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# Genetic diversity, virulence and fitness evolution in an obligate fungal parasite of bees

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## Abstract

Within-host competition is predicted to drive the evolution of virulence in parasites, but the precise outcomes of such interactions are often unpredictable due to many factors including the biology of the host and the parasite, stochastic events and co-evolutionary interactions. Here, we use a serial passage experiment (SPE) with three strains of a heterothallic fungal parasite (*Ascosphaera apis*) of the Honey bee (*Apis mellifera*) to assess how evolving under increasing competitive pressure affects parasite virulence and fitness evolution. The results show an increase in virulence after successive generations of selection and consequently faster production of spores. This faster sporulation, however, did not translate into more spores being produced during this longer window of sporulation; rather, it appeared to induce a loss of fitness in terms of total spore production. There was no evidence to suggest that a greater diversity of competing strains was a driver of this increased virulence and subsequent fitness cost, but rather that strain-specific competitive interactions influenced the evolutionary outcomes of mixed infections. It is possible that the parasite may have evolved to avoid competition with multiple strains because of its heterothallic mode of reproduction, which highlights the importance of understanding parasite biology when predicting disease dynamics.

## Introduction

The evolutionary arms race between hosts and parasites is considered a fundamental driver of evolution (Haldane, 1949; Kochin *et al.*, 2010). In nature, most parasite infections consist of multiple strains or species, which will ultimately exert different selection pressures on the host than would single infections (Schmid-Hempel, 1998; Alizon *et al.*, 2013). When infections involve a single parasite strain, its fitness will generally be maximized by prudent exploitation of the host's resources. This leads to a characteristic life-history strategy by the parasite to optimize its fitness, which will change depending on the prevailing environmental and biological conditions affecting both host and parasite (Anderson & May, 1981; Levin & Pimentel, 1981; May & Anderson, 1983; Frank, 1996). However, host co-infections by several strains of the same parasite species will influence this optimum and may result in prudent parasite strains being outcompeted or suppressed by faster-growing or more virulent strains (Read & Taylor, 2001; Balmer *et al.*, 2009; Alizon *et al.*, 2013).

A large body of theoretical evidence and epidemiological models predict that mixed strain infections can select for higher virulence, providing that virulence confers a within-host competitive advantage to the parasite (van Baalen & Sabeilis, 1995; May & Nowak, 1995; Frank, 1996; Read & Taylor, 2001; Alizon *et al.*, 2013). However, mixed infections have also been shown to select for less virulent strains when host exploitation depends on cooperation by parasites (Brown *et al.*, 2002; Hughes & Boomsma, 2004; Buckling & Brockhurst, 2008). For example, when infections involve sexually reproducing parasites, such as heterothallic fungi where two compatible partners are required to produce sexual spores (Debuchy *et al.*, 2010), an element of

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cooperation may be incorporated into the within-host dynamics. The evolutionary outcomes of such mixed infections can thus lead to a variety of effects on the characteristics of the parasites involved (Read & Taylor, 2001; Balmer & Tanner, 2011).

The timing of transmission by a parasite can also influence the evolution of parasite virulence. Theory predicts that a saturating trade-off between transmission rate and virulence should lead to the existence of an optimal level of virulence (Anderson & May, 1982; Ewald, 1994; van Baalen & Sabeilis, 1995; Frank, 1996; de Roode *et al.*, 2008; Alizon *et al.*, 2009), which will maximize parasite fitness. Slower production of infective propagules can allow a parasite to utilize its host's tissues more efficiently, whereas rapid production through higher virulence may increase replication rate by the parasite, but may decrease fitness due to a truncation in its host's lifespan (Cooper *et al.*, 2002). Consequently, there may be a trade-off between virulence (i.e. the speed of kill by a parasite) and fitness (total reproductive output of the parasite through propagule production), and so an increase in virulence driven by competitive pressures may come at a fitness cost to competing parasites (de Roode *et al.*, 2008; Alizon *et al.*, 2009). When cooperative interactions are considered, the evolutionary relationships between virulence and transmission then become even more complex (Alizon & Lion, 2011; Alizon *et al.*, 2013). It is clear that more empirical studies of the effects on virulence and transmission in parasites evolving under cooperative, as well as competitive, selection pressures are needed to understand virulence evolution in natural systems, especially considering that mixed infections are common in nature (Read & Taylor, 2001).

Experimental evolution through serial passage experiments (SPE) allows assessment of how parasites adapt across successive generations; parasites are transferred from one host to another several times to develop a derived strain, the virulence and fitness of which can then be compared directly to the ancestral strain (Ebert, 2000). Serial passage experiments generally result in an increase in parasite virulence as a result of more virulent strains outcompeting less virulent strains in mixed infections, or relaxed selection on transmission because it is carried out by the experimenter (Aizawa, 1971; Ferron, 1985; Ebert, 1998, 2000; Hughes & Boomsma, 2006). What is currently less clear, however, is how virulence evolves over successive generations when within-host competition or cooperation is involved. Single-generation experiments are useful for determining the processes by which parasites interact, but do not lead to firm conclusions about the direction or magnitude of their evolution or virulence (Alizon *et al.*, 2013).

Here, we use a serial passage experiment to investigate empirically the effect of mixed strain infections on the evolution of parasite virulence (defined here as the proportion of hosts killed by the parasite and the speed with which it killed them) and fitness (defined here as the speed of sporulation and the number of spores produced by the parasite). We use as our study system the entomopathogenic fungus, *Ascosphaera apis*, which is the causative agent of chalkbrood disease in Honey bee (*Apis mellifera*) larvae, naturally caused by co-infections of multiple strains (e.g. Gilliam *et al.* (1997) found 20 strains in 8 larvae). Spores are ingested by larvae and germinate in the alimentary tract, with hyphae then growing into surrounding tissues, killing the host. Following host death, hyphae grow out through the cuticle over several days, utilizing the host's tissue for growth and reproduction, with spores forming externally when two mating types meet (Chorbiński, 2004). The parasite is thus an 'obligate killer' that only achieves fitness by producing spores semelparously following host death (Ebert & Weisser, 1997). This also means fitness is easy to quantify and that the co-evolutionary host–parasite dynamics are likely to be particularly strong (Evison *et al.*, 2013). Importantly, *Asc. apis* is a heterothallic fungus and only achieves fitness following recombination between two opposite mating types within the host. Consequently, the within-host interaction between parasites may include cooperation to achieve sexual recombination rather than being solely competitive, unlike the host–parasite interactions most commonly studied previously. This system therefore provides a unique opportunity to assess how within-host parasite diversity impacts on virulence evolution when parasite strains obligately require sexual reproduction. We predict that parasite strain diversity during co-infection will either drive the evolution of greater virulence because of competition, with a consequent trade-off of a drop in fitness possibly occurring (Alizon *et al.*, 2009), or alternatively parasite diversity may allow the parasite to achieve greater fitness, even when competition for the host's resources occurs, because of increased opportunity for sexual recombination with different strains when multistrain infections are involved.

## Materials and methods

### Laboratory rearing of larvae

We collected larvae from ten colonies of the European Honey bee *Apis mellifera*, each headed by a naturally mated queen and situated in an experimental apiary at the University of Leeds farm. Larvae were reared individually in 48-well tissue culture plates on a diet of 50% royal jelly (apitherapy), 6% D-glucose, 6% D-fructose and sterile deionized water. One- to two-day-old larvae were removed from the comb and transferred onto a droplet of larval diet within a cell culture plate. The plates were placed in sealed boxes containing a pool of 0.04% $K_2SO_4$ to establish high

humidity and maintained at 34 °C. Larvae were treated with parasites 24 h after collection when they were 2–3 days old and fed daily following a feeding regime which ensured they were under no nutritional stress (Foley *et al.*, 2012; Evison *et al.*, 2013), until they began to defecate (which only occurs shortly before pupation); the wells were then cleaned with a cotton bud and the larvae fed no further.

### Parasite treatments

Spores were harvested from media plates (Sabouraud dextrose agar) of three different strains of *A. apis*, each of which was formed by the mating of complementary mating type hyphae grown from two single-spore isolates (termed mating plates; see Fig. S2 for a typical mating plate): strain I by isolates ARSEF 7405 + 7406; strain E by isolates KVL 0798 + 06 117; and strain F by isolates KVL 06 123 + 06 132. The parasite strains were obtained from infected larvae from different regions, either from the USA (ARSEF isolates) or from Denmark (KVL isolates; Vojvodic *et al.*, 2011), and were thus naive to any of the host genotypes used in this experiment, which were from the UK. Each of the pairs of isolates came from a distinct clade of *A. apis* and were therefore more closely related to each other than to the isolates used for the other strains (Fig. S1; Vojvodic *et al.*, 2011). Spore suspensions were made by transferring a small amount of spore material ($\approx$ 0.01 g) from the plate to a glass tissue homogenizer, and grinding with 50 μL deinonized water. Released spores were made up to a volume of 1 mL with sterile deionized water and allowed to settle for 20 min to obtain a medium spore density solution (allowing any clumped spores to collect to the bottom of the solution, thus making spore quantification reliable), a 0.5-mL sample of which was taken and stored in a separate Eppendorf tube. The concentrations of the spore solutions were determined using FastRead disposable haemocytometers (Immune Systems) and were adjusted to account for any differences in spore germination rates between the three strains (determined as detailed in Vojvodic *et al.*, 2011; average rates = strain E 38%; strain F 36%; strain I 51%). The adjusted spore solutions from each of the three strains were then mixed to form seven parasite treatments: three single-strain treatments (E, F and I alone), three double-strain treatments (EF, EI and FI mixed in equal ratio) and one triple-strain treatment (EFI mixed in equal ratio). Each treatment was adjusted to a concentration of $5.0 \times 10^5$ spores per mL (2500 spores per 5 μL dose), containing equal proportions of spores from each strain in the double- and triple-strain treatments. Double-strain treatments therefore contained twice the genetic diversity, and triple-strain treatments contained three times the genetic diversity of single-strain treatments.

### Serial passage procedure

Spore suspensions of each treatment were applied in 5 μL doses directly to the mouth of the larvae. Twelve larvae from each of ten different colonies were inoculated with each of the seven treatments and a control solution of 5 μL sterile deionized water. Thus, at the beginning of the experimental procedure (infection round one), each of the seven treatments was identical between the ten replicate lines (colonies) in that they were taken from the same mating plates described in 'parasite treatments'. For example, the genetic make-up of the spores of strain E (a single genotype of *Asc. apis*) in all replicate lines of treatments E, EI, EF and EFI was identical. Mortality and subsequent evidence of infection (hyphal growth and then sporulation) of each individual larva were monitored daily using a stereomicroscope for 10 days. Upon the external appearance of hyphal growth and sporulation, larvae were transferred to an individual media plate (Sabouraud dextrose agar) and incubated at 30 °C for a further 10 days to encourage hyphal growth to continue and allow spores to mature, to enable harvesting of sufficient spore quantities for the next infection round. This is necessary because the spores are not immediately infective, but if infected larvae are left longer under the *in vitro* rearing conditions used here, the cadavers are overcome with bacteria, and harvesting the spores becomes impossible. Spores were then harvested from the media plates (one plate per replicate line), to produce the spore suspensions for each of the still extant replicate lines of the seven treatments for the next round of infection (as described in 'parasite treatments', using the same dosages, each adjusted for any differences in germination rates). A total of 70 replicate lines (seven treatments applied to larvae from each of ten colonies, with each treatment assayed in each replicate host colony) were serial passaged in this way a total of three times (three rounds of infections, with two transfers). Each infection round used larvae from a different colony to the previous infection round to ensure that each replicate line within each treatment encountered a different host genotype during each infection round to prevent host genotype adaptation by the parasite confounding the results. Laboratory conditions were constant throughout the entire SPE. There were a number of extinction events following the second and third infection round (meaning a single replicate line within a treatment failed to produce any sporulating larvae), but multiple replicate lines of each of the seven treatment combinations survived both transfers and thus all three rounds of infection, and the final number of replicate lines following the serial passage was 44 (giving a 37% extinction rate; see supplementary material for details of extinctions within treatments).

### Comparison of derived and ancestral treatments

The virulence and fitness of the remaining 44 lines of the seven derived treatments were compared to that of the seven ancestral treatments and a sterile water control, using host larvae from four different colonies, all of which were naïve to any of the parasites (i.e. had never been encountered during the SPE). This final assessment was conducted using the same infection protocol as was used throughout the SPE procedure, but on a larger scale. The assessment was carried out in four batches, with 12 larvae from each of the four colonies being treated with each of the 44 derived lines over the course of the four batches (i.e. 48 larvae treated per treatment replicate), as well as the seven ancestral treatments and the control treatment repeated during each batch. Thus, this gave $n = 192–384$ larvae per treatment (depending on the number of replicate lines within a derived treatment that survived the SPE procedure), and $N = 3648$ larvae in total. As during the SPE procedure, mortality and evidence of infection (external appearance of hyphal growth and subsequent sporulation) were monitored daily using a stereomicroscope for 10 days following inoculation. On the 10th day, sporulating cadavers were collected and total spore production was counted for four individuals per treatment (one from each colony), giving 12–32 spore counts per treatment in total (depending on the number of replicate lines within a derived treatment that survived the passaging procedure).

### Statistical analysis

All analyses were carried out using R statistical software (R Development Core Team, 2013). Differences in survival of infected larvae between treatments, and whether they were ancestral or derived strains, along with their interaction, were analysed using Kaplan–Meier survival models fitted with a Weibull distribution implemented using the survreg function of the survival package (Therneau, 2011). Survivors of the experiment were incorporated as right-censored data, and we used a frailty model to incorporate the random effects of colony and batch. Hazard ratios (HR) were extracted from the survival analyses using the control data as a reference value. *Post-hoc* pairwise comparisons were made with Breslow statistics. Differences in day of sporulation and the numbers of spores produced between the seven treatment types, and whether they were ancestral or derived treatments, were assessed using linear mixed effects models, implemented using the lmer function in the lme4 package (Bates & Maechler, 2010). We fitted colony within batch as the random term, and the day of death was included as a covariate to account for larval age or size influencing sporulation quantity. *Post-hoc* pairwise comparisons, the significance of which is indicated on each figure using asterisks if not included in the text, were corrected using sequential Bonferroni correction.

## Results

Serial passage of the three strains of chalkbrood parasite, whether alone, in competition with another strain, or two other strains, showed contrasting effects on the virulence (negative effect on host survival) and the fitness (timing and number of spores produced) characteristics of the parasites. A number of replicate lines of the treatments were also driven to extinction during the serial passages (Table S3). Control larvae always survived well and never developed any infection from the chalkbrood parasites, with 85.4% surviving until at least the pupation stage (6-7 days post-grafting; Fig. S3; Table S1).

### Effects of serial passage and diversity on parasite virulence characteristics

Comparison of derived and ancestral parasites showed serial passage had significant effects on the virulence of each of the seven parasite treatments. We found that each of the seven derived treatments had higher virulence than the ancestral treatments, always causing death faster ($\chi^2_1 = 124$, $P < 0.001$; Figs 1a and S4). There were also differences in host survival between our seven types of treatment ($\chi^2_6 = 17.8$, $P = 0.007$; Fig. S3), regardless of whether they were ancestral or derived; however, there was no significant interaction between treatment and whether the parasites were ancestral or derived ($\chi^2_6 = 9.02$, $P = 0.172$), reinforcing the finding that the virulence of each of the parasite treatments increased after the experimental evolution. This was further reinforced by significant pairwise comparisons between each ancestral and derived treatment (Fig. 1a). To assess how the virulence increase played out through the course of the serial passage experiment, we plotted the final assessment data together with the within-passage data (Fig. 2), which shows an oscillating change over the course of serial passage procedure (Figs 2 and S7a), that is that virulence did not increase incrementally during each infection round, but was dynamic.

### Effects of serial passage and diversity on parasite fitness characteristics

Comparison of derived and ancestral parasites showed serial passage also had significant effects on the fitness characteristics of the parasites as measured both by mean day of sporulation and total spore production. There was a significant interaction between the seven parasite treatments and whether they were ancestral or derived in both the speed of sporulation ($\chi^2_6 = 15.4$, $P = 0.018$; Fig. 1b) and the number of spores produced ($\chi^2_6 = 18.9$,

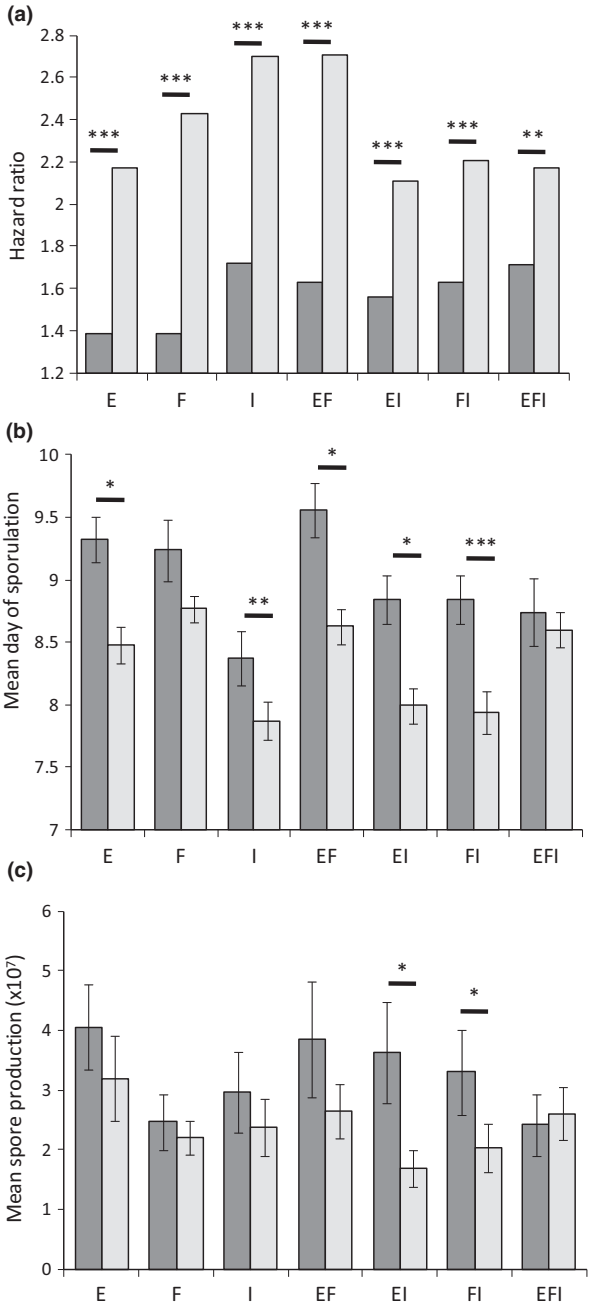


**Fig. 1** The effect of serial passage on the virulence and fitness characteristics of the seven parasite treatments. Bars show (a) the hazard ratio (HR) figure extracted from survival analysis, (b) the mean day of sporulation and (c) the mean spore production from parasite infections of larvae treated with ancestral strains (dark grey bars) compared to derived strains (light grey bars). Significant relationships are depicted by a bar and asterisks that show significance at the level $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***).

$P = 0.004$; Fig. 1c). In contrast to the effects on virulence, serial passage therefore affected fitness differently between strains and their mixes. There were also

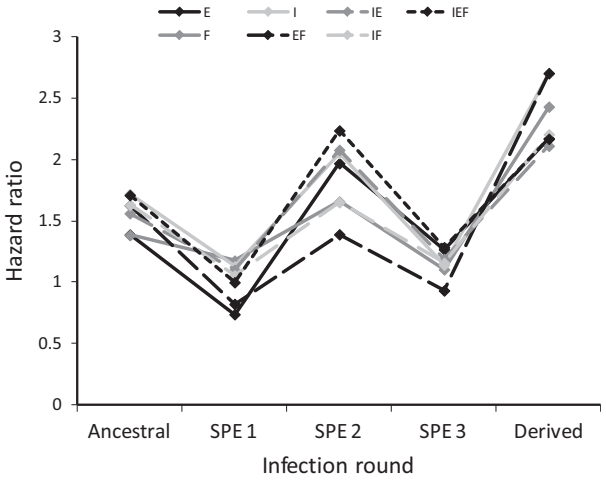


**Fig. 2** The dynamics of the virulence increase of the seven parasite treatments. Lines show the hazard ratio (HR) figure extracted from survival analysis from parasite infections of larvae treated with each of the rounds of infection during the entire study from left to right: the ancestral treatments (made during the final assessment), each infection round during the serial passage procedure (SPE1, SPE2, SPE3), and the derived treatments.

significant main effects of treatment type (sporulation day: $\chi^2_6 = 39.2$, $P < 0.001$, Fig. 1b; number of spores produced: $\chi^2_6 = 44.7$, $P < 0.001$, Fig 1c) and whether the treatments were ancestral or derived (sporulation day: $\chi^2_6 = 17.5$, $P < 0.001$, Fig. 1b; number of spores produced: $\chi^2_1 = 6.52$, $P = 0.012$, Fig. 1c) on both fitness measures. Pairwise comparisons showed that the interaction effects stemmed from specific treatments. For two of the single-strain treatments (E and I), sporulation was significantly faster in the derived parasites (Fig. 1b), but there was no significant drop in the numbers of spores produced (Fig. 1c), whereas for strain F, neither the decreases in time to sporulation nor the number of spores produced were significant. For each of the double-strain treatments (EF, EI and FI), sporulation was significantly faster (Fig. 1b), and significantly fewer spores were produced in treatments EI and FI (Fig. 1c). Despite the increase in virulence in treatment EFI (Fig. 1a), there was no significant change in sporulation time (Fig. 1b) or the number of spores produced (Fig. 1c).

## Strain-specific effects on parasite virulence and fitness

To gain a better understanding of the differences between treatments in virulence and fitness evolution of the parasites and assess how evolving under the pressures of different levels of diversity affected these, we looked at the pairwise comparisons of each strain and its combinations within a treatment (i.e. whether infections involved single strains or co-infections of one or two other strains). There were only significant differences

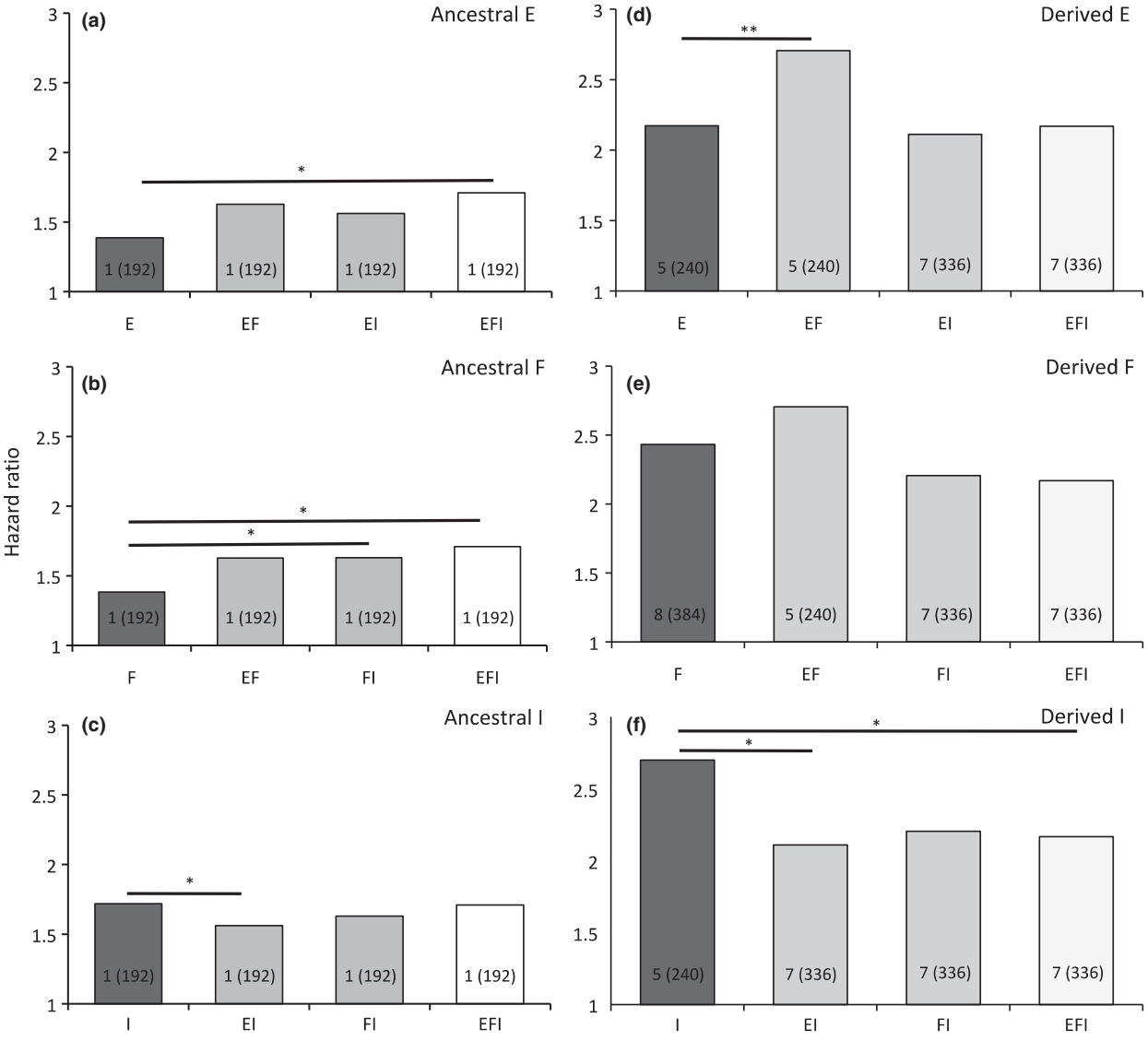


**Fig. 3** The strain-specific effects on parasite virulence of serial passage under differing competitive pressures. Bars show the hazard ratio (HR) figure extracted from survival analysis of larvae infected with parasite strains that had been passaged alone (dark grey bars) compared to when they had been passaged with one other strain (light grey bars), or both other strains (white bars) for the ancestral parasites of (a) strain E, (b) strain F and (c) strain I, and the derived parasites of (d) strain E, (e) strain F and (f) strain I. Significant relationships are depicted by a bar and asterisks that show significance at the level $P < 0.05$ (*), or $P < 0.01$ (**). Numbers on each treatment bar depict the number of replicate lines within each treatment type, and the total number of individual larvae infected in parentheses.

found here in survival (HR, our measure of virulence), but not in day of sporulation or the number of spores produced (our measures of fitness; see supplementary material Figs S8 and S9). This analysis was carried out on each ancestral strain and its combinations (Fig. 3a–c) and each derived strain and its combinations (Fig. 3d–f) and showed strain-specific differences in the direction of the effects of diversity on virulence. The ancestral strain E was involved in more virulent infections only when it infected together with both strains F and I (E vs. EFI: $\chi^2_1 = 5.154$, $P = 0.023$; Fig. 3a), but this was only the case for the EF combination in the derived parasites (E vs. EF: $\chi^2_1 = 7.645$, $P = 0.006$; Fig. 3d). The ancestral strain F was involved in more virulent infections when it infected together with strain I or with both E and I (F vs. FI: $\chi^2_1 = 4.253$, $P = 0.039$; F vs. EFI: $\chi^2_1 = 5.571$, $P = 0.010$; Fig. 3b), but these effects were not present in the derived parasites after serial passage. Conversely, we found that the ancestral strain I was involved in less virulent infections when it infected with strain E (I vs. EI: $\chi^2_1 = 4.365$, $P = 0.057$; Fig. 3c), and that this effect was maintained after the serial passage, (I vs. EI: $\chi^2_1 = 4.256$,

$P = 0.039$; Fig. 3f). Less virulent co-infections compared to the derived strain I alone was also seen in the derived treatment EFI (I vs. EFI: $\chi^2_1 = 6.703$, $P = 0.010$; Fig. 3f).

## Discussion

Serial passage of multiple strains of parasite, via both co-infection or alone, showed a variety of influences on the virulence and fitness characteristics of the parasites. Virulence was greater in all the derived parasites compared to the ancestral parasites, whereas fitness effects were variable between treatments, with lower spore production in some treatments and several replicate lines of the parasite treatments driven to extinction. The strength of these changes is particularly remarkable given that the experimental evolution lasted for only three rounds of infection (Yourth & Schmid-Hempel, 2006). Contrary to what theory would normally predict, there was not a clear effect of parasite diversity influencing the evolution of the parasites, with no evidence of greater within-host competition driving the evolution of greater virulence.

The results support the trade-off hypothesis (Anderson & May, 1982) in as much as a relaxed selection on transmission through the artificial method of transmission employed during the serial passage procedure is likely to have lead to the higher virulence seen in all derived treatments. However, the resulting effects on parasite fitness, faster sporulation and any associated drop in number of spores produced, was not consistent in all treatments, and notably, no impact on fitness was seen in the highest diversity group. Indeed, the patterns of virulence evolution appeared to be strain specific and suggest the opposite to what was expected based on most parasite theory that evolving under competitive pressure should increase virulence (van Baalen & Sabeilis, 1995; May & Nowak, 1995; Frank, 1996; Read & Taylor, 2001; Alizon *et al.*, 2013). Together, this suggests that specific interactions between strains, rather than competition alone, could have influenced the differences in the fitness characteristics of the derived parasite treatments. Whether this occurred due to ecological effects from interference competition or suppression (Levin & Bull, 1994; Read & Taylor, 2001; Balmer *et al.*, 2009), or by selection on the characteristics of the competing and/or cooperating parasite strains (Alizon *et al.*, 2013), it will have important consequences for the evolutionary dynamics of co-infections.

There was a general trend for an increase in virulence across all treatment groups, a common phenomenon in serial passage experiments (Aizawa, 1971; Ferron, 1985; Ebert, 1998, 2000). However, this increase was not linear across the infection rounds of the experiment, but rather showed oscillatory dynamics, with virulence increasing and then decreasing during the course of serial passages. It appears that as a consequence of the derived parasites killing their hosts faster, there was also faster sporulation in most treatments. Interestingly, this faster sporulation appeared to translate into a trend for a general drop in the number of spores produced, although in most cases, this was not significant. This impact on fitness is in fact greater than it appears when it is considered that the faster sporulation time resulted in a longer period for spore production (because the numbers of spores were counted out at a fixed time point at the end of the experiment). The spore production pattern may therefore also explain the oscillating nature of the virulence increase during serial passage. The increase in virulence during the second infection round (IR2) may be due to relaxed selection on transmission, whereas the subsequent drop in virulence during infection round three (IR3) might reflect loss of more virulent parasites here through loss of fitness. It is possible that although parasite virulence increases overall across serial passages, trade-offs between virulence and other fitness-related traits cause it to do so in an oscillatory rather than linear manner. In this case, the oscillatory dynamics may relate to the inclusion of a growth stage on media, which meant that parasites were under selection pressures to achieve fitness in two distinct environments (the host and on media). Many parasites naturally have to grow or survive in multiple environments, and it would be interesting to know whether oscillatory increases in virulence across serial passages are common. There was some evidence to suggest that the increase in the virulence of the derived parasites that were passaged together with only one other strain incurred the highest fitness costs, as the largest decreases in the numbers of spores produced were seen in these three double-strain treatments (although this was not significant in the case of treatment EF). When a parasite kills its host too quickly through higher virulence, the host will provide fewer resources for the parasite's growth and reproduction; hence, higher virulence often leads to lower fitness (Alizon *et al.*, 2009). However, if the parasite allocates resources specifically to quickened growth and reproduction to compete with another strain, it will again have fewer resources for production of spores (Hall *et al.*, 2012). Either or both these phenomena could explain the results found in this study, and the specific mechanisms involved require further investigation.

The notable exception to the general drop in fitness as a consequence of an increase in virulence was the highest diversity parasite treatment. Here, there was no change in timing of sporulation and no evidence of a drop in spore production, despite a significant increase in virulence. We looked at the characteristics of the parasites through the time course of the serial passage procedure to try and understand this effect further (data in supplementary material). The proportion of infections leading to sporulation events on individual hosts dramatically dropped during each infection round, almost to zero by the final passage. Theory predicts that

through time a co-evolving host–parasite relationship should lead to a level of virulence that is optimal to best exploit their host (Ewald, 1983; May & Anderson, 1983) due to the stochastic loss of any highly virulent strains that kill the host too fast to allow their own reproduction, which leads to a loss of parasite diversity (Bergstrom *et al.*, 1999). It is also known that recombination can be deleterious in the short term, which could also lead to loss of diversity when multiple strains recombine within the host or otherwise influence virulence evolution even in single-strain infections (López-Villavicencio *et al.*, 2013). These phenomena appear to be reflected in the within-passage results as the virulence initially increased during serial passage, but the proportion sporulating in all treatments dropped off dramatically, and several lines became extinct. It appears possible that only strains that most effectively utilized host resources remained. This would lead to the evolutionary trajectory of the highest diversity parasite treatments aligning with that of the single genotype treatments and may explain why, despite the increase in virulence in the highest diversity treatment EFI, there was no effect on transmission timing or quantity here, unlike in the other treatments. Considering the patterns of virulence and the extinction rates through the serial passages, it is possible that interacting strains quickly became outcompeted here, rather than the higher diversity providing increased opportunity for successful recombination, and consequently that there was no apparent effect on virulence or transmission in this group. To answer this question would require genetic inspection of the cocktail of parasites as they are passaged (something we could not do here).

Although we did not see strong or consistent evidence of higher genetic diversity driving increased virulence in the parasite treatments, the suggestion that only the those strains that most effectively utilize the host's resources remained in the cocktail of parasites as they were selected during the SPE is also supported by the strain-specific patterns of virulence evolution. When we looked at the strain-specific virulence effects, the three parasite strains appeared to interact in a different way when co-infecting with different partners, something that has been observed in other fungal parasites (e.g. Wille *et al.*, 2001). For example, infections involving the ancestral treatments of both strains E and F in isolation were of significantly lower virulence than those that evolved together with the two other strains. This effect was lost through the serial passage procedure, suggesting that the factors causing the higher virulence in the EFI treatment were lost through selection. Conversely, the ancestral strain I was involved in significantly more virulent infection when it infected alone compared to when it infected together with strain E, an effect that remained stable through serial passage. However, the derived isolates of strain I were more virulent in isolation than was the highest diversity cocktail after serial passage, perhaps suggesting that the highly virulent strain I was selected out of the EFI treatment during serial passage.

Such strain-specific differences may act as a mechanism to maintain parasite genetic polymorphism within in a population (Regoes *et al.*, 2000), as their varying life-history strategies allow co-existence and prevent the most virulent strains leading to short-sighted evolution and loss of multiple parasite genotypes from the population (Levin & Bull, 1994). Considering that the trade-off between virulence and fitness did not appear in the highest diversity group, it may be that the heightened competition within this treatment, or compensation by the greater diversity of the deleterious effects of recombination (López-Villavicencio *et al.*, 2013), prevented the effect. The results could also be due to effects of interference or suppression of competing parasites in the mixed strain treatments (Massey *et al.*, 2004; Balmer *et al.*, 2009). Either way, the evolutionary consequences of co-infections with multiple conspecific strains of a parasite are variable and appear to depend on the specific characteristics of the strains involved. This phenomenon demands further study; genetic analysis and quantification of strains as they interact, recombine and evolve are required to fully understand their interactions. For example, it is not known whether the parasite selectively mates with its own strain, or whether recombination between strains occurs at differing rates. We did not perform such analyses here because of this unknown potential for recombination within the host between strains in the multiple strain cocktails, leading to lack of distinction between strains after the first passage, and because we were primarily interested in the effect of parasite diversity on the evolution of the parasite's characteristics, rather than trying to track winning or losing strains. Considering that this parasite requires a phase of sexual reproduction to achieve fitness, it may have evolved to avoid competition with other strains, and thus, the highest diversity treatment did not respond to selection in the same way as the other treatments did. Selection through competition can be extremely important for how the virulence and fitness of a parasite changes over time. Specific aspects of the biology of a parasite, however, such as the requirement for sexual reproduction, and strain-specific differences in a Parasite's characteristics will influence these outcomes, reducing or changing the force of within-host competition. What is clear is that to disentangle the mechanisms of interactions between parasites, more information about infection dynamics, particularly in sexually recombining parasites, is needed.

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## Competing interests

There are no competing interests associated with the publication of this manuscript.

## Author contributions

SEFE participated in the design of and carried out the experimental procedures and drafted the manuscript. KF participated in collection of experimental Honey bee larvae. ABJ participated in the design of the study. WOHH conceived of the study, participated in its design and coordination and helped to draft the manuscript.

## References

Aizawa, K. 1971. Strain improvement and preservation of virulence. In: *Microbial Control of Insects and Mites* (H.D. Burges & N.W. Hussey, eds), pp. 655–672. Academic, New York.

Alizon, S. & Lion, S. 2011. Within-host parasite cooperation and the evolution of virulence. *Proc. R. Soc. Lond. B* **278**: 3738–3747.

Alizon, S., Hurford, A., Mideo, N. & van Baalen, M. 2009. Virulence evolution and the trade-off hypothesis: history, current state of affairs and the future. *J. Evol. Biol.* **22**: 245–259.

Alizon, S., de Roode, J.C. & Michalakis, Y. 2013. Multiple infections and the evolution of virulence. *Ecol. Lett.* **16**: 556–567.

Anderson, R.M. & May, R.M. 1981. The population dynamics of microparasites and their invertebrate hosts. *Phil. Trans. R. Soc. Lond. B* **291**: 451–524.

Anderson, R.M. & May, R.M. 1982. Coevolution of hosts and parasites. *Parasitology* **85**: 411–426.

van Baalen, M. & Sabeilis, M.W. 1995. The scope for virulence management: a comment on Ewald's view on the evolution of virulence. *Trends Microbiol.* **3**: 414–416.

Balmer, O. & Tanner, M. 2011. Prevalence and implications of multiple-strain infections. *Lancet. Infect. Dis.* **11**: 868–878.

Balmer, O., Stearns, S.C., Schötzau, A. & Brun, R. 2009. Intraspecific competition between co-infecting parasite strains enhances host survival in African trypanosomes. *Ecology* **90**: 3367–3378.

Bates, D. & Maechler, M. 2010. lme4: linear mixed effects models using S4 classes. Available at: http://cran.r-project.org/web/packages/lme4/

Bergstrom, C.T., McElhany, P. & Real, L.A. 1999. Transmission bottlenecks as determinants of virulence in rapidly evolving pathogens. *Proc. Natl. Acad. Sci. USA* **96**: 5095–5100.

Brown, S.P., Hochberg, M.E. & Grenfell, B.T. 2002. Does multiple infection select for raised virulence? *Trends Microbiol.* **10**: 401–405.

Buckling, A. & Brockhurst, M.A. 2008. Kin selection and the evolution of virulence. *Heredity* **100**: 484–488.

Chorbiński, P. 2004. The development of the infection of *Apis mellifera* larvae by *Ascosphaera apis*. *Elec. J. Pol. Agri. Univ. Vet. Med.* **7**: 3. http://www.ejpau.media.pl/volume7/issue2/veterinary/art-03.html. (Online)

Cooper, V.S., Reiskind, M.H., Miller, J.A., Shelton, K.A., Walther, B.A., Elkinton, J.S. *et al.* 2002. Timing of transmission and the evolution of virulence of an insect virum. *Proc. R. Soc. Lond. B* **269**: 1161–1165.

Debuchy, R., Berteaux-Lecellier, V. & Silar, P. 2010. Mating systems and sexual morphogenesis in ascomycetes. In: *Cellular and Molecular Biology of Filamentous Fungi* (K.A. Borkovich & D.J. Ebbole, eds), pp. 501–535. American Society for Microbiology Press, Washington, DC.

Ebert, D. 1998. Experimental evolution of parasites. *Science* **282**: 1432–1435.

Ebert, D. 2000. Experimental evidence for rapid parasite adaptation and its consequences for the evolution of virulence. In: *Evolutionary Biology of Host-Parasite Relationships: Theory Meets Reality* (R. Poulin, S. Morand & A. Skoping, eds), pp. 163–184. Elsevier, Amsterdam, The Netherlands.

Ebert, D. & Weisser, W.W. 1997. Optimal killing for obligate killers: the evolution of life histories and virulence of semelparous parasites. *Proc. R. Soc. Lond. B* **264**: 985–991.

Evison, S.E.F., Fazio, G., Chappell, P., Foley, K., Jensen, A.B. & Hughes, W.O.H. 2013. Host-parasite genotypic interactions in the honeybee: the dynamics of diversity. *Ecol. Evol.* **3**: 2214–2222.

Ewald, P.W. 1983. Host-parasite relations, vectors, and the evolution of disease severity. *Ann. Rev. Ecol. Syst.* **14**: 465–485.

Ewald, P.W. 1994. *Evolution of Infectious Disease*. Oxford University Press, Oxford, UK.

Ferron, P. 1985. Fungal control. In: *Comprehensive Insect Physiology, Biochemistry and Pharmacology: Insect Control*, vol. **12** (G.A. Kerkut & L.I. Gilbert, eds), pp. 313–346. Pergamon Press, Oxford.

Foley, K., Fazio, G., Jensen, A.B. & Hughes, W.O.H. 2012. Nutritional limitation and resistance to opportunistic *Aspergillus* parasites in honey bee larvae. *J. Invert. Pathol.* **111**: 68–73.

Frank, S.A. 1996. Models of parasite virulence. *Q. Rev. Biol.* **71**: 37–78.

Gilliam, M., Lorenz, B.J., Wenner, A.M. & Thorp, R.W. 1997. Occurrence and distribution of *Ascosphaera apis* in North America: chalkbrood in feral honey bee colonies that has been in isolation on Santa Cruz Island, California for over 110 years. *Apidologie* **28**: 329–338.

Haldane, J.B.S. 1949. Disease and evolution. *Ric. Sci.* **19**(Suppl A): 68–75.

Hall, S.R., Becker, C.R., Duffy, M.A. & Cáceres, C.E. 2012. A power-efficiency trade-off in resource use alters epidemiological relationships. *Ecology* **93**: 645–656.

Hughes, W.O.H. & Boomsma, J.J. 2004. Let your enemy do the work: within-host interactions between two fungal parasites of leaf-cutting ants. *Proc. Biol. Sci.* **271**(Suppl 3): S104–S106.

Hughes, W.O.H. & Boomsma, J.J. 2006. Does genetic diversity hinder parasite evolution in social insect colonies? *J. Evol. Biol.* **19**: 132–143.

Kochin, B.F., Bull, J.J. & Antia, R. 2010. Parasite evolution and life history theory. *PLoS Biol.* **8**: e1000524.

Levin, B.R. & Bull, J.J. 1994. Short-sighted evolution and the virulence of pathogenic microorganisms. *Trends Microbiol.* **2**: 76–81.

Levin, S. & Pimentel, D. 1981. Selection of intermediate rates of increase in parasite-host systems. *Am. Nat.* **117**: 308–315.

López-Villavicencio, M., Debets, A.J.M., Slakhorst, M., Giraud, T. & Schoustra, S.E. 2013. Deleterious effects of recombina-

tion and possible nonrecombinatorial advantages of sex in a fungal model. *J. Evol. Biol.* **26**: 1968–1978.
Massey, R.C., Buckling, A. & ffrench Constant R. 2004. Interference competition and parasite virulence. *Proc. R. Soc. Lond. B* **271**: 785–788.
May, R.M. & Anderson, R.M. 1983. Epidemiology and genetics in the coevolution of parasites and hosts. *Proc. R. Soc. Lond. B* **219**: 281–313.
May, R.M. & Nowak, M.A. 1995. Coinfection and the evolution of parasite virulence. *Proc. R. Soc. Lond. B* **261**: 209–215.
R Development Core Team: R 2013. *A Language and Environment for Statistical Computing*. R foundation for Statistical Computing, Vienna.
Read, A.F. & Taylor, L.H. 2001. The ecology of genetically diverse infections. *Science* **292**: 1099–1102.
Regoes, R.R., Nowak, M.A. & Bonhoeffer, S. 2000. Evolution of virulence in a heterogeneous host population. *Evolution* **54**: 64–71.
de Roode, J.C., Yates, A.J. & Altizer, S. 2008. Virulence–transmission trade-offs and population divergence in virulence in a naturally occurring butterfly parasite. *Proc. Natl. Acad. Sci. USA* **105**: 7489–7494.
Schmid-Hempel, P. 1998. *Parasites in Social Insects*. Princeton University Press, Princeton, NJ.
Therneau, T.M. 2011. A package for survival analysis in S. Technical report series 53. Section of Biostatistics, Mayo Clinic.
Vojvodic, S., Jensen, A.B., Markussen, B., Eilenberg, J. & Boomsma, J.J. 2011. Genetic variation in virulence among chalkbrood strains infecting Honeybees. *PLoS ONE* **6**: e25035.
Wille, P., Boller, T. & Kaltz, O. 2001. Mixed inoculation alters infection success of strains of the endophyte *Epichloë bromicola* on its grass host *Bromus erectus*. *Proc. R. Soc. Lond. B* **269**: 397–402.
Yourth, C.P. & Schmid-Hempel, P. 2006. Serial passage of the parasite *Crithidia bombi* within a colony of its host, *Bombus terrestris*, reduces success in unrelated hosts. *Proc. R. Soc. Lond. B* **273**: 655–659.

## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Figure S1** Molecular Phylogenetic analysis by Maximum Likelihood method of the *Ascosphaera apis* strains used to form the parasite treatment. Strain I (mating of isolates ARSEF 7405 + 7406), strain E (mating of isolates KVL 0798 + 06 117) and strain F (mating of isolates KVL 06 123 + 06 132).

**Figure S2** A typical mating plate (on SDA media) showing the zone of sporulation (black area) where the hyphae of opposite mating types meet and recombination occurs to produce the spores.

**Figure S3** Survival curve showing hazard of each treatment (ancestral and derived combined) over the 10-day observation period compared to the control.

**Figure S4** Survival curve showing hazard of each type of treatment (ancestral or derived) over the 10-day period compared to the control.

**Figure S5** Survival curve showing hazard of each colony used in the final assessment of ancestral and derived treatments.

**Figure S6** Survival curve showing hazard of each colony used in the serial passage infection rounds 1 (a), 2 (b), and 3 (c).

**Figure S7** The virulence and fitness characteristics of the parasites during serial passage.

**Figure S8** Data on day of sporulation to indicate the patterns of strain specific effects on parasite fitness.

**Figure S9** Data on mean spore production to indicate the patterns of strain specific effects on parasite fitness.

**Table S1** The virulence and fitness characteristics of the ancestral parasite treatments compared to the control parasite treatments with reference to the control larvae.

**Table S2** The virulence and fitness characteristics of the seven parasite treatments during the serial passage procedure (recorded within a 10-day period).

**Table S3** The number of extinction events during the course of the serial passage procedure (IR = infection round), and the remaining extant replicate lines tested during the final assessment.

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