placed into a well containing acetonitrile. Columns of the plate were sealed as soon as they were full and as soon as a plate was complete, it was immediately returned to the fridge. Several wells of each plate were left without beetles to act as blanks in the analysis. Plates containing beetles were kept in the fridge for a further 24 hours in order to allow quinones to dissolve into the acetonitrile.

Following this 24 hour period of refrigeration, 120 μ l of the contents of each well was transferred to the respective well of a 96-well quartz plate (Hellma, Muellheim, Germany). Upon transfer, the quartz plate was read immediately using a BioTek EON plate reader over a spectrum of 220-300 nm in steps of 2 nm, as benzoquinones have previously been shown to peak in OD at 246 nm (Loconti and Roth, 1953). The area under the curve of each well was calculated over the range of 220-262nm, to give a measurement of the quantity of quinones present.

Quinone measurement at the end of the experiment:

Quinone measurement was carried out on five week old F2 adults, which had been kept under pathogen free conditions, at the end of the experiment. 45 uninfected adults of each line were cold shocked on ice and the benzoquinones in the quinone glands extracted and measured as described above.

Haemolymph extraction:

Haemolymph was extracted from 10 adults from each treatment population at transfers 1 and 11. Prior to extraction of haemolymph, PCR plates were prepared by pipetting 20 μ l of BisTris buffer (0.1M, pH 7.5) (Sigma-Aldrich, Steinheim, Germany) into each well. These plates were kept in the fridge until required for use. During haemolymph extraction the plate was kept on ice. For haemolymph extraction adult beetles were cold shocked on ice for around 10 minutes in order to immobilise them. Following this each beetle or larva was pricked with a 0.2 mm diameter needle (Bioform, Nuremberg, Germany) under a dissecting microscope and haemolymph extracted from the wound using a 1 μ l end-to-end capillary (Hirschmann, Eberstadt, Germany) that had been cooled on ice. Collected haemolymph was then transferred to a well of the PCR plate. Samples were finally diluted in BisTris buffer to a concentration of 0.1 μ l per 20 μ l and stored at -80°C until needed.

Phenoloxidase activity measurement:

Phenoloxidase activity was analysed for haemolymph samples from 10 adult beetles of each treatment at transfers 1 and 11. Haemolymph samples were

defrosted on ice prior to use and PO activity measured following the methods of (Roth *et al.*, 2010). Briefly, 50 μ l of deionised water and 50 μ l of BisTris buffer were pipetted into a flat bottom 96-well plate (Sarstedt, Nuembrecht, Germany) on ice. 20 μ l of each sample was then transferred to a single well of the flat bottom plate. Several blanks were also included for which 20 μ l of BisTris buffer was added instead of haemolymph. Finally 50 μ l of L-DOPA (Sigma-Aldrich, Steinheim, Germany) was pipetted into each well. The plate was then immediately transferred to a BioTek EON plate reader and read at 490 nm every 2 minutes for 90 minutes at 37°C. The V_{\max} of the linear phase of the reaction was used as a measure of PO activity. PO activity was measured for generations 1 and 11 of the evolution experiment.

Phenoloxidase activity at the end of the experiment

Phenoloxidase activity at the end of the experiment was carried out on F2 individuals of a mixed control or mixed intermediate line population, due to feasibility of handling the treatment combinations. PO activity was measured in approximately 2 week old larvae of the mixed populations, as larvae have been shown to be the most susceptible stage to oral infection in a number of beetle pathogens (Blaser and Schmid-Hempel, 2005; Milutinović *et al.*, 2013b). The larvae were ground in 20 μ l of BisTris as they were too small to extract haemolymph from. PO was then measured as described above.

In this case, however, prior to haemolymph extraction, individuals were challenged with either *B. bassiana* or a pathogen free control substance, either flour or BisTris buffer depending on the method of infection, through three different infection methods, pricking, dipping or feeding. Individuals in the pricked treatment were pricked with a 0.2 mm diameter needle that had either been dipped in BisTris buffer alone or BisTris buffer containing 10^8 spores per ml of the ancestral *B. bassiana* strain. Dipped individuals were fully immersed in either one of the same solutions and orally infected individuals were placed in a single well of a 96 well plate containing approximately 0.1 g of flour plus 5% yeast and either *B. bassiana* at a concentration of 10^8 spores per gram or non-inoculated flour.

Survival assay at the end of the experiment

Survival assays were carried out with F2 larvae from all of the evolved beetle lines against all evolved *B. bassiana* strains along with a mixed control population, compare *chapter 3*. Survival assays were carried out on populations of 50 individuals and carried out for 28 days. Detailed methods for the survival assay can be found in *chapter 3*. Survival was tested against both the ancestral and coevolved fungus strains isolated at the end of the experiment. Details of co-isolation of the fungus can be found in *chapter 3*.

Number of pupae throughout the experiment

At every transfer of the evolution experiment, the number of pupae was counted and recorded in order to track how the populations changed over time.

Development time at the end of the experiment

F1 adults were allowed to oviposit for 5 days, following the oviposition methods described above. Subsequently, jars were sieved and young larvae removed. Larvae were transferred to individual 55 X 11.6 mm glass vials (Assistant, Sondheim/Rhoen, Germany) closed with a cotton wool stopper (Herenz, Hamburg, Germany) each containing 0.17g of flour, representing the amount of flour per larva present during the evolution experiment. Larvae were kept individually throughout the experiment. Development was checked every other day and the number of days to eclosion calculated +/- 1 day.

Statistical analysis

The quinone production data were compared for each time point using Kruskal-Wallis tests. The data over time did not conform to any single distribution, with the distribution showing a consistent bimodal pattern over time. In order to get an impression of how the dynamics of these two peaks change over time the data were converted into binomial form, taking the lowest point of the entire distribution of all values as the cut off for what was classed as 1 and what as 0. These binomial data were then analysed with a nested Generalized Linear Model with a binomial error distribution and with transfer number and the nested effect of population

within treatment as random factors. Quinone data from F2 beetles were also analysed using both a Kruskal-Wallis test and a nested Generalized Linear Model with a binomial distribution.

PO measurements taken during the experiment were square root transformed to fit a Gaussian distribution and compared using a nested linear model, with transfer number and the nested factor assigned as random factors, followed by Tukey multiple comparison tests. PO data from the end of the experiment were log transformed to fit a Gaussian distribution then analysed using a Generalized Linear Model (GLM) followed by Tukey multiple comparison tests.

End point survival data were analysed using a Kruskal-Wallis test when testing survival on the ancestral pathogen and pairwise Wilcoxon tests when testing against coevolved pathogens. The data for the number of pupae over time were analysed using a nested linear model, again with transfer number and the nested effect of population within treatment assigned as random factors. Finally, development time data were analysed using a nested GLM with a Poisson distribution.

Wherever appropriate across analyses p values were corrected with the false discovery rate (fdr) method. The R packages “lme4” and “car” were used to create and analyse all nested models and the packages “lsmeans” and “multcomp” were used for post hoc tests on all linear and generalised linear models. All statistics were carried out in R version 3.0.2 (R core development team 2013) and all graphs were created using the ggplot2 package for R and edited in Inkscape version 0.48.

Results

Quinones throughout the experiment

When considering the mean quinone production values for each population, there were no significant differences at any time point during the evolution experiment among treatments (Kruskal-Wallis tests) (Table 1) (Figure 1). Quinone production, however, shows a bimodal distribution, consistently in all populations and at all

time points of the experiment. Consequently, a lot of information is lost when taking the mean as there are in fact two distributions, relating to two distinct strategies within each population, with one peak being around zero and the other much higher. To attempt to establish whether the frequencies of the two phenotypes changed over the course of the experiment, we converted the data into binomial format. These data showed that there was indeed a difference between treatments (nested GLM with binomial distribution: $\chi^2= 9.2641$, d.f=3, $p=0.026$) (Table 2), with the significant interactions coming from the high treatment tending to have fewer low producers (Table 3). No significant effect was seen across time nor was the interaction between treatment and time significant (nested GLM with binomial distribution, Table 2).

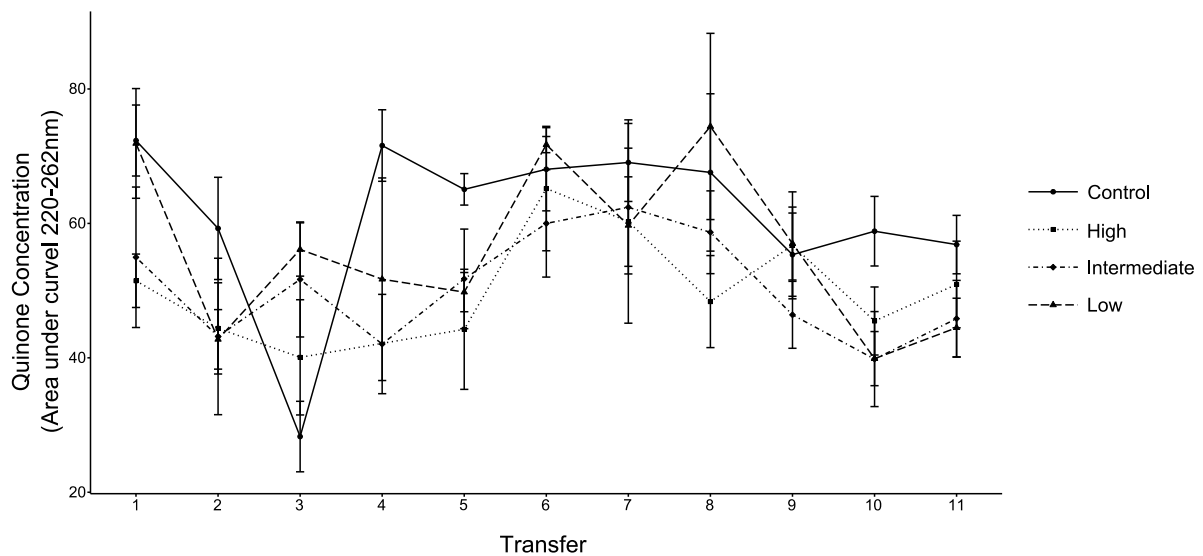


Figure 1. Population quinone production means throughout the evolution experiment. Error bars represent standard error of the mean.

Table 1. Results of Kruskal Wallis tests on quinone concentrations comparing treatments within each transfer of the evolution experiment. P values corrected using the fdr method.

Transfe	χ^2	d.f.¹	Adjusted p value
1	5.5942,	3	0.366025
2	3.2778,	3	0.5511
3	8.4138,	3	0.210045
4	3.2222,	2	0.43934
5	8.6939,	3	0.210045
6	1.9333,	3	0.716711
7	0.8727,	3	0.9152
8	1.9788,	3	0.716711
9	0.3616,	3	0.9481
10	7.2702,	3	0.233823
11	3.5681,	3	0.5511

¹ Degrees of freedom

Table 2. ANOVA results of the nested generalized linear model with binomial distribution for the concentration of quinones produced by beetles over the course of the experiment.¹

Effect	χ^2	d.f.²	p value
<i>B. bassiana</i> concentration	9.2641	3	0.02598
Transfer	0.1037	1	0.74739
<i>B. bassiana</i> concentration:Transfer	0.2138	3	0.97534

¹ In this model quinone production as a binomial classification of high or low was taken as the response variable. *B. bassiana* concentration was taken as a fixed factor and transfer and the nested effect of transfer within treatment were taken as random factors.

² Degrees of freedom

Table 3. Results of Tukey multiple comparison tests for differences in quinone concentration between *B.bassiana* concentration treatments throughout the evolution experiment.

Comaparison	Z value	p value
High:Control	2.71	0.034
Intermediate:Contro	2.466	0.0656
Low:Control	1.598	0.3793
Intermediate:High	-0.425	0.9742
Low:High	-1.191	0.6323
Low:Intermediate	-0.83	0.8402

Quinones at the end of the experiment

To establish whether the significant difference between treatments was an effect of evolution with significance of the time factor being masked by the variation between time points or whether this was an effect of pathogen exposure alone, quinones were extracted from F2 beetles of the last generation of the evolution experiment. Again, the population means did not differ significantly between treatments (Kruskal-Wallis test: $\chi^2= 0.8321$, d.f. = 3, p-value = 0.8418) (Figure 2). Results were converted into a binomial format to test whether the dynamics of the two peaks differed between treatments. These data were tested with a nested GLM with the binomial classification of high and low producers as the response variable, treatment as a fixed factor and the effect of replicate population within treatment as a random factor, which showed there to be no significant difference between treatments (ANOVA on nested GLM with binomial distribution: $\chi^2= 18.918$, d.f.=20, p=0.5272).

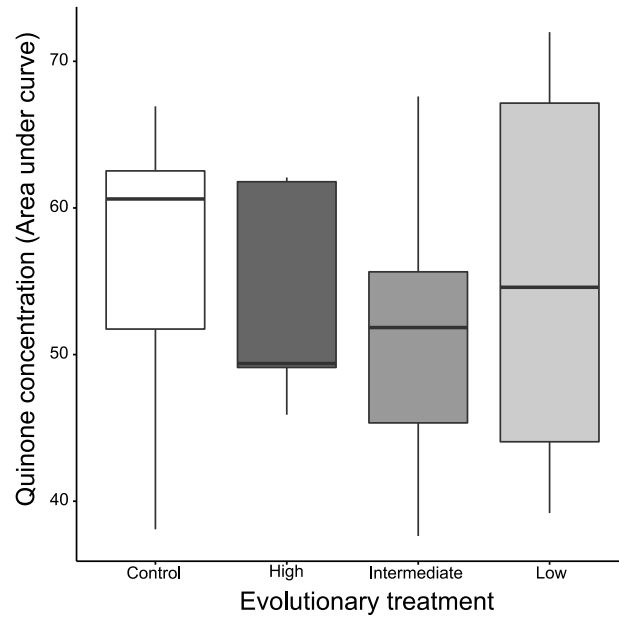


Figure 2. Box plots of population quinone production means of the F2 beetles from each treatment at the end of the experiment

PO throughout the experiment

PO activity was measured for beetles taken from generations one and 11 of the experiment. In all treatments PO activity was significantly higher in generation 11 than generation 1 (nested linear model: $\chi^2=48.3$, d.f=1, $p<0.001$) (Tables 4 and 5), however, this was a trend seen across all treatments including the control (Figure 3) (Table 5). The effect of treatment alone was not significant (Table 4), however, there was a significant interaction between transfer and treatment (nested linear model: $\chi^2=8$, d.f=3, $p=0.046$). In spite of this interaction, treatments did not differ significantly from one another at either time point (Tukey multiple comparison tests, Table 5).

Table 4. ANOVA results of the nested linear model comparing PO levels at the beginning and end of the experiment across treatments.¹

Effect	χ^2	d.f.²	p value
Transfer	48.298	1	<0.0001
Treatment	1.9405	3	0.58485
Transfer:Treatment	7.9805	3	0.04642

¹ In this model square root transformed PO values were taken as the response variable. Treatment and transfer were taken as fixed effects and the nested effect of beetle population within treatment was taken as a random effect.

² Degrees of freedom

Table 5. Tukey multiple comparisons of PO levels among treatments within each transfer and within treatments between transfers.

Subset	Comparison	d.f.¹	t ratio	p value
Transfer = 1:	Control:High	47.96	0.096	0.9997
	Control:Intermediate	61.79	-0.135	0.9991
	Control:Low	44.7	1.363	0.5287
	High:Intermediate	63.72	-0.199	0.9972
	High:Low	50.8	1.048	0.722
	Intermediate:Low	64.18	1.218	0.6181
Transfer= 11:	Control:High	58.48	-0.773	0.8664
	Control:Intermediate	52.01	-2.07	0.1764
	Control:Low	46.25	-2.151	0.1526
	High:Intermediate	57.54	-1.081	0.7021
	High:Low	52.63	-1.116	0.6813
	Intermediate:Low	46.14	-0.004	1
Control	1-11	328.93	2.179	0.03
High	1-11	334.94	2.468	0.0141
Intermediate	1-11	314.05	3.312	0.001
Low	1-11	325.95	5.848	<0.0001

¹ Degrees of freedom

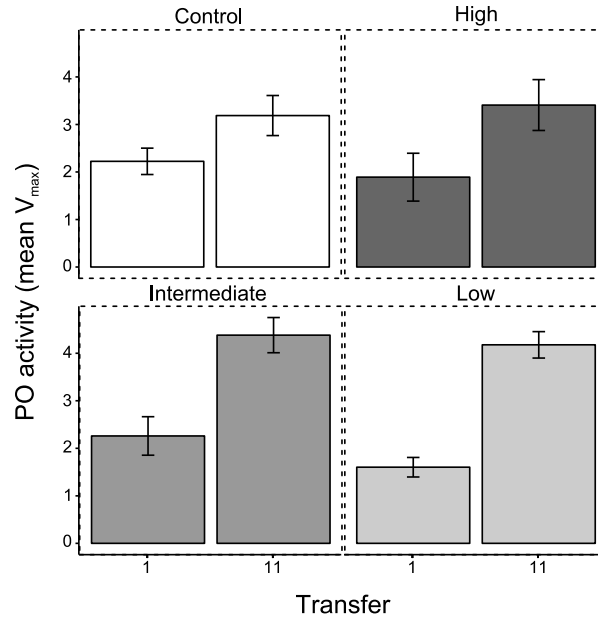


Figure 3. PO activity measured as V_{\max} from beetles taken directly from the experiment at transfers 1 and 11. Subtitles within the graph indicate the evolutionary treatment. Error bars represent standard error of the mean.

PO activity at the end of the experiment

The PO activity in the larvae of the mixed control and the mixed intermediate populations showed a non-significant trend in their PO responses to infection with *B. bassiana* as opposed to a control treatment (GLM: $\chi^2=3.7$, d.f.=1, $p=0.06$) (Table 6). Infection method, however, as well as being significant alone (GLM: $\chi^2=173.4$, d.f.=2, $p<0.001$) (Table 6) showed a significant interaction with beetle line (GLM: $\chi^2=7.5$, d.f.=2, $p=0.02$) (Table 6) meaning that evolved and control beetles responded in a significantly different way to different methods of pathogen challenge. When infected with a pathogen orally, coevolved beetles showed a non-significant trend towards down-regulating their PO responses in comparison to control beetles, whereas when infected via pricking, PO activity was significantly up-regulated in evolved beetles in comparison to control line beetles (Tukey multiple comparison tests, Table 7) (Figure 4).

Table 6. ANOVA results of the generalised linear model comparing PO activity of F2 control and intermediate treatment beetles following either infection or sham control via three different infection methods.¹

Effect	χ^2	d.f.²	p value
Pathogen	0.479	1	0.48879
Infection mode	173.394	2	<0.0001
Beetle line	1.812	1	0.17832
Pathogen: Infection mode	1.776	2	0.41152
Pathogen: Beetle line	3.669	1	0.05544
Infection Mode: Beetle line	7.468	2	0.0239
Pathogen :Infection mode: Beetle line	0.009	2	0.99564

¹ In this model log transformed Vmax measurements of PO activity were taken as response variables. Infection mode, Beetle line and Pathogen were taken as fixed effects.

² Degrees of freedom

Table 7. Tukey multiple comparison tests comparing PO activity between beetle lines within each of three modes of infection, dipping, feeding or pricking.

Subset	Comparison	Z ratio	p value
Dipping	Control:	-0.9693427	0.3324
Feeding	Control:	0.9595175	0.3373
Pricking	Control:	-2.7274502	0.0064

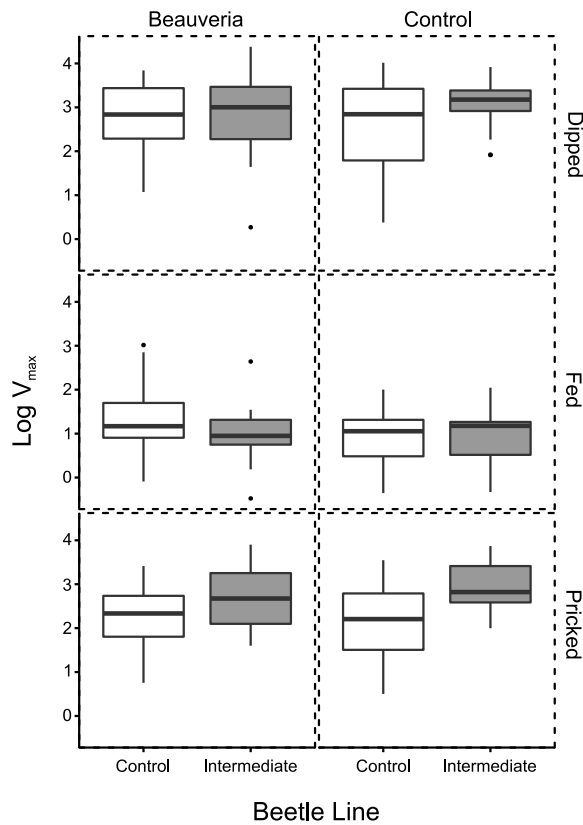


Figure 4. PO responses ($\text{Log } V_{\max}$) of F2 beetles from the Control (C) and Intermediate treatment (I) mixed populations of the evolution experiment upon exposure to *B. bassiana* or a control substance via three different infection methods.

Survival at the end of the experiment

There were no significant differences in survival of the evolved beetle lines to one another on the ancestral (Kruskal-Wallis test: $\chi^2=7.2$, $df=8$, $p=0.5$) (Figure 5) or coevolved (pairwise Wilcoxon tests, Table 8) lines of the fungus.

Table 8. Pairwise comparisons of survival of evolved and control beetle lines on coevolved *B. bassiana* co-isolated from the beetle line against which it is tested.

Beetle line compared to the control	W	p value
High, replicate 1	3	0.8
High, replicate 2	8	0.46666667
Intermediate, replicate 2	4	0.8
Intermediate, replicate 3	0	0.46666667
Intermediate, replicate 5	7	0.469525
Low, replicate 6	6	0.46666667
Low, replicate 3	6	0.70924

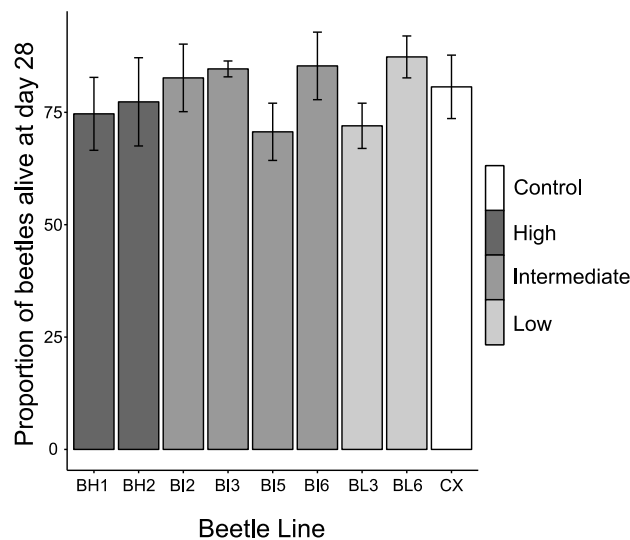


Figure 5. The proportion of beetles alive from each of the coevolved populations at the end of the survival assay following exposure to the ancestral *B. bassiana* strain. Error bars represent standard error of the mean. BH1 and 2 = high treatment lines, BI2, 3, 5 and 6 = intermediate treatment lines, BL3 and 6 = low treatment lines and CX = the mixed control population.

Number of pupae over time

The number of pupae present at each transfer of the experiment changed both over time (Nested Linear Model: $\chi^2=4.49$, d.f.=1, $p=0.034$) (Table 9) and differed between treatments (Nested Linear Model: $\chi^2=11.27$, d.f.=3, $p=0.01$) (Table 9). Furthermore,

the interaction between treatment and transfer differed highly significantly (Nested Linear Model: $\chi^2=24.96$, d.f.=3, $p<0.0001$) (Table 9) meaning that the level of change over time differed dramatically between treatments, with the high treatment in particular changing from having the least number of pupae at the beginning to the most at the end of the experiment (Figure 6) (Table 10).

Table 9. ANOVA results of the nested linear model comparing the number of pupae present in each experimental population at each transfer of the experiment.¹

Effect	χ^2	d.f.²	p value
Treatment	11.27	3	0.01036
Transfer	4.49	1	0.034
Treatment: Transfer	24.96	3	<0.0001

¹ In this model the number of pupae for each population was taken as the response variable. Treatment and the interaction between treatment and transfer were taken as fixed factors. Transfer alone and the nested effect of population within treatment were taken as random factors.

² Degrees of freedom

Table 10. Multiple comparisons of the number of pupae in each population among treatments at each transfer throughout the experiment. P values are fdr corrected.

Subset	Comparison	d.f.¹	t ratio	p value
Transfer 1	Control - High	250.73	1.553	0.2433
	Control - Intermediate	250.73	2.423	0.0485
	Control - Low	250.73	2.537	0.0485
	High - Intermediate	250.73	0.724	0.5637
	High - Low	250.73	0.829	0.5637
	Intermediate - Low	250.73	0.11	0.9125
Transfer 2	Control - High	250.73	-0.972	0.7073
	Control - Intermediate	250.73	-1.611	0.6508
	Control - Low	250.73	-0.869	0.7073
	High - Intermediate	250.73	-0.54	0.7073
	High - Low	250.73	0.142	0.8875
	Intermediate - Low	250.73	0.715	0.7073
Transfer 3	Control - High	250.73	1.753	0.2425
	Control - Intermediate	250.73	0.74	0.5518
	Control - Low	250.73	2.068	0.2384
	High - Intermediate	250.73	-1.015	0.4665
	High - Low	250.73	0.205	0.838
	Intermediate - Low	250.73	1.28	0.404
Transfer 4	Control - High	250.73	1.221	0.4467
	Control - Intermediate	250.73	1.513	0.4467
	Control - Low	250.73	1.285	0.4467
	High - Intermediate	250.73	0.21	1
	High - Low	250.73	0	1
	Intermediate - Low	250.73	-0.22	1

Transfer 5	Control - High	250.73	-0.856	0.4716
	Control - Intermediate	250.73	-1.446	0.2242
	Control - Low	250.73	-3.231	0.0085
	High - Intermediate	250.73	-0.501	0.6168
	High - Low	250.73	-2.141	0.1
	Intermediate - Low	250.73	-1.72	0.1736
Transfer 6	Control - High	250.73	-1.33	0.5077
	Control - Intermediate	250.73	-0.155	0.9789
	Control - Low	250.73	-0.027	0.9789
	High - Intermediate	250.73	1.144	0.5077
	High - Low	250.73	1.262	0.5077
	Intermediate - Low	250.73	0.124	0.9789
Transfer 7	Control - High	250.73	-1.849	0.1315
	Control - Intermediate	250.73	-2.031	0.1315
	Control - Low	250.73	-1.946	0.1315
	High - Intermediate	250.73	-0.079	1
	High - Low	250.73	0	1
	Intermediate - Low	250.73	0.083	1
Transfer 8	Control - High	250.73	-2.339	0.1209
	Control - Intermediate	250.73	-0.612	0.6494
	Control - Low	250.73	-0.883	0.5671
	High - Intermediate	250.73	1.7	0.2714
	High - Low	250.73	1.451	0.2963
	Intermediate - Low	250.73	-0.261	0.794

Transfer 9	Control - High	250.73	-1.186	0.7123
	Control - Intermediate	250.73	-1.091	0.7123
	Control - Low	250.73	-0.534	0.7123
	High - Intermediate	250.73	0.144	0.8854
	High - Low	250.73	0.656	0.7123
	Intermediate - Low	250.73	0.537	0.7123
Transfer 10	Control - High	250.73	-3.022	0.0166
	Control - Intermediate	250.73	-1.901	0.1168
	Control - Low	250.73	-0.707	0.4803
	High - Intermediate	250.73	1.176	0.301
	High - Low	250.73	2.273	0.0716
	Intermediate - Low	250.73	1.151	0.301
Transfer 11	Control - High	250.73	-2.552	0.0678
	Control - Intermediate	250.73	-1.873	0.1245
	Control - Low	250.73	-0.508	0.612
	High - Intermediate	250.73	0.747	0.5467
	High - Low	250.73	2.001	0.1245
	Intermediate - Low	250.73	1.315	0.2844
Transfer 12	Control - High	250.73	-1.83	0.2055
	Control - Intermediate	250.73	0.49	0.6409
	Control - Low	250.73	-0.467	0.6409
	High - Intermediate	250.73	2.22	0.1645
	High - Low	250.73	1.341	0.3627
	Intermediate - Low	250.73	-0.922	0.5364

Transfer 13	Control - High	250.73	-3.56	0.0013
	Control - Intermediate	250.73	-1.212	0.2723
	Control - Low	250.73	0.545	0.5866
	High - Intermediate	250.73	2.33	0.0414
	High - Low	250.73	3.943	0.0006
	Intermediate - Low	250.73	1.692	0.1379

¹ Degrees of freedom

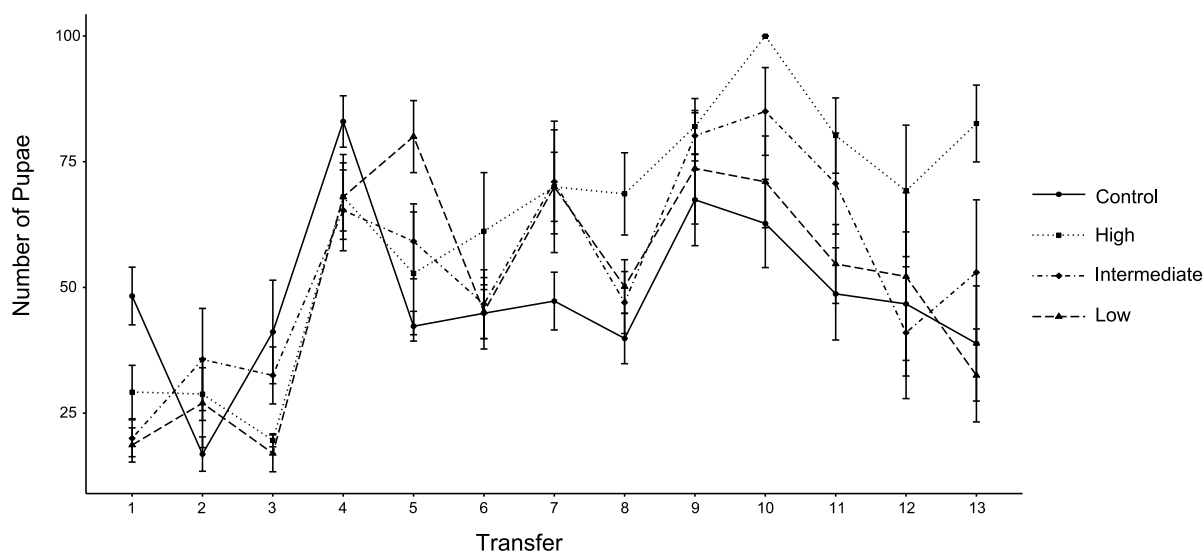


Figure 6. Mean number of pupae present at each transfer of the experiment for each treatment. Error bars represent standard error of the mean.

Development time at the end of the evolution experiment

Development time did not differ significantly between the F2 of the intermediate coevolved treatment and the control treatment or among populations within treatment (ANOVA on nested GLM with Poisson error distribution: $\chi^2=0.1638$, d.f=1, $p=0.6857$).

Discussion

As a consequence of host-parasite co-evolution, resistance to *B. bassiana*, in terms of increased survival did not appear to change in our experiment, even when hosts

were exposed to virulent coevolved isolates. Neither was a change seen in development time, mean population quinone production or background phenoloxidase activity, contrary to our initial predictions. We did, however, see that the number of pupae produced each generation increased throughout the experiment with coevolving populations in particular appearing to buffer the effects of parasite exposure on the number of pupae produced as the experiment progressed. As we did not see an evolved change in development time among treatments or lines, the number of pupae can be taken as an indirect measure of the number of offspring and therefore also as an indirect measure of fitness, indicating that fitness of the coevolving population increased throughout the experiment. Furthermore, although background PO levels did not differ among treatments, there were differences in PO activity between the control and intermediate coevolved treatment lines as a response to differing infection methods, indicating that the coevolved beetles are better able to regulate their PO activity in response to specific threats.

The fact that we saw an apparent increase in fitness during the experiment, but failed to see an increase in survival, is consistent with the findings of (Kraaijeveld and Godfray, 2008), who failed to find an increase in survival or life time reproductive success as a result of artificial selection for resistance to *B. bassiana* in *Drosophila melanogaster*. They did, however, find an increase in late life reproduction, relevant for fitness in their experimental design, which, consistently with our results indicates that resistance to *B. bassiana* can be subtle and perhaps somewhat complicated to obtain. Similar to the study by Kraaijeveld and Godfray (2008), this increase in number of pupae we observe here may be an indicator of increased tolerance to *B. bassiana* rather than increased resistance *per se* (Råberg *et al.*, 2009), although we cannot draw any definite conclusions on this from the data we present here. It is possible that beetles may have evolved to buffer fitness costs upon infection without reducing the infection success or within host reproduction rate of the pathogen by optimising their reproductive rate for the experimental conditions without necessarily increasing survival. In contrast to our findings and the findings of Kraaijeveld and Godfray (2008), in a different evolution experiment,

Dubovskiy *et al.* (2013b) found increased resistance to evolve in *Galleria mellonella* after 25 generations of one sided adaptation to *B. bassiana*. The contradictory results of Dubovskiy *et al.* (2013b) could potentially either be due to the *G. mellonella* population being somehow more predisposed to evolve increased antifungal defences, or that *G. mellonella* may have been under stronger selection by *B. bassiana* as, at least in comparison to *T. castaneum*, *B. bassiana* displayed higher virulence towards *G. mellonella*.

The observed increased ability to cope with *B. bassiana* under the experimental conditions may be a result of an increased ability to regulate PO activity. We observed that in the coevolved beetles from our experiment, PO activity was down-regulated upon exposure to *B. bassiana* via feeding, but up-regulated upon penetration of the cuticle via pricking, even without a pathogen present. Background PO activity, however, did not differ between control and coevolved beetles. It has been shown in the ancestral beetle stock used to start this experiment (Cro1) that the immune response to *Bacillus thuringiensis* (Bt) differs depending on infection route (Behrens *et al.*, 2014), potentially providing the genetic basis for more strongly differentiated responses to evolve, as we see here. Interestingly, the change in PO activity under specific conditions that we see here is somewhat consistent with the results of Dubovskiy *et al.* (2013b) who found that *G. mellonella* adapted to *B. bassiana* did not show an increase in background PO activity but did show heightened PO activity in the cuticle in the early stages of *B. bassiana* infection.

The finding that PO activity differed in the coevolved beetle lines dependent on exposure method is particularly interesting in light of the findings of the *B. bassiana* genome sequencing project, where genes were found that appeared to be very similar to Bt cry like toxins (Xiao *et al.*, 2012). Bt infects solely orally, with infection being dependent on cry like toxins which form pores in the gut (Schnepf *et al.*, 1998). The fact that we see differential responses in PO activity upon infection via feeding and pricking through the cuticle indicates that the beetles have adapted to two different methods of infection. It is possible that in the case of oral infections a

more specific immune response is initiated and PO activity reduced in order to save resources for these more specific responses (Haine *et al.*, 2008). In the case of penetration through the cuticle, on the other hand, it could make sense to quickly close the hole and encapsulate any invading fungal cells, a response that would be dependent on the PO cascade and something that has indeed been shown to increase as a result of evolved resistance to *B. bassiana* in the past (Dubovskiy *et al.*, 2013b). Our finding that PO activity is differentially regulated depending on infection method in beetles coevolved with *B. bassiana* is the first experimental indication, to our knowledge, that *B. bassiana* does indeed have two methods of infection.

If it is the case that *B. bassiana* is able to infect via these two routes, it may go some way to explaining why both evidence of evolved resistance (Kraaijeveld and Godfray, 2008) and evidence of acquired resistance to *B. bassiana* (Reber and Chapuisat, 2012) have been difficult to demonstrate in the past and also why we do not see a clear pattern of resistance here. Particularly if the two infection routes require different types of immune response, it may be both costly, in terms of required resources, and difficult, in terms of the number of alleles required, to convey complete resistance. This could be why the result we see here and the results of Kraaijeveld and Godfray (2008) are more indicative of evolved tolerance than evolved resistance and also why even when resistance does evolve, immune responses seem to be either somewhat specific, as in both our case and that of Dubovskiy *et al.* (2013b), or localised (Dubovskiy *et al.*, 2013b).

Contrary to our expectations, we did not see a change in mean quinone production among populations. This is somewhat surprising considering that we observed increased quinone resistance in the coevolved *B. bassiana* (chapter 3), meaning that, at least initially, the beetles' external immune secretions were an important barrier to overcome for the fungus. This adaptation did, however, come rapidly, within 7 host generations and it could be that increased quinone production was of little benefit to the beetles after *B. bassiana* had achieved resistance, particularly as in high concentrations, quinones can be toxic to the larvae of the beetles themselves

(Chapman, 1926, Joop et al. 2014). Although we had speculated that we would see inverse patterns of change in quinone production and PO levels, due to a putative trade-off based on the fact that both cascades are tyrosine dependent, the fact that we did not see change in quinone production does support the findings of Joop *et al.* (2014) who found that selecting quinone production away from the natural state in either direction reduced PO activity. Together with the findings of Li et al. (2013) and Joop *et al.* (2014), this suggests that the interaction between the two aspects of the immune system could be in a delicate balance, with any move from the current stage having negative effects. Although we did see changes in PO activity these were in opposite directions dependent on the method of infection. If infection happened equally through both routes in the experiment, mean PO activity would not have differed, providing a potential explanation for why we did not see any correlated change in quinone production.

All in all, although we did not find evidence of increased survival in the presence of *B. bassiana* in *T. castaneum* as a result of host-parasite coevolution, we did find some evidence potentially indicating an increased ability of coevolving populations to cope with the presence of *B. bassiana* as a consequence of a more flexible PO response. This result demonstrates, consistently with the work of Kraaijeveld and Godfray (2008) that *B. bassiana* may be more difficult for insects to evolve resistance to than other parasite species, potentially as a result of having two mechanisms of infection (Xiao *et al.*, 2012). The fact that we did see infection method dependent PO responses in coevolved beetles exposed to *B. bassiana* does provide the first empirical indication that *B. bassiana* may indeed be able to infect orally, which is something that certainly warrants further attention. Furthermore, in order to observe evolved change we had to look at traits under very specific conditions that showed no change on a general level, highlighting the importance of very carefully selecting which phenotypes to examine at the end of evolution experiments.

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Summary and Outlook

To re-entangle a disentangled bank...

I carried out a host-parasite coevolution experiment where *Tribolium castaneum* was allowed to coevolve separately with two of its parasites, *Paranosema whitei* and *Beauveria bassiana*. *Tribolium castaneum* secretes antimicrobial compounds into its environment (Prendeville and Stevens, 2002; Yezerski *et al.*, 2005; Joop *et al.*, 2014) and both *B. bassiana* and *P. whitei* are able to persist outside of the beetle hosts (Milner, 1972; Hong *et al.*, 1997). The evolution experiment was carried out with three different concentration treatments for each parasite, low, intermediate and high, representing three different levels of parasite induced mortality and consequently infection probability. The three concentrations were predicted to have differing effects on the trajectory of evolution in the host immune system (see *General Introduction*). This experiment aimed to fill a gap in the literature by allowing the external immune compounds of the beetles and persistent parasite stages to play a role in the coevolutionary interaction, rather than limiting the interaction to processes occurring within the host.

I found that as a consequence of coevolution, both parasites increased in virulence. I only saw subtle changes, however, in *T. castaneum* beetles coevolved with *B. bassiana*. Coevolving beetle populations showed an apparent increase in ability to cope with *B. bassiana* throughout the experiment and an increased ability to up or down regulate phenoloxidase (PO) activity dependent on the mode of infection. *P. whitei*, particularly in the low treatment, rapidly increased in virulence contributing to a collapse of the host populations and eventually to host extinction. *B. bassiana* also showed a rapid increase in virulence, having substantially increased its induced mortality of the host in all treatments by the mid-point of the experiment. Fascinatingly, we were able to demonstrate for *B. bassiana* that increased virulence

was achieved via increased resistance to the external immune compounds of the beetles.

The mechanism of transmission is likely to have been vital in allowing for increased virulence to evolve in the case of both parasites. This is consistent with my first objective in the *General Introduction* where I hypothesised that allowing for more natural parasite transmission to occur across host generations and for transmissive parasite stages to be allowed to persist in the environment, that an increase in virulence would be seen in my experiment (objective i). This is particularly interesting in the case of *P. whitei* as it provides exact opposite results from a previous experiment in the same system where a decrease in virulence occurred (Béréños *et al.*, 2009). Here transmission mode can be highlighted as a clear factor influencing differences in results that we see. Whereas in our experiment a portion of the environment was transferred from generation to generation meaning that spores from hosts that burst across a range of time points would make it to the next generation, Béréños *et al.* (2009) took spores from intact hosts at a relatively late time point for the parasite (Milner, 1972a). This meant in the case of Béréños *et al.* (2009) transmission could be maximised by being relatively benign to the host and reproducing at a slower rate (Figure 1).

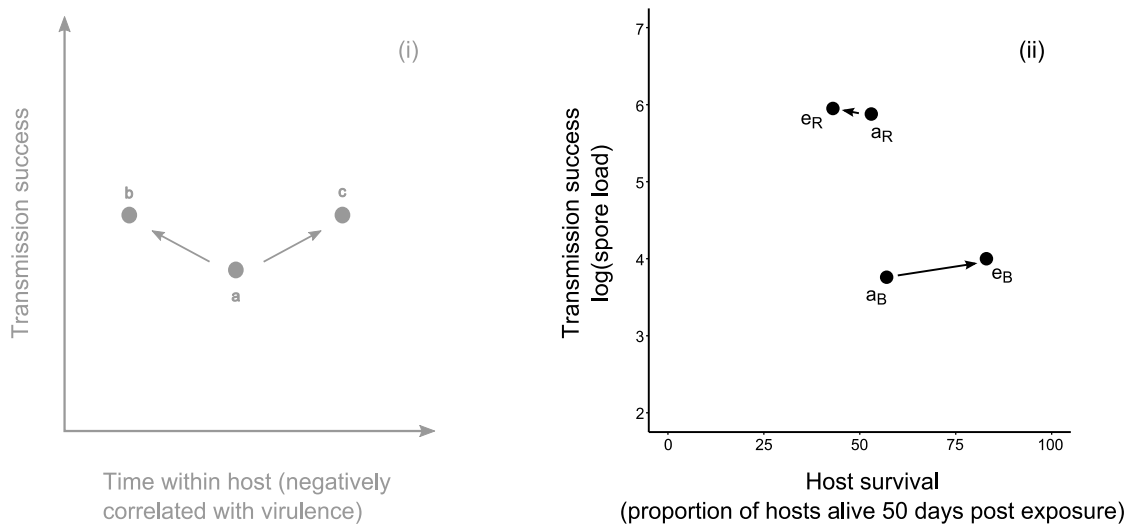


Figure 1. Predictions vs observations for the evolution of transmission success in evolution experiments. (i) under natural conditions a parasite is predicted to evolve increased transmission success in comparison to the ancestral state (a) by reproducing more rapidly within the host, reducing time spent within the host and increasing virulence (b) whereas if parasite transmissive stages are artificially taken from hosts by the experimenter at a relatively late time point transmission success is likely to be increased by reproducing more slowly within the host, remaining in the host up until the point of extraction thereby reducing virulence (c). (ii) shows experimental results from *chapter 2* and Béréños *et al.* (2009) on comparable axes. Here we show that in my experiment with closer to natural transmission, transmission success was increased from the ancestral state (a_R) to the coevolved state (e_R) by increasing virulence, whereas in the case of Béréños *et al.* (2009) transmission success was increased by decreasing virulence from the ancestral state (a_B) to the coevolved state (e_B).

Theory predicts that virulence will increase as a consequence of host-parasite coevolution (Gandon *et al.*, 2002), a fact supported by natural observations (Ebert, 1994; Sloan *et al.*, 2008). Allowing for more natural transmission to occur, both through the environment and through having an overlap in generations, is likely to be responsible for the fact that our results for both parasites more closely mirror both the theoretical predictions and results from natural systems (Ebert, 1994; Gandon *et al.*, 2002; Sloan *et al.*, 2008; *chapter 1*) (Figure 1) than previous coevolution experiments (Lohse *et al.*, 2006; Béréños *et al.*, 2009; Kashiwagi and Yomo, 2011; Béréños *et al.*, 2011). Our experimental design is likely to have reduced the potential confounding effects of parasite adaptation to the transmission strategy imposed by

the experiment, which may have been responsible for reduced virulence being observed in previous coevolution experiments (*chapter 1*).

Further to allowance for more natural transmission alone, through our experimental design we allowed parasite spores to persist in the environment across multiple host generations. When a parasites longevity exceeds that of its host increased, or even extreme, virulence is predicted to be selected for under certain circumstances (Bonhoeffer *et al.*, 1996), a prediction known as “the curse of the pharaoh hypothesis”. In nature, *P. whitei* spores can persist for over 6 months outside of a host (Milner, 1972) and *B. bassiana* spores, although not being as long lived as *P. whitei* can also survive outside of a host for substantially longer than an experimental host generation (Hong *et al.*, 1997). Furthermore, the external immune compounds of the beetles are effective against both pathogens (Ruther *et al.*, 2001; Joop *et al.*, 2014; *chapter 3*), potentially drastically reducing the longevity of the spores in the environment. Consequently, any increase in longevity in the presence of quinones could have provided the decrease in spore mortality predicted by the curse of the pharaoh model to select for increased virulence (Bonhoeffer *et al.*, 1996). Although I did not test this prediction implicitly it may have played a role in the increased virulence that I observed and at least in the case of *P. whitei* the modelling results showed that it could have potentially contributed to the observed change (*chapter 1*).

In *B. bassiana* we were able to ascertain how increased virulence was attained. The fact that we were able to identify the precise barrier of the immune system overcome in order for an increase in virulence to be achieved is novel and something that has not been shown in previous evolution experiments where increased parasite virulence evolved (Kolodny-Hirsch and Van Beek, 1997; Schulte *et al.*, 2010; Garbutt *et al.*, 2011). *Beauveria spp.* are relatively strongly affected by quinone secretions (Ruther *et al.*, 2001; *chapter 2*) and as predicted in objective (ii) (see *General Introduction*) more resistant and therefore more infective genotypes appear to have been favoured by selection (Figure 2). The fact that *B. bassiana* evolved to overcome the external immune system of *T. castaneum* indicates both

that external immunity is important for the beetles in defence against entomopathogenic fungi and that it presents an important barrier for coevolving parasites to overcome. Furthermore, it is impressive that the fungus was able to evolve resistance to such a broadly cytotoxic compound, showing that, although being a strong barrier to infection; external immunity is not a barrier that cannot be overcome. It would be interesting to more closely examine the effects of external immunity on coevolution in other insect species with antimicrobial secretions such as burying beetles (Cotter *et al.*, 2010), termites (Rosengaus *et al.*, 2004; Hamilton *et al.*, 2011) and ants (Poulsen *et al.*, 2002; Kay *et al.*, 2014), to see if the outcomes we observed here hold true in other systems.

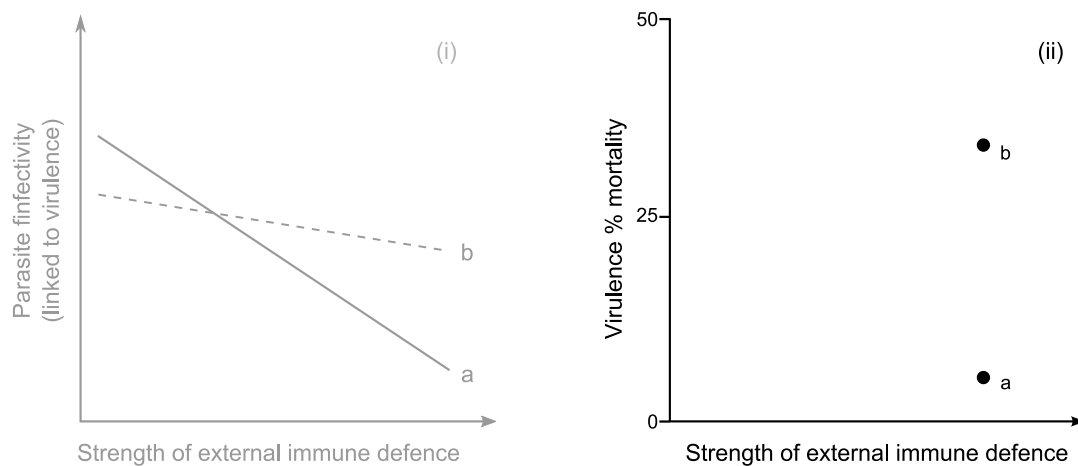


Figure 2. (i) represents the prediction made in the *General Introduction* that at increasingly strong levels of external immune defence resistance genotypes (b) would be favoured by selection due to fitness benefits of increased infectivity, resulting in increased virulence. (ii) shows my data for mean virulence of ancestral (a) and coevolved (b) isolates of *B. bassiana* (chapter 3), as external immune defences did not change during the course of the experiment I only have data for one level of host immune defence. I assume that *T. castaneum* external immune defences exert relatively strong effect on *B. bassiana* due to the low levels of virulence in the ancestral strain (a) as predicted increased virulence evolved as a consequence of increased resistance to *T. castaneum* external immune compounds (b).

Despite seeing such profound change in both parasites, changes in the host were not so distinct. Hosts were driven to complete extinction as a result of coevolution

with *P. whitei* after very few generations. Consequently, any change that had occurred could not be measured at the end of the experiment. Extinction itself, however, indicates that there was no substantial increase in host fitness. In the *B. bassiana* experiment some subtle changes were seen in the host but not on the scale that has been seen in previous coevolution experiments with *T. castaneum* (Béréanos *et al.*, 2009; Kerstes *et al.*, 2012) or in published coevolution experiments in general (reviewed in Brockhurst and Koskella, 2013).

The two parasites used in our experiments represented opposite ends of the virulence scale, with *P. whitei* showing relatively high virulence to *T. castaneum* and *B. bassiana* relatively low. Consequently, it could be that *P. whitei* induced little change in the host as it simply increased in virulence and environmental concentration too rapidly for the host to respond. Conversely, *B. bassiana* may have initially only put a weak selection pressure on *T. castaneum*. After increasing in virulence, however, it may have presented a complex evolutionary challenge for the beetles to overcome. If *B. bassiana* is indeed able to infect both orally and through the cuticle (Xiao *et al.*, 2012, chapter 4), this may have further complicated the host reaction, causing evolutionary compromises between differing aspects of immune defence to be made. This could explain the evolution of apparent increased flexibility in PO response, which in itself indicates that *B. bassiana* is able to infect both orally and through the cuticle, providing the first experimental indication of the suggestion arising from the sequenced genome of *B. bassiana* (Xiao *et al.*, 2012).

My third objective, objective (iii), proposed that external immunity would be traded -off against internal immunity. I found no evidence to support or contradict this hypothesis. External immunity was neither selected to increase nor decrease during the course of coevolution and although PO responses (the primary component of the internal immune system predicted to be traded-off against external immunity) did appear, to an extent, to be under selection no overall change in background PO activity was observed across treatments. Consequently, it appears that neither component of the immune system predicted to be involved in the trade-off

followed a clear trajectory of evolution and it is therefore impossible to draw conclusions on the hypothesised trade-off.

In objective (iv) I proposed that the environmental concentration of the parasite would affect how resources were invested into each arm of the host immune system over time. Parasite concentration, however, appeared to have little or no effect on host immune allocation over evolutionary time in this experiment. This hypothesis is, however, in itself reliant on the prediction that the components of the immune system are traded-off against one-another and although they have been shown to be linked, no clear evidence of a trade-off has been found (Joop *et al.*, 2014). If the trade-off does not stand, it would explain why concentration did not have an effect here. A further potential explanation is that either the starting parasite concentrations did not sufficiently differ from one another to induce the evolutionary effects predicted or that, as the parasites both rapidly increased in virulence and consequently in reproductive success, that parasite concentration simply increased too rapidly for host external immunity to be able to control it, resulting in all treatments being at a “high” concentration. This is certainly true in the case of *P. whitei* where we found parasite concentrations to not differ significantly from one another across treatments at the end of the experiment, i.e. the point of host extinction, however, as the host went extinct I do not have data on host immunity. Nevertheless, it is certainly plausible that *B. bassiana* concentration followed a similar trajectory. Investment in increased quinone production is potentially costly in general as in high concentrations quinones can be toxic to the larvae of the beetles themselves (Chapman, 1926). This means that increases above a certain level come at an extreme fitness cost. It could also have been that quinone concentrations are already at the optimal level in terms of a trade-off between defence against parasites and inadvertent killing of larvae (Joop *et al.* 2014).

All in all, our results, particularly those relating to virulence evolution, differ from those from other coevolution experiments (Lohse *et al.*, 2006; Béréños *et al.*, 2009; Béréños *et al.*, 2011; Kashiwagi and Yomo, 2011). In *chapter 2* I presented a mathematical model demonstrating that the presence of transmissive parasite

stages likely contributed to the increase in virulence that I observed in *P. whitei*, predictions that may well hold in *B. bassiana*. Furthermore, I demonstrated in *chapter 3* that host external immune compounds had a profound effect on the trajectory of evolution in *B. bassiana*. Together, the results presented in this thesis strongly indicate that inclusion of parasite transmissive stages and the consideration of extended immune phenotypes affect the course of host-parasite coevolution. These results have both contributed to our general understanding of coevolutionary interactions and highlighted several areas of research that require further attention. We have found evidence indicating that *B. bassiana* does indeed infect both orally and through the cuticle, as suggested by (Xiao *et al.*, 2012). This is something that is most certainly of future interest as it may explain both how *B. bassiana* succeeds so well as a generalist and why it appears so complicated to evolve and acquire resistance to (Kraaijeveld and Godfray, 2008; Reber and Chapuisat, 2012). Most significantly, we have demonstrated that experiments continuing to include the effects of extended phenotypes and in particular more carefully considering the mode of parasite transmission are needed in order to gain a more comprehensive understanding of host-parasite interactions. My experiments have provided a clear step in this direction.

A wide range of animal taxa are able to manipulate their environment via extended immune phenotypes including birds (Shawkey *et al.*, 2003), amphibians (Fleming *et al.*, 2009), social (Rosengaus *et al.*, 2004) and non-social (Cotter *et al.*, 2010) insects and fish (Giacomello *et al.*, 2006) (reviewed in Cotter and Kilner, 2010). Similarly, microparasites able to persist in the environment span a number of taxa, covering bacteria (Decaestecker *et al.*, 2007), microsporidia (Milner, 1972b), fungi (Hong *et al.*, 1997) and viruses (Yates *et al.*, 1985) among others. In order to understand how interactions evolve in nature in organisms that possess such traits, we require more experiments which allow environmental manipulation by host and parasite and, critically, transmission to occur in a way that closely resembles the natural state. Only then can we answer some of the open questions that remain, such as does the curse of the pharaoh hypotheses hold true when empirically tested? Does external immunity in general select for more virulent parasites? How often are external

immune traits under selection as a result of host-parasite coevolution? How often do trade-offs between virulence and transmission hold true? These and many other questions can only be addressed when all aspects of the hosts and the parasites ecology are taken into account.

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Rafaluk, Schulenburg and Joop. (unpublished manuscript) Chemical warfare does not always win – rapid evolution of increased virulence in *Beauveria bassiana*. – Chapter 3 of thesis

Rafaluk, Wagner, Schulenburg and Joop (unpublished manuscript). Cryptic changes in immune response and fitness in *Tribolium castaneum* as a consequence of coevolution with *Beauveria bassiana*. – Chapter 4 of thesis

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Appendix

List of chemicals and disposables and suppliers:

Disposable/ chemical	Supplier
8-lid strips	Sarstedt
15ml tubes	Sarstedt
2ml tubes	Sarstedt
50ml tubes	Sarstedt
6-well plates	Greiner Bio-one
96-well plates	Sarstedt
Agar-Agar Kobe 1	Roth
Amphotericin B	Sigma-Aldrich
BisTris	Sigma-Aldrich
1 µl end to end capillaries	Eydam
Chloramphenicol	Roth
Cotton wool fillers	Eydam
96 well flat bottom plates	Sarstedt
Flour, Alnatura type 405	DM-drogerie markt
Glass vials	Eydam
Glycerol	Roth
Beetle rearing jars	Bardenhewer, Kiel
L-DOPA	Sigma-Aldrich
MBQ	Sigma-Aldrich
Methanol	Roth
Needles	Bioform.de
Pasteur pippettes	Sarstedt
PCR plates	Sarstedt
Petri dishes	Sarstedt
qPCR plates	Sarstedt
Tween 80	Roth
Yeast	Leiber